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Comparison of Central Effects of L-Ornithine Metabolites on the Stress Responses of Neonatal Chicks

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Abstract: Recently, we observed that central administration of L-arginine attenuated the stress responses of neonatal chicks by inducing a sedative and hypnotic effect. In addition, L-ornithine, which is produced from L-arginine in the brain, appeared to interact with L-arginine during a stress response. Several putative metabolites from L-ornithine, including L-citrulline and D-ornithine, were therefore investigated in the present study. The effects of intracerebroventricular injection of L-ornithine, L-citrulline and D-ornithine were compared in chicks under an isolation-induced stress. L-ornithine greatly attenuated the stress response and induced sedative and hypnotic effects. D-ornithine weakly attenuated the stress responses, while L-citrulline had no effect.

Key words: L-ornithine, D-ornithine, L-citrulline, intracerebroventricular injection, social separation stress, neonatal chick

INTRODUCTION

L-arginine is classified as an essential amino acid for birds, carnivores and young mammals and is a conditionally essential amino acid for adult humans Recently, we observed that intracerebroventricular (i.c.v.) injection of L-arginine induced sedative and/or hypnotic effects in chicks exposed to a social isolation stress (Suenaga *et al*., 2008a). 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). Among these metabolites, i.c.v. injections of L-ornithine (Suenaga *et al*., 2008b), L-proline (Hamasu *et al*., 2009a, b), L-glutamate (Yamane *et al*., 2009b) and L-glutamate had a similar function as observed by i.c.v. injection of L-arginine (Suenaga *et al*., 2008a), but the contribution of NO (Suenaga *et al*., 2008a) and agmatine was low (Suenaga *et al*., 2008b).

L-ornithine is converted to pro-proliferative polyamines via ornithine decarboxylase. 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). We showed that polyamines, unlike L-ornithine, do not induce hypnotic effects, whereas only putrescine caused a sedative effect among three polyamines (Kurauchi). Other L-ornithine metabolites include L-citrulline and D-ornithine. Following condensation with carbamyl phosphate, L-ornithine is converted to L-citrulline in the ornithine transcarbamylase reaction. D-Ornithine is produced from L-ornithine through the action of ornithine racemase (EC 5.1.1.12).

There is no information on the contribution of L-citrulline or D-ornithine on the sedative and hypnotic effect of L-ornithine. The effect of L-citrulline and D-ornithine was compared with that of L-ornithine in neonatal chicks under an acute stressful condition.

MATERIALS AND METHODS

Animals and food: Day-old male layer chicks (Julia; Murata Hatchery, Fukuoka, Japan) were 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 continuous light, until the experimental day. Chicks were the same age and

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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, 4-5 days of age 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-ornithine monohydrochloride was provided by Kyowa Hakko Bio Co., Ltd. (Tokyo, Japan). L-citrulline (Sigma, St. Louis, MO, USA) and D-ornithine monohydrochloride (Wako, Osaka, Japan) were used in the present study. 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, chicks were immediately and gently individually placed into acrylic glass chambers (40×30×20 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 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: active wakefulness; standing/sitting motionless with eyes open; standing motionless with eyes closed; 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, L-citrulline and D-ornithine. Saline was used as a control.

At the conclusion of the behavioral observations, 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 spontaneous activity and distress vocalization were statistically analyzed by repeated measure two-way Analysis of Variance (ANOVA) and 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±SEM. Statistical analysis was made using a commercially available package, StatView (1998). All data were first subjected to Grubs-Smirnov rejection test to eliminate outliers. The remaining data were used.

RESULTS

Figure 1 shows, the effect of i.c.v. injection of L-ornithine, L-citrulline and D-ornithine on spontaneous activity (upper panel) and the number of distress vocalizations (lower panel) in chicks during the 10 min social separation stress. There was no significant effect of drugs ($F(3,24) = 2.240$, $p>0.05$), time after injection ($F(9,216) = 1.001$, $p>0.05$), or interaction between drugs and time ($F(27,216) = 0.873$, $p>0.05$) on spontaneous activity. For distress vocalizations, a significant effect of drugs ($F(3,24) = 4.024$, $p<0.05$) and an

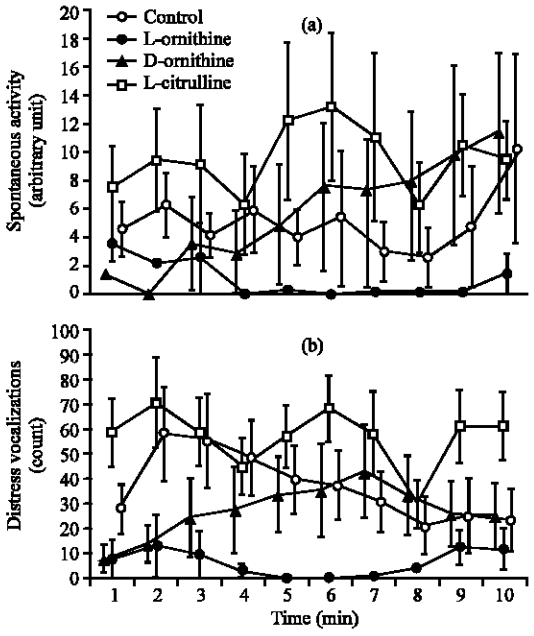

Fig. 1: (a and b) Effect of i.c.v. injection of L-ornithine, L-citrulline and D-ornithine on spontaneous activity (upper panel) and vocalizations (lower panel) during a 10 min social separation stress in neonatal chicks. Values are means with SEM. The number of chicks used in each group was 7

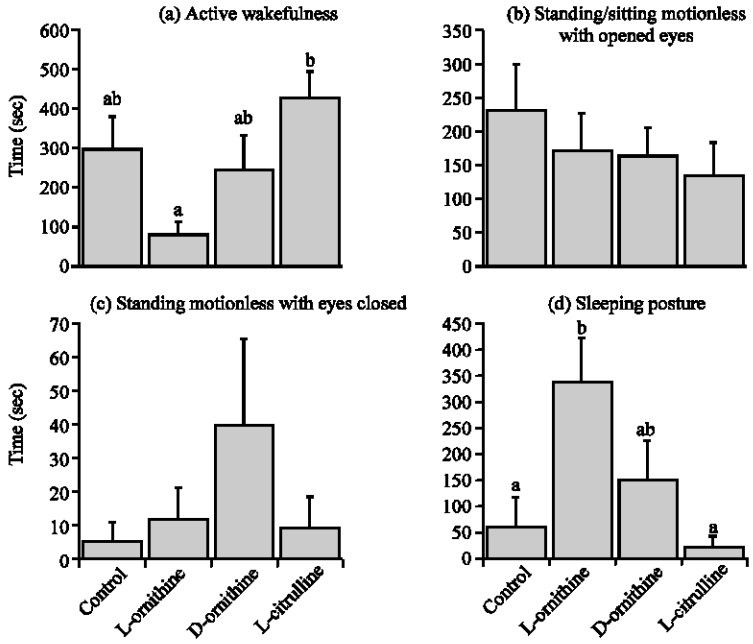

Fig. 2: Effect of i.c.v. injection of L-ornithine, L-citrulline and D-ornithine on various behaviors of neonatal chicks. Values are means with SEM. Different letters indicate significant differences at $p<0.05$. The number of chicks used in each group was 7

interaction between drugs and time ($F(27,216) = 1.855$, $p<0.01$) were detected. No significant ($F(9,216) = 1.650$, $p>0.05$) effect of time was observed. L-ornithine decreased the numbers of distress vocalizations compared to L-citrulline. L-ornithine strongly inhibited distress vocalization through 10 min post-injection whereas the inhibition caused by D-ornithine rapidly disappeared.

Figure 2 shows, the responses within the various behavioral categories during the 10 min social separation stress following the i.c.v. injection of L-ornithine, L-citrulline or D-ornithine. Significant effects were observed in active wakefulness ($F(3,24) = 4.635$, $p<0.05$) and sleeping posture ($F(3,24) = 4.642$, $p<0.05$), while no effect was observed in standing/sitting motionless with eyes open ($F(3,24) = 0.598$, $p>0.05$) or standing motionless with eyes closed ($F(3,24) = 1.115$, $p>0.05$). Time for active wakefulness was significantly ($p<0.05$) decreased by L-ornithine compared to L-citrulline. In sleeping posture, time for L-ornithine was significantly longer than that for the control or L-citrulline groups.

DISCUSSION

Suenaga *et al.* (2008b) concluded that L-ornithine, produced by arginase from L-arginine in the brain, mainly plays an important role in the sedative and hypnotic effects of L-arginine observed during a stress response. However, L-ornithine is further metabolized to L-citrulline and D-ornithine. Accordingly to clarify the contribution of L-ornithine metabolites on the sedative and hypnotic effect of L-ornithine, we compared the effect of L-ornithine, L-citrulline and D-ornithine in the present study.

First, there is an influence of the optic isomerism. For instance, L-serine might be converted to the enantiomer D-serine by serine racemase, which was purified from the mammalian brain (Konno, 2003; Wolosker *et al.*, 1999). D-serine is present in the brain of several vertebrate species including carp, frog, mice, rat and chick (Nagata *et al.*, 1994) and is in particularly high concentration in the mammalian brain where it acts as an endogenous ligand for an N-Methyl-D-Aspatate (NMDA) receptor-related glycine site (Contreras, 1990). According to Asechi *et al.* (2006), the i.c.v. injection of L-serine and its derivatives, including glycine and L-cysteine, induced sedative and hypnotic effects under isolation-induced stress in neonatal chicks. However, D-serine did not show any effects.

Recently, it was demonstrated that L-serine induces sedative and hypnotic effects by enhancing inhibitory neurotransmission via $GABA_A$ receptors (Shigemi *et al.*, 2008). This fact implies that the optic isomerism alters the receptor, at which each amino acid acts. This was the case for proline, since L-and D-proline differentially induce

sedative and hypnotic effects through NMDA and glycine receptors, respectively (Hamasu *et al*., 2009b). On the other hand, the optic isomerism influenced the efficacy for the sedative and hypnotic effect. The i.c.v. injection of L- and D-cysteine decreased both distress vocalization and spontaneous activity induced by isolation (Yamane *et al*., 2009a). However, the two cysteine isomers induced different behaviors. L-cysteine increased sleep-like behavior, while D-cysteine caused abnormal behavior including syncope as well as sleep-like behavior. In the present study, the hypnotic effect of D-ornithine was weaker than that of L-ornithine. To the researchers knowledge, the presence of D-ornithine and/or ornithine racemase in the brain has been unclear. Therefore, sedation and hypnosis induced by ornithine was mainly regulated by the L-form.

Second, L-citrulline is made from L-ornithine and carbamoyl phosphate in one of the central reactions in the urea cycle. It is also produced, from arginine as a by-product of the reaction catalyzed by NO Synthase (NOS) family. L-arginine is first oxidized into N-hydroxyl-arginine, which is then further oxidized to citrulline concomitant with the release of nitric oxide. In the latter case, the i.c.v. injection of L-arginine had no effect on NO x (NO_2 + NO_3) concentration, an index of NO production, at various brain sites (Suenaga *et al*., 2008a). These results suggest that the contribution of NO generation via NOS may be low in the sedative and hypnotic actions of L-arginine. In other words, the effect of L-arginine was not associated with the production of L-citrulline through the NO pathway. In the former case, we have not confirmed that central L-citrulline concentration increased after i.c.v. injection of L-ornithine, but we confirmed that i.c.v. injection of L-arginine dose-dependently increased brain L-ornithine levels (Suenaga *et al*., 2008b). In their study, however, L-citrulline levels were increased by L-arginine but this effect was not significant ($F(2,17) = 1.570$, $p>0.05$). In the present study, no sedative and hypnotic effects were confirmed in L-citrulline. Accordingly, if L-citrulline was produced from L-ornithine, it was not involved with the sedative and hypnotic effect. Kurauchi found that polyamines, which are metabolites of L-ornithine had no hypnotic effect. Among putrescine, spermidine and spermine, only putrescine caused the sedative effects. Therefore, taken together, L-ornithine, but not its metabolites, is a major regulator for sedation and hypnosis in the brain.

CONCLUSION

It appears that L-ornithine, but not its metabolites, is a major regulator in the brain for sedation and hypnosis.

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REFERENCES

Asechi, M., S. Tomonaga, T. Tachibana, L. Han, K. Hayamizu, D.M. Denbow and M. Furuse, 2006. Intracerebroventricular injection of L-serine analogs and derivatives induces sedative and hypnotic effects under an acute stressful condition in neonatal chicks. Behav. Brain Res., 170: 71-77. PMID: 16574253.

Contreras, P.C., 1990. D-Serine antagonized phencyclidine and MK-801-induced stereotyped behavior and ataxia. Neuropharmacology, 29: 291-293. PMID: 2109276.

Davis, J.L., D.T. Masuoka, L.K. Gerbrandt and A. Cherkin, 1979. Autoradiographic distribution of L-proline in chicks after intracerebral injection. Physiol. Behav., 22: 693-695. PMID: 482410.

Hamasu, K., T. Haraguchi, Y. Kabuki, N. Adachi, S. Tomonaga, H. Sato, D.M. Denbow and M. Furuse, 2009a. L-proline is a sedative regulator of acute stress in the brain of neonatal chicks. Amino Acids, 37: 377-382. PMID: 18696178.

Hamasu, K., K. Shigemi, Y. Tsuneyoshi, H. Yamane, H. Sato, D.M. Denbow and M. Furuse, 2009b. Intracerebroventricular injection of L-proline and D-proline induces sedative and hypnotic effects by different mechanisms under an acute stressful condition in chicks. Amino Acids in Press. PMID: 19023642.

Konno, R., 2003. Rat cerebral serine racemase: Amino acid deletion and truncation at carboxy terminus. Neurosci. Lett., 349: 111-114. PMID: 12946565.

Koutoku, T., H. Takahashi, S. Tomonaga, D. Oikawa, S. Saito, T. Tachibana, L. Han, K. Hayamizu, D.M. Denbow and M. Furuse, 2005. Central administration of phosphatidylserine attenuates isolation stress-induced behavior in chicks. Neurochem. Int., 47: 183-189. PMID: 15916832.

Morris, S.M. Jr., 2004. Enzymes of arginine metabolism. J. Nutr., 134: 2743S-2747S. PMID: 15465778.

Nagata, Y., K. Horiike and T. Maeda, 1994. Distribution of free D-serine in vertebrate brains. Brain Res., 634: 291-295. PMID: 8131079.

Pegg, A.E. and P.P. McCann, 1982. Polyamine metabolism and function. Am. J. Physiol., 243: C212-C221. PMID: 6814260.

StatView, 1998. Version 5, SAS Institute, Cary USA.

Shigemi, K., Y. Tsuneyoshi, K. Hamasu, L. Han, K. Hayamizu, D.M. Denbow and M. Furuse, 2008. L-Serine induces sedative-hypnotic effects via $GABA_A$ receptor in neonatal chicks. Eur. J. Pharmacol., 599: 86-90. PMID: 18929557.

Suenaga, R., S. Tomonaga, H. Yamane, I. Kurauchi, Y. Tsuneyoshi, H. Sato, D.M. Denbow and M. Furuse, 2008a. Intracerebroventricular injection of L-arginine induces sedative and hypnotic effects under an acute stress in neonatal chicks. Amino Acids, 35: 139-146. PMID: 18163184.

Suenaga, R., H. Yamane, S. Tomonaga, M. Asechi, N. Adachi, Y. Tsuneyoshi, I. Kurauchi, H. Sato, D.M. Denbow and M. Furuse, 2008b. Central L-arginine reduced stress responses are mediated by L-ornithine in neonatal chicks. Amino Acids, 35: 107-113. PMID: 18219550.

Tabor, C.W. and H. Tabor, 1984. Polyamines. Annu. Rev. Biochem., 53: 749-790. PMID: 6206782.

van Luijtelaar, E.L.J.M., Van der C.P.M. Grinten, H.J. Blokhuis and A.M.L. Coenen, 1987. Sleep in the domestic hen (*Gallus domesticus*). Physiol. Behav., 41: 409-414. PMID: 3432393.

Wolosker, H., S. Blackshaw and S.H. Snyder, 1999. Serine racemase: A glial enzyme synthesizing D-serine to regulate glutamate-N-methyl-D-aspartate neurotransmission. Proc. Natl. Acad. Sci. USA., 96: 13409-13414. PMID: 10557334.

Yamane, H., M. Asechi, Y. Tsuneyoshi, D.M. Denbow and M. Furuse, 2009a. Central central L-cysteine induces sleep and D-cysteine induces sleep and abnormal behavior during acute stress in neonatal chicks. Anim. Sci. J., 80: 428-432. DOI: 10.1111/j.1740-0929.2009.00647.x.

Yamane, H., Y. Tsuneyoshi, D.M. Denbow and M. Furuse, 2009b. N-Methyl-D-aspartate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate receptors involved in the induction of sedative effects under an acute stress in neonatal chicks. Amino Acids in Press. PMID: 19018608.