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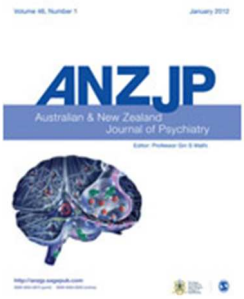

Changes in Cortical N-methyl-D-aspartate receptors and Postsynaptic Density Protein 95 in Schizophrenia, Mood Disorders and Suicide

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Changes in Cortical ~~the~~ N-methyl-D-aspartate receptors and Postsynaptic Density Protein 95 in Schizophrenia, Mood Disorders and Suicide

Running Title: Changes in Cortical NMDA Receptors and PSD 95 Protein in Schizophrenia, Mood Disorders and Suicide

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Abstract

Objectives: In humans, depending on dose, blocking the N-methyl-D-aspartate receptor (NMDAR) with ketamine can cause ~~has been shown to be either~~ psychomimetic or ~~to have~~ antidepressant effects. The overall outcome for drugs such as ketamine is depends dose and the number of its available binding sites in the CNS and to understand something of the latter variable we measure NMDAR in the frontal pole, dorsolateral prefrontal (DLPFC), anterior cingulate (ACx) and parietal cortices from people with schizophrenia, bipolar disorder (BD), major depressive disorders (MDD) and age / sex matched controls.

Method: We ~~The ketamine data in humans led us to~~ measured levels of NMDARs (using [^{3}H]MK-801 binding) and NMDAR sub-unit mRNAs (GRINS: using *in situ* hybridisation) as well as ~~in the frontal pole as well as the dorsolateral prefrontal (DLPFC), anterior cingulate (ACx) and parietal cortices from people with schizophrenia, bipolar disorder (BPD~~BD), ~~major depressive disorders (MDD) and age / sex matched controls. We also measured levels of~~ post-synaptic density protein 95 (PSD 95: ACx only; not MDD: a NMDAR post-synaptic associated protein) ~~in , a NMDAR post-synaptic associated protein, in ACx from people with BPD~~BD, schizophrenia and controls.

Results: Compared to controls, levels of NMDAR were lower in lamina I to III plus a portion of laminae IV in DLPFC (-17%, $p = 0.01$) in people with schizophrenia. In ~~BPD~~BD, levels of NMDAR in lamina IV – VI (-19%, $p < 0.01$) and GRIN2C mRNA (-27%, $p < 0.05$) were lower in the ACx and NMDAR was lower in lamina I to III plus a portion of laminae IV in the DLPFC (-19%, $p < 0.01$). In MDD, levels of GRIN2D mRNA were higher in frontal pole (+22%, $p < 0.05$). In suicide completers, levels of GRIN2B mRNA were higher in parietal cortex (+20%, $p < 0.01$) but lower (-35%, $p = 0.02$) in DLPFC whilst PSD 95 was higher (+26%, $p < 0.05$) in ACx.

Conclusions: These data suggest differences ~~diagnostic specific changes~~ in cortical NMDAR expression and PSD 95 are present in ~~could contribute to the aetiologies of~~ psychiatric disorders and suicide completion and may contribute to ~~, suicidal ideation and~~ different responses to ketamine.

Keywords: schizophrenia major depressive disorders bipolar disorders cortex NMDA

Highlights

- Lower NMDAR receptors in lamina I to III plus a portion of laminae IV in DLPFC from people with schizophrenia (-17%, $p = 0.01$).
- Lower NMDAR in laminae IV - VI in ACx (-19%, $p < 0.01$) and lamina I to III plus a portion of laminae IV in DLPFC from people with bipolar disorders (-19%, $p < 0.01$).
- Lower GRIN2C mRNA in ACx from people with bipolar disorders (-27%, $p < 0.05$).
- Higher GRIN2D mRNA in frontal pole from major depressive disorders (+22%, $p < 0.05$).
- Higher GRIN2B mRNA in parietal cortex (+20%, $p < 0.01$) from suicide completers.
- Lower GRIN2B mRNA in DLPFC (-35%, $p = 0.02$) from suicide completers.
- Higher levels of PSD 95 (+26%, $p < 0.05$) in ACx from suicide completers.

Abbreviations: ACx = anterior cingulate cortex, BA = Brodmann's area, ~~BPD~~BD = bipolar disorders, CNS = central nervous system, DLPFC = dorsolateral prefrontal cortex, DI = duration of illness, ETE = estimated tissue equivalent, fmol / g = femtomoles mRNA per gram tissue, FRADD = final recorded dose of antipsychotic drug, IC = internal control, LEAP = lifetime exposure to antipsychotic drugs, MDD = major depressive disorder, NMDAR = N-methyl-D-aspartate receptor, NSB = non-specific binding, PMI = post-mortem interval, PSD 95 = post synaptic density 95, TB = total binding.

1.0 Introduction

In humans, ketamine has been shown to have rapid acting antidepressant effects and to be psychomimetic (Abdallah et al., 2015).~~Recent studies have shown that, in humans, ketamine can be psychotomimetic (Malhotra et al., 1997) or have antidepressant effects (aan het Rot et al., 2012).~~ Significantly, the major action of ketamine is to block the N-methyl-D-aspartate receptor (NMDAR), an ionotropic glutamate receptor (Sucher et al. 1996). ~~Thus, these neuropsychopharmacological data add significant weight to the argument that the activity of the NMDAR can contribute to the severity of psychiatric symptoms. Moreover, the ability of the same drug to have different behavioural effects in humans suggests it may be acting differently in people with different psychiatric conditions, that is as a psychotomimetic in people who are not depressed and as an antidepressant in those with depression.~~

The NMDAR is a ligand gated ion channel made up of heteromeric complexes formed from different combinations of three families of sub-units, the families of sub-units have been given the nomenclatures of GRIN1, GRIN2 and GRIN3 (Paoletti and Neyton, 2007). It is now generally accepted that a functional NMDAR is a tetramer of sub-units that must include at least one GRIN1 and one GRIN2 with differing combinations of sub-units conferring different functionality (Paoletti and Neyton, 2007). This growing understanding of the structure of the NMDAR has allowed increasingly sophisticated studies using postmortem central nervous system (CNS) tissue designed to understand how the NMDAR may be affected by the aetiology of psychiatric disorders. The neuropsychopharmacological outcome from administering any drug is dependent on dose and the number of available targets sites for the drug in CNS (Ruffolo, Jr., 1982). Thus, whilst the clinical useful antidepressant outcome of ketamine is known to be dose dependent (Abdallah et al., 2015) less is known as to whether any of its effects could be due to diagnostic-dependent variation in levels of NMDAR.

Studies in schizophrenia have reported higher (Deakin et al., 1989; Grimwood et al., 1999; Ishimaru et al., 1994; Newell et al., 2005; Nudmamud and Reynolds, 2001; Zavitsanou et al., 2002), unchanged (Beneyto and Meador-Woodruff, 2008; Dean et al., 1999a; Kornhuber et al., 1989; Scarr et al., 2005) or lower (Nudmamud et al., 2003) levels of NMDAR in different cortical regions. There are equally varied outcomes from studies measuring levels of NMDAR sub-unit mRNA in the cortex of subjects with the disorder (Supplementary Table 1). Changes in the level of NMDAR sub-unit expression is only one mechanism by which the levels or activity of the NMDAR can occur (Gladding and Raymond, 2011). It is therefore significant that more recent studies have suggested changes in the post-translational processing of NMDAR sub-units may be occurring in the CNS of people with schizophrenia. For example, in the cortex of people with schizophrenia it has been reported there is a decreased GRIN2B and PSD 95 in the endoplasmic reticulum (Kristiansen et al., 2010), changes in *N*-linked glycosylation of NMDAR sub-units (Tucholski et al., 2013) and changes in the types of cells expressing NMDAR sub-units (Bitanihirwe et al., 2010; Woo et al., 2008). These post-translational modifications and / or differential cellular expression of NMDAR would clearly altered glutamate homeostasis in the CNS of people with the disorder.

There have been fewer studies of NMDAR in mood disorders. However, one study has reported lower levels of radioligand binding to the glycine binding site on the NMDAR in people with bipolar disorders (~~BPD~~BD) (Nudmamud-Thanoi and Reynolds, 2004) Outcomes from studies on NMDAR sub-units in ~~BPD~~BD are varied but the majority report levels of NMDAR sub-unit mRNA are not changed in the disorder (Supplementary Table 1). There is also a report that levels of NMDAR are not changed in major depressive disorder (MDD) (Holemans et al., 1993) and most studies report unchanged levels of NMDAR sub-unit mRNA (Supplementary Table 1).

Whilst there have been a number of studies on NMDAR in postmortem CNS, none seem to have measured levels of NMDAR and mRNA for its more abundant sub-units in tissue from subjects with schizophrenia, ~~BPD~~BD and MDD to begin to understand the changes in these markers in different cortical regions in people with different psychiatric disorders. We posited that such data could also help understand whether any of the effects of ketamine, or the dose of ketamine required to cause advantageous effects, could be influenced by ~~having different psychotropic outcomes because of diagnostic specific changes in~~ NMDAR levels. Hence, we decided to measure levels of NMDAR, its sub-units and a protein associated with NMDAR function (post synaptic density 95: PSD 95) in areas of the cortex from people with psychiatric disorders (see below).

2.0 Materials and methods

2.1 Materials

See Supplementary ~~Text~~Methods

2.2 Tissue Collection

For these studies, tissue was collected from frontal pole (Brodmann's Area (BA 10), the DLPFC (BA 46)), the anterior cingulate cortex (ACx: BA 24) and the parietal cortex (BA 40) from 10 people with ~~BPD~~BD, 10 people with MDD, 20 people with schizophrenia and 20 subjects with no history of psychiatric illness (controls) matched for age and sex to the psychiatric cases (Regions

defined in Supplementary ~~Text~~Methods). Suicide completion was recorded when suicide was formally recognised in the Coroner's report on cause of death. Tissue was obtained from the Victorian Brain Bank Network following approval from the Ethics Committee of the Victorian Institute of Forensic Medicine and the Mental Health Research and Ethics Committee of Melbourne Health.

Diagnoses were made according to DSM-IV criteria, following case history reviews using the Diagnostic Instrument for Brain Studies (DIBS) (Hill et al., 1996; Roberts et al., 1998), by consensus between a psychologist and a senior psychiatrist. While completing the DIBS, duration of illness (DI) was calculated as time from first clinical presentation to a psychiatric service to death. In addition, the final recorded dose of antipsychotic drug (FRADD) was recorded and converted to chlorpromazine equivalents using a well-established methodology (Foster, 1989), as was total lifetime exposure to these drugs (LEAP). Notably, all cadavers were refrigerated within 5 hr. of being found. When death was witnessed, post-mortem interval (PMI) was calculated as the time from death to autopsy. Where death was not witnessed, tissue was only collected from subjects who had been seen alive up to 5 hr. prior to being found dead; here the PMI was taken as the midpoint between the person being found and being last seen alive. As well as maintaining cases at low temperatures for most of their PMI, tissue was rapidly processed and frozen to -70^{o}C within 30 min of autopsy (Dean et al., 1999b); processing tissue in this way significantly slows autolytic changes (Ferrer et al., 2007). CNS pH was measured as described previously (Kingsbury et al., 1995) as this provides a good measure of overall tissue quality (Stan et al., 2006).

2.3 [^{3}H]MK-801 In Situ Radioligand Binding with Autoradiography

The conditions used for these studies were the same as our previous study (Scarr et al., 2003), meaing that [^{3}H]MK-801 would bind with high specificity to the ion channel of the NMDAR (Reynolds, 2001) (For full details see Supplementary Methods). ~~First, 5 x 20µm frozen tissue sections (3 x total binding (TB) and 2 x non specific binding (NSB)) were incubated in assay buffer (50 mM TRIS acetate, 100 µM glutamate, 50 µM glycine, 50 µM spermidine; pH 7.4) for 30 min at 4°C and then rinsed in water and dried. The washed sections were exposed to [^{3}H]MK-801 (0.2 µM) in the absence (TB) or presence (NSB) of 100µM MK-801 for 60 min at room temperature in assay buffer and then washed twice for 10 min in ice cold assay buffer, rinsed in ice cold water and dried in a stream of cool air before being partially fixed overnight in paraformaldehyde vapour (Pavey et al., 2002).~~

~~Partially fixed slides were apposed to a BAS-TR2025 plate with autoradiography [^{3}H]microscales™ for 7 days, the plates were then scanned in a BAS5000 high resolution phosphorimage and the resulting images analysed using AIS imaging software. The distribution of the binding of radioligand in each region of the cortex was carefully assessed, both visually and by placing a transept across each cortex to obtain a binding profile. This determined whether binding could be differentiated into distinct layers within the grey matter. Subsequently, radioligand binding was either taken as an integrated measurement across the whole cortex or a measure within each layer of binding. In all cases, signal intensity were calibrated against the microscales to allow the bound radioligand to be expressed as a function of an equivalent amount of tissue (estimated tissue equivalent (ETE)) (Dean et al., 1999b). Specific [^{3}H]MK-801 binding was calculated by subtracting NSB from TB for each subject.~~

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2.4 *In Situ* Hybridisation

Riboprobes for the NMDAR subunits GRIN1, GRIN2A–2D and GRIN3A were synthesized from previously characterized plasmid constructs (Beneyto et al., 2007) (For full details see Supplementary Methods). To prepare the riboprobes, 8 µl of [^{35}S] UTP was dried, suspended in a 10 µl reaction volume containing 1x transcription buffer, 0.01 M DTT, 3 mM ATP, CTP, and GTP, 2.0 µl linearized plasmid DNA, 0.5 µl RNase inhibitor, and 1.5 µl SP6 or T7 RNA polymerase and incubated for 2 h at 37°C. 1.0 µl DNase was added to the reaction mixture and incubated for 15 min at RT. Radiolabeled probes were purified with microspin chromatography columns. Two slides per subject for each antisense probe were fixed in 4% formaldehyde at RT for 1 hr. The slides were washed thrice in 2x SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.2) for 5 min, washed in water for 1 min and placed in 0.1 M triethanolamine, pH 8.0 / acetic anhydride, 400:1 (vol. : vol.) for 10 min. The slides were washed in 2x SSC for 5 min followed by dehydration through graded alcohols and air dried for 30 min. A cover slip with 200 µl of riboprobe (2 million c.p.m., 50% formamide buffer (50% formamide, 10% dextran sulfate, 3x SSC, 50 mM Na_2HPO_4 (pH 7.4), 10 mM dithiothreitol, 1x Denhardt's solution, 100 µg / ml yeast tRNA), and 0.01 M dithiothreitol) was placed on each slide. Slides were hybridized overnight at 55°C in a covered chamber containing 50% formamide saturated filter paper. Cover slips were removed and the slides were placed in 2x SSC for 5 min followed by RNase (200 µg / ml in 10 mM Tris–HCl (pH 8.0) and 0.5 M NaCl) at 37 °C for 30 min and then washed in 2x SSC for 15 min at RT; 2x SSC for 15 min at RT; 1x SSC for 10 min at RT; 0.5 x SSC for 5 min at RT; 0.1 x SSC for 60 min at 55 °C; 0.1 x SSC for 60 min at 55°C; and 0.5 x SSC for 15 min at RT. The slides were dehydrated in graded ethanol solutions, air dried, placed in X-ray cassettes, and apposed to Kodak XAR-5 film for 2–60 days alongside a [^{14}C] microscale standard.

~~Film was developed and used for quantitative computer image analysis. An integrated measure was taken across the cortical gray matter with tissue background readings being subtracted from grayscale values. Background corrected values were averaged, providing one mean value per region, per subject, for each probe. Background adjusted, averaged grayscale values were expressed as femtomoles mRNA per gram tissue (fmol /g).~~

2. 5 Western Blotting

As levels of PSD-95 can be associated with NMDAR density we followed~~Following~~ the lead from the region-specific changes in [^{3}H]MK-801 binding and ~~we~~ measured PSD 95 in the ACx from people with schizophrenia, ~~BPD~~BD and controls. Levels of PSD 95 were essentially measured using methodologies we have developed (Dean et al., 2006; Dean et al., 2010; Scarr et al., 2006) to address the ~~that acknowledge the~~ difficulties in finding reference proteins to normalize data (Eaton et al., 2013) in human CNS (for full details see Supplementary Methods). ~~Thus, to maximise the reproducibility of the data, total protein levels were carefully measured in each homogenate that underwent electrophoresis after tissue from each case was homogenized tissue into 10 mM Tris (pH 7.4) 1% SDS, 1 mM Na_3VO_4. A sample of each homogenate containing 20 µg protein was loaded, in duplicate, onto a 7.5% polyacrylamide gel along with a protein homogenate from an independent cortical sample (internal control: IC (Dean et al., 2006)) that had previously undergone electrophoresis, transfer and visualization to establish and expected intra- and inter-gel variation. The protein in each gel were then transferred overnight to a nitrocellulose membrane and each membrane was stained with Ponceau S solution and examined to further ensure no major variations in protein loading. After destaining, the membranes were blocked for 1 hr. at RT in 1x TTBS + 5% NFMP (20 mM TRIS HCl, pH 7.6, 137 mM NaCl, 0.1% Tween 20, 5% non-fat milk powder). The membranes were incubated in 1:1000 rabbit anti-~~

~~PSD 95 for 2 hrs., washed thrice for 5 min in 1x TTBS + 5% NFMP incubated for 1 hr. in 1:2000 goat anti rabbit secondary antibody, washed thrice for 5 min in 1x TTBS + 5% NFMP and rinsed in 1x TTBS. The membranes were then exposed to Supersignal West Pico ECL substrate and the resulting sum intensity of the immunogenic band was quantified as a ratio of the intensity of the internal control using a Kodak Image Station with Kodak 1D Image Analysis Software. As a final quality control, for each gel, the intensity of the IC needed to be within the previously defined range determined during the methodological optimization; data was discarded from gels where the IC were outside of the acceptable range.~~

2.6 Statistics

Given well recognised difficulties in assessing the distribution of data when sample sizes are small (D'Agostino et al., 1990), and that biological variables tend to be normally distributed (McKillup, 2006), statistical analyses were completed using parametric analyses. Thus, variation in experimental, donor demographic, drug and CNS collection data were assessed using Graphpad Prism and either one-way ANOVA followed by a post hoc Dunnett's multiple comparison test comparing data for each diagnoses to control for experimental data and Tukey's comparing all columns for other data or student t-tests (if 2 cohorts). The sex ratios and rates of suicide in the different cohorts were compared using a Fisher's exact test. Relationships between experimental variables and demographic, tissue collection and pharmacological data were assessed using linear regression. Small sample sizes mean that strong relationships, as assessed using linear regression analyses, need to have an $r^2 > 0.49$ (Cook and Weisberg, 1999). If strong relationships were revealed, and there was no variation in the non-experimental data with diagnoses (Miller and Chapman, 2001), the variation in the experimental data was re-assessed using the General Linear Model with the appropriate non-experimental data as a covariant(s).

3.0 Results

3.1 Demographic Data

Mean age ($F = 0.68$, d.f. = 3,55, $p = 0.57$), PMI ($F = 0.97$, d.f. = 3,55, $p = 0.41$), CNS pH ($F = 0.48$, d.f. = 3,55, $p = 0.69$) and DI ($F = 0.27$, d.f. = 3,55, $p = 0.77$) did not vary between diagnostic cohorts (Table 1: full details Supplementary Table 2).

There was no difference in the ratio of males to females between the diagnostic groups ($p = 0.99$: Table 1). There were significant differences in rates of suicide between diagnostic cohorts ($p < 0.0001$) due to a significantly higher incidence of suicide in subjects with schizophrenia and MDD compared to controls. As suicide was an unresolvable confound, all experimental data was also analysed with suicide completion, independent of diagnoses, as the primary variable.

3.2 General Findings

In the human cortex two layers of [^{3}H]MK-801 binding were detectable (Figure 1A and 1B) and thus a section from each case was stained with Nissl stain and viewed under a microscope to determine which laminae of the cortex were encompassed by the outer and inner layer of [^{3}H]MK801 binding. This showed that the outer layer of [^{3}H]MK-801 binding overlaid cortical laminae I to III plus a portion of laminae IV. The inner layer of [^{3}H]MK-801 binding overlaid the remainder of laminae IV plus laminae V and VI.

As described previously, levels of GRIN1 mRNA was highest in all cortical regions studied (Scherzer et al., 1998). Consistent with this previous study, levels of the other NMDAR sub-unit mRNA varied between cortical regions where they were present at low to medium levels.

3.3 Schizophrenia

There were lower levels of [^{3}H]MK-801 binding (-17%; p = 0.01) in the outer, but not inner, layer of the DLPFC from people with schizophrenia (Figure 1C; Supplementary Table ~~2~~3). There were no other significant variations in levels of [^{3}H]MK-801 binding in people with the disorder (Supplementary Table ~~2~~3). There were no changes in the levels of hybridisation of radioactive probes bound to NMDAR sub-unit mRNA in cortex from people with schizophrenia (Supplementary Table ~~3~~4). Levels of PSD 95 were not significantly different in the ACx from people with the disorder (Control = 0.62 ± 0.05 vs. schizophrenia = 0.70 ± 0.06 ratio internal control: p = 0.31).

3.3 Bipolar Disorders

There were lower levels of [^{3}H]MK-801 binding in the outer, but not inner, layers of in the DLPFC (- 19 %; p < 0.01) and the inner layer of the ACx (-19 %; p < 0.01) from people with ~~BPD~~BD (Figure 1C; Supplementary Table ~~2~~3). There was no other significant variation in [^{3}H]MK-801 binding in ~~BPD~~BD (Supplementary Table ~~2~~3). The level of hybridisation of radioactive probes to GRIN2C mRNA was lower (-13%) in ACx from people with ~~BPD~~BD (Figure 2; Supplementary Table ~~3~~4); there were no other significant changes in the levels of

hybridisation to NMDAR sub-unit mRNA. Levels of PSD 95 were not significantly different in the ACx from people with the disorder (Control = 0.62 ± 0.05 vs. vs. ~~BPD~~BD = 0.57 ± 0.08 ratio internal control: p = 0.61).

3.4 Major Depressive Disorders

There was no significant variation in levels of [^{3}H]MK-801 binding in the outer or inner layer in any cortical region from people with MDD (Supplementary Table ~~2~~3). By contrast, levels radioactive probe hybridisation to GRIN2D mRNA was higher (+29%) in frontal pole from people with MDD (Figure 2; Supplementary Table ~~3~~4), there were no changes in the levels of hybridisation in MDD.

3.5 Suicide

Levels of [^{3}H]MK-801 binding did not differ between people who had, or had not, died by suicide (Supplementary Table ~~4~~5). By contrast, levels of radioactive probe hybridized to the GRIN2B sub-unit of the NMDAR was higher (25%) in parietal cortex and lower (-35%) in DLPFC from suicide completers (Figure 3; Supplementary Table ~~5~~6), there were no other significant changes in levels of oligonucleotide riboprobes with suicide. The differences in levels of GRIN2B mRNA remained significant when analysed after removal of data from the controls, none of whom had died by suicide (parietal cortex 0.13 ± 0.01 vs 0.18 ± 0.02; $p < 0.01$; DLPFC 0.40 ± 0.02 vs. 0.31 ± 0.02; p = 0.05). In the ACx, levels of PSD 95 were higher (26%) in suicide completers compared to those who had died by other causes (Figure 4). The differences in levels of PSD 95 remained significant after removal of data from the controls, none of whom had died by suicide (ACx 0.84 ± 0.06 vs 0.60 ± 0.06; $p < 0.05$). Whilst it would have been interesting to analyse the

suicide effect within each diagnoses sample sizes were not large enough to allow such an analyses to have any predictive power.

3.6 Potential Confounds

Levels of [^{3}H]MK-801 binding, hybridisation of radioactive probes to NMDA receptor sub-unit mRNA or PSD 95 (Male = 0.70 ± 0.05 vs. Female = 0.58 ± 0.05 ratio internal control; p = 0.11) did not differ with gender in any of the cortical regions examined (Supplementary Table ~~6~~ 7 and ~~7~~8). There were no strong correlations between [^{3}H]MK-801 binding (Supplementary Table ~~8~~9), hybridisation of radioactive probes to NMDA receptor sub-unit mRNA (Supplementary Table ~~9~~10) or PSD 95 (age (r^2 = 0.39; p < 0.001), PMI (r^2 < 0.001; p = 0.93), DI (r^2 = 0.09; p = 0.93), CNS pH (r^2 = 0.003; p = 0.69), FRADD (r^2 = 0.03; p = 0.69) or LEAP (r^2 = 0.007; p = 0.13)) with any potential confounds.

There were a number of correlations between experimental variables where a regression line defining the relationships deviated significantly from the horizontal (i.e. p < 0.05: Supplementary Table ~~10~~11). One correlation where there was a p < 0.05 across all cortical regions was between the level of [^{3}H]MK-801 binding in the two cortical binding layers which showed a strong levels of correlation in the frontal pole, ACx and DLPFC. The other correlation with p < 0.05 across all regions was level of GRIN1 and GRIN2B mRNA (from r^2 = 0.11 to 0.16).

4.0 Discussion

A major finding of this study is that there were significantly lower levels of [^{3}H]MK-801 binding to the outer lamina of the DLPFC from people with schizophrenia, and as [^{3}H]MK-801 would have been almost exclusively binding to the NMDAR (Wong et al., 1986), our data suggests there are lower levels of NMDAR in the outer cortical lamina in DLPFC from people with the disorder. As our findings are restricted to specific cortical laminae within certain cortical regions any comparisons to other studies must be tentative but worthwhile to put our findings into some context. Thus, t~~T~~hese data differ from our previous studies that showed no changes in NMDAR in the DLPFC from people with schizophrenia (Dean et al., 1999a; Scarr et al., 2005). This could be because our current study is in BA 46 whereas our previous studies were in BA 9 (Dean et al., 1999a; Scarr et al., 2005) and that loss of NMDAR only occurs in a portion of the DLPFC. Thus, it is also possible that previous reports of higher (Deakin et al., 1989; Grimwood et al., 1999; Ishimaru et al., 1994; Newell et al., 2005; Nudmamud and Reynolds, 2001; Zavitsanou et al., 2002), unchanged (Beneyto and Meador-Woodruff, 2008; Dean et al., 1999a; Kornhuber et al., 1989; Scarr et al., 2005) or lower (Nudmamud et al., 2003) levels of NMDAR in different cortical regions could be reflecting these regional selective changes. Our current data also suggests that the lower levels of NMDAR in the outer lamina of DLPFC from people with schizophrenia is not associated with any significant changes in levels of receptor sub-unit mRNA. This suggests the changes in NMDAR in the outer layers of the DLPFC is not due to decreased NMDAR sub-unit expression and that our data is in agreement with other studies reporting no change in NMDAR sub-unit mRNA in in the cortex of people with schizophrenia (Supplementary Table 1) but does not agree with other studies that report changes in levels of GRIN mRNAs (Akbarian et al., 1996; Dracheva et al., 2001; Kristiansen et al., 2006; Mueller and Meador-Woodruff, 2004; Woo et al., 2008b).

Contrary to a previous study (Funk et al., 2009), we did not find lower levels of PSD 95 in the ACx from people with schizophrenia. Significantly, PSD 95 has been reported to be higher in the

occipital cortex (Dracheva et al., 2001) and lower in the DLPFC (Kristiansen et al., 2010) from people with schizophrenia. Thus, it would seem that, as for other components of the glutamatergic system, there are regionally selective changes in PSD 95 in the cortex of people with schizophrenia.

Another major finding of this study is that there were significantly lower levels of NMDAR in the inner lamina of the ACx and outer lamina of the DLPFC from people with ~~BPD~~BD. . This finding is in line with an earlier study that reported lower levels of radioligand binding to the glycine binding site on the NMDAR in the in people with ~~BPD~~BD (Nudmamud-Thanoi and Reynolds, 2004). We also showed lower levels of GRIN2C in ACx from people with ~~BPD~~BD, which raises the possibility the lower NMDAR in that cortical region, but not the DLPFC, is partly due to decreased gene expression. It has been suggested that the GRIN2 family of sub-units are important in NMDAR modulation and desensitization (McIlhinney et al., 2003). Thus our data is consistent with both a lower level of NMDAR and possibly a change in the ability of these receptors to desensitize in the ACx from people with ~~BPD~~BD. As our study is the first to report on NMDAR mRNA in the ACx this is a particularly novel finding. We did not find changes in the levels of PDS 95 in the ACx from people with ~~BPD~~BD which agrees with a previous report of this protein not being changed in the DLPFC from people with ~~BPD~~BD (Beneyto and Meador-Woodruff, 2008).

In MDD, we found higher levels of GRIN2D mRNA in the frontal pole, where there was no difference in levels of NMDAR. These data are consistent with the notion that a change in gene expression will not necessarily be associated with changes for the encoded protein of that gene.

Finally, we report higher levels of GRIN2B mRNA in parietal cortex and lower levels of that

mRNA in the DLPFC from suicide completers. In addition, levels of PSD 95 were increased in ACx from suicide completers. These data open up a possible role for abnormal signaling through the NMDAR in the CNS of suicide completers. It is also intriguing that a previous a study had reported lower levels of PSD 95 and GRIN2B mRNA in the hippocampus from suicide completers (Sowa-Kucma et al., 2013). These and our findings suggest a further investigation into levels of GRIN2B mRNA and PSD 95 in suicide completion would be worthwhile to better understand the extent of changes in these measures across the CNS. Notably, the study in the hippocampus also reported a significant increase in zinc mediated EC_{50} for [^{3}H]MK-801 binding, but not total [^{3}H]MK-801 binding, in suicide completers (Sowa-Kucma et al., 2013). These latter data suggest a lower bioavailability of the NMDAR in the CNS of suicide completers, possibly due to conformational changes in the assembled receptor.

5.0 Conclusion

Our studies have certain limitations. Thus, although our cohort sizes are not unusually small for postmortem CNS studies they are still a low representation from each diagnosis and our findings need replicating in larger cohorts. Moreover, like many studies in psychiatry treatment remains a potential confound. We did not observe any strong relationships between types of drug or drug doses and any of our experimental variables however, at this point, it is not possible to exclude changes in glutamatergic markers in the cortex being part of the neuropsychopharmacological outcome of psychotropic drug treatments. Nonetheless, given the somewhat homogenous neurochemical nature of the human cortex it is somewhat difficult to explain how psychotropic drugs, acting on the same targets throughout the cortex, could produce the region-specific outcomes we observed in different psychiatric disorders. Whilst rates of suicide completion

differed between diagnose, our sample sizes were too small to determine if suicide completion within specific diagnoses was associated with changes in glutamatergic markers in the cortex.

In interpreting the significance of our finding it is important to note that changed levels of [^{3}H]MK801 binding in rodents have been linked to changes in functions such as learning and memory (Basham et al., 1996; Stecher et al., 1997) This suggests that the changes in levels of NMDAR in the DLPFC and ACx from people with schizophrenia and ~~BPD~~BD could have behavioral / functional consequences. However, the complex changes in glutamatergic markers in schizophrenia, ~~BPD~~BD and MDD will require further study to better understand the complex regional changes in the CNS that may be altering glutamatergic function in psychiatric disorders and in suicide. Moreover, given the regional variation in changes in NMDAR in people with different psychiatric disorders, continuing to unravel these complex changes in the glutamatergic system in the CNS should help to better understand the role of NMDAR in the pathophysiologies of schizophrenia, BD and MDD as well as the dose-dependent outcomes from ketamine administration in humans~~explain the varying outcomes relating to ketamine in humans~~.

6.0 Conflict of Interest Statement

None of the authors have any conflict of interest and the research described in this manuscript was completed in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest. ES has previously received honorarium from Astra-Zeneca and travel support from GSK that were unrelated to this research.

7.0 Author and Contributors

All authors made a substantial contribution to editing the iterations of this manuscript leading to submission for publication. AG, SB and AU completed all of the experimental work described in the manuscript. BD, ES, JMW and RM provided oversight to the experimental activities. BD completed all the statistical analyses described in the manuscript and took the lead role in writing the manuscript.

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10.0 Figure legends

Figure 1: A typical autoradiograph showing the binding of [^{3}H]MK-801 to the NMDAR in human cortex in the absence (**A**: Total binding (TB)) or presence (**B**: non-specific binding (NSB)) of 100μM MK-801 and **C:** the levels of specific binding of [^{3}H]MK-801 (mean ± SEM) in the inner radioligand binding layer of the anterior cingulate (ACx) and the outer radioligand binding layer of the dorsolateral prefrontal cortex (DLPFC) from the controls and people with schizophrenia (Sz) and bipolar disorder (~~BPD~~BD). Specific binding was calculated as TB – NSB. **p = 0.01; ***p < 0.01.

Figure 2: Levels of [^{35}S]riboprobe hybridisation (mean ± SEM) to the GRIN2D sub-unit in the frontal pole from people with major depressive disorders (MDD) and GRIN2D sub-unit in the ACx from people with bipolar disorder (~~BPD~~BD) compared to that in the same regions from age / sex matched controls. *p < 0.05.

Figure 3: Levels of [^{35}S]riboprobe hybridisation (mean ± SEM) to the GRIN2B sub-unit in the frontal pole and dorsolateral prefrontal cortex (DLPFC) from suicide completers and people who had died from other causes. **p < 0.01, ***p = 0.02.

Figure 4A: A typical Western blot showing PSD 95 immunogenic bands in 2 cases and the internal control (I.C.). The I.C. is run on every gel to control for gel to gel variation and **B:** Levels of PSD 95 (mean ± SEM) in the ACx from suicide completers and people who had died from other causes.

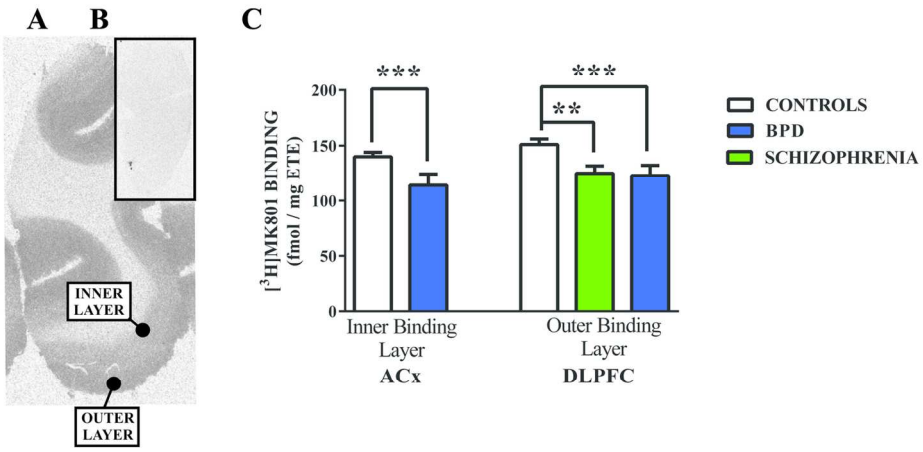

Figure 1: A typical autoradiograph showing the binding of [3H]MK-801 to the NMDAR in human cortex in the absence (A: Total binding (TB)) or presence (B: non-specific binding (NSB)) of 100μM MK-801 and C: the levels of specific binding of [3H]MK-801 (mean ± SEM) in the inner radioligand binding layer of the anterior cingulate (ACx) from bipolar disorders (BD) and controls and the outer radioligand binding layer of the dorsolateral prefrontal cortex (DLPFC) from the controls and people with schizophrenia (Sz), BD and controls. Specific binding was calculated as TB – NSB. **p = 0.01; ***p < 0.01.
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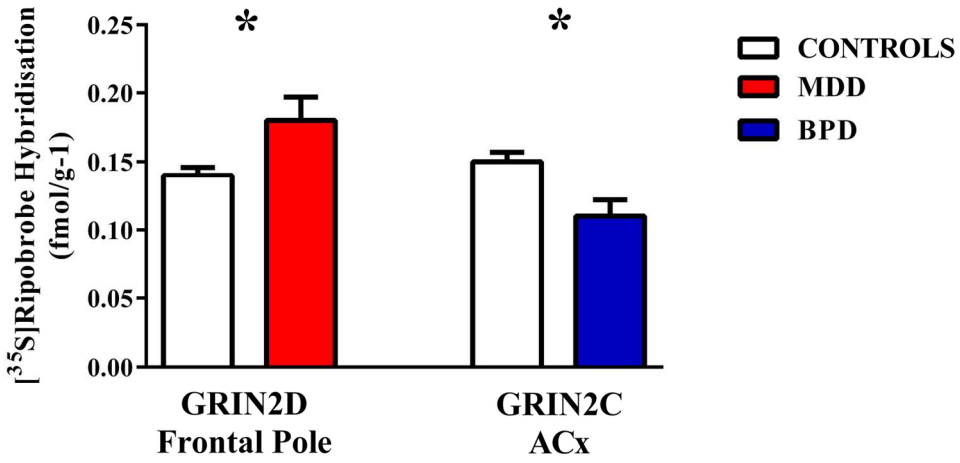

Figure 2: Levels of [35S]riboprobe hybridization (mean ± SEM) to the GRIN2D sub-unit in the frontal pole from people with major depressive disorders (MDD) and GRIN2D sub-unit in the ACx from people with bipolar disorder (BD) compared to that in the same regions from age / sex matched controls. *p < 0.05.
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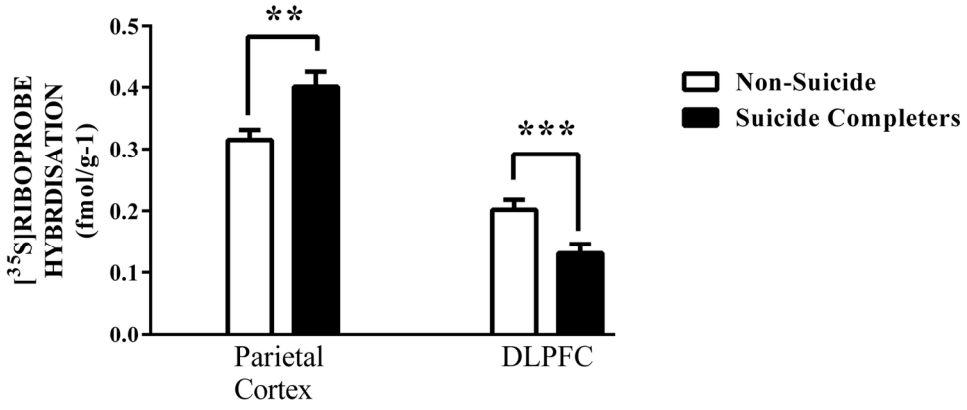

Figure 3: Levels of [35S]riboprobe hybridization (mean ± SEM) to the GRIN2B sub-unit in the frontal pole and dorsolateral prefrontal cortex (DLPFC) from suicide completers and people who had died from other causes. **p < 0.01, ***p = 0.02.
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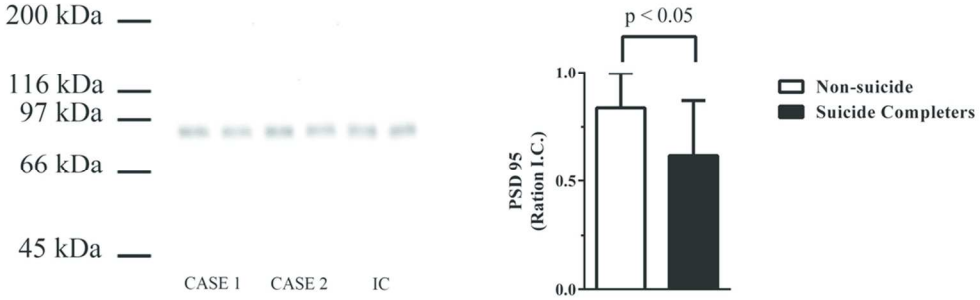

Figure 4A: A typical Western blot showing PSD95 immunogenic bands in 2 cases and the internal control (I.C.). The I.C. is run on every gel to control for gel to gel variation and B: Levels of PSD95 (mean ± SEM) in the ACx from suicide completers and people who had died from other causes.
98x37mm (300 x 300 DPI)

Table 1: Summary of demographic and tissue collection data for cases in this study.

	Gender (M / F)	Sui	Age (yrs)		DI (yrs)		PMI (hrs)		pH	
			mean	SEM	mean	SEM	mean	SEM	mean	SEM
Controls	11 / 9	0	50	4.1			42	3.9	6.29	0.05
Schizophrenia	11 / 9	5	51	4.1	21	2.6	40	2.6	6.30	0.05
Bipolar Disorders	5 / 4	1	60	3.3	20	4.6	38	4.7	6.33	0.06
Major Depressive Disorders	5 / 5	8	52	5.2	17	3.3	49	5.1	6.4	0.01

Abbreviations: Sui = suicide completion; PMI = postmortem interval; DI = duration of illness