Author's response to reviews

Title: Glucocorticoid receptor mRNA and protein isoform alterations in the orbitofrontal cortex in schizophrenia and bipolar disorder

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Author's response to reviews: see over
The Editor,
BMC Psychiatry

Dear Sir, Madam,

Please consider our revised manuscript entitled ‘Glucocorticoid receptor mRNA and protein isoform alterations in the orbitofrontal cortex in schizophrenia and bipolar disorder’ by D Sinclair, M Webster, J Fullerton and C Shannon Weickert for publication as an original research paper in BMC Psychiatry.

We have responded individually to each of the issues raised by the reviewers, and made a number of changes to the manuscript. These are outlined in a point-by-point fashion below.

Thank you for considering our manuscript for publication in BMC Psychiatry.

Yours sincerely,

Duncan Sinclair

Reviewer 1

1. The Materials and Methods Section gives no details of diagnostic criteria used (DSM-IV? ICD-10?), and how donors were selected. Was a post-mortem review of the donor’s medical history performed to confirm the diagnosis? This is essential information that should be included. It is important to reassure the reader that in order to have such an impressively large number of post-mortem samples (35 schizophrenia, 34 bipolar disorder, and 35 controls) that there was no unnecessary loosening of the diagnostic criteria.

We appreciate this constructive feedback on our manuscript. A post-mortem review of the donors’ medical history was performed. In order to clarify how the cohort was compiled, we
have added the following paragraph to describe the diagnostic process employed by the Stanley Medical Research Institute (Methods, pp7-8):

‘Brain samples from the Stanley Foundation Array Cohort were collected by pathologists in the Office of the Medical Examiner in several states [42]. The selection process, clinical information, diagnoses of patients and processing of tissues have been described previously [42]. DSM-IV diagnoses were made independently by two senior psychiatrists based on medical records and, when necessary, telephone interviews with family members. Exclusion criteria included anyone over age 70, anyone with a history of seizures or other neurologic disorders that might affect brain pathology, and anyone with evidence of such conditions on neuropathologic examination.’

In addition, the diagnostic subtypes of individuals within each diagnostic group have now been included in Table 1 (see below).

**Table 1 - Demographic details of cases used in this study**

<table>
<thead>
<tr>
<th></th>
<th>Control group (n=35)</th>
<th>Bipolar disorder group (n=34)</th>
<th>Schizophrenia group (n=35)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diagnostic subtype</strong></td>
<td>-</td>
<td>BP1=27, BP2=4, BPNOS=2, schizoaffective=1</td>
<td>SCZ(disorganised)=1, SCZ(undifferentiated)=26</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>44.2 (31-60)</td>
<td>45.4 (19-64)</td>
<td>42.6 (19-59)</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td>9F, 26M</td>
<td>18F, 16M</td>
<td>9F, 26M</td>
</tr>
<tr>
<td><strong>Hemisphere</strong></td>
<td>16L, 19R</td>
<td>19L, 15R</td>
<td>17L, 18R</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td>6.61 +/- 0.27</td>
<td>6.43 +/- 0.30</td>
<td>6.48 +/- 0.24</td>
</tr>
<tr>
<td><strong>PMI (hours)</strong></td>
<td>29.4 +/- 12.9</td>
<td>37.9 +/- 18.6</td>
<td>31.4 +/- 15.5</td>
</tr>
<tr>
<td><strong>RIN</strong></td>
<td>7.23 +/- 0.87</td>
<td>7.34 +/- 0.88</td>
<td>7.36 +/- 0.61</td>
</tr>
<tr>
<td><strong>Manner of death</strong></td>
<td>natural=35</td>
<td>natural=19, suicide=15</td>
<td>natural=28, suicide=7</td>
</tr>
<tr>
<td><strong>Age of onset (years)</strong></td>
<td>-</td>
<td>25.3 +/- 9.2</td>
<td>21.3 +/- 6.1</td>
</tr>
<tr>
<td><strong>Duration of illness</strong></td>
<td>-</td>
<td>20.2 +/- 9.6</td>
<td>21.3 +/- 10.2</td>
</tr>
<tr>
<td><strong>Lifetime antipsychotics</strong></td>
<td>-</td>
<td>10212 +/- 22871</td>
<td>85004 +/- 100335</td>
</tr>
<tr>
<td><strong>(fluphenazine equivalents, mg)</strong></td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Antidepressant use</strong></td>
<td>yes=0, no=35</td>
<td>yes=19, no=15</td>
<td>yes=9, no=26</td>
</tr>
<tr>
<td><strong>Type of antidepressant</strong>*</td>
<td>-</td>
<td>SSRI=9 (fluoxetine=5)</td>
<td>SSRI=4 (fluoxetine=2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SNRI=0, SARI=2, TCA=6</td>
<td>SNRI=4, SARI=5, TCA=6</td>
</tr>
<tr>
<td><strong>Smoking around time of death</strong></td>
<td>yes=9, no=9, unknown=17</td>
<td>yes=15, no=6, unknown=13</td>
<td>yes=23, no=4, unknown=8</td>
</tr>
</tbody>
</table>
Demographic details of controls, bipolar disorder cases and schizophrenia cases used in this study. *Note- some individuals took multiple antidepressant medications. Abbreviations: BP1=bipolar disorder type 1, BP2=bipolar disorder type 2, BPNOS=bipolar disorder not otherwise specified, SCZ=schizophrenia, M=male, F=female, L=left, R=right, PMI=post-mortem interval, RIN=RNA integrity number, SSRI=selective serotonin reuptake inhibitor, SNRI=serotonin-norepinephrine reuptake inhibitor, SARI=serotonin antagonist and reuptake inhibitor, TCA=tricyclic antidepressant. Data quoted are mean (range) ± standard deviation.

2. The authors observe a decrease in transcripts containing exons 1B and 1H. However, total GR levels remain unchanged. Is there an explanation for this, given that the other first exons, including 1D, 1E, and 1F were present, but below the minimum detectable level (MDL) of their PCR assay? Was any attempt made to see if, for example, exon 1F increased to levels above the MDL, especially since Yau et al have shown that the rodent orthologue of exon 1F, exon 1-7, is upregulated by antidepressants.

We agree that quantification of GR-1F may clarify the observation of unchanged pan GR mRNA levels despite decreased GR-1B mRNA in bipolar disorder. When initial attempts were made to quantify GR-1F mRNA expression by qPCR, no successful amplification from OFC cDNA was observed in any of the subset of samples tested, nor in universal human cDNA. Therefore, we have redesigned the GR-1F Taqman probe, in case this arose due to failure of the probe, rather than absence of the transcript. Using this new probe, we have successfully quantified GR-1F mRNA expression in the lateral OFC. We did not find significant diagnostic differences in GR-1F mRNA expression. This is described on p13:

‘A trend towards decreased GR-1F mRNA expression in schizophrenia and bipolar disorder cases was seen [ANCOVA F(2, 90)=2.56, p=0.08, co-varying for RIN and brain pH; Figure 2D], but no significant group differences were observed by post-hoc test.’

However, an effect (of trend-level significance) of suicide on GR-1F mRNA expression was seen. This is described on p14:

‘A trend towards an effect of suicide on GR-1F mRNA was also seen [ANCOVA F(4, 88)=1.92, co-varying for RIN, p=0.11; Figure 2G]. By post-hoc test, suicide-negative bipolar disorder cases displayed 24.6% lower GR-1F mRNA than controls (p<0.05), and 25.6% lower than suicide-positive bipolar cases (p<0.05).’

As a result of these findings, we believe that changes in GR-1F, GR-1C or GR-1H mRNA expression do not account for the fact that total GR mRNA levels were stable despite decreased GR-1B expression in bipolar disorder. We believe that levels of GR-1A, GR-1D and GR-1E are very low in the lateral OFC (please see new Figure 1, included below in responses to reviewer 2). Therefore, we believe that diagnostic differences were not present in pan GR mRNA expression, because decreases in GR-1B mRNA expression may have been diluted by other transcript variants such as GR-1C. We have drawn on calculations of relative proportions of GR transcripts (please see point 5 below), and added the following statement to the discussion on p19:
‘Although GR-1B expression (which represents approximately 32% of total measured GR mRNA) was decreased in bipolar disorder, there was no difference in pan GR mRNA levels between bipolar disorder cases and controls. This may arise because other GR mRNA transcripts including GR-1C (which represents approximately 66% of total measured GR mRNA) may have diluted this diagnostic effect, despite themselves being unchanged in bipolar disorder.’

3. Similarly, since it has been previously reported that anti-depressant medication, especially fluoxetine affects GR transcript levels more detailed information on antidepressants taken would be appreciated in Table 1. This is important since the regulation of GR was specific to fluoxetine. If there are enough donors, an analysis of the effect of fluoxetine on GR transcript levels is warranted. Similarly, if in any donors exon 1F levels are above the MDL, the effect of fluoxetine intake on GR 1F levels should be investigated.

We have now added further information about the antidepressant use of cases in the cohort to Table 1 (see above). There were a total of 7 individuals taking fluoxetine (5 bipolar disorder, 2 schizophrenia). A significant effect of fluoxetine on GR-1C, but not GR-1F, mRNA expression was seen, as described on pp14-15:

‘When the schizophrenia and bipolar disorder groups were combined and divided according to fluoxetine use, no group differences in pan GR, GR-1B, GR-1F or GR-1H GR mRNA expression of individuals on fluoxetine (n=7), compared with individuals not on fluoxetine (n=58), were observed. However a significant 16.3% increase in GR-1C mRNA expression in individuals on fluoxetine (n=7) relative to with individuals not on fluoxetine was seen [ANCOVA F(1, 59)=6.72, p<0.05].’

This is further discussed on p20:

‘The effects of antidepressant use in general, and fluoxetine use in particular, were explored in this study, since previous work has showed selective effects of fluoxetine on total GR and GR-1F mRNA expression in rodent hippocampus [50]. In our study, GR-1C mRNA expression was increased in fluoxetine users relative to non-users. The direction of this change is consistent with the previous study. However, unlike previously reported, we observed no effects of fluoxetine on GR-1F mRNA expression in the OFC, suggesting that antidepressant effects may vary between species and/or brain regions.’

4. Given the significance of the isoform specific upregulation of GRalpha-D1, the authors should include an extra lane in Figure 2B where the antibody has been pre-incubated with its cognate (blocking) peptide to demonstrate the specificity of the GR immunoreactivity.

Pre-incubation of the P-20 anti-GRα primary antibody with blocking peptide has been performed and reported in two of our previous published papers [1, 2]. The antibody batch used for preabsorption in our recent paper in Neuropsychopharmacology was the same as the batch used in this study, although different brain samples (from the DLPFC) were used (please see figure below, taken from [1]).
Figure S1 [1]. Preabsorption of GRα antibody sc1002X with blocking peptide. Increasing ratios (vol:vol) of blocking resulted in almost complete amelioration of antibody binding, while increasing ratios of unrelated peptide had no effect. IR- immunoreactive, kDa- kilodalton, Ab-antibody, Con- control case, Scz- schizophrenia case.

Therefore, we are confident of the specificity of this antibody. We have added a statement in the manuscript referring to, and referencing, the pre-incubation performed in the above study as follows (pp9-10):

‘Antibody specificity has been previously demonstrated, with amelioration of GRα immunoreactivity by pre-incubation of this same P-20 antibody batch with blocking peptide [1].’

5. The importance of the 1H transcript needs to be discussed. Previous reports suggest that in the 5 brain regions so far studied exon 1H represents only 1-3% of the total GR transcripts (Alt et al 2010). The proportion of the total GR represented by exon 1H should be measured, and if the levels are as low as previously reported, then the relevance of the down regulation of such a small percentage of the total GR should be discussed.

We agree that the GR mRNA changes we report need to be interpreted in the context of the relative abundances of GR variants in the lateral OFC. Therefore, we have used the ∆∆Ct method to approximately compare the relative abundances of GR mRNA transcript variants. We found that GR-1B, GR-1C, GR-1F and GR-1H represented approximately 31.6%, 65.6%, 1.6% and 1.2% of total measured GR mRNA in the OFC respectively. This data is now included in Figure 2F, and on p14:

‘The relative abundances of each GR mRNA transcript variant, relative to total measured GR mRNA, were estimated within each individual. On average, the GR-1B, GR-1C, GR-1F and GR-1H mRNA transcript variants represented approximately 31.6%, 65.6%, 1.6% and 1.2% of total measured GR mRNA in the OFC respectively (Figure 2F).’
These figures are approximately consistent with the endpoint PCR used to detect the variants (please see responses to reviewer 2 below, and new Figure 1). The importance of GR-1H mRNA changes in schizophrenia, given the small portion of total GR represented by this variant, are now discussed on p19:

‘GR-1H mRNA represents only a small fraction (approximately 1.2%) of total measured GR mRNA in this study, as in other studies [3]. As a result, the selective GR-1H mRNA deficits which we observe may have limited impact on GR signalling in the lateral OFC in schizophrenia.’

6. The authors observe significant down regulation of transcripts containing exons 1B and 1H. The promoters of both exons were genotyped for rs 5871845, and rs10482614 respectively. However the functional promoter 1B SNPs rs3806854 and -5 were not investigated, neither were the additional SNPs in promoter 1H (rs11244544 and rs112794517). These should be investigated and included in the manuscript.

We appreciate that other polymorphisms, particularly those in the 1B and 1H promoter regions, may prove interesting to explore. When designing the genotyping experiment, we chose SNPs with >10% minor allele frequency, focusing on functional SNPs or those in the vicinity of the 1B promoter. We did attempt to genotype the rs3806854 and -5 SNPs, but unfortunately they were not compatible with other SNPs in the multiplex assay. We did not explore 1H promoter SNPs, however in future studies we intend to continue to explore the effect of these and other NR3C1 polymorphisms on GR gene and protein expression.

7. Please use the name of the antibody clone used for the Western Blot, rather than the catalogue number, as the P20 clone is a well known clone (p9 line 9).

The name of the antibody clone has now been added to the text (p9):

‘The P-20 anti-GRα primary antibody (sc-1002X, Santa Cruz Biotechnology, Santa Cruz, CA) was used for detection of GRα in this study.’

8. Previously the authors have reported differences in the intensity of the different molecular weight GR immunoreactive band with different batches of the P20 antibody. The authors need to confirm that all their western blots were performed using the same batch of antibody. (Sinclair et al 2011)

All western blotting runs were conducted with the same batch of each antibody, including P-20 anti-GRα primary antibody. This is now referred to in the text on p10:

‘Duplicate samples were run in separate experimental runs, with the same batch of each antibody used for all runs’

9. The legend to Figure 2 needs to include all the abbreviations used, especially since Bp (in this case I assume Bipolar disorder) is often used in western blots to indicate that the antibody has been pre-included with a blocking peptide.

The abbreviations used in Figure 2 (now Figure 3) have now been added to the figure legend: ‘Abbreviations: Bp- bipolar disorder, Scz- schizophrenia, Con- control’.
Reviewer 2

1. The authors should show the data for the endpoint PCR that they describe indicates the subset of GR splice variants most robustly expressed in the OFC, because these data provide a rationale for proceeding with the analysis of only these splice variants.

We agree that this data should be provided, and have added an additional figure (Figure 1), which contains the endpoint PCR results of GR exon 1 variant detection in OFC cDNA and universal human cDNA.

![Figure 1 – Endpoint PCR detection of GR exon 1 mRNA transcript variants in the OFC](image)

- A) Strong amplification of the GR-1B, GR-1C and GR-1H variants was evident in OFC cDNA and in universal human cDNA.
- B) Weaker amplification of the GR-1F variant was observed in the OFC, with strong amplification in universal human cDNA.
- C) No amplification of GR-1E was seen in OFC cDNA, while weak amplification was seen in universal human cDNA.
- D) No amplification of GR-1A or GR-1D in OFC or universal human cDNA was detected. Univ- universal human cDNA, bp- base pairs.

The description in the text is as follows (p12):
‘We determined by endpoint PCR that the GR-1B, GR-1C, GR-1F and GR-1H mRNA transcript variants are expressed in the OFC (Figure 1). The GR-1B, GR-1C, GR-1F and GR-1H mRNA transcripts were abundant in universal human cDNA, which was used as a positive control. GR-1E was not detected in OFC tissue but was present in universal cDNA. GR-1A1-3 and GR-1D were not detected in OFC tissue or universal cDNA (Figure 1).’

2. Please fix reference #41.

We have updated reference #41, which has been published since initial submission of this manuscript.

3. We seem to be missing a piece of the puzzle – that of the relationship between the untranslated splice variants shown to altered and the expression of the GRα-D1 isoform. Is there a known relationship between these?

We agree that it would be helpful to include a brief analysis and discussion of the relationships between GR mRNA and protein measures in the lateral OFC. Therefore, we have included an analysis of the correlations between levels of GR mRNA transcript variants and GRα protein isoforms on p16:

‘GR mRNA expression levels in the lateral OFC did not correlate significantly with abundance of GRα protein isoforms, with the exception of pan GR mRNA expression, which correlated positively with GRα IR band 5 abundance (r=0.23, p<0.05).’

The discussion of the relationship between GR mRNA expression and GRα protein isoform abundance on p20 has been expanded to include mention of this finding:

‘In vitro experiments have revealed that the abundance of the GRα-D1 isoform is determined not only by mRNA transcript levels, but also by post-transcriptional mechanisms [2, 4]. Consistent with these findings, over-expression of the GRα-D1 isoform was observed in both the DLPFC and lateral OFC in bipolar disorder and schizophrenia, despite divergent patterns of GR mRNA dysregulation in both regions. The absence of consistent correlations between GR mRNA and GRα protein measures in this study also suggests post-transcriptional regulation of GRα protein abundance.’

4. One possible implication of the differences seen among the groups in the absence of genotype differences is the presence of epigenetic alterations, which may deserve mention here, as they have been shown in human and animal studies to be involved in the regulation of GR as a function of early life factors.

In recognition of the potential role of GR promoter methylation in mediating GR mRNA deficits, the following sentence has been added to the discussion on p18:

‘GR promoter methylation may also play a role, since GR promoter hyper-methylation has been associated with decreased GR-1B mRNA expression in the hippocampus of child abuse sufferers [5].’
References


