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Enhanced GABAergic inhibition in the premammillary nucleus of photorefractory turkey hens via $GABA_A$ receptor upregulation

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ABSTRACT

The premammillary nucleus (PMM) of the turkey mediobasal hypothalamus, where dopamine-melatonin (DA-Mel) neurons are localized, is a site for photoreception and photoperiodic time measurement, which is essential for the initiation of avian reproductive seasonality. In addition, this area could also be responsible for the onset and maintenance of photorefractoriness at the end of the breeding season due to the enhanced inhibitory effect of γ-aminobutyric acid (GABA). GABA is an inhibitory neurotransmitter in the central nervous system which interferes with the photosexual response in the turkey, a seasonally breeding bird. Here, we further characterized the $GABA_A$ receptor subunits in the PMM DA-Mel neurons related to reproductive seasonality and the onset of photorefractoriness. $GABA_A$ receptor subunits and GABA synthesis enzymes in the PMM of photosensitive and photorefractory turkey hens were identified using real-time qRT-PCR. The upregulation of $GABA_A$ receptor α1-3, β2-3, γ1-3, ρ1-3, δ, and θ mRNA expression were observed in the PMM of photorefractory birds when compared to those of photosensitive ones while there is no change observed in the GABA synthesis enzymes, glutamate decarboxylase 1 and 2. Those upregulated $GABA_A$ receptor subunits were further examined using immunohistochemical staining and they appeared to be co-localized within the PMM DA-Mel neurons. The upregulation of $GABA_A$ receptor subunits observed in the PMM of photorefractory birds coincides with a lack of responsiveness to a light stimulus provided during the photosensitive phase. This is supported by the absence of *c-fos* induction and *TH* upregulation in the PMM and a subsequence inhibition of *c-fos* and *GnRH-I* expression in the nucleus commissurae pallii. The augmented $GABA_A$ receptor subunits expression may mediate an enhancement of inhibitory GABAergic neurotransmission and the subsequent interference with the photosexual response. This could contribute to the state of photorefractoriness and the termination of breeding activities in the turkey, a temperate zone bird.

1. Introduction

Photorefractoriness is a key stage of the reproductive life cycle in temperate zone breeding birds associated with a loss of response to a gonad stimulatory photoperiod, the suppression of reproductive hormones, regression of the reproductive system, and a complete molt (Cho et al., 1998; Dawson, 2013; Dawson et al., 2001; Kosonsiriluk et al., 2013). Seasonally breeding birds become photorefractory after prolonged exposure to the stimulating photoperiod and cease their reproductive neuroendocrine responses (Dawson and Goldsmith, 1984; Dixit and Singh, 2011). Recent findings from our laboratory reveal a substantial component that may contribute to the onset of photorefractoriness, the inhibitory neurotransmitter γ-aminobutyric acid (GABA) (Kosonsiriluk et al., in press). GABA is a major inhibitory neurotransmitter in the mammalian central nervous system and plays an important role in regulating neuronal activity in the mammalian master clock, the suprachiasmatic nucleus (SCN) (Albus et al., 2005; Liu and Reppert, 2000; Shirakawa et al., 2000). The presence of the GABAergic system has been identified in the turkey premammillary nucleus (PMM) which is located in the upper most segment of the mediobasal hypothalamus (MBH) (Kosonsiriluk et al., in press).

Abbreviations: AANAT, aralkylamine N-acetyltransferase; β-Actin, actin, beta; CT, circadian time; DA-Mel, dopamine-melatonin; GABA, γ-aminobutyric acid; GAD, glutamate decarboxylase; GnRH-I, gonadotropin-releasing hormone-I; HPRT1, hypoxanthine phosphoribosyltransferase 1; IHC, immunohistochemistry; MBH, mediobasal hypothalamus; nCPA, nucleus commissurae pallii; PMM, premammillary nucleus; PR, photorefractory; PS, photosensitive; real-time RT-qPCR, real-time quantitative reverse transcription polymerase chain reaction; SCN, suprachiasmatic nucleus; TH, tyrosine hydroxylase; TPH1, tryptophan hydroxylase 1.

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The turkey PMM is a site for photoreception, photoperiodic time measurement, and the transmission of the encoded photoperiodic information to the reproductive neuroendocrine system (Kosonsiriluk et al., 2013). The photic information is conveyed by melanopsin photopigment (Kang et al., 2010) expressed in PMM dopamine-melatonin (DA-Mel) neurons (Kang et al., 2007). Clock genes are also identified in this area and display rhythmic activity with *Per3* and *Cry1* being responsive to the photic stimulus during the photoinducible phase (Leclerc et al., 2010). The encoded photoperiodic information transmits to the reproductive neuroendocrine system, the pars tuberalis of the pituitary, via DAergic fibers projecting from DA-Mel neurons in the PMM (Kang et al., 2010). A light pulse provided during the photoinducible phase at circadian time (CT) 14 enhances DAergic activity in the PMM and the release of gonadotropin-releasing hormone-I (GnRH-I) from the nucleus commissurae pallii (nCPA) (Thayananuphat et al., 2007). Considering the PMM as an initial site for the photoperiodic reproductive neuroendocrine responses, we believe that this nucleus is also responsible for the onset of photorefractoriness and the termination of reproductive activities in avian seasonal breeders.

It is well established that GABA plays a role in modulating neuronal activity in the SCN including circadian responses to light (Gillespie et al., 1997, 1999; Hummer et al., 2015). Enhanced GABAergic inhibition has been shown to interfere with light transduction in both the mammalian SCN (Colwell et al., 1993) and the avian PMM (Kosonsiriluk et al., in press). Administration of GABAergic agonists prevents light-induced *c-fos* expression and causes phase shift circadian rhythms in the mammalian SCN (Colwell et al., 1993; Moldavan and Allen, 2013; Ralph and Menaker, 1989). Likewise, the injection of a GABA receptor agonist blocks light-induced *c-fos* expression in the turkey PMM together with the subsequent photoperiodic reproductive neuroendocrine responses (Kosonsiriluk et al., in press). GABA receptors and synthetic enzymes are expressed in the turkey PMM and the $GABA_A$ receptor, α2 and α4, subunits and the $GABA_B$ receptor 2 are found to be upregulated in photorefractory birds (Kosonsiriluk et al., in press).

$GABA_A$ receptors in the mammalian brain are heterogeneous and exist as pentameric constructs of 19 subunits containing α1-6, β1-3, γ1-3, ρ1-3, δ, π, θ, and ε as reviewed by Barnard et al. (1998). Functional receptors are formed from specific arrangements of subunits with the particular combination of subunits determining the pharmacological profile and/or cellular location of the receptor (Rabow et al., 1995). Our recent findings suggest that GABAergic neurotransmission of PMM DA-Mel neurons in the MBH might be involved in the reproductive neuroendocrine system's responsiveness to light, which regulates reproductive seasonality (Kosonsiriluk et al., in press). We therefore hypothesized that different subpopulations of $GABA_A$ receptor subunits in PMM DA-Mel neurons are responsible for the action of GABA on the state of photorefractoriness.

To further investigate the PMM control of the reproductive neuroendocrine system, we focused on the changes in GABAergic neurotransmission and their effects on the reproductive neuroendocrine system by examining the expression of $GABA_A$ receptor subunits in the turkey PMM and their association with the state of photorefractoriness.

2. Materials and methods

2.1. Animals

Somatically mature female Hybrid turkeys (*Meleagris gallopavo*) at 24 weeks of age, provided by Willmar Poultry Company, Willmar, MN, were housed in floor pens under a lighting regimen of 6 hours light/18 hours dark (6L:18D). At 28 weeks of age, birds were subjected to long day (18L:6D) and maintained through a complete reproductive cycle (i.e. laying, incubation, and photorefractory). Only those birds that entered photorefractory state were further used in the experiment. Feed and water were available *ad libitum*. All animals were treated in accordance with University of Minnesota Institutional Animal Care and Use Committee Guidelines.

For the gene expression analysis experiment, thirty-two of these photorefractory birds were exposed to short day (6L:18D) for 8 weeks to regain photosensitivity and then divided into 2 groups: 1) photosensitive group was maintained under short day light regimen and 2) photorefractory group was subjected to long day and maintained through a complete egg laying period and the initiation of molt. Birds in the photorefractory group were exposed to short day regimen one day before photostimulation. Six additional photorefractory birds were used for immunohistochemical staining to locate $GABA_A$ receptor subunits in the PMM.

2.2. Photostimulation

Birds in the photosensitive and photorefractory groups were further divided into 2 groups: 1) photosensitive – dark control (PS dark); 2) photosensitive – light pulse (PS light pulse); 3) photorefractory – dark control (PR dark); and 4) photorefractory – light pulse (PR light pulse). Birds in the PS light pulse and PR light pulse groups were exposed to a 30 min light pulse during the photoinducible phase for the stimulation of reproductive neuroendocrine system at CT14 while birds in the PS dark and PR dark groups were not exposed. Four birds, one from each treatment group, were euthanized in the same day at CT 14.5. Each treatment group contained eight birds.

2.3. Tissue preparation

The nCPA and PMM tissue preparation for real-time quantitative reverse transcription polymerase chain reaction (real-time qRT-PCR) was performed as previously described by Kosonsiriluk et al. (in press). Briefly, Birds were euthanized by intravenous injection of sodium pentobarbital (Euthasol, Virbac AH, Inc., Fort Worth, TX, USA). The whole brain was removed from the skull, frozen in powderized dry ice, and stored at −80 °C until microdissection. Ten consecutive sections (40 μm thickness, encompassing the entire nCPA and PMM as defined by Youngren, unpublished turkey atlas) were manually microdissected for the nCPA and the PMM with a magnifier using a hypodermic needle (I.D. 0.889 mm, Small Parts Inc., San Clemente, CA, USA).

Tissue preparation for double immunohistochemistry (IHC) was performed as previously described by Thayananuphat et al. (2007). Briefly, birds were injected with 5 ml of heparin (NDC25021-400-10, Sagent Pharmaceuticals, Schaumburg, IL, USA), before euthanized with sodium pentobarbital (Euthasol, Virbac AH, Inc.). A decapitated head was immediately pressure-perfused via the carotid arteries for 5 min with 0.1 M phosphate-buffered saline, followed by freshly prepared 4% paraformaldehyde solution in 0.1 M phosphate buffer (pH. 7.4) for 30 min. Brains were extracted and placed in 20% sucrose in PBS at 4 °C until saturated for cryoprotection, then frozen in powderized dry ice, and stored at −80 °C. Brains were sectioned in a cryostat (−20 °C) at 16 μm thickness, then mounted onto microscope slides (ProbeOn Plus, Thermo Fisher Scientific, Waltham, MA, USA) and stored desiccated at −80 °C until further processed for immunohistochemical staining.