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Central L-arginine reduced stress responses are mediated by L-ornithine in neonatal chicks

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Summary. Recently, we observed that central administration of L-arginine attenuated stress responses in neonatal chicks, but the contribution of nitric oxide (NO) to this response was minimal. The sedative and hypnotic effects of L-arginine may be due to L-arginine itself and/or its metabolites, excluding NO. To clarify the mechanism, the effect of intracerebroventricular (i.c.v.) injection of L-arginine metabolites on behavior under social separation stress was investigated. The i.c.v. injection of agmatine, a guanidino metabolite of L-arginine, had no effect during a 10 min behavioral test. In contrast, the i.c.v. injection of L-ornithine clearly attenuated the stress response in a dose-dependent manner, and induced sleep-like behavior. The L-ornithine concentration in the telencephalon and diencephalon increased following the i.c.v. injection of L-arginine. In addition, several free amino acids including L-alanine, glycine, L-proline and L-glutamic acid concentrations increased in the telencephalon. In conclusion, it appears that L-ornithine, produced by arginase from L-arginine in the brain, plays an important role in the sedative and hypnotic effects of L-arginine observed during a stress response. In addition, several other amino acids having a sedative effect might partly participate in the sedative and hypnotic effects of L-arginine.

Keywords: L-Ornithine – L-Arginine – Agmatine – Intracerebroventricular injection – Social separation stress – Neonatal chick

Introduction

L-Arginine exerts its metabolic roles through the production of diverse metabolites, including nitric oxide (NO), L-ornithine, polyamines, L-proline, L-glutamate, L-glutamine, creatine and agmatine (Morris, 2004). Recently, we observed that intracerebroventricular (i.c.v.) injection of L-arginine induced sedative or hypnotic effects in chicks exposed to a social isoloation stress (Suenaga et al., 2008). Although NO is a major physiological mediator of arginine-induced responses, it appears to play a minimal role in this response. Consequently, the sedative and hypnotic effects of L-arginine may be due to L-arginine itself and/or other arginine metabolites.

L-Arginine is metabolized by arginase to yield L-ornithine. The availability of L-ornithine may regulate the synthesis of polyamines, which are essential for cell proliferation and differentiation processes. The metabolic roles of L-ornithine are likely cell and tissue specific.

Agmatine is a ligand for α_2-adrenergic and imidazoline receptors in the rat brain, thus serving as a signaling molecule (Reis and Regunathan, 2000). Agmatine has a guanidino component as a common structure with L-arginine and creatine. Koga et al. (2005) reported that creatine in the central nervous system (CNS) has sedative and hypnotic effects. Agmatine and putrescine have antidepressant effects (Li et al., 2003; Zomkowski et al., 2006).

L-Arginine metabolites may have important roles in nutrition and physiology. To clarify the mechanism by which L-arginine induces sedative and hypnotic effects, we investigated the effects of (1) agmatine and (2) L-ornithine on sedative and hypnotic effects under social isolation stress. Furthermore, we determined whether the i.c.v. injection of L-arginine influenced the free amino acid concentrations in several brain sites.

In the present study, we used the social separation stress model. This stress model is frequently used for the study of anxiety. Chicks feel comfortable when living in a group, but develop anxiety when isolated. Social separation stress increases spontaneous activity and vocalization

of chicks (Feltenstein et al., 2003). Therefore, this social separation stress paradigm has been used for developing antianxiety agents using vocalization and spontaneous activity as indicators of their effectiveness. Additionally, this model is beneficial since chicks are inexpensive to purchase and maintain, and require small quantities of drugs in the screening process (Watson et al., 1999).

Materials and methods

Animals and food

Day-old male layer chicks (Julia; Murata Hatchery, Fukuoka, Japan) were housed in a wire-meshed cage ($50 \times 35 \times 33$ cm) in a group (20–25 birds) at a constant temperature of $30 \pm 1\,^{\circ}C$ and continuous light until the experimental day. Chicks were the same age and housed without an adult. Diet (AX, Toyohashi Feed and Mills Co. Ltd., Aichi, Japan) and water were available ad libitum. On the day of the experiment, chicks (5–6 days 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

Agmatine sulfate and L-ornithine monohydrochloride were purchased from Sigma (St. Louis, MO, U.S.A.). L-Arginine monohydrochloride was a gift from Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan. Drugs were dissolved in 0.85% saline containing a 0.1% Evans Blue solution.

Experimental procedure

Drugs were injected i.c.v. into the left lateral ventricle of the chicks in a volume of 10 µl using a microsyringe according to the method of Davis et al. (1979). The stress and pain suffered by this method is minimal as described elsewhere (Koutoku et al., 2005). After injection, in Experiments 1 and 2, chicks were returned to a cage for 5 min to allow for sufficient drug diffusion after which they were gently placed alone into acrylic glass chambers ($40 \times 30 \times 20$ cm) with paper on the floor for 10 min in a separate room at a constant temperature of 30 °C. In Experiment 3, chicks were placed in the chamber immediately after i.c.v. injection. They were deprived of water and diet, and spontaneous activity and vocalizations were recorded. Spontaneous activity was automatically determined utilizing infrared beam sensors (NS-AS01; Neuroscience Inc., Tokyo, Japan) placed about 20 cm above the center of the floor of the monitoring cage and analyzed by the software DAS-008 (Neuroscience Inc., Tokyo, Japan). The number of vocalizations were simultaneously recorded and counted using a computer with Gretchen software (Excla Inc., Saitama, Japan). Chick behaviors were recorded by three video cameras positioned at different directions. According to the method of van Luijtelaar et al. (1987), the recorded chick behaviors were classified into four categories: (1) active wakefulness; (2) standing/sitting motionless with eyes open; (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.

In Experiment 1, the effect of i.c.v. injection of agmatine on behaviors of chicks under social separation stress was investigated. Birds (6 days old) were injected i.c.v. with 0.125, 0.25, or 0.5 µmol of agmatine.

The effect of i.c.v. injection of L-ornithine on behaviors of chicks under social separation stress was investigated in Experiments 2 (5 min after i.c.v. injection) and 3 (immediately after i.c.v. injection). Birds (4–6 days old) were injected i.c.v. with 0.125, 0.25, or 0.5 µmol of L-ornithine. Saline was used as a control in all experiment.

Finally, the birds were decapitated following 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 4, the effect of i.c.v. injection of L-arginine on the levels of amino acids in the brain was determined. Birds (4 days old) were injected i.c.v. with either saline as a control, L-arginine (1.9 and 3.8 µmol). After 10 min i.c.v. injection, 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. Chicks were deprived of water and diet to coordinate with behavior experiments.

Sample preparation

The brains were carefully removed and placed on a cold glass dish in Experiment 4. According to the atlas of the chicken brain (Kuenzel and Masson, 1988), two parts of the brain, the telencephalon and diencephalon, were dissected and weighed, respectively. The brain tissue samples were placed in new plastic tubes. Quadruple volumes of 10 mM phosphate buffered saline (PBS) (pH 7.4) were added (Han et al., 2002), and then the brain tissue samples were homogenized. All samples were centrifuged at $10{,}000 \times g$ for 20 min at 4 °C. The supernatant was subjected to ultrafiltration. Interference due to hemoglobin as well as other proteins was prevented by filtering the supernatant through a 10,000 MW cut-off microcentrifuge filters (Microcon YM-10, Amicon Bioseparations, Millipore Co., Bedford, MA, U.S.A.) by centrifugation at $14.000 \times g$ for 60 min at 4 °C (Al-Rejaie and Dar, 2006). A colorless filtrate was obtained after filtration. An equal quantity of PBS was added into filtrates. Samples were stored at 4 °C prior to assay.

Amino acid analysis

Free amino acids in the telencephalon and diencephalon were determined using high-performance liquid chromatography (HPLC) (Waters, Milford, MA, U.S.A.) using the Pico Tag method (Rubio, 2003). Twenty µl of samples and standard solutions were dried under vacuum in a centrifugal vaporizer (CVE-200D, Tokyo Rikakikai Co. Ltd., Tokyo, Japan) at 25 °C. Nitrogen gas was applied under vacuum to prevent oxidation during sample drying. After dried, samples and standards were re-dissolved in 10 µl of a 1 M sodium acetate:methanol:triethylamine (2:2:1) solution, vortexed, and re-dried. Samples and standards were added in 20 µl of a methanol:-water:triethylamine:phenylisothyocyanate (7:1:1:1) derivatizing solution, vortexed, allowed to react for 20 min at room temperature, and vacuum-dried. Dried samples and standards were stored at −80 °C prior to analysis. Samples and standards were re-dissolved in 100 µl of Pico Tag sample diluent for free amino acid analysis, vortexed, and the solution cleaned through a membrane filter (Ultrafree-MC, pore size 0.22 µm, Amicon Bioseparations, Millipore Co., Bedford, MA, U.S.A.) by centrifugation at $10{,}000 \times g$ for 2 min at 4 °C. Then, 10 µl of solution were injected for HPLC analysis. Analysis was done using a Waters HPLC system. The apparatus consisted of a temperature control module (maintained at 46 °C), a separations module (model 2690), a dual λ absorbance detector (model 2487, controlled at 254 nm filter) and an interface module. Analytical method development, data collection and data integration were performed using Millennium^{32} Chromatography Manager software from Waters run on a personal computer. The column used was a 3.9×300 mm Pico Tag column (WAT 010950).

Statistical analysis

Regression equations were fitted for data on spontaneous activity, vocalizations, and the times for various behavioral categories, respectively.

Further, data were statistically analyzed by one-way analysis of variance (ANOVA) and a Tukey–Kramer test was done as a post hoc test. Significant differences implied $P < 0.05$. Values are presented as means ± SEM. Statistical analysis was made using a commercially available package, StatView (Version 5, SAS Institute, Cary, U.S.A., 1998). All data were first subjected to Grubs-Smirnov rejection test to eliminate outliers. Then remaining data were used.

Results

Figure 1 shows the effect of i.c.v. injection of agmatine on spontaneous activity (upper panel) and the number of vocalizations (lower panel) in chicks during the 10 min social separation stress (Experiment 1). The effect of agmatine on spontaneous activity was significant ($F(3, 21) = 3.570$, $P < 0.05$) with a reverse U shape dose-response. The effect of agmatine on the number of vocalizations was not significant, but showed a similar yet weak pattern as that observed in spontaneous activity. Table 1

Table 1. Effect of of agmatine on various behavioral categories of 6-day-old chicks exposed to social separation stress for 10 min beginning 5 min after i.c.v. injection

Agmatine (μmol)	0	0.125	0.25	0.5
Active wakefulness	503 ± 41	491 ± 98	595 ± 5	548 ± 52
Standing/sitting motionless with eyes open	97 ± 41	109 ± 98	5 ± 5	52 ± 52
Standing motionless with eyes closed	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Sitting motionless with head drooped (sleeping posture)	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Total	600	600	600	600

Values are mean ± SEM in seconds
The number of chicks used in each group was as follows: 0 μmol, 6; 0.125 μmol, 6; 0.25 μmol, 7; and 0.5 μmol, 6

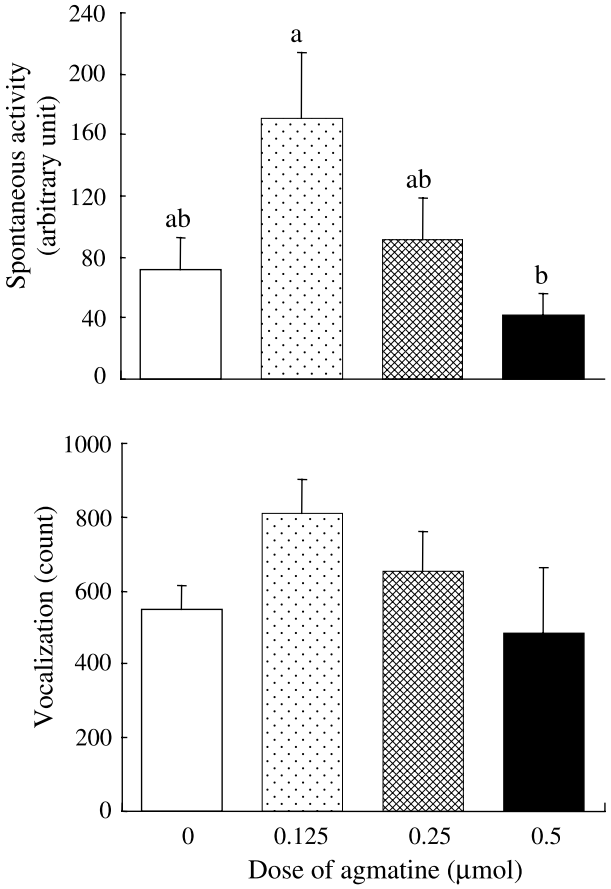

Fig. 1. Effect of i.c.v. injection of several doses of agmatine on spontaneous activity (*upper panel*) and vocalizations (*lower panel*) during a 10 min social separation stress in 6-day-old layer chicks. Values are means with SEM. Different letters indicate significant differences at $P < 0.05$. The number of chicks used in each group was as follows: 0 μmol, 6; 0.125 μmol, 6; 0.25 μmol, 7; and 0.5 μmol, 6

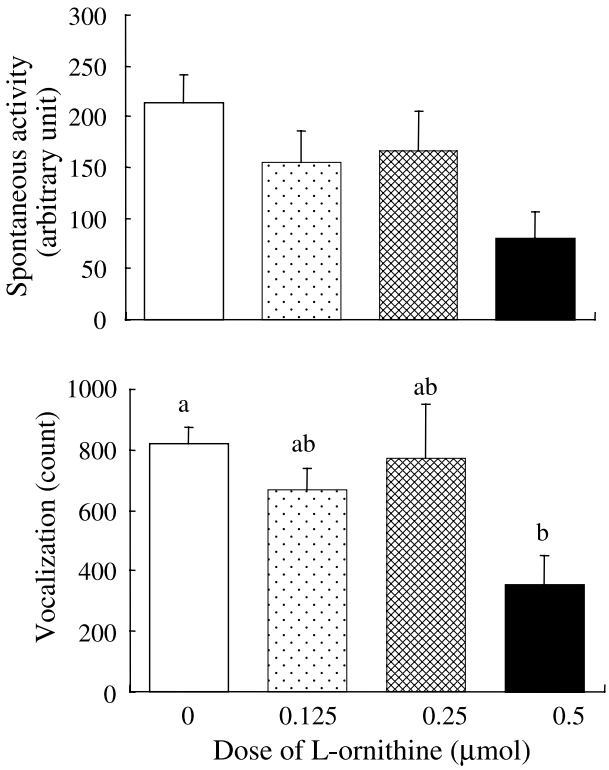

Fig. 2. Effect of i.c.v. injection of several doses of L-ornithine on spontaneous activity (*upper panel*) and vocalizations (*lower panel*) during a 10 min social separation stress beginning 5 min of i.c.v. injection in 4-day-old layer chicks. Values are means with SEM. Different letters indicate significant differences at $P < 0.05$. The number of chicks used in each group was as follows: 0 μmol, 5; 0.125 μmol, 7; 0.25 μmol, 6; and 0.5 μmol, 6. Spontaneous activity (arbitrary unit/10 min) = 20.445 (SE = 1.373) −23.995 (SE = 4.776) × ($R^2 = 0.096$, $P < 0.0001$). The number of vocalizations (count/10 min) = 841.309 (SE = 90.043) − 867.796 (SE = 313.145) × ($R^2 = 0.259$, $P < 0.05$)

shows the effect of i.c.v. injection of agmatine on various behavioral categories of chicks during a 10 min behavioral observation under social separation stress. No behavioral changes were induced by agmatine.

Figure 2 shows the effect of i.c.v. injection of L-ornithine on spontaneous activity (upper panel) and the number of vocalizations (lower panel) in chicks during the 10 min social separation stress beginning 5 min after i.c.v. injection. The i.c.v. injection of L-ornithine clearly attenuated spontaneous activity. Significant negative correlations between the dose of L-ornithine and spontaneous activity ($P<0.0001$) were detected. Significant negative correlations between the dose of L-ornithine and the number of vocalizations ($P<0.05$) were also detected. The effect of L-ornithine on the number of vocalizations was significant ($F(3, 20)=3.404$, $P<0.05$). The i.c.v. injection of L-ornithine attenuated the number of vocalizations. Table 2 shows the effect of L-ornithine on various behavioral categories of chicks during the 10 min behavioral observation under social separation stress beginning 5 min after i.c.v. injection. The time for active wakefulness was reduced with increasing L-ornithine. Significant negative correlations between the dose of L-ornithine and the time for active wakefulness ($P<0.01$) were detected.

Figure 3 shows the effect of i.c.v. injection of L-ornithine on spontaneous activity (upper panel) and the number of vocalizations (lower panel) in chicks during the 10 min under social separation stress just after i.c.v. injection. Significant negative correlations between the dose of L-ornithine and spontaneous activity ($P<0.05$) were detected. The effect of L-ornithine on spontaneous activity

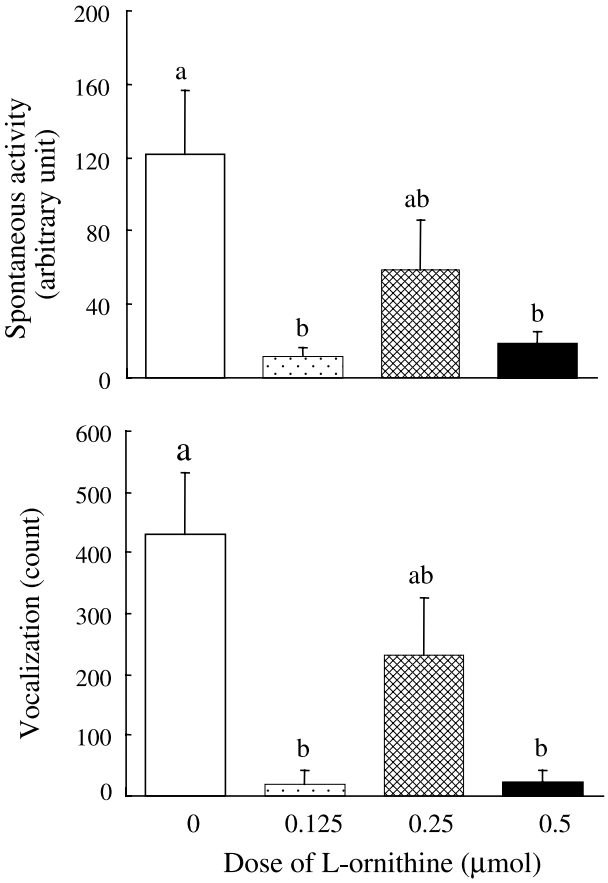

Fig. 3. Effect of i.c.v. injection of several doses of L-ornithine on spontaneous activity (*upper panel*) and vocalizations (*lower panel*) during a 10 min social separation stress just after i.c.v. injection in 6-day-old layer chicks. Values are means with SEM. Different letters indicate significant differences at $P<0.05$. The number of chicks used in each group was as follows: 0 μmol, 7; 0.125 μmol, 6; 0.25 μmol, 7; and 0.5 μmol, 6. Spontaneous activity (arbitrary unit/10 min) = 90.174 (SE = 21.292) −165.472 (SE = 79.767) × ($R^2=0.158$, $P<0.05$). The number of vocalizations (count/10 min) = 322.035 (SE = 68.148) −628.709 (SE = 243.812) × ($R^2=0.217$, $P<0.05$)

Table 2. Effect of several doses of L-ornithine on various behavioral categories of 4-day-old chicks exposed to social separation stress for 10 min beginning 5 min of i.c.v. injection

L-Ornithine (μmol)	0	0.125	0.25	0.5
Active wakefulness	600 ± 0	540 ± 33	491 ± 98	339 ± 70
Standing/sitting motionless with eyes open	0 ± 0	27 ± 15	50 ± 41	110 ± 28
Standing motionless with eyes closed	0 ± 0	28 ± 21	0 ± 0	110 ± 49
Sitting motionless with head drooped (sleeping posture)	0 ± 0	4 ± 4	57 ± 57	39 ± 39
Total	600	600	600	600

Values are mean ± SEM in seconds
The number of chicks used seven in each group
Active wakefulness (second/10 min) = 606.935 (SE = 48.533) – 520.454 (SE = 168.785) × ($R^2=0.302$, $P<0.01$)

was significant ($F(3, 22)=4.400$, $P<0.05$). The i.c.v. injection of L-ornithine clearly attenuate spontaneous activity. Significant negative correlations between the dose of L-ornithine and the number of vocalizations ($P<0.05$) were detected. The effect of L-ornithine on the number of vocalizations was significant ($F(3, 22)=6.768$, $P<0.01$). The i.c.v. injection of L-ornithine attenuated the number of vocalizations. Table 3 shows the effect of L-ornithine on various behavioral categories of chicks during the 10 min behavioral observation under social separation stress immediately after i.c.v. injection. The time for active wakefulness was reduced with increasing L-ornithine. Significant negative correlations between the dose of L-ornithine and the time for active wakefulness ($P<0.05$) were detected. The posture for standing motionless with

Table 3. Effect of several doses of L-ornithine on various behavioral categories of 6-day-old chicks exposed to social separation stress for 10 min beginning immediatley after i.c.v. injection

L-Ornithine (μmol)	0	0.125	0.25	0.5
Active wakefulness	475 ± 49^{a}	31 ± 31^{b}	$291 \pm 103^{a,b}$	69 ± 42^{b}
Standing/sitting motionless with eyes open	113 ± 40^{b}	360 ± 71^{a}	146 ± 34^{b}	$302 \pm 67^{a,b}$
Standing motionless with eyes closed	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}	0 ± 0^{a}
Sitting motionless with head drooped (sleeping posture)	12 ± 12^{a}	209 ± 72^{a}	163 ± 80^{a}	229 ± 90^{a}
Total	600	600	600	600

Values are mean ± SEM in seconds
The number of chicks used in each group was as follows: 0 μmol, 7; 0.125 μmol, 6; 0.25 μmol; 7, and 0.5 μmol, 6
Different letters indicate significant difference at $P < 0.05$
Active wakefulness (second/10 min) = 356.864 (SE = 66.037) − 601.54 (SE = 236.26) × ($R^2 = 0.213$, $P < 0.05$)

Table 4. Effect of several doses of L-arginine on free amino acid concentrations of the telencephalon of 4-day-old chicks 10 min just after i.c.v. injection

L-Arginine (μmol)	Intact	0	1.9	3.8
L-Arginine	182 ± 15^{c}	175 ± 7^{c}	1658 ± 204^{b}	3350 ± 219^{a}
L-Ornithine	23 ± 3^{b}	28 ± 3^{b}	57 ± 8^{a}	73 ± 6^{a}
γ-Aminobutyric acid	1729 ± 108^{a}	1820 ± 91^{a}	1888 ± 137^{a}	2017 ± 110^{a}
L-Alanine	821 ± 58^{b}	853 ± 40^{b}	$1009 \pm 98^{a,b}$	1158 ± 96^{a}
L-Proline	158 ± 9^{b}	$173 \pm 7^{a,b}$	$240 \pm 36^{a,b}$	256 ± 26^{a}
L-Lysine	219 ± 21^{b}	$263 \pm 39^{a,b}$	$428 \pm 99^{a,b}$	507 ± 90^{a}
Glycine	1222 ± 38^{a}	1243 ± 52^{a}	1368 ± 83^{a}	1490 ± 93^{a}
L-Threonine	305 ± 11^{b}	$397 \pm 31^{a,b}$	473 ± 54^{a}	$437 \pm 42^{a,b}$
L-Valine	136 ± 33^{a}	146 ± 34^{a}	291 ± 77^{a}	326 ± 43^{a}
L-Glutamic acid	$7340 \pm 187^{a,b}$	7042 ± 123^{b}	$7523 \pm 139^{a,b}$	7860 ± 263^{a}

Values are means ± SEM in nmol/g wet tissue
The number of chicks used in each group was as follows: Intact, 6; 0 μmol, 7; 1.9 μmol, 6; and 3.8 μmol, 7
Different letters indicate significant difference at $P < 0.05$

Table 5. Effect of several doses of L-arginine on free amino acids concentrations of the diencephalon of 4-day-old chicks for 10 min just after i.c.v. injection

L-Arginine (μmol)	Intact	0	1.9	3.8
L-Arginine	123 ± 16^{c}	130 ± 16^{c}	846 ± 142^{b}	1618 ± 240^{a}
L-Ornithine	14 ± 2^{c}	$23 \pm 4^{b,c}$	$49 \pm 12^{a,b}$	55 ± 9^{a}
γ-Aminobutyric acid	1596 ± 135^{a}	1578 ± 161^{a}	1692 ± 258^{a}	1855 ± 195^{a}
L-Alanine	545 ± 55^{a}	602 ± 70^{a}	561 ± 53^{a}	621 ± 82^{a}
L-Proline	103 ± 8^{a}	108 ± 9^{a}	122 ± 17^{a}	123 ± 5^{a}
L-Lysine	178 ± 14^{a}	145 ± 39^{a}	188 ± 44^{a}	199 ± 25^{a}
Glycine	982 ± 88^{a}	1045 ± 102^{a}	1027 ± 127^{a}	1088 ± 130^{a}
L-Threonine	197 ± 14^{a}	223 ± 21^{a}	262 ± 46^{a}	211 ± 14^{a}
L-Valine	103 ± 8^{a}	108 ± 9^{a}	122 ± 17^{a}	123 ± 5^{a}
L-Glutamic acid	2566 ± 231^{a}	2558 ± 248^{a}	2623 ± 342^{a}	2721 ± 309^{a}

Values are means ± SEM in nmol/g wet tissue
The number of chicks used in each group was as follows: Intact, 6; 0 μmol, 7; 1.9 μmol, 6; 3.8 μmol, 7
Different letters indicate significant difference at $P < 0.05$

eyes open significantly increased by L-ornithine. A similar tendency was observed in sleeping posture, but was not significant.

Table 4 shows the effect of i.c.v. injection of L-arginine on free amino acid concentrations in the telencephalon. The effect of L-arginine on telencephalon L-arginine concentration was significant ($F(3, 22) = 103.114$, $P < 0.0001$). Significant ($P < 0.0001$) positive correlations between the dose of L-arginine and L-arginine concentration were detected. L-Arginine also increased telencephaon L-ornithine ($F(3, 22) = 17.720$, $P < 0.0001$). Significant ($P < 0.0001$) positive correlations between the dose of L-arginine and L-ornithine were detected. The inhibitory neurotransmitter GABA was not influenced. Other amino acids including L-alanine, L-proline, L-lysine, L-threonine, and L-glutamic acid significantly increased.

Table 5 shows the effect of i.c.v. injection of L-arginine on free amino acid concentrations in the diencephalon. L-Arginine injection increased diencephalon L-arginine concentration ($F(3, 22) = 24.706$, $P < 0.0001$). Significant ($P < 0.0001$) positive correlations between the dose of L-arginine and diencephalons L-arginine concentration were detected. The effect of i.c.v. L-arginine injection on diencephalons L-ornithine was significant ($F(3, 22) = 6.003$, $P < 0.0001$). Significant ($P < 0.01$) positive correlations between the dose of L-arginine and L-ornithine were detected. The effect of L-arginine on other amino acid concentrations was not significant.

Discussion

To clarify the mechanism by which central L-arginine attenuates stress responses in chicks, we investigated the central role of L-arginine metabolites. Thereafter, the effects of i.c.v. injection of L-arginine on free amino acid concentrations in two brain regions was determined.

In Experiment 1, the i.c.v. injection of agmatine did not induce sedative and hypnotic effects (Fig. 1, Table 1). Agmatine has a guanidino component. Guanidino compounds are known to have a relation to GABA-A receptors (Neu et al., 2002). Creatine, which has a guanidino component, attenuates the acute stress response by acting through GABA-A receptors (Koga et al., 2005). However, the effect of i.c.v. injection of agmatine differed from these reports. The i.c.v. injection of L-ornithine, which does not have a guanidino component, induced sedative and hypnotic effects in Experiments 2 and 3. These data suggest that the function of L-arginine might not be associated with GABA-A receptor activation by guanidine compounds. Furthermore, L-arginine did not modify brain GABA concentrations (Experiment 4). Accordingly, sedative and hypnotic effects of central L-arginine may not be associated with the GABAergic system.

In Experiment 2, L-ornithine attenuated spontaneous activity and the number of vocalizations (Fig. 2), and slightly increased the time in sleeping posture (Table 2) in the observation period beginning 5 min after i.c.v. injection. The effect of L-ornithine immediately after i.c.v. injection in Experiment 3 was stronger than that seen in Experiment 2. The difference in response between Experiments 2 and 3 indicated that L-ornithine was rapidly metabolized to the next metabolite. Accordingly, L-ornithine itself may have an important role for the induction of sedative and hypnotic effects. The i.c.v. injection of putrescine, an L-ornithine metabolite, produced antidepressant-like effects in mice that seem to be mediated through its interaction with the polyamine-site of NMDA receptors (Zomkowski et al., 2006). The structure of L-ornithine is similar to that of putrescine. This suggests the possibility that L-ornithine might act on the polyamine-site of NMDA receptors if it is metabolized quickly enough.

In Experiment 4, the i.c.v. injected L-arginine increased both L-arginine and L-ornithine concentrations of the telencephalon and diencephalon in chicks 10 min post-injection (Tables 4 and 5). In addition, L-ornithine concentration was proportionally increased by L-arginine injection suggesting that L-arginine was metabolized by arginase in the brain. These results support the chick behavior results observed following the i.c.v. injection of L-ornithine under social separation stress. Therefore, it indicates that the sedative and hypnotic effects of L-arginine were mainly caused by L-ornithine. So far, no reports are available showing a sedative effect of L-ornithine under stressful conditions. The present result are the first report that i.c.v. injected L-ornithine attenuates the stressful response in chicks.

In the present study, various amino acids increased in the telencephalon following L-arginine injection (Table 4). Increased amino acids including L-alanine (Kurauchi et al., 2006), L-proline (Hamasu et al., unpublished data) and L-glutamic acid (Yamane et al., unpublished data) have been observed to have sedative or hypnotic effects. These results suggest that effects of L-arginine might be supported by these amino acids.

In conclusion, guanidine-containing metabolites of L-arginine did not function through the GABAergic system. The effect of L-arginine was mainly caused by increases in L-ornithine. In addition, increases in several amino acids having a sedative effect might partly participate in

the sedative and hypnotic effects of L-arginine. However, the present results were obtained by exogenous L-arginine. The function of endogenous L-arginine to stress responses remains to be studied by investigating the changes in brain L-arginine contents and the effect of arginase inhibitor in the future.

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