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ORIGINAL ARTICLE

Changes in free amino acids in the brain during embryonic development in layer and broiler chickens

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Abstract Developmental changes in the levels of the excitatory amino acids L-glutamate (Glu) and L-Aspartate (Asp) and inhibitory amino acids glycine (Gly) and γ-amino butyric acid (GABA), as well as taurine and its related amino acids L-methionine (Met), L-cysteine (Cys) and L-serine (Ser) in the brain and pectoralis muscle at various embryonic stages and hatch in broiler and layer type chickens were determined. Brain concentrations of Asp, GABA and taurine were higher than those in the muscle, but the difference in the two types was small. The concentrations of the precursors of taurine including Met, Cys and Ser were lower than that of taurine. In conclusion, the synthesis of some amino acids and their metabolites such as Asp, GABA and taurine in the chick embryo is very high in order to support brain development.

Keywords Brain · Chicken · Embryo · Free amino acid · Skeletal muscle · Taurine

Introduction

The amino acids L-glutamate (Glu) and L-aspartate (Asp), which serve as excitatory neurotransmitters, play major roles in the development of normal synaptic connections in the brain. On the other hand, γ-amino butyric acid (GABA) and glycine (Gly) act as major inhibitory neurotransmitters in the brain. Lundgren et al. (1995) reported that the development of GABAergic neurons in the prosencephalon and telencephalon of chicken embryos on days 4–14 (E4–E14) is rapid, being abundant at E8 and "overexpressed" at E10–E11. This is due to the high activity of the GABA-synthesizing enzyme glutamate decarboxylase (GAD), since its activity increased approximately 25-fold from E3 to E17 (Ahman et al. 1996). Ahman et al. (1996), showing that GABA indeed accumulates during embryogenesis, whereas the levels of Glu, the substrate for GAD, were more or less unchanged up to later developmental stages.

In addition to excitatory and inhibitory amino acids, amino acid metabolites also act as developmental signals or regulators in the immature developing fetal brain. L-cysteine (Cys), either from exogenous sources or formed by the transsulfation pathway using L-serine (Ser) and the sulfur of L-methionine (Met), is metabolized to taurine (Stipanuk 1986). Taurine is postulated to be a neurotrophic factor in brain development, because proliferation and differentiation of neurons are increased in pure cerebral fetal human brain cultures containing taurine (Chen et al. 1998). Furthermore, abnormal kitten cortex development is observed in response to maternal dietary taurine deprivation (Palackal et al. 1986). On the other hand, Ser as the precursor of Cys, is a glia-derived trophic factor, is Gly (Furuya et al. 2000). Taurine and Ser, in addition to Gly, can activate the Gly receptor (Schmieden and Betz 1995). Thus, these amino acids interact closely in the brain.

Since broilers are selected for rapid growth and high meat yield, their embryonic development is actually faster

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than that of layers selected for egg production (Muramatsu et al. 1990a; Ohta et al. 2004; Sato et al. 2006). However, whole body protein turnover, both in terms of fractional and absolute rates, was significantly faster in dwarf than in broiler embryos, with intermediate values in layer embryos (Muramatsu et al. 1990a). For protein turnover, a two-compartment model has been applied, i.e., a free amino acid pool and a protein pool. Protein turnover of chick embryos is affected by the egg albumen content (Muramatsu et al. 1990b). Furthermore, in ovo injection of amino acids increased chick weight through an increase in amino acid utilization by the embryo (Al-Murrani 1982; Ohta et al. 1999). These facts imply that the free amino acid pool has an important role in embryonic growth. In addition, taurine is an important amino acid for growth. Actually, intraperitoneal administration of beta-alanine, a transport antagonist of taurine, to the natural dams through the lactation period induced a slower growth rate of pups (Hu et al. 2000). Therefore, there may be differences in taurine concentration between broiler and layer embryos.

The objectives of the present study were: (1) to clarify the developmental changes in excitatory and inhibitory amino acids and taurine-related amino acids in the brain between broiler and layer chickens at embryonic stages and hatch, and (2) to confirm whether these changes are specific to the brain compared with changes in skeletal muscles.

Materials and methods

Sample preparation

Fertilized eggs were obtained from Mori hatchery, Fukuoka, Japan (broiler: Chanky) and GHEN Corporation Gifu, Japan (layer: Julia), and were incubated at 37.6°C and a relative humidity of 58–68%. They were candled before incubation to remove chapped and broken eggs. Non-chapped and non-broken eggs were weighed individually. All experimental procedures were performed according to the National Research Council publication, Guide for Care and Use of Laboratory Animals and the Guidance for Experiments in the Faculty of Agriculture and in the Graduate Course of Kyushu University, and the Law (No.105) and Notification (No.6) of the Japanese Government.

Tissue

Samples were obtained at the following stages: E14, embryonic day of 18 (E18) and within 2 h after hatching (P0). Whole brain and pectoralis muscle were dissected rapidly. Tissues were blotted, placed in liquid nitrogen and stored at −80°C until analysis.

Analysis of free amino acids by high-performance liquid chromatography (HPLC)

Brain and pectoralis muscle were homogenized with distilled water. They were then centrifuged at 10,000*g* for 20 min. The supernatants were deproteinized by filtration through a 10,000 dalton molecular weight cut-off filter (Millipore, Bedford, MA, USA) via centrifugation at 10,000*g* for 60 min. The samples (40 µl) were then completely dried under reduced pressure. Dried residues were dissolved in 10 µl of 1 M sodium acetate–methanol–triethylamine (2: 2: 1) solution. The samples were re-dried and disssolved in 20 µl of derivatization solution [methanol–water–triethylamine–phenylisothiocyanate (7: 1: 1: 1)]. A period of 20 min at room temperature was allowed for the reaction of phenylisothiocyanate with amino groups to produce phenylthiocarbamyl amino acid residues. The samples were dried again. The dried samples were dissolved in 100 µl of Pico-Tag diluent (Waters, Milford, MA, USA). These diluted samples were filtered through a 0.45 µm filter (Millipore, Bedford, MA, USA). The same method was applied to standard solutions prepared by diluting a commercially available L-amino acid solution (type AN II and type B; Wako, Osaka, Japan) with distilled water. These derivatized samples (brain: 20 µl, skeletal muscle: 20 µl, and standard solution: 5 µl) were applied to a Waters HPLC system (Pico-Tag free amino acid analysis column (3.9 × 300 mm), Alliance 2690 separation module, 2487 dual-wavelength UV detector, and Millennium 32 chromatography manager; Waters, Milford, MA, USA). They were equilibrated with buffer A [70 mM sodium acetate (pH 6.45 with 10% acetic acid)–acetonitrile (975: 25)] and eluted with a linear gradient of buffer B [water–acetonitrile–methanol (40: 45: 15)] (0, 3, 6, 9, 40 and 100%) at a flow rate of 1 ml/min at 46°C. The absorbance at 254 nm was measured, and concentrations of free amino acids were determined. Triethylamine and sodium acetate trihydrate were purchased from Wako (Osaka, Japan), while other drugs for which no manufacturer is noted were purchased from Sigma (St Louis, MO, USA).

Statistical analysis

Data were analyzed by three-way analysis of variance (ANOVA) with respect to tissues (brain and muscle), types (broiler and layer) and developmental stages (E14, E18 and P0). Statements of significance were based on $P < 0.05$. Data were expressed as means ± SEM.

Results

Changes in the concentrations of excitatory and inhibitory amino acids in the brain and muscle of the two types of chick embryos are shown in Fig. 1. For Asp, significant effects of stage [(2,45) = 49.122, $P < 0.0001$] and tissue [(1,45) = 443.180, $P < 0.0001$], and significant interactions between stage × tissue [(2,45) = 44.933, $P < 0.0001$] were detected. Asp was higher in the brain than in the muscle, and dramatically increased in the brain at hatch. The concentration in muscle slowly increased in broilers with age, but the reverse was true in layers. Significant effects of stage [(2,44) = 23.879, $P < 0.0001$] and tissue [(1,44) = 147.930, $P < 0.0001$], and a significant interaction between stage × tissue [(2,44) = 12.358, $P < 0.0001$] were found in GABA. GABA rapidly increased with age in the brain. For Glu, there were significant effects of stage [(2,44) = 24.786, $P < 0.0001$], and significant interactions between stage × type [(2,44) = 4.144, $P < 0.05$] and between stage × tissue [(2,44) = 74.138, $P < 0.0001$]. Glu increased in the brain with age, but the maximum increase was reached in the muscle at E18. Significant effects of type [(1,46) = 12.362, $P < 0.0001$] and tissue [(1,46) = 34.179, $P < 0.0001$], and significant interactions between stage × tissue [(2,46) = 35.843, $P < 0.0001$] and between type × tissue [(2,46) = 35.843, $P < 0.0001$] were detected in Gly. Gly in the brain increased slowly with age. Furthermore, Gly concentration in the brain was lower than that in the muscle during embryogenesis. Gly decreased at hatch with strong reduction in broiler.

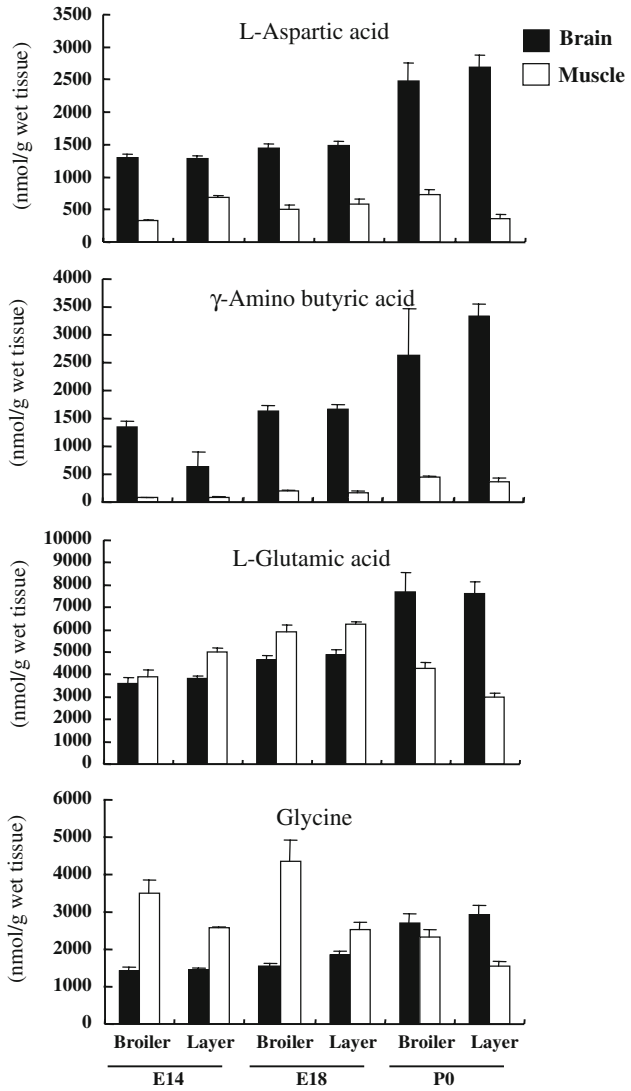

Fig. 1 Changes in excitatory and inhibitory amino acids in the brain between two types of chicken embryos in comparison with those in the skeletal muscle. The values are means ± SEM

Figure 2 shows changes in taurine and its related amino acids in the brain and muscle of two types of chicken embryos. Met significantly [(2,46) = 18.970, $P < 0.0001$] increased with aging. Significant effects of stage [(2,44) = 14.506, $P < 0.0001$] and type [(1,44) = 5.712, $P < 0.05$], and significant interactions between stage × type [(2,44) = 7.794, $P < 0.0001$] and among stage × type × tissue [(2,44) = 4.498, $P < 0.05$] were found in Ser. Ser was the highest at E18. Cys concentration was decreased from E14 to E18 and was increased from E18 to P0 [(2,47) = 8.447, $P < 0.001$]. A significant interaction between type × tissue [(1,47) = 4.856, $P < 0.05$] indicated that Cys concentrations of broilers in the brain was low, but those in the muscle was comparable to layers. There was significant interaction among stage × type × tissue [(2,47) = 8.537, $P < 0.001$] indicating that Cys concentration was identical in both tissues between layers and broilers except for E18. In E18, taurine concentration of broilers was lower in the brain than in the muscle, but the reverse was true for layers. These interactions were influenced by the concentrations of amino acids in the brain of broilers and in the muscle of layers at E18.

Taurine concentrations were altered by all three main effects (stage [(2,44) = 46.400, $P < 0.0001$], type [(1,44) = 4.738, $P < 0.05$]) and tissue [(1,44) = 139.172, $P < 0.0001$]). Significant interactions between stage × tissue [(2,44) = 4.554, $P < 0.05$] and among stage × type × tissue [(2,44) = 4.480, $P < 0.05$] existed. Taurine concentration was always higher in the brain than in the muscle. The values in the muscle increased with age in broilers.

Discussion

The concentration of the excitatory amino acid Asp and the inhibitory amino acid GABA was extremely high in the

brain during the incubation period, and at P0. This implies that the central nervous system in the chick embryo requires large amounts of both amino acids. Ahman et al. (1996) reported that GABA accumulated during embryogenesis, whereas the levels of Glu, the substrate for GAD, were relatively unchanged up to later developmental stages. This increase in GABA could be explained by GAD activity, since the activity increased approximately 25-fold from E3 to E17. In the present study, Glu level was comparable between E14 and E18. This observation supports the report by Ahman et al. (1996). Glu concentration was somewhat higher in muscle during the incubation period, but this trend was reversed at P0. The reasons for this change are not clear.

The response in Gly was similar to Glu, but the magnitude of the difference during incubation was large with Gly levels being much lower in the brain. The immediate precursor of Gly is Ser, which is converted to Gly by the the enzyme Ser hydroxymethyltransferase. As shown in Fig. 2, Ser levels in the brain, as well as in muscle, were relatively low. In contrast, although Ser is a precursor for taurine (Stipanuk 1986), the concentration of taurine was much higher in the brain than in the muscle (Fig. 2). Therefore, Ser might be utilized for taurine synthesis rather than Gly synthesis during embryonic stages and as a result Gly levels kept low.

GABA and GABA-releasing synapses are formed before glutamatergic contacts and provide the principle excitatory drive in developmental immature neurons (Represa and Ben-Ari 2005). GABA, acting via $GABA_A$ receptor-mediated depolarization, alters resting cell membrane potentials sufficiently to release the voltage-dependent magnesium block on *N*-methyl-D-aspartate channels to allow calcium influx into the cell (Ben-Ari et al. 1989). Thus, GABA acts as an excitatory amino acid in the immature developing CNS. In addition, taurine triggers inward currents and induces membrane depolarization resulting in elevation in intracellular calcium concentrations in the rat embryonic cortex (Flint et al. 1998). Taken together, these observations suggest that GABA and taurine act to increase calcium influx into the neuron providing the main excitatory drive within fetal brain development.

The levels of taurine precursors such as Met, Cys and Ser in the brain were comparable with those in the muscle and were not high compared with taurine. However, brain Ser concentrations of embryos in the present study were high compared with those of young chicks reported by Tomonaga et al. (2005).

Ser is recognized as an important amino acid for the development of the nervous system, and inhibiting serine synthesis can result in mental disabilities (Koning et al. 2004). Therefore, Ser is an important amino acid for development and the activity of Ser synthase may be high during embryogenesis.

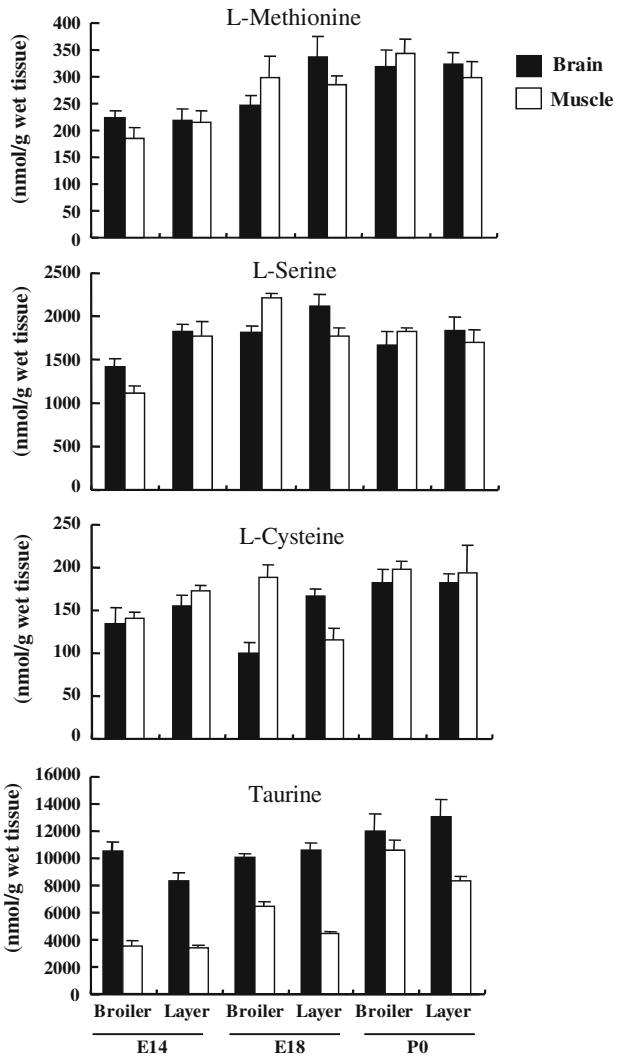

Fig. 2 Changes in taurine and its related amino acids in the brain between two types of chicken embryos in comparison with those in the skeletal muscle. The values are means ± SEM

Brain taurine levels at embryonic stages in the present study were also high compared with those of young (7 days old) chicks reported by Tomonaga et al. (2005). Turner et al. (1994) reported that in rats, brain taurine level was high during fetal and neonatal stages and significantly higher when compared with adult levels. Taurine mostly existed only in the yolk, but not in the albumen. Taurine may be absorbed well in the embryo, since mRNA of taurine transporter during embryonic stages was highly expressed (Kim et al. 2006). The synthesis of taurine from Met or Cys has been reported in the liver, brain, lung and muscle tissues (Ensunsa et al. 1993; Sharma et al. 1995). The rate-limiting enzyme for taurine biosynthesis is cysteine-sulfinate decarboxylase (CSD). In the chicken embryo,

CSD expression was detected as early as E2 and CSD mRNA appeared at 12 h after incubation (Kim et al. 2006). Pasantes-Morales et al. (1976) reported that CSD activity in the retina increased threefold from E10 to P0 and during postnatal development in rats. The high activity of CSD at embryonic stages resulted in high brain taurine levels in the present study. These facts suggest that brain taurine has an important role for brain development in animals.

Skeletal muscle concentration of taurine increased with embryonic stages in both types of chickens, although this increase was delayed in layers. Skeletal muscle taurine was also high compared with other free amino acids from E18, and was higher in embryonic stages than in 7-day-old chicks (Tomonaga et al. 2005). Taurine has many functions such as conjugation of bile acids in the liver (Vessey 1978), maintenance of osmolarity (Huxtable 1992), modulation of calcium levels (Timbrell et al. 1995), stabilization of membranes (Huxtable and Lippincott 1983), antiarrhythmic activity in the heart (Read and Welty 1963), regulation of ion fluxes (Huxtable 1992) and stimulation of sperm motility (Mrsny et al. 1979). In addition, β-alanine is a transport antagonist of taurine. Intraperitoneal administration of β-alanine to dams through the lactational period induced a slower growth rate of pups (Hu et al. 2000). Therefore, taurine may help in the rapid growth of broiler embryos (Muramatsu et al. 1990a; Ohta et al. 2004; Sato et al. 2006).

In conclusion, the patterns of free neuronal amino acids levels during brain development differed among amino acids, but the difference between broilers and layers was small. In the muscle, taurine concentration was higher in broiler than in layer embryos. The level of taurine was particularly high, suggesting an important role for taurine in brain development.

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