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$GABA_A$ Receptor Subunit Transcriptional Regulation, Expression Organization, and Mediated Calmodulin Signaling in Prefrontal Cortex of Rats Showing Testosterone-Mediated Impulsive Behavior

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Testosterone can induce impulsivity, a behavioral impairment associated with various psychiatric illnesses. The molecular mechanisms associated with testosterone-induced impulsivity are unclear. Our earlier studies showed that supraphysiological doses of testosterone to rats induced impulsive behavior, impacted hypothalamic-pituitary-adrenal axis (HPA) and hypothalamic-pituitary-gonadal axis interactions, and altered α_{2A} adrenergic receptors in prefrontal cortex (PFC). Owing to the importance of GABAergic system in impulsivity and memory, the present study examines whether testosterone-mediated impulsivity is associated with changes in the expression of Gamma-Aminobutyric Acid (GABA) A and B receptor subunit transcripts (*Gabra1, Gabra2, Gabra2 transcript variant 2, Gabra3, Gabra4, Gabra5, Gabra6, Gabrb1, Gabrb2, Gabrb3, Gabrg1, Gabrg2, Gabrg3, Gabbr1, Gabbr2*) in rat PFC, and whether testosterone influences $GABA_A$ receptor subunit organization. We studied GABA receptor functions by examining GABA receptor-mediated calcium/calmodulin-dependent kinase signaling genes (*Calm1, Calm2, Calm3, Camk2a, Camk2b, Camk2g, Camk2d, Camk4*) in the testosterone-induced impulsivity model. Rats were left untreated as controls (C), gonadectomized (GDX), or GDX and injected with supraphysiological doses of testosterone (T). Impulsive behavior was examined using the go/no-go paradigm. Gene expression was studied using qRT-PCR and $GABA_A$ subunit reorganization using cross correlation. Our findings show that expressions of select $GABA_A$ receptor subunits (*Gabra3, Gabra5, Gabra6*) were significantly upregulated in PFC of T group compared to GDX or C groups. $GABA_A$ receptor subunit organization was different in C, T, and GDX groups. Additionally, *Camk4* expression was significantly downregulated in T compared to C group. Our findings suggest that specific $GABA_A$ receptor subunit expression, their reorganization, and *Camk4*-mediated functions may be associated with testosterone-mediated impulsivity.

Keywords: impulsivity, testosterone, GABAA receptor, rodent model, transcript level

INTRODUCTION

Impulsivity is described as decision-making or acting without regard to prior thinking. Being a non-unitary construct, impulsivity is often defined as a multidimensional concept related to maladaptive personality traits critical to many neuropsychiatric conditions. Attention-deficit hyperactivity disorder, mood disorders, addiction-related disorders, impulse-control disorders, non-suicidal self-injury, and suicidal behavior are some of the neuropsychiatric conditions that are significantly associated with impulsivity (Bakhshani, 2014; Liu et al., 2017). Despite its significance in neuropsychiatric illnesses, the underlying mechanisms associated with impulsivity are not clearly understood. Recent evidence suggests that increased levels of testosterone and impulsivity are highly correlated in humans (Wu et al., 2019). It has been shown that there is a significant correlation between increased testosterone level and suicide attempts in men (Stefansson et al., 2016). Past studies also suggest an association of increased use of androgen-enhancing drugs, known as anabolic-androgenic steroids, with psychopathologies, such as a blunted hypothalamic-pituitary-adrenal axis (HPA) response and increased risk of suicide attempts (Thiblin et al., 1999; Trenton and Currier, 2005; Melhem et al., 2016). In fact, we have earlier shown that testosterone not only increases impulsivity in rats, but also heightens the interaction of HPA and hypothalamic-pituitary-gonadal axis (HPG) axes in the brains of these rats (Ludwig et al., 2019). In addition, we found that the α_{2A} adrenergic signaling pathway is significantly impacted in the prefrontal cortex (PFC) of rats given supraphysiological doses of testosterone, which was correlated with impulsivity behavior (Agrawal et al., 2019). Our study indicates that neurochemical changes may be central to testosterone-mediated impulsivity.

Gamma-Aminobutyric Acid (GABA) is the main inhibitory neurotransmitter in the mammalian central nervous system. In conjunction with excitatory glutamate, GABA is involved in balancing excitatory and inhibitory response, critical in proper brain functioning (Petroff, 2002). GABA binds to GABA receptors to mediate its functions. There are two main types of GABA receptors: (1) GABA$_A$ receptor subtypes, which are ligand-gated ion channels and (2) GABA$_B$ receptor subtype, which are G-protein-coupled receptors (Hayes et al., 2014). GABA$_A$ is the primary GABA receptor in the brain, which, due to its chloride ion channel activity, quickly hyperpolarizes the post-synaptic neurons, causing an inhibitory effect. GABA$_A$ receptors are pentameric, composed of α, β, γ, δ, and ρ subunits. They have been found to be localized in post-synaptic and extrasynaptic locations (Shrivastava et al., 2011; Hammond, 2015). Based on transcription patterns, GABA$_A$ subunits α1 and β2 are co-regulated (Goetz et al., 2007). GABA binds between the α and β subunits, and β subunits regulate ion selectivity of the GABA$_A$ receptor (Goetz et al., 2007). The γ2 subunit plays a role in anchoring the receptor to the synapse. Subunit composition determines the GABA$_A$ receptors' conductance and deactivation rate (Goetz et al., 2007). On the other hand, GABA$_B$ receptors are composed of GABA$_{B1}$ and GABA$_{B2}$ subunits, forming a heterodimer. GABA$_B$ receptors localize pre-synaptically, post-synaptically, and potentially extrasynaptically. GABA$_B$ receptors have a slower response than GABA$_A$ receptors and play a role in regulating neurotransmission. GABA$_{B1}$ and GABA$_{B2}$ receptor subunits must be co-expressed to render a functional GABA$_B$ receptor (Mott, 2015).

Recent studies have shown that GABA may be involved in impulsive behavior. For example, it has been shown that impulsive choice or impulsive action can be associated with expression changes in GABA receptor subunits in rat orbitofrontal cortex (Ucha et al., 2019). Also, GABA level may predict individual differences in rash impulsivity (Boy et al., 2011). A past study examined gonadectomy (GDX) and testosterone treatment on GABA receptors in rats. An alteration in the GABA$_A$/benzodiazepine receptors was reported in testosterone-treated animals (Svensson et al., 2000). $^{36}Cl^-$ uptake in synaptoneurosomes was increased in the testosterone-treated compared to control rats, suggesting that testosterone may increase GABA$_A$ receptor function in this group (Svensson et al., 2003). However, this study did not observe receptor subunit differences between the groups. Furthermore, whether impulsivity induced by testosterone is associated with GABA receptor subunits and their functions has not been studied.

In the present study, we hypothesize that testosterone will disrupt the GABA receptor signaling by increasing their expression in testosterone-treated (T) group compared to control (C) and gonadectomized (GDX) groups. GABA$_A$ α, β, and γ subunits were chosen for this study because they comprise the majority of GABA$_A$ receptors. Both subunits of GABA$_B$ receptors were studied (Shrivastava et al., 2011). We also tested whether testosterone influences GABA$_A$ subunit organization by correlating various GABA$_A$ receptor subunits in individual groups of rats, as subunit correlations have been found to be disrupted in the brains of subjects with psychiatric disorders such as depression and depressed individuals who died by suicide (Merali et al., 2004). Additionally, we examined the expression of calmodulin (Cal) and calcium/calmodulin-dependent (CaM) protein kinase 2 (CaMk2) as they are considered to be active downstream transducers of GABA receptor functions (Churn and DeLorenzo, 1998; Churn et al., 2002). Furthermore, CaM kinase 4 (CaMk4) gene was studied for its role in GABA receptor expression changes. These studies were performed under the supraphysiological influence of testosterone in PFC in testosterone-induced impulsivity rat model. The PFC was chosen because of its well-studied role in motor control (Robbins and Dalley, 2017; Sholler et al., 2020) decision making (Hiser and Koenigs, 2018; Saberi Moghadam et al., 2019), and emotional processing and regulation (Dixon et al., 2017). Our previous study also indicated that PFC plays a significant role in testosterone-mediated impulsivity (Agrawal et al., 2019).

MATERIALS AND METHODS

Experimental Group Design, Behavioral Testing, and Tissue Collection

Rodent Impulsivity Model Preparation

A total of 30 Male Long Evans rats were received from Envigo Laboratories and maintained as described in our previous studies (Agrawal et al., 2019; Ludwig et al., 2019). They were individually

housed in a reversed 14 h of light: 10 h of dark photoperiod. They were weighed daily and given food *ad libitum*. The rats were acclimatized for 5 days before starting the experiment. They were divided into a control (C) group and two experimental groups, both of which received gonadectomies to eliminate testosterone. The age of rats was ∼6 weeks at the time of gonadectomy. One of the experimental groups was given daily injections of excessive testosterone and is known as the testosterone (T) group. The group with the gonadectomy only is known as the gonadectomized (GDX) group. Experimental procedures were approved by the IACUC of the University of Alabama at Birmingham and all procedures were conducted in strict adherence to the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals.

Rats receiving a GDX or testosterone injections were subjected to the same protocol used previously in this lab (Agrawal et al., 2019; Ludwig et al., 2019). To summarize, rats received isoflurane as an anesthetic (5% induction, 1–3% maintenance), and their vitals were checked regularly to minimize the risk of cardiorespiratory failure. Rats also received carprofen (5 mg/kg, subcutaneous) and buprenorphine (0.1 mg/kg, subcutaneous) before incision. For the incision, the aseptic technique was followed. Rats' abdomen were shaved, and betadine was used to cleanse. Then, a single transverse incision in the caudal abdomen was made, and using blunt forceps, the testicular fat pad on one side was pulled through the incision. Both testes and epididymis were removed using a hemostat and ligature for control. Monocryl sutures were used to close the incisions. During and after surgery, rats' body temperatures were monitored using a heating pad. 24 h post-surgery, rats received a second subcutaneous injection of carprofen (5 mg/kg).

A schematic diagram showing the experimental protocol is mentioned in **Figure 1**. One day after surgery, GDX rats were randomly assigned to receive testosterone injections and form the testosterone (T) group. They were subcutaneously injected with testosterone propionate (7.5 mg/kg, dissolved in 0.1 mL corn oil). This dose is equivalent to a heavy steroid dose in humans, and has been used in previous studies (Wood et al., 2013; Cooper et al., 2014). Injections were given daily for a 24-week period. GDX rats, not receiving testosterone, received corn oil injections of equal volume and for the same duration. Rats were weighed every 2 weeks, and the daily dose of testosterone was adjusted for their weight.

Behavioral Testing

After 24 weeks of testosterone administration, rats were subjected to the go/no-go task paradigm to test their impulsive behavior. This paradigm is described in our previous study (Agrawal et al., 2019). Rats placed in sound-attenuated and ventilated chambers were autoshaped for 15 days to learn that pressing the lever in the chamber would result in the dispensing of 45 mg sucrose pellets. Rats who successfully learned (defined as pressing the lever at least 20 times during 30 min of the "go" phase, when the light was on) continued testing, and those who did not were removed from testing.

Rats that continued testing moved to the "go/no go" task phase of the experiment. Rats were to press the lever to dispense sucrose pellets during the "go" phase, which was signaled by a bright white light and continued with a house light. They were not to press the lever during the "no-go" phase, which was signaled by a red light and continued with no light. Each phase was 10 min, with 10 s timeouts between phases. In total, there were 4 "go" and 4 "no-go" phases which alternated for a total of an 80 m session. Rats underwent 3 sessions that were included in results, and they completed one warm-up session of this paradigm that was not included in results. The sessions were controlled by Med-PC IV® software ran on a computer. Impulsive behavior was defined as pressing the lever during the "no-go" phase. Impulsive behavior was indexed by the go/no-go ratio, which was the number of times the lever was pressed during the "go" phase compared to the number of times it was pressed during the "no-go" phase. A high ratio indicated low impulsive behavior, while a low ratio indicated high impulsive behavior. If variance in this ratio between the 3 sessions was <10% for any individual rat, results were recorded. Individual responses were averaged based on experimental group (C, T, and GDX).

Twenty-four hours after the last session, rats were decapitated, trunk blood was collected, and brains were flash frozen and stored at −80°C. The PFC was dissected out from 300 μm sections prepared from the frozen brain on a cryostat (Leica

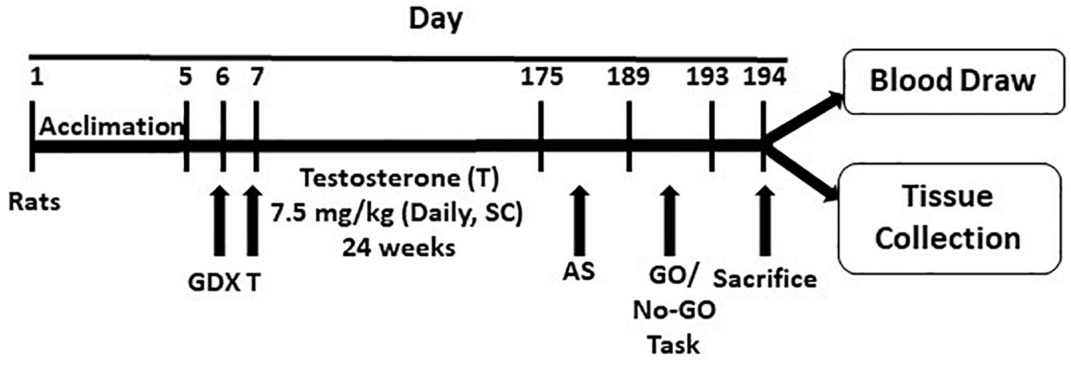

FIGURE 1 | Schematic diagram showing the experimental protocol. The protocol is detailed in the text. GDX, gonadectomy; T, testosterone; AS, autoshaping; SC, subcutaneous.

TABLE 1 | Rat-specific primers for amplifying mRNA transcripts.

Genes	Forward (5′–3′)	Reverse (5′–3′)
Gabra1	TGC CTG TGT TTC CCT AAA ACG	AGG CAG GAC CAA ATC AAA CAA T
Gabra2	CCT CTG GCC TGG TTG CTT TA	GCT CTC TCC TTA TGT GTG TCA AG
Gabra2-transcript variant 2	ATC CTT CTG TCC CAC CCT TTT AG	CAT CAA CAT AAT CCC CAG CAC T
Gabra3	GGC ATG ATC CGC AAA CAG TAG	CTC TGG GGT TTG GGA TTT GGA
Gabra4	CCT TCT GGA TCT GGC ACA AGT	AAT GCC CCA AAT GTG ACT GG
Gabra5	CCA TTT TTC CCA GCC AAC AGA	TGT ACC CGA GGA TCT TTG CTT T
Gabra6	AGC TGT ATG CTT TGC GTT TGT	CTT TCG GCT TTC TGG GAC TG
Gabrb1	TCC TTT CCT CCT CGC TTG TTT	AAC TGG AAG GCG GAA TCT CTT
Gabrb2	TGT CAA CAA GAT GGA CCC ACA	ATG CTG GAG GCA TCA TAG GC
Gabrb3	TAC GCA TAC ATA CCA TAC ATT TTG C	TGT GTT TCT CGC CCT CAT TCT
Gabrg1	AGA CTT GGT TCG ATA GCC GTT	GGC GAT TGG GCG TTG TTA TC
Gabrg2	TCA CAG CAA TGG ATC TCT TCG T	TGG TAG GGG CAG GGT TTT TC
Gabrg3	TCG AAA GCC AAC CAT CAG GAA	GTG GGG GTC TCA TAT CCA GG
Gabbr1	CTG CGT CGT GGT TTC CTT TC	TCC GTG CCT TCA TTT GGT CA
Gabbr2	AAC AAG CCC CAC AAA GTG ATT	TCT AGG AAA TGG ACC GTG TGT T
Calm1	CTC TGA TGG GG GAC CAA CTC	CGC AGT TAG AGA TGA AAG GCT G
Calm2	TGG AGT TGG TCA AAT GAG GGA	TGT CCA TAG TCC ACG CAG AG
Calm3	CAA TGT GGG CAG TTC AGT CG	GTC CCA GGA AAA GCC ACT TG
Camk2a	CAG CCG ATG AAG GAG CAA AC	AGG AGT CCA GCC AGT GAC TAT
Camk2b	CCC CTA CAA ATC AAG CCA AGG	ACG GAA GAT GGT GTC CAC TC
Camk2d	CTC TTG TTT TGC TGT TGG GCT	TGC TGA GAC ATT TGA GTC CGA
Camk2g	GCT GGT GCT TGG ATT TAG CC	AAG CCA GCA AAA CGA AAC CC
Camk4	ACA GAT GCA AAC AGA AGG GGA	TTG GAT GTG AGA GGC GAA GAA

CM1950, Leica, Germany) and PFC was carved out precisely by scalpel using the Rat Brain Atlas and stored at −80°C until further analyses.

Testosterone Levels

Serum isolated from trunk blood was tested for testosterone levels using an ELISA kit (Abcam, MA, United States). One-way ANOVA and Student's *t*-tests were performed between groups to determine variance and statistical significance. Data are presented as testosterone concentration values in ng/mL.

Expression Levels of GABA Receptor Subunits

Primer Design

Primers were designed using Rat Genomic Database (RGD) and NCBI BLAST search tool. Primer sequences were designed to target the 3′ end of the gene and accounted for all the transcript variants for each gene. Primers were designed to have low self-complementarity, low self-3′ complementarity, low GC%, and melt temperatures within 2°C of each other. A list of all primers used in the gene expression assay can be found in **Table 1**.

RNA Isolation and qPCR-Based Gene Expression Analysis of GABA Receptor Subunits and Signaling Genes in Rat PFC

RNA was isolated following TRIzol® (Life Technologies, United States) method as described earlier (Roy et al., 2017). RNA purity was determined by measuring the optical density with an absorbance ratio of 260/280 (NanoDrop 2000c, Thermo-Scientific, Waltham, MA, United States), and integrity was tested following denaturing agarose gel electrophoresis. All samples had 260/280 ratio >1.8 and demonstrated 28S:18S rRNA ratio of 2:1 on agarose gel.

The single-stranded cDNA was prepared following the previously described method (Timberlake et al., 2018). Following the preparation of mRNA specific first strand cDNA, relative transcript abundance of coding genes was measured with quantitative real-time PCR (Stratagene MxPro3005, La Jolla, CA, United States) method. With the help of EvaGreen chemistry (Applied Biological Material Inc., Canada), qPCR amplification for the specific gene was performed using gene-specific forward and reverse primers as mentioned in **Table 1**. Primers were designed for the following genes: GABA$_A$ receptor subunits α1–6 (*Gabra1*, *Gabra2, Gabra2-transcript variant 2, Gabra3, Gabra4, Gabra5, Gabra6*), GABA$_A$ receptor subunits β1–3 (*Gabrb1, Gabrb2, Gabrb3*), GABA$_A$ receptor subunits γ1–3 (*Gabrg1, Gabrg2, Gabrg3*), GABA$_B$ receptor subunits 1–2 (*Gabbr1, Gabbr2*), calmodulin genes 1–3 (*Calm1, Calm2, Calm3*), calcium/calmodulin-dependent protein kinase type II subunits α, β, γ, and δ (*Camk2a, Camk2b, Camk2g, Camk2d*), and calcium/calmodulin-dependent protein kinase type IV (*Camk4*). The possibility of primer dimer formation and secondary product amplification was ruled out by running template-free samples. *Gapdh* normalized gene expression values were used to determine the relative gene expression levels of individual transcripts following Livak's ΔΔCt calculation method (Livak and Schmittgen, 2001).

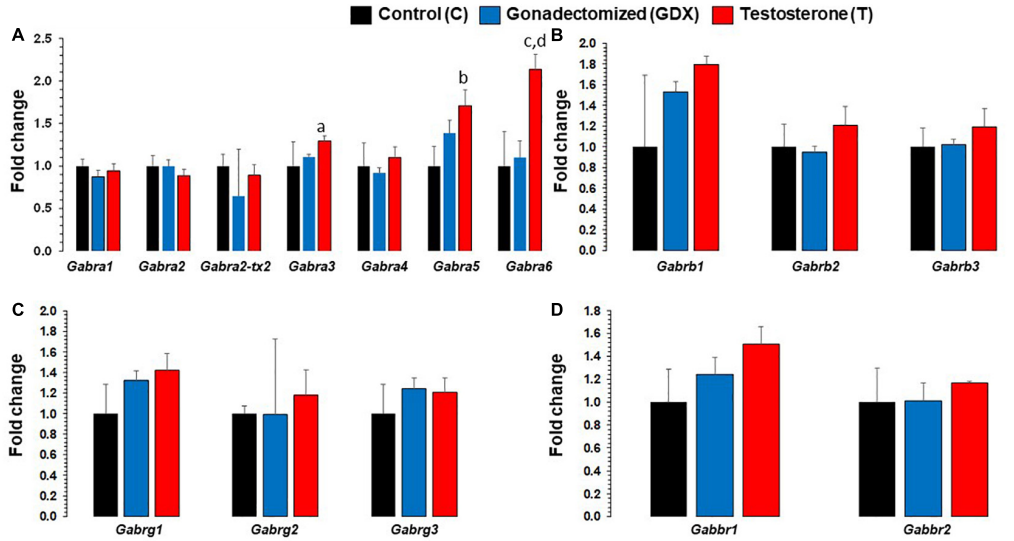

FIGURE 2 | Bar diagram showing gene expression results for GABA$_A$ and GABA$_B$ receptor subunits in prefrontal cortex across control (C, n = 4), gonadectomized (GDX, n = 5), and testosterone (T, n = 4) groups. Data are the mean ± SEM. **(A)** Expression changes in GABA$_A$ α receptor subunits across T and GDX groups. The T rats displayed significantly upregulated *Gabra3* mRNA transcript levels ($^a p = 0.0087$) compared with the GDX group. Similar significant expression upregulation was seen for *Gabra6* gene in T rats as compared to GDX rats ($^d p = 0.009$). The *Gabra5* ($^b p = 0.037$) and *Gabra6* ($^c p = 0.047$) were all found to be upregulated in the T group compared to the C group. No significant changes in *Gabra1, Gabra2, Gabra2-tx2, Gabra4* were noticed when comparing T group of rats with GDX rats, nor when comparing T rats with C rats. **(B)** Expression levels of GABA$_A$ β receptor subunits across C, T, and GDX groups. No significant changes in *Gabrb1, Gabrb2, and Gabrb3* were noted when comparing T group of rats with GDX rats, or T rats with C rats. **(C)** Expression levels of GABA$_A$ γ receptor subunits across C, T, and GDX groups. No significant differences in *Gabrg1, Gabrg2, and Gabrg3* were noted when comparing T group of rats with GDX rats, or T rats with C rats. **(D)** Expression levels of GABA$_B$ receptor subunits across C, T, and GDX groups. No significant changes in *Gabbr1 and Gabbr2* were noted when comparing T group of rats with GDX rats, or T rats with C rats.

GABA$_A$ Receptor Subunit Transcript Reorganization

GABA$_A$ receptor subunit mRNA reorganization was determined by correlating expression levels of various GABA$_A$ receptor subunits (*Gabra1*, *Gabra2, Gabra2-transcript variant 2, Gabra3, Gabra4, Gabra5, Gabra6*, *Gabrb1, Gabrb2, Gabrb3*, *Gabrg1, Gabrg2,* and *Gabrg3*) in C, T, and GDX groups independently using Pearson Product Moment Analysis.

RESULTS

Serum Testosterone Levels

As can be seen in our prior study (Agrawal et al., 2019), average testosterone concentration for the testosterone (T) group (n = 5) was 11.6 ng/mL (SEM = 4.67), compared to the average concentration for the control (C) group (n = 6) of 1.63 ng/mL (SEM = 0.4), and the average concentration for the gonadectomized (GDX) group (n = 6) of 0 ng/mL (SEM = 0.0061). T group concentration was significantly higher than both C group (p = 0.04) and GDX group (p = 0.02). GDX group concentration was significantly lower than C group (p = 0.001).

Impulsive Behavior Testing Results

As detailed in our previous study, T group rats had significantly lower go/no-go ratios than those in the GDX and C groups (Agrawal et al., 2019). T group (n = 5) had a ratio of 0.54 (SEM = 0.035), C group (n = 6) had a ratio of 0.87 (SEM = 0.073), and GDX group (n = 6) had a ratio of 0.76 (SEM = 0.075). T group's ratio was significantly lower than the ratio for C group (p = 0.004) and GDX group (p = 0.03). There was no significant difference between the ratios of C group and GDX group (p = 0.32).

GABA Receptor Gene Expression Analysis

Gene expression analysis was done in rat PFC to determine the effect of a supraphysiological dose of testosterone (T) on GABA receptor subunit genes and genes associated with calcium-dependent calmodulin kinase signaling pathway compared to rats with normal levels of testosterone (C) and rats with no testosterone due to gonadectomy (GDX).

Expression level changes of all GABA$_A$ and GABA$_B$ receptor subunit transcripts in PFC are presented in **Figure 2**. Fold changes between groups were standardized with C group as the reference (fold change = 1). *Gabra3* fold change was 1.29 (SEM = 0.058) for T group and 1.10 (SEM = 0.035) for GDX group, compared to 1 (SEM = 0.28) for C group. T group's *Gabra3* expression was significantly upregulated compared to GDX (p = 0.008), but not significant compared to C group (p = 0.244). *Gabra5* fold change was 1.71 (SEM = 0.18) for T group and 1.39 (SEM = 0.15) for GDX group, compared to 1 (SEM = 0.22) for C group. T group's *Gabra5* expression was significantly upregulated

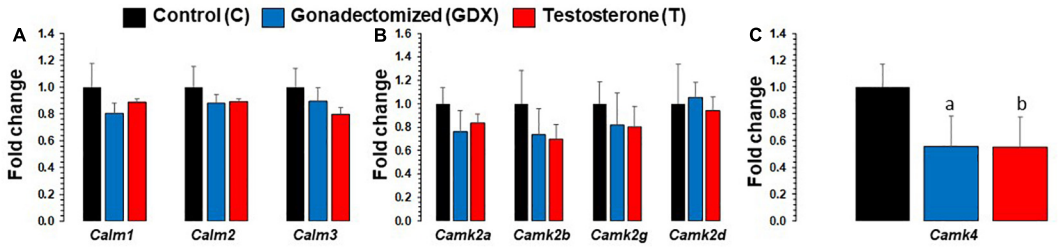

FIGURE 3 | Bar diagram showing gene expression results for calmodulin and Calcium/calmodulin-dependent protein kinase subunits in prefrontal cortex across control (C, $n = 4$), gonadectomized (GDX, $n = 5$), and testosterone (T, $n = 4$) groups. Data are the mean ± SEM. **(A)** Expression levels of *Calm* genes across C, T, and GDX groups. No significant differences were noted in *Calm1 and Calm2 genes, however, calm3* was nearly significant ($p = 0.06$) when comparing T rats to C rats. **(B)** Expression levels of *Camk2* genes across C, T, and GDX groups. No significant differences were found in the following genes: *Camk2a, Camk2b, Camk2g, and Camk2d* when comparing T to GDX, T to C, or GDX to C. **(C)** Expression changes in *Camk4* across T and GDX groups. One-way ANOVA found significant differences across groups in the *Camk4* gene. The T rats displayed significantly downregulated *Camk4* mRNA transcript levels ($^b p = 0.02$) compared with the C group. Similar significant expression downregulation was seen for *Camk4* in GDX rats as compared to C rats ($^a p = 0.03$).

compared to C ($p = 0.038$), but not significant compared to GDX group ($p = 0.24$). *Gabra6* fold change was 2.14 (SEM = 0.17) for T group and 1.10 (SEM = 0.19) for GDX group, compared to 1 (SEM = 0.40) for C group. T group's *Gabra6* expression was significantly upregulated compared to both GDX ($p = 0.009$) and C ($p = 0.047$).

Other GABA$_A$ receptor subunits (*Gabra1, Gabra2, Gabra2-transcript variant 2, Gabra4, Gabrb1, Gabrb2, Gabrb3, Gabrg1, Gabrg2,* and *Gabrg3*) were not significantly altered between groups. Neither of the GABA$_B$ receptor subunits (*Gabbr1 and Gabbr2*) were significantly different between groups.

Calmodulin and CaM Kinase Gene Expression Analysis

mRNA expression changes of all calmodulin and CaMK subunit genes in PFC are presented in **Figure 3**. Based on ANOVA, significant expression differences were found across groups *in Camk4*. *Camk4* fold change was 0.55 (SEM = 0.222) for T group and 0.55 (SEM = 0.227) for GDX group, compared to 1 (SEM = 0.173) for C group. *Camk4* expression was significantly downregulated in T group compared to C group ($p = 0.02$). *Camk4* expression was also significantly downregulated in GDX group compared to C group ($p = 0.03$). No significant differences were found in *Camk4* expression between T and GDX groups. Additionally, *Calm3* expression was nearly significantly downregulated in T group compared to C group ($p = 0.069$), but not to GDX ($p = 0.233$). No significant differences were found in the expression levels of *Calm1, Calm2, Camk2a, Camk2b, Camk2g,* and *Camk2d.*

GABA$_A$ Receptor Subunit Transcript Organization

Figure 4 shows Pearson correlations for GABA$_A$ receptor subunit gene expression in the control group. Significant correlations were found in 17 different subunit combinations: *Gabra3* and *Gabra4*, *Gabra3* and *Gabra5*, *Gabra4* and *Gabra5*, *Gabra5* and *Gabrb1*, *Gabra3* and *Gabrb2*, *Gabra4* and *Gabrb2*, *Gabra5* and *Gabrb2*, *Gabrb1* and *Gabrb2*, *Gabra3* and *Gabrb3*, *Gabra4* and *Gabrb3*, *Gabra2* and *Gabrg1*, *Gabra2* and *Gabrg2*, *Gabrg1* and *Gabrg2*, *Gabra2* and *Gabrg3*, *Gabra3* and *Gabrg3*, *Gabrg1* and *Gabrg3*, and *Gabrg2* and *Gabrg3*.

Figure 5 shows Pearson correlations for the T group GABA$_A$ receptor subunit gene expression. Significant correlations were found in 9 different subunit combinations: *Gabra1* and *Gabra4*, *Gabra2-transcript variant 2* and *Gabra6*, *Gabra2-transcript variant 2* and *Gabrb3*, *Gabra6* and *Gabrb3*, *Gabra2-transcript variant 2* and *Gabrg1*, *Gabra6* and *Gabrg1, Gabrb2* and *Gabrg2, Gabra6* and *Gabrg3,* and *Gabrg1* and *Gabrg3*.

Figure 6 shows Pearson correlations for GDX group GABA$_A$ receptor subunit gene expression. Significant correlations were found in 5 different subunit combinations: *Gabra3* and *Gabra4*, *Gabra4* and *Gabra6*, *Gabra3* and *Gabrb3*, *Gabra2* and *Gabrg1*, and *Gabrb1* and *Gabrg2*.

DISCUSSION

In this study, we analyzed the effects of testosterone-induced impulsivity on GABA$_A$ and GABA$_B$ receptor subunit mRNA levels in rat PFC. As indicated in the results section, we found that only GABA$_A$, but not GABA$_B$ receptor transcript levels, showed changes. Within GABA$_A$ receptors, testosterone had a subunit-specific effect on *Gabra3, Gabra5,* and *Gabra6* transcript levels. We predict that changes in only GABA$_A$ but not in GABA$_B$ could be due to the underlying differences in receptor composition between these two receptors and their distinct mechanisms of interaction with specific ligands and consequent downstream functions (Hammond, 2015; Mott, 2015). GABA$_B$ receptors are inhibitory G-protein coupled receptors and are found in both pre- and post-synaptic locations (Wu and Sun, 2015). GABA$_A$ receptors are ionotropic, fast hyperpolarizing, and expressed throughout the brain. Of various GABA$_A$ receptor subunits, GABA$_A$ 4, 5, and 6 subunits are extrasynaptic (Wu and Sun, 2015). These receptors have a high GABA binding affinity and are persistently activated even at a low concentration of GABA, resulting in tonic inhibition (Farrant and Nusser, 2005). This tonic inhibition could be a compensatory mechanism shown in testosterone-induced impulsivity. Because testosterone is a

	gabra1	*gabra2*	*gabra2-transcript variant 2*	*gabra3*	*gabra4*	*gabra5*	*gabra6*	*gabrb1*	*gabrb2*	*gabrb3*	*gabrg1*	*gabrg2*	*gabrg3*
gabra1		-0.348, .567	-0.783, .117	.243, .694	.336, .58	.305, .618	.632, .253	.391, .515	.305, .618	.187, .763	-.401, .503	-.399, .506	-.219, .724
gabra2			0.746, 0.148	.703, .186	.605, .28	.643, .242	-0.077, .902	.579, .306	.622, .262	.747, .147	**.881, .048**	**.912, .031**	**.898, .038**
gabra2-transcript variant 2				.289, .637	.154, .804	.277, .652	-.331, .586	.258, .675	.236, .702	.199, .748	.637, .248	.802, .102	.669, .217
gabra3					**.988, .002**	**.984, .002**	.651, .235	.878, .050	**.992, .001**	**.913, .030**	.724, .167	.786, .115	**.891, .043**
gabra4						**.965, .008**	.736, .156	.836, .078	**.988, .002**	**.917, .028**	.677, .209	.703, .186	.828, .083
gabra5							.689, .199	**.942, .017**	**.993, .001**	.832, .081	.615, .269	.744, .150	.847, .070
gabra6								.587, .298	.728, .164	.469, .425	.113, .857	.172, .782	.321, .599
gabrb1									**.898, .039**	.665, .220	.402, .502	.623, .261	.716, .174
gabrb2										.867, .057	.650, .235	.739, .154	.850, .068
gabrb3											.823, .087	.723, .168	.836, .078
gabrg1												**.912, .031**	**.914, .030**
gabrg2													**.980, .003**
gabrg3													

FIGURE 4 | Pearson Correlations for C group GABA$_A$ receptor subunit gene expression. Data are presented as R and p. R is the correlation coefficient, and p is the *p*-value from student's *t*-test. 17 significant correlations were found between *Gabra3* and *Gabra4*, *Gabra3* and *Gabra5*, *Gabra4* and *Gabra5*, *Gabra5* and *Gabrb1*, *Gabra3* and *Gabrb2*, *Gabra4* and *Gabrb2*, *Gabra5* and *Gabrb2*, *Gabrb1* and *Gabrb2*, *Gabra3* and *Gabrb3*, *Gabra4* and *Gabrb3*, *Gabra2* and *Gabrg1*, *Gabra2* and *Gabrg2*, *Gabrg1* and *Gabrg2*, *Gabra2* and *Gabrg3*, *Gabra3* and *Gabrg3*, *Gabrg1* and *Gabrg3*, and *Gabrg2* and *Gabrg3*.

	gabra1	*gabra2*	*gabra2-transcript variant 2*	*gabra3*	*gabra4*	*gabra5*	*gabra6*	*gabrb1*	*gabrb2*	*gabrb3*	*gabrg1*	*gabrg2*	*gabrg3*
gabra1		.917, .083	.592, .408	.045, .955	**.988, .012**	.076, .924	.655, .345	.057, .943	.611, .389	.491, .509	.727, .273	-.505, .495	.829, .171
gabra2			.252, .748	-0.297, .703	.847, .153	-.157, .843	.319, .681	.071, .929	.306, .694	.151, .849	.431, .569	-.271, .729	.575, .425
gabra2-transcript variant 2				.826, .174	.670, .330	.742, .258	**.995, .005**	.347, .653	.662, .338	**.990, .010**	**.981, .019**	-.400, .600	.936, .064
gabra3					.136, .864	.919, .081	.770, .230	.495, .505	.315, .685	.892, .108	.719, .281	-0.048, .952	.595, .405
gabra4						.110, .890	.734, .266	-0.008, .992	.723, .277	.569, .431	.783, .217	-.610, .390	.868, .132
gabra5							.673, .327	.797, .203	.022, .978	.826, .174	.680, .320	.277, .723	.582, .418
gabra6								.278, .722	.720, .280	**.971, .029**	**.987, .013**	-.473, .527	**.955, .045**
gabrb1									-0.463, .537	.436, .564	.370, .630	.708, .292	.338, .662
gabrb2										.569, .431	.651, .349	**-.952, .048**	.654, .346
gabrb3											**.955, .045**	-.291, .709	.893, .107
gabrg1												-.394, .606	**.986, .014**
gabrg2													-.417, .583
gabrg3													

FIGURE 5 | Pearson Correlations for T group GABA$_A$ receptor subunit gene expression. Data are presented as R and p. R is the correlation coefficient, and p is the *p*-value from student's *t*-test. 9 significant correlations were found between *Gabra1* and *Gabra4*, *Gabra2-transcript variant 2* and *Gabra6*, *Gabra2-transcript variant 2* and *Gabrb3*, *Gabra6* and *Gabrb3*, *Gabra2-transcript variant 2* and *Gabrg1*, *Gabra6* and *Gabrg1, Gabrb2* and *Gabrg2, Gabra6* and *Gabrg3,* and *Gabrg1* and *Gabrg3*.

hydrophobic steroid hormone, it is able to cross the Blood-Brain Barrier and target GABA receptors This effect could be localized to several brain regions by signaling molecules directing testosterone's activity, or by increased binding affinity for location-specific receptors. Testosterone may target GABA receptors that are not present at the synapse itself but are still along the neuronal cell surface. Increased GABA$_A$ receptor activity in impulsivity is consistent with previous findings in rodent models. For example, a study found that increased GABA$_A$ receptor activity in the infralimbic PFC, rather than the prelimbic PFC, increased impulsive responding in rats (Murphy et al., 2012). Another study examined GABA receptors in gambling disorder and found that individuals with gambling disorder had higher levels of GABA$_A$ receptors in the right hippocampus compared to healthy controls (Mick et al., 2017). While this study focused on testosterone's effect on the PFC due to the PFC's well-studied role in impulsivity and our previous findings showing a PFC-specific alteration in noradrenergic signaling, it is important to note that other brain regions may also be involved in impulsivity. For instance, a study recently found a neural circuit connecting the ventral hippocampus to the PFC in food impulsivity inhibition (Hsu et al., 2018). Because our previous study indicated the localized effect that testosterone had on the PFC and not on the hippocampus or amygdala, we chose to continue focusing on the PFC in this study for testosterone-mediated alterations of other neurotransmitter receptor signaling pathways (Agrawal et al., 2019).

We observed that increased testosterone level was associated with altered expression of specific GABA$_A$ subunit mRNA expression. The reasons for the altered expression of specific subunit genes of GABA$_A$ receptors are not clear at the present time; however, several transcription factors have been found to be associated with promoter regions of these genes (Steiger and Russek, 2004). These include early growth response factors 1

	gabra1	gabra2	gabra2-transcript variant 2	gabra3	gabra4	gabra5	gabra6	gabrb1	gabrb2	gabrb3	gabrg1	gabrg2	gabrg3
gabra1		-.552, .334	.532, .357	.270, .661	-.132, .832	-.739, .154	.111, .859	-.084, .894	.758, .138	-.462, .433	-.559, .328	.080, .899	.182, .770
gabra2			.226, .715	-.517, .372	.726, .165	.158, .800	.659, .227	-.065, .918	-.751, .143	.318, .602	**.937, .019**	-.064, .919	-.715, .174
gabra2-transcript variant 2				-.62, .264	.761, .135	-.642, .243	.866, .057	-.320, .599	.094, .881	.308, .614	.341, .575	-.234, .704	-.411, .492
gabra3					**-.901, .037**	.081, .897	-.764, .132	.408, .495	.424, .476	**-.903, .036**	-.717, .173	.502, .389	.506, .385
gabra4						-.264, .667	**.958, .010**	-.391, .515	-.424, .477	.642, .242	.820, .089	-.395, .511	-.719, .171
gabra5							-.479, .415	.690, .198	-.728, .163	.257, .677	.264, .668	.524, .365	.479, .414
gabra6								-.417, .484	-.237, .701	.420, .482	.700, .188	-.355, .558	-.750, .144
gabrb1									-.443, .455	-.215, .728	.019, .976	**.973, .005**	.710, .179
gabrb2										-.503, .388	-.848, .069	-.329, .589	.116, .853
gabrb3											.585, .300	-.391, .515	-.177, .776
gabrg1												-.034, .957	-.563, .323
gabrg2													.641, .243
gabrg3													

FIGURE 6 | Pearson Correlations for GDX group GABA$_A$ receptor subunit gene expression. Data are presented as R and p. R is the correlation coefficient, and p is the *p*-value from student's *t*-test. 5 significant correlations were found between *Gabra3* and *Gabra4*, *Gabra4* and *Gabra6*, *Gabra3* and *Gabrb3*, *Gabra2* and *Gabrg1*, and *Gabrb1* and *Gabrg2*.

and 3, Myc associated growth factor, and zinc binding protein 9 and 89. Whether these transcription factors influence these subunits needs to be further studied. In a recent study, we observed that testosterone interacts with the HPA axis through molecules that influence stress response (Ludwig et al., 2019). For example, corticotropin-releasing hormone (Crh) and FK506 binding protein 5 (Fkbp5) genes were significantly upregulated in GDX rats. Interestingly, there is a significant interaction between CRH and GABA receptor systems. It has been reported that pharmacological agents that influence GABA can profoundly impact CRH systems (Cullinan and Wolfe, 2000; Skelton et al., 2000; Stout et al., 2001; Gilmor et al., 2003). Also, CRH is uniquely expressed in glutamic acid decarboxylase (GAD)-positive neurons (Cullinan and Wolfe, 2000). GAD is an enzyme responsible for the conversion of glutamate to GABA. Several GABA$_A$ receptor subunits are altered within CRH neurons (Cullinan and Wolfe, 2000). Thus, the possibility of HPA axis-responsive genes and changes in specific subunits of GABA by testosterone cannot be ruled out. Whereas an association between CRH-GABA has been established under stressful conditions (Yan et al., 1998), whether this interaction plays a role in impulsivity needs to be tested.

Our study also assessed cross-correlations between GABA$_A$ receptor subunit mRNA levels in control (C), testosterone (T), and gonadectomized (GDX) groups. We found that while C group rats had 17 significant correlations, T rats had 9, and GDX had only 5. These differences in subunit composition indicate a loss of co-regulated genes in GDX group and testosterone partially restored this loss. Interestingly, studies have examined the organization of various GABA receptor subunits and found that in certain psychiatric conditions, such as depression and suicide, not only are mRNA expression altered in specific GABA receptor subunits, but inter-relations between various subunits differ (Brambilla et al., 2003; Merali et al., 2004; Poulter et al., 2010; Yin et al., 2016). Although these studies did not correlate GABA subunit organization with impulsivity, it is quite possible that altered stoichiometric organization could influence neuronal firing patterns and their timing, which may be consequential in impulsivity.

Protein phosphorylation is one of the main mechanisms of regulating GABA receptor functions. It has been shown that CamKII-dependent phosphorylation can increase GABA receptor binding, and thus modulate GABA receptor-mediated chloride ion channel activity (Churn and DeLorenzo, 1998). It has also been reported that CaMKII activation can lead to an increase in specific GABA receptor subunits (Churn et al., 2002). Calmodulin genes *Calm1*, *Calm2*, and *Calm3* have been studied to produce identical calmodulin proteins. Calmodulin is a calcium-binding protein that plays a role in memory formation. The calcium/calmodulin dependent kinase IIa (*Camk2a)* has been found to play a role in integrating Ca^{2+} signals in dendritic spines (Chang et al., 2019), and increasing expression after long-term potentiation (Thomas et al., 1994). *Camk2b* is found to be more prevalent in sympathetic neurons (Ma et al., 2015), and to play a role in tethering the *Camk2* protein complex to dendritic spines (Shen et al., 1998). While *Camk2a* and *Camk2b* are primarily found in the nervous system, *Camk2g* and *Camk2d* are found throughout the body (Gray and Heller Brown, 2014). *Camk2g* functions to transport calmodulin from the cell surface to the nucleus (Malik et al., 2014). *Camk2d* is predominantly found in cardiac tissue, and its expression alters during cardiomyocyte differentiation, heart failure, and ischemia (Gray and Heller Brown, 2014). Also, both calmodulin and a *Camk2* inhibitor can block the potentiating effect of Ca^{2+} on Cl^- current gated by GABA$_A$ receptors (Aguayo et al., 1998). Moreover, glutamatergic synaptic activity is controlled by GABA$_A$ receptors by inhibiting glutamate release via Ca^{2+}/calmodulin-dependent signaling (Long et al., 2009). Although the relationship of GABA$_A$ receptors and CaMKIV is not well established, a histochemical study shows CaMKIV is expressed in a subgroup of GABAergic neurons in all layers of cortical interneurons of adult monkey area V1 in which parvalbumin was present (Lalonde et al., 2004). In this study, we examined the expression of calmodulin genes and various CaM kinases: *Calm1, Calm2, Calm3, Camk2a, Camk2b,*

Camk2g, Camk2d, and Camk4. It was observed that *Camk4* gene expression was significantly lower when comparing T rats to C rats and GDX rats to C rats. Additionally, *Calm3* was lower when comparing T rats to C rats, but it could not reach significance level. It appears that *Camk4* might be involved in differential regulation of *Gabra3, Gabra5*, and *Gabra6* receptors. However, changes in *Camk4* are rather surprising given that this kinase is not very well linked to GABA$_A$ receptor functioning (Houston et al., 2009). The possibility of GABA-independent functions of *Camk4* cannot be ruled out.

CONCLUSION AND LIMITATIONS

Altogether, this is the first GABA$_A$ and GABA$_B$ subunit gene expression analysis in an animal model of testosterone-induced impulsivity. We show that the impulsivity response may be GABA$_A$ subunit-specific when involving supraphysiological concentrations of testosterone. Also, the organization of GABA$_A$ receptor subunits is quite different between control, testosterone, and GDX rats. Further, this study shows that subunit-specific effects of testosterone may be associated with calmodulin/calcium-dependent kinases. Our study thus provides an interplay between testosterone, impulsivity and GABAergic functions. From a clinical perspective, GABA$_A$ receptors are more frequently used as therapeutic targets when treating disorders such as anxiety and epilepsy (Arin et al., 2018). In the future, it will be interesting to translate this study to a human level to examine if GABA receptor functions are altered during impulsivity, particularly in suicidal people where this interaction has been shown to play a critical role in attempted and completed suicide (Sher et al., 2017; Lenz et al., 2019). There were a few limitations to our study. This study did not have a group with a normal amount of testosterone (created by GDX + normal amount of testosterone). However, in the results section, testosterone level analyses from serum confirmed the following 3 groups: a testosterone-depleted GDX group, a group with supraphysiological levels of testosterone (T+) and another group with physiological levels of testosterone (C). Thus, the C rats were used for normal level of testosterone. One caveat of the study is that the results were derived from the transcript (mRNA) levels of GABA$_A$ receptor subunits and CaM kinases and not from protein levels. Thus, there is a possibility that changes in transcription may not necessarily reflect changes in protein. Similarly, CaM kinase activity was not studied. However, as mentioned above, based on mRNA expression levels, Poulter et al. (2010) showed that GABA$_A$ receptor organization was altered in the brain of depressed subjects. These investigators convincingly argued that the relative mRNA abundance of GABA$_A$ receptor subunits would be a mechanism that ensured proportional abundance of protein. In addition, Brooks-Kayal et al. (1999) demonstrated that subunit mRNA levels correlated closely with receptor pharmacology within individual dentate granule cells, which could be similar to those predicted by studies of recombinant receptors. Nevertheless, our present study needs to be confirmed at the protein level to reach a definite conclusion. In addition, though the most common GABA$_A$ receptors were chosen to study, other subunits such as δ and ρ could also be studied in the future.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The experimental procedures were approved by the IACUC of the University of Alabama at Birmingham.

AUTHOR CONTRIBUTIONS

JA performed the experiments and analyzed the data. YD conceptualized the idea. JA and YD co-wrote the manuscript. Both authors contributed to the article and approved the submitted version.

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