# Abnormal Glucocorticoid Receptor mRNA and Protein Isoform Expression in the Prefrontal Cortex in Psychiatric Illness

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Stress has been implicated in the onset and illness course of schizophrenia and bipolar disorder. The effects of stress in these disorders may be mediated by abnormalities of the hypothalamic–pituitary–adrenal axis, and its corticosteroid receptors. We investigated mRNA expression of the glucocorticoid receptor (GR) and mineralocorticoid receptor (MR), and protein expression of multiple GRα isoforms, in the prefrontal cortex of 37 schizophrenia cases and 37 matched controls. Quantitative real-time PCR, western blotting, and luciferase assays were employed. In multiple regression analysis, schizophrenia diagnosis was a significant predictor of total GR mRNA expression ($p < 0.05$), which was decreased (11.4%) in schizophrenia cases relative to controls. No significant effect of diagnosis on MR mRNA was detected. At the protein level, no significant predictors of total GRα protein or the full-length GRα isoform were identified. However, schizophrenia diagnosis was a strong predictor ($p < 0.0005$) of the abundance of a truncated $\sim 50\,kDa$ GRα protein isoform, putative GRα-D1, which was increased in schizophrenia cases (80.4%) relative to controls. This finding was replicated in a second cohort of 35 schizophrenia cases, 34 bipolar disorder cases, and 35 controls, in which both schizophrenia and bipolar disorder diagnoses were significant predictors of putative GRα-D1 abundance ($p < 0.05$ and $p = 0.005$, respectively). Full-length GRα was increased in bipolar disorder relative to schizophrenia cases. Luciferase assays demonstrated that the GRα-D1 isoform can activate transcription at glucocorticoid response elements. These findings confirm total GR mRNA reductions in schizophrenia and provide the first evidence of GR protein isoform abnormalities in schizophrenia and bipolar disorder.

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## INTRODUCTION

A role for stress in the onset and illness course of psychotic mental illness is suggested by three lines of evidence from epidemiological, clinical, and neurobiological studies. First, stressful events at critical life stages have been shown to increase risk of developing schizophrenia and bipolar disorder. Prenatally, maternal emotional trauma, metabolic stress due to malnutrition, and increased stress due to infection may increase schizophrenia risk (Brown *et al*, 2004; Brown *et al*, 2005; Khashan *et al*, 2008; St Clair *et al*, 2005; Susser *et al*, 1996; Susser and Lin, 1992; Van Os and Selten, 1998). Stress associated with birth, such as obstetric complications, has been shown to increase schizophrenia

risk (Cannon *et al*, 2002). Trauma during childhood has been associated with the development of psychotic symptoms at age 12, and with psychotic illness later in life (Arseneault *et al*, 2011; Fisher *et al*, 2010; Mortensen *et al*, 2003; Poulton *et al*, 2000; Read *et al*, 2005; Schreier *et al*, 2009). The incidence of early parental loss is increased in individuals with schizophrenia relative to controls, and to a lesser extent, in individuals with bipolar disorder (Agid *et al*, 1999). In young people at high risk of psychosis, impaired tolerance to normal stress has been shown to increase risk of transition to psychotic illness (Yung *et al*, 2005). After the onset of illness, stress can precipitate relapse into psychosis (Day *et al*, 1987; Fowles, 1992). Incorporating these observations, the diathesis–stress model of schizophrenia has become a leading theory on the interaction between genetic vulnerability and stress in the onset and progression of schizophrenia (Corcoran *et al*, 2003; Walker and Diforio, 1997).

Second, dysregulation of the hypothalamic–pituitary–adrenal (HPA) axis, which orchestrates hormonal stress responses, has been observed in schizophrenia and bipolar

disorder. Increased basal levels of the stress hormone cortisol have been demonstrated in medicated and unmedicated individuals with schizophrenia (Meltzer et al, 2001; Muck-Seler et al, 1999; Venkatasubramanian et al, 2010) and bipolar disorder (Gallagher et al, 2007; Watson et al, 2004). Increased cortisol forms part of a proposed biomarker signature for schizophrenia (Schwarz et al, 2011). Impaired negative feedback regulation of the HPA axis, as measured by the dexamethasone suppression test, has been reported in individuals with schizophrenia and individuals with bipolar disorder (Lammers et al, 1995; Plocka-Lewandowska et al, 2001; Watson et al, 2004; Yazici et al, 2002). Blunted cortisol responses by individuals with schizophrenia to psychosocial and metabolic stressors have also been described (Jansen et al, 2000; Marcelis et al, 2004; van Venrooij et al, 2010). The exact nature of HPA axis disturbances may be quite variable, as a range of other studies in schizophrenia have been conducted which failed to find HPA axis disturbances (for review see Bradley and Dinan, 2010).

Third, abnormal expression of the mineralocorticoid receptor (MR) and glucocorticoid receptor (GR) have been described in the brains of individuals with psychosis. The high affinity MR binds cortisol at basal levels and responds to circadian fluctuations of its secretion, whereas GR binds cortisol at higher concentrations and orchestrates cellular responses to stress-induced cortisol secretion. Decreased expression of the MR gene (NR3C2) in the prefrontal cortex in schizophrenia has been reported (Xing et al, 2004). Expression of the GR gene (NR3C1) is also decreased in the hippocampus, dorsolateral prefrontal cortex (DLPFC), inferior temporal cortex, and amygdala in schizophrenia, and in the hippocampus, entorhinal cortex, inferior temporal cortex, and amygdala in bipolar disorder (Perlman et al, 2004; Webster et al, 2002). The decrease in GR mRNA expression was one of the most robust findings arising from the Stanley Foundation postmortem brain cohort (Knable

et al, 2004; Knable et al, 2001). Decreased GR mRNA in the DLPFC is of particular relevance, because the DLPFC has been implicated widely in psychiatric illness (Lewis and Gonzalez-Burgos, 2008), and may be involved in negative feedback regulation of the HPA axis (Diorio et al, 1993; Mizoguchi et al, 2003). It is not known whether these changes in GR and MR mRNA expression in schizophrenia are reproducible, or if they are reflected in changes in receptor proteins and their functions.

To understand the role of stress in the pathogenesis of schizophrenia and bipolar disorder, a more detailed understanding of steroid receptor abnormalities in the prefrontal cortex at the protein level is required. The archetypal GR isoform, full-length GRα ($\sim$105 kDa), is expressed in the human prefrontal cortex, along with smaller GRα isoforms, approximately 67, 50, and 40 kDa in size (Sinclair et al, 2011). We have previously shown that the 50 and 40 kDa isoforms putatively represent the GRα-D1 and GRα-Dx isoforms, respectively (Sinclair et al, 2011), which arise from employment of alternate translation start sites in exon 2 (Lu and Cidlowski, 2005; Figure 1). However, it is not known whether other human GR isoforms such as GRβ, GR-'A' (Δ5–7), GR-P, and GRγ (Bamberger et al, 1995; Moalli et al, 1993; Rivers et al, 1999) are expressed in the prefrontal cortex. In this study, we set out to 1) confirm GR and MR mRNA alterations in schizophrenia, 2) determine whether expression levels of GRα protein isoforms are altered in schizophrenia and bipolar disorder, and 3) explore the mRNA expression of the GRβ and GR-'A' (Δ5–7) transcripts in human DLPFC.

## MATERIALS AND METHODS

### Tissue Collection

For mRNA and protein quantification, frozen tissue from the middle frontal gyrus of 30 schizophrenia cases, 7
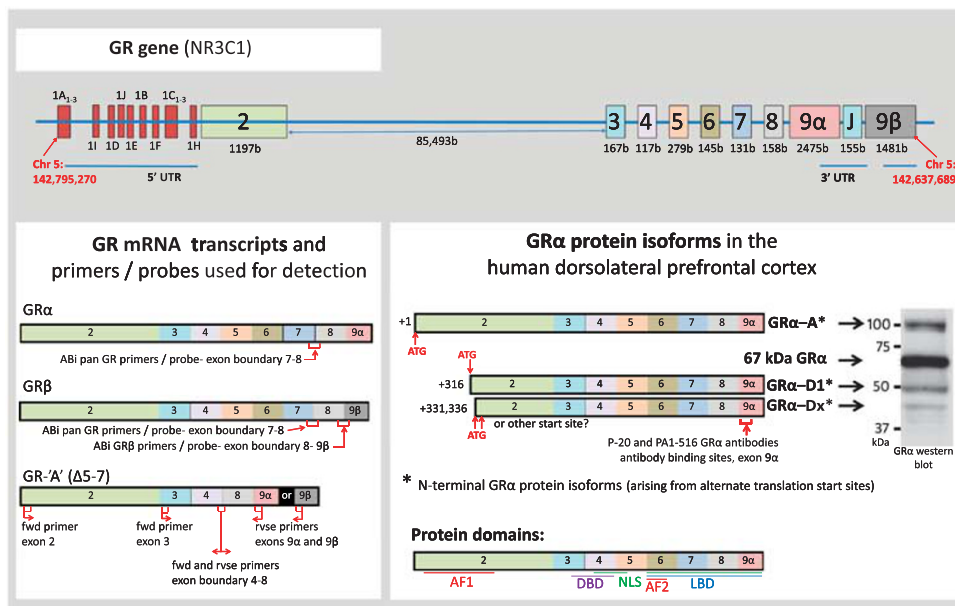


**Figure 1** Map of glucocorticoid receptor (GR) gene (NR3C1), GR mRNA transcripts, primer, and probes used for detection in this paper, and GRα protein isoforms in the dorsolateral prefrontal cortex (DLPFC). Abi, Applied Biosystems; AF, activation function; ATG, methionine (start) codon; Chr, chromosome; DBD, DNA binding domain; fwd, forward; LBD, ligand binding domain; NLS, nuclear localisation signal; rvse, reverse; UTR, untranslated region.

schizoaffective cases, and 37 well-matched control individuals was provided by the New South Wales Tissue Resource Centre (TRC cohort; Table 1). Characterisation of this Australian TRC schizophrenia cohort has been described previously (Weickert et al, 2010).

For replication of protein findings, the Stanley Foundation array cohort was employed. Crude protein homogenate from the middle frontal gyrus of 35 schizophrenia cases, 34 bipolar disorder cases, and 35 control individuals was supplied by the Stanley Medical Research Institute (Table 2)

### Quantitative Real-Time PCR (qPCR) Analysis

qPCR analysis was conducted as reported (Weickert et al, 2010). Briefly, total RNA was extracted from 300 mg gray matter using Trizol (Invitrogen, Carlsbad, CA), and its

**Table 1** Demographic Detail of Cases from the TRC Cohort Used in this Study

| | Control group (n = 37) | Schizophrenia group (n = 37) |
|---|---|---|
| Age (years) | 51.1 (18–78) | 51.3 (27–75) |
| Gender (F, M) | 7, 30 | 13, 24 |
| Hemisphere (R, L) | 23, 14 | 17, 20 |
| PH | 6.66 +/− 0.29 | 6.61 +/− 0.30 |
| PMI (h) | 24.8 +/− 10.97 | 28.8 +/− 14.07 |
| RIN | 7.3 +/− 0.57 | 7.3 +/− 0.58 |
| Manner of death | Natural = 37 | Natural = 29, suicide = 8 |
| Age of onset (years) | — | 23.7 ± 0.10 |
| Duration of illness (years) | — | 27.6 ± 2.3 |
| Daily chlorpromazine mean (mg) | — | 692 ± 502 |
| Last recorded chlorpromazine dose (mg) | — | 542 ± 374 |
| Lifetime chlorpromazine dose (g) | — | 7908 ± 7954 |
| Antidepressant use | Yes = 0, No = 37 | Yes = 19, No = 18 |

Abbreviations: F, female; L, left; M, male; R, right.
Data quoted are mean (range) ± SD.

quality determined using the Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). Samples had average RNA integrity number (RIN) of 7.3 (all samples had RIN $\geqslant 6.0$). cDNA was synthesised using the Superscript First-Strand Synthesis Kit (Invitrogen). Inventoried TaqMan gene expression assays (Applied Biosystems, Foster City, CA) were used for $\beta$-actin, GAPDH, TBP, UBC, NR3C2 (MR), and NR3C1 (GR; separate assays for pan GR and GR$\beta$) (Supplementary Table S1). Two custom assays were designed for detection of the GR-'A' transcript variant of NR3C1. None of the housekeeping genes, nor the geometric mean of the four, varied between schizophrenia patients and controls (for geometric mean, $t = 0.448$, df = 72, $p = 0.656$). Serial dilutions of pooled cDNA (from six samples, three per diagnostic group) were included on every qPCR plate for quantification of sample expression by the relative standard curve method ($R^2$ values of 0.93–0.99).

All qPCR reactions were performed in triplicate. For each sample, the most distant triplicate was removed if the SD of the triplicates was greater than 40% of their mean, and the sample excluded if the SD of the duplicates remained greater than 40% of their mean. No samples were excluded at this stage from the pan GR analysis, whereas four schizophrenia cases were excluded from the MR assay. Expression levels were then normalised to the geometric mean of four housekeeper genes, and population outliers excluded if their normalised expression values were greater than 2 SDs from the group mean. Three population outliers were removed at this stage from the pan GR assay (one schizophrenia case, two controls), whereas five population outliers were removed from the MR analysis (two schizophrenia cases, three controls).

### Western Blotting

Western blotting was conducted as previously described (Sinclair et al, 2011). The P-20 anti-GR$\alpha$ primary antibody (sc-1002X, Santa Cruz Biotechnology, Santa Cruz, CA), which targets a region within amino acids 720–770 of the GR$\alpha$ protein, was used. Standard curves were generated by loading 3–15 µg of crude protein homogenate, whereas sample analysis was conducted using 7 µg of protein homogenate. Proteins were heated (95 °C, 5 min), run on

**Table 2** Demographic Detail of Cases from the Stanley Foundation Array Cohort Used in this Study

| | Control group (n = 35) | Bipolar disorder group (n = 34) | Schizophrenia group (n = 35) |
|---|---|---|---|
| Age (years) | 44.2 (31–60) | 45.4 (19–64) | 42.6 (19–59) |
| Gender (F, M) | 9, 26 | 18, 16 | 9, 26 |
| Hemisphere (R, L) | 19, 16 | 15, 19 | 18, 17 |
| PH | 6.61 +/− 0.27 | 6.43 +/− 0.30 | 6.48 +/− 0.24 |
| PMI (h) | 29.4 +/− 12.9 | 37.9 +/− 18.6 | 31.4 +/− 15.5 |
| Manner of death | Natural = 35 | Natural = 19, suicide = 15 | Natural = 28, suicide = 7 |
| Age of onset (years) | — | 25.3 ± 9.2 | 21.3 ± 6.1 |
| Duration of illness (years) | — | 20.2 ± 9.6 | 21.3 ± 10.2 |
| Lifetime antipsychotics (fluphenazine equivalents, mg) | — | 10 212 ± 22 871 | 85 004 ± 100 335 |
| Antidepressant use | Yes = 0, No = 35 | Yes = 19, No = 15 | Yes = 9, No = 26 |

Abbreviations: F, female; L, left; M, male; R, right.
Data quoted are mean (range) ± SD.

10% bis-tris polyacrylamide gels (Bio-Rad, Hercules, CA), and transferred onto nitrocellulose membranes (Bio-Rad). Transfer was performed at 100V for 120 min. Blots were probed with P-20 anti-GRα (1:2000 dilution in 5% skim milk) and goat anti-rabbit secondary (1:2000; Millipore, Billerica, MA), before being stripped (stripping buffer 25 mM glycine, 1.5% SDS, pH 2.0) and reprobed with anti-$\beta$-actin primary (1:10 000; MAB1501, Millipore) and goat anti-mouse secondary antibodies (1:5000; Millipore). Pre-absorption to determine the specificity of the P-20 sc1002X GRα antibody under our running conditions was conducted with the sc1002P blocking peptide (Santa Cruz; mapping to the C-terminus 50 amino acids of GRα), or unrelated peptide (SOX-5, sc-17329, Santa Cruz) at 4 °C for 24 h prior to application to the blot. The P-20 GRα antibody was previously confirmed by cloning and *in vitro* transfection to recognise GRα isoforms (Sinclair *et al*, 2011).

All blots were imaged using the Chemidoc XRS Molecular Imager (Bio-Rad). A substantial difference in intensity between the 67-kDa immunoreactive (IR) band and the other IR bands was observed. As a result, a shorter exposure time (10 s) was used to quantify the 67-kDa IR band to ensure that this band was not overexposed. A longer exposure time (90 s) was used to quantify total GRα protein and all other IR bands, to increase sensitivity of detection. All GRα IR bands were within a linear range of detection.

The loading curve and samples were run in duplicate in separate experimental runs. For each sample run, the quantity of each immunoreactive band was normalised to the $\beta$-actin band detected in the same lane, and an internal control (pooled sample from entire cohort) loaded onto the same gel. Population outliers within each run were excluded if the normalised geomean value was greater than 2 SDs from the group mean. The geometric mean of both runs was then calculated and expressed as a percentage of the control mean for each band. For the TRC cohort, a minimum of 36 cases per diagnostic group were analysed for each immunoreactive band, whereas for the Stanley cohort, a minimum of 33 cases per diagnostic group for each immunoreactive band were analysed. Overall, the internal standard displayed an average variability of ± 11.5% from blot-to-blot.

## Cell Culture and Luciferase Reporter Assay

Plasmids were constructed containing DNA fragments encoding GRα N-terminal variants, GRα-A and GRα-D1, with V5 tags as previously described (Sinclair *et al*, 2011). CHOK1 cell-lines were obtained from the American Type Culture Collection, grown at 37 °C in a 5% $CO_2$ atmosphere, and cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) FBS supplemented with glutamax (2 mM). On day 1, cells were seeded in uncoated 48-well plates at a density of $2 \times 10^4$ cells/well. On day 2, media was replaced with phenol red-free media, and cells co-transfected for 24 h with Cignal glucocorticoid response elements (GRE) reporter (100 ng/well; SABiosciences, Frederick, MD), and either pcDNA3.1-V5-His-GRα-A, pcDNA3.1-V5-His-GRα-D1 (all 125 ng/well), or both pcDNA3.1-V5-His-GRα-A and pcDNA3.1-V5-His-GRα-D1 (125 ng/well each), using Lipofectamine LTX transfection reagent (Invitrogen). Negative controls consisted of either GRE reporter co-transfected with empty pcDNA3.1-V5-His vector, or GRE-negative control co-transfected with pcDNA3.1-V5-His-GRα-A. On day 3, cells were treated for 24 h with dexamethasone (cat #D4902, Sigma) with two sets of concentrations, either 0, 0.1, 1, 10, 100 nM (low/narrow range), or 0, 10, 50, 100, 500, 1000 nM (high/wide range). On day 4, cells were washed and resuspended in 65 μl 1 × passive lysis buffer before luciferase assays were performed using the dual-luciferase reporter assay system (Promega, Madison, WI) on a Fluostar OPTIMA (BMG Labtech, Offenburg, Germany).

Treatments were performed in triplicate in each experiment. Within each assay, the results for each well, expressed as the ratio of luciferase : renilla activity in that well, were normalised to the mean of all wells in the assay. Results presented are the mean normalised data from two independent experiments using a low/narrow range of dexamethasone concentrations, or four independent experiments using a high/wide range of dexamethasone concentrations.

## Statistical Analysis

Stepwise multiple regression was used for analysis of pan GR and MR mRNA expression, to determine whether diagnosis, age, gender, brain pH, post-mortem interval (PMI), or RIN were significant predictors of gene expression. Suicide and antidepressant use were included in a second regression analysis for each experiment, as additional independent variables. Suicide and non-suicide cases within the schizophrenia (scz) group were coded as separate 'dummy' variables ('suicide-negative schizophrenia' group (scz non-suicide = 1, scz suicide = 0, and controls = 0), 'suicide-positive schizophrenia' group (scz suicide = 1, scz non-suicide = 0 and controls = 0)). Antidepressant use was coded in the same way. For GRα western blotting, stepwise multiple regressions were also used, with diagnosis, age, gender, brain pH, PMI, suicide, and antidepressant use included as independent variables. Schizophrenia and bipolar disorder diagnoses were coded as separate independent variables (schizophrenia group (scz = 1, bp = 0 and controls = 0), bipolar disorder group (bp = 1, scz = 0 and controls = 0)). For all mRNA and protein measures, Pearson correlation analyses were conducted with age of onset, duration of illness, and antipsychotic drug measures, within schizophrenia cases only. For luciferase assay, the effect of dexamethasone treatment on GRα-transfected cells was determined by repeated measures ANOVA with Bonferroni post-hoc analysis.

## PCR Detection of GR-'A'

Endpoint PCR was performed to amplify the GR-'A' mRNA transcript variant in pooled DLPFC cDNA (from three schizophrenia and three control cases) and universal human cDNA (from normal human tissues, Biotaq, Gaithersburg, MD). Primers used were anchored in GR exons 2, 3, 4/8 junction, 9α, and 9β (Figure 1 and Supplementary Table S1). Each reaction contained $MgCl_2$ (2 mM), dNTPs (0.2 mM), forward and reverse primers (0.2 mM), cDNA (approximately 4.5 ng/μl), and RedHot DNA polymerase (0.5 U; Thermo Scientific, Waltham, MA) in 1 × reaction buffer.

The PCR protocol used involved incubation at 94 °C for 3 min, then 40 consecutive cycles of 94 °C (30 s), 58–62 °C (30 s, or 90 s for exon 2–exon 4/8), and 72 °C (30 s), then incubation at 72 °C for 10 min and 4 °C overnight. Products were run on a 1% agarose gel alongside a 1-kb ladder (Fermentas, Waltham, MA), and visualised on the Chemi-doc XRS Molecular Imager (Bio-Rad).

## RESULTS

### GR mRNA Expression in the DLPFC in Schizophrenia

To confirm GR mRNA abnormalities in schizophrenia, NR3C1 gene expression was quantified in the DLPFC in schizophrenia and control cases. The relative contributions of schizophrenia diagnosis, gender, brain pH, PMI, and RIN to variation in pan GR mRNA expression were determined by stepwise multiple regression. Data were normally distributed. A significant model, which incorporated RIN and diagnosis, was able to explain 37% of the variance in pan GR mRNA ($F(2, 68) = 21.12$, $p < 0.0005$). Both RIN and diagnosis were significant predictors of pan GR mRNA expression ($p < 0.0005$ and $p < 0.05$, respectively), whereas age, pH, PMI, and gender were not (Table 3). When the means of schizophrenia and control groups were compared, an 11.4% decrease in pan GR mRNA expression in schizophrenia cases relative to controls was observed (Figure 2a).

### Death by Suicide, Antidepressant Use, and GR mRNA Expression

The effects of suicide and antidepressant use on pan GR mRNA expression were also investigated. When suicide and antidepressant use status were taken into account, a significant model emerged ($F(2, 68) = 24.98$, $p < 0.0005$) in which RIN and 'suicide-negative schizophrenia' status were significant predictors of pan GR mRNA expression ($p < 0.0005$ and $p < 0.005$, respectively; Table 2). When means of the 'suicide-negative schizophrenia',
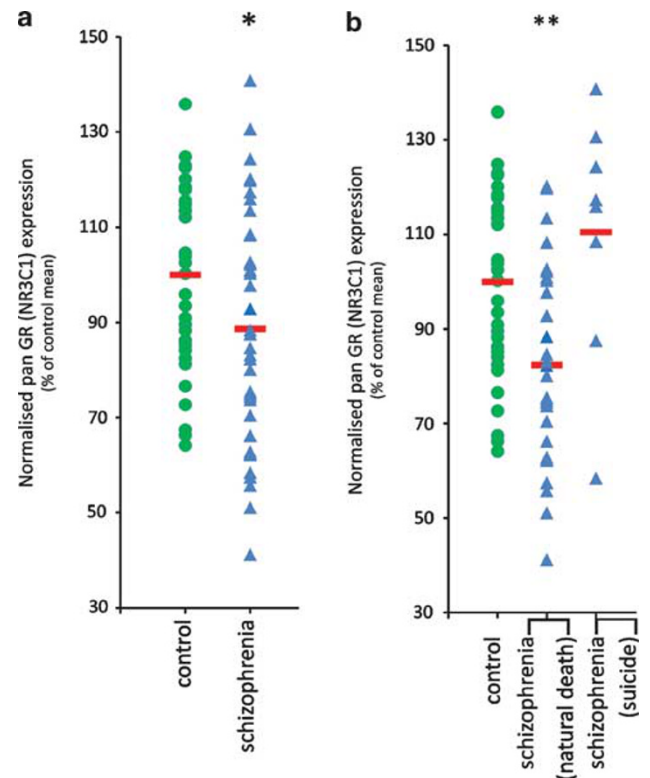


**Figure 2** Pan glucocorticoid receptor (GR) mRNA expression in the dorsolateral prefrontal cortex (DLPFC) in controls (green circles), and schizophrenia cases (blue triangles). (a) Schizophrenia diagnosis was a significant predictor ($p < 0.05$) of pan GR mRNA expression in the regression model ($F(2, 68) = 21.12$, $p < 0.0005$). Comparison of the means of schizophrenia and control groups revealed an 11.4% decrease in pan GR mRNA expression in the schizophrenia cases relative to controls; (b) when included in multiple regression analysis, 'suicide-negative schizophrenia' status was a significant predictor ($p < 0.005$) of pan GR mRNA expression in the resultant regression model ($F(2, 68) = 24.98$, $p < 0.0005$). Comparison of their means revealed that the 'suicide-negative schizophrenia' group ($n = 28$) displayed 25.4% lower pan GR mRNA expression than the 'suicide-positive schizophrenia' group ($n = 8$), and 17.6% lower pan GR mRNA expression than controls. $*p < 0.05$, $**p < 0.005$.

**Table 3** Regression Modelling of the Effects of Diagnosis and Other Demographic Variables on Pan GR and MR mRNA Expression in the DLPFC in Schizophrenia and Control Cases

| Dependent variable | Predictor(s) | B | SE | β | Adjusted $R^2$ |
|---|---|---|---|---|---|
| pan GR mRNA expression | Constant | −70.55 | 28.84 | | |
| | RIN | 23.33 | 3.92 | 0.567*** | |
| | Diagnosis (schizophrenia) | −10.2 | 4.35 | −0.224* | 0.365 |
| pan GR mRNA expression | Constant | −52.92 | 28.78 | | |
| | RIN | 20.98 | 3.88 | 0.510*** | |
| | Schizophrenia (suicide-negative) | −14.37 | 4.40 | −0.308** | 0.407 |
| MR mRNA expression | Constant | 3.71 | 1.47 | | |
| | Age | 0.078 | 0.028 | 0.334* | 0.097 |

Diagnosis (schizophrenia) was coded as (control = 0, schizophrenia = 1). Suicide and non-suicide cases within the schizophrenia group were coded as follows: 'suicide-negative schizophrenia' group (non-suicide schizophrenia = 1, suicide schizophrenia = 0, and controls = 0), and 'suicide-positive schizophrenia' group (suicide schizophrenia = 1, non-suicide schizophrenia = 0, and controls = 0).
$*p < 0.05$, $**p < 0.005$, $***p < 0.0005$.

'suicide-positive schizophrenia', and control groups were compared, 'suicide-negative schizophrenia' cases ($n = 28$) displayed 25.4% lower pan GR mRNA expression than 'suicide-positive schizophrenia' cases ($n = 8$), and 17.6% lower pan GR mRNA expression than controls (Figure 2b). Causes of death in the 'suicide-negative schizophrenia' group included heart disease (46/66 cases), peritonitis, and asthma. Although 'antidepressant use' groups were not included as predictors in the significant regression model, the predictive value of 'antidepressant-positive schizophrenia' and 'antidepressant-negative schizophrenia' groups approached significance ($p = 0.053$ and $p = 0.069$, respectively). Pan GR mRNA expression of antidepressant-negative schizophrenia cases was 18.1% lower than controls, and 15.7% lower than antidepressant-positive schizophrenia cases. Antidepressant use was not more prevalent in those who died by suicide than those who died by natural causes ($\chi^2 = 0.39$, df = 1, $p = 0.532$).

GR mRNA expression within schizophrenia cases was not significantly correlated with age of onset, duration of illness, daily, last, or lifetime chlorpromazine-equivalent antipsychotic doses.

## MR mRNA Expression in the DLPFC in Schizophrenia

NR3C2 (MR) gene expression was also analysed by stepwise multiple regression. Age was a significant predictor of MR mRNA expression ($p < 0.05$) in the resultant model (F(1, 63) = 7.91, $p < 0.01$), revealing that MR mRNA expression increases with age. Schizophrenia diagnosis, gender, PMI, brain pH, RIN, antidepressant use and manner of death were not significant predictors of MR mRNA expression (Table 3).

No significant correlations of MR mRNA expression within schizophrenia cases with age of onset, duration of illness, daily, last, or lifetime chlorpromazine-equivalent antipsychotic doses were seen.

## GRα Protein Isoforms in the DLPFC in Schizophrenia

To explore the specificity of the P-20 anti-GRα antibody used, preabsorption was conducted. We found that antibody preabsorption with GRα C-terminus immunogenic peptide resulted in almost complete blocking of P-20 antibody binding in western blots of homogenates from both schizophrenia and control cases (Supplementary Figure S1).

Western blotting of DLPFC samples from schizophrenia and control cases using the anti-GRα (P-20) antibody was conducted, revealing five GRα IR bands of approximately 105, 67, 50, 40, and 25 kDa (Figure 3b). This banding pattern was consistent with the pattern observed using this antibody previously (Sinclair *et al*, 2011). After cloning of distinct GR mRNAs and *in vitro* expression studies, we previously reported that IR band 1, 3, and 4, correspond to full-length GRα, GRα-D1, and GRα-Dx (another GRα-D isoform), respectively. IR bands 2 and 5 represent 67 and 25-kDa GRα isoforms, which have yet to be characterised. Loading standard curves were conducted to establish that all bands were within a linear range of detection when loading 7 μg of protein (Figure 3a and c).

For analysis of western blotting results, the data for IR band intensities were transformed by taking the square root, so that they were approximately normally distributed (skewness from $-1.19$ to 0.44). Stepwise multiple regressions were then performed to analyse the effect of diagnosis, gender, age, brain pH, and PMI, on intensities of each IR band, and on the total of IR bands 1–5. For IR bands 1, 2, 5, and total bands 1–5, there were no significant models explaining variance in band intensity, with neither diagnosis, age, pH, PMI nor gender emerging as significant predictors. However, for band 3, a significant model which incorporated diagnosis was able to account for 17% of the variance (F(1,72) = 15.96, $p < 0.0005$; Table 4). In this model, diagnosis was the only significant predictor of GRα band 3 abundance ($p < 0.0005$). When the means of schizophrenia and control groups were compared, the mean IR band intensity of schizophrenia cases was 80.4% greater than controls (Figure 3d). For band 4, there was also a significant model, which accounted for 6% of the variance (F(1, 72) = 5.34, $p < 0.05$; Table 4), in which PMI was the only significant predictor of IR band 4 intensity ($p < 0.05$).

## Relationships of Demographic Variables with GRα Protein Expression

In contrast to findings at the mRNA level, neither suicide status nor antidepressant use were significant predictors of IR band intensity in multiple regression modelling.

Within schizophrenia cases, GRα IR band 1 intensity correlated positively with last recorded antipsychotic (chlorpromazine equivalent) dose ($r = 0.49$, $p < 0.01$) and lifetime antipsychotic dose (chlorpromazine equivalent; $r = 0.42$, $p < 0.05$). No other significant correlations of GRα protein levels within schizophrenia cases with age of onset, duration of illness, daily, last, or lifetime chlorpromazine doses were seen.

Pan GRα mRNA expression was not significantly correlated with total GRα protein, nor the abundance of any GRα IR band individually.

## Replication of Western Blotting Findings: GRα Protein Isoforms in Schizophrenia and Bipolar Disorder

To confirm altered GRα-D1 protein isoform abundance in schizophrenia, additional western blotting was performed with the same P-20 antibody in a second independent cohort, the Stanley Foundation array cohort. Western blots in duplicate runs with this cohort displayed the same GRα banding pattern as observed in the TRC cohort (Figure 4a). Data for IR band intensities were approximately normally distributed (skewness from 0.10 to 1.07), and were analysed using stepwise multiple regressions. For IR band 1, the diagnosis of bipolar disorder predicted 5% of the variance in band intensity (F(1, 101) = 6.48, $p < 0.05$; Table 5), whereas schizophrenia diagnosis, age, gender, PMI, and brain pH did not significantly predict band intensity. When their group means were compared, bipolar disorder cases displayed a 25.7% increase in IR band 1 intensity relative to controls, and a 55.1% increase relative to schizophrenia cases (Figure 4b). For IR band 3, a significant model emerged which predicted 7% of the variance (F(2, 101) = 4.56, $p < 0.05$; Table 5), in which both schizophrenia
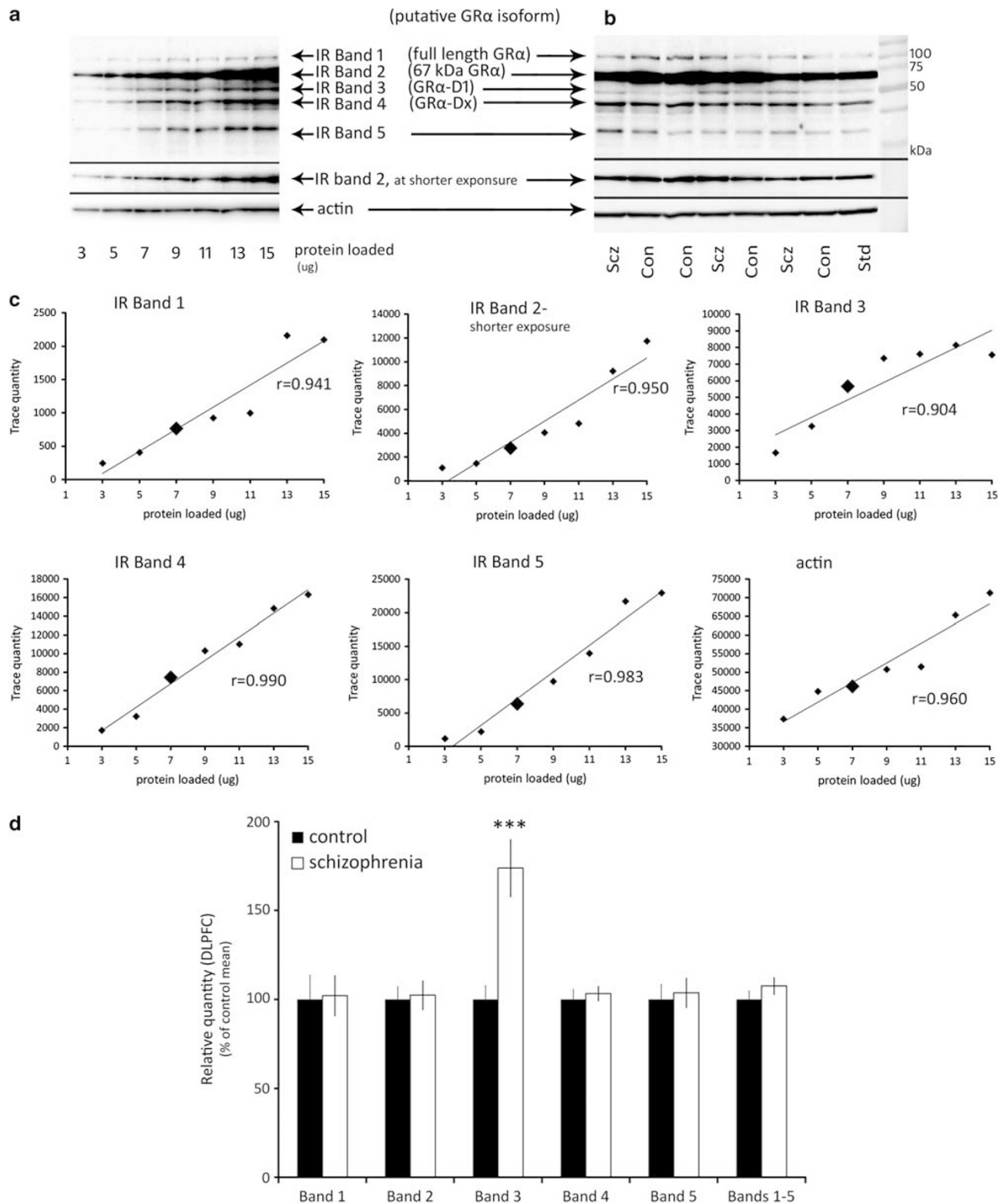
**Figure 3** Glucocorticoid receptor (GR)α protein expression in the dorsolateral prefrontal cortex (DLPFC) of Tissue Resource Centre (TRC) schizophrenia and control cases. (a) Loading standard curve western blot. Crude protein homogenate of 3–15 μg was loaded, and the blot probed with P-20 anti-GRα antibody; (b) western blot of representative control (Con), schizophrenia (Scz), and internal control (Std) samples probed with P-20 anti-GRα antibody, displaying five GRα immunoreactive bands (IR bands 1–5) of approximately 105, 67, 50, 40, and 25 kDa; (c) standard curves for IR bands 1–5 and β-actin, demonstrating linear increases in intensity with increasing protein. Large diamonds represent 7 μg of protein, the amount loaded for quantification of the schizophrenia cohort. (d) Average intensities of GRα IR bands in controls (black bars), and schizophrenia cases (white bars). Comparison of the means of schizophrenia and control groups revealed an 80.4% increase in schizophrenia cases relative to controls. By stepwise multiple regression, diagnosis was the only significant predictor of GRα IR band three abundance in the significant regression model ($F(1, 72) = 15.96$, $p < 0.0005$). Normalised quantities are the geometric mean of normalised duplicate runs, displayed as a percentage of the mean of the control group. ***$p < 0.0005$. Error bars represent SEM.

**Table 4** Regression Modelling of the Effects of Diagnosis and Demographic Variables on GRα Protein Abundance in the DLPFC in TRC Schizophrenia and Control Cases

| Dependent variable | Predictor(s) | B | SE | β | Adjusted $R^2$ |
|---|---|---|---|---|---|
| IR band 1 intensity | None | — | — | — | — |
| IR band 2 intensity | None | — | — | — | — |
| IR band 3 intensity | Constant | 0.908 | 0.053 | | |
| | Diagnosis (schizophrenia) | 0.297 | 0.074 | 0.426*** | 0.170 |
| IR band 4 intensity | Constant | 0.884 | 0.049 | | |
| | PMI | 0.004 | 0.002 | 0.263* | 0.069 |
| IR band 5 intensity | None | — | — | — | — |
| Total IR bands 1-5 intensity | None | — | — | — | — |

Diagnosis (schizophrenia) was coded as (control = 0, schizophrenia = 1).
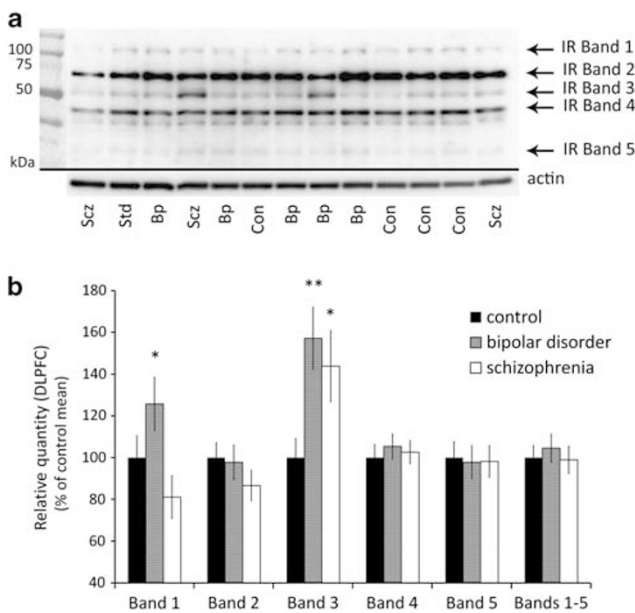*$p < 0.05$, ***$p < 0.0005$.



**Figure 4** Intensities of glucocorticoid receptor (GR)α immunoreactive (IR) bands in western blotting of dorsolateral prefrontal cortex (DLPFC) samples from the Stanley Array cohort. (a) Representative western blot of control (Con), schizophrenia (Scz), bipolar disorder (Bp), and internal control (Std) samples probed with P-20 anti-GRα antibody, displaying the same banding pattern as observed in the Tissue Resource Centre (TRC) cohort; (b) Average intensities of GRα IR bands in controls (black bars), bipolar disorder cases (grey bars), and schizophrenia cases (white bars). Bipolar disorder diagnosis was the only significant predictor of IR band 1 intensity (F(1, 101) = 6.48, $p < 0.05$). Mean IR band 1 intensity was increased (55.1%) in bipolar disorder cases relative to schizophrenia cases, and 25.7% increased in bipolar disorder cases relative to controls. Both schizophrenia diagnosis and bipolar diagnosis were significant predictors of IR band 3 intensity ($p < 0.05$ and $p = 0.005$, respectively, F(2, 101) = 4.56, $p < 0.05$). Mean IR band 3 intensity was 57.2% increased in bipolar disorder cases relative to control cases, and 43.8% increased in schizophrenia cases relative to controls. Error bars represent SEM. *$p < 0.05$, **$p = 0.005$.

diagnosis and bipolar diagnosis were significant predictors of GRα band 3 intensity ($p < 0.05$ and $p = 0.005$, respectively). Age, gender, PMI, and brain pH did not predict band 3 intensity. The mean IR band 3 intensity of bipolar disorder cases was 57.2% greater than controls, whereas the mean IR band 3 intensity of schizophrenia cases was 43.8% greater than controls (Figure 4b). The intensities of bands 2,

4, 5, or bands 1–5, were not predicted in any significant models containing diagnosis, age, gender, PMI, or brain pH. Neither suicide status nor antidepressant use were significant predictors of IR band intensity in the Stanley cohort when included in multiple regression modelling. No significant correlations of GRα protein levels within schizophrenia cases with age of onset, duration of illness, or lifetime fluphenazine equivalent antipsychotic doses were seen.

## Functional Activity of GRα Isoforms

To determine the functional activity of the GRα-D1 isoform, which is increased in schizophrenia and bipolar disorder, we assessed the relative capacity of the full-length GRα and GRα-D1 isoforms to activate transcription of target genes at GREs. Two ranges of dexamethasone concentrations were used, to delineate the effects of both low (0.1–100 nM) and high (10–1000 nM) concentrations of dexamethasone on GRα isoforms. In assays using a narrow range of lower dexamethasone concentrations (0, 0.1, 1, 10, and 100 nM), repeated measures ANOVA revealed a significant effect of dexamethasone concentration (F(4,68) = 23.22, $p < 0.0005$), and a significant interaction of dexamethasone concentration with GRα isoform (F(12, 68) = 2.87, $p < 0.005$; Figure 5a). At very low dexamethasone concentration (0.1 nM), no significant differences in luciferase activity between cells transfected with empty vector, full-length GRα and GRα-D1 were observed. However, at dexamethasone concentrations of 10 nM and 100 nM, the luciferase activities of both full-length GRα and GRα-D1-transfected cells were significantly increased (up to 390%), relative to corresponding empty vector controls (Bonferroni post-hoc, $p \leqslant 0.05$). For cells co-transfected with both full-length GRα and GRα-D1, a significant increase (227%) in luciferase activity relative to empty vector control was observed at 100 nM dexamethasone ($p < 0.005$), suggesting that the presence of GRα-D1 does not interfere with full-length GRα function. Assays were repeated with a wide range of higher dexamethasone concentrations (0, 10, 50, 100, 500, and 1000 nM). A significant effect of dexamethasone treatment (F(5, 220) = 42.57, $p < 0.0005$), and a significant interaction of dexamethasone concentration with GRα isoform (F(15, 220) = 3.81, $p < 0.0005$) were observed (Figure 5b). There were significant differences between empty vector and GRα isoforms (alone or in

**Table 5** Regression Modelling of the Effects of Diagnosis and Demographic Variables on GRα Protein Abundance in the DLPFC in Stanley Foundation Schizophrenia, Bipolar Disorder, and Control Cases

| Dependent variable | Predictor(s) | B | SE | β | Adjusted $R^2$ |
|---|---|---|---|---|---|
| IR band 1 intensity | Constant | 76.55 | 6.87 | | |
| | Diagnosis (bipolar disorder) | 30.89 | 12.13 | 0.246* | 0.051 |
| IR band 2 intensity | None | — | — | — | — |
| IR band 3 intensity | Constant | 86.84 | 12.11 | | |
| | Diagnosis (bipolar disorder) | 49.64 | 17.25 | 0.316** | |
| | Diagnosis (schizophrenia) | 38.03 | 17.13 | 0.244* | 0.065 |
| IR band 4 intensity | None | — | — | — | — |
| IR band 5 intensity | None | — | — | — | — |
| total IR bands 1-5 intensity | None | — | — | — | — |

Diagnosis (bipolar disorder) was coded as (control = 0, schizophrenia = 0, bipolar disorder = 1), and diagnosis (schizophrenia) was coded as (control = 0, schizophrenia = 1, bipolar disorder = 0).
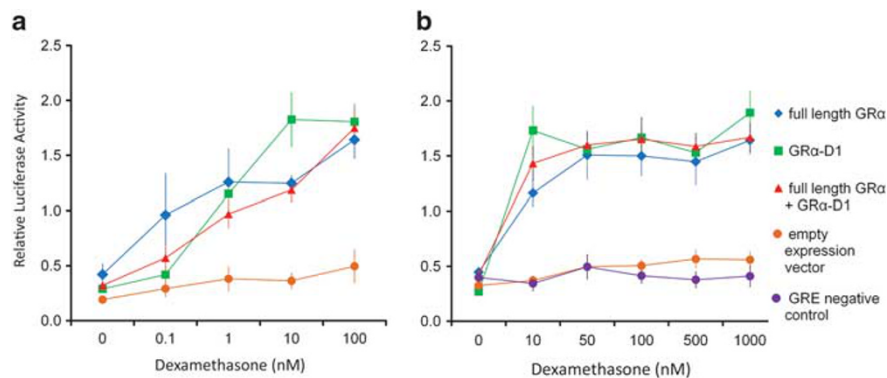*$p < 0.05$, **$p = 0.005$.



**Figure 5** Luciferase reporter assays of full-length glucocorticoid receptor (GR)α (blue diamonds), truncated GRα-D1 isoform (green squares), and combination of both full-length GRα and truncated GRα-D1 (red triangles). (a) GRα isoform transfections with a narrow range of dexamethasone concentrations—0, 0.1, 1, 10, and 100 nM; (b) GRα isoform transfections with a wider range of dexamethasone concentrations— 0, 10, 50, 100, 500, and 1000 nM. In both assays, the full-length GRα, and the truncated GRα-D1 isoforms increased transcription at glucocorticoid response elements (GREs) in response to dexamethasone, when compared with an empty vector control. No additional effect was observed when co-transfecting both the full-length GRα and truncated GRα-D1 isoforms. Results are the normalised mean of (a) two or (b) four independent experiments, with treatments performed in triplicate in each experiment. Error bars represent SEM.

combination) at all dexamethasone concentrations ⩾ 100 nM (Bonferroni post-hoc $p < 0.001$). In both sets of experiments, there were no significant differences between cells transfected with full-length GRα, GRα-D1 or full-length GRα + GRα-D1 at any dexamethasone concentration. Thus, co-expression of GRα-D1 with full-length GRα did not significantly increase or decrease luciferase activity above or below the levels of luciferase activity induced by full-length GRα or GRα-D1 individually.

### Expression of GR-β and GR-'A' (Δ5–7) mRNA Transcript Variants

Endpoint PCR detection of GR-'A', a truncated GRα variant which is missing exons 5–7, was performed as a first step in determining whether the 67-kDa GRα isoform (IR band 2) may represent the GR-'A' isoform. A truncated transcript, missing exons 5–7, and consistent with GR-'A', was amplified in pooled schizophrenia/control DLPFC tissue. Some limited amplification from GR exon 2 (Figure 6a) and exon 3 (Figure 6b) to the exon 4–8 junction, was observed in DLPFC

tissue, whereas robust amplification with these same primers was achieved with universal human cDNA. In addition, transcripts containing the sequence from the exon 4–8 junction to exon 9α were readily detected in both the DLPFC and universal human cDNA (Figure 6c). In contrast, transcripts containing sequence spanning the exon 4–8 junction, but ending in exon 9β, were detected neither in DLPFC cDNA nor universal human cDNA, supporting our qPCR results (Figure 6d). In an attempt to quantify GR-'A' in our cohort, two custom GR-'A' Taqman qPCR primer/probes were tested. However, because the sequence around the exon 4/8 boundary was not amenable to Taqman primer/probe design, these custom probes failed to amplify GR-'A' in DLPFC, or positive control cDNA. As a result, GR-'A' expression in the schizophrenia cohort was not quantified.

GRβ expression in the DLPFC was also investigated. It was below the limit of detection in the prefrontal cortex by qPCR (Supplementary Figure S2B), so quantification in the entire cohort was not attempted. GRβ was detected in HeLa cell cDNA used as a positive control for the GRβ qPCR (Supplementary Figure S2C).
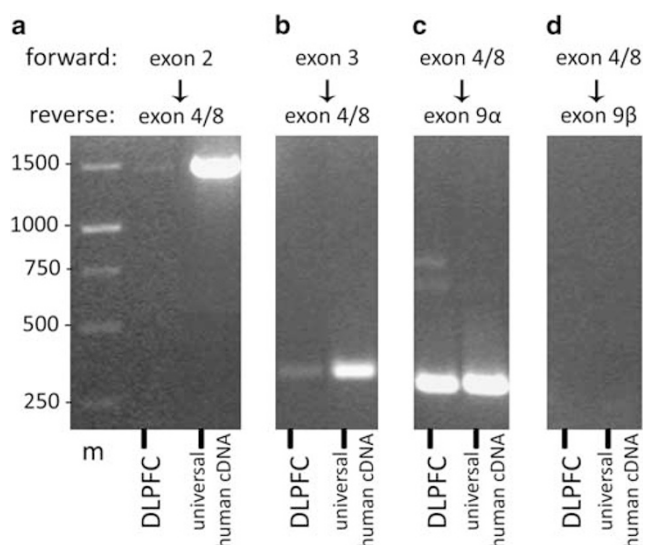
**Figure 6** PCR amplification of a glucocorticoid receptor (GR)-'A' (Δ5–7) transcript in dorsolateral prefrontal cortex (DLPFC) cDNA, using primers spanning the GR exon 4–8 junction. (a) Limited amplification was achieved from the start of GR exon 2 to the exon 4–8 junction; (b) some amplification was evident from the start of GR exon 3 to the exon 4–8 junction; (c) strong amplification was seen from the exon 4–8 junction to exon 9α. Robust amplification of these transcripts from universal human cDNA was observed. (d) No amplification from the exon 4–8 junction to exon 9β was seen using both DLPFC, and universal human cDNAs. Expected band sizes were 1480 bp (exon 2–exon 4/8), 289 bp (exon 3–exon 4/8), 254 bp (exon 4/8–exon 9α), and 209 bp (exon 4/8–exon 9β). m, marker.

## DISCUSSION

In this study, we provide further evidence that decreased GR mRNA expression in the DLPFC is a feature of schizophrenia. We also report an increase in abundance of the truncated GRα-D1 protein isoform in schizophrenia, based on observations from two schizophrenia cohorts. In the second cohort, we extended this finding to bipolar disorder, in which increased full-length GRα was also seen. We did not find evidence of altered MR mRNA expression in schizophrenia in this study, nor were we able to detect GRβ in the DLPFC of schizophrenia or control cases. The GR-'A' transcript variant, ending in exon 9α, was identified in the DLPFC in schizophrenia and control cases, but further work is required to determine if levels of this GRα transcript are altered in schizophrenia.

The observation of stable MR mRNA expression in the DLPFC in schizophrenia in this study is consistent with the other published report of MR mRNA expression in schizophrenia (Xing et al, 2004). Xing et al, (2004) reported decreased MR expression in Brodmann's area (BA)-9, but found no differences in BA46, which incorporates the region of the middle frontal gyrus sampled in this study. Although decreased MR mRNA expression has been only demonstrated in BA9, decreased GR mRNA expression has been observed in multiple brain regions implicated in the pathogenesis of schizophrenia (Perlman et al, 2004; Webster et al, 2002).

Two possible explanations for decreased GR mRNA expression in schizophrenia may be considered. First, it is possible that the decreased GR mRNA expression in schizophrenia arises due to increased basal cortisol levels caused by increased stress associated with the illness. GR expression has been shown to decrease under the influence of increased cortisol due to stress in both rodents (Sapolsky et al, 1984) and primates (Patel et al, 2008). Second, stress around birth and in infancy may be responsible for decreased GR mRNA in schizophrenia. Early life stress has been shown to decrease GR mRNA expression in both rodent and primate in multiple brain regions (Arabadzisz et al, 2010; Maniam and Morris, 2010; Meaney et al, 1996; Patel et al, 2008). Given the effect of early life stress on schizophrenia risk, it is possible that GR mRNA decreases are a hallmark of early life stress in the illness. Further work is required to determine the relative contributions of early life stress and later life stress to GR deficits in schizophrenia.

Analysis of cause of death revealed that decreased pan GR mRNA expression was driven by deficits in individuals with schizophrenia, who died of natural causes, and not by deficits in those who died by suicide. Increased GR mRNA expression associated with suicide has been previously reported in bipolar disorder cases in the frontal cortex, temporal cortex, and hippocampus (Webster et al, 2002). It may be that those with schizophrenia dying from suicide experience an increase in GR mRNA expression from a lower baseline state found in the disease. However, the mechanism underlying any effects of suicide on GR mRNA expression in schizophrenia is not known.

Although decreases in pan GR mRNA in schizophrenia were observed in this study, no decreases in GRα protein were observed. Unexpectedly, a significant increase in truncated GRα-D1 isoform abundance was detected in both schizophrenia and bipolar disorder. Levels of truncated GRα-D protein isoforms are not only regulated at the mRNA transcript level, but also by a translational mechanism. All GRα-D isoforms are generated, by means of ribosomal shunting, from the same mRNA transcript as the full-length GRα receptor (Lu and Cidlowski, 2005;see Figure 1). As a result, we would not expect to predict GRα-D isoform abundance based on levels of pan GR mRNA transcript alone. In the western blotting data in this study, there was appreciable variability, which was not predicted by demographic variables. This may arise in part due to the protein detection and quantification steps employed in the western blotting technique. The relative intensities of IR bands were consistent within each cohort's duplicate quantification runs (which used the same antibody batch), and were also consistent between the TRC and Stanley cohorts. However, relative intensities of IR bands were seen to vary between batches of antibody in other experiments (such as preabsorption), both for the P-20 antibody used here (also described in Sinclair et al, 2011), and for another GRα antibody tested (PA1–516, Thermo Fisher, Rockford, IL; data not shown). Despite the variability inherent in western blotting, reproducible diagnostic differences in GRα-D1 protein isoform abundance were observed.

A number of structural and functional properties of the truncated GRα-D1 isoform have been identified. GRα-D isoforms lack the entire N-terminus AF1 transactivation domain, which is present in the full-length GRα receptor (Lu and Cidlowski, 2005). In the COS-1 and U-2 OS cell lines, which lack endogenous GR, these GRα-D isoforms

have a decreased capacity to induce transcription at GREs in response to glucocorticoids (Lu and Cidlowski, 2005). However, in the CHOK1 cell line, which expresses GR endogenously, we demonstrate that the GRα-D1 isoform can activate transcription at GREs in response to dexamethasone to the same extent as the full-length GRα. The mechanism facilitating GRα-D1 transactivation in CHOK1 cells, but not COS-1 and U-2 OS cells, has yet to be identified. In agreement with previous work, we show that the full-length and truncated GRα isoforms do not function synergistically at GREs. The capacity of truncated GRα-D isoforms to induce transcription at GREs in response to glucocorticoids suggests a possible biological role for these isoforms in normal brain function and psychotic illness.

Endpoint PCR detection of the 'GR-A' transcript was conducted as a first step in determining whether the 67-kDa GRα isoform, which was abundant in the DLPFC, may represent the GR-'A' isoform. GR-'A' is a GRα variant that contains the C-terminus, features an in-frame deletion of exons 5–7, and is translated into a protein similar in size to the 67-kDa GRα protein isoform (Moalli *et al*, 1993). In this study, we robustly amplified an mRNA transcript in which exons 5–7 were deleted. Such a transcript was present in both pooled schizophrenia and control DLPFC tissue. Although GR-'A' expression was not quantified in this study, the presence of GR-'A' mRNA suggests that this isoform may be among the GRα isoforms detected by western blotting. It remains to be determined whether the 67-kDa GRα isoform observed in western blotting in this study represents the GR-'A' (Δ5–7) isoform, an N-terminal GRα isoform, or another GRα isoform altogether. GRβ mRNA was expressed below the level of detection in the DLPFC in this study, consistent with other studies in which GRβ was detected only at very low levels in human brain (DeRijk *et al*, 2003; Pujols *et al*, 2002).

Our findings confirm that altered cortical GR expression occurs in schizophrenia at the mRNA and protein levels, potentially impacting the responsivity of cortical neurons to stress-induced cortisol secretion. The GRα-D1 isoform, which is functionally active and elevated in schizophrenia and bipolar disorder, may represent a new molecular target to modify stress responsivity in schizophrenia.

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Supplementary Information accompanies the paper on the Neuropsychopharmacology website (http://www.nature.com/npp)