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Intracerebroventricular injection of orexin-A stimulates monoamine metabolism but not HPA axis in neonatal chicks

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ABSTRACT

We investigated the effects of intracerebroventricular (ICV) injection of orexin-A on plasma corticosterone (CORT) concentration and brain monoamine metabolism to clarify the mechanism by which ICV orexin-A induced arousal in chicks. In Experiment 1, plasma CORT concentrations were measured as an indicator of hypothalamic-pituitary-adrenal (HPA) axis activity. There was no significant difference in CORT concentration between the control and orexin-A administered groups. In Experiment 2, the concentrations of monoamines (norepinephrine, dopamine and serotonin), their metabolites, and their metabolic turnover rates in the telencephalon, mesencephalon, and diencephalon were investigated. All metabolic turnover rates studied were increased at all brain sites after ICV orexin-A injection. In conclusion, the HPA axis does not appear to be involved in arousal-inducing mechanisms of orexin-A in neonatal chicks; however, several monoaminergic systems do.

Orexin-A and orexin-B are peptidergic neurotransmitters that were first isolated through a search for ligands of previously orphan receptors now identified as OX1 and OX2 [31]. Orexin-A and orexin-B are alternatively known as hypocretin-1 and hypocretin-2, respectively, because they are found in the hypothalamus and are related to the incretin family of neurotransmitters [6]. Since their isolation over a decade ago, the orexins have been implicated in a few processes including food intake and energy expenditure. In addition, the orexins are most noted for their roles in wakefulness: central injection of orexin induces arousal, activity and decreases non-rapid eye movement (REM) and REM sleep [10]. Recent evidence supports that orexins are important mediators of sleep/wake cycles. However, most reports on orexins are based on rodent studies.

Our group has tested the orexins in two stocks of chicks: layers (egg-type) and broilers (meat-type). We demonstrated that under *ad lib* feeding, orexin-A did not affect food intake and orexin-B was associated with decreased food intake of broiler chicks [9]. More recently, we have demonstrated that orexin-A increased arousal in layer, but not in broiler chicks, and that orexin-B is not likely involved in sleep/wake cycles [14]. Possible mechanisms associated with these behavioral differences may involve the hypothalamic-pituitary-adrenal (HPA) axis and/or activation of monoamine neurons.

The HPA axis is activated under stressful conditions in chicks [8]. With activation of this axis, corticotropin-releasing factor (CRF) in the hypothalamus induces the release of adrenocorticotropic hormone (ACTH) in the pituitary, which enhances release of corticosterone (CORT) from the adrenal cortex. Orexins may be related to the stress response [7]. Increased plasma ACTH and CORT after central administration of orexin suggests that orexin activates the HPA axis in rats [29]. Alternatively, CRF-containing neurons conversely innervates orexin neurons [36]. Arousal mechanisms of orexin-A in neonatal chicks are unreported, but we thought that the behavioral differences after orexin-A administration may contribute to activation of the HPA axis. Saito et al. [30] demonstrated that plasma CORT concentrations after the ICV injection of CRF were significantly higher in layers than broilers. Recently, the differences between layers and broilers were suggested to be due to differential regulation of the HPA axis, but not synthesis of associated neuropeptides [37].

The relationships between orexins and monoamines are reported by many researchers (reviewed by Ohno and Sakurai [27]). Orexin neurons innervate many areas of the brain that are important in the activity of each monoamine. The locus coeruleus (LC) has a dense concentration of norepinephrine (NE), the dorsal raphe nucleus (DRN), and median raphe nucleus (MRN) are areas with high serotonin (5-HT) concentration, and the brain stem is where orexins are synthesized and also has an abundant concentration of dopamine (DA) [15]. NE and 5-HT are known as waking-active,

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and their activity is different depending on sleep status [32]. In the state of vigilance, orexins stimulate these monoamines to keep animals awake, and have a suppressed firing rate [27]. However, to our knowledge there is no report of relationships between the orexins and monoamines to explain the arousal effect of orexin-A in layer neonatal chicks.

As a primary step in the investigation of mechanisms of arousal by orexin-A, we studied the effects of central injection of orexin-A on blood CORT (Experiment 1) and brain monoamine contents in neonatal chicks (Experiment 2). The structure of orexin peptides is highly conserved between different mammalian species [31]. According to Ohkubo et al. [25], chicken orexin-A and orexin-B showed approximately 85% and 65% similarities with the corresponding mammalian sequence at the amino acid level. Katayama et al. [14] reported that mammalian orexin-A was functioned and increased arousal in chicks. We also applied mammalian orexin-A in the present study.

Day-old male layer chicks (Julia) were purchased from local hatchery (Murata Hatchery, Fukuoka, Japan). The chicks were maintained in a windowless room at the temperature of 30 ± 1 °C with continuous lighting. They were given free access to a commercial starter diet (Toyohashi Feed and Mills Co. Ltd., Aichi, Japan) and water unless otherwise mentioned. When they were divided into groups for experiments, body weights were distributed uniformly across groups. Experimental procedures followed the guidelines set forth by the Animal Experiments in Faculty of Agriculture, the Graduate Course of Kyushu University, Japanese Law No. 105 and Notification No. 6 of the Japanese Government.

Chicks were ICV injected with human orexin-A (Peptide Institute, Osaka, Japan) dissolved in vehicle solution at a volume of 10 μl using a microsyringe according to the method of Davis et al. [5]. The stress and discomfort by this method is minimal as described elsewhere [9,17]. The vehicle consisted of 0.85% saline containing 0.1% Evans blue that was made on the day of the experiments. At the end of the experiments, birds were sacrificed by cervical dislocation after which the location of the injection site was confirmed. Any chicks without Evans blue dye in the lateral ventricle were eliminated from analysis.

In Experiment 1, layer chicks (5-day old) were acclimated to individual cages for 1 day prior to the experiment. Chicks were ICV injected with vehicle (control) or 2 nmol orexin-A. Jugular blood was collected at 30 and 60 min after injection and was immediately treated with heparin, then centrifuged at 11,000 × *g*, 4 °C for 4 min. Plasma samples were stored at −80 °C until analysis. Plasma CORT was determined using a corticosterone enzyme immunoassay kit (Assay Designs Inc., USA).

In Experiment 2, layer chicks (6-day old) were acclimated to individual cages 1 day before the experiment. They were ICV injected with either vehicle (control) or 2 nmol orexin-A according to the previous studies [9,14]. At 30 and 60 min after injection, the whole brain was rapidly removed, and was dissected into three parts according to the atlas of the chicken brain [18]: mesencephalon, telencephalon and diencephalons. Diencephalon was divided by cutting between stria medullaris and posterior commissure. Mesencephalon was separated by cutting connective tissue in the posterior commissure. They were immediately removed, weighed, and kept at −80 °C until analysis.

The concentrations of monoamines and their metabolites (contents/g wet tissue) were analyzed by modified method based on Kabuki et al. [13]. Briefly, the tissue was homogenized in 0.2 nmol/l perchloric acid containing 100 μM EDTA 2Na. The homogenate was left for 30 min to allow complete deproteinization. Then, the homogenate was centrifuged at 20,000 × *g* for 15 min at 0 °C. After centrifugation, the pH of the supernatant was adjusted to approximately 3.0 by adding 1 M sodium acetate. The supernatant was then centrifuged with a centrifuge–filtration unit (Ultra Free C3-GV Millipore, Bedford, USA) at 10,000 × *g* for 5 min at 0 °C. A 30 μl portion of filtrate was applied to a high-performance liquid chromatography system (Eicom, Kyoto, Japan) with a 150 mm × 3.0 mm octadecyl silane column (SC-50DS, Eicom) and an electrochemical detector (ECD-300, Eicom) at an applied potential of +0.75 V versus Ag/AgCl reference analytical electrode. Changes in electric current (nA) were recorded in a computer using an interface system (Power Chrom ver 2.3.2.j, AD Instruments, Tokyo, Japan). The mobile phase consisted of 100 mM aceto-citric acid buffer (pH 3.5), methanol, 460 mM sodium 1-octane sulfonate, and 15 mM disodium ethylenediaminetetraacetic acid (830:170:1.1:1) at a flow rate of 0.5 ml/min. The concentrations of monoamines and metabolites including NE, DA, 5-HT, the NE metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG),

Table 1
The effect of intracerebroventricular injection of orexin-A on the monoamine assessment of diencephalons.

Treatment	Time after injection (min)				*F* value	*P* value
	30		60		Time (1,24)	Time
	Control	Orexin	Control	Orexin	Treatment (1,24)	Treatment
NE	7429 ± 398	6438 ± 435	6741 ± 236	5362 ± 343	5.515	*
					9.967	**
MHPG	782 ± 60	877 ± 30	754 ± 25	768 ± 74	1.616	NS
					1.011	NS
MHPG/NE	0.107 ± 0.009	0.139 ± 0.007	0.113 ± 0.006	0.143 ± 0.011	0.299	NS
					12.713	**
DA	1546 ± 117	1354 ± 117	1202 ± 81	862 ± 65	16.981	**
					6.881	*
DOPAC	372 ± 18	366 ± 14	249 ± 14	262 ± 36	24.582	**
					0.023	NS
DOPAC/DA	0.246 ± 0.015	0.281 ± 0.022	0.210 ± 0.014	0.297 ± 0.022	0.277	NS
					10.199	**
5-HT	8058 ± 456	7067 ± 434	6584 ± 195	5283 ± 289	18.223	**
					9.022	**
5-HIAA	1897 ± 32	2253 ± 122	2218 ± 90	2479 ± 226	4.062	NS
					5.172	*
5-HIAA/5-HT	0.240 ± 0.013	0.322 ± 0.018	0.338 ± 0.016	0.466 ± 0.023	45.902	**
					34.717	**

Values are means ± SEM in pg/g wet tissue. The numbers of chicks were: saline 30 min 8; orexin 30 min 7; saline 60 min 6; orexin 60 min 7. NE: norepinephrine, 5-HT: serotonin, DA: dopamine, MHPG: 3-methoxy-4-hydroxyphenylglycol, DOPAC: 3,4-dihydroxyphenylacetic acid, 5-HIAA:5-hydroxyindoleacetic acid.

* $P < 0.05$.

** $P < 0.01$.