Amino Acids (2008) 34: 55–60
DOI 10.1007/s00726-007-0596-y
Printed in The Netherlands

Amino Acids

Relationships between the sedative and hypnotic effects of intracerebroventricular administration of L-serine and its metabolites, pyruvate and the derivative amino acids contents in the neonatal chicks under acute stressful conditions

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Received April 10, 2007
Accepted July 26, 2007
Published online October 2, 2007;

Summary. Intracerebroventricular (i.c.v.) injection of L-serine was shown to have sedative and hypnotic effects on neonatal chicks under acute stressful conditions. To clarify the central mechanism of these effects of L-serine, two experiments were done. First, we focused on the glycogenic pathway in which L-serine is converted into pyruvate and finally glucose. I.c.v. administration of pyruvate (0.84 μmol) did not induce any behavioral and endocrinological changes, while L-serine and glucose triggered sedative and hypnotic effects. Secondly, the relationship between the sedation by L-serine and the metabolism into other amino acids which have sedative effects was investigated in the telencephalon and diencephalon. In both brain areas, a dose-dependent increase was seen in L-serine, although other amino acids were not changed. In the present study, it was concluded that the sedative action of L-serine was not due to the action of its metabolite pyruvate, or to the action of other amino acids.

Keywords: L-serine – Pyruvate – Intracerebroventricular injection – Social separation stress – Amino acids contents

Introduction

One of the dispensable amino acids L-serine is biosynthesized as an intermediate of the glycolytic pathway, and is at equilibrium with glycine. L-Serine also contributes to the formation of L-cysteine and lipids such as phosphatidylserine and ceramide. In addition, L-serine has been implicated as a novel neurotrophic factor (Mitoma et al., 1998; Furuya et al., 2000). A congenital disease due to an inborn error of L-serine biosynthetic was discovered (Jaken et al., 1996; De Koning et al., 2002, 2004). Patients with this serine-deficiency syndrome suffer from serious neurological symptoms such as congenital microcephaly, seizures and severe psychomotor retardation, and serine and glycine levels in their plasma and cerebrospinal fluid are very low. These facts indicate the importance of L-serine in the central nervous system.

We deomonstrated the sedative and hypnotic effects of intracerebroventricular (i.c.v.) injection of L-serine and its derivatives including glycine and L-cysteine using an acute stressful model of the neonatal chicks (Asechi et al., 2006). This behavioral model is based on the response of neonatal chicks to acute stress (Panksepp et al., 1980; Sahley et al., 1981; Feltenstein et al., 2003). In brief, chicks exhibit less stress-related behavior when they are in crowds. However, when chicks are isolated, this induces acute stress. This social separation stress increases spontaneous activity and vocalization of chicks. Additionally, this model has a high utility in that chicks are relatively inexpensive to purchase and maintain. For these reasons, this social separation-stress paradigm has been used as a model to screen anxiolytics by monitoring there ability to alter spontaneous activity and vocalizations as the index of stress.

To investigate the central mechanism of the sedative and hypnotic effects of L-serine, two experiments were done based on its metabolic pathways. Besides the roles as a precursor of other amino acids and membrane lipids, L-serine is a glycogenic amino acid. Thus, L-serine is metabolized to pyruvate, and finally glucose. There are

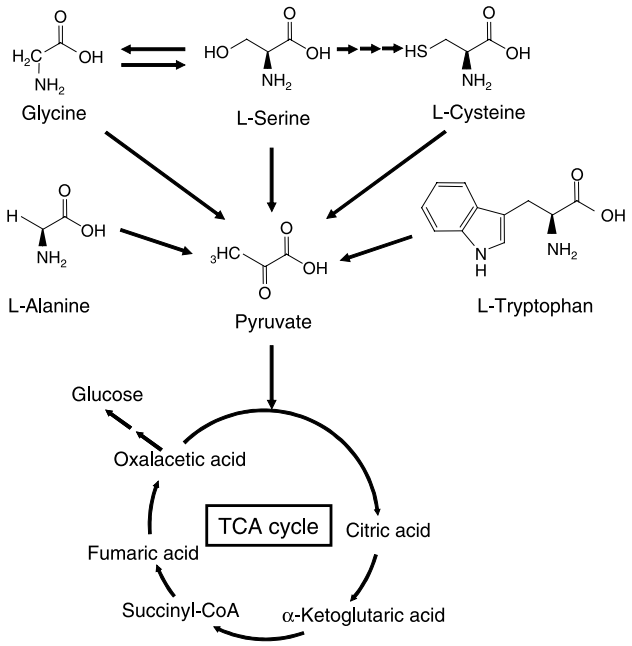

Fig. 1. Metabolic pathway of the glycogenic amino acids that were previously reported to have sedative effects in neonatal chicks under acute stressful conditions

other glycogenic amino acids that are primarily converted into pyruvate, e.g., glycine, L-cysteine, L-alanine and L-tryptophan (Berg et al., 2002). Among these four amino acids, sedative effects have been reported (Asechi et al., 2006, Kurauchi et al., 2006, 2007) (Fig. 1). Therefore, it was hypothesized that the conversion of these five glycogenic amino acids into pyruvate might be a trigger for sedative effects. Studies using adiposis rats revealed that pyruvate inhibited lipid synthesis and weight gain (Stanko and Adibi, 1986), and pyruvate-containing supplementations acted as diet food (Cupp and Tracy, 2003). However, the central function of pyruvate has not been clarified.

On the other hand, there is no report for the metabolism of L-serine into the other amino acids in the brain of the neonatal chicks. It is possible that the sedative effects of L-serine might be related to the production of the five amino acids mentioned above.

The purposes of the present study were 1) to confirm the effect of i.c.v. injection of pyruvate and glucose using the social-separation model of neonatal chicks (Experiment 1), and 2) to research the effect of the dose-dependent i.c.v. administration of L-serine on the amino acids content in the telencephalon and diencephalon 10 min post-injection (Experiment 2). The telencephalon regulates higher brain functions such as memory, language and emotion, while the diencephalon is involved in consciousness, emotion, feeding and drinking in humans. Among amino acids, the focus was on changes in the content of the metabolites of L-serine (glycine and L-cysteine), major inhibitory neurotransmitters (glycine and γ-aminobutyric acid (GABA)), and amino acids having sedative effects (L-alanine; Kurauchi et al., 2006, DL-tryptophan; Kurauchi et al., 2007).

Materials and methods

Animals and food

One-day-old male layer type chicks (Julia) were purchased from a local hatchery (Murata Hatchery, Fukuoka, Japan) and housed in a windowless room at a constant temperature of $30 \pm 1\,^{\circ}C$. Continuous lighting was provided. The birds were given free access to a commercial starter diet (AX, Toyohashi Feed and Mills Co Ltd., Aichi, Japan) and water. In Experiment 1, chicks were reared in a group (20–25/cage) till the start of the experiment. In Experiment 2, chicks were reared individually the day prior to the experiment for acclimatization. On the day of the experiment, chicks (5- or 6-day-old) were assigned to treatment groups based on their body weight in order to produce uniform treatment groups. Experimental procedures followed the guidance for Animal Experiments in Faculty of Agriculture and in the Graduate Course of Kyushu University and the Law (No. 105) and Notification (No. 6) of the Government.

Preparation of drugs

L-Serine and sodium pyruvate were purchased from Wako Pure Chemical Industries, Ltd., (Osaka, Japan) and D-glucose from Sigma (St. Louis, MO). These reagents were dissolved in 0.85% saline containing 0.1% Evans Blue solution and vortexed.

Intracerebroventricular (i.c.v.) injection and behavioral observation

I.c.v. injections were made using a microsyringe according to the method of Davis et al. (1979) and Koutoku et al. (2005). The stress and pain suffered by this method is minimal as described elsewhere (Koutoku et al., 2005). The control group was administrated 0.85% saline containing 0.1% Evans Blue solution. The injected volume was 10 µl.

In Experiment 1, chicks were given i.c.v. injections of 0.84 µmol of L-serine, sodium pyruvate or D-glucose. After the injection, chicks were immediately placed in an acrylic monitoring cage ($40\,cm \times 30\,cm \times 20\,cm$), and behavioral observations were made for 10 min. During this period, chicks were deprived of water and diet. Spontaneous activity was automatically determined by utilizing infrared beam sensors (Neuroscience Inc., Tokyo, Japan) placed above the center of the monitoring cage and analyzed by the software DAS-008 (Neuroscience Inc.). Chick vocalizations were simultaneously recorded using a computer with the software Windows Media Player (Microsoft Corporation, USA) and the number of distress vocalizations was counted using Gretchen software (Excla Inc., Japan). Video cameras were positioned to record the behavior of chicks from three different directions on digital versatile discs. Based on the method by Van Luijtelaar et al. (1987), by watching the digital versatile disc, chick behaviors were classified into four categories: (1) active wakefulness; (2) standing/sitting motionless with eyes opened; (3) standing motionless with eyes closed; and (4) sitting motionless with head drooped (sleeping posture). The monitoring systems were set in a separate room to avoid disturbing the animals. Blood was collected from the jugular vein into heparinized syringes at the conclusion of the behavioral tests.

The blood was centrifuged at $4\,^{\circ}C$ and $8000 \times g$ for 4 min, and the plasma was collected and stored at $-30\,^{\circ}C$ until analysis. Plasma corticosterone was determined using a corticosterone enzyme immunoassay kit (Assay Designs Inc., U.S.A.).

Finally, the birds were decapitated after an overdose of sodium pentobarbital. The brains were removed and the location of the Evans Blue dye was confirmed. Data of chicks without dye in the lateral ventricle were deleted.

In Experiment 2, chicks were given i.c.v. injections of 0, 0.21, 0.42 and 0.84 µmol of L-serine and returned to individual cages. Ten minutes after the injection, chicks were decapitated and the location of the Evans Blue dye was confirmed. The chicks without dye in the lateral ventricle were deleted. Both sides of the telencephalon and diencephalon were quickly divided and weighed. These samples were dropped into liquid nitrogen for flash freezing and stored at −80 °C in the deep freezer until amino acids analysis.

Amino acid analysis

The brain tissues were homogenized in phosphate-buffered saline (137 mM NaCl, 8.10 mM Na_2HPO_4, 2.68 mM KCl, and 1.47 mM KH_2PO_4, pH 7.4). The homogenate was centrifuged at 10,000 × *g* for 20 min at 4 °C. Then the supernatant was centrifuged with a centrifuge-filtration unit (Ultracel, YM-10, Millipore, Bedford, MA, USA) at 14,000 × *g* for 60 min at 4 °C. The 20 µl filtrate was dried under reduced pressure. The dried samples were neutralized with the mixed solution containing 40% of 1 M sodium acetate (Wako, Osaka, Japan), 40% of methanol (Sigma Aldrich Japan, Osaka, Japan) and 20% of triethylamine (Piece, Rockford, IL, USA), and then dried under reduced pressure. After drying, the derivatization was done with the reaction solution that consisted of 70% of methanol, 10% of triethylamine, 10% of phenyl isothiocyanate (Sigma, St. Louis, MO, USA) and 10% of ultrapure water. After a 20 min reaction time, the liquid-containing substances were dried under reduced pressure. Prior to analysis, the dried samples were dissolved in Pico-tag sample diluent (Waters, Milford, MA, USA) and centrifuged with a centrifuge-filtration unit (Ultra Free C3-GV, Millipore, Bedford, MA, USA) at 10,000 × *g* for 2 min at 4 °C to remove the solid contents. The 20 µl of the filtrate was applied to an HPLC system (Pico-Tag™, Waters, Milford, MA, USA) for the measurement of amino acid contents. The standard solution was prepared by diluting a commercially available L-amino acid solution (Type AN II and Type B; Wako, Osaka, Japan). The detection limit of this system for all amino acids was 5 pg/sample. Date processing was done using Millennium[32] Chromatography Manager software from Waters.

Statistical analysis

In Experiment 1, data were statistically analyzed by one-way analysis of variance (ANOVA) and a Tukey-Kramer test was done as a post hoc test. Regression analysis for dose of L-serine and amounts of each amino acid was done in Experiment 2. Significant differences implied $P < 0.05$. Values are presented as mean ± S.E.M. Statistical analysis was made using commercially available package, Stat View (Version 5, SAS Institute, Cary, U.S.A., 1998).

Results

The upper panel in Fig. 2 shows the effect of i.c.v. administration of saline, L-serine, pyruvate and glucose on total spontaneous activity during the 10 min isolation. The effect of the drugs on spontaneous activity was significant ($F(3,25) = 7.629$, $P < 0.001$). L-Serine and glucose significantly decreased spontaneous activity compared to the control group while the value for pyruvate was almost identical to the control.

The lower panel in Fig. 2 shows the effect of i.c.v. administration of saline, L-serine, pyruvate and glucose on total number of distress vocalizations during 10 min

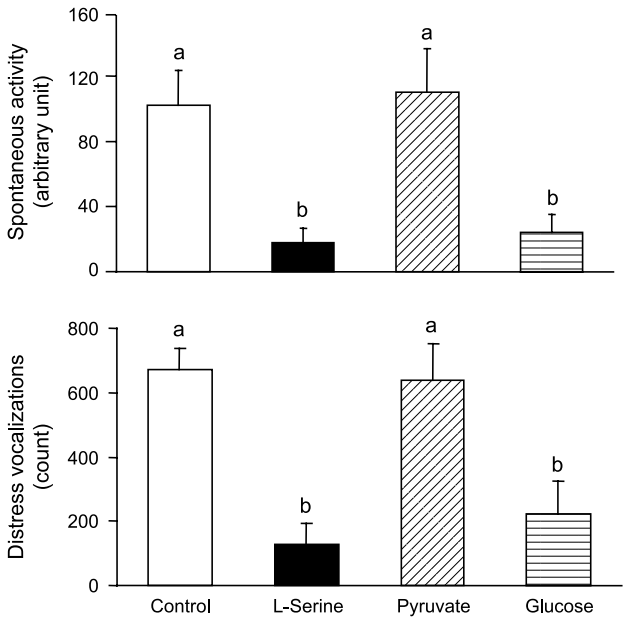

Fig. 2. Effect of i.c.v. injection of saline, L-serine, pyruvate and glucose on total spontaneous activity (upper panel) and distress vocalizations (lower panel) during 10 min isolation in 6-day-old layer chicks. Results are expressed as means ± S.E.M. The number of chicks used in each group was as follows: control, 7; L-serine, 8; pyruvate, 7; glucose, 7. Groups with different letters are significantly different ($P < 0.05$)

Table 1. Effect of i.c.v. injection of saline, L-serine, pyruvate and glucose on various behavioral categories of 6-day-old chicks after 10 min post injection

	Control	L-Serine	Pyruvate	Glucose
Active wakefulness	537 ± 30^{a}	243 ± 86^{b}	$490 \pm 82^{a,b}$	$257 \pm 77^{a,b}$
Standing/sitting motionless with eyes open	42 ± 18^{a}	216 ± 61^{b}	38 ± 11^{a}	$169 \pm 40^{a,b}$
Standing motionless with eyes closed	20 ± 20	2 ± 2	0 ± 0	12 ± 12
Sitting motionless with head drooped (sleeping posture)	0 ± 0	139 ± 65	71 ± 71	161 ± 80
Total	600	600	600	600

Values are means ± S.E.M. in seconds. The number of chicks used in each group was as follows: control, 7; L-serine, 8; pyruvate, 7 and glucose, 7. Groups with different letters are significantly different ($P < 0.05$)

isolation. A significant ($F(3, 25) = 10.072$, $P < 0.001$) effect of the drugs on distress vocalizations was detected, with the results being similar to those obtained in spontaneous activity.

Table 1 shows the effect on various behavioral categories of chicks 10 min post injection. Significant drug effects were detected in time spent for active wakefulness ($F(3,25) = 4.358$, $P < 0.05$) and standing/sitting motionless with eyes open ($F(3,25) = 5.057$, $P < 0.01$). The L-serine treated group spent significantly less time in active wakefulness compared to the control. The time for standing/sitting motionless with eyes open was significantly increased in the L-serine- and glucose-treated groups, and that for sitting motionless with head drooped (sleeping posture) tended to increase in these two groups.

Figure 3 shows the effect of i.c.v. administration of saline, L-serine, pyruvate and glucose on plasma corticosterone concentration immediately after 10 min of isolation. In the intact (not stressful, e.g., rearing in flock) group, plasma corticosterone concentration was 1.83 ± 0.43 ng/ml. L-Serine and glucose significantly lowered ($F(3,25) = 7.76$, $P < 0.001$) the corticosterone level compared to the control group, and these values were as low as that of the intact group. Pyruvate had no effect on plasma corticosterone level.

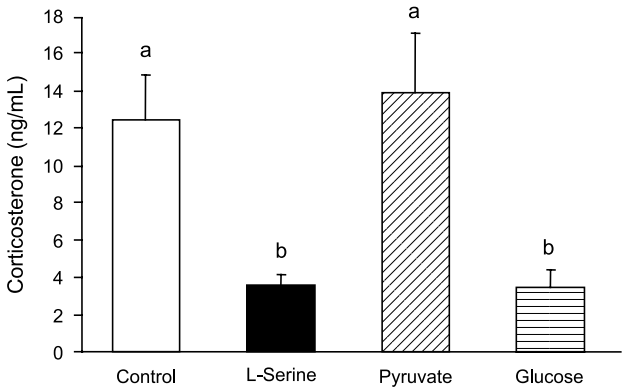

Fig. 3. Effect of i.c.v. injection of saline, L-serine, pyruvate and glucose on plasma corticosterone concentration immediately after a 10 min isolation in 6-day-old layer chicks. Results are expressed as means ± S.E.M. The number of chicks used in each group was as follows: control, 7; L-serine, 8; pyruvate, 7; glucose, 7. Groups with different letters are significantly different ($P < 0.05$)

Tables 2 and 3 show the effects of i.c.v. administration of 0, 0.21, 0.42, and 0.84 µmol of L-serine on the amino acid content in the telencephalon and diencephalon

Table 2. Influence of i.c.v. injection of L-serine on amino acids contents in the telencephalon in 5-day-old chicks

		L-Serine (µmol)			
	Intact	0	0.21	0.42	0.84
L-Serine	695 ± 54	792 ± 145	689 ± 25	845 ± 36	1121 ± 114
Glycine	1631 ± 100	1572 ± 161	1520 ± 76	1609 ± 104	1892 ± 160
γ-Aminobutyric acid	2421 ± 140	3056 ± 593	2227 ± 114	2295 ± 104	2718 ± 137
L-Alanine	333 ± 33	331 ± 28	317 ± 27	326 ± 18	383 ± 34
L-Tryptophan	26 ± 3	25 ± 1	19 ± 2	22 ± 1	26 ± 3

Values are means ± S.E.M. in nmol/g wet tissue. The number of chicks used in each group was as follows: intact, 7; L-serine 0 µmol, 6; L-serine 0.21 µmol, 6; L-serine 0.42 µmol, 6 and L-serine 0.84 µmol, 7. L-Serine (nmol/g wet tissue) = 703 (SE 78.3) + 447 (SE 159.5)X ($R^2 = 0.247$, $P < 0.01$)

Table 3. Influence of i.c.v. injection of L-serine on amino acids contents in the diencephalon in 5-day-old chicks

		L-Serine (µmol)			
	Intact	0	0.21	0.42	0.84
L-Serine	433 ± 36	477 ± 56	474 ± 59	522 ± 56	670 ± 94
Glycine	1091 ± 122	1211 ± 165	1027 ± 116	1150 ± 122	1155 ± 84
γ-Aminobutyric acid	1185 ± 112	1267 ± 89	1210 ± 84	1326 ± 139	1228 ± 46
L-Alanine	327 ± 36	394 ± 45	370 ± 42	397 ± 42	331 ± 39
L-Tryptophan	13 ± 3	14 ± 3	14 ± 2	14 ± 4	9 ± 1

Values are means ± S.E.M. in nmol/g wet tissue. The number of chicks used in each group was as follows: intact, 7; L-serine 0 µmol, 6; L-serine 0.21 µmol, 6; L-serine 0.42 µmol, 6 and L-serine 0.84 µmol, 7. L-Serine (nmol/g wet tissue) = 448 (SE 51.8) + 244 (SE 106)X ($R^2 = 0.182$, $P < 0.05$)

10 min post injection, respectively. A significant positive correlation between the dose and content of L-serine was detected in the telencephalon ($P<0.01$) and diencephalon ($P<0.05$), while other amino acids contents did not changed significantly. The content of L-cysteine could not be detected correctly because of the overlap of its peak with L-cystine, a dimmer of L-cysteine.

Discussion

In the present study, pyruvate appeared to have little involvement with the sedative effect of glucogenic amino acids including L-serine, glycine, L-alanine and L-tryptophan. On the other hand, sedation and induction of sleep-like behavior were seen in the chicks given D-glucose, and its potency was as strong as that of L-serine. Further, i.c.v. administration of L-serine did not influence the concentration of these four amino acids or the inhibitory neurotransmitter GABA in the telencephalon or hypothalamus 10 min after the administration.

Pyruvate metabolism in the rat brain using ^{13}C-labelled pyruvate and glucose has investigated (Hassel, 2001; Gonzalez et al., 2005). Intravenous (i.v.) injection of 2.25–18 mmol/kg of [3-^{13}C] pyruvate and 1.125, 2.25, 4.5, and 9 mmol/kg of [1-^{13}C] glucose lead to dose-dependent labeling of brain alanine, and GABA (Gonzalez et al., 2005). The i.c.v. dose of pyruvate in the present study, 0.84 μmol/10 μl, could be converted to 0.012 mmol/kg body weight based on the fact that average body weight of chick was 70 g. Although it is difficult to compare categorically because of difference in administration route, the i.c.v. dose used in the present study might be too low to trigger neurotransmission and behavioral changes in chicks.

The i.c.v. injection of glucose attenuated stress-related behavior of chicks, and appeared to induce a hypnotic effect and inhibit plasma corticosterone release. These effects seem to be almost identical to L-serine. I.v. administration of [1-^{13}C] glucose increased related amino acids labeled dose-dependently, and this reaction was very similar to that of [3-^{13}C] pyruvate (Gonzalez et al., 2005). Since sedative effects were observed following the i.c.v. administration of glucose, but not pyruvate, it is possible that an increase in brain ketone bodies resulting from hyperglycemia might trigger the sedative effect. However, this seems unlikely since the synthesis of ketone bodies from glucose involves exchange to pyruvate. In addition, the place for ketone body production is limited at the liver. The immediate sedative effect of glucose after the i.c.v. injection further argues against a role for ketone bodies since the amount of glucose administered was too small to produce ketone bodies in the liver. Furthermore, if a large amount of glucose was administered to the brain, it would take a relatively long time for it to reach the liver and come back to the brain through the blood stream.

Taken together, it was suggested that the metabolic pathway from L-serine to glucose via pyruvate has less involvement in the sedative and hypnotic effect of L-serine, since the intermediate product pyruvate has no influence on the stress-related behaviors and plasma corticosterone level. Further, the sedative effects of glucose seem independent of that of L-serine. This can be explained by the fact that pyruvate itself is the precursor of glucose as well as L-serine.

Regarding plasma corticosterone levels, the i.c.v. injection of L-serine showed a significant inhibitory effect. This is in contrast to the results of our previous report that demonstrated a poor ability for L-serine to attenuate the release of corticosterone from adrenal glands through the hypothalamic-pituitary-adrenal (HPA) axis (Asechi et al., 2006). Although it was confirmed that social separation stress increases plasma corticosterone concentration, the intensity sometimes fluctuant with each experiment. Considering that plasma corticosterone concentrations in L-serine-treated groups tended to be lower than that in the control groups in our previous work, it seems possible that the i.c.v. injection of L-serine attenuates release of corticosterone from the adrenal glands.

L-Serine acts as a precursor of protein, nucleotides, membrane lipids, and other amino acids such as glycine, D-serine, and L-cysteine. Recent research has shown that glycine, known as the main inhibitory neurotransmitter and the allosteric modulator for the N-methyl-D-aspartate (NMDA) receptor, improved sleep quantity (Inagawa et al., 2006). However, the present study indicates less involvement of glycine in the sedative and hypnotic effects of L-serine, since there is no significant change in the content of glycine after i.c.v. injection of L-serine. Although the measurement of L-cysteine and D-serine was impossible in the present experiment, its synthesis L-cysteine 10 min after the injection of L-serine might be slight because the metabolism of L-serine to L-cysteine is not direct. Additionally, it has already been demonstrated that administration of 0.84 μmol of D-serine did not affect chick behavior (Asechi et al., 2006).

The present study suggested that i.c.v. administration of L-serine did not influence the content of other amino acids in the telencephalon and diencephalon of chicks. However, the change of release of these amino acids into the extracellular was not determined. Since the sedative and hypnotic effects of 0.84 μmol L-serine appeared im-

mediately after the i.c.v. injection, some kinds of receptor-related response might be involved in the inhibitory effect by L-serine. Further, a unique reaction for L-serine under stressful conditions may exist in the CNS. For example, phosphatidylserine (PS) had a similar effect as observed for L-serine (Koutoku et al., 2005), and L-serine is a constituent of PS. L-serine may function via incorporation into PS.

In conclusion, i.c.v. injection of pyruvate did not affect behavior or the plasma level of corticosterone in neonatal chicks under acute stressful conditions. Therefore, it is suggested that sedation and hypnosis triggered by L-serine were not due to its metabolite, i.e., pyruvate. Furthermore, i.c.v. administration of L-serine increased the concentration of L-serine itself in a dose-dependent manner in the telencephalon and diencephalon 10 min post injection. Other amino acids concentrations, including glycine, GABA, L-alanine, and L-trypthophan were not significantly changed. These results indicate that the sedative and hypnotic effects might be due to the action by L-serine itself, and not due to pyruvate. However, it is still unclear how the i.c.v. administrated L-serine reduces the stress-related responses and induces the sleeping-like behavior in chicks. Further study is needed to understand the relationships between L-serine and the neurotransmission systems.

Acknowledgements

This work was supported by a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science. The authors are grateful to the staffs and students of Laboratory of Chemistry and Technology of Animal Products in Kyushu University for their assistance in analysis of amino acids.

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