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Reverse structure of carnosine-induced sedative and hypnotic effects in the chick under acute stress

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ABSTRACT

In the central nervous system, β-alanine is thought to act as an inhibitory neurotransmitter, but the role or precise mechanism of β-alanine in the brain has not been clearly defined. β-Alanine is found in high levels in the chicken brain as a component of the dipeptides carnosine (β-alanyl-L-histidine) and anserine, or as a free amino acid. We focused on the position of β-alanine, i.e., at the carboxyl terminus. In Experiment 1, the central effects of glycyl-β-alanine, L-histidyl-β-alanine and L-valyl-β-alanine were compared with a saline control in chicks. L-Histidyl-β-alanine significantly induced sedative and hypnotic effects. In Experiment 2, the effects of carnosine, its reverse (L-histidyl-β-alanine), and their combination were investigated. Central carnosine-induced hyperactivity while reverse carnosine-induced hypoactivity, and the behaviors were intermediate following the combination of the two peptides. Finally, the central effect of reverse carnosine was compared with β-alanine alone and L-seryl-β-alanine in Experiment 3. Reverse carnosine showed similar effects to β-alanine. In conclusion, L-histidyl-β-alanine not only has the reverse structure of carnosine, but also reverse function. Thus, we propose to name reverse carnosine (L-histidyl-β-alanine) rev-carnosine.

Introduction

In the central nervous system (CNS), glycine and γ-aminobutyric acid (GABA) act as inhibitory neurotransmitters which bind to the strychnine-sensitive glycine receptors and GABA, respectively (Donato and Nistri, 2000). β-Alanine is a structural analog of GABA and glycine (Choquet and Korn, 1988), and is naturally found in the vertebrate CNS. Furthermore, it has been found that β-alanine can be taken up into vesicles by the same transporter as GABA and glycine, and be released in a Ca^{2+}-dependent manner (Fykse and Fonnum, 1996; Saransaari and Oja, 1993). Thus, it has been suggested that β-alanine can activate the glycine and $GABA_A$ receptors (Wu et al., 1993), and act as an inhibitory transmitter in certain CNS regions (Fykse and Fonnum, 1996). In fact, Tomonaga et al. (2004) demonstrated that intracerebroventricular (i.c.v.) injection of β-alanine induced hypoactivity manifested as sleep-like behavior in the chick brain.

However, the role or precise mechanism of β-alanine has not been clarified in the brain. β-Alanine is rich in the brain and breast muscle of chickens as components of carnosine (β-alanyl-L-histidne) and anserine (β-alanyl-1-methyl-L-histidine), or as a free amino acids (Tomonaga et al., 2005a) even though β-alanine is a non-proteinaceous amino acid. Carnosine is thought to be a putative neurotransmitter in olfactory receptor neurons (Bonfanti et al., 1999), and we previously demonstrated that i.c.v. injection of carnosine-induced hyperactivity such as increasing spontaneous activity and distress vocalization in chicks (Tomonaga et al., 2004). Therefore, the effects of β-alanine and β-alanyl-dipeptides are different in the CNS. So we focused on the specificity of β-alanine, and speculated that β-alanine related dipeptides could function as neurotransmitter in the brain. We recently reported the effect of carnosine related dipeptides in which the carboxyl terminal histidine was replaced with other amino acids. β-Alanyl-branched chain amino acids induced hyperactivity in chicks as observed in carnosine (Tsuneyoshi et al., 2007). Accordingly, we investigated carboxyl β-alanine related dipeptides in which terminal amino acids were replaced with other amino acids in the present study. The experiments were done by screening these dipeptides with special reference to their effects on behavior in chicks.

Materials and methods

Animals and food

Day-old male layer chicks (Julia strain) were purchased from a local hatchery (Murata Hatchery, Fukuoka, Japan). The chicks were maintained in a windowless room at constant temperature 30±1 °C and continuous lighting. We have confirmed that chicks reared in a

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group under no light-off conditions kept plasma corticosterone levels very low. However, when they were separated from the group, their plasma corticosterone levels greatly enhanced. Therefore, we believed that our lighting condition may have not serious problem for the stress. We have also reported that chicks slept well under these conditions (Saito et al., 2003). Food (Toyohashi Feed and Mills Co. Ltd., Aichi, Japan) and water were freely accessible. The chicks were raised in a group (20–25 per cage) until being placed on experiment. On the day of the experiment, the chicks (5- or 6-old-day) were distributed into experimental groups based on their body weight so that the average body weight of each group was as uniform as possible. Experimental procedures followed the guides for animal experiments in the Faculty of Agriculture and the Graduate Course of Kyushu University, as well as the Law (No. 105) and Notification (No. 6) of the Government.

Preparation of drugs

Glycyl-β-alanine (Gly-β-Ala), L-valyl-β-alanine (L-Val-β-Ala), L-histidyl-β-alanine (L-His-β-Ala) and L-seryl-β-alanine (L-Ser-β-Ala) were gifted from Kyowa Hakko Kogyo (Tokyo, Japan). β-Alanine and carnosine were purchased from Sigma (St. Louis, MO, USA). Drugs were dissolved in 0.85% saline containing 0.1% Evans Blue solution and control groups were given the saline solution. The i.c.v. injections were 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 are minimal as described elsewhere (Koutoku et al., 2005). The molality and volume applied were determined by a previous study (Tsuneyoshi et al., 2007). A head holder with a hole in the head plate accommodated the 26-gauge needle of a Hamilton microsyringe directed into the lateral ventricle was used to make i.c.v. injections. The injection depth was approximately 0.6 cm from the bottom of the head plate. At the end of the experiment, the birds were sacrificed with an overdose of sodium pentobarbital after which the location of the injection was verified. Data from individuals not having Evans Blue dye present in the lateral ventricle were deleted.

Experimental procedure

In Experiment 1, the effects of Gly-β-Ala, L-Val-β-Ala and L-His-β-Ala injections were investigated. Chicks were divided into four groups, and given i.c.v. injections of saline as a control and 2.8 μmol of Gly-β-Ala, L-Val-β-Ala and L-His-β-Ala. Tomonaga et al. (2004) investigated the effect of i.c.v. injection of several doses (0, 0.8, 3.2 and 6.4 μmol) of carnosine on behavior. The highest dose (6.4 μmol) of carnosine caused abnormal behavior, and the second dose (3.2 μmol) of carnosine-induced hyperactivity. Thereafter, we have used the level around the 3.2 μmol for chick behavior and previously confirmed that the effect of i.c.v. injected β-alanyl-BCAAs caused hyperactivity in chicks (Tsuneyoshi et al., 2007). Then, we also selected this dose in the present study. After the injection, chicks were immediately placed in an acrylic monitoring cage (*W*40 cm×*D*30 cm×*H*20 cm), and behavioral observations were made for 10 min. The monitoring systems were set in a separate room to avoid disturbing the chick with man and other chicks. Spontaneous activity was automatically determined utilizing infrared beam sensors (Neuroscience Inc., Tokyo, Japan) placed above the center of the monitoring cage and analyzed by the software DAS-008 (Neuroscience Inc.). The measuring device nonspecifically counted chick behavior when chick moved, stood, sit and so on. The numbers of distress vocalization, which are shrill and intense calls, were simultaneously recorded and counted, using a computer with Gretchen software (Excla, Inc.). Feltenstein et al. (2003) demonstrated that the number of distress vocalization in isolated condition significantly increased when compared to in the social group. Chicks were recorded by three video cameras positioned in different directions. Based on the method by van Luijtelaar et al. (1987), the behavior was classified into four categories by watching the videotapes: (1) active wakefulness; (2) standing/sitting motionless with eyes opened; (3) standing motionless with eyes closed; and (4) sitting motionless with the head drooped (sleeping posture). A direct correlation exists between sleeping posture and electrophysiological sleep with EEG measurement (van Luijtelaar et al., 1987). We defined a sedative effect as a total of the classified behaviors from (2) to (4) and a hypnotic effect as the classified behavior (4). During the monitoring period, chicks were not given food or water.

Experiment 2 was done to investigate the combination effect of carnosine and L-His-β-Ala which caused behavioral effects opposite of carnosine in Experiment 1. Chicks were given i.c.v. injections of saline, carnosine (2.8 μmol), L-His-β-Ala (2.8 μmol) or carnosine (2.8 μmol) plus L-His-β-Ala (2.8 μmol). Spontaneous activity, distress vocalization and behavior observation are analyzed as described in Experiment 1.

In Experiment 3, we investigated the effect of i.c.v. injection of saline, β-Ala (2.8 μmol), L-His-β-Ala (2.8 μmol) and L-Ser-β-Ala (2.8 μmol). β-Alanine and L-serine are known to induce hypoactivity in chicks (Tomonaga et al., 2004; Asech et al., 2006). Thus, the effect of dipeptide by these amino acids was investigated. Spontaneous activity, distress vocalization and behavior observation are analyzed as described in Experiment 1.

Statistical analysis

Data were statistically analyzed by one-way analysis of variance (ANOVA) and a Tukey–Kramer test was done to compare mean values among each group. Significant differences implied $P<0.05$. Values are presented as means±S.E.M. Statistical analysis was made using commercially available package, Stat View (Version 5, SAS institute, Cary, USA, 1998).

Results

Fig. 1 shows the effect of i.c.v. injection of Gly-β-Ala, L-His-β-Ala and L-Val-β-Ala on total spontaneous activity (upper panel) and total distress vocalization (lower panel) during the 10 min isolation (Experiment 1). Though no significant effects were observed in total spontaneous activity ($F(3, 24)=2.272$, $P=0.106$), L-His-β-Ala tended to decrease the spontaneous activity. Significant effects on total distress vocalization ($F(3, 24)=7.025$, $P<0.01$) were detected, and L-His-β-Ala alone decreased distress vocalization.

Table 1 shows the effect of i.c.v. injections of Gly-β-Ala, L-His-β-Ala and L-Val-β-Ala on behavioral categories of chicks during the 10 min observational period. Significant effects were observed in active wakefulness ($F(3, 24)=10.639$, $P<0.001$) and sleeping posture ($F(3, 24)=10.654$, $P<0.001$). L-His-β-Ala induced sedative and hypnotic effects.

Fig. 2 demonstrates the effect of i.c.v. injection of carnosine, L-His-β-Ala and carnosine plus L-His-β-Ala (Car+L-His-β-Ala) on total spontaneous activity (upper panel) and total distress vocalization (lower panel) during the 10 min isolation (Experiment 2). Significant effects on total spontaneous activity ($F(3, 24)=13.78$, $P<0.0001$) and total distress vocalization ($F(3, 24)=27.419$, $P<0.0001$) were detected. Carnosine-induced hyperactivity while L-His-β-Ala conversely induced hypoactivity. In addition, carnosine plus L-His-β-Ala induced intermittent values of the effect of both dipeptides.

Table 1 shows the effect of i.c.v. injection of carnosine, L-His-β-Ala and carnosine plus L-His-β-Ala on behavioral categories of chicks during 10 min behavior observation. Significant effects were observed in active wakefulness ($F(3, 24)=29.865$, $P<0.0001$) and sleeping posture ($F(3, 24)=77.743$, $P<0.0001$). L-His-β-Ala caused lower active wakefulness and higher sleeping posture. The effect of combination was similar to that of carnosine rather than that of L-His-β-Ala.