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_ARs) 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_ARs 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 Exogenous ovarian steroids were administered and then withdrawn from turkey hens to mimic the decline of ovarian steroids levels at the end of a breeding season.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_AR $\alpha 3$, $\alpha 4$, δ , π , and $\gamma 2$ -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; Dawson, 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 post-synaptic potential is mediated by a gated chloride channel, an intrinsic property of the GABA_A receptors (GABA_ARs) (Pritchett et al., 1989). GABA_ARs mediate most fast inhibitory action in the brain (Jacob et al., 2008). It can be assembled as multiple subunits including 19 subunits designated as α 1-6, β 1-3, γ 1-3, δ , ϵ , θ , ρ 1-3, and π (MacKenzie and Maguire, 2014). The upregulation of GABA_ARs 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_ARs 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 (3 α , 5 α -THP) in the brain (Smith et al., 1998a). The

primary targets of allopregnanolone are members of the GABA_AR 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 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; Kang et al., 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., in press; Kosonsiriluk et al., 2016).

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 hours of light and 18 hours 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 lux); (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 lux) 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 lux) 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., in press).

The expression levels of GABA_ARs (α 1-6, β 1-3, γ 1-3, δ , ϵ , θ , ρ 1-3, 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 Technologies Inc., Santa Clara, CA, USA). The reaction was run at 95°C for 3 min, then 40 cycles of 95°C for 15 sec, and 60°C for 20 sec followed by a melting curve analysis and agarose gel electrophoresis after each run to confirm a single product at the expected size. Relative mRNA levels were calculated by the $\Delta\Delta CT$ method (Livak and Schmittgen, 2001) using hypoxanthine phosphoribosyltransferase 1 (HPRT1) and β -actin (ACTB) as reference genes. The amplification efficiency of all the primers was between 95-105% with $R^2 > 0.99$. Quality controls for qPCR analyzes include for all runs were: (1) negative control, containing all of the RT-qPCR reagents but no-template (NTC) and no-reverse transcriptase (no RT) controls, (2) Inter-plate control, containing a pooled sample from all groups were included in each run to ensure the reproducibility. All primer sequences and GenBank accession numbers of gene products can be found in a previous study (Kosonsiriluk et al., 2016).

2.7. Statistical analysis

Real-time qRT-PCR results were expressed as mean $\pm$ SEM (n=8 per group). The data were subjected to One-way Analysis of Variance (ANOVA) and the significant differences among treatments group were determined by a Bonferroni Post hoc test using GraphPad Prism[®] software version 6.0h (La Jolla, CA, USA). A P value <0.05 was considered statistically significant.

3. Results

3.1. Effect of ovarian steroid hormones implantation and withdrawal on the physiological and behavioral changes associated with photorefractoriness in turkey hens

Sexual receptivity (squatting behavior) as noted in layers is prominent during the priming with EB in the steroid withdrawal group. The squatting hens typically were calm and crouched down close to the ground with gently outstretched wing feathers when approached. After administration of P, the squatting behavior was suppressed, and the hens showed nesting activity. The molting occurred during the withdrawal periods, which began nearly 2 weeks after withdrawal and continued until 3-5 weeks after the initiation of molt. The degree of molting in each hen is indicated in Table S1. The steroid withdrawal hens on short-day with the molting score of +6 showed the most molting during the withdrawal period when compared to the short-day photosensitive hens as shown in Fig. 1A-B. During the late molting stages, the steroid withdrawal hens exhibited at finished molt with new pin feathers which was comparable to that observed in the natural photorefractory hens (Fig. 1C-D).

The expected physiological and behavioral changes associated with photorefractoriness, including nervous habits, escaping when approached, and molting, was expressed in the steroid withdrawal hens as observed in the photorefractory hens. The behavioral excitability when approached and the shedding of feathers with new pin feather growth following the withdrawal period was video recorded to document this behavior and phenotype and is shown in the supplementary electronic materials.

3.2. Hypothalamic mRNA expressions of GnRH-I in the nCPA and TH, OPN4x, AANAT, TPH1, circadian clock genes (Per2, Per3, and Cry1) in the PMM of the steroid withdrawal turkey hens.

We examined the levels of the reproductive neuroendocrine response genes including *GnRH-I* in the nCPA, and *TH*, *AANAT*, *TPH1*, *OPN4x*, and circadian clock genes (*Per2*, *Per3*, and *Cry1*) in the PMM of the steroid withdrawal light stimulated (SWD-LS) hens, photorefractory light stimulated (PR-LS) hens, and short-day light stimulated (SD-LS) hens compared to the control group, short-day non-photostimulated dark-control hens (SD-DC). As shown in Fig. 2 and Supplementary Table S2, the levels of *GnRH-I*, *TH*, *OPN4x*, *Per2*, *Per3*, and *Cry1* mRNA did not differ significantly between the groups SD-DC, PR-LS, and SWD-LS, but were significantly increased in SD-LS group. Even though the *AANAT* and *TPH1* mRNA were trending toward higher expression levels in PR-LS and SWD-LS groups as compared to the SD-DC and SD-LS groups, these differences were not statistically significant. The gene expression pattern of GnRH-I neurons in the nCPA and all the genes (*TH*, *AANAT*, *TPH1*, *OPN4x*, *Per2*, *Per3*, and *Cry1*) in the PMM DA-Mel neurons of steroid withdrawal hens was comparable to that expressed in the photorefractory hens indicating that this steroid withdrawal model in turkey can be model or recapitulate the state of photorefractoriness study. We also examined the expression

of the GABA synthesis enzymes in the PMM DA-Mel neurons of the photorefractory and steroid withdrawal hens.

3.3. Effect of ovarian steroid withdrawal on GABA synthetic enzymes and GABA_ARs expression in turkey hens

Evaluation of GABA_ARs as candidate genes for photorefractoriness by ovarian steroid withdrawal in seasonally breeding turkey hens was investigated using real-time qRT-PCR. All subunits were screened to determine the subunit upregulation (Fig. 3A-E and Supplementary Table S3). A one-way ANOVA with Bonferroni Post hoc comparisons at $P < 0.05$ demonstrated that the expression levels of GABA_AR $\alpha 3$, $\alpha 4$, δ , π , and $\gamma 2$ -subunits was significantly upregulated when ovarian steroid hormone levels fell in SWD-LS and PR-LS groups as compared to the SD-LS group.

To evaluate if steroid treatment followed by withdrawal could manipulate GABAergic activity through a shift in the synthesis of this inhibitory neurotransmitter, the expression of the mRNAs encoding the synthetic enzymes, glutamate decarboxylase 1 (GAD1) and glutamate decarboxylase 2 (GAD2), was examined. We found no significant difference in GAD1 or GAD2 mRNA expressed in the PMM of photorefractory or steroid withdrawal hens as compared to the short-day light stimulated hens (Supplementary Table S2).

4. Discussion

The present study investigated the effect of diminishing exogenously administered ovarian steroids (EB and P) on reproductive behavior and the modulation of GABAergic

inhibition in the turkey, a temperate zone bird. The observed physiological and behavioral changes proved that sexually receptive behavior (i.e. squatting) and nesting activity could be induced in experimental turkey hens when EB was implanted alone and followed by a combination of EB and P as described in a previous study (El Halawani et al., 1986). In addition, the steroid withdrawal hens manifested an average period of 3-5 weeks of molting after the initiation of molt until a complete molt, comparable to that observed in the natural photorefractory hens. These findings suggest that a physiological state resembling photorefractoriness was induced in our steroid withdrawal turkey hen model.

In seasonally breeding temperate zone birds, including the domestic turkey hen, the changes in the reproductive neuroendocrine pathway and the subsequent manifestation of photorefractoriness terminate the reproductive season (MacDougall-Shackleton et al., 2006; Siopes, 2001; Siopes and Proudman, 2009). In the present study, we observed that the expression patterns of genes in this neuroendocrine pathway including *GnRH-I* in the nCPA (Kang et al., 2006; Thayanunphat et al., 2007), *TH* (DA synthesis), *OPN4x* (deep brain photopigment) (Kang et al., 2007; Kang et al., 2010), and circadian clock genes (*Per2*, *Per3*, *Cry1*) in the PMM where DA-Mel neurons are localized (Leclerc et al., 2010) were all downregulated in the steroid withdrawal hens as in photorefractory hens. As expected, in photosensitive hens, these genes were upregulated following exposure to a light pulse provided during the photoinducible phase. In contrast, *TPH1* and *AANAT* of the MELergic system, which controls the night/day rhythm in the PMM, are upregulated and comparable in both steroid withdrawal and photorefractory hens (Kang et al., 2007; Kang et al., 2010).

The evidence from our prior studies (Kosonsiriluk et al., in press; Kosonsiriluk et al., 2016) of enhanced GABAergic activity during the state of photorefractoriness showed that

GABAergic neurotransmission in the PMM modulates reproductive seasonality and the initiation of photorefractoriness in turkey hens. In the present study, we show that the decreases in levels of implanted ovarian steroid hormones result in the termination of sexual receptivity (squatting behavior) and the onset of molt which are associated with an upregulation of GABA_AR subunits ($\alpha 3$, $\alpha 4$, δ , π , and $\gamma 2$) in the PMM. These results suggest the possibility that ovarian regression at the end of the breeding season may contribute to the upregulation of GABA_ARs in PMM DA-Mel neurons, and this could be a potential defining characteristic of natural photorefractoriness in turkey hens. For the GABA synthesis enzymes, we have revealed that both of GAD1 and GAD2 were not changed in the PMM of both the steroid withdrawal induced and natural photorefractory hens. Our data are in line with the earlier work (Kosonsiriluk et al., 2016) suggesting that the inhibitory effect of GABA may be enhanced by the upregulation of GABA_A receptors in the PMM. The subset of GABA_A receptor subunits mRNA upregulated in this study was different from our previous work conducted with natural photorefractory turkeys (Kosonsiriluk et al., 2016). But, it is interesting to note that most of the subunit mRNAs, that were observed to be significantly upregulated in the work reported here ($\alpha 3$, δ , π , and $\gamma 2$; **not** $\alpha 4$) were also found to be upregulated by Kosonsiriluk and colleagues (Kosonsiriluk et al., 2016). We suggest that factors in addition to steroids withdrawal may also be involved in the expression of photorefractoriness including age and/or the number of molting cycles and these could contribute to the differences between these studies. For example, in this study, we used birds in their first reproductive cycle therefore they had not ever progressed through a complete molt.

The GABA_AR $\alpha 3$, $\alpha 4$, δ , π , and $\gamma 2$ -subunits upregulation associated with the depletion of implanted estrogen and progesterone hormones in this study has also been reported in human and rat brains (Genazzani et al., 2000; Maggi and Perez, 1984). The depletion of estrogen following

ovariectomy in rats increases the GABA_A receptor α 2-, α 3- and α 4-subunits expression in the midbrain (Daendee et al., 2013). Several studies on progesterone withdrawal showed a significant increase in the expression of GABA_AR α 4 and δ -subunits in hippocampus and midbrain of rats (Gulinello et al., 2002; Lovick et al., 2005; Smith et al., 1998a; Smith et al., 1998b; Smith et al., 2007). And GABA_AR δ -subunit is highly sensitive to neurosteroid modulation, and low concentrations of neurosteroid can enhance the tonic inhibition conductance mediated by δ -subunit (Belelli and Lambert, 2005; Comenencia-Ortiz et al., 2014). Moreover, the results of a recent study have shown that the δ -subunit is expressed in the suprachiasmatic nucleus (SCN) and inhibits the effects of light during the dark phase of the mammalian circadian cycle (Ehlen and Paul, 2009). We suggest that the upregulation of these subunits, in particular the δ -subunit, in the steroid withdrawal birds in this study could potentially augment the inhibitory effects of GABAergic neurotransmission in the PMM and could be associated with the state of photorefractoriness.

The functional form of GABA_ARs is composed of the heterogeneity of different subunit compositions with the different physiological properties and differentially expressed throughout the brain. Most GABA_ARs are comprised of two α -subunits, two β -subunits, and one γ -subunit (or one δ -subunit) (Jacob et al., 2008; Rudolph and Mohler, 2004). However, the γ 2-subunit which shares the utmost sequence identity with α - and β -subunits its mRNA is expressed in neurons throughout the brain of rat and also co-expressed with α - and β -subunits (Pritchett et al., 1989). Likewise, the π -subunit is expressed in the brain and non-neuronal tissues in human and rat. This subunit is most closely related to β and δ -subunits, and can combine with α , β , or γ -subunits to form functional GABA_ARs, and alters the sensitivity of recombinant receptors to pregnanolone (Hedblom and Kirkness, 1997; Neelands and Macdonald, 1999). Taken together,

these findings suggest that the upregulation of GABA_AR π and $\gamma 2$ -subunits in this study may play a role in the subunit stoichiometry of the receptor to form a functional GABA_AR in the expression of photorefractoriness.

Although the GABA_AR $\alpha 3$ -subunit has been previously reported to display the lowest sensitivities to endogenous GABA of all GABA_ARs targeting to cellular domains *in vitro* (Bohme et al., 2004), our data demonstrate that the GABA_AR $\alpha 3$ -subunit is highly upregulated in the photorefractory and steroid withdrawal hens. This result leads to the suggestion that ovarian steroid withdrawal may play a role in the upregulation of GABA_AR $\alpha 3$ -subunit, and could be related to the state of photorefractoriness in turkey hen. Consistent with our previous study (Kosonsiriluk et al., 2016), the upregulation of δ -, $\gamma 2$ - and $\alpha 3$ - subunit were observed.

Taken together, these findings suggest that all the upregulated subunits of GABA_ARs in this study ($\alpha 3$, $\alpha 4$, δ , π , and $\gamma 2$) may show the functional form of the heterogeneity of GABA_ARs on the DA-Mel PMM neurons diminishing the reproductive neuroendocrine responses and favoring the state of photorefractoriness during the withdrawal of ovarian steroids at the end of breeding season.

4.1 Conclusion

The withdrawal of exogenously administered ovarian steroids enhances the GABAergic inhibition in the PMM through the upregulation of GABA_ARs which diminishes the photoperiodic neuroendocrine responses, resulting in a condition resembling the state of photorefractoriness in seasonally breeding birds.

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Figure legends and Table captions

Fig. 1. Photographs showing the effect of steroid withdrawal on physiological changes in steroid withdrawal hens compared to photosensitive and photorefractory hens. (A) Full-feathered short-day photosensitive hen showed no sign of molting. (B) Short-day steroid withdrawal hen with molting score +6 (feathers completely or partially dropped/lost) (C) Natural photorefractory hen during late molting stages with new pin feathers. (D) Short-day steroid withdrawal hen during late molting stages with new pin feathers.

Fig. 2. Graphs show the mRNA expression of: gonadotropin-releasing hormone-I (*GnRH-I*) in the nCPA and tyrosine hydroxylase (*TH*), melanopsin (*OPN4x*), arylalkylamine N-acetyltransferase (*AANAT*), tryptophan hydroxylase 1 (*TPH1*), circadian clock genes (*Per2*, *Per3*, and *Cry1*) in the PMM in short-day non-photostimulated dark control hens (SD-DC), short-day light stimulated hens (SD-LS), photorefractory-light stimulated hens (PR-LS), and steroid withdrawal-light stimulated hens (SWD-LS). Fold change of short-day non-

photostimulated dark control (SD-DC) hens is set to 1. Significant differences between means are indicated by difference letters at $P < 0.05$ ($n = 8$ per group).

Fig. 3. The expression changes of GABA_AR subunits including: (A) ALPHA = $\alpha 1-5$; (B) BETA = $\beta 1-3$; (C) GAMMA = $\gamma 1-3$; (D) RHO = $\rho 1-2$; and (E) DELTA, PI, THETA, EPSILON = δ, π, θ , and ϵ in short-day light stimulated hens (SD-LS), steroid withdrawal-light stimulated hens (SWD-LS), and photorefractory-light stimulated hens (PR-LS). The expression of $\alpha 6$ and $\rho 3$ -subunits are undetectable in this study. Data (mean $\pm$ SEM, $n = 8$ per group) are expressed as fold change of relative expression level, with a fold change of short-day light stimulated control (SD-LS) hens set to 1. Significant differences between means are indicated by an asterisk (*) at $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Supplementary Data

Supplementary electronic materials

A video illustrating the physiological and behavioral changes of the photorefractory and steroid withdrawal turkey hens can be observed by viewing the attached video files.

Table S1

The efficiency of shedding of primary wing feathers of steroid withdrawal turkey hens (Primary molt scores in each bird were recorded at the end of molt).

Table S2

Statistical comparison of GnRH-I gene expression in the commissurae pallii (nCPA) and TH, AANAT, TPH1, OPN4x, Per2, Per3, and Cry1 gene expression in the premammillary nucleus (PMM). Analyses conducted to determine the effect of ovarian steroid withdrawal on the reproductive neuroendocrine response in turkey hens between the short-day non-photostimulated dark-control (SD-DC), short-day light stimulated (SD-LS), photorefractory lightstimulated (PR-LS), and steroid withdrawal light stimulated (SWD-LS) groups (One-way ANOVA, Bonferroni post-hoc tests).

Table S3

Statistical comparison of GABA_A receptors mRNA expression within the premammillary nucleus (PMM). Analyses conducted to determine the effect of ovarian steroid withdrawal on the expression of GABA_A receptors between the short-day light stimulated (SD-LS), photorefractory light stimulated (PR-LS), and steroid withdrawal light stimulated (SWD-LS) groups (One-way ANOVA, Bonferroni post-hoc tests).

Author Contributions V.C. and S.K. performed the research and analyzed data; M.E.H., L.J.M., V.C., and S.K. designed the research study; M.E.H. and L.J.M. contributed essential tools; V.C. and M.E.H. wrote the paper with the help from all authors.

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Fig. 1

Fig. 2

Fig. 3

(A) ALPHA

(B) BETA

(C) GAMMA

(D) RHO

(E) DELTA, PI, THETA, EPSILON