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Central L-ornithine, but not polyamines, induces a hypnotic effect in neonatal chicks under acute stress

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ABSTRACT

To clarify whether L-ornithine and/or its metabolite involves sedative and hypnotic effects under social separation stress, the effects of intracerebroventricular (i.c.v.) injection of L-ornithine and polyamines (putrescine, spermidine and spermine) were compared in chicks. Birds were injected i.c.v. with 0.5 μmol of L-ornithine, putrescine, spermidine, spermine or saline (control). After injection, chicks were immediately separated from the flock and monitored for the number of distress vocalizations and various postures. L-Ornithine greatly attenuated the stress response and caused sedative and hypnotic effects. Among the polyamines, only putrescine attenuated distress vocalizations but did not induce sleep. In conclusion, the sedative and hypnotic effect of L- ornithine was mainly induced by L-ornithine itself, while the polyamines contributed to the sedative, but not hypnotic, effect under social separation stress.

Keywords: L-Ornithine, putrescine, polyamine, intracerebroventricular injection, social separation stress, neonatal chick

Introduction

L-Arginine (2-amino-5-guanidinovaleric acid) exerts its metabolic roles through the production of diverse metabolites including nitric oxide (NO), L-ornithine, polyamines, L-proline, L-glutamate, 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 isolation stress (Suenaga et al., 2008a). 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.

In mammals, the i.c.v. injection of γ–amino butyric acid (GABA) increased slow-wave sleep with no rebound effect (Karadzic, 1966). Among L-arginine metabolites, agmatine has a guanidino component. Guanidino compounds are known to have a relationship to $GABA_A$ receptors (Neu et al., 2002). Creatine, which has a guanidino component, attenuates the response under social separation stress by acting through $GABA_A$ receptors (Koga et al., 2005). However, i.c.v. injection of agmatine did not attenuate the response under social separation stress (Suenaga et al., 2008b). L-Proline induced a sedative and hypnotic effect (Hamasu et al., 2009a) acting partially through the *N*-methyl-D-aspartate glutamate receptor (NMDA receptor) (Hamasu et al., 2009b). L-Glutamate also induced a sedative and hypnotic effect acting through NMDA and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors (Yamane et al., 2009).

Among L-arginine metabolites, only L-ornithine had a sedative and hypnotic effect after i.c.v. injection, and the L-ornithine level was increased in the brain after i.c.v. injection of L-arginine (Suenaga et al., 2008b). This fact implies that L-ornithine is a major mediator of L-arginine-induced reduction in the stress response under social separation stress.

Polyamines such as putrescine, spermidine and spermine are small ubiquitous cationic molecules required for cell growth and homeostasis (Pegg and McCann, 1982; Tabor and Tabor, 1984). L-Ornithine is converted to pro-proliferative polyamines via ornithine decarboxylase (ODC), and to proline, a constituent of the extracellular matrix, via ornithine aminotransferase. ODC, one of the most highly regulated eukaryotic enzymes, is the first and rate limiting enzyme of biosynthesis of ornithine to putrescine, the first polyamine. Spermidine is synthesized from putrescine using an aminopropylic group from decarboxylated S-adenosyl-L-methionine. The reaction is catalyzed by spermidine synthase. Spermine is synthesized from the reaction of spermidine with S-adenosyl-L-methionine in the presence of the enzyme spermine synthase.

The effect of polyamines on the stress response has not been extensively examined. Hayashi et al. (2004) suggested that polyamine metabolism is linked to psychological stress in mice. On the other hand, Hamasu et al. (2009a) reported that L-arginine and L-proline were decreased in the telencephalon and diencephalon of chicks exposed to either restraint with isolation-induced or fasting stress. These facts suggest that L-proline production from L-ornithine via ornithine aminotransferase is low and polyamine production from L-ornithine via ODC may be dominant in the brain under stressful conditions.

Taken together, the sedative and hypnotic effect of L-ornithine may be associated with its polyamine metabolites. To clarify this hypothesis, in the present study we compared the effect of L-ornithine and its metabolites putrescine, spermidine and spermine under the social separation stress model.

Materials and methods

Animals and food

Day-old male layer chicks (Julia; Murata Hatchery, Fukuoka, Japan) were housed

in a wire-meshed cage (50 x 35 x 33 cm) in a group (20-25 birds) at a constant temperature of 30±1°C and continuous light until the experimental day. Chicks were the same age and housed without an adult. Feed (AX, Toyohashi Feed and Mills Co. Ltd., Aichi, Japan) and water were available *ad libitum*. On the day of the experiment, chicks (4-5 days old) were assigned to five 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-Ornithine monohydrochloride (Kyowa Hakko Bio Co., LTD, Tokyo, Japan), putrescine (1,4-butanediammonium dichloride) and spermidine trihydrochloride (Wako, Osaka, Japan), and spermine tetrahydrochloride (Calbiochem, Darmstadt, Germany) were used in the present study. Drugs were dissolved in 0.85% saline containing a 0.1% Evans Blue solution, which served as the control.

Experimental procedure

Drugs were injected i.c.v. into the left lateral ventricle of 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, chicks were immediately and gently placed alone into acrylic glass chambers (40 x 30 x 20 cm) with paper on the floor for 10 min in a separate room at a constant temperature of 30°C. They were deprived of water and diet, and vocalizations were recorded. 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.

Birds were injected i.c.v. with 0.5 μmol of L-ornithine, putrescine, spermidine or spermine. Saline was injected i.c.v. for the control group.

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.

Statistical analysis

Data for distress vocalization were statistically analyzed by repeated measure two-way analysis of variance (ANOVA) and for the postures were analyzed by one-way ANOVA. When significant effects were determined, comparisons between means were made using Fisher's LSD as a post hoc test. 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, NC, U.S.A., 1998). All data were first subjected to Grubs-Smirnov rejection test to eliminate outliers, and the remaining data were used.

Results

Fig. 1 shows the effect of i.c.v. injection of L-ornithine and polymines on the number of vocalizations in chicks during the 10 min social separation stress. The effect of L-ornithine and polymines on the number of vocalizations was significant ($F(4, 35)=6.050$, $P<0.001$). In the overall means, L-ornithine and putrescine significantly

lowered the number of distress vocalization compared with the control and spermidine. The values for L-ornithine were the lowest and those for putrescine were followed. There were a significant effect ($F(36, 315)=2.619$, $P<0.01$) of time and an interaction ($F(36, 315)=1.901$, $P<0.01$) between drugs and time. The control birds constantly vocalized at a rate of approximately 80 per min over the experimental period while the values for other groups were lower at the commencement of separation. Vocalizations of chicks receiving L-ornithine remained lower through 10 min with the putrescine group being the next lowest. The vocalization rate of chicks receiving spermidine and spermine gradually increased.

Table I shows the effect of i.c.v. injection of L-ornithine and polyamines on various behavioral categories of chicks during a 10 min behavioral observation during social separation stress. Active wakefulness was significantly ($F(4, 35)=4.957$, $P<0.01$) decreased by L-ornithine compared with other groups. The reverse was true for sleeping posture with the L-ornithine group having the highest value ($F(4, 35)=2.819$, $P<0.05$). These facts suggest that a hypnotic effect was induced by L-ornithine, but not by its metabolites. Time for standing/sitting motionless with eyes open was longest in the putrescine group followed by the L-ornithine group ($F(4, 35)=3.113$, $P<0.05$), indicating that putrescine and L-ornithine have a sedative effect.

Discussion

Suenaga et al. (2008b) reported that i.c.v. injected L-arginine increased both L-arginine and L-ornithine concentrations of the telencephalon and diencephalon of chicks 10 min post-injection. In addition, L-ornithine concentration was proportionally increased by L-arginine injection, suggesting that L-arginine was metabolized by arginase in the brain. They concluded that the sedative and hypnotic effects of L-arginine were mainly caused by L-ornithine. In addition, various other amino acids increased in the telencephalon following L-arginine injection (Suenaga et

al., 2008b). Increased amino acids in the brain including L-alanine (Kurauchi et al., 2006), L-proline (Hamasu et al., 2009a) and L-glutamate (Yamane et al., 2009) have been observed to have sedative or hypnotic effects. These results suggest that L-arginine and its metabolites have an important role in the CNS.

In the present study, the sedative and hypnotic effects of L-ornithine were confirmed as reported by Suenaga et al. (2008b). However, beneficial effects of polyamines on the stress response were not clear except for putrescine on distress vocalizations. Although putrescine is the first polyamine synthesized from ornithine and may be partly involved in sedative effect of L-ornithine, it is suggested that the sedative and hypnotic effects of L-ornithine are induced by L-ornithine itself and not its metabolite polyamines. Putrescine synthesis does not have to occur via L-ornithine, but can also be synthesized via agmatine. Arginine decarboxylase converts arginine to agmatine. However, agmatine did not attenuate the stress response under an acute stress (Suenaga et al., 2008b). Further studies are needed to clarify the mechanism by which L-ornithine induces its hypnotic effect, and both L-ornithine and putrescine induce a sedative effect under acute stress conditions.

One of the polyamines, putrescine, induced an anxiolytic effect as evidenced from the reduction in distress vocalization. However, further metabolized forms of putrescine, i.e., spermidine and spermine, did not show any functions in the stress response. None of the polyamines induced the hypnotic effect in the present study.

In conclusion, the hypnotic effect induced by i.c.v. L-arginine may be mediated by its metabolism to L-ornithine, but not polyamines.

Acknowledgements

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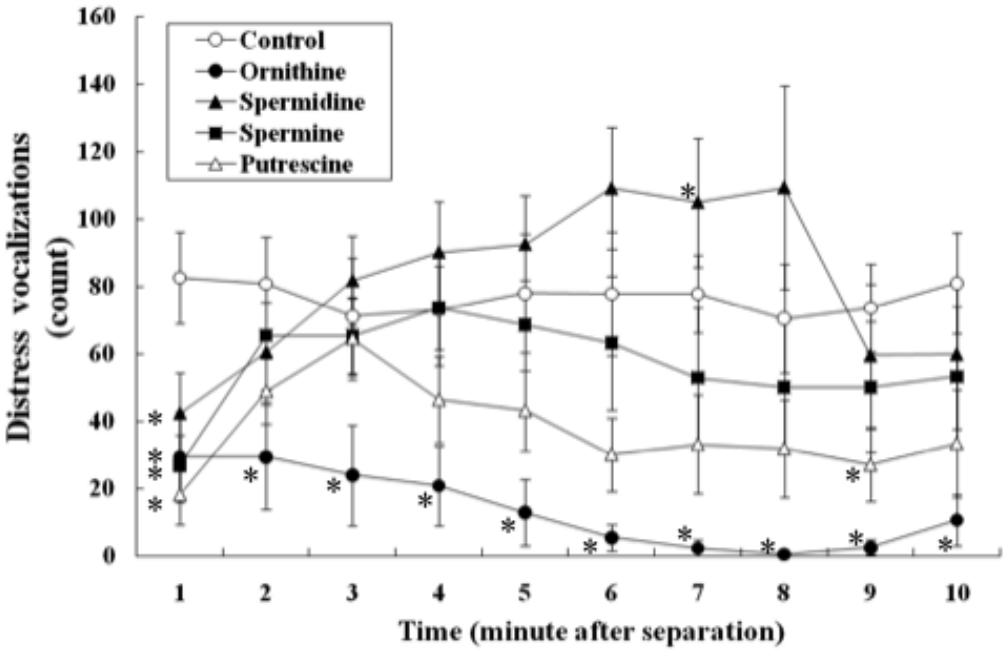
Legends to figures

Fig. 1. Effect of i.c.v. injection of L-ornithine and polyamines on vocalizations during a 10 min social separation stress in layer chicks (4- or 5-day-old). Values are means with S.E.M. The number of chicks used in each group was 8. * Significantly different from the control at $P<0.05$.


Control
Ornithine
Spermidine
Spermine
Putrescine
Distress vocalizations (count)
160
140
120
100
80
60
40
20
0
1
2
3
4
5
6
7
8
9
10
Time (minute after separation)

1

Table I.

Effect of L-ornithine and polyamines (0.5 μmol) on various behavioral categories of chicks (4- or 5-day-old) exposed to social separation stress for 10 min after i.c.v. injection[1,2]

	Control	L-Ornithine	Putrescine	Spermidine	Spermine
Active wakefulness	437±70[a]	114±37[b]	302±52[a]	443±66[a]	380±73[a]
Standing/sitting motionless with eyes open	82±31[b]	179±37[a,b]	223±44[a]	93±18[b]	107±37[b]
Standing motionless with eyes closed	9±9	30±16	19±17	0±0	0±0
Sitting motionless with head drooped (sleeping posture)	72±48[b]	277±68[a]	56±28[b]	64±64[b]	113±57[b]
Total	600	600	600	600	600

[1] Values are mean±S.E.M. in seconds.

[2] The number of chicks used in each group was 8.

[a,b] Values within a row with different superscripts are significantly different ($P<0.05$).

1