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

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Running Title: GABA_A receptors in the turkey premammillary nucleus

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 α 1-3, β 2-3, γ 1-3, ρ 1-3, 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 subsequent 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.

Keywords: Birds; dopamine-melatonin neuron; GABA_A receptors; photorefractoriness; premammillary nucleus; reproduction

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

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., 2015). 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., 2015).

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; Gillespie et al., 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., 2015). 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., 2015). GABA receptors and synthetic enzymes are expressed in the turkey PMM and the GABA_A receptor, $\alpha 2$ and $\alpha 4$, subunits and the GABA_B receptor 2 are found to be upregulated in photorefractory birds (Kosonsiriluk et al., 2015).

GABA_A receptors in the mammalian brain are heterogeneous and exist as pentameric constructs of 19 subunits containing $\alpha 1-6$, $\beta 1-3$, $\gamma 1-3$, $\rho 1-3$, 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., 2015). 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. (2015). 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 powdered 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 Thayanunphat 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.

2.4. Real-time qRT-PCR analysis

The expression level of all the GABA_A receptor subunits (α 1-6, β 1-3, γ 1-3, ρ 1-3, δ , π , θ , and ϵ), GABA synthesis enzymes (glutamate decarboxylase 1 (GAD1) and GAD2) in the PMM were compared between PS dark vs. PR dark (n=8 per group). To confirm the reproductive stages and photosensitivity of birds, the expression of melatonin synthesis enzymes, tryptophan hydroxylase 1 (TPH1) and aralkylamine N-acetyltransferase (AANAT) together with *c-fos* and tyrosine hydroxylase (*TH*; the rate-limiting enzyme of DA synthesis) in the PMM and *c-fos* and *GnRH-I* in the nCPA were examined in each group (n=8 per group).

Total RNA extraction, cDNA synthesis, and real-time qRT-PCR were performed as previously described (Kosonsiriluk et al., 2015). Briefly, total RNA was isolated using RNAqueous-Micro Kit followed by DNase I treatment according to manufacturer's protocol (Applied Biosystems, Austin, TX, USA) and reverse-transcribed using SuperScript VILO™ cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). Real-time qRT-PCR was executed in triplicate with no-template and no-reverse transcriptase controls included for each gene using Brilliant III Ultra-Fast SYBR® Green QPCR Master Mix and Stratagene MX3005P (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. Melting curve analysis and agarose gel electrophoresis were carried out after each run to confirm a single product at the expected size. Nucleotide sequence analysis of amplified products was performed by ACGT Inc. (Wheeling, IL, USA). Relative mRNA level of each gene was evaluated by the $\Delta\Delta CT$ method (Livak and Schmittgen, 2001) using hypoxanthine phosphoribosyltransferase 1 (HPRT1) and actin, beta (β -actin) as reference genes. The efficiency of all primer pairs was between 95%-105% with $R^2 > 0.99$. Primer sequences and GenBank accession numbers of gene products can be found in Table 1.

2.5. Double IHC

Some of the GABA_A receptor subunits were selected for IHC to confirm the protein expression and localization in the PMM. An antigen retrieval step using sodium citrate buffer was carried out before processing through a double IHC as previously described (Kosonsiriluk et al., 2015). Primary antibodies directed against GABA_A receptor subunits were used as follows: anti-GABA_A receptor $\alpha 1$ (1:100; ab33299, Abcam Inc., Cambridge, MA, USA); $\beta 2$ (1:300; NBP-1-51214, Novus biologicals, LLC, Littleton, CO, USA); $\beta 3$ (1 μ g/ml; ab125302, Abcam Inc.); $\gamma 1$

(1:50; NBP1-91003, Novus biologicals, LLC); γ 2 (1:300; NB300-151, Novus biologicals, LLC); γ 3 (1:50; ab174242, Abcam Inc.); ρ 1 (1:50; bs-12084R, Bioss Inc., Woburn, MA, USA); ρ 2 (1 μ g/ml; ab83223, Abcam Inc.); ρ 3 (1:100; ab170654, Abcam Inc.); δ (1:100; ab111048, Abcam Inc.); ϵ (1:1000; ab35971, Abcam Inc.); TH (1:1000; 22941, ImmunoStar Inc., Hudson, WI, USA). Cy3 anti-mouse IgG (1:1000; Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA.) and FITC anti-rabbit IgG (1:200; Jackson ImmunoResearch Laboratories Inc.) were used as secondary antibodies. The anti-TH antibody was validated in our previous study (Kang et al., 2007). No autofluorescence was detected in tissue sections prior to staining. No staining was shown in single immunohistochemical staining when replacing the anti-GABA_A receptor antibodies with normal serum (the same species as the antibodies were raised). Image acquisition and processing for double IHC were performed as previously described (Kosonsiriluk et al., 2015).

2.6. Statistical analysis

Levene's test for equality of variances was performed to assess the homoscedasticity and the statistical comparison of means between the two groups was further computed using unpaired two-tailed *t*-test with $p < 0.05$ considered statistically significant. Data (mean $\pm$ SEM, $n=8$ per group) were presented as fold change of relative expression level, with fold change of PS groups set to 1. Statistical comparison across treatment groups was performed using ANOVA and the significance of the difference among treatment groups was further computed using Tukey's HSD test with $p < 0.05$ considered statistically significant. Data (mean $\pm$ SEM, $n=8$ per group) were presented as fold change of relative expression level, with fold change of PS dark groups set to 1.

3. Results

3.1. The mRNA expression of GABA_A receptor subunits in the PMM of photosensitive and photorefractory turkey hens

As shown in Fig. 1 and Supplementary table 1, an upregulation of GABA_A receptor α 1-3, β 2-3, γ 1-3, ρ 1-3, δ , and θ mRNAs were observed in the PMM of photorefractory birds when compared to their corresponding photosensitive birds while there were no changes observed in α 5-6 and β 1. Unlike those subunits, α 4, π , and ϵ were found to be downregulated in photorefractory birds.

3.2. The mRNA expression of GABA synthesis enzymes, TPH1, and AANAT in the PMM of photosensitive and photorefractory turkey hens

No changes were noted in either of the GABA synthesis enzymes (GAD1 and GAD2). The enzymes in melatonin synthesis pathway, TPH1 and AANAT, were significantly increased in the photorefractory group when compared to the photosensitive group, upholding the physiological differences between the two reproductive states (Fig. 2 and Supplementary table 2).

3.3. Responses of c-fos and TH mRNAs in the PMM and c-fos and GnRH-I mRNAs in the nCPA to 30-min light pulse during photosensitive phase of photosensitive and photorefractory turkey hens

Light-induced *c-fos* mRNA expression in the PMM of photorefractory birds was repressed when compared to those of photosensitive ones (fig. 3A and Supplementary table 3). An absence of *TH* response to light stimulus in the PMM and a lack of *c-fos* as well as *GnRH-I* induction in the nCPA were observed in photorefractory birds, while these neurons responded to the light stimulus in the photosensitive group (fig. 3B-D and Supplementary table 3).

3.4. Double IHC of *TH* and GABA receptor subunits

The localization of the GABA_A receptor subunits on PMM DA-Mel neurons is further supported by double immunohistochemical staining of *TH* and GABA_A receptor α , β , γ , ρ , δ , and ϵ subunits. As shown in Figs. 4-7, GABA_A receptor subunits were co-localized with DA neurons in the PMM of photorefractory birds.

4. Discussion

Our present findings indicate alterations in the expression of GABA_A receptor subunits in the PMM of the turkey mediobasal hypothalamus during the transition from the photosensitive phase to photorefractoriness of the reproductive cycle. The upregulation of GABA_A receptor α 1-3, β 2-3, γ 1-3, ρ 1-3, δ , and θ coincided with an absence of light responsiveness during the photoinducible phase, as indicated by the interference with *c-fos* induction and *TH* upregulation in the PMM, and the subsequent photoperiodic neuroendocrine responses including the diminished *c-fos* induction and *GnRH-I* expression in the nCPA. Consistent with our previous study (Kosonsiriluk et al., 2015), there are no changes observed in the GABA synthesis enzymes, GAD1 and GAD2, suggesting that the enhanced inhibitory effect of GABA is provoked by the upregulation of GABA receptors in the PMM.

The changes in the photorefractory PMM are presumably related to the altered expression of genes encoding different GABA_A receptor subunits. Here, we suggest that the upregulation in the expression of those subunits favor the state of photorefractoriness in the turkey, a temperate zone bird. These results are supported by our recent findings revealing the interference of the GABAergic system with light reception in the PMM (Kosonsiriluk et al., 2015) and suggest the

involvement of these upregulated GABA_A receptor subunits in the alteration of the photoperiodic neuroendocrine responses which are responsible for the state of photorefractoriness in the turkey. Even though we showed an upregulation of the $\alpha 4$ subunit in photorefractory hens in our previous study (Kosonsiriluk et al., 2015), it was down regulated in the present study. The differences in experimental designs between the two studies i.e. the shift of sampling times, breeding cycle of birds, and the variation of the physiological states may have caused the discrepancy of the result and could suggest that the $\alpha 4$ subunit does not play a direct role in regulating the state of photorefractoriness.

A recent study has shown that the mammalian SCN also integrates environmental stimuli by filtering light input through a specific subset of GABA_A receptor containing δ -subunit (Ehlen and Paul, 2009; Myung et al., 2015). GABA plays a major role in regulating SCN neuronal activities including circadian time synchronization (DeWoskin et al., 2015; Liu and Reppert, 2000; Shirakawa et al., 2000) and circadian responses to light (Ehlen and Paul, 2009; Gillespie et al., 1999; Ralph and Menaker, 1989). The δ subunit-containing GABA_A receptors are located extrasynaptic and respond to ambient GABA in the extracellular space mediating tonic inhibition. The activation of this extrasynaptic subpopulation of GABA_A receptors prevents photic entrainment and modulates the response of SCN neuronal activities to a light stimulus during the dark phase (Ehlen and Paul, 2009). This system may exist in the turkey PMM as the upregulation of δ subunit-containing receptors observed in photorefractory birds may enhance the tonic inhibitory effect of GABA_A receptor on DA-Mel neurons and prevent a photoperiodic entrainment during the photoinducible phase.

An upregulation of all three ρ subunits was observed in the PMM of photorefractory birds. These receptor subunits have been shown to be expressed in the retina and other brain region in the visual pathways of mammals (Enz et al., 1995; Ogurusu et al., 1997; O'Hara et al., 1995; Wegelius et al., 1998) indicating an association exists between the ρ subunits and photo transmission. In the present study, the ρ subunits upregulation in photorefractory hens may contribute, at least in part, to the blockage of light sensing ability in the PMM via enhanced inhibitory action of GABA on the DA-Mel neurons. Likewise, all the γ subunits which are required for normal channel function and postsynaptic localization of GABA_A receptor (Gunther et al., 1995; Kerti-Szigeti et al., 2014; Schweizer et al., 2003) were found to be upregulated in the photorefractory state and could assist in promoting the inhibitory effect of GABA neurotransmission in the brain area during the photorefractory state.

Peripheral steroid hormones, i.e. sex steroids, are able to cross blood-brain barriers and target brain tissues to regulate reproductive activities (Gore, 2001; Herbison, 1998). Several studies in mammals have shown that the withdrawal of sex steroids upregulates GABA_A receptors (Hsu and Smith, 2003; Lovick, 2008; Mascia et al., 2002; Smith et al., 1998b; Suda et al., 2008) and the upregulation of GABA_A receptor $\alpha 4$ and δ subunits were noted after steroids withdrawal (Hsu and Smith, 2003; Smith et al., 1998a; Smith et al., 2006). We suggest this may also be the case in birds since sex steroid hormones fluctuate throughout the reproductive cycle (Ellsworth et al., 2014; Seiler et al., 1992; Sockman and Schwabl, 1999; Williams, 1992; Wingfield, 1984) and the elevated steroid hormones observed during the breeding season decline sharply when birds enter the photorefractory state (Blache et al., 2001; Dawson, 1983; Dawson et al., 1985; Sharp et al., 1998; Wingfield and Farner, 1978) which would correspond with the upregulation of several GABA_A receptor subunits observed in this study.

Several studies in mammals have shown that DAergic neurons are regulated by GABAergic transmission (Bolam and Smith, 1990; Celada et al., 1999; Lobb et al., 2010; Paladini and Tepper, 1999) and we believe this relationship exists true in avian species. Close relationships occur between DAergic and GABAergic activities of DA-Mel neurons in the PMM. Enhanced GABAergic activity by the upregulation of GABA_A receptor subunits as observed in photorefractory birds appears to antagonize DAergic actions by interfering with light transmission in the PMM and the onset of photorefractoriness (Kosonsiriluk et al., 2015). It is well established that the DAergic system plays an important role in the reproductive cycle of seasonally breeding birds (Kang et al., 2007; Millam et al., 1980; Sartsoongnoen et al., 2008; Sharp et al., 1989). Hence, the interference with light transmission in the PMM of photorefractory birds may have been caused by the augmented inhibitory GABAergic neurotransmission through the upregulated GABA_A receptor and in turn prevents the subsequent neuroendocrine responses. The results of the present study describe GABA_A receptor subunits upregulation enhancing the inhibitory effect of GABA on the photoperiodic response in the PMM DA-Mel neurons during the state of photorefractoriness in the turkey, a seasonally breeding bird.

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Table 1 Real-time RT-qPCR primer sequences, amplicon length, and GenBank accession number of nucleotide products

Gene name	Gene symbol	Primer sequence (5'-3')	Amplicon length (bp)	GenBank accession no.
Aralkylamine N-acetyltransferase	AANAT	F: gtgttcgagatcgagaggaag R: gtactgcaggtatcgccacat	297	KM610224
Actin, beta	ACTB	F: ggagccgggtaccaattactggtgt R: actcctagactgtgggggactgta	194	NM205518

c-fos	c-fos	F: ttaactggtgctgggtatctcc R: gtcgttgctaagtcacagaacac	192	AY873804
GABA_A receptor α1	GABRA1	F: aaacaaagcctgcagaaccc R: aggctcccggtttaaataggtg	134	KJ634397
GABA_A receptor α2	GABRA2	F: tgcctatgcagtggctgttg R: tgcttctgtggctttgctt	130	JF273559
GABA_A receptor α3	GABRA3	F: agcgcaagaaactcgttacc R: agcgcaagaaacacaaaggc	89	KJ634398
GABA_A receptor α4	GABRA4	F: gcaagacactcactgccaaa R: ttgacagcagcaaacctgat	105	JF420839
GABA_A receptor α5	GABRA5	F: ttaccagttttggccctgtg R: ctttcgtcctccagcttg	82	XM004938470
GABA_A receptor α6	GABRA6	F: atggacaccagacacgtttt R: tgtccatccattgggaagtt	171	KM610220
GABA_A receptor β1	GABRB1	F: ggagggacaaaaggctctct R: cgaatcatccggttttcac	151	KM610221
GABA_A receptor β2	GABRB2	F: atccgctggatgaacagaac R: tcccgtgactgcattatcac	104	KJ634399
GABA_A receptor β3	GABRB3	F: tcctcagttctccattgtgg R: atggagggcattgtaggttg	141	KJ634400
GABA_A receptor δ	GABRD	F: tgtgacatggacctctccaa R: ttatccagcccatggatctc	134	XR795842
GABA_A receptor ϵ	GABRE	F: ttcaacagcaccttgaaggc R: aatgcggaggagctgatttg	135	KJ634407
GABA_A receptor γ1	GABRG1	F: ttgccagacttggtatgacag R: agttccggaagaaagtgtctgg	114	KJ634401
GABA_A receptor γ2	GABRG2	F: ttctgcccaaactgtggtatg R: tcttcccaacctgttgctg	86	KJ634402
GABA_A receptor γ3	GABRG3	F: tttgtctcagacgtggacag R: agtgtgcttctgctgtcttg	137	KJ634403
GABA_A receptor π	GABRP	F: ttggagacctatgtgccatc R: tactccaatgcaggttctcg	99	KJ634408
GABA_A receptor θ	GABRQ	F: aacgcctggcatataacgac R: atgcgattcttcaccgtcac	136	KJ634406
GABA_A receptor ρ1	GABRR1	F: ccgcgtttctcatcaaaag R: ttatcccgaggtttcctctc	138	KJ634404
GABA_A receptor ρ2	GABRR2	F: atacgcggcagtgattacc R: tgaatgaggtatcccacacg	94	KJ634405

GABA_A receptor ρ3	GABRR3	F: cttttgacggctgctaccat R: ctgcccacatttccttaaa	160	KM610222
Glutamate decarboxylase 1	GAD1	F: tggttgatgtggaaggcaaa R: tctgccagttccagcattt	71	KJ442692
Glutamate decarboxylase 2	GAD2	F: gccttggaattggtacaga R: ccacatgcacccagattttg	220	KM610223
Gonadotropin-releasing hormone-I	GnRH-I	F: atggaaaatcttggggaagc R: ctggcttctccttcgatcag	110	KM610225
Hypoxanthine phosphoribosyltransferase 1	HPRT1	F: tgtgggatatcgccagact R: tccccgtctcactgatcaca	125	NM204848
Tyrosine hydroxylase	TH	F: acgagccctacacccacagcatcg R: gcagtgcaaatgtttggagacaac	207	AY169720
Tryptophan hydroxylase 1	TPH1	F: gccagtggtggaatgagctacgtcatgagctgg R: gacgttacgtgcctagaagagacaagtgg	161	DQ886025

Figure legends

Fig. 1. Differential mRNA expression of the GABA_A receptor subunits in the premammillary nucleus of photosensitive (PS) and photorefractory (PR) birds. Most of GABA_A receptor subunits were regulated in the photorefractory group (black bar) when compared to those of photosensitive birds (white bar) including α 1-3, β 2-3, γ 1-3, ρ 1-3, δ , and θ . Data (mean $\pm$ SEM, n=8 per group) are presented as fold change of relative expression level, with fold change of photosensitive groups set to 1. Statistical comparison between two groups was performed using an unpaired two-tailed *t*-test. Significant differences are denoted as **p*<0.05 and ***p*<0.01 (See supplementary table 1).

Fig. 2. Differential mRNA expression of GABA synthesis enzymes and melatonin synthesis enzymes in the premammillary nucleus of photosensitive (PS) and photorefractory (PR) birds. No changes were observed in both of GABA synthesis enzymes, glutamate decarboxylase

1 and 2 (GAD1 and GAD2) between photosensitive (PS) and photorefractory (PR) group. Both of melatonin synthesis enzymes, tryptophan hydroxylase 1 (TPH-1) and aralkylamine N-acetyltransferase (AANAT) were upregulated in the photorefractory group. Data (mean $\pm$ SEM, n=8 per group) are presented as fold change of relative expression level, with fold change of photosensitive groups set to 1. Statistical comparison between two groups was performed using an unpaired two-tailed *t*-test. Significant differences are denoted as $**p<0.01$ (See supplementary table 2).

Fig. 3. Photoperiodic responses of light-responsive genes in the premammillary nucleus (PMM) and the nucleus commissurae pallii (nCPA) of photosensitive (PS) and photorefractory (PR) birds. Fig 3A showed a repressed expression of *c-fos* in PR birds exposed to light stimulus (PR light pulse) during photoinducible phase at circadian time 14 when compared to those of PS light pulse group. A lack of light-induced *tyrosine hydroxylase* (*TH*) response in the PMM (B) *c-fos* (C) and gonadotropin-releasing hormone I (*GnRH-I*; D) responses in the nCPA of PR light pulse were observed, while all the genes responded normally to the light stimulus in the PS light pulse group. Data (mean $\pm$ SEM, n=8 per group) are presented as fold change of relative expression level and fold change of PS dark groups is set to 1. The comparison across treatment groups were performed using ANOVA and the significance of the difference among treatment groups was further computed using Tukey's HSD test with a $p<0.05$ was considered statistically significant (See supplementary table 3). Significant differences are denoted with different letters.

Fig. 4. The expression of GABA_A receptor $\alpha 1$, ϵ , and δ within tyrosine hydroxylase-immunoreactive (TH-ir) neurons in the premammillary nucleus (PMM) of photorefractory

birds. Double immunohistochemistry staining of TH (A, D, and G) and GABA_A receptor $\alpha 1$ (B), ϵ (E), and δ (H) shows co-localization of dopamine neurons and GABA_A receptors (merged image; C, F, and I) in the PMM of turkey mediobasal hypothalamus. Scale bars = 20 μ m.

Fig. 5. The expression of GABA_A receptor $\beta 2$ and $\beta 3$ within tyrosine hydroxylase-immunoreactive (TH-ir) neurons in the premammillary nucleus (PMM) of photorefractory

birds. Double immunohistochemistry staining of TH (A and D) and GABA_A receptor $\beta 2$ (B) and $\beta 3$ (E) shows co-localization of dopamine neurons and GABA_A receptors (merged image; C and F) in the PMM of turkey mediobasal hypothalamus. Scale bars = 20 μ m.

Fig. 6. The expression of GABA_A receptor $\gamma 1-3$ within tyrosine hydroxylase-immunoreactive (TH-ir) neurons in the premammillary nucleus (PMM) of photorefractory

birds. Double immunohistochemistry staining of TH (A, D, and G) and GABA_A receptor $\gamma 1-3$ (B, E, and H) shows co-localization of dopamine neurons and GABA_A receptors (merged image; C, F, and I) in the PMM of the turkey mediobasal hypothalamus. Scale bars = 20 μ m.

Fig. 7. The expression of GABA_A receptor $\rho 1-3$ within tyrosine hydroxylase-immunoreactive (TH-ir) neurons in the premammillary nucleus (PMM) of photorefractory

birds. Double immunohistochemistry staining of TH (A, D, and G) and GABA_A receptor $\rho 1-3$ (B, E, and H) shows co-localization of dopamine neurons and GABA_A receptors (merged image; C, F, and I) in the PMM of the turkey mediobasal hypothalamus. Scale bars = 20 μ m.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7