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Involvement of Gaba and Cannabinoid Receptors in Central Food Intake Regulation in Neonatal Layer Chicks: Role of CB_1 and $GABA_A$ Receptors

■Author(s)

Zendehdel M[I]
Tirgari F[II]
Shohre B[II]
Deldar H[II]
Hassanpour S[III]

[I] Department of Basic Sciences, Faculty of Veterinary Medicine, University of Tehran, 14155-6453, Tehran, Iran
[II] Department of Animal Science, Sari Agricultural Sciences & Natural Resources University, Sari, Iran
[III] Department of Basic Sciences, Faculty of Veterinary Medicine, Science and Research Branch, Islamic Azad University, Tehran, Iran

■Mail Address

Corresponding author e-mail address
Morteza Zendehdel
Department of Basic Sciences, Faculty of Veterinary Medicine, University of Tehran, 14155-6453, Tehran, Iran
Tel: +98-21-61117186
Email: zendedel@ut.ac.ir

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ABSTRACT

Feeding behavior is regulated via a complex network which interacts via diverse signals from central and peripheral tissues. Endocannabinoids modulate release of GABA in a variety of regions of the central nervous system. Endocannabinoids and GABAergic system have an important role in the central regulation of appetite. Thus, the present study examines the possible interaction of central canabinoidergic and GABAergic systems on food intake in 3-h food-deprived (FD_3) neonatal layer-type chicks. The results of this study showed that intracerebroventricular (ICV) injection of 2-AG (2-Arachidonoylglycerol, selective CB_1 receptors agonist, 2µg) significantly increased food intake and this effect of 2-AG was attenuated by Picrotoxin ($GABA_A$ antagonist, 0.5µg) ($P<0.001$); but 21ng CGP54626 ($GABA_B$ antagonist) had no effect ($p>0.05$). Also, hyperphagic effect of CB65 (CB_2 receptors agonist, 1.25µg) was not affected by Picrotoxin or CGP54626 ($p>0.05$). Moreover, the food intake of chicks was significantly increased by ICV injection of $GABA_A$ agonist (Gaboxadol, 0.2 µg) and SR141716A (CB_1 receptors antagonist, 6.25µg) significantly decreased Gaboxadol-induced hyperphagia ($P<0.001$) but CB_2 receptors antagonist (AM630, 1.25µg) had no effect. In contrast, co-injection of SR141716A or AM630 with $GABA_B$ agonist (baclofen, 0.2µg) had no effect on the hyperphagia induced by baclofen ($p>0.05$). These data showed there might be an interaction between central cannabinoidergic and GABAergic systems via CB_1 and $GABA_A$ receptors in control of food intake in neonatal layer chicks.

INTRODUCTION

Feeding behavior is a complex physiologic phenomenon which interacts via various signals from central and peripheral tissues. In the central nervous system (CNS), several neurotransmitters interact by a wide distributed neurological network on food intake regulation (Zendehdel *et al.,* 2014; Honda, 2016). γ-aminobutyric acid (GABA) is one of the most abundant neurotransmitter in mammalian brain. The GABAergic is known as an inhibitory neurotransmitter in the CNS (Bonaventura *et al.,* 2012). GABA exerts its effect via two distinct receptors including the bicuculline-sensitive $GABA_A$ receptor and the bicuculline-insensitive $GABA_B$ receptors (Stratford and Wirtshafter, 2013). The $GABA_A$ and $GABA_C$ receptors are part of a macromolecular complex coupled to a Cl^- ionophore while $GABA_B$ is metabotropic receptor belong to G-protein coupled receptors (GPCRs) (Jonaidi *et al.,* 2012).

The appetite-stimulating effects of marijuana (Cannabis sativa) are known in science for the past decades. Animal tissues express at least two cannabinoidergic (CBergic) receptors: cannabinoid type 1 (CB_1) and type 2 (CB_2) receptors which belong to the GPCRs (Kangas

et al., 2013). CB_1 receptors mainly expressed in the presynaptic terminals of the brain (Sharkey *et al.,* 2014) and CB_2 receptors formerly exist on cells and organs of the immune system in the peripheral nervous system (PNS) but they also expressed in the CNS (Onaivi *et al.,* 2008). The endocannabinoids (ECBs) mediates many physiological functions like pain relief, motor control, learning and memory formation as well as appetite regulation (D'Addario *et al.,* 2014).

GABA and CBs involved control of food intake (Volkow *et al.,* 2011). It appears GABA has orexigenic effect in domestic fowls (Jonaidi *et al.,* 2012). ICV injection of Muscimol (a $GABA_A$ agonist) and Baclofen (a $GABA_B$ agonist) increased food consumption in rats (Ebenezer, 1990), turkeys (Denbow, 1991), meat-type (broiler) and layer-type chicks (hens) (Jonaidi *et al.,* 2002). Both CB_1 and CB_2 receptors have a role in food intake regulation in the rat (Chen et al., 2006). Scarce information exists about the role of ECBs on feeding behavior in domestic fowl. Reports claim just CB_1 receptors have a regulatory role on appetite in broilers (*et al.,* 2011; Novoseletsky *et al.,* 2011). Recent reports revealed both CB_1 and CB_2 receptors have hyperphagic effect in neonatal layer-type chicken (Alizadeh *et al.,* 2015; Hassanpour *et al.,* 2015).

There is central evidence mechanisms for food intake regulation are different between mammalian and birds (Zendehdel & Hassanpour 2014). Comparative physiological studies suggested there are differences award pathways between the meat-type and layer-type chicks (Alizadeh *et al.,* 2015). For example, food intake mediates via both CB_1 and CB_2 receptors in layer-type chicken (Alizadeh *et al.,* 2015) but only CB_2 receptors interact on feeding in meat-type chicken (Emadi *et al.,* 2011; Novoseletsky *et al.,* 2011). For instance, ICV injection of Baclofen had no effect on food intake in broilers (Jonaidi *et al.,* 2002; Tajalli *et al.,* 2006; Zendehdel *et al.,* 2008, 2009).

During the last several years, GABA and ECBs have been the subject of an increasing number of behavioral studies. Several types of researches are done to find the mediatory effect of neurotransmitters on feeding behavior in mammals but aspects of food intake regulation in avian are still unclear (Zendehdel & Hassanpour, 2014). ECBs modulate the release of GABA in a variety of regions of the CNS, including the hippocampus, basal ganglia, cerebellum, and brainstem. Anatomical investigations have shown high levels of CB_1 receptor immune-reactivity and mRNA associated with GABAergic neurons (Irving *et al.,* 2002). In the brain, CB_1 receptors is predominantly expressed presynaptically and modulating the release of neurotransmitters, such as GABA, dopamine, noradrenaline, glutamate and serotonin (Cota *et al.,* 2003). To the best of our knowledge, no report exists on the interconnection of the CBergic and GABAergic systems on food intake regulation and energy balance in neonatal layer type chicken. So, the aim of this study was to investigate the possible involvement of the CBergic and GABAergic systems on feeding behavior in 3 h food deprived (FD_3) neonatal layer type chicken.

MATERIALS AND METHODS

Animals

In this study, a total 384 one-day-old layer-type chickens (Hy-Line) purchased from a local hatchery (Morghak Co. Iran). Birds were kept as flocks for two days then randomly transferred into individual cages at a temperature of 30 ± 1°C with 50 ± 2 percent humidity (Olanrewaju *et al.,* 2006). A commercial diet provided during the study containing 21 % crude protein and 2850 kcal/kg of metabolizable energy (Chineh Co. Iran). All birds received *ad libitum* food and fresh water during the study. Just three h prior the ICV injections, chicken, were food deprived (FD_3) but had free access to water. The injections were applied to all birds at five days of age. Animal handling and experimental procedures were performed according to the Guide for the Care and Use of Laboratory animals by the National Institutes of Health (USA) and the current laws of the Iranian government for animal care.

Experimental Drugs

Gaboxadol ($GABA_A$ agonist), Baclofen ($GABA_B$ agonist), Picrotoxin ($GABA_A$ antagonist), CGP54626 ($GABA_B$ antagonist), 2-AG (2-Arachidonoylglycerol, a selective CB_1 receptors agonist), CB65 (a selective CB_2 receptors agonist), SR141716A (a selective CB_1 receptors antagonist), AM630 (a selective CB_2 receptors antagonist) and Evans Blue purchased from Sigma Co. (Sigma, USA). Drugs except 2-AG at first dissolved in absolute dimethyl sulfoxide (DMSO) then diluted with 0.85% saline containing Evans blue at a ratio of 1/250. 2-AG solved in 0.1 % Evans blue solution which was prepared in 0.85 % saline. DMSO with this ratio does not have a cytotoxic effect (Blevins *et al.,* 2002; Qi *et al.,* 2008).

ICV injection procedures

In this study, eight experiments designed to investigate interconnection of CBergic and GABAergic systems on cumulative food intake in neonatal layer-type birds (each includes four groups within 12

replicates in each group). Before each treatment, the chicks were weighed and based on their body weight allocated into groups so the average weight between treatment groups was as uniform as possible. ICV injection was applied using a microsyringe (Hamilton, Switzerland) without anesthesia according to the technique previously described by Davis *et al.,* (1979) and Furuse *et al.,* (1997) in which the head of the birds was held with an acrylic device while the bill holder was 45° and calvarium parallel to the surface of table (Van Tienhoven and Juhasz, 1962). In a plate, a hole was drilled in the skull over the right lateral ventricle and immediately overlaid through this plate. A microsyringe inserted into the right ventricle via the hole and tip of the needle penetrated 4 mm beneath the skin of the skull. It is revealed that there is no injection-induced physiological stress using this method in neonatal chicks (Saito *et al.,* 2005). Each bird received an ICV injection (with vehicle or drug solution) in a volume of 10 µL. Right away after the injection, FD_3 birds returned to their individual cages and offered fresh water and food (pre-weighed). Cumulative food intake (gr) was measured at 30, 60 and 120 min after the injection. Food consumption was calculated as a percentage of body weight to minimize the effect of body weight on the amount of food intake. Each bird was just used once in each experimental group. At the end, accuracy of placement of the injection in the ventricle was verified by the presence of Evans Blue followed by slicing the frozen brain tissue. In each group, 12 birds received the injection, but just data of those individuals where the dye was present in their lateral ventricle (12 chickens per group), was used for the analysis. All experimental procedures were done from 8:00 A.M. until 3:30 P.M. Also, the time course for feeding behavior was selected by previous studies (Jonaidi *et al.,* 2012; Zendehdel & Hassanpour 2014; Alimohammadi *et al.,* 2015; Alizadeh *et al.,* 2015; Hassanpour *et al.,* 2015).

Feeding experiments

In this study, eight experiments were designed, each with four treatment groups: A, B, C and D groups (n=48 in each). In experiment 1, chickens received intracerebroventricular (ICV) injection of (A) saline, (B) 2-AG (2-Arachidonoylglycerol, selective CB_1 receptors agonist, 2µg), (C) Picrotoxin ($GABA_A$ antagonist, 0.5µg) and (D) co-injection of 2-AG+Picrotoxin. In experiment 2, birds injected with (A) saline, (B) 2-AG (2µg), (C) CGP54626 ($GABA_B$ antagonist, 21ng) and (D) co-injection of 2-AG+CGP54626. In experiment 3, animals received: (A) saline, (B) CB65 (selective CB_2 receptors agonist, 1.25µg), (C) Picrotoxin (0.5µg) and (D) CB65+ Picrotoxin. In experiment four, chicks ICV injected with (A) saline, (B) CB65 (1.25µg), (c)

Table 1 – Treatments procedure in experiments 1-8

Exp. 1	ICV Injection
Treatment groups	
A	CS*
B	2-AG (2µg)
C	Picrotoxin (0.5µg)
D	2-AG (2µg)+ Picrotoxin (0.5µg)
Exp. 2	ICV Injection
Treatment groups	
A	CS*
B	2-AG (2µg)
C	CGP54626 (21ng)
D	2-AG (2µg)+ CGP54626 (21ng)
Exp. 3	ICV Injection
Treatment groups	
A	CS*
B	CB65 (1.25µg)
C	Picrotoxin (0.5µg)
D	CB65 (1.25µg) + Picrotoxin (0.5µg)
Exp. 4	ICV Injection
Treatment groups	
A	CS*
B	CB65 (1.25µg)
C	CGP54626 (21ng)
D	CB65 (1.25µg) +CGP54626 (21ng)
Exp. 5	ICV Injection
Treatment groups	
A	CS *
B	Gaboxadol (0.2µg)
C	SR141716A (6.25µg)
D	Gaboxadol (0.2µg) + SR141716A (6.25µg)
Exp. 6	ICV Injection
Treatment groups	
A	CS *
B	Gaboxadol (0.2µg)
C	AM630 (1.25µg)
D	Gaboxadol (0.2µg)+ AM630 (1.25µg)
Exp. 7	ICV Injection
Treatment groups	
A	CS *
B	baclofen (0.2µg)
C	SR141716A (6.25µg)
D	baclofen (0.2µg) + SR141716A (6.25µg)
Exp. 8	ICV Injection
Treatment groups	
A	CS *
B	baclofen (0.2µg)
C	AM630 (1.25µg)
D	baclofen (0.2µg) + AM630 (1.25µg)

CS: control solution, 2-AG: 2-Arachidonoylglycerol, selective CB_1 receptors agonist, Picrotoxin: $GABA_A$ antagonist, CGP54626: GABAB antagonist, CB65: selective CB_2 receptors agonist,CGP54626: $GABA_B$ antagonist, Gaboxadol: $GABA_A$ agonist, SR141716A: selective CB_1 receptors antagonist, AM630: selective CB2 receptors antagonist, Baclofen: $GABA_B$ agonist.

CGP54626 (21ng) and (D) co-administration of CB65+CGP54626. In experiment 5, birds injected with (A) saline, (B) Gaboxadol ($GABA_A$ agonist, 0.2µg), (C) SR141716A (selective CB_1 receptors antagonist, 6.25µg) and (D) Gaboxadol+ SR141716A injected. In experiment 6, ICV injection of (A) saline, (B) Gaboxadol (0.2µg), (C) AM630 (selective CB_2 receptors antagonist, 1.25µg) and (D) co-injection of Gaboxadol+AM630 were applied. In trial 7, avian injected with (A) saline, (B) baclofen ($GABA_B$ agonist, 0.2µg), (C) SR141716A (6.25µg) and (D) baclofen+SR141716A. In experiment 8, (A) saline, (B) baclofen ($GABA_B$, 0.2µg), (C) AM630 (1.25µg) and (D) co-injection of baclofen+AM630 used. The injection procedure is shown in Table 1. To find the possible interaction between these CBergic and GABAergic systems, effective and sub-effective doses of pharmacologic agents were administered to confront nullifying effects of the agents. In other words, when an effective dose of a system was administered, the sub-effective dose of the other system was considered. These doses of drugs were determined according to the previous studies (Jonaidi *et al.,* 2002; Chen *et al.,* 2006; Emadi *et al.,* 2011; Novoseletsky *et al.,* 2011; Alizadeh *et al.,* 2015; Hassanpour *et al.,* 2015).

Statistical analysis

Data is presented as mean ± SEM (standard error of the mean). Cumulative food intake (as the percentage of body weight) was analyzed by repeated measure two-way analysis of variance (ANOVA) using SPSS 16.0 for Windows (SPSS, Inc., Chicago, IL, USA). For treatment showing a main effect by ANOVA, means were compared by Tukey-Kramer test. $p<0.05$ was significant differences between treatments.

RESULTS

Effects and interactions of central CBergic and GABAergic systems on cumulative food intake in FD_3 neonatal layer-type bird are shown in Figures 1-8.

In experiment 1, ICV injection of effective dose of CB_1 receptors agonist (2-AG, 2µg) significantly increased food intake (% BW) in comparison with control group [$F(1,42)=67.02$; $p<0.001$]. Also, the ICV injection of sub effective dose of $GABA_A$ antagonist Picrotoxin (0.5µg) had no effect on food intake compared to control group [$F(1,42)=0.01$; $p>0.05$]. Co-injection of CB_1 receptors agonist plus $GABA_A$ antagonist (Picrotoxin + 2-AG) significantly decreased CB_1 receptors-induced hyperphagia [Time, $F(2,14)=93.45$, $p<0.001$; Picrotoxin × 2-AG interaction, $F(l,42)=48.95$, $p<0.001$] [Fig. 1].

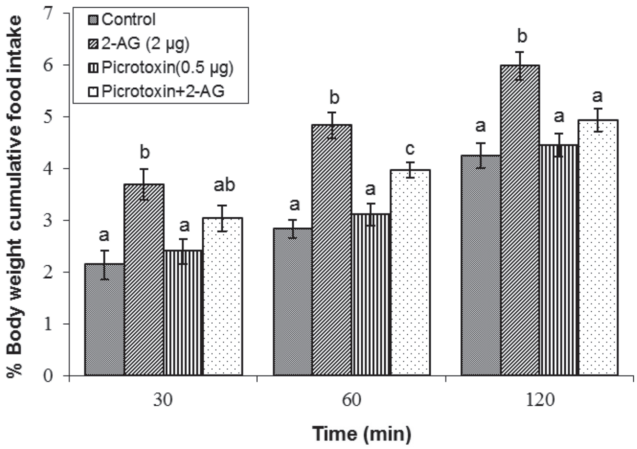

Figure 1 – Effect of ICV injection of 2-AG (2µg), Picrotoxin (0.5µg) and their combination on percent of body weight cumulative food intake in neonatal layer type chickens (n=48). 2-AG: 2-Arachidonoylglycerol, selective CB_1 receptors agonist, Picrotoxin: $GABA_A$ antagonist. Data are expressed as mean ± SEM. F and P value for within and between subject factors are as follows: Time, $F(2,14)=93.45$, $p<0.001$; 2-AG, $F(l,42)=67.02$, $p<0.001$; Picrotoxin, $F(l,42)=0.01$, $p>0.05$; Picrotoxin × 2-AG interaction, $F(l,42)=48.95$, $p<0.001$. Different letters (a, b and c) indicate significant differences between treatments ($p<0.001$). The average body weight for each group was as follows: control, 62.14 g; 2-AG, 61.71 g; Picrotoxin, 61.48 g; 2-AG + Picrotoxin, 62.83 g.

In experiment 2, ICV injection of 2-AG, (2µg) had hyperphagic effect compared to control group in neonatal layers [$F(1,38)=39.51$; $p<0.001$]. Also, ICV injection of sub effective dose of $GABA_B$ antagonist (CGP54626, 21ng) had no effect on food consumption [$F(1,38)=0.62$; $p>0.05$]. Co-injection of CGP54626 + 2-AG was not able to attenuated 2-AG-induced hyperphagia in neonatal layers [Time, $F(2,69)=112.04$, $p<0.001$; CGP54626× 2-AG interaction, $F(l,38)=0.08$, $p>0.05$] [Fig 2].

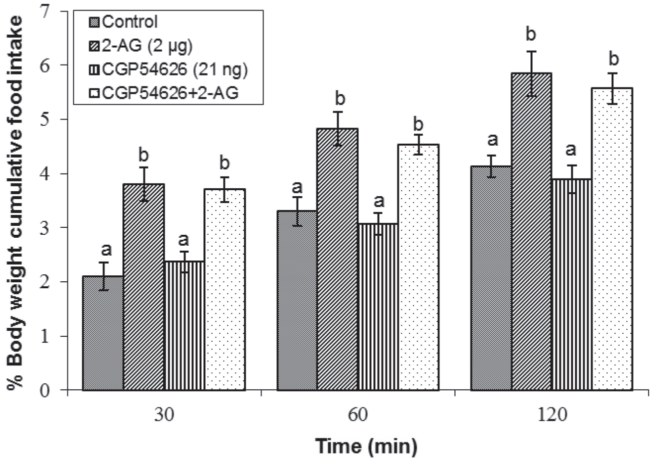

Figure 2 – Effect of ICV injection of2-AG (2µg), CGP54626 (21ng) and their combination on percent of body weight cumulative food intake in neonatal layer type chickens (n=48). 2-AG: 2-Arachidonoylglycerol, selective CB_1 receptors agonist, CGP54626: $GABA_B$ antagonist. Data are expressed as mean ± SEM. F and P value for within and between subject factors are as follows: Time, $F(2,69)=112.04$, $p<0.001$; 2-AG, $F(1,38)=39.51$, $p<0.001$; CGP54626, $F(1,38)=0.62$, $p>0.05$; CGP54626×2-AG interaction, $F(l,38)=0.08$, $p>0.05$. Different letters (a and b) indicate significant differences between treatments ($p<0.001$). The average body weight for each group was as follows: control, 64.26 g; 2-AG, 63.92 g; CGP54626, 63.19 g; CGP54626 + 2-AG, 62.38 g.

In experiment 3, ICV administration of CB65 (CB_2 receptors agonist, 1.25µg) significantly increased food

intake compared to control group [F(1,54)=76.01; p<0.001]. ICV administration of Picrotoxin (0.5µg) had no effect on food intake compared to control group [F(1,54)=0.08; p>0.05]. Additionally, co-injection of Picrotoxin+CB65 was not able to fluctuate hyperphagic effect of CB_2 receptors in neonatal layer-type chicks [Time, F(2,81)=67.52, p<0.001; Picrotoxin × CB65, F(1,54)=1.36; p>0.05] [Fig 3].

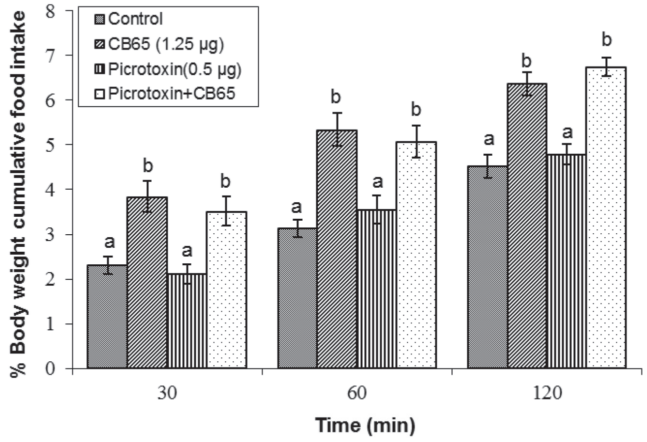

Figure 3 – Effect of ICV injection of CB65 (1.25µg), Picrotoxin (0.5µg) and their combination on percent of body weight cumulative food intake in neonatal layer type chickens (n=48). CB65: selective CB_2 receptors agonist, Picrotoxin: $GABA_A$ antagonist. Data are expressed as mean ± SEM. F and P value for within and between subject factors are as follows: Time, F(2,81)=67.52, p<0.001; CB65, F(1,54)=76.01, p<0.001; Picrotoxin, F(1,54)=0.08, p>0.05; Picrotoxin × CB65interaction, F(1,54)=1. 36; p>0.05. Different letters (a and b) indicate significant differences between treatments (p<0.001). The average body weight for each group was as follows: control, 63.41 g; CB65, 63.75 g; Picrotoxin, 62.74 g; CB65 + Picrotoxin, 62.51 g.

In experiment 4, ICV injection of CB65 (1.25µg) significantly amplified feeding behavior in neonatal layer-type chicks in comparison with control group [F(1,26)=47.15; p<0.001]. ICV injection of sub effective dose of $GABA_B$ antagonist, CGP54626 (21ng), had no role on food consumption compared to control group [F(1,26)=0.49; p>0.05]. Also, their combination (CGP54626+CB65) had no effect on CB_2 receptors agonist-induced hyperphagia [Time, F(2,55)=124.13, p<0.001; CGP54626× CB65interaction F(1,26)=1.27; p>0.05] [Fig 4].

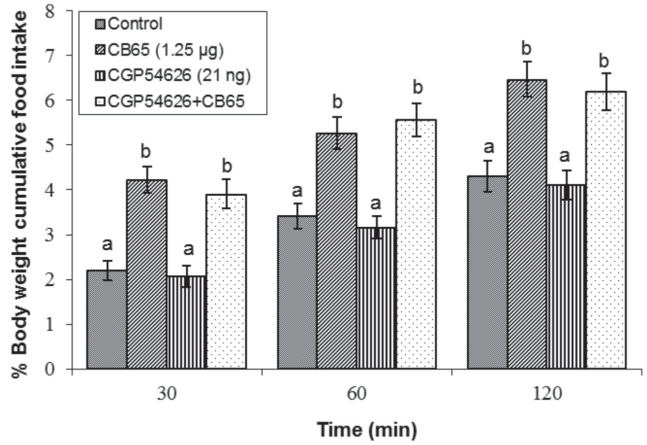

Figure 4 – Effect of ICV injection of CB65 (1.25µg), CGP54626 (21ng) and their combination on percent of body weight cumulative food intake in neonatal layer type chickens (n=48). CB65: selective CB_2 receptors agonist, CGP54626: $GABA_B$ antagonist. Data are expressed as mean ± SEM. F and P value for within and between subject factors are as follows: Time, F(2,55)=124.13, p<0.001; CB65, F(1,26)=47.15, p<0.001; CGP54626, F(1,26)=0. 49, p>0.05; CGP54626× CB65interaction, F(1,26)=1. 27; p>0.05. Different letters (a and b) indicate significant differences between treatments (p<0.001). The average body weight for each group was as follows: control, 61.43 g; CB65, 62.04 g; CGP54626, 63.27 g; CB65 + CGP54626, 62.17 g.

In experiment 5, ICV injection of effective dose of $GABA_A$ agonist, (Gaboxadol, 0.2 µg) significantly increased cumulative food intake compared to control group [F(1,53)=87.04; p<0.001]. ICV injection of sub effective dose of selective CB_1 receptors antagonist, (SR141716A, 6.25µg) had no effect on food intake [F(1,53)=0.31; p>0.05]. Also, co-administration of SR141716A + Gaboxadol significantly decreased Gaboxadol-induced hyperphagia at 60 and 120 min after the injection [Time, F(2,79)=56.27, p<0.001; SR141716A × Gaboxadol interaction, F(1,53)=65.93; p<0.001] [Fig 5].

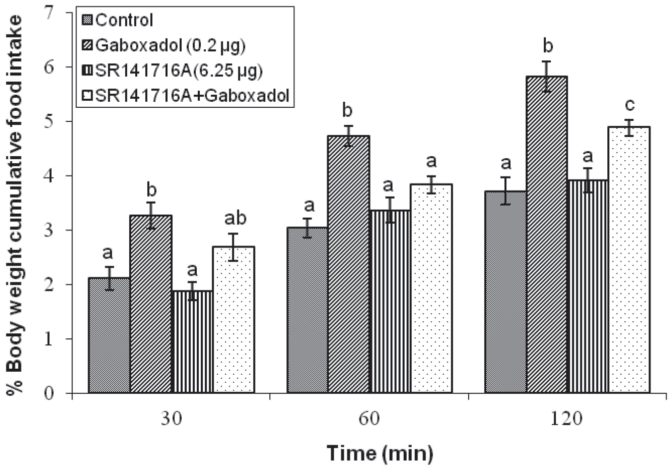

Figure 5 – Effect of ICV injection of Gaboxadol (0.2µg), SR141716A (6.25µg) and their combination on percent of body weight cumulative food intake in neonatal layer type chickens (n=48). Gaboxadol: $GABA_A$ agonist, SR141716A: selective CB_1 receptors antagonist. Data are expressed as mean ± SEM. F and P value for within and between subject factors are as follows: Time, F(2,79)=56.27, p<0.001; Gaboxadol, F(1,53)=87.04, p<0.001; SR141716A, F(1,53)=0.31, p>0.05; SR141716A × Gaboxadol interaction, F(1,53)=65.93; p<0. 001. Different letters (a, b and c) indicate significant differences between treatments (p<0.001). The average body weight for each group was as follows: control, 64.26 g; Gaboxadol, 64.74 g; SR141716A, 64.06 g; Gaboxadol + SR141716A, 63.81 g.

In experiment 6, ICV injection of Gaboxadol at dose of 0.2 µg amplified food consumption [F(1,21)=38.12; p<0.001] but ICV injection of sub effective dose of selective CB_2 receptors antagonist (AM630, 1.25µg) had no effect on feeding behavior in neonatal layer-type chicks [F(1,21)=1.02; p>0.05]. Co-injection of AM630+ Gaboxadol had no effect on $GABA_A$ receptors-induced hyperphagia [Time, F(2,17)=48.91, p<0.001; AM630 × Gaboxadol interaction, F(1,21)=2.37; p>0.05] [Fig 6].

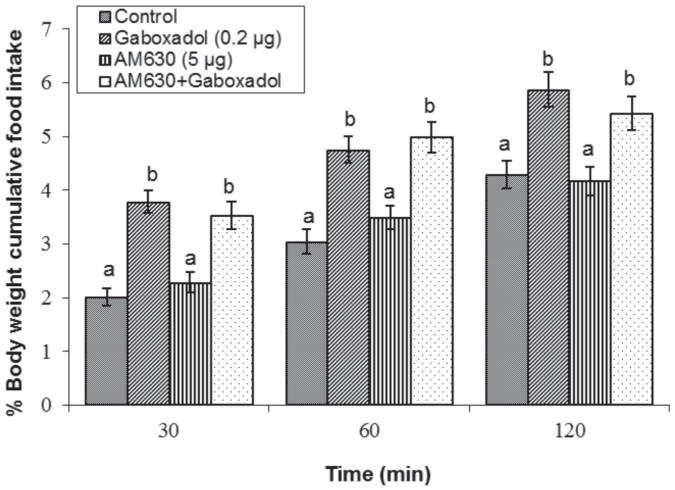

Figure 6 – Effect of ICV injection of Gaboxadol (0.2µg), AM630 (1.25µg) and their combination on percent of body weight cumulative food intake in neonatal layer type chickens (n=48). Gaboxadol: $GABA_A$ agonist, AM630: selective CB_2 receptors antagonist. Data are expressed as mean ± SEM. F and P value for within and between subject factors are as follows: Time, F(2,17)=48.91, p<0.001; Gaboxadol, F(1,21)=38.12, p<0.001; AM630, F(1,21)=1.02, p>0.05; AM630 × Gaboxadol interaction, F(1,21)=2.37; p>0.05. Different letters (a and b) indicate significant differences between treatments (p<0.001). The average body weight for each group was as follows: control, 62.35 g; Gaboxadol, 61.96 g; AM630, 61.47 g; Gaboxadol + AM630, 62.94 g.

In experiment 7, ICV injection of effective dose of $GABA_B$ agonist (baclofen, 0.2µg) significantly improved food intake in comparison to the control group [F(1,31)=46.73; p<0.001]. ICV injection of selective CB_1 receptors antagonist (SR141716A, 6.25µg) had no effect on food intake in neonatal layer-type chicks [F(1,31)=0.09; p>0.05]. Co-administration of SR141716A + baclofen had no effect on baclofen-induced hyperphagia [Time, F(2,83)=102.17, p<0.001; SR141716A × baclofen interaction, F(1,31)=0.04; p>0.05] [Fig 7].

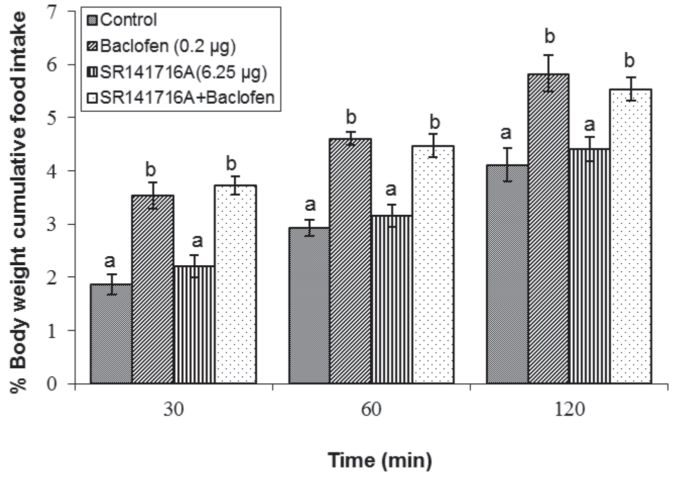

Figure 7 – Effect of ICV injection of Baclofen (0.2µg), SR141716A (6.25µg) and their combination on percent of body weight cumulative food intake in neonatal layer type chickens (n=48). Baclofen: $GABA_B$ agonist, SR141716A: selective CB_1 receptors antagonist. Data are expressed as mean ± SEM. F and P value for within and between subject factors are as follows: Time, F(2,83)=102.17, p<0.001; Baclofen, F(1,31)=46.73, p<0.001; SR141716A, F(1,31)=0.09, p>0.05; SR141716A × Baclofen interaction, F(1,31)=0.04; p>0.05. Different letters (a and b) indicate significant differences between treatments (p<0.001). The average body weight for each group was as follows: control, 64.11 g; baclofen, 64.26 g; SR141716A, 63.21 g; Baclofen + SR141716A, 62.75 g.

In experiment 8, ICV injection of baclofen (0.2µg) showed hyperphagic effect [F(1,46)=63.01; p<0. 001]; but ICV injection of AM630 (selective CB_2 receptors antagonist, 1.25µg) had no effect on feeding behavior [F(1,46)=0.06; p>0.05]. Also, the combined injection of baclofen + AM630 was not able to fluctuate $GABA_B$ receptors-induced food intake in neonatal layer-type chicks [Time, F(2,65)=70.31, p<0.001; AM630 × Baclofen interaction, F(1,46)=0.83; p>0.05] [Fig 8].

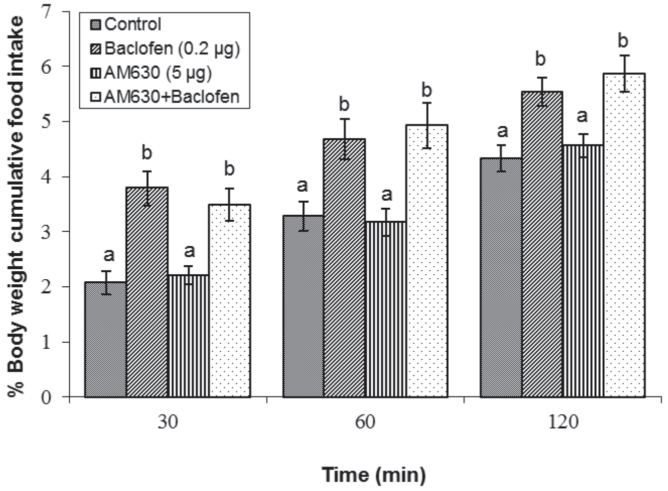

Figure 8 – Effect of ICV injection of Baclofen (0.2µg), AM630 (1.25µg) and their combination on percent of body weight cumulative food intake in neonatal layer type chickens (n=48). Baclofen: $GABA_B$ agonist, AM630 (selective CB_2 receptors antagonist). Data are expressed as mean ± SEM. F and P value for within and between subject factors are as follows: Time, F(2,65)=70.31, p<0.001; Baclofen, F(1,46)=63.01, p<0.001; AM630, F(1,46)=0.06, p>0.05; AM630 × Baclofen interaction, F(1,46)=0.83; p>0.05. Different letters (a and b) indicate significant differences between treatments (p<0.001). The average body weight for each group was as follows: control, 61.48 g; baclofen, 61.92 g; AM630, 63.19 g; baclofen + AM630, 62.38 g.

DISCUSSION

So far, numerous researches were done to investigate the role of neurotransmitters on appetite regulation in mammals, but aspects of food intake regulation in avian are still unclear (Zendehdel & Hassanpour 2014). To the best of our knowledge, there are no studies describing the interaction between CBergic and GABAergic systems on food intake in FD_3 neonatal layer-type chickens. According to the results, ICV injection of CB_1 and CB_2 agonists amplified food consumption in layer-type chicks which was similar to mammals (Chen *et al.,* 2006; Wiley *et al.,* 2012) and recent studies on layer-type chicks (Alizadeh *et al.*, 2015; Hassanpour *et al.,* 2015). However, these results were dissimilar compared to broilers which only CB_2 receptors were responsible for feeding (Emadi *et al.,* 2011; Novoseletsky *et al.,* 2011). The ECBs is an important modulator in the central and peripheral regulation of energy and

metabolism (Bellocchio *et al.,* 2013). Broilers are genetically selected for rapid muscle and body weight gain while layer has indirectly been selected for slow growth and low body weight. So, presumably, genetic selection altered chicken's brain neurological pathways associated with food intake (Denbow 1994; Richards 2003). It is a hypothetical effect of ECBs on appetite regulation which mediates at two levels. First, ECBs reinforces the motivation to find and eating, possibly via interaction with the mesolimbic pathways involved in reward mechanisms. Second, it activated 'on demand' in the hypothalamus after short-term food deprivation, then transiently regulates the action of the other orexigenic and anorectic neurotransmitters. The dual action hypothesis in mesolimbic and hypothalamic regions revealed by the finding that injection of ECBs into these brain areas provoke food intake in rodents (Di Marzo & Matias, 2005).

Based on the results, ICV injection of $GABA_A$ and $GABA_B$ receptors agonists increased food intake in neonatal layer-type chicks. These results were in agreement with observation in rats (Di Marzo *et al.*, 2004), broilers (Jonaidi *et al.,* 2002), turkeys (Denbow, 1991) sheep (Seone *et al.,* 1984) and pigs (Baldwin *et al.,* 1990). Regarding the site of action of GABA, with rats and sheep suggest GABA might alter food intake on some intra hypothalamic or extra-hypothalamic structures (Jonaidi *et al.,* 2002). Several possible mechanisms introduced the mechanism of action of GABA. For example, it may inhibit the satiety centers e.g. ventromedial hypothalamus (VMH). GABA may act via dis-inhibition of the brain feeding centers by inhibitory GABAergic projection from the medial accumbens to the lateral hypothalamus (LH). Finally, GABA can exert orexigenic effect via a direct excitatory effect in some regions of the brain including the hypothalamus (Jonaidi *et al.,* 2002).

As observed in this study, hyperphagic effects of 2-AG (CB_1 receptors agonist) attenuated by co-injection of 2-AG + Picrotoxin ($GABA_A$ receptors antagonist). CB_1 receptors control the activity of many neurotransmitter systems involved in central regulation of food intake (Pagotto *et al.,* 2006). In the CNS, CB_1 is predominantly expressed presynaptically, modulating the release of neurotransmitters, such as GABA, dopamine, noradrenaline, glutamate and serotonin (Cota *et al.,* 2003). These data showed there might be an interaction between central CBergic and GABAergic systems via CB_1 and $GABA_A$ receptors. The broad distribution of CB_1 receptors in the CNS, along with its modulatory role on other neurotransmitter systems, complicates the mechanisms underlying its effect on food intake regulation (Rey *et al.,* 2012).

Retrograde signaling by ECBs released from depolarized postsynaptic neurons inhibits presynaptic GABA release in the lateral hypothalamus and arcuate nucleus (ARC) (Di Marzo *et al.,* 2004). The ARC plays a crucial role in the integration of signals regulating appetite. The ARC is accessible to circulating signals of energy balance via the median eminence, this is not protected by the blood-brain barrier. In the ARC two types of neurons identified which integrates signals of nutritional status and influence energy homeostasis: pro-opiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART) which are anorexigenic neuropeptides while another neuronal circuit stimulates feed intake via neuropeptide Y (NPY) and agoutirelated protein (AgRP) (Zendehdel & Hassanpour 2014). The AgRP, POMC and NPY contain the $GABA_A$ R alpha (3) subunit in the ventromedial and ventrolateral part of ARC (Jonaidi *et al.,* 2002). ECBs impress their hyperphagic effect by modulating POMC neurons via CB_1 receptors in the ARC (Hentges *et al.,* 2005). Also, ICV injection of the CB_1 receptors agonist increases NPY levels (D'Addario *et al.,* 2014). A strong indication exists for CB_1 receptors on modulating appetite by enhancement of NPY (Cota *et al.,* 2003). One potential target for cannabinoids in the arcuate nucleus is NPY/ AgRP /GABA neurons (Menzies *et al.,* 2010). This is most likely a direct inhibitory effect on spontaneous release mediated via pre-synaptic CB_1 receptors on NPY/ AgRP /GABA neurons or other GABAergic terminals in the explanation. It is reported that CB_1 receptor mRNA are not expressed by arcuate NPY/ AgRP /GABA neurons and target probably underlies the direct effect (Menzies *et al.,* 2010). CB_1 receptors are expressed in presynaptic terminals of excitatory neurons (Sharkey *et al.,* 2014). Functionally, $GABA_A$ receptors seem to be located both pre- and post-synapses (Tellez *et al.,* 2012). $GABA_A$ receptors are ionotropic receptors which couple to a Cl^- ionophore (Jonaidi *et al.,* 2012). ECBs synthesized in postsynaptic neurons and binds to CB receptors on the presynaptic membrane. These receptors belong to G protein, inhibits Ca^{2+} influx which decreases the release of other neurotransmitters (Williams and Kirkham 2002). CBs modulate GABA release in a variety of CNS regions, including the hippocampus, basal ganglia, cerebellum, and brainstem. Activation of CB_1 receptors modulates the release of GABA in the brain. However, minute information exists about the mechanism behind the interaction of CB_1 with $GABA_A$ receptors.

Anatomical investigations have shown high levels of CB_1 receptor immune reactivity and mRNA associated with GABAergic neurons. CBs modulate GABA release by a presynaptic mechanism. Presumably, effect of CB_1 receptors on $GABA_A$ release is mediated via inhibition of presynaptic Ca^{2+} channels (Irving *et al.,* 2002).

To the best of our knowledge, CB_1 receptors mRNA expressed by POMC neurons and CBs directly inhibit arcuate POMC neurons. In turn, POMC neurons inhibit GABA release from NPY/AgRP neurons (Menzies *et al.,* 2010). As observed in this study, the hyperphagic effect of CB_1 receptors diminished by $GABA_A$ receptors antagonist (experiment 1). For instance, hyperphagic effects of $GABA_A$ receptors minimized by CB_1 receptors antagonist (experiment 5). Presumably, ECBs, by inhibition of the hypothalamic POMC, leads to increasing GABA release in the CNS of neonatal layer-type chicken. So, it seems both CBergic and GABAergic systems impress their hyperphagic effects via CB_1 receptors in neonatal layer-type chicken. Also, perhaps CBergic and GABAergic systems interact with other systems such as opioidergic system (Menzies *et al.,* 2010).

Most research on central food intake regulation was done on rat models because few investigations were in avian. These results can use as base information on central appetite regulation in layer-type chicken. Finally, the authors recommend merit investigation needed to find direct cellular and molecular signaling pathways of CBergic and GABAergic mechanism with other neurotransmitters in the physiology of eating behavior in poultry.

CONFLICT OF INTEREST

Authors declare that they have no conflict of interest.

INFORMED CONSENT

This manuscript does not contain any studies with human subjects performed by any of the authors.

HUMAN AND ANIMAL RIGHTS

All experiments executed according to the Guide for the Care and Use of Laboratory Animals and approved by the institutional animal ethics committee.

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