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Clorgyline Inhibits Orexin-A-Induced Arousal in Layer-Type Chicks

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ABSTRACT. We previously demonstrated that intracerebroventricular (ICV) injection of orexin-A induces arousal and increased metabolic turnover rates of norepinephrine, dopamine, and serotonin in layer (egg-type) chicks. Because monoamine oxidase-A (MAO-A) is a potent degrading enzyme of these monoamines, we hypothesized that orexin-A may mediate its arousal-inducing effects through MAO-A. Therefore, we simultaneously injected clorgyline, a specific inhibitor of MAO-A, with orexin-A and examined behavior of chicks. Behaviors associated with arousal were attenuated in the group of chicks that received clorgyline and orexin-A compared with those that received orexin-A alone. For the monoamine turnover rate, enhancement of the turnover rate of serotonin by orexin-A was attenuated by clorgyline. Therefore, we conclude that orexin-A-induced arousal is dependent upon monoamine neural activities stimulated by MAO-A in chicks.

KEY WORDS: arousal, chick, clorgyline, monoamine oxidase-A, orexin-A.

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Orexin peptides were first identified as neurotransmitters by the process of reverse-pharmacology while searching for ligands of then-orphan receptors by Sakurai *et al.* [11]. Around the same time, de Lecca *et al.* [2] also identified these same peptides and named them hypocretins (hypocretin-1 and hypocretin-2) based on the brain regions where they were found and synthesized. Soon after their identification, it was reported that central injection of orexin induces arousal and activity and decreases non-REM and REM sleep [6]. More recently, orexins have been implicated as important mediators of the sleep/wake cycle.

Recently, we reported that orexin-A increased arousal in layer, but not broiler, chicks and that orexin-B is not likely to be involved in chick sleep/wake cycles [8]. Possible mechanisms associated with these behavioral affects may involve the hypothalamic-pituitary-adrenal (HPA) axis and/or activation of monoamine neurons. Additionally, we demonstrated that the HPA axis is unlikely to be associated with arousal-inducing mechanisms of orexin-A in chicks, but that several monoaminergic systems including norepinephrine (NE), dopamine (DA) and serotonin (5-HT) are [9]. That orexin-A induces arousal of neonatal chicks is consistent with mammalian reports [10]. However, the central mechanisms that mediate these responses are poorly understood in all species.

Therefore, we designed the experiment reported here to better elucidate the central mechanisms associated with NE, DA, 5-HT and orexin-A-induced arousal through inhibition of a potent monoaminergic degrading enzyme, monoamine oxidase (MAO). MAO is found in two forms within in the brains of humans and rats: MAO-A and MAO-B [7]. These enzymes exist in mitochondria and oxidize monoamines in neurons containing them [14]. Specifically, both NE and 5-HT are metabolized by MAO-A, and DA is metabolized by both MAO-A and MAO-B. MAO-B mainly metabolizes DA, histamine, benzylamine, phenylethylamine and tyramine (reviewed by Finberg and Youdim [4]). We speculated that orexin-A can affect the activity of MAO-A because the metabolic turnover rates of NE, DA, and 5-HT increased in all brain regions measured after intracerebroventricular (ICV) injection of orexin-A [9]. Therefore, we decided to investigate the relationships between orexin-A and MAO-A using clorgyline [N-methyl-N-propargyl-3-(2,4-dichlorophenoxy) propylamine], a specific inhibitor of MAO-A [3]. The results reported here may contribute to a greater understanding of the pathways of action through which the orexins induce arousal.

Day-old male layer chicks (Julia) were purchased from a local hatchery (Murata Hatchery, Fukuoka, Japan). The chicks were maintained in a windowless room at a temperature of 30 ± 1°C with continuous lighting. They were given free access to commercial starter diet (Toyohashi Feed and Mills Co., Ltd., Aichi, Japan) and water unless otherwise mentioned. When they were divided into groups for the experiment, the body weights were distributed as uniformly as possible. 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 Japanese Government.

Chicks were ICV-injected (10 μl) using a microsyringe according to the methods by Davis *et al.* [1], with injection starting at 09:30. Orexin-A (human, rat, mouse and bovine) was purchased from the Peptide Institute (Osaka, Japan). N-Methyl-N-propargyl-3-(2,4-dichlorophenoxy) propylamine

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hydrochloride (clorgyline hydrochloride) was purchased from Sigma (St. Louis, MO, U.S.A.). They were dissolved in 0.85% saline containing 0.1% Evans blue on the day of the experiment. At the end of the experiment, the birds were euthanized by cervical dislocation, and the location of the injection site was confirmed by dissection. Data from any chick in which Evans blue dye was not found in the lateral ventricle excluded from the analysis.

The day before the experiment, the chicks were moved from a group cage to individual transparent acrylic cages. On the day of the experiment, the chicks were 5 or 6 days old. Barriers between cages prevented visual contact between chicks. Chicks were randomly assigned to receive an ICV injection of either vehicle only (0.85% saline), 0.2 nmol orexin-A or 0.2 nmol orexin-A + 81 nmol clorgyline. The behavior of the chicks was recorded following injection by a digital video recorder (SELCO Corporation, Kyoto, Japan). Food intake was determined at 60 min postinjection. Behaviors during the 60-min observation period postinjection were later categorized into the following four mutually exclusive categories according to the report of van Luijtelaar *et al.* [13]: (1) standing with eyes opened (active wakefulness), (2) sitting with eyes open, (3) standing with eyes closed and (4) sitting with eyes closed (sleep-like posture). In addition to these categories, active wakefulness was further distinguished as either with or without feeding behavior as described by Takagi *et al.* [12].

At 60 min after injection, the whole brain was rapidly removed, and the telencephalon was immediately removed, weighed and kept at –80°C until analysis.

The concentrations of monoamines and their metabolites (contents/g wet tissue) were analyzed by a modified method based on that of Katayama *et al.* [9]. 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, MA, U.S.A.) at 10,000× *g* for 5 min at 0°C. A 30-μ*l* portion of filtrate was applied to a high-performance liquid chromatography system (Eicom, Kyoto, Japan) with a 150 × 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 an 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 m*l*/min. The concentrations of monoamines and metabolites including NE, DA, 5-HT, the NE metabolite 3-methoxy-4-hydroxyphenylglycol (MHPG), the DA metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) and the 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA) were determined, and their concentrations in each brain region were calculated. The turnover rate of each monoamine (DOPAC/DA, MHPG/NE and 5-HIAA/5-HT) was also calculated. The limit of detection of the system for all monoamines was 0.1 *p*g/sample.

The time spent in each posture for feeding, awake (eyes open), sleeping (eyes closed), food intake, and the monoamine concentrations were examined by two-way analysis of variance (ANOVA). The behavior and monoamines were then analyzed by *t*-test when significant interactions were observed. Significant differences implied $P<0.05$. Statistical analysis was conducted using the commercially available package, StatView package (version 5, SAS Institute, Cary, NC, U.S.A., 1998).

Food intake was not affected by any treatment in the present study (saline, 2.376 ± 0.696; orexin-A, 1.189 ± 0.256; clorgyline, 0.770 ± 0.191; orexin-A + clorgyline, 1.213 ± 0.432), which supports previous reports in which orexin-A did not alter food intake in chicks [5, 8].

In our previous study, the concentrations of monoamines and metabolites including NE, DA, 5-HT, the NE metabolite MHPG, the DA metabolite DOPAC and the 5-HT metabolite 5-HIAA were determined, and the turnover rate of each monoamine (DOPAC/DA, MHPG/NE and 5-HIAA/5-HT) was also calculated [9]. All of these turnover rates increased after orexin-A injection. We hypothesized that these changes were associated with increased monoamine oxidase-A (MAO-A) and monoamine neuron activity, since MAO-A degrades amine neurotransmitters, such as DA, NE and 5-HT. Thus, we evaluated the hypothesis that orexin-A-induced arousal was associated with MAO-A in the present study through behavioral observation.

Table 1 shows the results for behavioral changes. The amount of time spent feeding and in active wakefulness was not significantly affected by treatments. There was no change in the amount of the time spent standing/sitting motionless with eyes open. However, when the two wakeful states were combined (active wakefulness total and sitting with eyes open), the group of chicks treated with orexin-A spent a significantly more time in a wakeful state than the other treatment groups. Threshold affects were observed for behaviors associated with eyes closed. None of the orexin-A-treated chicks spent time with their eyes open, but chicks from all the other treatment groups did. When both sleeping states were combined (standing with eyes closed and sitting with eyes closed), the threshold affect was still present, and it could be interpreted that the effect of orexin-A was blocked by clorgyline. To confirm that the orexin-A-induced metabolic turnover rates of NE, DA and 5-HT were also attenuated with clorgyline, we determined the monoamines and their metabolites in the brain. Table 2 shows the effect of ICV injection of orexin-A and/or clorgyline on the monoamine assessment of the telencephalon. NE, MHPG and MHPG/NE were not significantly different among groups. DA was not significantly different, but DOPAC was significantly decreased by clor-

Table 1. Amount of time spent (in sec) per behavior after intracerebroventricular injection of orexin-A and/or clorgyline in layer-type chicks

	Control	Clorgyline	Orexin-A	Orexin-A + Clorgyline
1) Active wakefulness (total)	1,560 ± 438	1,675 ± 367	2,161 ± 208	1,685 ± 335
Feeding behavior	439 ± 125	303 ± 64	120 ± 45	326 ± 136
Without feeding behavior	1,121 ± 315	1,372 ± 358	2,041 ± 170	1,359 ± 319
2) Sitting with eyes open	1,235 ± 290	1,336 ± 252	1,439 ± 208	1,191 ± 169
Wakeful state (1+2)	2,795 ± 211[a)]	3,011 ± 211[a)]	3,600 ± 0[b)]	2,876 ± 283[a)]
3) Standing with eyes closed	13 ± 13	155 ± 83	0	252 ± 133
4) Sitting with eyes closed (sleeping posture)	791 ± 204	434 ± 165	0	471 ± 273
Sleeping state (3+4)	805 ± 211	589 ± 211	0	724 ± 283
Total (1+2+3+4)	3,600	3,600	3,600	3,600

The numbers of chicks in the control, clorgyline, orexin-A and orexin-A + clorgyline groups were 5, 7, 6 and 7, respectively. Values with different superscripts are significantly different (P<0.05).

Table 2. The effect of intracerebroventricular injection of orexin-A and/or clorgyline on the monoamine assessment of the telencephalon

Treatment	Control	Clorgyline	Orexin-A	Orexin-A + Clorgyline	F value Orexin (1,21) Clorgyline (1,21)	P value Orexin Clorgyline
NE	184,104 ± 18,696	197,330 ± 12,434	152,129 ± 10,775	183,432 ± 13,119	2.799 2.638	NS NS
MHPG	90,838 ± 8,689	105,579 ± 5,655	127,200 ± 39,831	92,408 ± 5,988	0.333 0.249	NS NS
MHPG/NE	0.496 ± 0.030	0.542 ± 0.032	0.824 ± 0.215	0.507 ± 0.019	1.873 1.612	NS NS
DA	432,800 ± 36,876	422,480 ± 16,620	393,120 ± 17,582	413,628 ± 22,297	1.100 0.048	NS NS
DOPAC	23,318 ± 2,622	11,431 ± 457	24,296 ± 920	12,117 ± 561	0.482 100.859	NS P<0.0001
DOPAC/DA	0.054 ± 0.003	0.027 ± 0.001	0.063 ± 0.005	0.030 ± 0.002	4.473 117.247	P=0.05 P<0.0001
5-HT	567,055 ± 45,373	634,906 ± 9,989	496,074 ± 21,040	627,629 ± 19,628	2.635 17.106	NS P=0.0005
5-HIAA	70,324 ± 7,887	47,976 ± 2,139	84,160 ± 10,145	47,403 ± 3,448	1.127 22.387	NS P=0.0001
5-HIAA/5-HT	0.123 ± 0.006[b)]	0.076 ± 0.004[a)]	0.171 ± 0.022[c)]	0.075 ± 0.004[a)]	4.551 41.157	P=0.05 P<0.0001

Values are means ± SEM in pg/g wet tissue. Values with different superscripts are significantly different (P<0.05). The numbers of chicks in the control, clorgyline, orexin-A and orexin-A + clorgyline groups were 5, 7, 6 and 7, respectively.

gyline. DOPAC/DA was significantly increased by orexin-A, but decreased by clorgyline, without a significant interaction. Clorgyline significantly increased 5-HT, and 5-HIAA was decreased. Orexin-A significantly increased 5-HIAA/5-HT in the telencephalon, but clorgyline decreased it. A significant interaction was detected in the 5-HT turnover rate.

Therefore, we conclude that MAO-A is partially involved in the arousal-inducing effect of orexin-A.

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