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Research paper

Ovarian steroid withdrawal results in $GABA_A$ receptor upregulation in the photoperiodic neuroendocrine pathways of the turkey hen

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ABSTRACT

The mechanism(s) underlying photorefractoriness in temperate zone seasonally breeding birds remains undetermined. Our recent findings reveal a link between the upregulation of $GABA_A$ receptors ($GABA_A$Rs) in the premammillary nucleus (PMM) and the state of photorefractoriness. Gonadal steroid levels fluctuate during the breeding season; increasing after gonadal recrudescence and declining sharply once gonadal regression begins. Here, we examined the effect of gonadal steroid withdrawal on the expression of $GABA_A$Rs in the turkey PMM. Exogenous ovarian steroids were administered and then withdrawn from turkey hens to mimic the decline of ovarian steroids levels at the end of a breeding season. The upregulation of $GABA_A$R α3, α4, δ, π, and γ2-subunits was observed in the PMM of the steroid withdrawal group when compared to the non-steroid treatment group. The level of tyrosine hydroxylase, photopigment melanopsin, and circadian clock genes in the PMM of the steroid withdrawal group resembled the levels observed in the natural photorefractory hens and were significantly lower than those of the short-day light stimulated group. A reduction in gonadotropin-releasing hormone-I mRNA expressed within the nucleus commissurae pallii was also observed in hens undergoing steroid withdrawal. These results suggest that the natural decline in circulating ovarian steroid levels may modulate the GABAergic system in the PMM through the upregulation of $GABA_A$ receptors. This, in turn, could diminish the reproductive neuroendocrine responses to light and favor a condition resembling the state of photorefractoriness.

1. Introduction

Photorefractoriness is an essential stage in the reproductive cycle of temperate zone birds insuring that the young are hatched at the appropriate time of the year for optimal survival. In temperate zone birds, molting and photorefractoriness are critical stages, necessary for survival and breeding (Coppack and Pulido, 2004). Photorefractoriness is characterized by the inability to decode light information as a sexual stimulation, marked by a decrease in hypothalamic gonadotropin-releasing hormone-I (GnRH-I), luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and gonadal regression (Dawson et al., 2001; Dawson and Sharp, 2007; Dawson, 2008, 2015). The domestic turkey is a seasonally breeding bird with a reproductive cycle regulated by photoperiod and terminated by the state of photorefractoriness (Siopes, 2001).

The neurotransmitter gamma-aminobutyric acid (GABA) is a major inhibitory molecule in the brain. Its inhibitory postsynaptic potential is mediated by a gated chloride channel, an intrinsic property of the $GABA_A$ receptors ($GABA_A$Rs) (Pritchett et al., 1989). $GABA_A$Rs mediate most fast inhibitory action in the brain (Jacob et al., 2008). It can be assembled as multiple subunits including 19 subunits designated as α1-6, β1-3, γ1-3, δ, ε, θ, ρ1-3, and π (MacKenzie and Maguire, 2014). The upregulation of $GABA_A$-Rs during ovarian steroid hormone withdrawal has been observed in several studies in rat brain (Maggi and Perez, 1984; Smith, 2002; Smith et al., 2007). An increase in the $GABA_A$Rs in the dopaminergic areas of the rat brain is observed after ovariectomy and is corrected by estradiol treatment (Bosse and DiPaolo, 1996). The number of $GABA_A$ receptor binding sites is increased in the mediobasal hypothalamus (MBH) of ovariectomized rats (Juptner et al., 1991). In addition, progesterone is converted to allopregnanolone (3alpha, 5alpha-THP) in the brain (Smith et al., 1998a). The primary targets of allopregnanolone are members of the $GABA_A$R subunit family (Belelli and Lambert, 2005; Belelli et al., 2009). Both estrogen and progesterone have been shown to modulate the GABAergic system in the brain of the rat and the human (Genazzani et al., 2000; Maggi and Perez, 1984).

Previous studies from our laboratory have explored the mechanisms involved in photoreception in seasonally breeding turkey hens. The dopamine-melatonin (DA-Mel) neurons in the premammillary nucleus (PMM) were identified as putative hypothalamic photoreceptive cells which regulate reproductive seasonality in

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the turkey (Kang et al., 2007). Circadian clock genes, including the photoinducible genes Per3 and Cry1, and the photopigment gene melanopsin (OPN4x), are localized within these neurons (El Halawani et al., 2009; Kang et al., 2007, 2010; Kosonsiriluk et al., 2013; Leclerc et al., 2010). More recently, we found the upregulation of $GABA_AR$ subunits within the PMM DA-Mel neurons of photorefractory turkey hens (Kosonsiriluk et al., 2016b,a).

In the study described in this article, we propose that the enhanced inhibitory action of the GABAergic system in the PMM is caused by ovarian steroid withdrawal through the upregulation of $GABA_ARs$. Notably, the modulation of $GABA_ARs$ during ovarian steroid hormone withdrawal at the end of the breeding season may be crucial in understanding the state of photorefractoriness in seasonally breeding birds. Thus, we concentrated on the influence of withdrawing exogenously administered ovarian steroids on $GABA_AR$ expression.

2. Materials and methods

2.1. Experimental animals

Hybrid turkey hens (*Meleagris gallopavo*) provided by Willmar Poultry Company, Willmar, MN were raised and used at 33 weeks of age. All the birds were housed and treated in accordance with University of Minnesota Institutional Animal Care and Use Committee Guidelines.

The hens were housed in floor pens with feed and water available *ad libitum*. The light controlled rooms were lit by incandescent bulbs and provided with nests to monitor nesting behavior. The hens were housed under a light regime of 6 h of light and 18 h of darkness (6L:18D, short-day). The four experimental groups (each n = 8) were: (1) short-day non-photostimulated dark-control (SD-DC) – these hens remained under the light regime of 6L:18D and sampling took place under dim blue light (less than 2 lx); (2) short-day light stimulated hens (SD-LS) – these hens remained under the light regime of 6L:8D and were light stimulated as described below; (3) short-day steroid withdrawal light stimulated hens (SWD-LS) – these hens remained under the light regime of 6L:18D, were treated with steroids (described in Section 2.2 below) followed by steroid withdrawal and were light stimulated as described below; and (4) photorefractory-light stimulated hens (PR-LS) – these hens were allowed to progress through a normal reproductive cycle and handled as described below. Due to the extended length of the hen's reproductive cycle and to allow the collection of all groups at the same time, the hens placed in the PR-LS group were acquired 5 months prior to the other hens so a reproductive cycle could be completed. This insured that the hens were naturally photorefractory and had completed a molt.

The SD-LS and SWD-LS groups were housed under 6L:18D throughout the experiment, and received a single 30 min light pulse (25 lx) during the photoinducible phase for gonad stimulation at circadian time 14 (CT14; adequate for induction of sexual development in turkey (Siopes, 1984)). The PR-LS group was housed under 16L:8D and maintained through a complete cycle of egg laying and molt, then subjected to short-day (6L:18D) for 24 h before a photostimulation of a single 30 min light pulse (25 lx) at CT14. One bird of each group of four experimental groups was terminated at the same time during the same time period post-light pulse at CT14.

2.2. Ovarian steroid hormone implantation regimen in short-day steroid withdrawal group

To mimic the profile of circulating steroid hormones during the reproductive cycle, turkey hens were primed with 17-beta-estradiol-3-benzoate (EB), followed by the combinations of EB and progesterone (P), then P alone. And, to eliminate the confounding effects of light, the reproductive quiescence short-day photosensitive hens in the SWD-LS group were employed and subcutaneously implanted at the dorsum of the neck with the steroid hormone time release pellets containing EB and/or P (Innovative Research of America, Sarasota, FL, USA): EB 0.2 mg/kg/day (14-day release, Day 0 – Day 14); EB 0.04 mg/kg/day + P 2 mg/kg/day (10-day release, Day 15 – Day 24); and P 2 mg/kg/day (20-day release, Day 25 – Day 44) respectively. The subcutaneously time-release sterilized pellets were designed to activate immediately and slow-release when contacted with fluids inside the body after implantation. The dosage of EB and P hormones were modified from the previous study (El Halawani et al., 1986).

2.3. Physiological and behavioral observations

Squatting behavior and molting in response to hormone treatments and withdrawal were monitored. The characteristic that usually expressed by photorefractory birds (i.e. molt, nervous habits, and escapes when approached) were observed. Postmortem examination of reproductive organs was also performed to verify the reproductive stages of photosensitive and photorefractory hens. Molt stages were observed on the basis of feathers shedding from head, neck, breast, wings, and tails (Paguia et al., 2012). These molt stages characteristics were compared between the steroid withdrawal hens and the photorefractory hens.

2.4. Tissue collection, preparation, and nCPA/PMM microdissection

The hens were euthanized using an overdose of sodium pentobarbital injected intravenously (Midwest Veterinary Supply Inc., Lakeville, MN, USA). The whole brain was removed from the skull, frozen in powdered dry ice, and stored at −80 °C. For the real-time quantitative reverse transcription polymerase chain reaction (real-time qRT-PCR) experiments, the nCPA and the PMM were microdissected using a needle tubing punch (I.D. 0.889 mm, Small Parts Inc., San Clemente, CA, USA). The PMM and nCPA microdissection were performed as previously described (Kosonsiriluk et al., 2016b).

The expression levels of $GABA_ARs$ (α1-6, β1-3, γ1-3, δ, ε, θ, ρ1-3, π) and the biosynthetic enzymes for GABA, glutamate decarboxylase 1 (GAD1) and glutamate decarboxylase 2(GAD2) were compared among the steroid withdrawal, photorefractory, and photosensitive groups. Tyrosine hydroxylase (TH), arylalkylamine N-acetyltransferase (AANAT), tryptophan hydroxylase 1 (TPH1), OPN4x, and circadian clock genes (Per2, Per3, and Cry1) mRNA expression in the PMM, and GnRH-I mRNA expression in the nCPA were also monitored to verify the reproductive neuroendocrine response to light.

2.5. RNA isolation and cDNA synthesis

Total RNA from microdissected nCPA and PMM tissues was extracted using RNAqueous-Micro Kit followed by DNase I treatment according to manufacturer's protocol (Ambion®, Life Technologies, Grand Island, NY, USA) and reverse-transcribed to cDNA using SuperScript VILO™cDNA Synthesis Kit (Invitrogen, Life Technologies, Grand Island, NY, USA) according to the manufacturer's instructions, and stored at −20 °C until further processed.

2.6. Real-time qRT-PCR analysis

Real-time qRT-PCR was performed in triplicates using Stratagene™ Mx3005P (Agilent Technologies Inc., Santa Clara, CA, USA), and Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix (Agilent