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ORIGINAL ARTICLE

Central administration of L- and D-aspartate attenuates stress behaviors by social isolation and CRF in neonatal chicks

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Abstract Intracerebroventricular (i.c.v.) administration of L-aspartate (L-Asp) attenuates stress responses in neonatal chicks, but the mechanism has not been clarified. In the present study, three behavioral experiments were carried out under socially isolated stressful conditions exacerbated by the use of corticotrophin-releasing factor (CRF). In Experiment 1, i.c.v. injection of L-Asp attenuated behavioral stress responses (distress vocalization and active wakefulness) in a dose-dependent manner. Furthermore, L-Asp increased time spent standing/sitting motionless with eyes open and sitting motionless with head dropped (sleeping posture) in comparison with the group receiving CRF alone. In Experiment 2, i.c.v. injection of D-Asp dose-dependently decreased the number of distress vocalizations and the amount of time spent in active wakefulness. D-Asp increased the time spent standing/sitting motionless with eyes open compared with the group receiving CRF alone. In Experiment 3, we directly compared the effect of L-Asp with that of D-Asp. Both L- and D-Asp induced sedative effects under an acutely stressful condition. However, L-Asp, but not D-Asp, increased the time spent in a sleeping posture. These results indicate that both L- and D-Asp, when present in the brain, could induce a sedative effect, while the mechanism for hypnosis in neonatal chicks may be different for L-Asp in comparison with D-Asp.

Keywords L-Aspartate · D-Aspartate · Intracerebroventricular injection · Social separation stress · Neonatal chick

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Introduction

The amino acid L-aspartate (L-Asp) and its enantiomer D-aspartate (D-Asp) occur in the central nervous system of various species, including chickens (Neidle and Dunlop 1990), rats (Hashimoto et al. 1995), pigeons (Kera et al. 1996), and humans (Dunlop et al. 1986). L-Asp functions as a neurotransmitter to stimulate the *N*-methyl-D-aspartate (NMDA) receptor (NMDA-R), one of the ionotropic L-glutamate receptors, even though its binding capacity for NMDA-R is weaker than that of L-glutamate (Chen et al. 2005). D-Asp, synthesized from L-Asp by aspartate racemase, can also directly stimulate the NMDA-R (D'Aniello et al. 2000; Woloskor et al. 2000). We have previously demonstrated that under acute social separation stress, intracerebroventricular (i.c.v.) injection of L-Asp causes a sedative effect in neonatal chicks (Yamane et al. 2009a). Furthermore, we have confirmed that L-glutamate and NMDA have similar effects to those observed with L-Asp (Yamane et al. 2009b) during acutely stressful conditions. From these findings, it can be hypothesized that both L-Asp and D-Asp play an important role in the regulation of physiological response under acutely stressful conditions via the NMDA-R.

Behavioral experiments have been carried out using neonatal chicks undergoing social isolation stress (Feltenstein et al. 2003; Panksepp et al. 1980; Sahley et al. 1981).

When chicks are isolated, they express characteristic stress-related behaviors, which include increased distress vocalizations (DVs), active wakefulness, and a decrease in sleeping behavior. Hence, the effect of drugs which have an anti-anxiety effect can be screened by observing the behavior of chicks undergoing isolation stress.

Corticotrophin-releasing factor (CRF), a 41-amino acid peptide hormone produced in the hypothalamus, is a key regulator of brain excitability associated with stress (Ehlers et al. 1983). This peptide has multiple biological effects and plays a central regulatory role in the hypothalamic–pituitary–adrenal (HPA) axis. It has been shown that the magnitude of anxiety induced by social separation stress is increased by CRF (Zhang et al. 2003, 2004; Kurata et al. 2011). CRF administered intracerebroventricularly or intracerebrally at specific brain sites produces a wide variety of behavioral effects, all of which are characterized by increases in arousal or by behavioral manifestations of a stressful state (Koob et al. 1984; Dunn and Berridge 1990).

Little is known about the central effects of either L- or D-Asp on the stress response, or about the mechanism by which these effects occur. In particular, no information is available on the possible role of isomerization of D-Asp from L-Asp in terms of the stress response. Therefore, to test the hypothesis that central L-Asp acts on the stress responses not only directly but also via its metabolites, represented by D-Asp, three behavioral experiments were carried out in the present study: (1) the dose-dependent effects of L-Asp were examined; (2) the dose-dependent effects of D-Asp were examined; and (3) a direct comparison was made between L- and D-Asp. To help clarify the mechanisms by which the central effects occur, in Experiment 3 the effects of L-Asp and D-Asp on levels of brain monoamines (dopamine [DA], serotonin [5-HT], and their metabolites) were also investigated because these monoamines have been recognized as regulators of behaviors and/or of stress responses in chicks (Hamasu et al. 2009b; Saito et al. 2004; Zhang et al. 2003).

Materials and methods

Animals and drugs

One-day-old layer chicks were purchased from a local hatchery (Murata hatchery, Fukuoka, Japan) and group-housed in a wire-meshed cage (50 × 35 × 33 cm) in a group (20–25 birds) at a constant temperature of 30 ± 1 °C and with continuous light until the experimental day. Chicks were all of the same age and were housed without an adult. Food (AX, Toyohashi Feed and Mills Co. Ltd., Aichi, Japan) and water were available ad libitum. On the day of the experiment, chicks (6 days old) were assigned to treatment groups on the basis of their body weight in order to produce uniform treatment groups. The number of animals used in each group was kept to the minimum that would still ensure adequate statistical power. This study was performed according to the guidance for Animals Experiments in the Faculty of Agriculture and in the Graduate Course of Kyushu University and Law No. 105 and Notification No. 6 of the government.

L- and D-Asp were purchased from Wako Pure Chemical Industries (Osaka, Japan). Rat CRF was purchased from Peptide Institute, Inc. (Osaka, Japan). The drugs were dissolved in a vehicle of 0.85 % saline containing 0.1 % Evans Blue (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

Procedure for behavioral test

Drugs were injected intracerebroventricularly by microsyringe into the left lateral ventricle of the chicks in a 10-µl dose, using the method of Davis et al. (1979). Minimal stress and pain are suffered when this method is used, as described elsewhere (Koutoku et al. 2005). In Experiment 1, chicks were administered either vehicle CRF (0.01 µg) or CRF plus L-Asp (0.42, 0.84 or 1.68 µmol). In Experiment 2, chicks were injected with either vehicle CRF (0.01 µg) or CRF plus D-Asp (0.42, 0.84 or 1.68 µmol). The doses of L-Asp and D-Asp were based on a previous report (Yamane et al. 2009a). In Experiment 3, chicks were injected with either CRF (0.01 µg), CRF plus L-Asp (0.84 or 1.68 µmol) or CRF plus D-Asp (0.84 or 1.68 µmol).

After injection, chicks were immediately placed in a monitoring cage (40 cm × 30 cm × 20 cm acrylic glass) with paper (changed for each animal) on the floor. Video cameras were positioned to record on digital versatile disc (DVD), the behavior of chicks from three different directions. The postures recorded during a 10-min period were characterized as (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 time spent in each posture was determined by watching the DVDs. In young adult hens, during both the sleeping posture (head tucked under a wing) and the resting postures with eyes closed, electrophysiological sleep was nearly always found to occur (van Luijtelaar et al. 1987). During the monitoring period, chicks were deprived of water and food. DVs of the chicks were simultaneously recorded and counted using Gretchen software (Excla Inc., Japan). The monitoring systems were in a separate room to avoid disturbing the animals. At the conclusion of the experiments, the birds were decapitated following anesthesia with isoflurane (Mylan Inc., Japan). The brains were removed and the location of the Evans Blue dye was confirmed. Data from chicks without dye in the lateral ventricle were excluded from analysis.

Analysis of monoamines in the brain

In Experiment 3, the brains were carefully removed and placed on a cold glass dish. They were divided into two parts (telencephalon and diencephalon), which were collected and stored at −80 °C until analysis. Monoamines (DA, 5-HT and their metabolites, homovanillic acid [HVA], 3,4-dihydroxyphenylacetic acid [DOPAC], and 5-hydroxyindoleacetic acid [5-HIAA]) in these brain regions were determined as described elsewhere (Tomonaga et al. 2008), with some modifications. The tissues were weighed and homogenized in 0.2 M ice-cold perchloric acid solution containing 0.01 mM EDTA 2Na. Samples were allowed to sit for 30 min on ice for deproteinization. The homogenate was centrifuged at 20,000*g* for 15 min. Supernatants were adjusted to pH 3 with 1 M sodium acetate and were filtered through a 0.2-μm filter (Millipore, Bedford, MA, USA). A 30-μl aliquot of filtrate was analyzed using a high-performance liquid chromatography system (Eicom, Kyoto, Japan) with a 150 × 3.0 mm ODS column (SC-5ODS, Eicom) and an electrochemical detector (ECD-300, Eicom) at an applied potential of +750 mV versus an Ag/AgCl reference analytical electrode. The mobile phase was 0.1 M sodium phosphate buffer, 2.3 mM sodium 1-octane sulfonante, 0.1 mM disodium ethylenediaminetetraacetic acid, and 17 % methanol, at pH 3.5. The external standard was used to identify peaks eluting in the chromatogram relating to retention time and conformation. The detection limits of the system for all monoamines were 0.1 pg/sample.

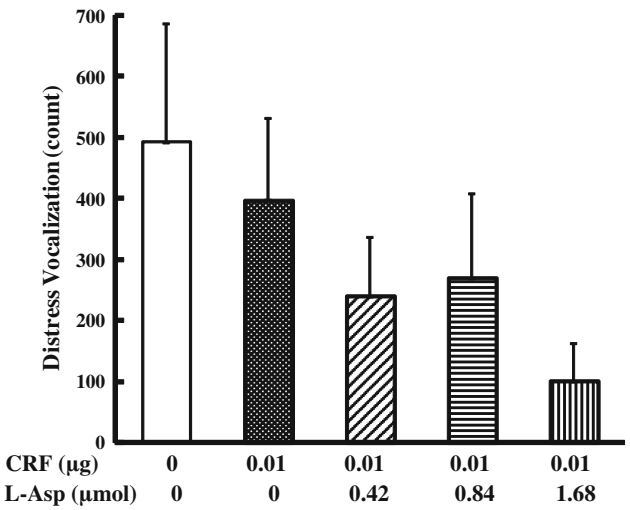

Fig. 1 Effects of several doses of L-Asp on DVs in chicks exposed to social separation stress. Values are means with SEM

Statistical analysis

In Experiments 1 and 2, regression equations were fitted for data relating to the DVs and the time spent exhibiting various types of behavior. In all experiments, 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 mean ± SEM. Statistical analysis was made using the commercially available package StatView (Version 5, SAS Institute, Cary, USA, 1998). All data were first subjected to a Thompson rejection test to eliminate outliers ($p < 0.01$). The remaining data were used.

Results

Experiment 1: Effects of i.c.v. injection of L-Asp on the behavior of chicks

Figure 1 shows the effects of i.c.v. injection of several doses of L-Asp with CRF on the number of DVs for 10 min post injection—a measure of social separation stress. A significant ($p < 0.05$) negative correlation between the dose of L-Asp and the number of DVs was detected (DVs [count/10 min] = 408 [SE 86] − 189 [SE 88]X, $R^2 = 0.181$). No significant effect ($F[4, 23] = 1.57$, $p = 0.217$) was found between different treatments in terms of DVs. Table 1 shows the effect of i.c.v. injection of several doses of L-Asp with CRF on various behavioral categories of

Table 1 Effects of i.c.v. injection of several doses of L-Asp and CRF on various behavioral categories of 6-day-old chicks exposed to social separation stress for 10 min

CRF (μg)	0	0.01	0.01	0.01	0.01
L-Asp (μmol)	0	0	0.42	0.84	1.68
Active wakefulness	398 ± 99^{ab}	479 ± 46^{a}	338 ± 64^{ab}	279 ± 98^{ab}	127 ± 35^{b}
Standing/sitting motionless with eyes open	157 ± 69^{b}	121 ± 46^{b}	257 ± 61^{ab}	259 ± 71^{ab}	401 ± 25^{a}
Standing motionless with eyes closed	6 ± 6	0 ± 0	0 ± 0	24 ± 24	0 ± 0
Sitting motionless with head drooped (sleeping posture)	40 ± 40	0 ± 0	5 ± 5	39 ± 30	73 ± 36
Total	600	600	600	600	600

Values are mean ± SEM in seconds. The number of chicks used in each group was 5–6

Means with different superscripts were significantly different at $p < 0.05$

chicks undergoing social separation stress during the 10-min behavioral observation. There was a significant effect ($F[4, 23] = 3.62$, $p < 0.05$) between treatments in terms of the time spent in active wakefulness, and a significant negative correlation ($p < 0.001$) was observed between the dose of L-Asp and the time spent in this posture (active wakefulness [second/10 min] = 455 [SE = 49] − 202 [SE = 50]X, $R^2 = 0.437$). A significant positive correlation ($p < 0.005$) between the dose of L-Asp and a posture involving standing or sitting motionless with eyes open was found (standing/sitting motionless with eyes open [second/10 min] = 144 [SE = 40] + 154 [SE = 41]X, $R^2 = 0.404$), and a significant effect ($F[4, 23] = 3.98$, $p < 0.05$) was detected among treatments in terms of this posture. In addition, a significant positive correlation ($p < 0.05$) was also detected between the dose of L-Asp and sitting motionless with head dropped (sleep-like behavior, sleeping posture [second/10 min] = −4 [SE = 18] + 46 [SE = 19]X, $R^2 = 0.222$), while no significant effect ($F[4, 23] = 1.19$, $p = 0.342$) was observed between treatments. No correlation was found between the dose of L-Asp and time spent standing motionless with eyes closed, and no significant effect was observed between treatments.

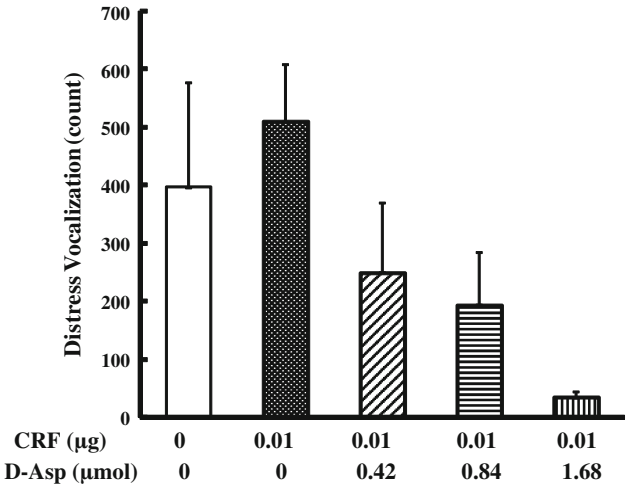

Fig. 2 Effects of several doses of D-Asp on DVs in chicks exposed to social separation stress. Values are means with SEM

Experiment 2: Effects of i.c.v. injection of D-Asp on the behavior of chicks

Figure 2 shows the effect of i.c.v. injection of several doses of D-Asp with CRF on the number of DVs during the 10 min of social separation stress. A significant negative correlation ($p < 0.005$) between the dose of D-Asp and the number of DVs was detected (DVs [count/10 min] = 453 [SE 67] − 270 [SE 72]X, $R^2 = 0.428$), while no significant effect ($F[4, 22] = 2.62$, $p = 0.063$) was found among treatments in terms of time spent on this behavior. Table 2 shows the effect of i.c.v. injection of several doses of D-Asp with CRF on various behavioral categories of chicks during the 10-min behavioral observation under conditions of social separation stress. A significant negative correlation ($p < 0.0001$) between the dose of D-Asp and active wakefulness was found (active wakefulness [second/10 min] = 522 [SE = 40] − 295 [SE = 43]X, $R^2 = 0.73$), and a significant effect ($F[4, 20] = 7.35$, $p < 0.001$) was detected in this posture among treatments. There was a significant effect ($F[4, 21] = 9.39, p < 0.0005$) in time spent standing or sitting motionless with eyes open among treatments, and a significant positive correlation ($p < 0.0001$) was observed between the dose of D-Asp and the time spent in this posture (standing/sitting motionless with eyes open [second/10 min] = 56 [SE = 40] − 232 [SE = 41]X, $R^2 = 0.639$). There was no significant effect ($F[4, 22] = 0.86$, $p = 0.506$) in time spent standing motionless with eyes closed. There was no significant effect ($F[4, 22] = 0.74, p = 0.573$) between treatments, and no correlation was found between the dose of D-Asp and time spent on sleep-like behavior.

Table 2 Effects of i.c.v. injection of several doses of D-Asp and CRF on various behavioral categories of 6-day-old chicks exposed to social separation stress for 10 min

CRF (µg)	0	0.01	0.01	0.01	0.01
D-Asp (µmol)	0	0	0.42	0.84	1.68
Active wakefulness	351 ± 90^{a}	561 ± 20^{a}	329 ± 72^{a}	300 ± 59^{ab}	32 ± 12^{b}
Standing/sitting motionless with eyes open	112 ± 36^{b}	39 ± 20^{b}	178 ± 58^{b}	246 ± 55^{ab}	442 ± 68^{a}
Standing motionless with eyes closed	2 ± 2	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Sitting motionless with head drooped (sleeping posture)	136 ± 76	12 ± 12	93 ± 44	54 ± 50	87 ± 72
Total	600	600	600	600	600

Values are mean ± SEM in seconds. The number of chicks used in each group was 4–6

Means with different superscripts were significantly different at $p < 0.05$

Experiment 3: Comparison of the effects of L-Asp with those of D-Asp on the behavior of chicks

Figure 3 shows the effect of i.c.v. injection of several doses of L-Asp or D-Asp with CRF on the number of DVs during 10 min of social separation stress. A significant effect ($F[4, 20] = 4.32$, $p < 0.05$) was detected among treatments. The number of DVs was reduced with increasing doses of L- or D-Asp. Table 3 shows the effect of i.c.v. injection of several doses of L- or D-Asp with CRF on various behaviors of chicks during 10 min behavioral observation under conditions of isolation stress. The time spent in active wakefulness was significantly reduced ($F[4, 20] = 8.38$, $p < 0.001$) with i.c.v. injection of L- or D-Asp. Likewise, i.c.v. injection of either L- or D-Asp significantly increased ($F[4, 21] = 3.36$, $p < 0.05$) the time spent standing or sitting motionless with eyes open. L-Asp caused significant increases ($F[4, 18] = 3.81$, $p < 0.05$) in time spent in sleep-like behavior. Tables 4 and 5 show the effect of i.c.v. injection of several doses of L- or D-Asp with CRF on the monoamine content in the diencephalon and telencephalon. There were no significant effects on levels of monoamines in either brain region among treatments, apart from 5-HIAA in the diencephalon ($F[5, 24] = 3.31$, $p < 0.05$).

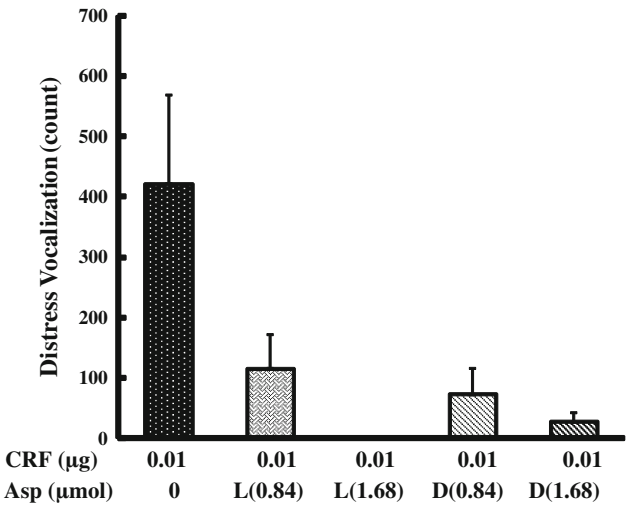

Fig. 3 Effects of L- and D-Asp on DVs in chicks exposed to social separation stress. Values are means with SEM

Discussion

We confirmed that i.c.v. injection of L-Asp can attenuate the stress response even under acute and highly stressful conditions induced by i.c.v. injected CRF and isolation stress (Fig. 1; Table 1). These findings are consistent with an earlier report that i.c.v. injection of L-Asp caused sedative and hypnotic effects under conditions of acute social isolation stress without treatment with CRF (Yamane et al. 2009a). Hamasu et al. (2009a) revealed that several amino acids, including Asp, were decreased in the diencephalon of neonatal chicks exposed to both restraint with isolation and fasting stress. This fact suggests that central free L-Asp might have an important role in the stress response. Significant negative correlations between the dose of L-Asp and a CRF-stimulated stress response (Fig. 1; Table 1) clearly suggest that L-Asp might attenuate CRF-induced stress behaviors.

Even though the function of D-Asp during the stress response in both mammalian and avian brains has not been well clarified, mounting evidence indicates that this D-amino acid may act as a putative neuromodulator or neurotransmitter of the glutamatergic system (Schell et al. 1997; Errico et al. 2008). Here, we investigated whether i.c.v. injection of D-Asp could attenuate the stress response induced by CRF and isolation stress. The i.c.v. injection of D-Asp dose-dependently decreased DVs and time spent in active wakefulness. On the other hand, D-Asp increased the time spent standing/sitting motionless with eyes open in comparison with the group receiving CRF alone. D-Asp and L-Asp both have a clear function regarding stress responses.

Table 3 Effects of i.c.v. injection of several doses of L- and D-Asp and CRF on various behavioral categories of 6-day-old chicks exposed to social separation stress for 10 min

CRF (μg)	0.01	0.01	0.01	0.01	0.01
L-Asp (μmol)	0	0.84	1.68	0	0
D-Asp (μmol)	0	0	0	0.84	1.68
Active wakefulness	406 ± 78^{a}	120 ± 40^{b}	10 ± 3^{b}	122 ± 37^{b}	107 ± 48^{b}
Standing/sitting motionless with eyes open	134 ± 45	322 ± 60	364 ± 42	356 ± 56	356 ± 75
Standing motionless with eyes closed	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Sitting motionless with head drooped (sleeping posture)	30 ± 18^{b}	158 ± 77^{ab}	262 ± 26^{a}	122 ± 43^{ab}	46 ± 22^{b}
Total	600	600	600	600	600

Values are mean ± SEM in seconds. The number of chicks used in each group was 4–6

Means with different superscripts were significantly different at $p < 0.05$

Table 4 Effects of i.c.v. injection of several doses of L- and D-Asp and CRF on monoamine content (pg/mg wet tissue) in the diencephalon of chicks exposed to social separation stress for 10 min

	Intact	CRF (0.01 μg)	CRF (0.01 μg) + L-Asp (0.84 μmol)	CRF (0.01 μg) + L-Asp (1.68 μmol)	CRF (0.01 μg) + D-Asp (0.84 μmol)	CRF (0.01 μg) + D-Asp (1.68 μmol)
DA	1,487 ± 202	1,950 ± 480	1,543 ± 117	2,028 ± 226	1,932 ± 55	1,640 ± 256
DOPAC	42 ± 8	62 ± 12	45 ± 3	58 ± 8	45 ± 3	55 ± 6
HVA	119 ± 16	140 ± 15	105 ± 12	149 ± 30	122 ± 10	111 ± 12
5-HT	497 ± 74	458 ± 60	563 ± 41	443 ± 76	461 ± 36	527 ± 38
5-HIAA	273 ± 10^{b}	395 ± 25^{a}	285 ± 33^{ab}	344 ± 40^{ab}	305 ± 6^{b}	306 ± 14^{ab}

Values are mean ± SEM. The number of chicks used in each group was 4–6

Means with different superscripts were significantly different at $p < 0.05$

Table 5 Effects of i.c.v. injection of several doses of L- and D-Asp and CRF on monoamine content (pg/mg wet tissue) in the telencephalon of 6-day-old chicks exposed to social separation stress for 10 min

	Intact	CRF (0.01 μg)	CRF (0.01 μg) + L-Asp (0.84 μmol)	CRF (0.01 μg) + L-Asp (1.68 μmol)	CRF (0.01 μg) + D-Asp (0.84 μmol)	CRF (0.01 μg) + D-Asp (1.68 μmol)
DA	545 ± 21	608 ± 26	623 ± 26	574 ± 18	613 ± 44	574 ± 18
DOPAC	28 ± 2	32 ± 1	29 ± 2	31 ± 3	28 ± 1	29 ± 4
HVA	66 ± 6^{b}	76 ± 2^{ab}	69 ± 2^{ab}	83 ± 3^{a}	69 ± 2^{ab}	80 ± 4^{ab}
5-HT	441 ± 13	452 ± 23	499 ± 13	453 ± 5	453 ± 30	457 ± 17
5-HIAA	123 ± 8	141 ± 5	133 ± 7	130 ± 4	140 ± 5	145 ± 10

Values are mean ± SEM. The number of chicks used in each group was 4–6

Means with different superscripts were significantly different at $p < 0.05$

In Experiment 3, we confirmed that i.c.v. injection of L- or D-Asp induced sedation in chicks, while the behavioral results for the two isomers of Asp vary. L-Asp decreased the time spent in active wakefulness and increased the time spent standing/sitting with eyes open and the time spent in the sleeping posture. On the other hand, D-Asp decreased the time spent in active wakefulness and increased the time spent standing/sitting with eyes open, while there was no significant effect on sleep-like behavior. According to Hamasu et al. (2010), L-proline and D-proline differentially induced sedative and hypnotic effects through NMDA and glycine receptors, respectively. Differences in receptors between isomers may also be involved in the findings for Asp. The binding sites of NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) are related to the brain regions stimulated during the stress response. In general, the NMDA receptor usually coexists with the AMPA receptor in the postsynaptic membrane (Nadler 2007). The agonist for both the NMDA-R and the AMPA-R induced sedative effects in chicks (Yamane et al. 2009b). L-Asp appears to recognize only NMDA-R, being inactive at the AMPA receptor and probably also at the kainate receptor (Dingledine and McBain 1999), and it can act as a selective NMDA-R agonist in avians (Kubrusly et al. 1998). Hence, it appears likely that the hypnotic and sedative effects of L-Asp may be due to the activation of the NMDA-R. On the other hand, Molinaro et al. (2010) suggest that D-Asp, but not NMDA, activates the metabotropic glutamate receptor 5 (mGluR5) coupled to polyphosphoinositide hydrolysis in early postnatal rat brain slices. The mGluR5 is highly expressed in the early postnatal brain and is also found in the embryonic brain (Di Giorgi Gerevini et al. 2004). The mGluR5 could play various roles in the stress response because stress-induced hyperthermia was reduced in mGluR5 knockout mice (Brodkin et al. 2002). Therefore, behavioral stress responses may be regulated by brain D-Asp to stimulate both NMDA-R and mGluR5, especially in the developmental period. However, these possibilities should be clarified in further experiments. On the other hand, in rodents, activation of excitatory neurotransmission involving the NMDA-R is linked to the stimulation of the HPA-axis (Zelena et al. 2005). Therefore, further work should be done to investigate whether L- and D-Asp in chicks possess same physiological functions, by focusing on, for example, secretion of corticosterone, a major glucocorticoid in avians.

When focusing on monoamine levels in the brain, no significant changes were observed, except for that involving 5-HIAA in the diencephalon. Although some experiments suggested that the NMDA-R stimulation is linked to the regulation of the monoaminergic system in the brain (Hamasu et al. 2009b; Karlsson et al. 2006; Hanania and

Zahniser 2002), we observed a significant effect only on 5-HIAA in the diencephalon. Thus, it remains unclear whether there is a relationship between L- or D-Asp and monoamines, because their levels in the brain do not always reflect the activity of their neurons. Further study focusing on monoaminergic systems in the brain is necessary.

Focusing on injected solutions, the pH decreased to some extent with L-Asp and with D-Asp (saline 5.93, CRF 5.73, L-Asp [0.42 μmol] + CRF 3.22, L-Asp [0.84 μmol] + CRF 3.14, L-Asp [1.68 μmol] + CRF 3.16, D-Asp [0.42 μmol] + CRF 3.25, D-Asp [0.84 μmol] + CRF 3.12, D-Asp [1.68 μmol] + CRF 3.15). However, the influence seems minimal when focusing on the difference in effects between L- and D-Asp, which was the major concern in the present study. On the other hand, in terms of the interpretation of the dose-dependent behavioral effects of L- or D-Asp, we cannot deny the possibility that a decrease of pH might affect the results, even though the dose-dependent decrease rate of pH seems slight compared with the dose-dependent behavioral effects of L- and D-Asp. It is possible that changes in pH themselves influenced the results by affecting, for example, receptor sensitivity. Further studies therefore need to be carried out.

In conclusion, central administration of L-Asp attenuated stress-induced behaviors in neonatal chicks. Furthermore, it was demonstrated for the first time that D-Asp seems to have a similar, although not identical, sedative effect. Further investigation focusing on NMDA-R, mGluR5, and monoamines may be necessary to gain a better understanding of the actions and mechanisms of L- and D-Asp in the brain.

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