MEDWELL PUBLISHING
Journal of Animal and Veterinary Advances 8 (6): 1207-1211, 2009
ISSN: 1680-5593

Cholinergic Neurotransmission is not Involved in Sedation Induced by L-Proline in Neonatal Chicks

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Abstract: The aim of the present study was to determine whether the sedative effects of L-proline are associated with the modulation of cholinergic neurotransmission. We investigated the effect of co-injection of L-proline with scopolamine, a Muscarinic Acetylcholine Receptor (M-AChR) antagonist, on behavior of neonatal chicks under isolation-induced stress. Intracerebroventricular (i.c.v.) injection of L-proline reduced spontaneous activity and the number of distress vocalizations, while co-injected scopolamine did not attenuate this effect implying that the M-AChR was not involved in the sedative effects induced by L-proline. In addition, the effect of L-proline on acetylcholineesterase activity in the telencephalon and diencephalon of chicks was investigated. No significant changes in acetylcholineesterase activity were observed in either the telencephalon or diencephalon. These results indicate that the sedative effects induced by L-proline are not mediated by the cholinergic system.

Key words: L-proline, acetylcholinesterase, stress, intracerebroventricular injection, neonatal chick

INTRODUCTION

Numerous studies have shown that the cholinergic systems are involved in emotionally guided behavior. For examples, there is evidence that Acetylcholine (ACh) Receptor (AChR) antagonists increase fear reactions (Hess and Blozovski , 1987; Smythe *et al*., 1998), whereas, increasing cholinergic activity reduced fear reactions (Degroot and Parent, 2001; Degroot *et al*., 2001). In addition, hippocampal cholinergic blockade enhanced the Hypothalamic-Pituitary-Adrenal (HPA) response to stress (Bhatnagar *et al*., 1997). These facts indicate that the hippocampal cholinergic system regulates stress-induced HPA activity and serves to coordinate behavioral responses to stress. Furthermore, the cholinergic systems play an important role in the stress response of chicks in which AChR agonists decreased isolation stress-induced vocalizations while antagonists had the opposite effect (Panksepp *et al*., 1980a; Sahley *et al*., 1981).

Ach Esterase (AChE) plays a key role in cholinergic transmission in the central nervous system, terminating the synaptic action of ACh. Delwing *et al*. (2003) showed that L-proline has an inhibitory effect of AChE in the brain suggesting that L-proline increases synaptic ACh levels and facilitates cholinergic activity.

A previous study has shown that intracerebroventricular (i.c.v.) injection of L-proline induces sedative effects in neonatal chicks mediated by the N-methyl-D-aspartate glutamate receptor (NMDA receptor) under an acute stressful condition (Hamasu *et al*., 2009a, b). It was predicted that the cholinergic systems might also be involved in the effect of L-proline. The aim of present study was to clarify whether the sedative effects of L-proline are associated with modulation of cholinergic neurotransmission. Accordingly, we investigated, the effect of the Muscarinic ACh Receptor (M-AChR) antagonist scopolamine on sedative effects induced by L-proline and the effect of L-proline on AChE activity in the telencephalon and diencephalon of chicks.

The effect of i.c.v. injection of scopolamine was observed using an acute stressful model with neonatal

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chicks. This behavioral model is based on the response of neonatal chicks to acute stress (Panksepp *et al*., 1980a; Sahley *et al*., 1981; Feltenstein *et al*., 2003a, b). In brief, chicks remain comfortable when in crowds, but feel acute stress when isolated. Isolation-induced stress increases spontaneous activity and vocalization of chicks.

MATERIALS AND METHODS

Animals and food: One-day-old male layer chicks (Julia) purchased from a local hatchery (Murata Hatchery, Fukuoka, Japan) were maintained in a windowless room at a constant temperature of 30±1°C. Continuously lighting was provided. Chicks were given free access to a commercial starter diet (Toyohashi Feed and Mills Co. Ltd., Aichi, Japan) and water. Chicks were distributed into experimental groups based on body weight, so that the average body weight in each treatment group was as uniform as possible within the same experiment. Testing was performed when chicks were 4 or 5 days of age. Experimental procedures followed the guidance for animal Experiments in the Faculty of Agriculture and in the Graduate Course of Kyushu University and the Law (No. 105) and Notification (No. 6) of the Japanese Government.

Preparation of drug: L-proline was a gift from Kyowa Hakko Kogyo (Tokyo, Japan). Scopolamine hydrobromide was purchased from Wako (Tokyo, Japan). In experiment 1, L-proline and scopolamine hydrobromide were dissolved in 0.85% saline containing 0.1% Evans Blue solution and control groups were given the saline solution. 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.

I.c.v. injection and behavioral observation: In experiment 1, drugs were injected i.c.v. into the left lateral ventricle of the chicks. I.c.v. injections were made 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 the injection, chicks were placed in an acrylic glass monitoring cage (40×30×20 cm) with paper on the floor and behavioral observations were made for 10 min at a constant temperature of 30±1°C. The monitoring systems were set in a separate room to avoid disturbing the animals. Spontaneous activity was automatically determined by 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 number of distress vocalizations, which are shrill and intense calls, was simultaneously recorded and counted using a computer with Gretchen software (Excla Inc., Japan). During the monitoring period, chicks were not given food or water. These behavioral experiments were conducted according to the method of Koutoku *et al*. (2005) and Asechi *et al*. (2006).

Determination of AChE activity: AChE activity was estimated according to the method of Ellman *et al*. (1961) with some modifications as a kinetic assay using a recording spectrophotometer (V-530, Jasco, Tokyo, Japan) fitted with a thermostated cuvette holder (EHC-477T, EHC-477S, Jasco, Tokyo, Japan). Diluted brain homogenates (50-80 μg protein) were preincubated at 25°C for 10 min in 0.1M potassium phosphate buffer, pH7.5, 0.52 mM 5,5′-Dithiobis-2-Nitrobenzoic acid (DTNB) to a final volume of 0.96 mL.

The reaction was started by addition of acetylthiocholine iodide as a substrate to a final concentration of 0.78 mM and the absorbance was read at 412 nm for 3 min (intervals of 30 s). All samples were run in duplicate.

Experiment 1

Effect of scopolamine on sedative effect induced by L-proline: Chicks were divided into 4 groups that received either saline, 1.81 pmol of scopolamine, 0.50 μmol of L-proline, or scopolamine plus L-proline. This dosage of scopolamine was based on the experiment of Koutoku *et al*. (2005). After the injection, behavioral observations were conducted.

Experiment 2

Effect of L-proline on AChE activity in telencephalon and diencephalon of chick: Chicks were injected i.c.v. with 0.5, 1.0 or 2.0 μmol/10 μL of L-proline, or saline as the control. This dosage of L-proline was determined according to the previous experiment (Hamasu *et al*., 2009a). After the injection, chicks were placed in a monitoring cage for 10 min. Thereafter, birds were sacrificed with an overdose of sodium pentobarbital and the location of the injection was verified.

After decapitation, the brain was rapidly removed and the telencephalon and diencephalon were dissected. The diencephalon was dissected at the anterior commissure (Youngren and Phillips, 1978). These samples were homogenized (about 1:10 w v^{-1} in

0.1M potassium phosphate buffer, pH7.5) and centrifuged at 1000 × g for 10 min. The supernatant was used for enzymatic analyses.

Statistical analysis: In experiment 1 data were statistically analyzed by 2-way Analysis of Variance (ANOVA). Experiment 2 was statistically analyzed by one-way ANOVA. Statistical analysis was conducted using the commercially available package StatView (version 5, SAS Institute, Cary, U.S.A. 1998).

RESULTS AND DISCUSSION

To examine whether the sedative effects of L-proline are associated with modulation of cholinergic neurotransmission, the effect of L-proline co-injected with the M-AChR antagonist scopolamine on the behaviour of neonatal chicks under isolation-induced stress was investigated. In chicks, the AChR plays an important role in the control of separation stress-induced vocalization. Non-selective AChR agonists decreased isolation stress-induced vocalization while antagonists had the opposite effect (Panksepp *et al*., 1980a, b). The AChR has two subtypes, M-AChR and Nicotinic AChR (N-AChR). Sahley *et al*. (1981) suggested that M-AChR was involved in controlling vocalizations more than the N-AChR. Therefore, we used the M-AChR antagonist scopolamine in the present study.

Figure 1 shows the effect of i.c.v. injection of L-proline with or without scopolamine on spontaneous activity (upper panel) and distress vocalizations (lower panel) during the 10 min isolation-induced stress. L-Proline significantly decreased spontaneous activity ($F(1, 22) = 18.051$, $p<0.001$) and the number of vocalizations ($F(1, 22) = 25.578$, $p<0.0001$). However, no significant effects of scopolamine were observed in spontaneous activity ($F(1, 22) = 0.311$, $p>0.05$) or the number of vocalizations ($F(1, 22) = 0.936$, $p>0.05$). Interactions between L-proline and scopolamine were not detected in either spontaneous activity ($F(1, 22) = 0.955$, $p>0.05$) or the number of vocalizations ($F(1, 22) = 0.929$, $p>0.05$). These results imply that the effect of L-proline could not be attenuated by scopolamine. Therefore, the M-AChR does not appear to be involved in the sedative effects induced by L-proline.

Delwing *et al*. (2003) showed that L-proline reduces AChE activity in the cerebral cortex of rats. The AChE activity was significantly decreased in the rat brain following a single s.c. injection of L-proline. Furthermore, L-proline also inhibited AChE activity *in vitro*. Therefore,

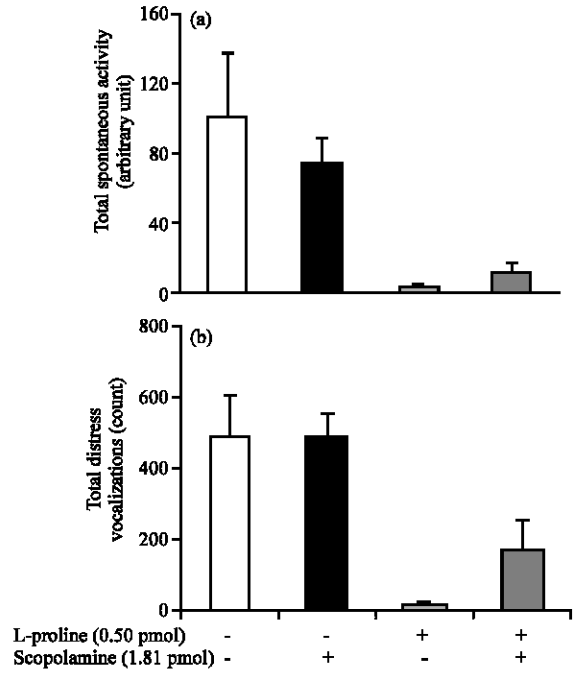

Fig. 1: Effect of i.c.v. injection of either 1.81 pmol of scopolamine, 0.50 μmoL of L-proline, or scopolamine plus L-proline on total spontaneous activity (upper panel) and distress vocalizations (lower panel) during 10 min isolation in 4- or 5-day-old layer chicks. Results are expressed as means±SEM. The number of chicks used in each group was as follows: control, 6; scopolamine, 7; L-proline, 6; scopolamine+L-proline, 7

we investigated the effect of L-proline on AChE activity in the telencephalon and diencephalon of chicks. Figure 2 shows the effect of i.c.v. injection of several doses of L-proline on AChE activity in the telencephalon (upper panel) and diencephalon (lower panel) of chicks. L-Proline had no significant effect on either telencephalon ($F(3, 24) = 0.460$, $p>0.05$) or diencephalon ($F(3, 24) = 0.873$, $p>0.05$) AChE activity. These results differed from those by Delwing *et al*. (2003). We injected L-proline directly to the lateral ventricle, while Delwing *et al*. (2003) injected s.c. Accordingly, some peripheral routes might be involved in brain AChE activities. Furthermore, these varying results might be attributed to the concentration of L-proline, interval of time after injection, effect of isolation stress, or species differences.

The results obtained here indicate that the cholinergic systems are not involved in the sedative effects induced by L-proline. In contrast, the NMDA receptor is involved in mediating the sedative effect induced by L-proline in chicks (Hamasu *et al*., 2009b).

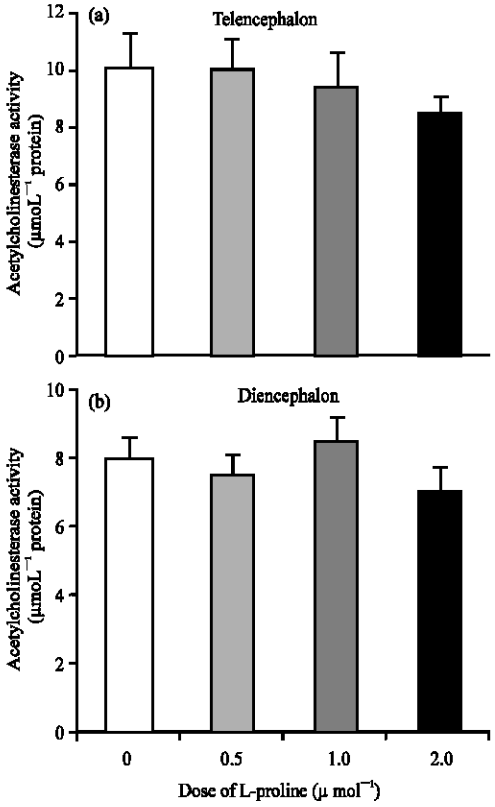

Fig. 2: Effect of i.c.v. injection of several doses of L-proine on AChE activity in the telencephalon (upper panel) and diencephalon (lower panel) of 4- or 5-day-old layer chicks. Results are expressed as means±SEM. The number of chicks used in each group was 7

CONCLUSION

The M-AChR was not involved in the sedative effect induced by L-proline. In addition, i.c.v. injection of several doses of L-proline has no effect on AChE activity in the telencephalon or diencephalon.

These results indicate that the mechanism of the sedative effects induced by L-proline does not involved the cholinergic systems.

ACKNOWLEDGEMENT

This research was supported by a Grant-in-Aid for Scientific Research from Japan Society for the Promotion of Science (No. 18208023) and the SKYLARK Food Science Institute.

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