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Evidence for enhanced androgen action in the prefrontal cortex of people with bipolar disorder but not schizophrenia or major depressive disorder

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ABSTRACT

Anxiety and depressive disorders are more prevalent in hypogonadal men. Low testosterone levels are associated with greater negative symptoms and impaired cognition in men with schizophrenia. Thus, androgens may contribute to brain pathophysiology in psychiatric disorders. We investigated androgen-related mRNAs in post-mortem dorsolateral prefrontal cortex of psychiatric disorders. We also assessed androgen receptor (AR) CAG trinucleotide repeat length, a functional AR gene variant associated with AR gene expression, receptor activity, and circulating testosterone. AR CAG repeat length was determined from genomic DNA and AR and 5α-reductase mRNAs measured using quantitative PCR in schizophrenia, bipolar disorder and control cases [n = 35/group; Stanley Medical Research Institute (SMRI) Array collection]. Layer-specific AR gene expression was determined using *in situ* hybridisation in schizophrenia, bipolar disorder, major depressive disorder and control cases (n = 15/group; SMRI Neuropathology Consortium). AR mRNA was increased in bipolar disorder, but was unchanged in schizophrenia, relative to controls. AR and 5α-reductase mRNAs were significantly positively correlated in bipolar disorder. AR CAG repeat length was significantly shorter in bipolar disorder relative to schizophrenia. AR mRNA expression was highest in cortical layers IV and V, but no layer-specific diagnostic differences were detected. Together, our results suggest enhanced cortical androgen action in people with bipolar disorder.

1. Introduction

Schizophrenia and mood disorders, such as bipolar disorder and major depressive disorder, are debilitating psychiatric disorders and leading contributors to global disease burden (Whiteford et al., 2013). Prominent sex differences exist in both vulnerability to and symptomatology of these psychiatric disorders including onset, incidence, symptoms and course of illness (Abel et al., 2010; Häfner, 2003; Marcus et al., 2008; Merikangas et al., 2007). Symptoms of schizophrenia, bipolar disorder and depression fluctuate with hormonal changes in females (Freeman et al., 2006; Huber et al., 2004; Munk-Olsen et al., 2006; Perich et al., 2017). These observations suggest a potential role for sex steroid hormones (estrogen and testosterone) in both the aetiology and pathophysiology of these disorders. Indeed, estrogen has been proposed as having a neuroprotective effect in the brains of people with psychiatric disorders (Riecher-Rössler, 2017); however, the role of testosterone in terms of ameliorating or exaggerating psychiatric illnesses is less clear.

Clinical evidence indicates testosterone can mediate neuropsychiatric symptomatology. Age-related decline of circulating testosterone in older men is associated with increased depressive symptoms (Ford et al., 2016; Morsink et al., 2007). Anxiety and depressive disorders are more prevalent in hypogonadal men (Zarrouf et al., 2009) and in men treated with androgen-depleting drugs (Lee et al., 2015). Low peripheral testosterone levels are also associated with greater negative symptoms (Akhondzadeh et al., 2006; Ko et al., 2007; Shirayama et al., 2002), impaired emotion processing (Ji et al., 2015) and worse cognition (Li et al., 2015; Moore et al., 2013) in males with schizophrenia. Collectively, these studies indicate higher circulating testosterone may be beneficial against mood-related symptom expression and impaired

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cognition in people with psychiatric disorders.

Conversely, higher testosterone levels may be detrimental for psychotic symptoms. Evidence from anabolic androgenic steroid users suggest a relationship between increased androgen action and psychotic illness (Daria et al., 2015). Additionally, inhibition of a key steroidogenic enzyme involved in the conversion of testosterone to the more potent androgen dihydrotestosterone is shown to produce antipsychotic-like effects in rodents (Bortolato et al., 2008; Devoto et al., 2012; Frau et al., 2016). Adolescent testosterone can increase dopamine-related measures in subcortical brain regions (Purves-Tyson et al., 2012, 2014) and, since the typical age of disease onset coincides with increased testosterone, another possibility is that increased testosterone may contribute to psychosis (Owens et al., 2017).

Current research has been unable to demonstrate a consistent relationship between circulating testosterone and cognition in both healthy and clinical samples (Holland et al., 2011). Some cross-sectional and longitudinal studies find a range of contrasting associations between circulating testosterone and measures of cognitive ability in older men, such as executive function, spatial memory, and global cognitive function (Hogervorst et al., 2004; LeBlanc et al., 2010; Matousek and Sherwin, 2010; Muller et al., 2005; Yaffe et al., 2002). In men with schizophrenia, studies report either a positive association (Li et al., 2015; Moore et al., 2013) or no association (Halari et al., 2004) between higher testosterone levels and better cognition. Trials of testosterone treatment also find varying effects dependent on the cognitive outcome measured (Yeap, 2014). Some trials find improvements in spatial memory, verbal memory or attention with testosterone treatment (Cherrier et al., 2015; Gray et al., 2005), while others find no change to cognitive measures with testosterone administration (Emmelot-Vonk et al., 2008; Huang et al., 2016; Resnick et al., 2017). One of the most well-powered studies of testosterone treatment found no effect on multiple measures of cognition, such as delayed paragraph recall, executive function and spatial ability, in older men with age-associated memory impairment (Resnick et al., 2017). However, it is difficult to interpret these mixed findings due to differences in the methodology between trials, including dose, duration, sample size, degree of hypogonadism, cognitive impairment and outcomes assessed.

The lack of uniform or robust alterations to cognition following peripheral testosterone treatment may stem from multiple factors influencing the brain's response to testosterone, such as brain biosynthesis, metabolism, and brain androgen and receptor levels. One way to ascertain how testosterone may affect cognition is by investigating molecules that reflect the brain's responsiveness to androgens. Testosterone can signal directly via androgen receptor (AR) but is also a precursor for other neurosteroids and can be converted by brain aromatase to estrogen, eliciting effects via estrogen receptors (ERα and ERβ) (Celotti et al., 1997). Testosterone is also converted by the enzyme 5α-reductase in brain tissue to the more potent androgen dihydrotestosterone which is also a ligand for AR (Celotti et al., 1997). ARs are members of the nuclear receptor superfamily and are ligand-activated transcription factors that regulate gene expression in target cells and can also induce intracellular signalling cascades at the cell surface (Bennett et al., 2010). In the mammalian central nervous system, ARs are localized to regions critical for cognition and mood including the frontal cortex, hippocampus, amygdala and hypothalamus (Beyenburg et al., 2000; Finley and Kritzer, 1999; Lorenz et al., 2005; Simerly et al., 1990). Notably, ARs are also found in regions involved in psychosis, including dopamine neurons of the midbrain (Morris et al., 2015; Purves-Tyson et al., 2012). Some evidence for an abnormal ability to respond to sex steroids in major mental illness is found in differential and aberrant ERα gene expression in the prefrontal cortex, hippocampus and amygdala of people with schizophrenia, bipolar disorder and depression (Perlman et al., 2005, 2004; Weickert et al., 2008). Since circulating testosterone can act in the brain through both AR and ERs, we asked if the extent to which the brain's ability to respond to androgens through AR synthesis differs in people with schizophrenia, bipolar disorder and depression.

In humans, the amount of AR synthesised is partially determined by genetics. Exon 1 of the human AR gene, located on chromosome Xq11-12, contains a polymorphic trinucleotide (CAG) repeat sequence that encodes a polyglutamine tract in the N-terminal transactivation domain (Chamberlain et al., 1994) with a normal range of approximately 11–31 repeats (Edwards et al., 1992). CAG repeat length is considered a functional AR gene variant as shorter CAG repeat length is associated with greater AR gene expression and increased receptor activity in experimental models (Chamberlain et al., 1994; Choong et al., 1996; Simanainen et al., 2011). Mixed associations have been found between CAG repeat length and psychiatric symptomatology, largely dependent on the diagnostic status of the group. Shorter CAG repeat length has been associated with major depressive disorder and depressive symptoms in adolescent males (Su et al., 2007) and with higher perceived stress in males with schizophrenia (Owens et al., 2018). In contrast, longer CAG repeat length has been associated with lower cognitive function in healthy older men (Yaffe et al., 2003) and with greater depressive symptoms and anxiety in older hypogonadal men (Schneider et al., 2011a,b). Yet, whether the relationship between AR CAG repeat length and AR gene expression is also altered in people with psychiatric disorders has not been explored.

Considering the evidence for a modulating effect of testosterone on affective symptoms, cognition and psychosis, we investigated the potential ability of the brain to respond to testosterone in schizophrenia, bipolar disorder and major depressive disorder. We examined whether AR and 5α-reductase gene expression, molecules important for the signal transduction and metabolism of androgens, are altered in post-mortem dorsolateral prefrontal cortex (DLPFC) of people with schizophrenia and mood disorders. Additionally, we examined if cortical AR gene expression was determined by CAG repeat length in the AR gene. We hypothesised that AR and 5α-reductase mRNAs in the DLPFC would be differentially altered in people with schizophrenia and bipolar disorder relative to healthy controls. We also expected cortical layer-specific differences in AR mRNA expression levels between people with schizophrenia, bipolar disorder and major depressive disorder compared to healthy controls. Furthermore, we hypothesised that longer AR CAG repeat length would predict less cortical AR gene expression overall as previously shown in experimental models.

2. Materials and methods

2.1. Cohort tissue collection and demographic characteristics

Studies involving human tissue were carried out with approval by the University of New South Wales Human Research Ethics Committee (HREC 12435). Genomic DNA and total RNA from post-mortem DLPFC (Brodmann Area 46) were obtained from the Stanley Medical Research Institute (SMRI) Array collection. RNA was available from 35 schizophrenia cases, 31 bipolar cases and 34 healthy control cases (Table 1). Genomic DNA was available from a subset of the samples used for RNA analyses (32 schizophrenia, 30 bipolar disorder and 32 healthy control cases). Tissue sections used for *in situ* hybridisation were supplied from the SMRI Neuropathology Consortium (Torrey et al., 2000) from 15 cases each with schizophrenia, bipolar disorder, major depressive disorder and healthy controls (Table 2).

2.2. Quantitative real-time PCR

Total RNA was extracted from post-mortem DLPFC (SMRI Array collection) using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) and supplied by the SMRI. RNA quality was measured using an Agilent Bioanalyzer 2100 (Agilent technologies, Santa Clara, CA, USA) and all RNA integrity numbers (RIN) were >6. Complementary DNA (cDNA) was synthesised from total RNA using the Superscript First-Strand Synthesis Kit (Life Technologies, Scoresby, VIC, Australia). Due to the

Table 1
Demographic detail of cases from the Stanley Medical Research Institute Array Collection used in this study.

Demographic variable	Control (n = 34)	Schizophrenia (n = 35)	Bipolar disorder (n = 31)
Age (years)	43.8 (31–60)	42.6 (19–59)	44.9 (19–64)
Sex (Male/Female)	25/9	26/9	15/16
Brain pH	6.6 ± 0.3	6.5 ± 0.2	6.5 ± 0.3
Post-mortem interval (h)	29.5 ± 13.1	31.4 ± 15.5	36.6 ± 18.1
RNA integrity number	8.3 ± 0.7	8.5 ± 0.6	8.3 ± 0.8
Manner of death (natural/suicide)	34/0	28/7	17/14
Duration of illness (years)	–	21.3 ± 10.2	20.16 ± 9.9
Lifetime antipsychotic dose (fluphenazine equiv., mg)	–	85,004.3 ± 100,335.3	10,296.8 ± 23,865.0
Antidepressant use (Yes/No)	–	9/26	18/13
Ethnicity (Caucasian/Non-Caucasian)	34/0	34/1	30/1

Data are mean ± SD (range).

availability of cDNA, two schizophrenia and one bipolar disorder case were excluded. Transcript levels were measured using quantitative real-time PCR (qPCR) using the ABI Prism 7900HT system (Applied Biosystems, Life Technologies) as previously described (Weickert et al., 2010). TaqMan gene expression assays (Applied Biosystems, Life Technologies) were used to measure AR mRNA (AR, Hs00171172_m1), 5α-reductase mRNA (SRD5A1, Hs00602694_mH) and four housekeeper genes (Tata-binding protein, Hs00427620_m1; β-2 microglobulin, Hs99999907_m1; β-actin, Hs99999903_m1; ubiquitin C, Hs00824723_m1). None of the housekeeper genes or the geometric mean of the four housekeeper genes varied by diagnostic group ($p > 0.05$; data not shown). Serial dilutions of cDNA pooled from all samples were included on all qPCR plates and sample expression was quantified by the relative standard curve method using Sequence Detector Software (v2.4, Applied Biosystems). All qPCR reactions were performed in triplicate. Mean triplicate expression levels for AR and 5α-reductase were normalised to the geometric mean of the housekeeper genes (Vandesompele et al., 2002).

2.3. Genotyping

Genomic DNA was supplied from the SMRI Array collection. Analysis of the AR gene CAG microsatellite was performed by the Australian Genomic Research Facility, Melbourne, VIC, Australia. Briefly, 50 ng of DNA from each case was used to amplify the AR gene by PCR using the FAM labelled forward primer 5′-TCCAGAATCTGTT CCAGAGCGTGC-3′ and the unlabelled reverse primer 5′-GCTGTGAAG GTTGCTGTTCCTCA-3′. Automated capillary electrophoresis using an ABI3730 DNA Analyzer (Applied Biosystems, Inc, Foster City, CA, USA) was used to analyse fluorescently labelled DNA fragments by size. GeneMapper Software (v4.1; Applied Biosystems) was used to quantify the length of the CAG repeat region for each sample. CAG repeat length was indeterminable for one schizophrenia case and two bipolar disorder cases. The AR gene is located on the X-chromosome; consequently, we averaged the CAG repeat lengths for the two alleles present in females to account for heterozygosity. Unexpected expression of two alleles was found for three phenotypic males (one bipolar disorder and two control cases) who were excluded from CAG repeat length analyses.

2.4. In situ hybridisation

In situ hybridisation was conducted blind to diagnosis. Human cDNA for AR (corresponding to nucleotides 1485–1973 of accession number NM_000044.2) was amplified by PCR and inserted into the TA cloning site of the PCRII vector of 3.9Kb (Invitrogen). Antisense and sense riboprobes were generated using T7 and Sp6 polymerase, respectively, using an *in vitro* transcription kit (Promega, Madison, WI, USA) and radiolabelled with ^{35}S-UTP (PerkinElmer Waltham, MA, USA) with a specific activity of 1×10^9 cpm/μg. Two 14 μm sections per case were thawed, fixed, acetylated, delipidated and dehydrated as previously described (Whitfield et al., 1990). Each section was incubated overnight with hybridization buffer containing ^{35}S-UTP labelled AR ribroprobe (5 ng/mL) at 55 °C in humidified chambers. Sense strand AR riboprobes were applied to additional sections as a control. Following *in situ* hybridisation, slides and a ^{14}C standards slide (American Radiolabeled Chemicals, St. Louis, MO, USA) were exposed to Biomax MR autoradiographic films (Kodak, Rochester, NY, USA) for 30 days and developed.

Quantitation of *in situ* hybridisation signals was conducted blind to diagnosis. Autoradiographic films were scanned and digital images cropped for each case. Images were calibrated using NIH Imaging software (v1.63) based on the standard Rodbard Curve obtained from the ^{14}C radioactive standards. Optical density measurements (nCi/g) were quantified using ImageJ (v1.50b). For each section, continuous optical density measurements were recorded along the length of three consecutive rectangular boxes placed perpendicular to the pial surface and spanning the entire cortical grey matter. Two sections per case were measured and averaged. Data for AR mRNA expression in individual cortical layers in the DLPFC were determined using the percentage of the total cortical width occupied by each layer as previously

Table 2
Demographic detail of cases from the Stanley Medical Research Institute Neuropathology Consortium used in this study.

Demographic variable	Control (n = 15)	Schizophrenia (n = 15)	Bipolar disorder (n = 15)	Major depressive disorder (n = 15)
Age (years)	48.1 (29–68)	44.5 (25–62)	42.3 (25–61)	46.5 (30–65)
Sex (Male/Female)	9/6	9/6	9/6	9/6
Brain pH	6.3 ± 0.2	6.2 ± 0.3	6.2 ± 0.2	6.2 ± 0.2
Post-mortem interval (h)	23.7 ± 10.0	33.7 ± 14.6	32.5 ± 16.1	27.5 ± 10.7
Manner of death (natural/suicide)	15/0	11/4	6/9	8/7
Duration of illness (years)	–	21.3 ± 11.4	20.9 ± 10.2	12.6 ± 11.2
Lifetime antipsychotics (fluphenazine equiv., mg)	–	55,533.3 ± 54,208.4	20,830.0 ± 24,013.0	–
Antidepressant use (Yes/No)	–	5/10	8/7	12/3
Ethnicity (Caucasian/Non-Caucasian)	14/1	12/3	14/1	15/0

Data are mean ± SD (range).

described (Rajkowska and Goldman-Rakic, 1995; Webster et al., 2002). Two bipolar disorder cases were excluded due to poor tissue quality.

2.5. Statistics

Statistical tests were conducted with IBM SPSS v24 (Armonk, NY, USA) and $p \leq 0.05$ was considered statistically significant. Data are expressed as the mean ± SEM. Population outliers were excluded if values were greater than two standard deviations from the group mean (0–2/group). Data were tested for normality using the Shapiro–Wilk test. One-way analysis of variance (ANOVA), independent samples 2-tailed *t*-tests or χ^2-tests were used to determine diagnostic differences in cohort demographics.

Pearson's product-moment correlations were performed between mRNA levels and demographic variables [age, brain pH, post-mortem interval (PMI), RIN]. Spearman's rank correlations were performed to assess the relationship between mRNA levels and lifetime fluphenazine-equivalent antipsychotic dose and duration of illness separately for each psychiatric diagnosis. Factorial ANOVA was used to determine any effects of manner of death (natural/suicide) or of antidepressant use (positive/negative) on mRNA levels. Diagnostic differences in mRNA expression levels were determined using one-way ANOVA or ANCOVA (if a correlation was detected between mRNA levels and demographic variables) followed by Fisher's least significant differences (LSD) *post hoc* tests. Two-way ANOVAs, with diagnosis and sex as independent factors, were used to identify the effects of sex and any sex-diagnosis interactions on mRNA expression. Pearson's product-moment correlations were conducted between AR and 5α-reductase mRNA levels by diagnostic group. Diagnostic differences in CAG repeat length were determined by one-way ANOVA followed by Fisher's LSD *post hoc* tests. Pearson's product-moment correlations were performed between CAG repeat length and AR mRNA expression levels for the full cohort and by diagnostic group. A mixed design ANOVA, with diagnosis as the between-group factor and cortical layer as the within-group factor, was performed to determine diagnostic differences for layer-specific AR mRNA expression followed by multiple comparisons, adjusted using the Benjamini–Hochberg method for false discovery rate correction, if significant effects were detected. Due to low sample size (females, $n = 4$/diagnostic group) sex was not included as a grouping factor when analysing layer-specific AR mRNA expression.

3. Results

3.1. AR mRNA expression was increased and correlated with 5α-reductase mRNA expression in bipolar disorder cases

AR mRNA levels [$F(2,87) = 3.10$, $p = 0.05$; Fig. 1A] were significantly increased by 15.5% in bipolar disorder cases relative to controls ($p = 0.05$) and by 18.1% relative to schizophrenia cases ($p = 0.02$). AR mRNA levels were unchanged in schizophrenia cases relative to controls ($p = 0.74$). 5α-reductase mRNA levels were unchanged between diagnostic groups [$F(2,92) = 0.05$, $p = 0.95$; Fig. 1B]. There was no significant main effect of sex [AR: $F(1,87) = 0.09$, $p = 0.77$; 5α-reductase: $F(1,89) = 1.46$, $p = 0.23$] as can also be discerned from the graphs (Fig. 1A and B) demonstrating an overlapping distribution for both males (closed circles) and females (open circles) in each diagnostic group. We did not detect an interaction between diagnostic group and sex [AR: $F(2,87) = 0.05$, $p = 0.95$; 5α-reductase: $F(2,89) = 0.27$, $p = 0.77$] on mRNA levels.

There was a trend level positive correlation between AR and 5α-reductase mRNA expression in control cases ($r = 0.34$, $p = 0.07$; Fig. 1C), but no correlation was detected in schizophrenia cases ($r = -0.13$, $p = 0.49$; Fig. 1D). In contrast, AR mRNA and 5α-reductase mRNA levels were significantly and positively correlated in bipolar disorder cases ($r = 0.42$, $p = 0.02$; Fig. 1E).

We assessed the impact of demographic (age, brain pH, PMI, RIN) and clinical variables (duration of illness, fluphenazine-equivalent antipsychotic dose, manner of death, antidepressant use) on AR and 5α-reductase gene expression levels. AR mRNA expression did not correlate with age, brain pH, PMI or RIN (all $p > 0.05$) in all control and psychiatric disorder cases, or with duration of illness or lifetime fluphenazine-equivalent antipsychotic dose in schizophrenia or bipolar disorder cases (both $p > 0.05$). 5α-reductase mRNA expression was significantly positively correlated with brain pH ($r = 0.35$, $p < 0.001$) and RIN ($r = 0.23$, $p = 0.02$), but not age or PMI (both $p > 0.05$), in all control and psychiatric disorder cases and were thus used as covariates when testing for diagnostic differences. 5α-reductase mRNA did not correlate with lifetime fluphenazine-equivalent antipsychotic dose or duration of illness in schizophrenia or bipolar disorder cases (both $p > 0.05$). There was no effect of manner of death or of antidepressant use on either AR or 5α-reductase mRNA levels (all $p > 0.05$).

3.2. AR CAG repeat length was significantly different between bipolar disorder and schizophrenia cases but was not associated with AR gene expression

The frequency distribution of averaged CAG repeat length alleles in schizophrenia, bipolar disorder and control cases is shown in Fig. 2A. CAG repeat length in our samples ranged between 14 and 30 repeats with 58% of samples between 20 and 23 repeats (Table 3). We found an overall trend effect of diagnosis on CAG repeat length [$F(2, 85) = 2.75$, $p = 0.07$]. Since we found a significant diagnostic difference in AR mRNA levels (Fig. 1A) and showed opposite directions of correlations between diagnostic groups (schizophrenia and bipolar disorder, Fig. 1D and E), we specifically contrasted CAG repeat length between diagnostic groups. We discovered that CAG repeat length was significantly lower in bipolar disorder relative to schizophrenia cases ($p = 0.02$). There was no significant difference in CAG repeat length between healthy control and either schizophrenia ($p = 0.26$) or bipolar disorder cases ($p = 0.22$). In contrast to our hypothesis, no significant correlation was detected between CAG repeat length and cortical AR mRNA expression when considering all cases ($r = -0.02$, $p = 0.86$; Fig. 2B) or when focusing on a specific diagnostic group (control: $r = 0.15$, $p = 0.44$; schizophrenia: $r = 0.07$, $p = 0.74$; bipolar disorder: $r = -0.10$, $p = 0.63$).

3.3. Cortical AR mRNA expression was lower in layer I and higher in layers IV and V, but did not differ by diagnosis

Qualitative observation of the autoradiographic films showed AR mRNA hybridisation signal in all cortical layers with low expression in layer I, higher expression in mid-cortical layers (IV and V), and low expression in the white matter areas below the grey matter for all cases (Fig. 3A, control; Fig. 3B, schizophrenia; Fig. 3C, bipolar disorder; Fig. 3D, major depressive disorder). AR sense control strand had weak low levels of hybridisation signal (Fig. 3E).

There was a significant main effect of layer, irrespective of diagnosis, on AR mRNA expression levels [$F(1.87, 98.9) = 158.33$, $p < 0.001$; Fig. 3G]. Pairwise comparisons revealed AR mRNA levels in layer I were significantly lower compared to all other layers (II-VI; all $p < 0.001$; Fig. 3G). AR mRNA expression of layer IV and of layer V were significantly higher compared to all other layers (I-III and VI; all $p < 0.001$; Fig. 3G). AR mRNA expression did not significantly differ between layer IV and layer V ($p > 0.05$; Fig. 3G). The highest mean intensity of AR mRNA was found in the bipolar group (purple circles, Fig. 3F and G), supporting our homogenate-based assay of AR mRNA being elevated in individuals with bipolar disorder. However, total AR mRNA expression [I-VI; $F(3, 53) = 0.64$, $p = 0.59$; Fig. 3F] was not significantly different between diagnostic groups. There was no significant diagnostic difference of layer-specific AR mRNA levels [$F(5.6, 98.9) = 0.43$, $p = 0.85$; Fig. 3G]. Both the total and mean AR mRNA expression across all cortical layers (I-VI) did not correlate with age,

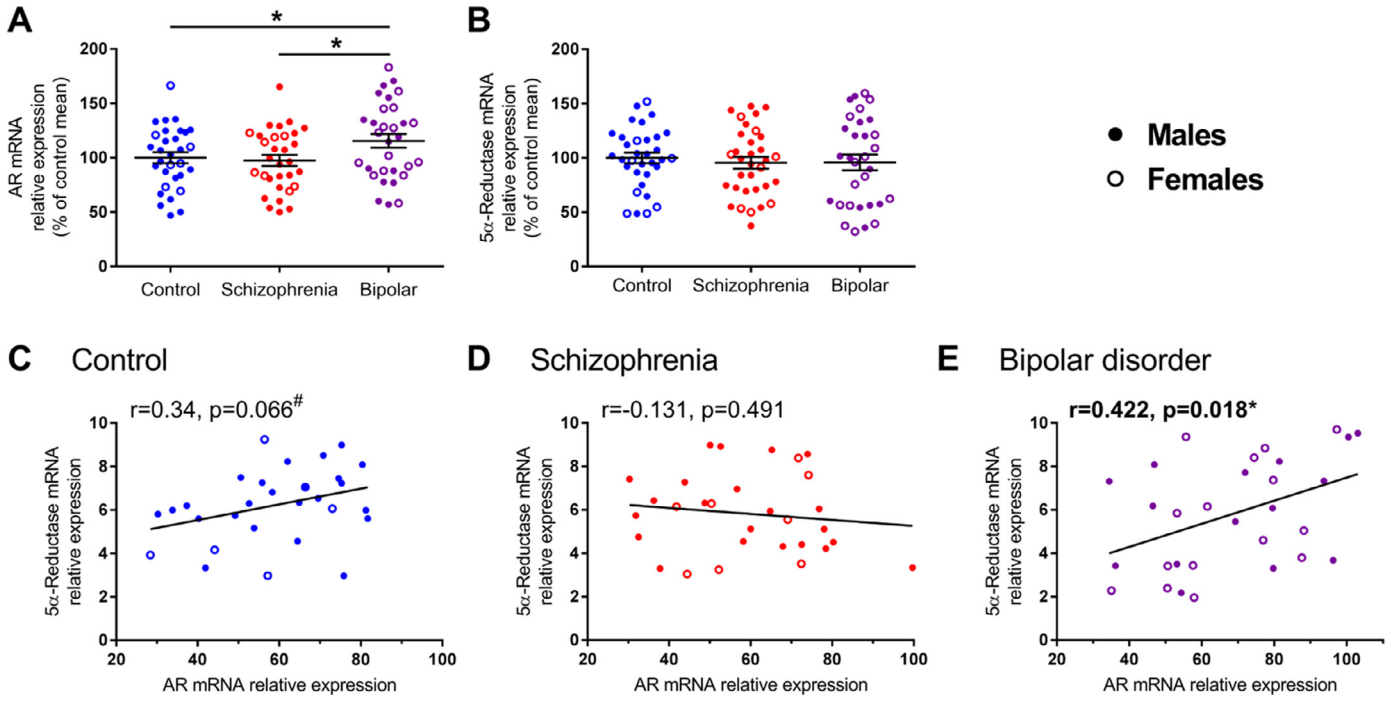

Fig. 1. Gene expression of androgen receptor (AR) and 5α-reductase in the dorsolateral prefrontal cortex of controls (blue), schizophrenia (red) and bipolar disorder (purple) cases of both sexes (males, closed circles; females, open circles) from the Stanley Medical Research Institute Array collection. (A) AR mRNA expression levels [$F(2,92) = 3.10, p = 0.05$] were significantly increased in bipolar disorder cases relative to control ($p = 0.05$) and schizophrenia cases ($p = 0.02$), but unchanged in schizophrenia relative to control cases ($p = 0.74$). (B) No significant diagnostic differences in 5α-reductase mRNA expression levels were detected [$F(2,92) = 0.05$, $p = 0.95$]. AR and 5α-reductase mRNA expression levels were not significantly correlated in (C) healthy controls or (D) schizophrenia cases, but were significantly positively correlated in (E) bipolar disorder cases. Data is mean ± SEM, $\#p < 0.10$, $*p \leq 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

brain pH, PMI or RIN ($p > 0.05$) in all cases, or with lifetime fluphenazine-equivalent antipsychotic dose or duration of illness ($p > 0.05$) in psychiatric disorder cases, and no effect of antidepressant use ($p > 0.05$) on AR mRNA expression.

4. Discussion

For the first time, we have discovered three different lines of evidence to support that there may be enhanced androgen action in the brains of people with bipolar disorder. Moreover, we have contrasted our findings in people with bipolar disorder to those diagnosed with schizophrenia and major depressive disorder, which have both similar

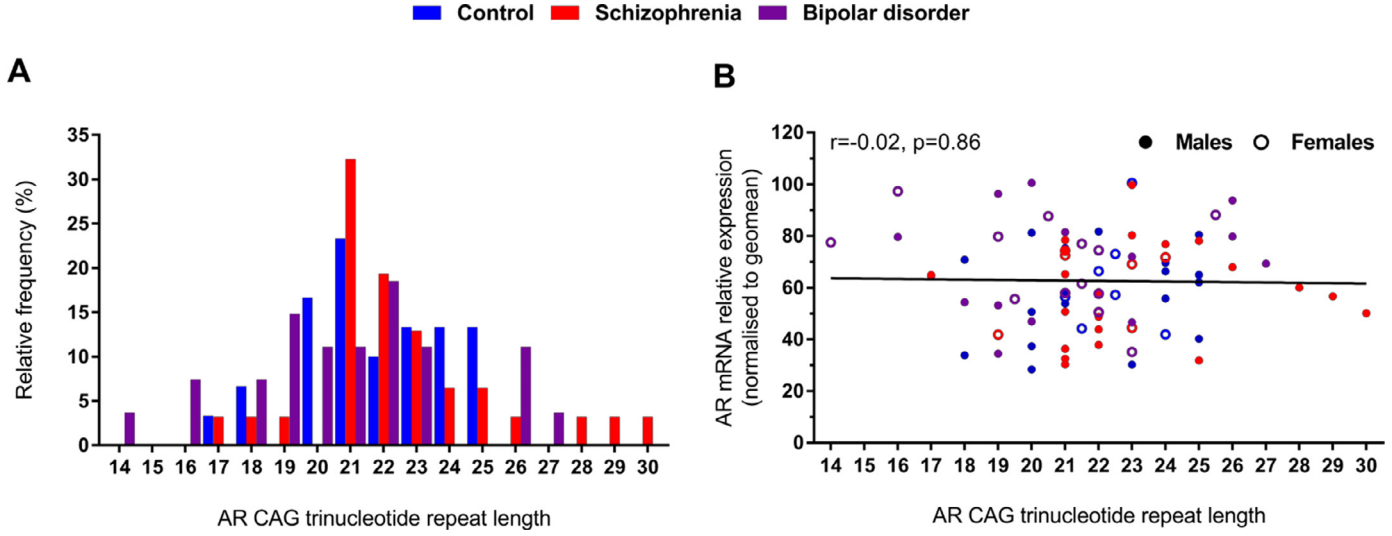

Fig. 2. Androgen receptor (AR) CAG trinucleotide repeat length frequency of control (blue), schizophrenia (red) and bipolar disorder (purple) cases and correlation with AR mRNA expression levels in the dorsolateral prefrontal cortex of all cases (males, closed circles; females, open circles) from the Stanley Medical Research Institute Array collection. (A) Frequency distributions of AR CAG trinucleotide repeat length in our sample (see also Table 3). (B) AR mRNA expression levels were not significantly correlated with CAG repeat length in all cases. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Distribution of androgen receptor gene polyglutamine length for schizophrenia, bipolar disorder and healthy controls cases.

Diagnosis	*N*	Range	Median	Mean (SD)
Control	30	17–25	21.3	21.7 (2.2)
Schizophrenia	31	17–30	22	22.5 (2.9)
Bipolar disorder	27	14–27	21	20.8 (3.2)

SD, standard deviation.

and distinct features. First, we detected increased AR mRNA expression in the DLPFC of people with bipolar disorder; and second, we found a positive association between AR and 5α-reductase mRNA expression, suggesting that as AR signalling increases testosterone is more likely converted to the more potent dihydrotestosterone in those with bipolar disorder. Thirdly, we showed that shorter AR CAG trinucleotide repeat length, which is known to bias cells towards greater AR activity (Chamberlain et al., 1994; Choong et al., 1996; Simanainen et al., 2011), was more common in bipolar disorder compared to schizophrenia cases. However, we did not find a direct correspondence between this AR genetic variant and brain AR mRNA levels, indicating that AR regulation is more complex in brain and not solely due to CAG repeat length. *In situ* hybridisation revealed widespread distribution and a neuronal pattern of AR gene expression throughout the DLPFC. Lower levels of AR gene expression were found in layer I and higher levels in layers IV and V, paralleling neuronal density in the cortex; however, we were unable to detect layer-specific diagnostic differences in AR gene expression possibly due to reduced number of individuals in the bipolar group which led to reduced power ($n = 15$ compared to $n = 31$). Together, our findings suggest that there is increased androgen action in the prefrontal cortex of people with bipolar disorder but not schizophrenia.

Our finding of increased AR mRNA expression in the DLPFC of people with bipolar disorder was unexpected as low testosterone levels, suggesting decreased rather than increased androgen action, has been associated with more depressive symptoms in healthy men (Ford et al., 2016; Morsink et al., 2007) and impaired cognition in men with schizophrenia (Li et al., 2015; Moore et al., 2013). However, women with polycystic ovary syndrome (PCOS), characterised by hyperandrogenemia, have a higher co-occurrence of psychiatric disorders including schizophrenia, bipolar disorder and depressive and anxiety disorders (Cesta et al., 2016). There is also evidence for increased psychotic illness (Daria et al., 2015) and impaired cognition (Kanayama et al., 2013) in anabolic androgenic steroid users. This suggests that increased androgen action may also have detrimental effects in psychiatric disorders. Some studies show a curvilinear association between circulating testosterone and cognition in healthy older men (Matousek and Sherwin, 2010; Muller et al., 2005) and depressive symptoms in women with PCOS (Weiner et al., 2004), suggesting an optimal level of androgen action may be necessary to maintain healthy human brain processes. This is further supported by research showing detrimental effects of both increased (Kanayama et al., 2013; Wallin and Wood, 2015) and reduced (Gonzalez et al., 2015; Yang et al., 2015) androgen action on cognitive function. Although it is difficult to conclude whether the increased AR mRNA levels observed in the DLPFC of people with bipolar disorder are beneficial or detrimental, these changes are indicative of abnormal androgen action and suggest alterations to the optimal levels needed to maintain normal function.

A possible factor contributing to increased AR mRNA levels in bipolar disorder, but not in schizophrenia, may be the prevalence of comorbid conditions that are characterised by hyperandrogenism. Women with bipolar disorder have a higher prevalence of PCOS as compared to women with schizophrenia, as well as menstrual abnormalities and symptoms of reproductive dysfunction (Cesta et al., 2016; Rassi et al., 2010; Reynolds-May et al., 2014; Zhang et al., 2016). Indeed, it has been suggested that both bipolar disorder and PCOS have shared endophenotypes, such as insulin resistance, obesity and hypothalamus-pituitary-adrenal axis dysregulation (Jiang et al., 2009). These hyperandrogenic comorbid conditions may enhance androgen action in target tissues, like ovary and brain, through a positive feedback loop, whereby androgens increase both AR and 5α-reductase mRNA expression in brain (Purves-Tyson et al., 2012), and contribute to the increased AR mRNA levels that we find in the prefrontal cortex in women with bipolar disorder. It is also possible that increased AR mRNA levels are a result of treatment. Mood-stabilising drugs used to treat bipolar disorder, specifically valproate, have been associated with the development of PCOS and reproductive dysfunction, including increased testosterone levels in women (Reynolds et al., 2007; Zhang et al., 2016). However, a few studies show these symptoms are present in women prior to valproate treatment (Joffe et al., 2006; Reynolds-May et al., 2014), but it remains difficult to determine whether PCOS and the similar reproductive conditions found in women with bipolar disorder are a consequence of treatment or the disease itself. Future studies taking into consideration comorbid conditions, treatment, and circulating androgen levels may help to identify whether changes to AR mRNA expression in the cortex are a consequence of treatment or an underlying part of the pathophysiology of bipolar disorder.

We found that the mRNA expression of 5α-reductase, the enzyme responsible for converting testosterone to dihydrotestosterone (Celotti et al., 1997), did not differ between people with schizophrenia or bipolar disorder and healthy controls in the DLPFC. However, 5α-reductase may be involved in regulating cortical androgen action in people with depressive disorders, rather than in psychotic illnesses. This is supported by research finding reduced 5α-reductase levels in the prefrontal cortex of a small cohort of major depressive disorder cases (Agis-Balboa et al., 2014). Further, men treated with 5α-reductase inhibitors have increased depressive and anxiety symptoms (Melcangi et al., 2017), suggesting reduced action of this enzyme may have deleterious effects on mood-related symptoms. We were unable to measure 5α-reductase mRNA expression in major depressive disorder cases using homogenate-based assays (qPCR) and additional work is needed to confirm whether changes to androgen action are evident in the brains of people with depressive disorders.

Although we showed no diagnostic changes in 5α-reductase mRNA expression, we found a positive association between AR and 5α-reductase mRNA levels in the DLPFC in bipolar disorder. In contrast, healthy control cases had a small but non-significant, trend positive association while there was no association between AR and 5α-reductase mRNA levels in people with schizophrenia. Manipulation of sex steroid levels in normal male rodents show that increased circulating androgen levels can potentiate androgenic signalling, suggesting that androgens can act in a positive feedback loop in the mammalian brain (Purves-Tyson et al., 2012). In particular dihydrotestosterone, a potent androgen, can increase both AR and 5α-reductase mRNA levels whereas testosterone alone did not significantly change 5α-reductase mRNA levels (Purves-Tyson et al., 2012). The positive relationship in bipolar disorder between AR and 5α-reductase mRNA gene expression may indicate the potential for positive feedback on androgen action and increased androgen-related genomic action in bipolar disorder. It is possible that as AR signalling increases, testosterone is more likely to be converted to the more potent dihydrotestosterone through increased 5α-reductase mRNA levels, further potentiating testosterone action in the cortex of those with bipolar disorder. Circulating levels of testosterone and other androgens may influence these processes in people with bipolar disorder; however current research has failed to identify a consistent change in circulating testosterone levels in the disorder (Feng et al., 2019; Ozcan and Banoglu, 2003; Ramsey et al., 2013; Sher et al., 2012; Wooderson et al., 2015). Some studies suggest sex steroid levels may be differentially altered in males and females (Wooderson et al., 2015) or be state-dependent (manic, depressed or euthymic) (Mousavizadegan and Maroufi, 2018; Ozcan and Banoglu,

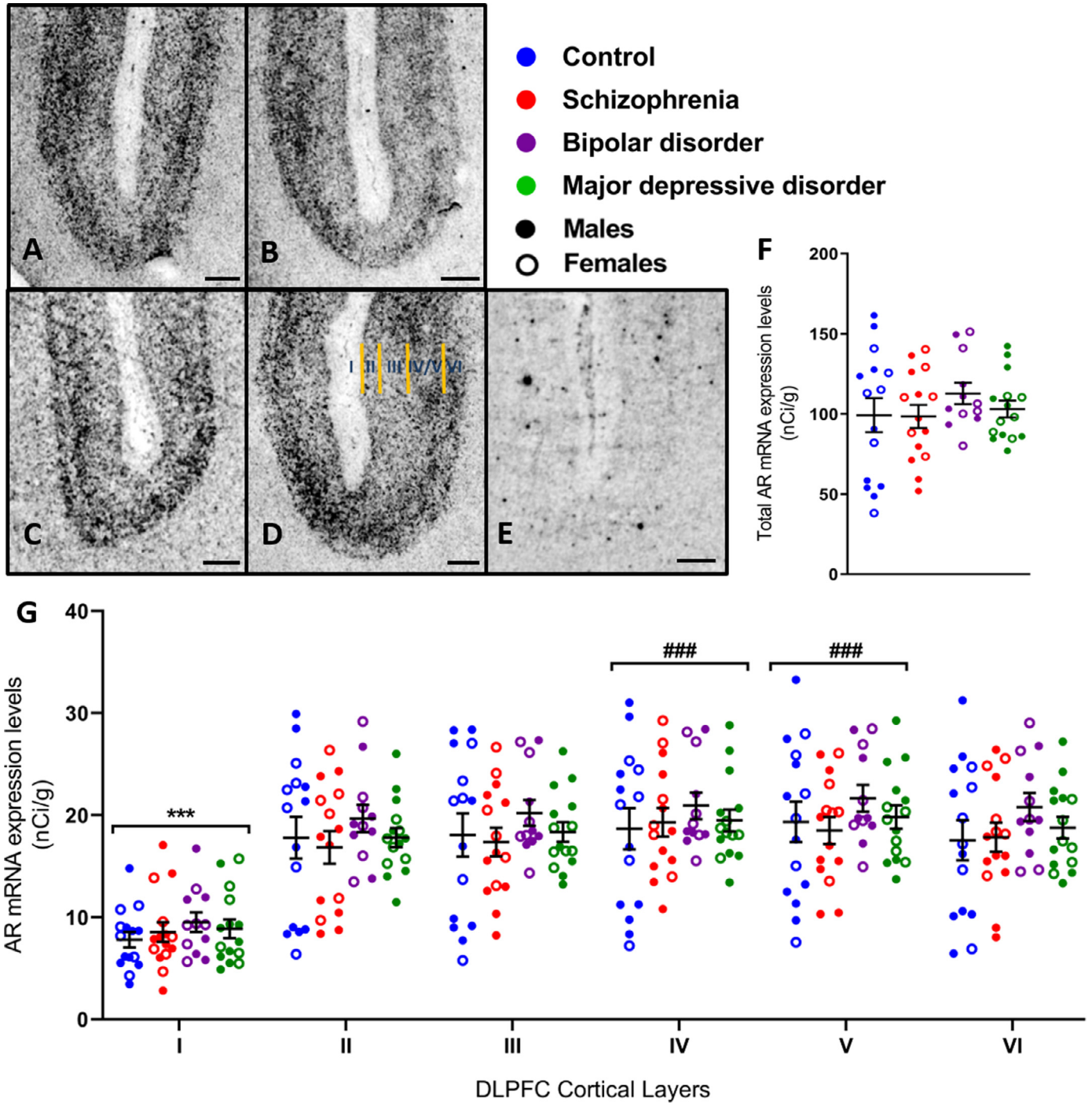

Fig. 3. Layer-specific androgen receptor (AR) mRNA expression in the dorsolateral prefrontal cortex (DLPFC) of controls (blue, $n = 15$), schizophrenia (red, $n = 15$), bipolar disorder (purple, $n = 12$) and major depressive disorder (green, $n = 15$) cases from the Stanley Medical Research Institute Neuropathology consortium. Representative *in situ* hybridisation images showing AR mRNA expression in post-mortem DLPFC (BA46) of (A) control, (B) schizophrenia, (C) bipolar disorder, (D) major depressive disorder cases and (E) control sense strand cases (Scale bar = 1000 μm). (F) Total AR mRNA expression (nCi/g) of all DLPFC layers was not significantly different between diagnoses [$F(3, 53) = 0.64$, $p = 0.59$]. (G) There was a significant main effect of layer, irrespective of diagnosis, on AR mRNA expression levels [nCi/g; $F(1.87, 98.9) = 158.33$, $p < 0.001$] in the DLPFC. AR mRNA expression in layer I was significantly lower compared to all other layers (II-VI; ***$p < 0.001$). AR mRNA expression of layer IV and layer V were significantly higher compared to all other layers (I-III and VI; ###$p < 0.001$), but did not significantly differ between layer IV and layer V ($p > 0.05$). Layer-specific AR mRNA expression [$F(5.6,98.9) = 0.43$, $p = 0.85$] was not significantly different between control, schizophrenia, bipolar disorder, and major depressive disorder cases. Data is mean ± SEM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2003). Additional research is needed to determine whether changes to circulating levels of testosterone or its conversion to dihydrotestosterone may potentiate androgen action in the brains of people with bipolar disorder.

AR CAG repeat length is a functional gene variant capable of mediating androgen sensitivity and action in target tissues. Research finding reduced AR function with longer CAG repeat lengths are typically conducted with a large range of CAG repeats (Albertelli et al.,

2006; Chamberlain et al., 1994; Choong et al., 1996; Simanainen et al., 2011); however, small changes to CAG repeat length may have cell or tissue specific effects. A study investigating a normal physiological range of CAG repeats (15, 24 and 31 repeats) in four different cell lines (prostatic and non-prostatic) showed that the effect of CAG repeat length on AR activity can be dependent on cell type (Beilin et al., 2000). In the brain, a longitudinal imaging study finds that CAG repeat length is associated with a greater rate of region-specific cortical grey matter thinning across adolescence in both healthy males and females (Raznahan et al., 2010). This study suggests that varying number of CAG repeats (9–28 repeats) within a normal physiological range can impact the human brain. We found that CAG repeat length was significantly shorter in people with bipolar disorder compared to schizophrenia by approximately two repeats. Similarly, previous studies have found shorter CAG repeat length, by approximately one to two repeats, was associated with major depressive disorder (Geng et al., 2007; Su et al., 2007) and depressive symptoms in adolescent males (Su et al., 2007). Shorter CAG repeat length in bipolar disorder may indicate increased androgen responsivity or action compared to the longer CAG repeat length found in schizophrenia; although, more research is required to determine the impact of CAG repeat length specifically in the brain.

Contrary to previous findings in experimental models (Albertelli et al., 2006; Choong et al., 1996), we observed no relationship between CAG repeat length and AR gene expression in the human cortex. This result should be interpreted with caution as it is likely that shorter AR CAG repeat length does bias the brain to synthesize more AR throughout life. However, since we only measured AR mRNA levels at death, more brain AR mRNA could have been produced at times that we have not sampled in our study. Alternatively, it is possible that AR CAG repeat length is not as brain penetrant, as transcription of the AR gene can also be regulated by several other tissue-dependent processes. For example, an unusually long 5′ untranslated region contains many regulatory elements that impact the binding of transcription factors and gene expression of the receptor (Hunter et al., 2018). A more comprehensive understanding of the regulatory processes controlling AR mRNA and protein expression in the brain is needed in order to determine if and how androgen signalling may be dysregulated in psychiatric disorders.

Irrespective of diagnosis, AR mRNA expression was significantly lower in layer I compared to all other layers and significantly higher in layers IV and V. Similar patterns of AR mRNA expression have also been shown in non-human primates (Finley and Kritzer, 1999) and may parallel the relative density of neurons in the cortex, which are sparsest in layer I and highest in layer IV (Rajkowska and Goldman-Rakic, 1995). Interestingly, layer V pyramidal cells in the prefrontal cortex that provide major afferent projections to the ventral tegmental area are highly AR-enriched in rodents, implicating these neurons in the regulation of dopaminergic neurotransmission (Aubele and Kritzer, 2012). Dysregulated dopaminergic neurotransmission is a hallmark of schizophrenia (Howes and Kapur, 2009) and the higher AR mRNA expression found in layer V in the current study may have implications for the androgenic regulation of dopamine signalling in humans.

The current study has limitations. It is important to recognise and consider confounding factors associated with post-mortem studies where the brains of patients are typically studied following chronic illness and treatment with antipsychotics and other psychiatric medications, making it difficult to determine if differences are due to a disease process or caused by other confounding factors. However, we found no correlations between the dependent variables examined and clinical variables (lifetime fluphenazine equivalents, duration of illness, and antidepressant use). Secondly, we have measured the gene expression of AR and 5α-reductase and cannot ascertain whether the changes observed in AR mRNA levels are reflected in measures of protein and receptor activity. Additional studies are needed to determine if the protein levels or enzymatic activity of these androgen-related molecules are altered in psychiatric disorders. We were also unable to measure 5α-reductase gene expression in our major depressive disorder cases using homogenate-based assays, which would be of interest given the evidence this enzyme may be involved in depression. Thirdly, our study had limited ability to assess the interaction between sex and diagnosis on AR and 5α-reductase mRNA expression due to the lower group numbers when separating males and females. The inclusion of more females would enable a more detailed examination of the effect of sex on androgen-related gene expression. Larger group sizes are also needed to confirm if there is a true genetic association between AR CAG repeat length and diagnosis of a psychiatric disorder. Finally, we do not have measures of either circulating or cortical levels of testosterone in our post-mortem cohorts. Determining circulating and cortical levels of testosterone and dihydrotestosterone would allow us to test if brain changes in AR are related to changes in sex hormone levels and may be indicative of widespread HPG axis dysregulation. This would allow us to determine how the brain and peripheral levels of androgens are related to one another in the human brain, especially given the evidence for local steroidogenesis in the brain (Melcangi et al., 2011).

In summary, we found increased AR mRNA gene expression, as well as a positive association between AR and 5α-reductase mRNA expression, in the prefrontal cortex of people with bipolar disorder. Our findings demonstrate evidence for increased androgen action in the brains of people with bipolar disorder. These observed changes in measures of androgen action in the brains of people with bipolar disorder (putatively hyperandrogenic) compared to schizophrenia may represent distinct differences between the two psychotic disorders. Studies in other brain regions implicated in psychiatric disorders, such as the hippocampus, amygdala and midbrain will help to determine whether there are global changes to androgen action and abnormal brain response to testosterone in people with psychiatric disorders. Further investigation of whether changes to AR gene expression are also evident in measures of protein and receptor activity and, in combination with clinical studies investigating the relationship between symptoms, cognition and androgen levels in people with bipolar disorder, will help to clarify if increased androgen action in the prefrontal cortex is deleterious or beneficial in people with this disease. This research may enable the development of sex steroid modulating therapies that can moderate androgen action in people with bipolar disorder.

Declaration of Competing Interest

CSW is on an advisory board for Lundbeck, Australia Pty Ltd and in collaboration with Astellas Pharma Inc., Japan. The authors declare no conflicts of interest in relation to this specific work.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psychres.2019.112503.

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