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Intracerebroventricular injection of L-arginine induces sedative and hypnotic effects under an acute stress in neonatal chicks

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Summary. L-Arginine participates in many important and diverse biochemical reactions associated with the normal physiology of the organism. In the present study, we investigated the effect of central administration of L-arginine on the stress response and its mechanism in neonatal chicks. Intracerebroventricular (i.c.v.) injection of L-arginine clearly attenuated the stress response in a dose-dependent manner, and induced sleep-like behavior during 10 min. To clarify the mechanism by which L-arginine induces sedative and hypnotic effects in chicks, we investigated the effects of nitric oxide (NO) synthase (NOS) inhibitors on L-arginine-induced sedative and hypnotic effects, and as well as the effects of a NO donor. L-Arginine-induced (1.9 μmol) sedative and hypnotic effects were attenuated by i.c.v. co-injection with a non-selective NOS inhibitor N^G-nitro-L-arginine methyl ester HCl (400 nmol). In addition, the effects of L-arginine were slightly attenuated by the inactive isomer of the NOS inhibitor N^G-nitro-D-arginine methyl ester HCl (400 nmol). The i.c.v. injection of 3-morpholinosylnomine hydrochloride, a spontaneous NO donor, had little effect on postures. The i.c.v. injection of L-arginine had no effect on NO_x concentration at various brain sites. These results suggested that the contribution of NO generation via NOS may be low in the sedative and hypnotic actions of L-arginine. Therefore, L-arginine and/or its metabolites, excluding NO, may be necessary for these actions.

Keywords: L-Arginine – L-NAME – D-NAME – SIN-1 – Nitric oxide – Intracerebroventricular injection – Social separation stress – Neonatal chick

Introduction

Research on the biochemistry and physiology of L-arginine has remained an attractive area for scientists over the last 100 years due to its diverse physiological functions in mammals. L-Arginine is classified as an essential amino acid for birds, carnivores and young mammals and a conditionally essential amino acid for adults. L-Arginine itself can stimulate growth hormone release when infused intravenously or orally administered (Chromiak and Antonio, 2002).

L-Arginine can be catabolized by four sets of enzymes in mammalian cells, resulting ultimately in the production of urea, proline, glutamate, polyamines, nitric oxide (NO), creatine, or agmatine (Morris, 2004). In particular, one of the L-arginine metabolites, NO, is produced by NO synthase (NOS) (Palmer et al., 1987). NO is the major endothelium derived relaxing factor, a mediator of immune responses, a neurotransmitter, a cytotoxic free radical, and a widespread signaling molecule in the body. Recently, it was shown that NO modulates various behaviors. For instance, NO donors produce defensive reactions characterized by wild running and jumping (De Oliveira et al., 2001). In contrast, NO increased slow wave sleep and reduced waking during the dark phase in adult rats (Monti and Jantos, 2004). Accordingly, it is believed that NO has biphasic effects (Colasanti and Suzuki, 2000; da Silva et al., 2000).

Birds lack carbamyl phosphate synthetase, one of the urea cycle enzymes necessary for the synthesis of citrulline from ornithine in the liver and kidney (Tamir and Ratner, 1963). Therefore, it is impossible to synthesize L-arginine in birds, and L-arginine is classified as an essential amino acid for birds. However, the role of L-arginine in the central nervous system (CNS) under stressful conditions has not been investigated in birds which, unlike mammals, lack a urea cycle. Therefore, in an effort to investigate a novel function of L-arginine on stress

responses, the aim of the present study was to clarify (1) whether intracerebroventricular (i.c.v.) injected L-arginine influences behavior under social separation stress, and (2) the mechanism by which L-arginine acts on the stress response in neonatal chicks.

In the present study, we used the social separation stress model. This stress model is frequently used for the study of anxiety for the following reason. Chicks are comfortable when living in a group, but exhibit 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 anti-anxiety agents using vocalization and spontaneous activity as parameters. Additionally, this model has a high utility since chicks are inexpensive to purchase and maintain, and they 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 (4–6 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

L-Arginine monohydrochloride was a gift from Kyowa Hakko Kogyo Co. Ltd. (Tokyo, Japan). N^G-nitro-L-arginine methyl estel HCl (L-NAME), a non-selective NOS inhibitor, N^G-nitro-D-arginine methyl ester HCl (D-NAME), an inactive isomer of the NOS inhibitor, and 3-morpholinosylnomine hydrochloride (SIN-1), a spontaneous NO donor, were purchased from Sigma (St. Louis, MO, USA). 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). Chicks were returned to a cage for 5 min post-injection to allow for drug diffusion. Thereafter, they were gently placed alone into acrylic glass chamber ($40 \times 30 \times 20$ cm) with paper on the floor for 10 min in a separate room at a constant temperature of 30 °C. Chicks were deprived of water and diet during this period, and behavior, spontaneous activity and vocalizations were recorded. Spontaneous activity was automatically determined utilizing infrared beam sensors (NS-AS01; Neuroscience Inc., Tokyo, Japan) placed 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 L-arginine on behaviors of chicks under social separation stress was investigated. Birds (6 days old) were injected i.c.v. with L-arginine (0.95, 1.9 and 3.8 µmol).

In Experiments 2 and 3, the effect of i.c.v. injection of a non-selective NOS inhibitor, L-NAME and an inactive isomer of the NOS inhibitor, D-NAME, on L-arginine-induced behaviors of chicks under social separation stress was studied. Birds (5 days old) were injected i.c.v. with L-NAME (400 nmol), L-arginine (1.9 µmol) or L-arginine (1.9 µmol) plus L-NAME (400 nmol) in Experiment 2 and with D-NAME (400 nmol), L-arginine (1.9 µmol) or L-arginine (1.9 µmol) plus D-NAME (400 nmol) in Experiment 3. The doses were determined in Experiment 1 and by Tomonaga et al. (2005), respectively.

In Experiment 4, the effect of i.c.v. injection of SIN-1, a spontaneous NO donor, on behaviors of chicks under social separation stress was studied. Birds (5 days old) were injected i.c.v. with 0.3, 0.6, or 1.2 µmol of SIN-1. Saline was used as a control in all experiments.

In Experiment 5, we investigated the effect of i.c.v. injection of L-arginine on NO*x* ($NO_2 + NO_3$), an index of NO production, of several brain sites. Birds (4 days old) were injected i.c.v. with either saline as a control, or L-arginine (1.9 and 3.8 µmol). Birds were injected with an overdose of sodium pentobarbital, decapitated, and the brains removed 10 min after the i.c.v. injection.

In all experiments, 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

In Experiment 5 the brains were carefully removed and placed on a cold glass dish. According to the atlas of the chicken brain (Kuenzel and Masson, 1988), the brains were divided into four parts (telencephalon, diencephalon, midbrain and cerebellum) and weighed. Since NO*x* is present in the air as well as dissolved in water the brain tissue samples were kept in new plastic tubes to prevent contamination with NO*x* in the air and water. Quadruple volumes of 10 mM phosphate buffered saline (PBS) (pH 7.4) were added (Han et al., 2002), and 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 ultra-filtration. The interference due to hemoglobin as well as other proteins was prevented by filtering the supernatant through a 10,000 MW cut-off microcentrifuge filter (Microcon YM-10, Amicon Bioseparations, Millipore Co., Bedford, MA) 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 volume of PBS was added to the filtrates. The samples were stored at 4 °C prior to assay.

NOx assay

Since NO has a very short half-life (<10 sec) (Archer, 1993) the assay determined NO*x*, the stable metabolites of NO. NO*x* was measured by a modification of the fluorometric assay (Al-Rejaie and Dar, 2006; Misko et al., 1993). The assay was based on reaction with 2, 3-diaminonaphthalene (DAN). The DAN reagent detects nitrite in a variety of biological solutions and displays greater sensitivity compared to the Griess method (Salter et al., 1996). The assays were performed immediately after the sample preparation.

The measurement of NO*x* concentration in the samples was done using a NO_2/NO_3 Assay Kit-FX (Dojindo Laboratiries Co. Ltd., Kumamoto, Japan). An 80 µl sample was placed in a well of a black 96-well flat bottom plate. The intensity of the fluorescent signal was immediately measured by a fluorometer (ARVOMX Multilabel Microplate Counter, Perkinelmer Inc., Wellesley, MA, USA) at $\lambda_{ex} = 355$ nm and $\lambda_{em} = 460$ nm. The concentration of NO*x* in each sample was calculated from a standard curve prepared by plotting the Fluorescent Units with sodium nitrite concentration in nmol using WorkOut2 software (DAZDAQ Ltd., Brighton, East Sussex, England) for linear regression and concentration analysis of the data. Results were expressed in nmol/g wet tissue.

Statistical analysis

Regression equations were calculated comparing dose of L-arginine to 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 (Experiments 1, 4 and 5). Data were analyzed by two-way ANOVA and a Tukey–Kramer test was done as a post hoc test (Experiments 2 and 3). Significant differences implied $P < 0.05$. Values are presented as means ± S.E.M. Statistical analysis was made using a commercially available package, StatView (Version 5, SAS Institute, Cary, USA, 1998). All data were first subjected to Grubs-Smirnov re-jection test to eliminate outliers, and the remaining data were used.

Results

Figure 1 shows the effect of i.c.v. injection of L-arginine on total spontaneous activity and vocalizations of chicks during the 10 min social separation stress (Experiment 1). The effect of L-arginine on total spontaneous activity ($F(3, 24) = 4.094$, $P < 0.05$) and vocalizations ($F(3, 24) = 8.219$, $P < 0.001$) was significant. The i.c.v. injection of L-arginine clearly attenuated total spontaneous activity and vocalizations. There were significant negative correlations between the dose of L-arginine and total spontaneous activity ($P < 0.05$) and vocalizations ($P < 0.0001$). Table 1 shows the effect of i.c.v. injection of L-arginine on various behavioral categories of chicks during the 10 min behavior observation under social separation stress. The time for active wakefulness was reduced with increasing doses of L-arginine. Significant negative correlations between the dose of L-arginine and the time for active wakefulness ($P < 0.05$) were detected. Additionally, the high dose of L-arginine increased the time for sleeping posture. Significant positive correlations between the dose of L-arginine and the time for sleeping posture ($P < 0.05$) were detected.

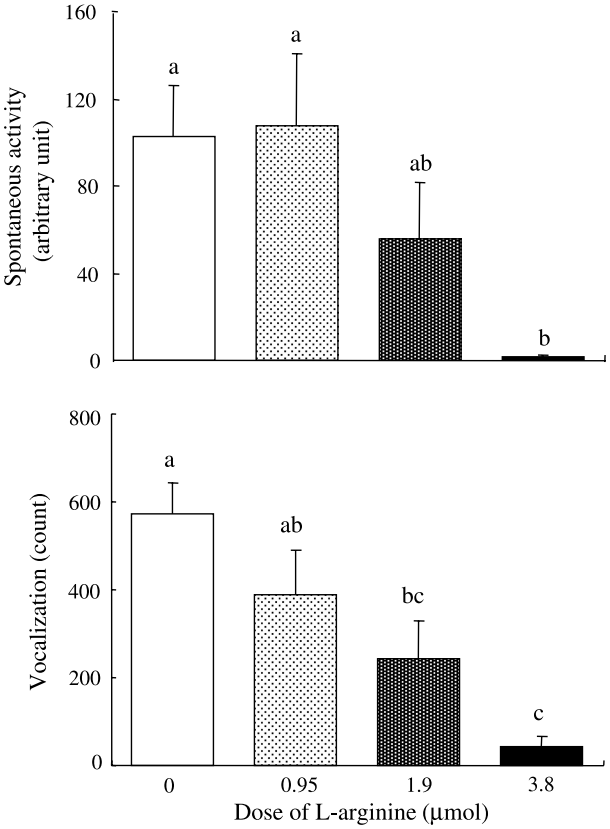

Fig. 1. Effect of i.c.v. injection of of L-arginine on spontaneous activity (*upper panel*) and vocalizations (*lower panel*) during a 10 min social separation stress in 6-day-old layer chicks. Values are means with S.E.M. Different letters indicate significant differences at $P < 0.05$. The number of birds used was seven in each group. Spontaneous activity (arbitrary unit/10 min) = 115.143 ($SE = 18.471$) − 0.138 ($SE = 0.04$) × ($R^2 = 0.311$, $P < 0.01$), the number of vocalizations (count/10 min) = 537.514 ($SE = 58.587$) − 0.646 ($SE = 0.128$) × ($R^2 = 0.495$, $P < 0.0001$)

Table 1. Effect of i.c.v. injection of L-arginine on various behaviors of 6-day-old chicks exposed to social separation stress for 10 min

L-Arginine (µmol)	0	0.95	1.9	3.8
Active wakefulness	551 ± 34[a]	426 ± 107[a]	360 ± 109[a,b]	86 ± 45[b]
Standing/sitting motionless with eyes open	49 ± 34	36 ± 25	15 ± 6	25 ± 9
Standing motionless with eyes closed	0 ± 0	0 ± 0	0 ± 0	5 ± 5
Sitting motionless with head drooped (sleeping posture)	0 ± 0[b]	138 ± 91[b]	225 ± 106[a,b]	484 ± 47[a]
Total	600	600	600	600

Values are mean ± S.E.M. in seconds. The number of chicks used seven in each group
Different letters indicate significant difference at $P < 0.05$
Active wakefulness (second/10 min) = 556 ($SE = 61$) − 0.571 ($SE = 0.133$) × ($R^2 = 0.413$, $P < 0.01$)
Sleeping posture (second/10 min) = 3.371 ($SE = 55.198$) + 0.594 ($SE = 0.12$) × ($R^2 = 0.485$ $P < 0.001$)

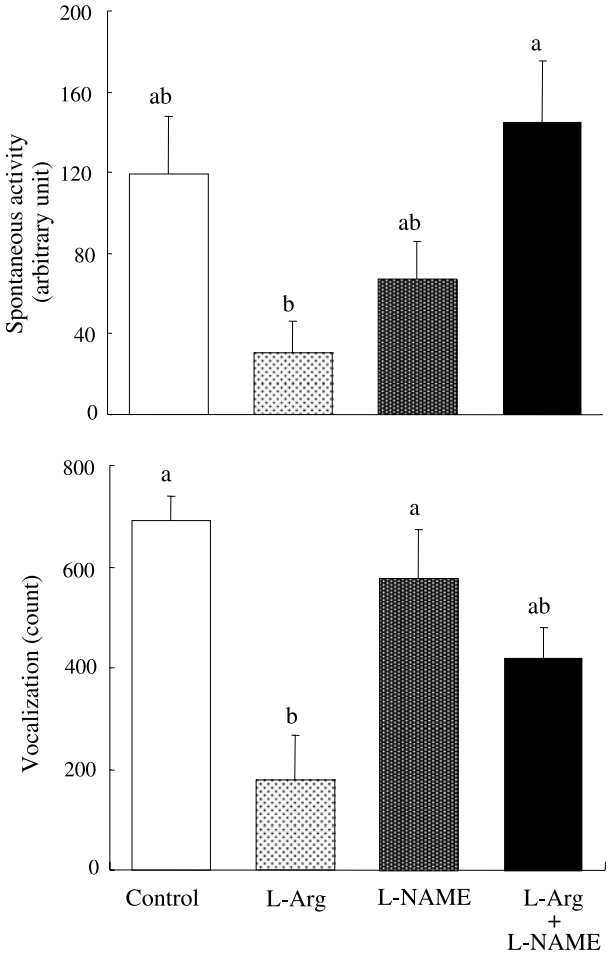

Fig. 2. Effect of N^G-nitro-L-arginine methyl ester HCl on L-arginine-induced spontaneous activity (*upper panel*) and vocalizations (*lower panel*) during a 10 min social separation stress in 5-day-old layer chicks. Values are means with S.E.M. Different letters indicate significant differences at $P<0.05$. The number of birds used was as follows: control (saline), 7; L-Arg, 7; L-NAME, 7; L-Arg + L-NAME, 6

Figure 2 shows the effect of L-NAME on the L-arginine-induced reduction in spontaneous activity and vocalizations in chicks during the 10 min social separation stress. An interaction between L-arginine and L-NAME was significant ($F(1, 24) = 8.611$, $P<0.05$), implying that the effect of L-arginine on spontaneous activity disappeared with co-injection of L-NAME. The effect of L-arginine on the number of vocalizations was significant ($F(1, 23) = 18.770$, $P<0.01$). An interaction between L-arginine and L-NAME was significant ($F(1, 23) = 5.152$, $P<0.05$) in spontaneous activity only. These results imply that the sedative effect of L-arginine was attenuated by L-NAME.

Table 2 shows the effect of L-arginine, L-NAME or both on various behavioral categories in chicks during the 10 min social separation stress. L-Arginine significantly ($F(1, 23) = 6.960$, $P<0.05$) decreased the time for active wakefulness. An interaction between L-arginine and L-NAME was significant ($F(1, 23) = 8.024$, $P<0.01$). L-Arginine significantly ($F(1, 23) = 8.686$, $P<0.01$) increased the time for sleeping posture. An interaction between L-arginine and L-NAME was significant ($F(1, 23) = 8.686$, $P<0.01$). These results indicated that behavioral changes induced by i.c.v. injection of L-arginine were attenuated by co-injection of L-NAME.

Table 2. Effect of i.c.v. injection of L-arginine, N^G-nitro-L-arginine methyl estel HCl or both on various behaviors of 5-day-old chicks exposed to social separation stress for 10 min

L-Arginine (μmol)	0	1.9	0	1.9
L-NAME (nmol)	0	0	400	400
Active wakefulness	585 ± 6^a	211 ± 106^b	499 ± 47^a	512 ± 69^a
Standing/sitting motionless with eyes open	15 ± 6	47 ± 16	78 ± 36	23 ± 9
Standing motionless with eyes closed	0 ± 0	42 ± 26	23 ± 23	65 ± 65
Sitting motionless with head drooped (sleeping posture)	0 ± 0^b	300 ± 97^a	0 ± 0^b	0 ± 0^b
Total	600	600	600	600

Values are mean ± S.E.M. in seconds
The number of chicks used in each group was as follows: control (saline), 7; L-arginine (1.9 μmol), 6; L-NAME (400 nmol), 7; and L-arginine (1.9 μmol) + L-NAME (400 nmol), 7
Different letters indicate significant difference at $P<0.05$

Figure 3 shows the effect of D-NAME on the L-arginine-induced reduction of spontaneous activity and vocalizations in chicks during the 10 min under social separation stress. An interaction between L-arginine and D-NAME was not significant, implying that the sedative effect of L-arginine was not attenuated by D-NAME.

Table 3 shows the effect of L-arginine, D-NAME or both on various behavioral categories in chicks during the 10 min social separation stress. L-Arginine significantly ($F(1, 23) = 64.314$, $P<0.01$) decreased the time for active wakefulness. An interaction between L-arginine and D-NAME was significant ($F(1, 23) = 4.460$, $P<0.05$). L-Arginine significantly ($F(1, 23) = 59.193$, $P<0.01$) increased the time for sleeping posture. An interaction between L-arginine and D-NAME was significant ($F(1, 23) = 9.735$, $P<0.01$). These results indicated that behavioral changes induced by i.c.v. injection of L-arginine were also attenuated by co-injection of D-NAME.

Figure 4 shows the effect of i.c.v. injection of SIN-1 on total spontaneous activity and vocalizations in chicks during the 10 min social separation stress. No significant correlations between the dose of SIN-1 and total spontaneous activity ($P = 0.059$) were detected. However, there was

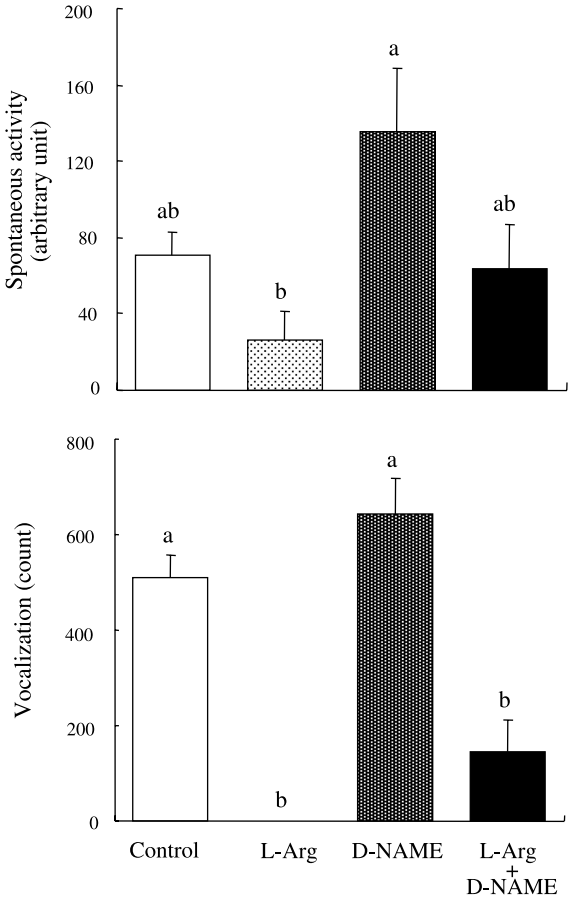

Fig. 3. Effect of N^G-nitro-D-arginine methyl ester HCl on L-arginine-induced spontaneous activity (*upper panel*) and vocalizations (*lower panel*) during a 10 min social separation stress in 5-day-old layer chicks. Values are means with S.E.M. Different letters indicate significant differences at $P < 0.05$. The number of birds used was as follows: control (saline), 7; L-Arg, 6; D-NAME, 7; L-Arg + D-NAME, 7

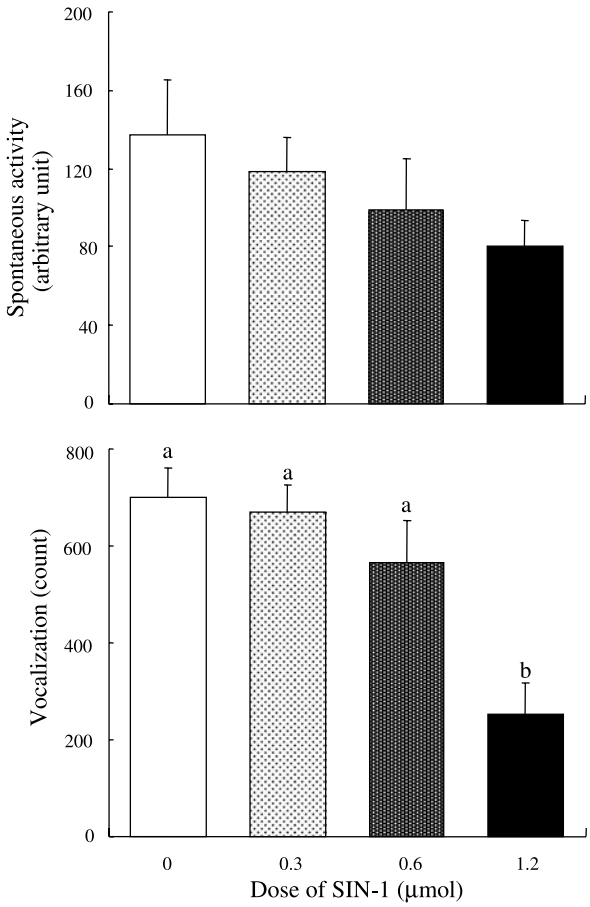

Fig. 4. Effect of i.c.v. injection of several doses of SIN-1 on spontaneous activity (*upper panel*) and vocalizations (*lower panel*) during a 10 min social separation stress in 5-day-old layer chicks. Values are means with S.E.M. Different letters indicate significant differences at $P < 0.05$. The number of chicks used in each group was as follows: 0 μmol, 7; 0.3 μmol, 6; 0.6 μmol, 7; and 1.2 μmol, 7. The number of vocalizations (count/10 min) = 749.391 ($SE = 52.768$) − 388.51 ($SE = 75.64$) × ($R^2 = 0.513$, $P < 0.0001$)

Table 3. Effect of i.c.v. injection of L-arginine, N^G-nitro-D-arginine methyl estel HCl or both on various behaviors of 5-day-old chicks exposed to social separation stress for 10 min

L-Arginine (μmol)	0	1.9	0	1.9
D-NAME (nmol)	0	0	400	400
Active wakefulness	526 ± 45^a	0 ± 0^b	544 ± 25^a	237 ± 85^c
Standing/sitting motionless with eyes open	44 ± 21	28 ± 6	50 ± 21	84 ± 26
Standing motionless with eyes closed	29 ± 29^a	0 ± 0^b	0 ± 0^a	31 ± 31^c
Sitting motionless with head drooped (sleeping posture)	0 ± 0	571 ± 6	5 ± 5	248 ± 101
Total	600	600	600	600

Values are mean ± S.E.M. in seconds
The number of chicks used in each group was as follows: control (saline), 7; L-arginine (1.9 μmol), 6; D-NAME (400 nmol), 7; and L-arginine (1.9 μmol) + D-NAME (400 nmol), 7
Different letters indicate significant difference at $P < 0.05$

Table 4. Effect of i.c.v. injection of 3-morpholinosylnomine hydrochloride (SIN-1) on various behaviors of 5-day-old chicks exposed to social separation stress for 10 min

SIN-1 (μmol)	0	0.3	0.6	1.2
Active wakefulness	581 ± 15	600 ± 0	577 ± 19	596 ± 2
Standing/sitting motionless with eyes open	19 ± 15	0 ± 0	23 ± 19	4 ± 2
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 ± S.E.M. in seconds
The number of chicks used in each group was as follows: 0 μmol, 7; 0.3 μmol, 6; 0.6 μmol, 7; and 1.2 μmol, 7
Different letters indicate significant difference at $P < 0.05$

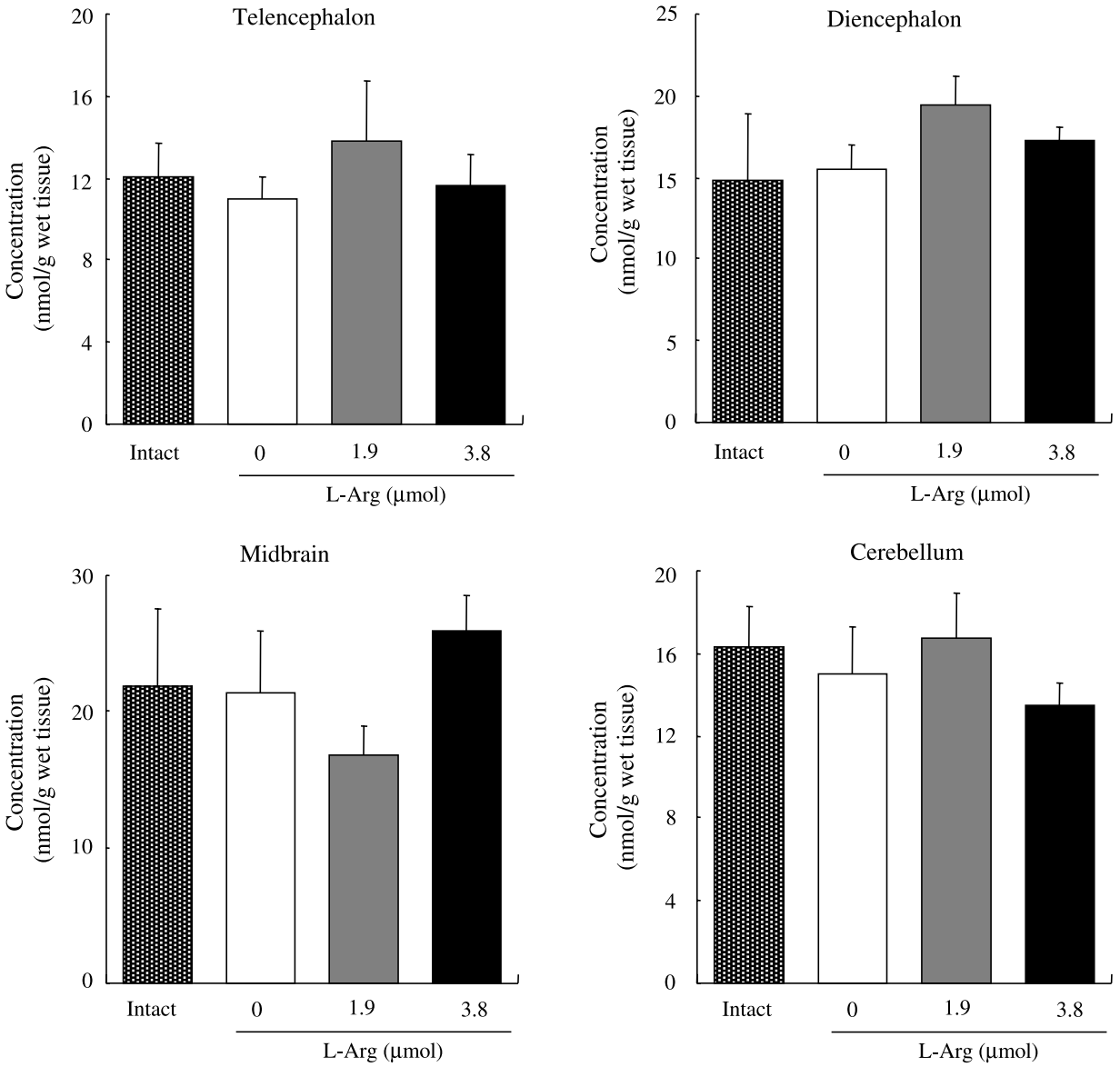

Fig. 5. Nitrites and nitrates (NO*x*) concentrations of chicks in the several brain sites injected intracerebroventricularly with saline, 1.9, or 3.8 μmol of L-arginine. The number of chicks used in each group was as follows: intact, 6; 0 μmol, 7; 1.9 μmol, 6; and 3.8 μmol, 7. Values are means with S.E.M

a significant negative correlation between the dose of SIN-1 and total vocalizations ($P < 0.0001$). The effect of SIN-1 on the number of vocalizations was significant ($F(3, 23) = 9.125$, $P < 0.001$).

Table 4 shows the effect of i.c.v. injection of SIN-1 on various behavioral categories of chicks during the 10 min behavioral observation under social separation stress. No significant changes were observed in any behavioral parameters.

The i.c.v. injection of L-arginine had no effect on NO*x* concentration in any brain site (Experiment 5). No significant correlations between the dose of L-arginine and NO*x* concentration were detected in any brain site (Fig. 5).

Discussion

After 5 min, the i.c.v. injection of L-arginine clearly attenuated spontaneous activity and the number of vocalizations compared with the control. In addition, L-arginine increased the time spent in sleeping posture. These results suggest that L-arginine has sedative and hypnotic effects (Experiment 1). In preliminary experiments, behavioral

tests were done immediately after the i.c.v. injection of L-arginine, but no significant effects of L-arginine were observed (data not shown). These results might suggest that a metabolite of L-arginine participates in the central function.

To determine the mechanism of the response to L-argnine, we first gave i.c.v. co-injections of L-NAME, a non-selective NOS inhibitor with L-arginine. The decrease in total spontaneous activity and vocalizations and the increase in the time of sleeping posture induced by L-arginine were attenuated to the control level by co-injection of L-NAME (Experiment 2). That is to say, NO, produced through NOS, could be involved in the sedative and hypnotic effects of L-arginine in chicks under social separation stress. The inactive isomer of the NOS inhibitor, D-NAME, did not attenuate L-arginine-induced sedation, although it did attenuate the L-arginine-induced sleep-like behavior (Experiment 3). In a previous study, D-NAME, even though it has been widely used as inactive isomer of the NOS inhibitor, was able to inhibit laminarin-stimulated NO production in primary haemocytes from the snail *Lymnaea stagnalis* (Wright et al., 2006). Therefore, there remains a possibility that D-NAME could also produce unexpected actions such as NOS inhibition.

SIN-1, an NO donor, did not show the sedative and hypnotic effects except for vocalization (Experiment 4). Results of co-injection of L-NAME with L-arginine are inconsistent with that of SIN-1, and NO*x* concentration in the several brain sites was not significantly increased by i.c.v. injection of L-arginine (Experiment 5). Even when L-arginine was not injected, results from Experiments 2, 4 and 5 show that the chick brain could produce NO. This endogenous NO production may be inhibited by L-NAME, since an adequate amount of NO was not released by exogenous L-arginine (Fig. 5).

It is believed that NO regulates sleep and waking (Hars, 1999). The i.c.v. injection of SIN-1 increased slow wave sleep and reduced waking during the dark phase in adult rats in a habituated cage (Monti and Jantos, 2004). In the present study, observation was done in non-habituated cages because chicks had to be exposed to acute social isolation stress. Differences in the experimental conditions and/or species might be linked to the different results. In the present study, NO*x* concentrations in the brain were not altered by i.c.v. injection of L-arginine. However, we can not deny the possibility that a very low, but undetectable level of NO was generated by L-arginine, which might be linked to its action. Therefore, there remains the possibility that NO, produced via NOS, could be partly involved in the sedative and hypnotic actions of L-arginine although the contribution of NO to these actions seemed, if any, low.

L-Arginine can be catabolized by four sets of enzymes in mammalian cells resulting ultimately in the production of urea, L-proline, L-glutamate, polyamines, NO, creatine, or agmatine (Morris, 2004). Creatine has sedative and hypnotic effects in the CNS under social separation stress in neonatal chicks. This action of creatine was mediated by the activation of GABA-A receptors (Koga et al., 2005). One of the possibilities was that creatine, made from L-arginine, acted in the CNS. Another possibility is that creatine, which has a similar structure to L-arginine, might be working together to induce these effects. In addition, L-arginine is metabolized by arginase to yield L-ornithine. L-Ornithine may regulate the synthesis of polyamines, which are essential for cell proliferation and differentiation processes. The metabolic roles of ornithine are likely cell- and tissue-specific. In fact, we have confirmed that the i.c.v. injection of ornithine attenuated stress responses in chicks (Suenaga et al., 2008).

In conclusion, these results suggest that NO may have a slight role in the effects of L-arginine in the neonatal chick. The sedative and hypnotic effects of L-arginine may be mainly due to L-arginine itself and/or its metabolites, excluding NO.

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