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Chronic Testosterone Increases Impulsivity and Influences the Transcriptional Activity of the Alpha-2A Adrenergic Receptor Signaling Pathway in Rat Brain

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Abstract
Testosterone is an anabolic androgenic steroid hormone involved in brain development, reproduction, and social behavior. Several studies have shown that testosterone can cause impulsivity in humans, which in turn, is linked with mood-related psychiatric disorders and higher risk of death by suicide. The mechanisms by which testosterone abuse influences impulsivity are unclear. The present study aims to understand how testosterone influences impulsivity in a rodent model both at behavioral and molecular levels. In this study, rats were either only gonadectomized or gonadectomized and injected with supraphysiological doses of testosterone. Their relative impulsivity levels were assessed using the go/no-go task. Serum level of testosterone was measured using ELISA. Transcript levels of alpha-2A adrenergic receptor (*Adra2a*), G proteins (stimulatory subunit-$G_{\alpha s}$ [*Gnas*], inhibitory subunit-$G_{i\alpha}$ [*Gnai1* and *Gnai2*]), and catalytic and regulatory subunits of protein kinase A (PKA) were examined using quantitative PCR (qPCR) in brain areas associated with limbic system (prefrontal cortex (PFC), hippocampus, and amygdala). The testosterone-treated (T) group showed significantly higher level of serum testosterone and displayed a lower go/no-go ratio, indicating greater impulsivity compared to the gonadectomized (GDX) group. The transcript levels *Adra2a and* $G_{\alpha s}$ genes and PKA subunits encoded by *Prkar1a*, *Prkar1b*, *Prkar2a*, and *Prkaca* genes were significantly upregulated in PFC of testosterone treated rats. The expression levels of these genes were not significantly altered in hippocampus. On the other hand, amygdala showed changes only in *Gnas* and *Prkar2a*. These results suggest that chronic testosterone influences impulsivity possibly via hyperactive alpha-2A adrenergic receptor-PKA signaling axis, specifically in the PFC.

Keywords Impulsivity · Testosterone · Noradrenergic system · Alpha-2A adrenergic receptor · Rodent model

Introduction

Impulsive behavior can be described as quick actions completed without anticipation that there will be negative consequences or risk associated with it. Impulsivity is a multifaceted personality construct and deemed as critical diagnostic criteria for several psychiatric disorders, such as conduct disorder, substance abuse, attention deficit hyperactivity disorder, and suicidal behavior [1, 2]. While many other factors such as insomnia and sleep disturbances are well established to the occurrence of para-suicidal and suicidal behavior [3], increased impulsivity is still being controversially discussed in this context [4]. Deficits in impulse control can also be present in affective disorder patients during manic episodes and in borderline personality disorder [5, 6]. Altered endocrine function with increased aggressiveness causing lack of impulse control has been a major concern in these disorders [7]. In this regard, the hypothesis considering the influence of androgens on impulsivity in psychiatric disorders seems to be of interest. Several studies have explored this association in the clinical context [8–10]. Studies in healthy men uphold the hypothesis that high levels of testosterone influence impulsive [11, 12] or risk-taking behavior [13]. In a recent study, it has been shown that early onset users under steroid rage are more impulsive and show deficits in affective processing, behavioral disinhibition, and planning, but not decision-making [14]. Although testosterone has been studied in relation to increase impulsivity and aggression [15], the molecular mechanisms associated with these effects are not clear.

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Previous studies have suggested the importance of the limbic system on impulsivity behavior, which is primarily associated with inability to perform executive functions [16, 17]. Alpha-2A adrenergic receptor (*Adra2a*) and associated adenylyl cyclase (AC)-cAMP signaling play significant roles in regulating prefrontal cortex (PFC)-related executive functions as well as in depression and impulsivity [18, 19]. Protein kinase A (PKA) is an important molecule in the adenylyl cyclase-cAMP signaling pathway. An earlier report has shown that functional deficiency in the catalytic subunits of PKA in dorsolateral prefrontal cortex (dlPFC) is associated with depression and suicidal behavior [20]. On the other hand, children with ADHD, oppositional-defiant, or conduct disorder have shown reduced impulsivity upon administration of clonidine, which acts as an agonist to *Adra2a*. The behavioral changes with clonidine treatment could be mediated via downregulated response in noradrenergic neurotransmission system [21, 22]. Although PKA dysregulation seems to be associated with pathophysiological behavior, the direct involvement of *Adra2a* signaling and the role of PKA have not been analyzed in the context of impulsivity. Moreover, this signaling cascade has not been fully studied in brains of subjects engaged in chronic testosterone abuse. Using a rat model system, the present study aims to examine testosterone-induced impulsivity and the extent to which the *Adra2a* and linked signaling pathway is involved in such a behavior. For this, the expression of *Adra2a* and expression of various catalytic and regulatory subunits of PKA as well as stimulatory and inhibitory subunit of G alpha protein were studied in PFC, hippocampus, and amygdala. It was hypothesized that T rats would show a lower go/no-go ratio, suggesting higher impulsivity compared to gonadectomized (GDX) rats and naïve controls. Furthermore, it was hypothesized that T rats would exhibit concomitant dysregulation in the adenylyl cyclase-cAMP signaling pathway.

Materials and Methods

Animals

Thirty male Long Evans rats (PND 25) were obtained from Envigo Laboratories (Indianapolis, IN, USA) and maintained as described previously [23]. After 5 days of acclimation, they were randomly divided into three groups: rats receiving gonadectomy with daily injections of testosterone propionate (Sigma-Aldrich, St. Louis, MS, USA) assigned as T rats ($n = 10$), gonadectomized with subsequent daily injections of vehicle only (corn oil) assigned as GDX rats (GDX; $n = 10$), and naïve controls assigned as C rats (C; $n = 10$). 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. The overall animal procedure has been represented with a schematic diagram in Fig. 1a.

Surgical Procedures and Testosterone Injection

For the gonadectomy and subsequent procedures involving the injection of testosterone, the animals were subjected to the same treatment as previously reported [23]. Briefly, rats were anesthetized with isoflurane (5% induction, 1–3% maintenance) and vitals were constantly monitored to minimize the occurrence of cardiorespiratory failure. Prior to incision, rats were administered carprofen (5 mg/kg, subcutaneous) and buprenorphine (0.1 mg/kg, subcutaneous). Using aseptic technique, the rat abdomen was shaved and cleaned with betadine, and a single transverse incision in the caudal abdomen was made; the testicular fat pad on the one side was pulled through the incision using blunt forceps. A hemostat was placed below the testes and epididymis, then a ligature was placed below the hemostat and both the testes and epididymis were removed. The incisions were closed with multiple monocryl sutures (Ethicon, Somerville, NJ, USA). The rats were monitored, and the body temperature was regulated during and after the procedure by a warming pad. A second subcutaneous dose of carprofen (5 mg/kg) was administered as an analgesic 24 h after the surgery.

On post-surgery, 10 GDX rats were randomly assigned to the testosterone group (T) and subcutaneously injected with daily dose of 7.5 mg/kg testosterone propionate (Sigma-Aldrich, MS, USA), dissolved in 0.1 mL of corn oil. Ten other GDX rats were administered equal volume of corn oil only. The testosterone injections were carried out for 24 weeks. Body weights were assessed every 2 weeks and the daily dose was adjusted according to the weight. The 7.5 mg/kg dose approximates a heavy steroid dose in humans and is based on previous literature [24, 25].

Impulsivity Testing

The experimental design to test for impulsivity is based on the go/no-go paradigm as modified from previous studies [25, 26]. Food-restricted rats (Fig. 1b) were tested on their ability to restrain from pressing the lever in this impulsivity behavior model. Two chambers with alternating plexiglass and stainless-steel walls (Med Associates Inc., VT, USA) were encased in a sound-attenuating box with a small ventilation fan. Each chamber contained an overhead white house light (4.6 W) and a retractable lever equipped with tri-colored stimulus lights (4.6 W). The retractable lever was connected to a food trough with 45-mg pellets. All experimental sessions were controlled by a computer running Med-PC IV® software (Med Associates Inc., VT, USA). The autoshaping phase lasted for 15 days. Rats that did not complete the learning goal

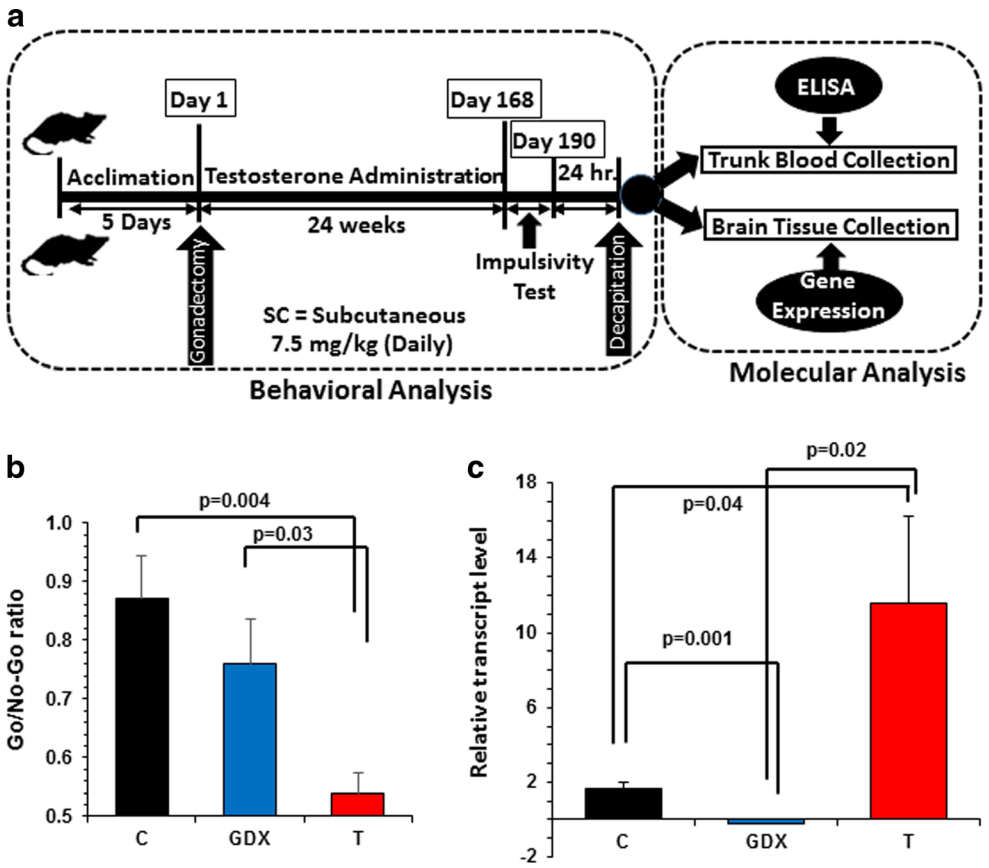

Fig. 1 Impulsivity model of gonadectomized rats treated with testosterone and serum level of testosterone in control (C), gonadectomized (GDX), and testosterone (T) rats. **a** schematic diagram of the timeline followed as part of testosterone treatment to induce impulsivity-related behavioral changes in GDX rats. **b** Bar diagram of go/no go impulsivity task ratio compared across control (C; $n = 6$), gonadectomized (GDX; $n = 6$), and testosterone (T; $n = 5$) groups. Data are the mean ± SEM. Single-factor ANOVA found significant differences across groups ($p = 0.013$). The ratio was found to be significantly down-regulated in T group compared to both GDX ($p = 0.03$) and to C ($p = 0.004$). No significant difference was found between GDX and control rats ($p = 0.32$). Results were taken after autoshaping, so all groups of rats were equally equipped to complete the task successfully. **c** Bar diagram comparing testosterone levels across C, GDX, and T groups based on serum-ELISA assay. Data are the mean ± SEM. One-way ANOVA found significant differences across groups ($p = 0.0085$). Individual groupwise comparison showed significantly increased serum testosterone level in the T group when individually compared with C ($p = 0.04$) or GDX group ($p = 0.02$). The serum testosterone concentration was significantly ($p = 0.001$) lower in the GDX compared to control (C) group

(pressing the lever 20× within 30 min during the go phase) within the time frame were excluded from further testing. Rats having met the autoshaping criteria were transferred to the go/no-go phase. During the go phase, a lever press produced a total of five 45-mg sucrose pellets. Pressing the lever during the no-go component was recorded as an index of impulsive action. The session started with the go phase and then moved to the no-go phase, alternating back and forth throughout the session. Each phase lasted 10 min with a 10-s timeout between components for an approximately 1-h 20-min sessions. Thus, there were four go alternating with four no-go components all in all. The main visual discriminants were *light* with the house light on for the go phase and *dark* with the house light off for the no-go phase. As additional cues, illumination of a bright white stimulus light over the lever marked the go phase of the paradigm, while activation of the red stimulus light marked the no-go component. The variable impulsivity was measured by calculating a ratio between adequate lever pressing during the go component and inadequate lever pressing during the no-go phase. A high ratio indicates a relatively high number of adequate responses in relation to inadequate responses and was interpreted as low impulsivity behavior. A low ratio indicates a low number of adequate responses in relation to inadequate responses and thus was interpreted as high impulsivity behavior. All rats had one warm-up session that was not included in the results. The following three sessions were recorded and only if the variance between the three ratios was less than 10%, the results were counted. Individual responses were averaged across the three experimental groups (GDX, C, and T).

Tissue Collection

Twenty-four hours after the final go/no-go testing session, rats were decapitated, and trunk blood was collected (08:00–12:00). Brains were removed and flash-frozen, then stored at

− 80 °C. Frozen brain samples were sectioned using cryostat (Leica, Wetzlar, Germany) and cut into 300-μM slices. The slices were then mounted on glass slide to dissect out the PFC, hippocampus, and amygdala. All tissue sections were returned to − 80 °C until further analyses.

Measurement of Testosterone Levels

Serum from trunk blood was isolated and stored at − 80 °C until the assays were performed. Serum levels of testosterone were measured using an ELISA kit (Abcam, MA, USA) according to the manufacturer's instructions. To determine the variability in serum testosterone levels, Student's *T* test was performed between groups, and a single-factor ANOVA was done across all groups. Data are presented as concentration values (ng/mL).

Primer Design

Rat-specific sequences were collected from the Rat Genomic Database (RGD) for designing gene expression primers. Primers were primarily designed close to the 3′ end of the transcripts and accounted for all the transcript variants for each gene. A complete list of primers used in the gene expression assays is provided in Table 1.

RNA Isolation and qPCR-based Gene Expression Analysis in Rat PFC, Amygdala, and Hippocampus

RNA isolation was completed using TRIzol® (Thermo-Scientific, MA, USA) method as described earlier [27]. RNA purity was determined by measuring the optical density with an absorbance ratio of 260/280 (NanoDrop 2000c, Thermo-Scientific, MA, USA). All samples had 260/280 ratio > 1.8. The quality of RNA was also determined by running them on agarose gel.

The single-stranded cDNA was prepared as described previously [28]. Following the preparation of mRNA-specific first-strand cDNA, 1 μg total RNA was reverse transcribed using M-MLV Reverse Transcriptase (Invitrogen, NY, USA) and oligo (dT) primer. The oligo dT primer annealing step was carried out at 5 μM concentration in the presence of 1 mM dNTPs by incubating the reaction at 65 °C for 5 min. Subsequently, the reaction was inactivated by incubating it for more than 2 min at 4 °C; 1× first strand synthesis buffer, 0.01 mM DTT, 2 U of RNaseOut, and 200 U of M-MLV Reverse Transcriptase were added, and the reaction was incubated at 37 °C for 50 min. The reaction was finally inactivated at 70 °C. Relative transcript abundance of coding genes was measured with a qPCR (Stratagene MxPro3005, La Jolla, CA, USA) instrument. With the help of Eva Green chemistry (Applied Biological Material Inc., Canada), the qPCR amplification for the specific gene was performed using gene-specific forward and reverse primers as mentioned in Table 1. Forty-fold diluted raw cDNA was used as template for qPCR amplification using a thermal parameter of initial denaturation at 95 °C for 10 min followed by repeating 40 cycles of denaturation at 95 °C for 10 s, primer annealing at 60 °C for 15 s, and an extension of amplicon at 72 °C for 20 s. The possibility of primer dimer formation and secondary product amplification was ruled out by running template-free samples. Relative gene expression levels were normalized with *Gapdh* and the fold-change values were determined following Livak's ΔΔCt calculation method [29].

Statistical Analysis

Data of behavioral tests were analyzed by one-way ANOVA followed by Student's *T* test to determine the effects of testosterone (GDX vs. C and C vs. T) on rats and to find out any significant impacts derived from the go/no go trial. For gene expression assays, *Gapdh* transcription levels were used as normalizer, which did not significantly differ between the three groups. Changes related to gene expression were used

Table 1 Oligo sequences used in qPCR-based gene expression assays

Genes	Forward (5′–3′)	Reverse (5′–3′)
Adra2a	AGC TCG CTG AAC CCT GTT AT	CTG ACC AGG GTC TGT AAG CA
Gnas	GGT GAG AAG GCC ACC AAA GT	AAC TGG TTC TCA GGG TTG GC
Gnai1	TTG GTT CTG TGT TTG GCA GTT	CAG GGT AAG GGG GTT GAC ATT
Gnai2	TCC CTG TCT AAA ACC CAC CTT	AGA AAA CCC GAA TGG ATG CC
Prkar1a	GCG TCG GTC AGA AAA CGA AG	ACG ATT CAT CAG GGC AA
Prakar1b	AAC CTG CCT ATT GGA GAC CC	GAG CAG AGA GGT TTG GGA GT
Prkar2a	CCG GGC AGT AGA TGT GAT GAA	GGT GTG TTC TTG TGG CTG AC
Prkar2b	CTG GCT CAT CCT TCT GTG TTC T	TCC ACA GGC ATT GGT TTC CG
Prkarca	CGT GTG AAA GGC CGA ACT TG	AAC CAG CCA TCT CGT AG
Prkarcb	GGG TTC GCC AAG AGA GTC AA	TAG CCA GCA GCC ATC TCG TA

to find differences following Student's *T* test. Additionally, one-way ANOVA followed by Bonferroni corrections was also used to determine the significant differences in-between groups for gene expression. The results were expressed as the mean ± SEM and the level of significance was $p \leq 0.05$. The data were analyzed by SPSS statistical package V.23 (IL, USA).

Results

Effect of Chronic Testosterone Administration on Impulsivity Test

A total of 11 rats did not meet the autoshaping criteria within the required time frame of 15 days and were subsequently excluded from further testing (4 T, 4 GDX, and 3 C rats). When testing for variance within the calculated ratios, two rats had to be excluded from further analyses because the variance between the calculated ratios exceeded 10% (one T and one GDX rat). The results from the impulsivity test related to testosterone administration are shown in Fig. 1b. In the go/no-go task, rats in the testosterone-treated group had a significantly lower go/no-go ratio compared with both the GDX ($p = 0.03$) and control ($p = 0.004$) groups. The lower go/no-go task ratio means that on average, T rats pressed the "no go" lever many more times compared with rats from other groups, not necessarily in total numbers but in relation to pressing the "go" lever. When comparing GDX rats to controls, there was no significant difference ($p = 0.32$) between the groups. These results were obtained after having learned that task in autoshaping; all groups of rats were equally equipped to successfully complete the task.

Measurement of Serum Testosterone Level

ELISA-based serum testosterone levels were determined in three groups of rats including control (C), GDX, and T groups. Based on optical density, serum testosterone concentration (ng/mL) were determined by comparing unknown values to a curve generated from standards with known concentrations. Mean concentrations with SEM values were calculated for each of the three groups, and significance between and across groups was determined with *T* tests and ANOVA, respectively. The average concentration for the C group ($n = 6$) was 1.63 ng/mL (SEM = 0.4). For the GDX group ($n = 6$), the average concentration was close to 0 ng/mL (SEM = 0.0061), whereas the maximum concentration of testosterone for the *T* group ($n = 5$) was noted with an average of 11.6 ng/mL (SEM = 4.67). Single-factor ANOVA found significant differences across the three groups ($p = 0.0085$). Individually, the concentration in T group was significantly higher than those for the C group ($p = 0.04$) and for the GDX group ($p = 0.02$). On the other hand, the GDX group's concentrations were significantly lower compared to that of the C group ($p = 0.001$). All ELISA results are shown in Fig. 1c.

Gene Expression Analysis

Gene expression analysis was done in behaviorally tested rat brain regions (PFC, amygdala, and hippocampus) to determine the effect of supraphysiological dose of testosterone on genes associated with alpha-2A adrenergic receptor signaling and PKA pathway compared to rats with normal levels of testosterone (C) and rats with no testosterone due to gonadectomy (GDX).

RNA level expression changes of all genes in PFC are presented in Fig. 2. Based on the single factor ANOVA, a PFC related significant expression differences were found across groups in the following genes: *Adra2a* ($p = 0.0022$), *Gnas* ($p < 0.000001$), *Prkaca* ($p = 0.00014$), *Prkacb* ($p = 0.0028$), *Prkar1a* ($p = 0.000104$), *Prkar1b* ($p = 0.0001$), and *Prkar2a* ($p = 0.000114$). The T rats ($n = 4$) displayed significantly upregulated *Adra2a* mRNA transcript levels ($p = 0.009$) compared with the GDX group of rats ($n = 5$). A similar significant expression upregulation was seen for *Gnas* ($p = 0.0004$), *Prkar1a* ($p = 0.0014$), *Prkar1b* ($p = 0.00018$), *Prkar2a* ($p = 0.0047$), *Prkar2b* ($p < 0.001$), and *Prkaca* ($p = 0.0015$) genes in T rats as compared to GDX group. However, no significant changes in *Gnai1*, *Gnai2*, and *Prkacb* were noticed while comparing T group of rats with GDX rats. Several genes demonstrated upregulated expression in T vs. C groups; these included *Adra2a* ($p = 0.0059$), *Gnas* ($p < 0.000001$), *Prkar1a* ($p = 0.00014$), *Prkar1b* ($p = 0.00013$), and *Prkar2a* ($p = 0.00018$) and *Prkar2b* ($p < 0.001$), *Prkaca* ($p = 0.00026$) and *Prkacb* ($p = 0.0047$). The GDX group had significantly upregulated expression of the *Gnas* gene compared to C ($p = 0.0012$). No other genes had significant expression-related differences in this pair of comparing groups (GDX vs. C).

In the amygdala, expression for *Adra2a* gene was not significantly altered in T rats ($n = 5$) compared to C rats. One-way ANOVA-based three-group comparison determined significant differences in expression of *Gnas* ($p = 0.0046$), *Prkar1a* ($p = 0.044$), and *Prkar2a* ($p = 0.0029$). Groupwise comparison showed significant upregulation in *Gnas* ($p = 0.004$), *Prkar1a* ($p = 0.006$), and *Prkar2a* ($p = 0.002$) in GDX rats ($n = 6$) compared to C rats ($n = 6$). When comparing T vs. C rats, the following genes showed significantly upregulated expression: *Gnas* ($p = 0.03$) and *Prkar2a* ($p = 0.03$). All amygdala-related qPCR results are presented in Fig. 3. However, no significant expression related changes were noticed for other genes when GDX rats were compared with T rats.

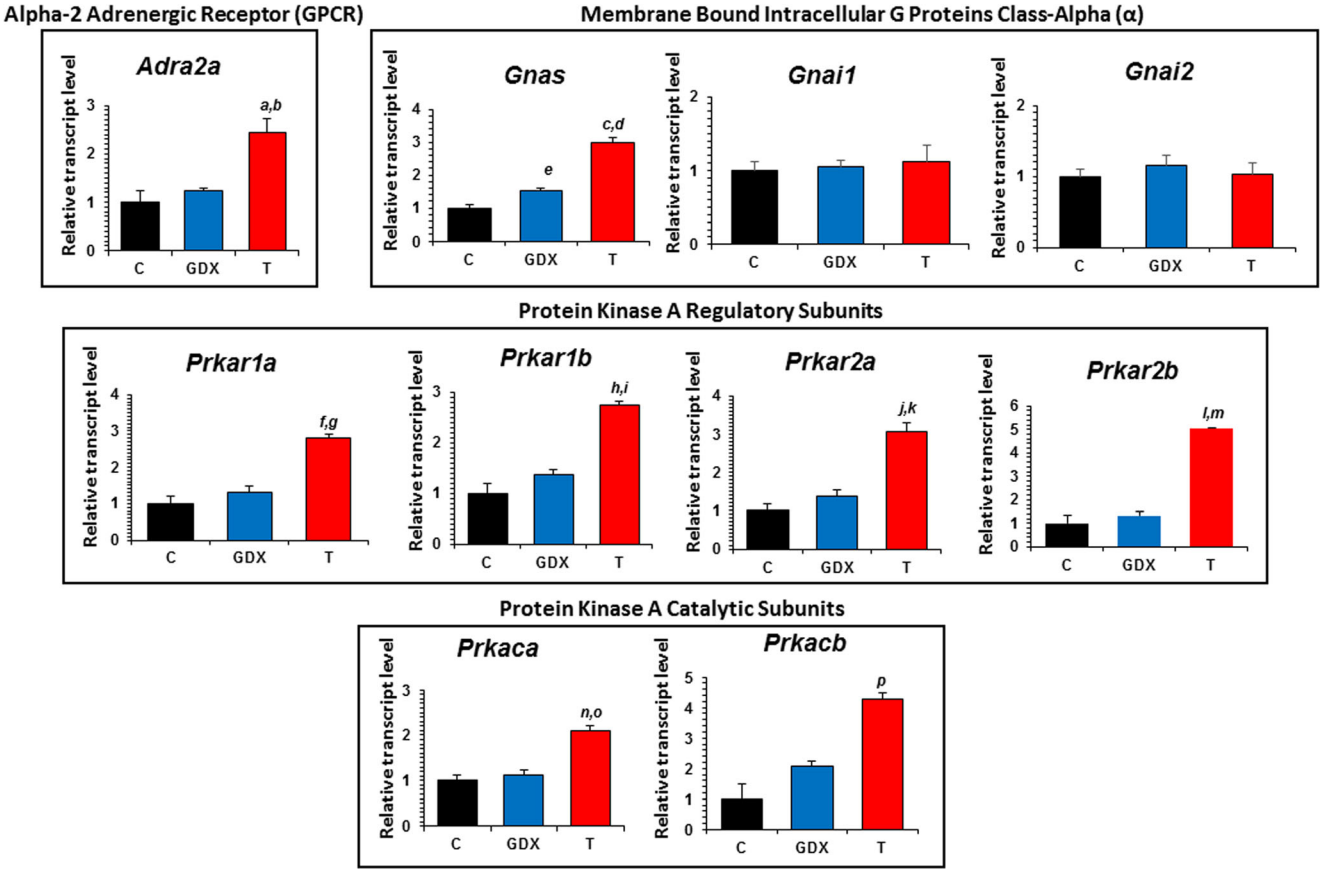

Fig. 2 Bar diagram comparing qPCR prefrontal cortex gene expression results across control (C), gonadectomized (GDX), and testosterone (T) groups. Data are the mean ± SEM. One-way ANOVA found significant differences across groups in the following genes: *Adra2a* ($p = 0.0022$), *Gnas* ($p < 0.000001$), *Prkaca* ($p = 0.00014$), *Prkacb* ($p = 0.0028$), *Prkar1a* ($p = 0.000104$), *Prkar1b* ($p = 0.0001$), *Prkar2a* ($p = 0.000114$), and *Prkar2b* ($p < 0.0001$). The T rats ($n = 4$) displayed significantly upregulated *Adra2a* mRNA transcript levels ($^{a}p = 0.009$) compared with the GDX group ($n = 5$). Similar significant expression upregulation was seen for other genes in T rats as compared to GDX rats [*Gnas* ($^{c}p = 0.0004$), *Prkar1a* ($^{f}p = 0.0014$), *Prkar1b* ($^{h}p = 0.00018$), *Prkar2a* ($^{j}p = 0.0047$), *Prkar2b* ($^{l}p < 0.001$), and *Prkaca* ($^{n}p = 0.0015$)]. However, no significant changes in *Gnai1*, *Gnai2*, and *Prkacb* were noticed when comparing T group of rats with GDX rats. The *Adra2a* ($^{b}p = 0.0059$), *Gnas* ($^{d}p < 0.000001$), *Prkar1a* ($^{g}p = 0.00014$), *Prkar1b* ($^{i}p = 0.00013$), *Prkar2a* ($^{k}p = 0.00018$), *Prkar2b* ($^{m}p < 0.001$), *Prkaca* ($^{o}p = 0.00026$), and *Prkacb* ($^{p}p = 0.0047$) were all found upregulated in the T group compared to the C group. The GDX group had upregulated expression of the *Gnas* gene compared to C ($^{e}p = 0.0012$)

In the hippocampus, single-factor ANOVA did not find significant differences across groups in any genes tested. All qPCR results for the hippocampus are presented in Fig. 4.

Discussion

The present study aimed to understand testosterone's influence on impulsivity and alpha-2A adrenergic receptor signaling in a rodent model. The higher serum levels of testosterone correlated significantly with a lower go/no-go ratio, suggesting higher impulsivity in testosterone-injected rats. This is consistent with previous findings showing a positive correlation between higher testosterone levels and impulsive behavior [11, 13, 30, 31]. Absence of significant association between testosterone level and impulsivity has also been reported [8, 25, 32, 33]. Different behavioral outcomes depend on factors such as dosages, form of administration, and long- vs. short-time administration of testosterone. It has been shown that the formation of different derivatives or metabolites of testosterone can lead to different behavioral effects [34].

Noradrenergic pathways play an important role in impulsivity. For example, it has been reported that repeated administration of selective α_{2A}-adrenergic receptor agonist significantly reduces impulsive choice behavior in rats, which is associated with direct stimulation of α_{2A}-adrenergic receptors [35]. In this study, we investigated the alpha-2A adrenergic receptor signaling pathway and found that expression of *Adra2a* gene was significantly higher in the PFC of testosterone rats compared to the GDX or control rats. However, this increase was not evident in other limbic brain regions such as

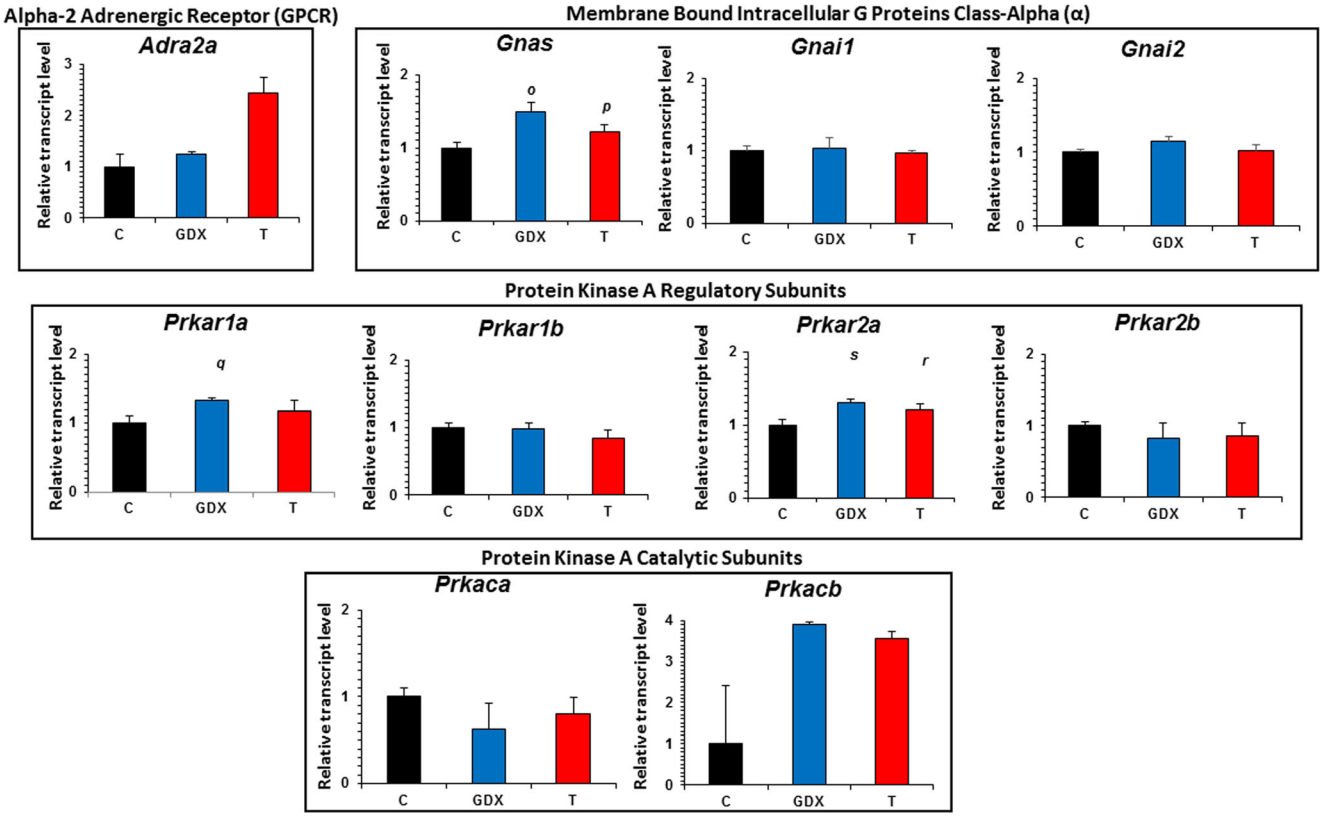

Fig. 3 Bar diagram comparing qPCR amygdala gene expression results across control (C), gonadectomized (GDX), and testosterone (T) groups. Data are the mean ± SEM. Significant differences were found for the following genes by using one-way ANOVA across groups: *Gnas* ($p = 0.0046$), *Prkar1a* ($p = 0.044$), and *Prkar2a* ($p = 0.0029$). Expression was significantly upregulated in *Gnas* ($^{o}p = 0.004$), *Prkar1a* ($^{q}p = 0.006$), and *Prkar2a* ($^{s}p = 0.002$) genes when GDX rats ($n = 6$) were compared with control rats ($n = 6$). When comparing T rats to control, only *Gnas* ($^{p}p = 0.03$) and *Prkar2a* ($^{r}p = 0.03$) showed significant expression upregulation. However, no significant changes were noticed in other genes while comparing T group of rats with GDX group

hippocampus and amygdala, indicating brain region-specific effects of testosterone and underlining the importance of the PFC for response inhibition. Moreover, reports based on binding studies have strongly suggested the high abundance of alpha-2A adrenergic receptors in superficial layers in primate PFC [36]. Further experiments revealed interesting results at the level of G proteins and various PKA subunit expressions associated with the intracellular signaling axis downstream to alpha adrenergic system. Alpha-2A adrenergic receptors belong to G protein-coupled receptor (GPCR) family and are specifically linked with the inhibitory $G_{\alpha i,}$ which blocks the adenylate cyclase activity and subsequently the formation of cAMP. Since *Adra2a* acts as an inhibitory pre-synaptic receptor, increased *Adra2a* expression indicates that norepinephrine is inhibited in testosterone treated rats [18]. Another interpretation might be that decreased norepinephrine may lead to increased expression of *Adra2a* in a compensatory fashion [18]. This is evident from our downstream examination of G proteins subunits in PFC of testosterone treated rats. When we examined the expression of $G_{\alpha i}1$ or $G_{\alpha i}2$ subunits, which encode for $G_{\alpha i}$, in the PFC of testosterone-treated rats, we did not find any significant alterations. However, another subunit of G protein, $G_{\alpha s,}$ which is stimulatory in nature, was significantly increased in PFC of testosterone treated rats. This change was paralleled with an increased expression of *Adra2a* gene. This demonstrates that at *Adra2a* and G protein levels, there is a compensatory response to the lower level of norepinephrine. Observations have been made in PFC of depressed and suicidal individuals where lower norepinephrine and higher *Adra2a* expression have been reported [37]. Earlier observations in depressed suicide brain have also shown that PFC specific $G_{\alpha s}$ increase is linked with induced adenylate cyclase activity [38]. Induced adenylate cyclase function, resulting from increased $G_{\alpha s}$ activity, possibly determines a norepinephrine based small feed-forward loop depending on the cellular availability of $G_{\alpha i}$, which is often decided by the release of another G protein subunit Gq [39].

Protein phosphorylation and dephosphorylation are important mediators of neural plasticity [40]. PKA is one of the key phosphorylating enzymes, which, upon activation, triggers a wide variety of physiologic responses in the brain including receptor desensitization, altered neurotransmitter release, cell

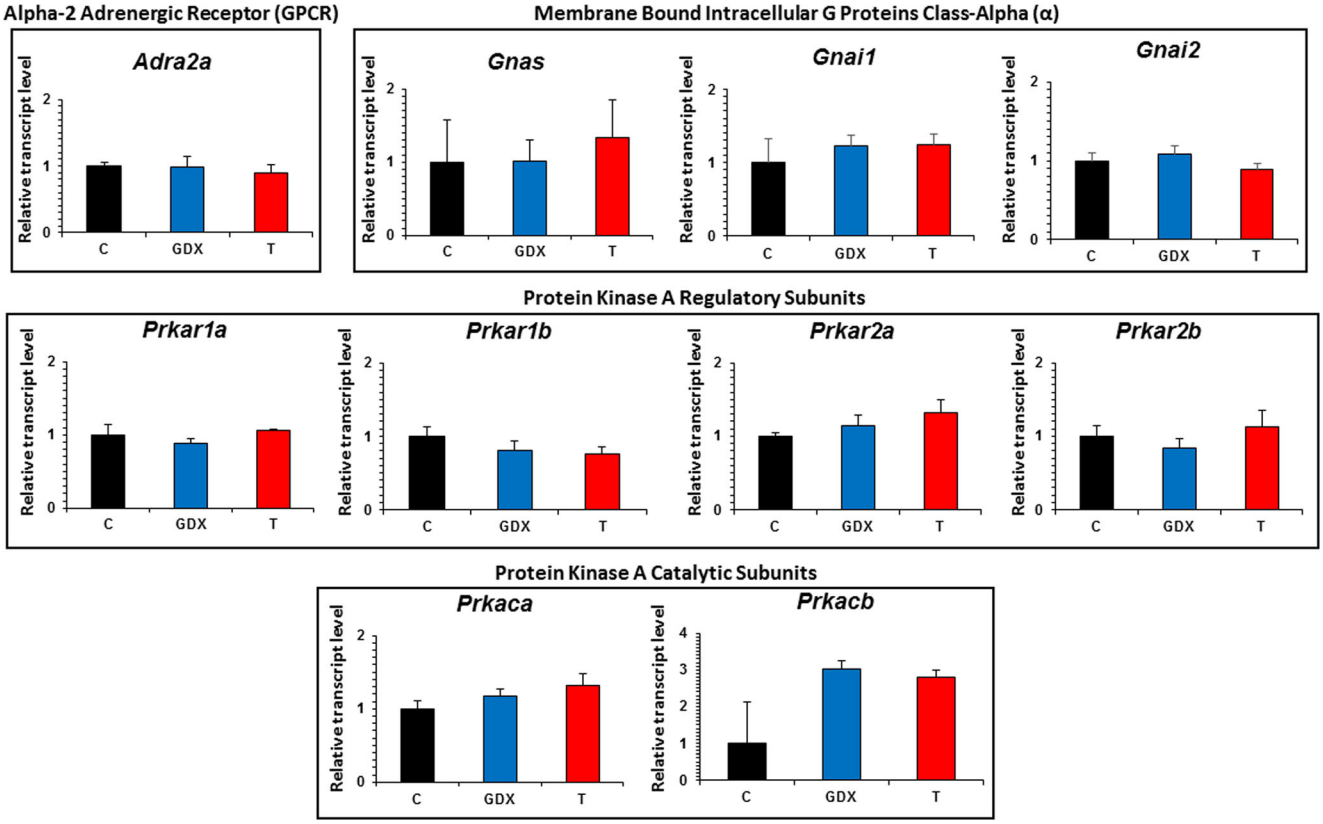

Fig. 4 Bar graph comparing qPCR hippocampus gene expression results across control (C), gonadectomized (GDX), and testosterone (T) groups. Data are the mean ± SEM. Single-factor ANOVA did not find significant differences across groups in any genes tested

growth, differentiation and survival, synaptic plasticity, and activation or repression of gene transcription. PKA is a tetrameric holoenzyme composed of a regulatory subunit dimer with a catalytic subunit bound to each regulatory subunit. When two molecules of cAMP bind to each regulatory subunit, the affinity of the regulatory subunits for the catalytic subunits decreases, causing the holoenzyme to dissociate from and release the two catalytic subunits and a regulatory subunit dimer, which then is capable of diffusing into the nucleus and phosphorylating substrates [41]. Multiple isoforms of PKA regulatory and catalytic subunits exist and are encoded by separate genes. Four regulatory (RIα, RIβ, RIIα, RIIβ) and two catalytic (Cα, Cβ) subunits have been reported in the CNS. Among them, RIIβ and Cβ are the predominant isoforms. When the expression of PKA catalytic and regulatory subunits were examined in various brain areas, it was observed that the expression levels of Cα, RIα, RIβ, and RIIα were significantly upregulated in PFC of testosterone-administered rats compared with GDX or control rats. On the other hand, Cβ subunit was significantly upregulated only in testosterone-administered rats compared with control rats. The hippocampal brain area did not show any such changes in any of the catalytic or regulatory subunits of PKA. Interestingly, a small increase was noted in the expression of RIIα in amygdala when both GDX and T group of rats were compared with controls. On the other hand expression of only RIα was significantly increased in GDX rats as compared to control. These results show that there was a consistent upregulation in PKA catalytic and regulatory subunits in PFC, particularly in the testosterone treated group, in which increases in *Adra2a* and $G_{\alpha s}$ were also found. It is pertinent to mention that whereas *Adra2a* is linked to PKA pathway in an inhibitory fashion, increased expression of PKA subunits demonstrate that the functions of the signaling components of *Adra2a* are hyperactive in testosterone treated rats, particularly in PFC. Further studies will be needed to examine the downstream target genes to assess the functional relevance of altered PKA subunits.

Our laboratory has previously studied the relevance of PKA in the context of depression and suicidal behavior. We have shown diminished adenylyl cyclase-PKA signaling in postmortem brains of depressed and suicidal individuals. We have reported reduced levels of specific PKA catalytic and regulatory subunits in PFC of depressed patients as well as in rats with depression-like behavior [20, 42, 43]. In addition, we have found that low PKA signaling was associated with increased cortisol [44]. This suggests that PKA signaling may be interacting at the hypothalamic-pituitary-adrenal (HPA) axis level in an inhibitory fashion and reducing PKA-mediated functions [44]. In the present study, we found opposite

changes such that PKA signaling was upregulated by testosterone. There is a possibility that in contrast to depression, testosterone-induced impulsivity may be associated with overstimulation of PKA signaling. It is important to note that both depression and impulsivity appear to upregulate alpha-2 adrenergic receptors [37]; however, downstream PKA signaling is differentially regulated in these two populations. An interesting point that needs further attention is that PKA subunits were not altered in PFC of GDX rats compared to control rats. This suggests that PKA is downregulated only when the level of testosterone exceeds a certain threshold.

Very recently, we studied the effect of testosterone on learned helplessness behavior and noted that supraphysiological doses of testosterone induced this behavior in rats [23]. In addition, we found that there was a significant interaction of hypothalamic-pituitary-adrenal (HPA) axis and hypothalamic-pituitary-gonadal (HPG) axis at the level of stress-related genes and androgen receptors such that HPA axis was inhibited by testosterone [23]. The present study provides important information of the contrasting pattern of PKA and the ones reported earlier in depressed individuals. There is a possibility that HPA hyperactivity may lower PKA and mediated functions, and the opposite can be seen when there is an inhibition of HPA axis mediated by testosterone. This is supported by another study where we observed that PKA is hyperactive in adrenalectomized rats, which was reversed by supplementation of corticosterone [44]. Thus, response of PKA signaling may depend upon the balance between HPA and HPG axes. There is a limitation in this study that the nature of interaction between the administered steroid and the dysregulations of the noradrenergic system has not been examined. It could be either a non-genomic effect of the administered steroids or the classical method (genomic effect) of action [45]. Moreover, previous reports on selective activation of α2-adrenoceptor have shown the genomic effect of testosterone in upregulating Adra2a mRNA in the cortical region of adult Sprague-Dawley rats [46]. Last but not least, it also seems important to mention that although stress conditions were exactly the same for T, GDX, and control rats, dysregulations in the noradrenergic neurotransmission can occur independently from testosterone administration. Stress might cause impairment in PFC function by activating the adrenergic system via alpha-1 adrenergic receptors [47] and/or activating cAMP second messenger system, which may eventually result in the activation of downstream PKA signaling pathway.

In conclusion, from a behavioral point of view, we found that chronic, excessive doses of testosterone increase impulsivity in rats. These results also validate previous findings in clinical populations where increased testosterone levels are associated with increased impulsivity and aggression [48]. From a molecular standpoint, it was found that administration of supraphysiological doses of testosterone increased expression of *Adra2a* gene transcript and associated PKA signaling at the level of specific catalytic and regulatory subunits in the PFC. Thus, our study provides important information of the interplay between testosterone and impulsivity and possible neurobiological mechanisms associated with these interactions.

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Compliance with ethical standards

Conflict of Interest The authors declare that they have no competing interests.

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