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# Effect of adolescent androgen manipulation on psychosis-like behaviour in adulthood in BDNF heterozygous and control mice

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ABSTRACT

*Rationale:* Males are more prone to psychosis, schizophrenia and substance abuse and addiction in adolescence and early adulthood than females. However, the role of androgens during this developmental period is poorly understood.
*Objectives:* This study aimed to examine how androgens in adolescence influence psychosis-like behaviour in adulthood and whether brain-derived neurotrophic factor (BDNF) is a mediator of these developmental effects.
*Methods:* Wild-type and BDNF heterozygous male mice were castrated at pre-pubescence and implanted with testosterone or dihydrotestosterone (DHT). In adulthood, we assessed amphetamine- and MK-801-induced hyperlocomotion as a model of psychosis-like behaviour. Western blot analysis was used to quantify levels of the dopamine transporter (DAT) and *N*-methyl-D-aspartate (NMDA) receptor subunits.
*Results:* While castration itself had little effect on behaviour, adolescent testosterone, but not DHT, significantly reduced amphetamine-induced hyperlocomotion, whereas both testosterone and DHT reduced the effect of MK-801. These effects were similar in mice of either genotype. In wildtype mice, both testosterone and DHT treatment reduced DAT expression in the medial prefrontal cortex (mPFC) but these effects were absent in BDNF heterozygous mice. There were no effects on NMDA receptor subunit levels.
*Conclusions:* The differential effect of adolescent testosterone and DHT on amphetamine-induced hyperlocomotion in adulthood suggests involvement of conversion of testosterone to estrogen and subsequent modulation of dopaminergic signalling. In contrast, the similar effect of testosterone and DHT treatment on NMDA receptor-mediated hyperlocomotion indicates it is mediated by androgen receptors. The involvement of BDNF in these hormone effects remains to be elucidated. These results demonstrate that, during adolescence, androgens significantly influence key pathways related to various mental illnesses prevalent in adolescence.

## 1. Introduction

Adolescence and/young adulthood is the peak onset period of several mental illnesses such as psychosis (Stevens et al., 2014), schizophrenia (Hafner, 2003), substance abuse and addiction (Jordan and Andersen, 2017). During this transitional period, sex hormone levels surge to impart dynamic and often sex-specific neurodevelopmental effects on brain development (Schulz et al., 2009). Hence, abnormal testosterone and estradiol levels, the major male and female sex hormones, are purported to contribute to these disorders. Interestingly, there are gender disparities in the presentation of these disorders, with overall male biases in the rate of onset and severity of psychosis (Pang et al., 2016; Spauwen et al., 2003), schizophrenia (Hafner et al., 1993; McGrath et al., 2004; Szymanski et al., 1995) and substance abuse and addiction (Cotto et al., 2010; Lopez-Quintero et al., 2011). The mechanism of how testosterone might influence this sex bias during adolescence in relation to these disorders is not fully elucidated.

Available evidence suggests that abnormal testosterone expression may modulate these disorders. Adolescent boys with prodromal symptoms of schizophrenia show reduced salivary testosterone levels (van Rijn et al., 2011). In schizophrenia patients, reduced testosterone levels correlate with reduced cognitive performance (Moore et al., 2013), and

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increased negative symptom severity (Akhondzadeh et al., 2006; Sisek-Sprem et al., 2015), while testosterone was also reported to be reduced in newly diagnosed men with non-affective psychosis (Fernandez-Egea et al., 2011). On the other hand, a recent study reported an association between earlier age of puberty, as measured by increases in testosterone, with increased risk of substance use (Marceau et al., 2018). A twin study found higher salivary testosterone in adolescent boys to be correlated with increased alcohol use and diagnosis of alcohol dependence (Eriksson et al., 2005). A recent study further showed that higher salivary testosterone levels in adolescence predicted higher alcohol use 2 years later (Braams et al., 2016). These findings suggest abnormal testosterone in either direction during adolescence can be detrimental and may play a part in mediating various mental illnesses.

Anomalies in both the dopaminergic and the glutamatergic neurotransmitter systems are firmly implicated in the pathophysiology of psychosis and schizophrenia and are replicated in various rodent models (Howes et al., 2015; Tost et al., 2010; van den Buuse, 2010). Likewise, both systems are central to the neurobiology of addiction (Adinoff, 2004). Testosterone can influence both pathways in their development and function (Howes et al., 2015). Abnormally elevated dopamine signalling has long been a major hypothesis in the pathophysiology of schizophrenia, particularly psychosis (Brisch et al., 2014). In rats, reduced pre-pubertal exposure to testosterone resulted in enhanced amphetamine-induced hyperlocomotion in adulthood (Purves-Tyson et al., 2015b). Also in rats, exposure to increased levels of testosterone sensitised animals to fenproporex, an amphetamine class drug, and cocaine, only during adolescence but not adulthood (Conceicao et al., 2017; Engi et al., 2015). These findings suggest an important developmental role of testosterone during adolescence that modulates sensitivity to dopamine signalling in adulthood. However, the extent of androgen's influence and the signalling pathways involved warrant further investigation.

Reduced glutamatergic signalling, especially through the *N*-methyl-D-aspartate (NMDA) receptor, contributes to schizophrenia. Plasma glutamate levels are reduced in patients compared to healthy controls (Palomino et al., 2007). NMDA receptor subunit mRNA expression is also reduced in post-mortem schizophrenia patient brains, in proportion to the severity of cognitive deficits (Humphries et al., 1996). Application of NMDA receptor antagonists, like ketamine and MK-801, as well as mouse models with genetically compromised NMDA receptor function, both result in behavioural changes analogous to schizophrenia such as psychosis (Krystal et al., 1994; Mohn et al., 1999). Loss of glutamatergic homeostasis is also a key feature of addiction (Kalivas, 2009). In adulthood, the non-aromatisable androgen dihydrotestosterone (DHT), increased NMDA receptor binding in the hippocampus of male rats (Romeo et al., 2005). However, testosterone's effects on the glutamatergic system during adolescence are not clear. Taken together, effects of androgens on the development of the dopaminergic and glutamatergic systems could underlie sex differences in disease manifestation and thus a better understanding of this relationship may uncover important disease mechanisms and treatment targets.

An important intermediary of testosterone's effects is brain-derived neurotrophic factor (BDNF). Levels of testosterone during adolescence have been shown to correlate with the expression of BDNF and its receptor, tropomyosin receptor kinase B (TrkB), in the frontal cortex of rats, monkeys (Purves-Tyson et al., 2015a) and mice (Hill et al., 2012). Furthermore, in rats and monkeys, BDNF has been shown to mediate the pro-proliferative effects of testosterone (Allen et al., 2015). Reduced BDNF is a hallmark of schizophrenia and its roles pervade multiple aspects of schizophrenia (Jindal et al., 2010; Weickert et al., 2003). Blood BDNF has also been found to be significantly reduced in people with alcohol use disorder (Garcia-Marchena et al., 2017); in methamphetamine abusers (Chen et al., 2014); and in adolescents diagnosed with crack-cocaine dependence (Pianca et al., 2017). BDNF aids the development of the dopamine system and the expression of dopamine receptors (Guillin et al., 2007). Conversely, abnormally increased dopamine in the frontal cortex of dopamine transporter (DAT) knockout mice resulted in a persistent reduction of BDNF gene expression from an early age, which may impair neurodevelopment and synaptic plasticity (Fumagalli et al., 2003). BDNF also drives NMDA receptor expression (Caldeira et al., 2007; Kim et al., 2012) and induces receptor subunit phosphorylation (Lin et al., 1998). In a feed-forward fashion, NMDA receptor activation is necessary in mediating activity-dependent BDNF secretion (Fumagalli et al., 2003; Park et al., 2014). Consequently, blocking the NMDA receptor induced loss of BDNF gene expression in primate and rodents (Elsworth et al., 2014; Katanuma et al., 2014). These findings posit BDNF as an important intermediary of androgen influences on the dopaminergic and glutamatergic neurotransmitter systems. How it interacts with androgens during adolescent brain development remains to be elucidated.

Here, we aim to clarify the relationship between androgen signalling, BDNF and the two neurotransmitter systems. We hypothesise that abnormal fluctuations in testosterone during adolescence can contribute to abnormal development of the dopaminergic and glutamatergic systems. This effect may be mediated by BDNF. Hence, reduced BDNF in the BDNF Het mice will render them more vulnerable to the effects of preadolescent gonadectomy, sensitising the dopaminergic and glutamatergic systems to the psychosis-like effects of amphetamine and MK-801, compared to wild-type mice. To this end, we used both wild-type (WT) and BDNF heterozygous (Het) male mice to examine the effects of hormone manipulation, through pre-pubescent castration and hormone implants (testosterone or DHT), on adult functioning of the dopaminergic and glutamatergic signalling pathways. We chose to compare testosterone and DHT to differentiate pure androgen effect of DHT, which cannot be aromatised into estrogen, from testosterone, which can be aromatised to estrogen. We challenged the animals with the dopamine-releaser, amphetamine, and the NMDA receptor antagonist, MK-801, respectively and measured the resulting locomotor hyperactivity as a measure of psychosis-like behaviour. The BDNF Het mouse has one copy of the BDNF gene deleted from its genome, reducing BDNF mRNA expression (Ernfors et al., 1994; Linnarsson et al., 1997). In our colony of BDNF Het mice, we have consistently shown significant reductions in the protein expression of mature BDNF in multiple brain regions including the hippocampus, frontal cortex and the striatum, compared to WT mice (Du et al., 2018; Hill and van den Buuse, 2011; Klug et al., 2012; Wu et al., 2015). Finally, we examined the protein expression of the dopamine transporter (DAT) and NMDA receptors in the medial prefrontal cortex (mPFC) and caudate putamen (CPu) – brain regions involved in the regulation of psychotropic drug-induced hyperlocomotion, to analyse molecular mechanisms involved.

## 2. Materials and methods

### 2.1. Animals

Male WT and BDNF Het mice on a C57Bl/6 background were derived from a breeding colony at the Florey Institute of Neuroscience and Mental Health. Genotyping was performed with tail tip tissue by Transnetyx, INC (Cordova, TN, USA). The probes used are Forward: GGGCGCCCGGTTCTT; Reverse: CCTCGTCCTGCAGTTCATTCA for the neomycin, and Forward: GATGCCGCAAACATGTCTATGAG; Reverse: CCACTCGCTAATACTGTCACACA for the wild-type BDNF gene. Mice were group-housed in individually-ventilated cages (IVC, Tecniplast, Italy) with a 12 h/12 h light/dark cycle and ad libitum access to water and chow. All experimental procedures were approved by the Animal Experimentation Ethics Committee of the Florey Institute of Neuroscience and Mental Health, University of Melbourne, Victoria, Australia.

### 2.2. Surgical techniques

All surgeries were performed between 9 am and 12 pm. Mice were

either sham-operated or castrated (Cast) at pre-pubescence (5–6 weeks), before the sharp rise in testosterone as previously established (Hill et al., 2012), and were simultaneously implanted with 2 cm of silastic tubing filled with testosterone (~35 mg, Sigma-Aldrich, St Louis, MO, USA), DHT (~35 mg, Sigma-Aldrich) or received placebo treatment (empty implant). Castrations were performed as previously described (Hill et al., 2012). Briefly, mice were anaesthetised with an isoflurane/oxygen mixture and were given an injection of the non-steroidal analgesic, carprofen (5 mg/kg, i.p., Rimadyl®), and of the antibiotic, enrofloxacin (5 mg/kg, s.c., Baytril®). Through a single incision on the scrotum, the testes were ligated and removed. The hormone implants were inserted subcutaneously through a small incision at the nape of the neck. Incisions were suture-closed and betadine ointment and Tricin® triple antibacterial powder were applied on the incisions. Mice were individually placed in recovery chambers maintained at 30 °C until fully conscious and moving freely before being returned to their home cages. Sham control surgeries were identical to castration surgeries except testes were left intact.

### 2.3. Locomotor activity

When the mice were 10 weeks of age, psychotropic drug-induced locomotor hyperactivity was analysed (Seamless Open Field Arena, Med Associates, Inc. Fairfax, VT, USA). Each mouse received three 3-hr sessions. Each session involved one hour of habituation followed by injection of either amphetamine (Amph) (3 mg/kg), MK-801 (0.15 mg/kg) or vehicle before being put back in the cell for a further 2 h. Each session was separated by at least 48 h to allow drug washout. The order in which the mice received the drugs was pseudo-randomised. Locomotor distance moved was averaged every 5-minute period.

### 2.4. Brain dissections

Following behavioural testing, mice were killed by cervical dislocation at 14 weeks of age (at least 48 h after the final session) between 1200 h and 1700 h and their brains were immediately frozen on dry ice and stored at −80 °C for further analysis. Collected brains (7/group) were then micro-dissected into multiple regions using a mouse brain mould. The brains, which were stored at −80 °C, were placed inverted on the cold mouse brain mould (~−8 °C). Three 2 mm coronal slices were taken from the forebrain. The second slice (Bregma 3.08–1.18 mm) contained the mPFC, including the cingulate cortex, infralimbic cortex and prelimbic cortex. From the third slice (Bregma 1.18- -0.22 mm), the CPu was dissected out and snap-frozen.

### 2.5. Protein extraction and Western blot analysis

Protein extraction and Western blot analysis were performed as described previously (Wu et al., 2014). Primary antibodies were rabbit anti-NMDAR1 antibody (Cell Signalling 4204), rabbit anti-NMDAR2A antibody (Cell Signalling 4205S), rabbit anti-NMDAR2B antibody (Abcam ab110), rat anti-DAT antibody (Millipore MAB369), mouse anti-α-tubulin antibody (Abcam ab7291) and mouse anti-β-actin (Sigma-Aldrich A2228). Secondary antibodies were anti-mouse HRP-conjugate (Cell Signalling, Daners, MA, USA), anti-rabbit HRP-conjugate (Cell Signalling) and anti-rat HRP-conjugate (Merck Millipore).

### 2.6. Statistical analysis

Organ weights were analysed with GraphPad Prism® 7 for Windows (Graphpad Software). Two-way analysis of variance (ANOVA) was performed with genotype (WT and BDNF Het) and hormone conditions (sham, Cast + placebo, Cast + testosterone, Cast + DHT) as main factors. If significant main effects or interactions were found, post-hoc multiple comparisons were performed. Brain weights were not corrected by bodyweight due to the elevated bodyweight of the BDNF Het mice. We are adequately numbered to detect medium sized effects (f = 0.25) with a power of 0.71 (G*Power 3.1.9.2).

Psychotropic drug-induced hyperlocomotion time-curves were analysed using SPSS® Statistics version 24 (IBM). Repeated-measures ANOVA was performed with Time and either amphetamine (Amph) or MK-801 vs. vehicle as repeated factors and the hormone conditions (Sham, Cast + placebo, Cast + testosterone, Cast + DHT) and genotype (WT and BDNF Het) as between-subject factors. For the amphetamine-treated group, the data for the 2 h post injection were analysed. For the MK-801-treated group, due to the shorter drug effect, only the first hour post-injection was analysed. The Greenhouse-Geisser correction was used to adjust for violation of sphericity. Main effects of time or interactions involving time are not reported here unless they include drug, genotype or hormone treatment effects. Relevant significant main effects and interactions were further explored by post-hoc comparisons with Bonferroni corrections.

Protein expression was analysed with Prism® 7 for Windows. Two-way ANOVA was performed with genotype (WT and BDNF Het) and hormone condition as main factors. If significant effects or an interaction were found, post-hoc multiple comparisons were performed. We have adequate number to detect medium sized effects (f = 0.25) with a power of 0.82 (G*Power 3.9.2). Statistical significance was set at P = 0.05.

## 3. Results

### 3.1. Body and organ weights

Two-way ANOVA of body weight showed significant effects of hormone condition ($F_{(3,79)} = 2.93$, $p = 0.039$, $\eta_p^2 = 0.068$) and genotype ($F_{(1,79)} = 35.7$, $p < 0.0001$, $\eta_p^2 = 0.277$), reflecting higher body weights in BDNF Het mice than in WT, but no hormone condition × genotype interactions (Fig. 1A). While post-hoc test revealed no significant differences between hormone condition groups, there was a non-significant trend for the Cast DHT group to have increased weight compared to the Cast placebo group (p = 0.077).

Examination of seminal vesicle weights (Fig. 1B) revealed a significant main effect for hormone condition ($F_{(3,\ 79)} = 210.9$, $p < 0.0001$, $\eta_p^2 = 0.884$) but no significant genotype effect or hormone condition × genotype interaction. Post-hoc tests show that the seminal vesicle weight in the Cast placebo group was significantly lower than the sham placebo group as expected ($p < 0.0001$, $d = 0.267$). Both testosterone and DHT implants in castrated mice resulted in upregulated seminal vesicle weight compared to sham placebo ($p < 0.0001$, $d = 0.178$ and $p = 0.008$, $d = 0.059$ respectively) and Cast placebo mice (testosterone $p < 0.0001$, $d = 0.445$; DHT $p < 0.0001$, $d = 0.326$). In addition, mice implanted with testosterone had heavier seminal vesicles than mice in the DHT implanted group ($p < 0.0001$, $d = 0.119$).

A significant main effect for genotype was found when comparing brain wet weight, expressed as percentage of bodyweight ($F_{(1,78)} = 50.32$, $p < 0.001$, $\eta_p^2 = 0.344$) with BDNF Het mice showing lower overall brain weights (Fig. 1C). There was also a significant main effect of hormone condition ($F_{(3,\ 78)} = 4.853$, $p = 0.0038$, $\eta_p^2 = 0.099$). No significant interaction was seen.

### 3.2. Locomotor hyperactivity

#### 3.2.1. Locomotor hyperactivity – Amph challenge

Investigating total distance travelled in the two hours following injection and comparing all four hormone conditions, revealed a significant main effect of Amph ($F_{(1,80)} = 282.45$, $p < 0.001$, $\eta_p^2 = 0.779$) and an Amph × time interaction ($F_{(4.04,322.97)} = 88.13$, $p < 0.001$, $\eta_p^2 = 0.822$), reflecting the expected increase in ambulation by Amph treatment compared to saline. There were no Amph × genotype or Amph × time × genotype interactions, indicating

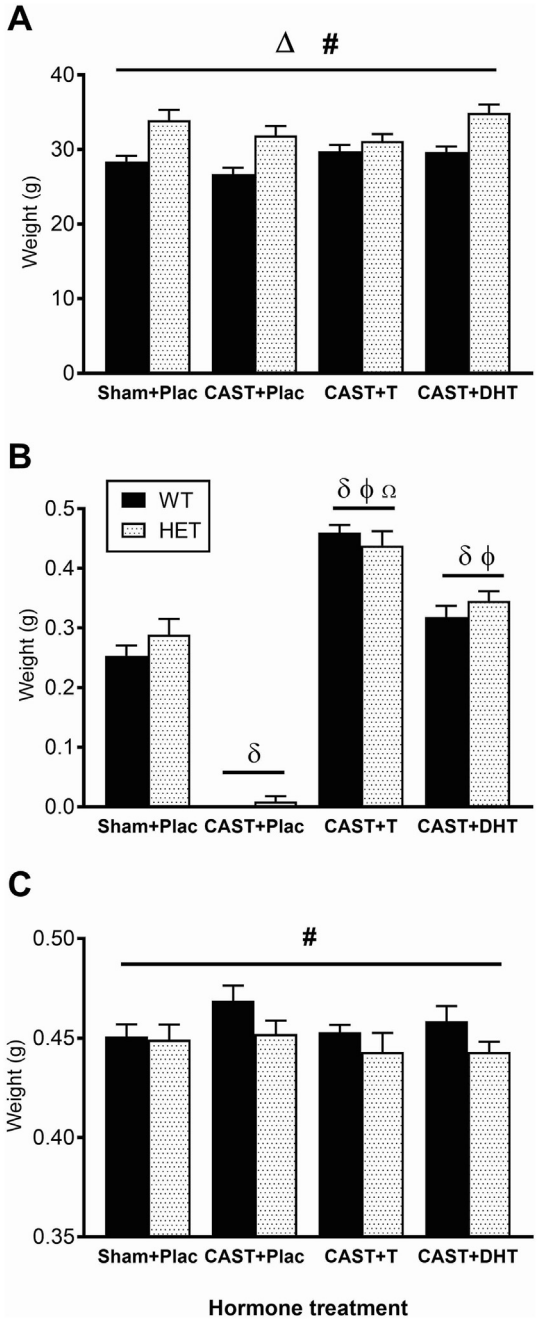



**Fig. 1.** Body, seminal vesicle and brain weights of mice. (A) BDNF Het mice had increased bodyweight compared to WT mice. There was also a main effect of hormone treatment on body weight although post-hoc analysis failed to show specific treatment effects. (B) Seminal vesicle weights did not differ between genotypes but there were significant effects according to hormone condition, with castration greatly reducing seminal vesicle weight and both testosterone (T) and DHT implants re-establishing this weight. Mice implanted with testosterone displayed significantly higher seminal vesicle weights than both sham-operated placebo-implanted mice and mice implanted with DHT. (C) Main effects of genotype and hormone treatment were observed for brain weight with BDNF Het mice exhibiting overall lower brain weights as proportion of bodyweight than WT control mice. N = 10–12/group, # main effect of genotype, Δ main effect of hormone treatment, δ significant difference vs. sham + placebo group, ϕ significant difference vs. Cast + placebo group, Ω significant difference vs. Cast + DHT group.

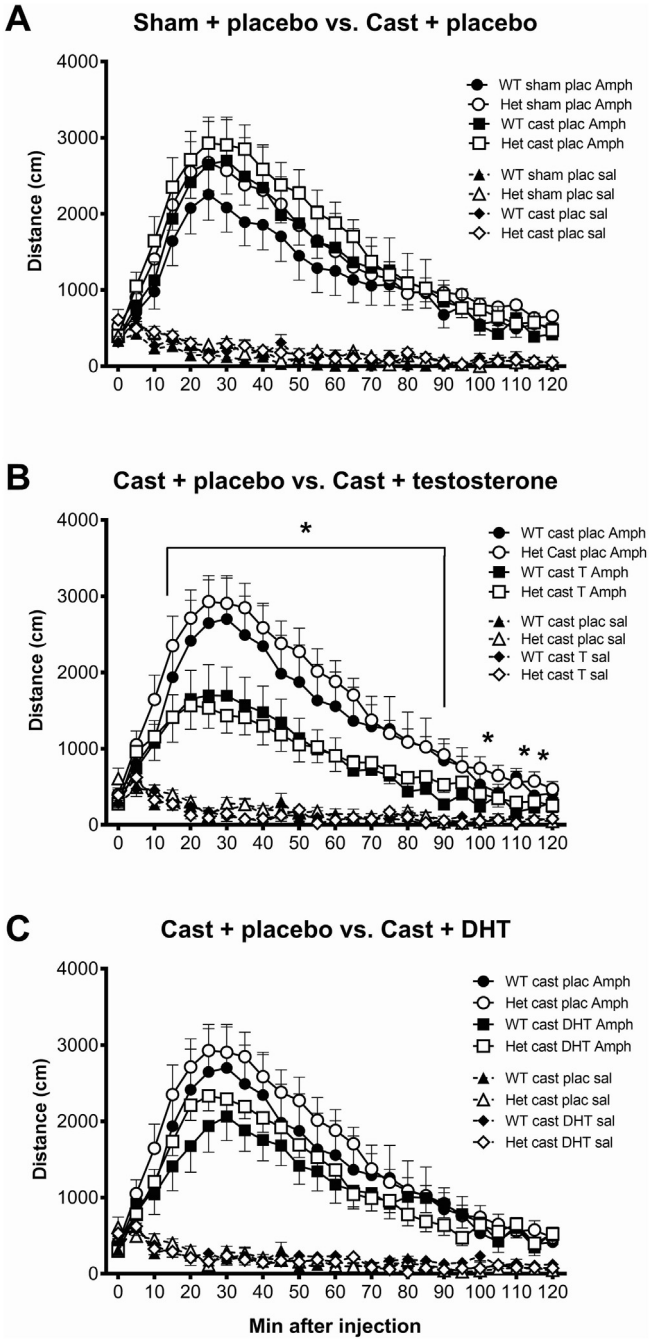



**Fig. 2.** Amphetamine-induced hyperlocomotion. (A) Hyperlocomotion induced by amphetamine (Amph) compared to saline (sal) was not different between sham-operated placebo (plac)-treated and castrated (Cast) placebo-treated mice across time. (B) Testosterone (T) treatment significantly reduced Amph-induced hyperlocomotion from 15 to 90 min post injection. (C) There was no significant effect of DHT on Amph-induced hyperlocomotion. There were no differences between BDNF Het mice and WT controls. N = 10–12 per group, *$p < 0.05$.

that BDNF Het mice responded to Amph similarly to WT mice. There was an Amph × hormone condition interaction ($F_{(3,80)} = 4.36$, $p = 0.007$, $\eta_p^2 = 0.14$), indicating modulation of the Amph response by the hormone condition of animals. This was further interrogated by pairwise ANOVAs.

Comparing placebo-treated sham vs. Cast animals (Fig. 2A), in addition to the expected main effect of Amph ($F_{(1,40)} = 137.11$, $p < 0.001$, $\eta_p^2 = 0.774$) and Amph × time interaction ($F_{(3.76, 150.24)} = 49.87$, $p < 0.001$, $\eta_p^2 = 0.555$), the analysis revealed that there was no Amph × Cast interaction, indicating castration having little effect on the animals' response to Amph. Again there were no effects of genotype or Amph × genotype interaction, reflecting similar

effects in WT and BDNF Het mice.

Comparing Cast + placebo vs. Cast + testosterone mice (Fig. 2B), in addition to the significant main effect of Amph ($F_{(1,38)} = 104.69$, $p < 0.001$, $\eta_p^2 = 0.734$) and Amph × time interaction ($F_{(3.371, 128.10)} = 39.60$, $p < 0.001$, $\eta_p^2 = 0.51$), there were significant Amph × testosterone ($F_{(1,38)} = 9.73$, $p = 0.003$, $\eta_p^2 = 0.204$) and Amph × time × testosterone interactions ($F_{(3.37,128.10)} = 3.38$, $p = 0.016$, $\eta_p^2 = 0.082$), reflecting a marked reduction of the response to Amph by testosterone. Post-hoc testing comparing Amph-treated Cast placebo vs. Cast + testosterone groups revealed significant differences from 15 to 90 min post-injection and at 100 min and 110-115 min post injection ($p < 0.05$). The lack of Amph × genotype and Amph × genotype × testosterone interactions indicates that there were no differences between the genotypes.

Comparing Cast vs Cast + DHT mice (Fig. 2C), again there were significant main effects of Amph ($F_{(1,40)} = 150.52$, $p < 0.001$, $\eta_p^2 = 0.79$) and an Amph × time interaction ($F_{(3.97, 158.67)} = 52.73$, $p < 0.001$, $\eta_p^2 = 0.569$). There was no significant interaction between Amph × DHT, nor a significant Amph × time × DHT interaction, suggesting that DHT did not significantly reduce the Amph response. The lack of Amph × genotype, or Amph × genotype × DHT interactions indicated that there were no differences between the genotypes.

The differential effect of testosterone and DHT on the Amph response was further analysed by comparing Cast + testosterone mice and Cast + DHT mice. In addition to the expected main effect of Amph ($F_{(1,40)} = 169.41$, $p < 0.001$, $\eta_p^2 = 0.809$) and Amph × time interaction ($F_{(4.034, 161.37)} = 38.54$, $p < 0.001$, $\eta_p^2 = 0.491$), there was an Amph × hormone condition interaction ($F_{(1,40)} = 5.26$, $p = 0.027$, $\eta_p^2 = 0.116$), suggesting the Amph response was significantly smaller in testosterone-treated mice compared to DHT-treated mice. Again this was independent of BDNF genotype (Fig. 2B and C).

### 3.2.2. Locomotor hyperactivity – MK-801 challenge

Examining the total distance travelled in the first hour following injection and comparing all four hormone conditions revealed a significant main effect of MK-801 ($F_{(1,80)} = 47.14$, $p < 0.001$, $\eta_p^2 = 0.371$) and an MK-801 × time interaction ($F_{(5.02, 401.63)} = 33.75$, $p < 0.001$, $\eta_p^2 = 0.612$), reflecting that MK-801 induces hyperlocomotion. The lack of MK-801 × genotype or MK-801 × time × genotype interaction suggested that WT and BDNF Het mice responded similarly to MK-801. In contrast, significant MK-801 × hormone condition ($F_{(3,80)} = 3.69$, $p = 0.015$, $\eta_p^2 = 0.122$) and MK-801 × time × hormone condition interactions ($F_{(15.06, 401.63)} = 2.64$, $p = 0.001$, $\eta_p^2 = 0.09$) suggested effects of hormone status on the MK-801 response. This was further analysed using pairwise ANOVAs.

Comparing sham vs. Cast mice (Fig. 3A), there were only a main effect of MK-801 ($F_{(1,40)} = 38.04$, $p < 0.001$, $\eta_p^2 = 0.487$) and an MK-801 × time interaction ($F_{(4.37, 174.67)} = 24.77$, $p < 0.001$, $\eta_p^2 = 0.382$), but no MK-801 × Cast interaction. There was an MK-801 × time × Cast interaction ($F_{(4.37, 174.67)} = 2.43$, $p = 0.044$, $\eta_p^2 = 0.057$), suggesting some effect of castration on the MK-801 response, but post-hoc analysis did not find any differences between the groups. There was also a lack of MK-801 × genotype and MK-801 × genotype × Cast interaction, pointing to similar responses in WT and BDNF Het mice.

Comparing Cast vs Cast + testosterone mice (Fig. 3B) again revealed a significant main effect of MK-801 ($F_{(1,38)} = 17.45$, $p = 0.001$, $\eta_p^2 = 0.315$) and a MK-801 × time interaction ($F_{(3.75, 142.60)} = 16.21$, $p < 0.001$, $\eta_p^2 = 0.299$). Significant MK-801 × testosterone ($F_{(1,38)} = 5.09$, $p = 0.030$, $\eta_p^2 = 0.118$) and MK-801 × Time × testosterone interactions ($F_{(3.75, 142.60)} = 2.99$, $p = 0.023$, $\eta_p^2 = 0.073$), signified that the MK-801 response was suppressed in testosterone-treated mice. Post-hoc analysis comparing Cast placebo vs. Cast + testosterone groups revealed significant differences from 15 to 45 min post injection ($p < 0.05$). There were no MK-801 × genotype, MK-801 × genotype × testosterone, nor MK-801 × Time × testosterone interactions, pointing to a lack of genotype difference.

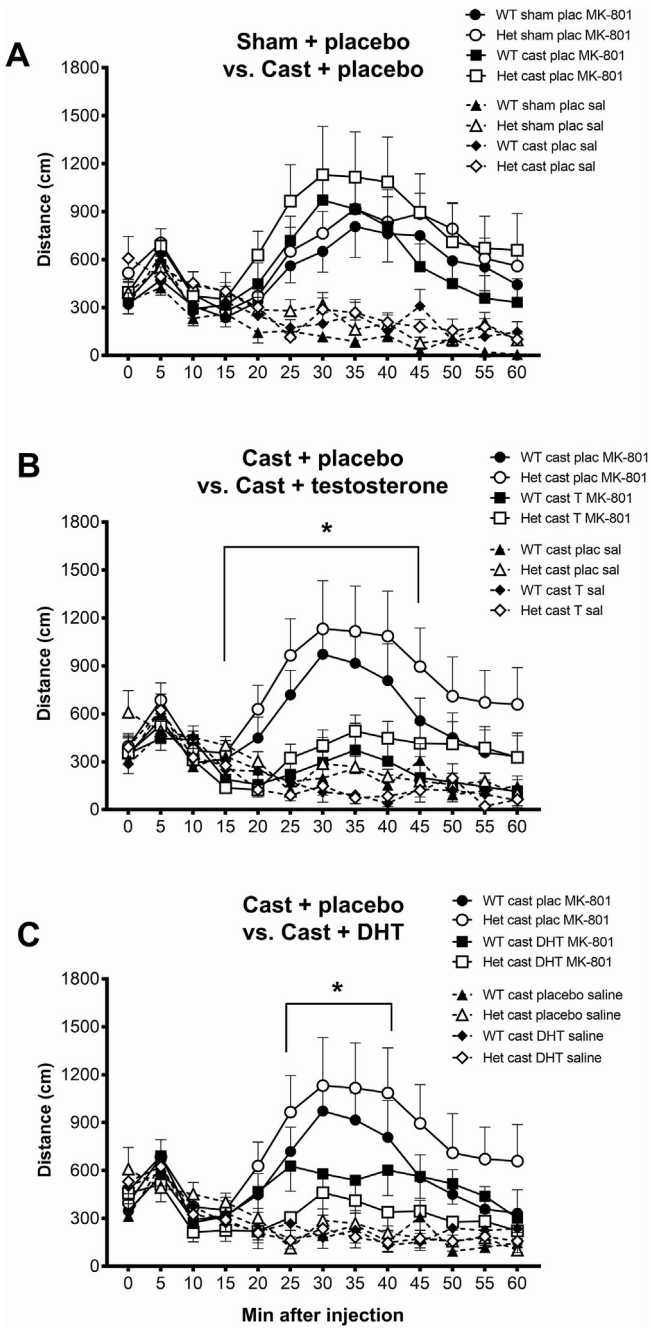



**Fig. 3.** MK-801-induced hyperlocomotion. (A) Hyperlocomotion induced by MK-801 was not different between sham-operated placebo (plac)-treated mice and castrated (Cast) placebo-treated mice across time. (B) Testosterone (T) treatment significantly reduced hyperlocomotion in response to MK-801 from 15 to 45 min post injection. (C) DHT significantly reduced MK-801-induced hyperambulation from 25 to 40 min post-injection. There were no differences between BDNF Het mice and WT controls. N = 10–12 per group. *$p < 0.05$.

Comparing Cast vs Cast + DHT mice (Fig. 3C), again revealed a significant main effect of MK-801 ($F_{(1,40)} = 21.13$, $p < 0.001$, $\eta_p^2 = 0.346$) and MK-801 × time interaction ($F_{(5.34, 213.46)} = 17.66$, $p < 0.001$, $\eta_p^2 = 0.306$). There were significant MK-801 × DHT ($F_{(1,40)} = 4.28$, $p = 0.045$, $\eta_p^2 = 0.097$) and MK-801 × time × DHT interactions ($F_{(5.34, 213.46)} = 2.81$, $p = 0.015$, $\eta_p^2 = 0.066$), indicating that DHT is able to moderate the MK-801 response. Post-hoc analysis showed that DHT treatment reduced MK-801-induced hyperactivity at 25–40 min post injection ($p < 0.05$). There were no statistical

interactions with genotype, again highlighting a lack of genotype difference.

Comparison of the Cast + testosterone and Cast + DHT groups only revealed a main effect of MK-801 ($F_{(1,40)} = 9.82$, $p = 0.003$, $\eta_p^2 = 0.197$) and an MK-801 × Time interaction ($F_{(5.38,\ 215.05)} = 9.59$, $p < 0.001$, $\eta_p^2 = 0.193$). The lack of any interaction with hormone condition suggested the Amph response was similar in testosterone and DHT-treated mice (Fig. 3B and C).

### 3.3. Molecular analysis

#### 3.3.1. DAT

In the mPFC, analysis of DAT expression found no significant effect of genotype, a trend for an effect of treatment ($F_{(3,\ 38)} = 2.62$, $p = 0.065$) and a significant genotype × treatment interaction ($F_{(3,\ 38)} = 3.10$, $p = 0.038$, $\eta_p^2 = 0.169$). In WT mice, despite a ~35% increase in DAT expression in WT Cast placebo mice vs. WT sham placebo mice, post-hoc analysis detected no significant difference between these groups. In contrast, the Cast testosterone ($p = 0.004$, $d = 0.046$) and the Cast DHT ($p = 0.034$, $d = 0.034$) treated mice had significantly lower expression of DAT protein compared to the WT Cast placebo mice. Notably, in BDNF Het mice, neither castration nor hormone treatments had any effect on DAT expression (Fig. 4A). Overall it appears that WT mice responded to castration by increasing DAT expression, and both testosterone and DHT treatment recovered this effect. However, this effect of hormone manipulation on mPFC DAT expression was absent in BDNF Het mice.

In the CPu, analysis of DAT expression revealed no significant effects of genotype or treatment. There was also no genotype × treatment interaction (Fig. 4B).

#### 3.3.2. NMDA receptor subunits

Expression of the NMDA receptor subunit 1 (GluN1) in the mPFC was not different between genotypes. There was no significant main effect of treatment nor significant genotype × treatment interaction (Fig. 4C). In the CPu, once again expression levels found to be comparable between genotypes. There was no significant effect of treatment nor a genotype × treatment interaction (Fig. 4D).

There was no significant main effect of treatment or genotype and there was no significant genotype × treatment interaction for NMDA receptor subunit 2A (GluN2A) expression in the mPFC (Fig. 4E). In the CPu, while there was an overall genotype effect ($F_{(1,31)} = 4.42$, $p = 0.044$, $\eta_p^2 = 0.114$), with expression of GluN2A higher in the BDNF Het mice compared to WT controls, there was no main effect of treatment nor a genotype × treatment interaction (Fig. 4F).

There were no significant group differences in GluN2B expression in the mPFC (Fig. 4G) or CPu (Fig. 4H).

## 4. Discussion

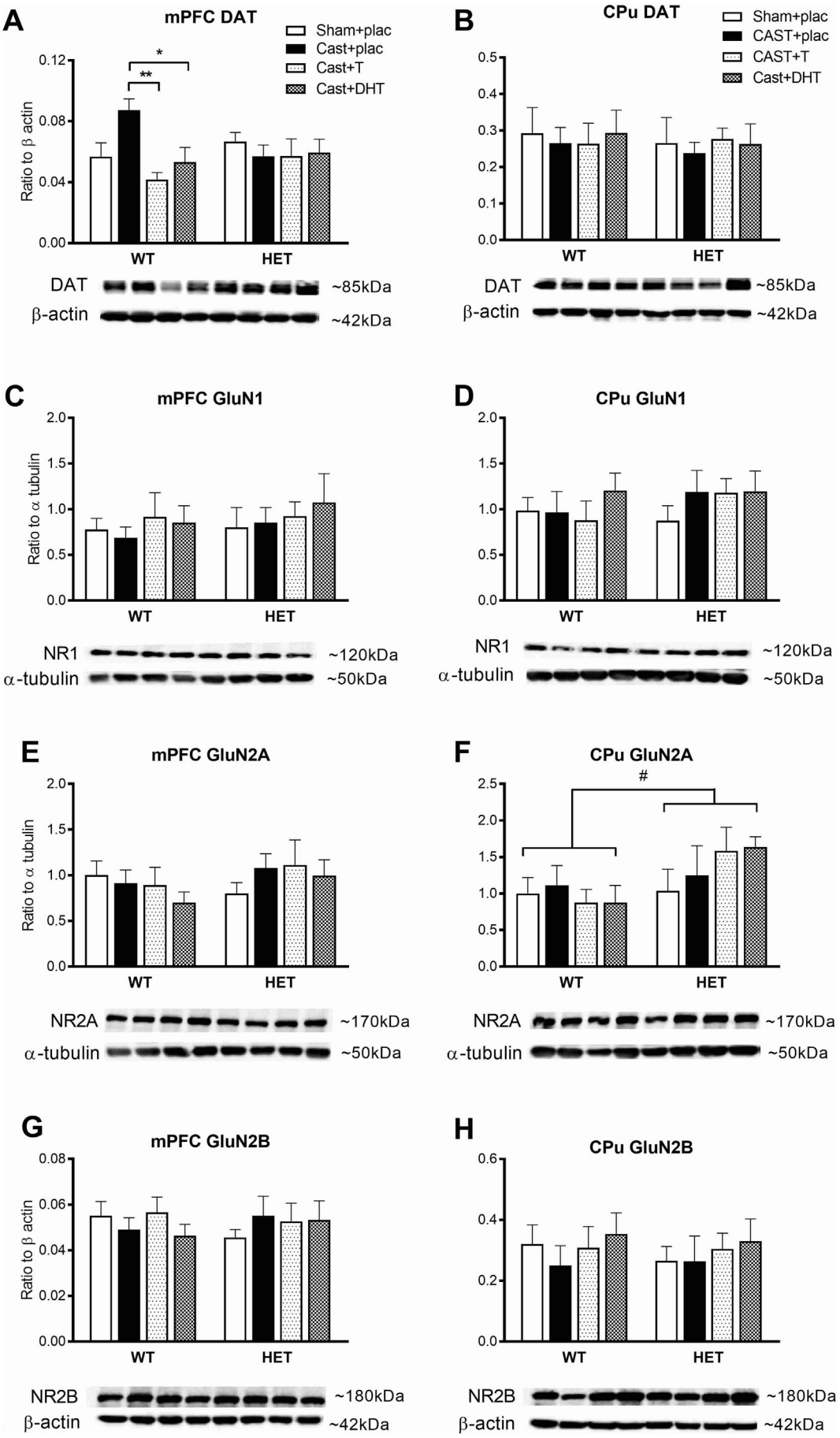
The role of androgens in male-biased disorders such as psychosis, schizophrenia, substance abuse and addiction, particularly during the vulnerable adolescent phase, is not well established. Testing two key neurotransmitter systems relevant to these disorders, our main finding is that high levels of androgens during adolescent development significantly dampen both amphetamine and MK-801-induced hyperlocomotor activity in adulthood – suggesting that loss of male sex hormones during this developmental period may sensitize the dopaminergic and glutamatergic pathways. Importantly, while testosterone significantly attenuated amphetamine-induced locomotor hyperactivity, DHT had no such effect. However, both testosterone and DHT attenuated MK-801-induced hyperlocomotor activity. BDNF heterozygosity did not interact with the effect of hormone treatments on adult behaviour.

While both testosterone and DHT supplements recovered SV weight in response to castration independent of genotype, testosterone resulted in higher SV weights than sham-operated mice, suggestive of supraphysiological levels. Given the higher affinity of DHT to androgen receptors compared to testosterone (Grino et al., 1990; Toth and Zakar, 1982), the higher SV weight in testosterone treated mice may reflect an additional influence of estradiol (converted from testosterone), which can synergistically increase SV weight with testosterone (Baines et al., 2005; Jackson et al., 1977; Li et al., 1989). Additionally, BDNF Het mice exhibited elevated body weight compared to WT as previously described (Klug and van den Buuse, 2013), as well as lower relative brain weights. As far as we know, this is the first report of reduced brain weight in BDNF Het mice compared to WT.

The behavioural data is summarised in Table 1. Pre-pubescent castration did not significantly change amphetamine-induced locomotor hyperactivity in adulthood. As we expected BDNF heterozygosity to sensitize the animals to the effects of amphetamine, in attempt to avoid a ceiling effect, a relatively low dose of the drug was chosen. It is possible that a higher dose may elicit an effect of pre-pubertal castration, as previously seen in rats (Purves-Tyson et al., 2015b). While it is beyond the scope of the current study, a dose curve would assist in the examination of pre-pubertal castration effects. In contrast, peri-adolescent testosterone, but not DHT supplementation inhibited amphetamine-induced locomotor hyperactivity in both WT and BDNF Het mice. The stronger effect of testosterone compared to DHT possibly reflects its ability to activate both androgen receptors and estrogen receptors. However, estraidol has been found to enhance amphetamine-induced locomotor activity in female ovariectomized rats (Forgie and Stewart, 1994), while no effect of ovariectomy was observed on amphetamine-induced locomotor hyperactivity in female mice (van den Buuse et al., 2017a). Likewise in humans, studies have shown estradiol to potentiate several positive subjective effects of d-amphetamine in women such as euphoria and increased energy (Justice and de Wit, 1999, 2000) and that men show higher levels of amphetamine-induced stimulation than women (White et al., 2002). Hence our results may point to specific organisational role of testosterone during male adolescence pertaining its maximal attenuating of amphetamine effects. Further studies assessing the effect of estradiol treatment in male mice or using combined testosterone + aromatase inhibitors such as letrozole, are therefore needed to dissect the precise role of androgen vs. estrogen receptor-mediated effects of adolescent testosterone treatment on amphetamine-induced locomotor hyperactivity in adulthood.

The lack of genotype difference in response to androgen manipulation suggests that this process is largely independent of BDNF level, or alternatively, that BDNF Het mice have developed compensatory mechanisms that may mask adolescent testosterone effects on psychotropic drug-induced locomotor hyperactivity. Further studies using both temporal and regional specific BDNF knockdown models may shed more light on the role of BDNF here.

The mPFC is a region critical in mediating amphetamine-induced hyperlocomotion (Bast et al., 2002; van den Buuse, 2010). In WT mice, androgen implants significantly reduced DAT expression in the mPFC compared to castrated mice. While DAT normally removes dopamine from the synaptic space, amphetamine reverses DAT action, resulting in dopamine efflux into the synaptic cleft (Jones et al., 1998). Mice overexpressing DAT exhibit increased locomotor responses to amphetamine and a 3-fold higher dopamine release compared to control mice (Salahpour et al., 2008). Hence the significant decrease in DAT expression observed in the WT mice after androgen treatment may contribute to the reduced hyperlocomotor response to amphetamine. In comparison, mPFC DAT levels in the BDNF Het mice did not alter in response to androgen manipulation, suggesting that androgen suppression of DAT levels is mediated via BDNF. However, despite this genotype difference at the level of DAT expression, the effect of testosterone on amphetamine-induced hyperactivity was similar in BDNF Het mice compared to WT mice. Here it is important to note that BDNF has the ability to modify several other components of dopaminergic signalling; for example, we have previously shown a significant




*(caption on next page)*

**Fig. 4.** Molecular analyses. (A) DAT protein expression in the mPFC was significantly higher in WT mice after castration compared to WT mice treated with testosterone and DHT. Surprisingly, no changes were observed in the Het animals. (B) In the CPu, DAT expression remained stable across genotype and treatment groups. (C) GluN1 expression did not alter across genotype or in response to hormone manipulations in the mPFC, (D) nor in the CPu. (E) GluN2A expression in the mPFC was comparable across genotype and hormone manipulations. (F) In the CPu, GluN2A protein expression level was significantly higher in BDNF Het mice compared to WT mice, although hormone manipulation did not alter its expression. (G) GluN2B expression was not significantly different between groups in the mPFC. (F) GluN2B expression in the CPu likewise did not significantly differ across genotypes or in response to hormone manipulation. N = 4–5 per group, # signifies genotype effect, * $p < 0.05$, ** $p < 0.01$.

**Table 1**
Summary of effects of hormone manipulation on drug-induced locomotion in comparison to sham castrated group.

| | WT | | Het | |
|---|---|---|---|---|
| | AMPH | MK801 | AMPH | MK801 |
| Castration | ⇔ | ⇔ | ⇔ | ⇔ |
| Testosterone | ⇩ | ⇩ | ⇩ | ⇩ |
| DHT | ⇔ | ⇩ | ⇔ | ⇩ |

correlation between BDNF and Drd2 and Drd3 mRNA expression in the medial prefrontal cortex of male but not female rats (Hill et al., 2014). We have also previously found a significant reduction in tyrosine hydroxylase expression in a humanized $BDNF^{met/met}$ transgenic mouse, which has reduced activity-dependent BDNF release (Notaras et al., 2017). Therefore it is possible that in BDNF Het mice, the lack of testosterone-induced changes in the expression of DAT is offset by other, as yet unknown changes in dopaminergic signalling in the mPFC or by changes in DAT expression in other brain regions, ultimately leading to a similar behavioural response to testosterone treatment in both genotypes. Further studies analysing the effect of adolescent testosterone on dopamine receptor expression and markers of dopamine synthesis, including tyrosine hydroxylase and catechol-*o*-methyl transferase, are needed to elucidate these additional mechanisms. Overall, the data suggest that modifying testosterone levels during adolescent development causes long term alterations in dopaminergic signalling, which attenuate amphetamine-induced hyperlocomotor activity in adulthood but that these molecular changes are different in WT and BDNF Het mice. In contrast to the mPFC, DAT level in the CPu, another region intimately involved with amphetamine responses, was not altered by either genotype or hormone manipulation. A study in rats also reported unaltered DAT expression in the dorsal striatum in response to prepubertal castration and hormone supplements (Purves-Tyson et al., 2014).

Adolescent castration did not alter the behavioural response to MK-801. Similar to the reason stipulated above for amphetamine treatment, a relatively low dose of MK-801 was chosen for this study. Therefore the possibility of a higher dose revealing an effect of castration cannot be overlooked. Both testosterone and DHT implants reduced MK-801-evoked hyperactivity and this influence apparently does not require BDNF. Indeed, a recent study examining the effect of BDNF deficiency (BDNF Het mice) and NMDA receptor hypofunction (MK-801) on evoked neuronal gamma oscillation also concluded that the two systems were not synergistic (Jones et al., 2017). Interestingly, a recent study found that adult castration in male mice decreased MK-801 evoked hyperlocomotion compared to sham-operated mice (van den Buuse et al., 2017b). Contrasting with their finding, our result, with both testosterone and DHT implants decreasing MK-801 evoked hyperlocomotion, highlights the importance of timing on the different responses elicited by adult versus adolescent hormone manipulation. Because in our paradigm the gonadectomy/hormone supplementation extends from pre-pubertal age to adulthood, in order to ensure that the differences between these studies can be attributed solely to hormone influences during adolescence per se, future studies should examine the effect of androgen and estrogen supplementation in adult gonadectomy model.

Moreover, the observation that both testosterone and DHT were equally effective in suppressing MK-801-induced locomotor hyperactivity suggests that this is mediated by an androgen receptor-mediated mechanism. This points to a major difference between the influence of adolescent hormone status on these two neurotransmitter systems with dopamine-mediated effects, triggered by amphetamine challenge, preferentially dampened by testosterone, whereas NMDA receptor-mediated effects, triggered by MK-801, was equally moderated by the two androgens. While testosterone's ability to be aromatised and evoke estrogen signalling is a possible explanation for the difference observed between the two pathways, it needs to be noted that the metabolite of DHT, 5α-androstane-3β, 17β-diol (3β-diol), has been found to selectively activate estrogen receptor β (Handa et al., 2008). To clarify this, future studies may include a testosterone + letrozole group to ensure full elimination of estrogen signalling effects.

There was a modest, but significant increase in GluN2A protein expression in the CPu of BDNF Het mice, which was most pronounced (albeit not significantly so) in the testosterone and DHT-treated groups. Previous studies have established a reciprocal relationship between BDNF and NMDA receptor, where activation of NMDA receptors induces BDNF secretion, and BDNF can increase NMDA receptor activity (Levine et al., 1998; Park et al., 2014). However, as the elevation in GluN2A expression in the BDNF Het mice in the present experiments did not confer to an altered behavioural response to MK-801, it would suggest that this molecular change is not pertinent to MK-801-induced hyperactivity nor adolescent androgens' ability to suppress it.

Taken together, our results demonstrate the important role played by sex hormones in pre-pubescent brain development of the dopaminergic and glutamatergic pathways. We show a clear attenuation of amphetamine-induced hyperlocomotion in mice exposed to high levels of testosterone, but not DHT during adolescence, while both testosterone and DHT reduced MK-801-induced hyperlocomotor responses. Thus the level of estrogen or androgen receptor stimulation during adolescence determines adult responses to psychomimetic drug-induced hyperlocomotor activity. Despite significant effects of genotype on both DAT and GluN2A protein expression levels, BDNF Het mice responded in a similar manner to both amphetamine and MK-801, suggesting other compensatory events may be at play here. Given the evidence of the involvement of these key neurotransmitter systems in the male-biased disorders of psychosis, schizophrenia, substance abuse and addiction, these data provide support for the positive association between male sex hormones exposure during adolescence and disease manifestation.

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## Declarations of interest

None.

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