Changes in levels of cortical metabotropic glutamate 2 receptors with gender and suicide but not psychiatric diagnoses.

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\textbf{Declaration of Interests:} None

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Abstract

Background: We previously reported that, compared to controls, there are lower levels of \[^3\text{H}]\text{LY341495} binding to metabotropic 2/3 receptors (GRM2/3) in Brodmann’s area (BA) 24, but not 17 or 46, from subjects with major depressive disorders (MDD) but not bipolar disorders (BD) or schizophrenia. To be able to better interpret these data we have now measured levels of GRM2 in two of these cortical regions.

Methods: Using a rabbit anti-metabotropic GRM2 monoclonal antibody with Western blotting we measured levels of GRM2 in BA 24 and 46 from subjects with MDD, BD, schizophrenia and controls (n = 15 per group).

Results: Compared to controls, levels of GRM2, normalised to β-actin, did not differ in BA 24 or 46 from subjects with MDD, BD or schizophrenia (p from 0.36 to 0.79). Levels of GRM2 in BA 46, but not BA 24, were significantly higher in males compared to females (p < 0.01) and in suicide completers (p < 0.01) compared to death by other causes.

Limitations: Our cohort sizes, whilst being comparable to many postmortem CNS studies, are relatively low.

Conclusions: Our data suggests levels of GRM2 are not altered in two cortical regions from subjects with mood disorders or schizophrenia. Given we have found lower levels of \[^3\text{H}]\text{LY341495} binding to GRM2/3 in BA 24 from subjects with MDD, our new data argues the lower levels of radioligand binding was due to lower levels of GRM3. Our data also suggests that glutamatergic activity through GRM2 in BA 46 may differ with gender and suicide ideation.
Introduction

We have recently reported that, compared to controls, there are lower levels of $[^3]H$LY341495 binding in Brodmann’s area (BA) 24, but not BA 17 or 46, from subjects with major depressive disorders (MDD) (McOmish et al., 2016). $[^3]H$LY341495 binding was not altered in those three cortical regions from subjects with bipolar disorders (BD) or schizophrenia. As $[^3]H$LY341495 binds to the mammalian metabotropic glutamate receptors (GRM) 2 and 3 (Johnson et al., 1999; Wright et al., 2001), our data is consistent with the notion that there is a decrease in the total number of GRM2 plus GRM3 in BA 24 from subjects with MDD. Our finding of lower levels of $[^3]H$LY341495 binding in BA 24 from subjects with MDD did not agree with a previous report that levels of $[^3]H$LY341495 binding was not different in BA 24 from subjects with psychotic and non-psychotic depression, BD or schizophrenia (Matosin et al., 2014).

In addition to radioligand binding, a number of studies have measured levels of total GRM2 plus GRM3 protein or GRM2 and GRM3 mRNA in the cortex of subjects with psychiatric disorders. Using antibodies that could not discriminate between GRM2 and GRM3, studies have reported higher levels of total GRM2/3 in BA 10 from subjects with MDD (Feyissa et al., 2010) and either no change (Crook et al., 2002) or higher (Gupta et al., 2005) levels of total GRM2/3 in BA 46 from subjects with schizophrenia. In addition, a study has reported lower levels of GRM3, but not GRM2, mRNA in BA 9 and no changes in levels of GRM2 or GRM3 mRNA in BA 4, 7, 11, 19, 24 and 38 from subjects with schizophrenia (Ghose et al., 2008). Hence, despite radioligand binding, mRNA and protein studies, there is a relative paucity of data specific to levels of cortical GRM2 or GRM3 in the cortex of subjects with psychiatric disorders. In addition, there appears to be no data on individual levels of GRM2 or GRM3 protein in the cortex of subjects with psychiatric disorders.
Given the lack of data on levels of GRM2 and GRM3 protein, and our data on $[^3]H$LY341495 binding, we decided to measure levels of GRM2 in the BA 24 and 46 from subjects with MDD, BD, schizophrenia and a group of age and sexmatched controls.
Experimental procedures

Tissue collection:

Consent to collect CNS tissue postmortem was from the Ethics Committee of the Victorian Institute of Forensic Medicine. Tissue collection and processing, case history reviews and diagnostics was as reported previously (McOmish et al., 2016). Hence, case histories were reviewed using the Diagnostic Instrument for Brain studies (Roberts et al., 1998); these data were used by two psychiatrists and a senior psychologist to reach a census diagnoses based on DSM IV criteria.- For each case, the left CNS hemisphere was collected, sliced according to a standardised protocol and frozen at -80°C to minimise autolytic degradation. Tissue was excised from Brodmann’s area (BA) 46, which is the lateral surface of the frontal lobe that is approximately constrained to the middle of the third of the middle frontal gyrus and the most rostral inferior frontal gyrus and BA24, which is the ventral anterior cingulate gyrus, around the most anterior aspect of the corpus callosum, from subjects with MDD (n = 15), BD (n = 15), schizophrenia (n = 15) and 15 subjects with no history of psychiatric disorders (Controls).

Western Blotting

For Western blotting, crude homogenates were prepared by homogenising tissue from BA 24 and BA 46 into 5% w/v in 10 mM Tris (pH 7.4) containing 1% SDS and 1 mM Na$_3$VO$_4$. The protein concentrations in these homogenates was determined using the Bio-Rad DC modified Lowry protein assay adapted for microplates prior to storage and stored at -80°C until required. On the day of electrophoresis was completed, samples of homogenates containing 15 µg protein were boiled for 5 mins before being resolved on a 10% polyacrylamide gel. To minimize
the impact of gel to gel variation between diagnoses, duplicate samples from a case from each diagnostic group and a matched control were loaded in consecutive lanes on the same gel (Supplementary Figure 1A). Molecular weight standards were resolved in a lane of each gel.

After electrophoresis, proteins were transferred on to nitrocellulose membranes (and protein transfer was confirmed by staining each membrane with 0.2% Ponceau S in 3% trichloroacetic acid). Each membrane was then blocked with 5% non-fat milk powder in 1X TTBS for 1hr at RT and the membranes were then incubated overnight at 4°C with rabbit anti-metabotropic GRM2 monoclonal antibody (#ab150387, Abcam) at 1:5000 dilution in 5% non-fat milk powder in 1X TTBS. Each membrane was then washed four times in 1X TTBS, and then incubated with horse radish peroxidase (HRP) conjugated goat anti-rabbit immunoglobulin antibody (1:2000, DAKO, Glostrup, Denmark; #P0448) for 1hr at RT and then washed four times in 1X TTBS. Antigenic bands were visualised using Pierce Supersignal West Pico chemiluminescent substrate (Thermo Scientific, Waltham, MA, USA) and an image of the chemiluminescent intensity measured was captured with a UVP BioSpectrum imaging system (Analytik Jena, Jena, Germany). Sum pixel intensities for mGluR2 were measured using the VisionWorks® Image Acquisition Analysis Software.

To measure for the analyses of β-actin, the nitrocellulose membranes were washed in distilled water for 5 mins at RT and then blocked with 1X TTBS for 1hr at RT. The membranes were then incubated with mouse anti-β-actin monoclonal antibody (MAB1501, Merck) at 1:200,000 dilution for 1hr at RT and then washed four times in 1X TTBS. Finally, the membranes were incubated with HRP-conjugated goat anti-mouse immunoglobulin antibody (1:4000, DAKO, Glostrup, Denmark; #P0447), washed four times in 1X TTBS and the antigenic bands visualised and quantified as per the GRM2 protocol.
All data were converted to the sum intensity of GRM2 normalised to the sum intensity of β-actin.

Statistics

Using GraphPad Prism, variance in demographic, clinical and CNS related collection and processing data with diagnoses was assessed using one-way ANOVA and sources of potential variance identified with a post hoc Dunnett’s tests comparing diagnostic groups to controls whilst frequency of gender and suicide completion was compared using the \(\chi^2\) test (GraphPad Prism).

As To accommodate the analyses of GRM2 data was normalised data (Dean et al., 2016b), all experimental data was analysed using the Mann-Whitney test in Graphpad Prism or a non-parametric one way ANOVA with appropriate covariates (Dean et al., 2016b; Quade, 1967) using Minitab 18. Spearman’s-rank correlations were used to identify correlations between levels of cortical GRM2 and demographic, clinical and CNS processing data.
Results

Antibody Validation

The strongest immunogenic band in human cortex for identified using the rabbit anti-GRM2 monoclonal antibody was ~ 95 kilodaltons (Supplementary Figure 1A), GRM2 the predicted molecular weight of GRM2. This antibody showed no immunogenic reaction with any protein in human serum or when human cortex was probed in the absence of either primary or secondary antibody. In addition, the immunogenic band of the same molecular weight was present in the membrane, but not the cytosolic, fraction of human cortical homogenate; consistent with GRM2 being a membrane bound protein (Niswender and Conn, 2010). As the predicted molecular weight of GRM3 is 99 kilodaltons, our data suggests measuring the intensity of the 95 kilodalton protein after Western blotting is a measure of the level of GRM2.

Potential Study Confounds

There were no significant differences in age (p = 0.98), sex ratio (p = 0.92), PMI, CNS pH (p = 0.94), PMI (p = 0.66) or DI (p = 0.60) did not vary with diagnoses (Table 1). There was a significant variation in the rates of suicide completion varied with diagnoses, whether or not data from all subjects or just those with psychiatric disorders were analysed.

Levels of GRM2 were higher in BA 46, but not BA 24, from males compared to females (Figure 4B1A, Supplementary Table 1A) and suicide completers compared to non-suicide those who died of other causes (Figure 4C1B, Supplementary Table 1B). Further analyses suggested there was no inter-relationship between the higher levels of GRM2 in males and in suicide completers (Supplementary Table 1D and E). There were no significant correlations between levels of GRM2
and age (BA 24 $r = -0.06$, $p = 0.66$; BA 46 $r = -0.16$, $p = 0.23$), CNS pH (BA 24 $r = 0.12$, $p = 0.36$; BA 46 $r = 0.18$, $p = 0.18$), PMI (BA 24 $r = 0.02$, $p = 0.90$; BA 46 $r = 0.06$, $p = 0.60$) or DI (BA 24 $r = 0.02$, $p = 0.90$; BA 46 $r = -0.29$, $p = 0.06$).

In respect to the data on potential confounding factors, subsequent analyses of data with respect to diagnoses was completed using gender and suicide as covariates.

*Levels of GRM2 in the cortex of subjects with psychiatric disorders*

There was no significant variance in levels of GRM2 in BA 24 or BA 46 with psychiatric diagnoses including (Figure 1D1C) or excluding covariates. Notably, there was also no variation in levels of GRM2 in BA 24 ($p = 0.69$) or BA 46 ($p = 0.55$) if the covariates of gender and suicide were omitted from the analyses.
Discussion

Our main finding is that, compared to controls, there were no differences in levels of GRM2 in BA 24 or BA 46 from subjects with MDD, BD or schizophrenia. We had previously reported lower levels of [³H]LY341495 binding in BA 24, but not BA 17 or 46, from subjects with MDD (McOmish et al., 2016). As [³H]LY341495 binds to both GRM2 and 3 (Johnson et al., 1999; Wright et al., 2001) our new data argues that the lower level of binding in BA 24 in subjects with MDD would be consistent with lower levels of GRM3 in that cortical region.

Our finding of no change in GRM2 protein levels in BA 24 and 46 from subjects with mood disorders adds to the finding of no change in GRM2 mRNA in the dorsolateral prefrontal cortex from subjects with MDD (Gray et al., 2015). Our finding also adds to the finding of an earlier study reporting no change in GRM2 mRNA in BA 4, 7, 9, 11, 19, 24 and 38 from subjects with schizophrenia (Ghose et al., 2008) to suggest there are no changes in either mRNA or protein from GRM2 in the cortex of subjects with that disorder. In addition, our data partly agrees with a study reporting that found no change in GRM2/3 protein levels in BA 46 from subjects with schizophrenia (Crook et al., 2002) or and no change in either monomeric or dimeric forms of GRM3 in the superior temporal cortex (Garcia-Bea et al., 2016) from subjects with schizophrenia. One another study has reported higher levels of GRM2/3 protein in BA 46 from subjects with the schizophrenia disorder (Gupta et al., 2005); our new data suggests the higher levels of immunogenicity in that study was must have been due to higher levels of GRM3. However, such a hypothesis is not consistent with our finding of no differences in levels of [³H]LY341495 binding to CHRM 2/3 in BA 46 in schizophrenia from subjects with schizophrenia. Our data does not allow comment on a report of higher levels of GRM2/3 in BA 10 from subjects with MDD (Feyissa et al., 2010).
Our report appears to be the first to suggest gender differences in levels of GRM2 in human cortex. Gender-related changes in levels of GRM2 may be associated with GRM2/3 activity being sensitive to the effects of oestrogen (Boulware et al., 2005) but such a general observation does not explain why there should be cortical region specific changes (i.e. BA 46 but not BA 24) in levels of GRM2.

This study may also seem to be the first to report increased levels of GRM2 protein in BA 46 from suicide completers and extends the finding of an earlier finding showing higher levels of GRM2 expression in dorsolateral prefrontal cortex from MDD suicide completers (Gray et al., 2015). We have previously reported higher levels of serotonin 1D receptor (BA 9) (Dean et al., 2006) and mRNA encoding the glutamate ionotropic receptor NMDA type subunit 2β (BA 10) (Dean et al., 2016a) as well as lower levels of mu opioid receptor (BA 24) (Scarr et al., 2012), serotonin 2A receptors (BA 46) (Dean et al., 2014), phospholipase C beta 1a (BA 9) (Udawela et al., 2017) and mRNA encoding the glutamate ionotropic receptor NMDA type subunit 2β (BA 24) (Dean et al., 2016a) in suicide completers. Hence, our new findings on GRM2 adds to the previous data to suggest there are complex changes the molecular architecture of the cortex of suicide completers, which in BA 46, may be particularly impacting on glutamatergic activity; a neurotransmitter that has been suggested to be affected in the CNS of suicide completers (Ernst et al., 2009; Sequeira et al., 2009).

Strengths and limitations

Strengths of this study are the use of a validated antibody and tissue from a well-recognised brain bank to, for the first time, measure levels of GRM2 protein in cortex from subjects with mood disorders and schizophrenia. Limitations of this study are that, although cohort sizes are typical of many studies of mood disorders they are still relatively small diagnostic cohorts which limits
exploration of differences in gender and suicide completion within psychiatric group. There is potential for drug treatment effects during life as a confound.

Conclusions

In conclusion, our data suggests there are higher levels of GRM2 in BA 46 from males compared to females and in suicide completers. Combining our the data showing no change in GRM2 in BA 24 from subjects with MDD with our data showing lower levels of $[^3H]LY341495$ binding to GRM2/3 suggest that GRM3 must be lower in BA 24 from subjects with MDD. Our data adds to the notion that others supporting a role for altered glutamatergic neurotransmission contributing to an increased level of suicide completion (Ernst et al., 2009).
Role of funding source

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References


Garcia-Bea, A., Walker, M.A., Hyde, T.M., Kleinman, J.E., Harrison, P.J., Lane, T.A., 2016. Metabotropic glutamate receptor 3 (mGlu3; mGluR3; GRM3) in schizophrenia: Antibody characterisation and a semi-quantitative western blot study. Schizophr. Res. 177, 18-27.


Matosin, N., Fernandez-Enright, F., Frank, E., Deng, C., Wong, J., Huang, X.F., Newell, K.A., 2014. Metabotropic glutamate receptor mGluR2/3 and mGluR5 binding in the anterior cingulate


Figure 1:

A—An image of a typical nitrocellulose membrane showing the immunogenic band used as the measure of the level of the metabotropic glutamate receptor (GRM2) in BA 24 and BA 46.

B-D—Levels (median ± interquartile range) of GRM2 normalise to β-actin in BA 24 and B 46 from females and males (BA), suicide completers and those dying from other causes including controls (CB) as well as subjects with mood disorders, schizophrenia—and age-sex matched controls (DC). **p < 0.01.
Table 1: Relevant details relating to the demographics and CNS collection details for subjects with schizophrenia, major depressive disorders, bipolar disorders, schizophrenia and age / gender matched controls used in this study.

<table>
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<th></th>
<th>Gender (f / m)</th>
<th>Age (yr)</th>
<th>CNS pH</th>
<th>PMI (Hr)</th>
<th>Suicide (Y / N)</th>
<th>Duration of illness (yr)</th>
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<td>Controls</td>
<td>8 / 7</td>
<td>61 ± 3.3</td>
<td>6.28 ± 0.06</td>
<td>44 ± 4.2</td>
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<td>58 ± 3.2</td>
<td>6.29 ± 0.04</td>
<td>39 ± 3.8</td>
<td>5 / 10</td>
<td>18 ± 3.1</td>
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<td>Major Depressive Disorders</td>
<td>8 / 7</td>
<td>60 ± 4.0</td>
<td>6.56 ± 0.04</td>
<td>44 ± 3.9</td>
<td>12 / 3</td>
<td>17 ± 2.4</td>
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<tr>
<td>Schizophrenia</td>
<td>8 / 7</td>
<td>56 ± 3.9</td>
<td>6.27 ± 0.06</td>
<td>47 ± 51</td>
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<td>F or χ²</td>
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<td>0.13</td>
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<td>p</td>
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Abbreviations: f = female, gms = grams, Hr = hours, m = male, N = no, Y = yes, yr = years.
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