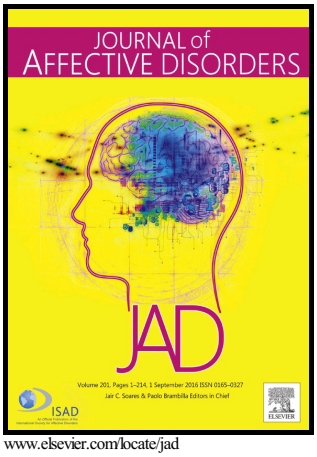
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Glutamatergic Hypofunction in Medication-Free Major Depression: Secondary Effects of Affective Diagnosis and Relationship to Peripheral Glutaminase

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Abstract

Background

There is uncertainty as to whether alterations in glutamatergic function in affective disorders differ between unipolar and bipolar disorders and between depressive and euthymic states. Additionally, there are currently no available blood-based markers of central glutamatergic function to support clinical diagnosis and aid brain based investigations.

Methods

In this study, we measured levels of glutamate in the dorsal anterior cingulate cortex in-vivo using 1H-Magnetic Resonance Spectroscopy in medication free unipolar and bipolar patients (n=29, 20 unipolar and 9 bipolar) experiencing a major depressive episode, in comparison with a group of matched healthy controls (n=20). We also analysed peripheral glutaminase measured in serum to examine the relationship between central and peripheral measures.

Results

Anterior cingulate glutamate levels were reduced in both unipolar and bipolar depression groups relative to healthy controls, although this only reached significance in the unipolar group. Peripheral glutaminase levels did not differentiate bipolar from unipolar depression and a positive correlation with central glutamate levels did not reach statistical significance.

Limitations

The sample of bipolar disorder patients was relatively small due to the difficulties involved in finding medication-free patients experiencing a depressive episode.

Conclusions

These results suggest that glutamatergic hypofunction might represent a state marker for a depressive episode irrespective of diagnosis. Peripheral glutaminase did not index central glutamate levels in this study, which could potentially reflect a small magnitude of the effect requiring larger samples for detection.

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Introduction

Major depression and bipolar disorder are common conditions with substantial negative consequences for sufferers (Kessler et al., 2003; Merikangas et al., 2007). Understanding the biological changes associated with these conditions is a major research priority, and a better understanding of their biological basis could lead to improved diagnosis and treatment.

Over the last two decades, neuroimaging has brought a deeper understanding of neurobiological alterations associated with these conditions, and researchers are moving towards developing these methods to diagnose and treat affective disorders (Wise et al., 2014). In particular there is now a substantial literature detailing neurochemical abnormalities in both major depression and bipolar disorder (Arnone et al., 2015a; Shrestha et al., 2012; Taylor, 2014), and research into glutamatergic neurotransmission specifically has been pursued with increasing interest spurred by studies demonstrating that ketamine, an NMDA receptor antagonist, is associated with a rapid (albeit not sustained in the long term) improvement in symptoms in both both unipolar and bipolar depression (McGirr et al., 2015). This research has been aided by developments in magnetic resonance spectroscopy (MRS), a method that allows non-invasive measurement of glutamate and glutamine levels *in vivo*.

Despite this, findings at present are inconsistent, and there are particularly divergent results from studies in major depression and bipolar disorder (Taylor, 2014). Glutamate and glutamine levels are typically found to be reduced in unipolar depression, most notably in prefrontal regions such as the anterior cingulate cortex (Arnone et al., 2015), while studies in bipolar disorder suggest an increase in glutamate in this region (Taylor, 2014). These results are difficult to reconcile with the efficacy of ketamine in treating both unipolar and bipolar depression. In particular, given the tendency of ketamine to increase glutamate levels (Stone et al., 2012), it is unclear why such a manipulation would improve depressive symptoms in

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bipolar disorder if it is indeed associated with increased glutamatergic tone. One possible explanation for these contradictory results is that studies examining glutamate levels in these disorders have typically used voxels placed in the perigenual anterior cingulate cortex, an area in which glutamate levels appear to be unaffected by ketamine administration (Taylor et al., 2012). Ketamine however appears to have marked effects in a more dorsal region of the anterior cingulate cortex (Stone et al., 2012) and it is possible that it is in this region that it exerts antidepressant effects.

Additionally, while MRS provides an accurate measure of brain glutamate concentration in vivo, the identification of an easily measurable peripheral marker of glutamatergic function capable of indicating an affective state would simplify the use of glutamate as a diagnostic or treatment marker. One marker of particular interest is glutaminase, an enzyme that catalyses the conversion of glutamine to glutamate. Genetic variation in the GLS1 gene, which codes for glutaminase, is associated with levels of central glutamate and glutamine as measured using MRS (Öngür et al., 2011), indicating that glutaminase may be a marker of brain glutamate levels.

Here we measured glutamate levels in a group of individuals with unipolar and bipolar major depression in the same voxel location where ketamine has been shown to affect glutamate levels (Stone et al., 2012). We predicted a reduction in glutamate levels associated with depressive state irrespective of affective diagnosis. Additionally, we measured peripheral glutaminase in serum and investigated changes in relation to brain glutamate. We hypothesised that peripheral glutaminase levels would positively correlate with anterior cingulate cortex glutamate levels. Importantly, we used a sample of medication-free patients to ensure that results were not influenced by current pharmacotherapy.

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Methods

Participants

Participants were recruited through public advertisements and from local psychological therapy services (Wise et al., 2016a). Patients met DSM-IV criteria for a current major depressive episode in the context of unipolar or bipolar disorders determined by a clinical interview with a psychiatrist based on the Mini International Neuropsychiatric Interview (MINI, Sheehan et al., 1998). Patients with any other DSM-IV diagnoses were excluded. Healthy volunteers did not meet criteria for any current or past psychiatric diagnoses, as assessed by the MINI, and reported no family psychiatric history in first-degree relatives. Patients were enrolled if they experienced 1) moderate to severe depressive symptoms (score of ≥18) established with the clinician-rated Montgomery-Åsberg Depression Rating Scale (MADRS, Montgomery & Asberg, 1979); 2) were psychotropic medication-free for ≥2 weeks (≥4 weeks for fluoxetine) and were not receiving any psychological intervention; and 3) did not experience current clinically significant symptoms of elation established by using the Young Mania Rating Scale (Young et al., 1978). Diagnoses of bipolar disorder were retrospective and supplemented by review of medical notes and collateral information where necessary. Historic hypomanic symptoms were assessed using the Hypomania Checklist 33-item (Angst et al., 2005; Feng et al., 2016). All participants were excluded if they reported any illicit substance use in the previous two months, had any physical health conditions or received pharmacotherapy that could affect safety, or interfere with data acquisition, analyses or interpretation. All participants were screened for MRI safety. The research was approved by the relevant local ethics committee and informed consent was obtained from each participant. Participants received a small financial compensation for taking part in the research.

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1H-Magnetic resonance spectroscopy data were acquired using a GE MR-750 3T system with a 12-channel head coil. A water supressed PRESS sequence was used (TR=3s, TE=30ms). As in previous work (Stone et al., 2012), a 20 x 20 x 20mm voxel was placed 16mm above the most anterior portion of the corpus callosum, on the midline, adjusted manually to ensure optimal coverage of grey matter. This resulted in coverage of the dorsal portion of Brodmann areas 24 and 32 (Figure 1). T1-weighted structural images were also collected to provide measures of intracranial volume (Echo time = 3.02ms, repetition time = 7.31ms, inversion time = 400ms, flip angle = 11°, 270mm x 270mm field of view, 196 slices. slice thickness = 1.2mm)(Jack et al., 2010).

Analysis of the MRS spectra was conducted using LCModel version 6.3 (Provencher, 2001), and spectra were visually inspected to ensure that the LCModel fit was acceptable. Glutamate concentrations were normalised to creatine to account for individual differences in the absolute volumes of metabolites.

Glutaminase measurement

To measure glutaminase levels in serum samples a commercial Enzyme-linked Immunosorbent Assay Kit for Glutaminase (Cloud-Clone Corp, Buckingham, UK) was used according to the manufacturer's instructions. The protein glutaminase standard was diluted in standard diluent to generate a standard curve with seven points, ranging from 0.312 to 20 ng/ml. Subsequently, 100 µl of prepared standards and samples (serum dilution 1:10) was added to the wells of the immunoassay plate pre-coated with a specific antibody against glutaminase and incubated at 37 °C for 2 h. The primary and secondary antibodies (1:100 dilution) were added to the wells and incubated at 37 °C for 1 h and 30 min respectively. Subsequently, 100 µl of 3,3′,5,5′-tetramethylbenzidine substrate (TMB) were added to each well and incubated for 15 min in the dark. Finally, 100 µl of Stop Solution was added and absorbance was measured at 450 nm with a microplate photometric reader (DV990BV4, GDV, Milan, Italy). Sample concentration was calculated by interpolating the sample

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measurement in the standard curve. The sensitivity of the human glutaminase ELISA kit was determined to be 0.312 ng/ml. Human glutaminase ELISA kit is specific for glutaminase and has been certified for the detection of human glutaminase. Blood samples were unable to be collected from one subject in the unipolar depression group and this subject was excluded from analyses involving glutaminase levels.

Intracranial volume

We compared intracranial volumes between groups to ensure that any group differences were not reflective of global alterations in brain volume. Intracranial volume was calculated by segmenting T1-weighted structural images from each subject using FreeSurfer (www.freesurfer.net).

Statistical analysis

All statistical analyses were performed using R (www.r-project.org). To assess whether depressed patients differed from controls we compared glutamate levels between both depressed groups combined and controls using an independent measures *t*-test, adjusted for age and sex. To test whether there were any differences between unipolar and bipolar depression, we compared the patient groups against one another using an independent measures t-test. To further explore diagnostic differences in central glutamate levels we used a one-way ANCOVA with age and sex as covariates. Significant effects were further explored using t-tests, corrected for multiple comparisons with the Benjamini-Hochberg method (Benjamini and Hochberg, 1995). Correlations between glutamate levels and clinical variables and peripheral markers were analysed using partial Pearson correlations, correcting for age and sex.

Results

Participants

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Twenty-nine patients with major depression (20 with unipolar and nine with bipolar disorder) and 20 healthy controls, matched on age, sex and handedness took part in the study. Of those with bipolar disorder, seven had bipolar disorder type II, and two had type I. Demographic and clinical details for the combined depressed group, as used in further analyses, are described in Table 1.

Magnetic resonance spectroscopy

The combined unipolar and bipolar depressed group had significantly lower glutamate levels in the anterior cingulate cortex than healthy volunteers ($t(46.62) = -2.82$, $p = 0.014$, $d = -0.79$). To test our hypothesis that this was not an effect of diagnosis, we compared unipolar vs. bipolar groups and found no significant difference in glutamate levels ($t(12.63) = -0.07.$, $p = 1$, $d = -0.03$). Total intracranial volume did not differ between controls and the depressed group ($t(41.60) = -1.71$, $p = 0.20$) or between unipolar and bipolar depression ($t(18) = 0.23$, $p = 1$, suggesting that this was not related to global brain volume. We also investigated potential differences in voxel composition that could affect our results by comparing grey matter volume within the voxel. This showed no significant differences between depressed patients and controls ($t(45.16) = -0.66$, $p = .50$, $d = -0.19$) or between depressed groups ($t(19.57) = -2.20$, $p = 0.08$, $d = -0.8$). Finally, to verify whether the observed reduction in glutamate was likely to be attributable to glutamine rather than glutamate per se, we repeated the critical comparison of patients versus controls by analysing Glx, a combined measure of glutamate and glutamine, and this showed no significant difference between groups ($t(45.16) = -0.66$, $p = 0.50$, $d = -0.19$).

We performed further exploratory analyses to investigate potential effects of diagnosis. A one-way ANCOVA revealed a main effect of diagnosis on glutamate levels ($F(2, 46) = 3.72$, $p = 0.032$, $\eta^2 = 0.14$, Figure 2). Post-hoc tests showed that the unipolar group had significantly reduced glutamate levels relative to controls ($t(38) = -2.63$, $p = 0.03$, $d = -0.90$).

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The bipolar group had reduced levels of glutamate relative to controls of similar effect size, but this was not statistically significant ($t(27) = -1.75$, $p = 0.14$, $d = -0.70$).

There was no significant correlation between glutamate levels and depression severity, as measured by the MÅDRS, in the combined depressed group ($R_{partial}(25) = 0.034$, $p = 0.87$). There was also no correlation between glutamate levels and self-reported hypomanic symptoms measured using the hypomania checklist ($R_{partial}(23) = 0.011$, $p = 0.62$).

Relationship between glutamate function and peripheral glutaminase

There were no differences in glutaminase levels between the combined unipolar and bipolar depressed groups and controls ($t(28.3) = -1.21$, $p = 0.48$, $d = -0.39$) or between unipolar and bipolar disorders ($t(13.76) = -0.96$, $p = 0.72$, $d = -0.40$). Analysis of the relationship between prefrontal glutamate levels and peripheral glutaminase indicated a positive but non-significant correlation between these measures ($R_{partial}(48) = 0.25$, $p = 0.09$, Figure 3).

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Discussion

We measured dorsal anterior cingulate cortex glutamate levels in unmedicated unipolar and bipolar major depression and found that glutamate was reduced relative to controls in the depressive state irrespective of diagnosis, although this effect only reached significance in the numerically larger unipolar group. Additionally, our results suggest that alternatives to peripheral glutaminase levels may be preferable to index central glutamate levels.

Our finding of reduced glutamate in unipolar depression is in line with previous research indicating glutamatergic hypofunction in unipolar depression (Arnone et al., 2015a). In bipolar disorder the majority of studies tend to indicate an increase in glutamate levels in the prefrontal cortex (Taylor, 2014), which contrasts with our finding of a reduction in glutamate levels. Although this reduction was not statistically significant, given the challenges associated with recruiting medication-free individuals with bipolar depression and the

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resulting issues with statistical power, it is important to consider these results in the context of previous research in similar patients. The only other study to date to investigate anterior cingulate cortex glutamate levels in medication-free bipolar disorder patients currently experiencing a depressive episode also reported a non-significant reduction in glutamate in the anterior cingulate cortex (Xu et al., 2013) and our results, taken together with this previous work, suggest that bipolar depression in the absence of medication may be associated with glutamatergic hypofunction. A reduction in glutamate levels as an expression of state rather than diagnosis concords with the clinical efficacy of ketamine in both unipolar and bipolar depression, known to increase glutamate levels in the region investigated in this study (Stone et al., 2012). In addition, topographical differences in prefrontal voxel placement might explain discrepancies between our findings and the literature. Most studies have used voxels placed in the perigenual anterior cingulate cortex, an area less affected by ketamine administration (Taylor et al., 2012). Nevertheless, it is not possible to determine the exact contributions of the unmedicated status of the patients and the voxel placement to the differences observed between our results and those of previous studies. These will be important questions for future research to address. In particular, the effects of traditional pharmacotherapy for unipolar and bipolar depression on glutamatergic function are poorly understood.

The absence of a significant difference in peripheral glutaminase levels in subjects with unipolar and bipolar depression vs. healthy controls suggests that variations in this enzyme might not necessarily reflect diagnostic differences in central glutamate levels. Additionally, the positive correlation between peripheral glutaminase levels and MRS-measured glutamatergic function did not reach statistical significance in our analysis. The finding might suggest that peripheral glutaminase does not reflect glutamatergic neurotransmission in the anterior cingulate cortex. This would be in agreement with preliminary studies in healthy individuals that measured glutamatergic function with MRS in a different location within the medial prefrontal cortex (Shulman et al., 2006). Alternatively, the trend towards significance

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in the correlation between peripheral glutaminase levels and MRS-measured glutamatergic function might reach significance in studies with larger samples. Further research with higher statistical power are required to confirm or reject our finding.

The most important implication of this work is that it provides a putative explanation for the effectiveness of fast acting glutamatergic agents such as ketamine, known to increase cortical glutamate release, in treating major depressive episodes (Rowland et al., 2005; Stone et al., 2012). Ketamine has been shown to be effective in both unipolar and bipolar depression (McGirr et al., 2015), and these results have been difficult to fully understand in the context of research showing increased glutamate levels in bipolar disorder (Taylor, 2014). We propose that the region within the prefrontal cortex centred around Brodmann area 32 might be key to understanding glutamatergic function in affective disorders. Notably, this region has been implicated in the processing of negative affect (Tolomeo et al., 2016), and the presence of glutamatergic hypofunction in this area may contribute to disturbances in affective regulation. This phenomenon is likely to be mediated by an increased baseline GABAergic interneuron tone, known to modulate glutamatergic hypofunction (Stone et al., 2012). In this context, ketamine may act by inhibiting GABAergic interneurons, resulting in increased glutamatergic release as demonstrated in healthy controls (Stone et al., 2012). Although these results are promising it is important to acknowledge that the literature on the glutamatergic effects of ketamine in humans is limited in both its extent and methodological rigour. The study by Stone and others (Stone et al., 2012), for example, was not placebo controlled. This means that its results must be interpreted with caution and that future work could employ a placebo controlled crossover design in healthy individuals or a pre-post treatment investigation in patients with both unipolar and bipolar depression to confirm our results.

It is also possible that decreased levels of glutamate in this region is a reflection of grey matter volume reduction, which we have recently demonstrated in both unipolar and bipolar depression (Arnone et al., 2016; Wise et al., 2016c). This seems unlikely however, as we

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used ratios of glutamine to creatine so that our results should not be a direct effect of reduced grey matter volume. Another factor to consider is that a reduction in the integrity of the white matter callosal pathways connecting the two hemispheres in affective disorders might also affect glutamatergic function (Wise et al., 2016b). This would be in agreement with recent theories suggesting that the metabolic interplay between glutamatergic neurons and astrocytes in the synapse might be central to understanding abnormalities in this neurotransmitter in affective disorders and could potentially contribute to the therapeutic effects of fast acting antidepressants (Arnone et al., 2015b).

Our findings indicate that the dorsal anterior cingulate cortex appears to be central to understanding the role of glutamatergic neurotransmission in affective disorders. Further investigations dissecting the functional neuroanatomy of this region are warranted, particularly in relation to the role played by GABAergic interneurons. Parvalbumin expressing GABAergic interneurons projecting from the medio-dorsal thalamic nucleus to the prefrontal cortex appear crucially important to investigate in future studies as they play a critical role in modulating the functional flexibility of the dorsal part of the anterior cingulate cortex when integrating sensory information (Delevich et al., 2015), and are regulated by glutamatergic neurotransmission (Homayoun and Moghaddam, 2007). Newly developed methods of spatially mapping glutamate may prove valuable in this respect (e.g. Cai et al., 2013).

The exclusion of the effects of pharmacological or psychological therapies on the data is a strength of this study. Although we cannot entirely exclude effects of previous treatments, we can be confident that our results are not an effect of current treatment. Additionally, many of our patients in the unipolar group were experiencing their first depressive episode and results are unlikely to represent “scar” effects. Diagnoses were also thoroughly examined for all patients to ensure that cases were not inappropriately classified.

Limitations include the small number of bipolar participants due to the difficulty of recruiting unmedicated, currently depressed, patients with this condition. Hence it is important to

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consider our findings in the context of previous research demonstrating a similar effect in unmedicated bipolar depression (Xu et al., 2013). The community sample we included in the research reflects a lower severity index than an inpatient sample or more complex forms of illness with comorbidities, which we excluded. Also, the bipolar sample most entirely comprised patients with bipolar type II. Hence, it is not possible to generalise findings to bipolar type I. For this reason we cannot entirely exclude the possibility that more severe cases and/or psychotic presentations may differ in relation to glutamatergic function, perhaps similarly to glutamatergic and morphometric alterations observed in schizophrenia (Arnone et al., 2009; Merritt et al., 2016). It is also not possible to comment on specific state effects as we did not include groups of patients experiencing euthymia or elated mood.

We also did not observe any reduction in Glx, a combined measure of glutamate and glutamine. It is possible that this is due to a less precise estimate of glutamine measurements at this field strength (Henry et al., 2011). Additionally, it is theoretically possible that our use of normalised glutamate levels (with creatine as a reference) introduced a bias if differences in the reference metabolite were present. Results from a recent meta-analysis however provide some reassurance by suggesting that differences in absolute levels of creatine do not significantly differ between patients with major depression and healthy controls (Arnone et al., 2015b).

In summary, we investigated glutamate levels in the dorsal anterior cingulate cortex in unmedicated subjects with bipolar and unipolar depression and demonstrated glutamatergic hypofunction in unipolar depression, with a trend towards a significant reduction in bipolar depression. Evaluation of circulating glutaminase indicated the need for larger studies to discard its utility or identification of an alternative proxy for central brain glutamate if accessible biomarkers for disease-related alterations in central glutamate are to be developed.

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Funding and disclosures

MJT reports personal fees from Sunovion, Otsuka, Lundbeck, outside the submitted work, and a family member has been an employee of GlaxoSmithKline. AJC has in the last three years received honoraria for speaking from Astra Zeneca (AZ), honoraria for consulting from Allergan, Livanova and Lundbeck, and research grant support from Lundbeck, the Medical Research Council (UK), Wellcome Trust (UK) and the National Institute for Health Research (UK). AHY has given paid lectures and sits on advisory boards for all major pharmaceutical companies with drugs used in affective and related disorders. DA has received travel grants from Janssen-Cilag and Servier. SCRW has received grant funding from the Medical Research Council (UK), Wellcome Trust (UK), National Institute for Health Research (UK) and support for investigator led studies from Takeda, Pfizer, Lundbeck, P1Vital, Roche and Eli Lilly. No other disclosures were reported.

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Contributors

DA, MJT, AJC, AHY, SCR, and TW designed the study. DA, TW, DJL, and AH contributed to methods development and collected data for the research. AMG and FC conducted laboratory analyses. TW, MJT and DA undertook the data analysis. TW, DA, AHY and LM wrote the first draft of the manuscript. All authors contributed to and approved the manuscript.

Role of the funding source

The funders had no role in the study design, data collection, analysis, interpretation, manuscript preparation or the decision to submit the article for publication.

Conflicts of interest

MJT reports personal fees from Sunovion, Otsuka, Lundbeck, outside the submitted work, and a family member has been an employee of GlaxoSmithKline. AJC has in the last three years received honoraria for speaking from Astra Zeneca (AZ), honoraria for consulting from Allergan, Livanova and Lundbeck, and research grant support from Lundbeck, the Medical Research Council (UK), Wellcome Trust (UK) and the National Institute for Health Research (UK). AHY has given paid lectures and sits on advisory boards for all major pharmaceutical companies with drugs used in affective and related disorders. DA has received travel grants from Janssen-Cilag and Servier. SCRW has received grant funding from the Medical Research Council (UK), Wellcome Trust (UK), National Institute for Health Research (UK)

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Figure 1: Illustration of anterior cingulate cortex MRS voxel location on a typical participant

Figure 2: Glutamate levels in anterior cingulate cortex of healthy controls, people with unipolar depression, and bipolar depression. Coloured areas represent a histogram of the data in each conditions, while circles represent the individual data points. Dashed lines represent means and standard errors.

Figure 3: Relationship between peripheral glutaminase levels and anterior cingulate glutamate measured using MRS.

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Table 1: Demographic and clinical information for all participants. Values are reported as mean (SD), except the number of episodes which is reported as median (SD) due to skewed data. *P* values in the table refer to comparison between the combined depressed group and healthy controls. MÅDRS: Montgomery-Åsberg Depression Rating Scale, YMRS: Young Mania Rating Scale; NA: Not Applicable. *Patients with bipolar depression had a higher proportion of males than the other groups (X^2 = 4.49, p = 0.034). **The bipolar group had experienced significantly more depressive episodes (W = 34.5, p = 0.007, non-parametric test used for non-normally distributed data). There were no other clinically significant differences between unipolar and bipolar groups.

	Major Depression			**Healthy control**	***p***
	Unipolar	**Bipolar**	**Combined**		
Age, Years	29.55 (6.59)	31.44 (8.23)	30.14 (7.04)	30.05 (6.71)	0.79
Sex: male, female	2, 18	4, 5	2, 18*	2, 18	0.041
MÅDRS score	27.25 (4.24)	25.11 (5.23)	6, 23	0.95 (1.39)	< .001
YMRS score	2.15 (1.04)	2.33 (3.0)	2.21 (1.82)	0.1 (0.31)	< .001
Duration of illness, Years	6.35 (6.41)	10.56 (5.64)	7.65 (6.40)	NA	0.092
Depressive episodes, Number	1.50 (2.50)	4 (20.10)	5.66 (11.94)**	NA	0.007
Total intracranial volume, ml	1391.58 (104.02)	1502.15 (195.72)	1425.89 (144.90)	1447.76 (113.11)	0.16

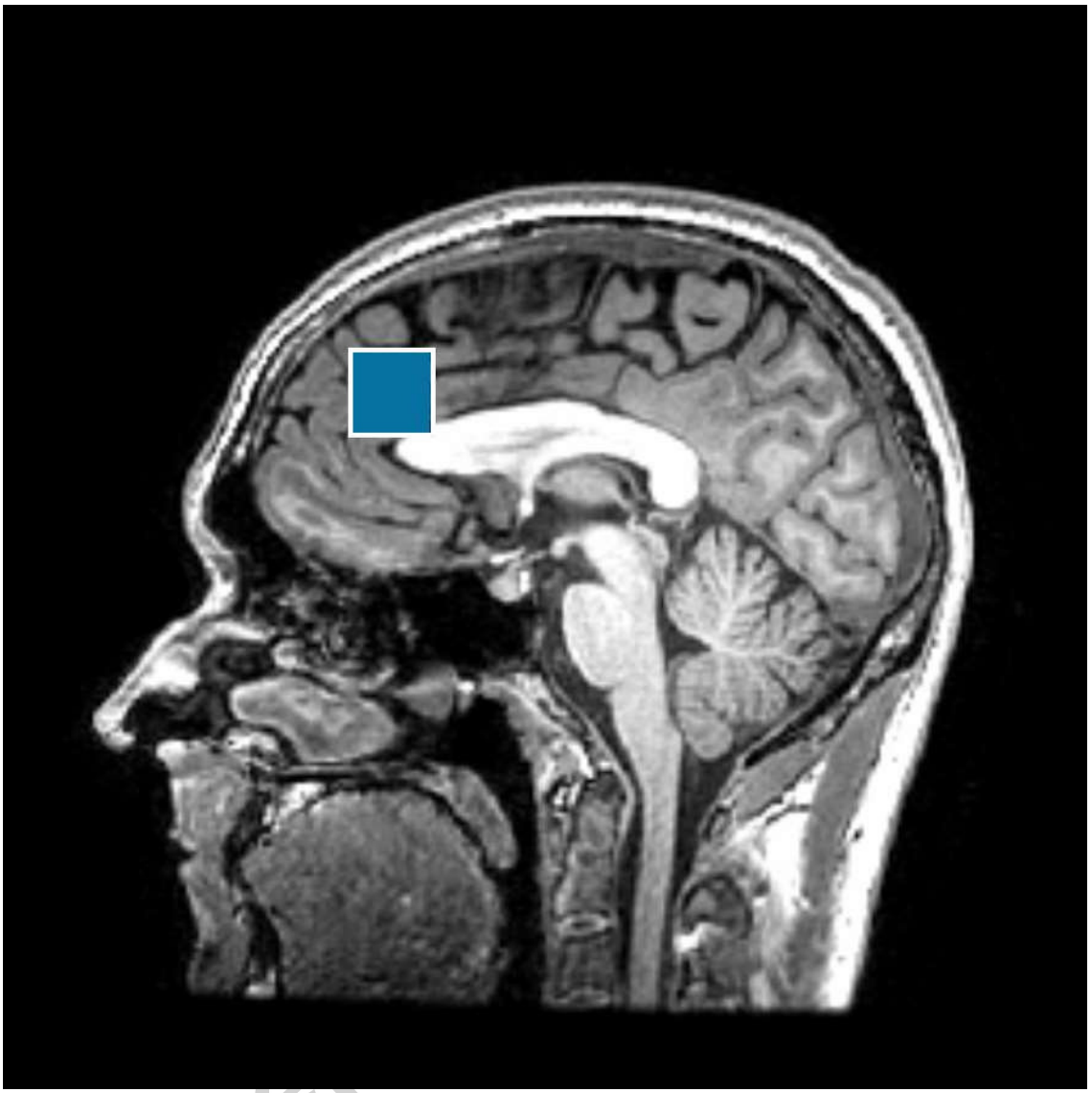
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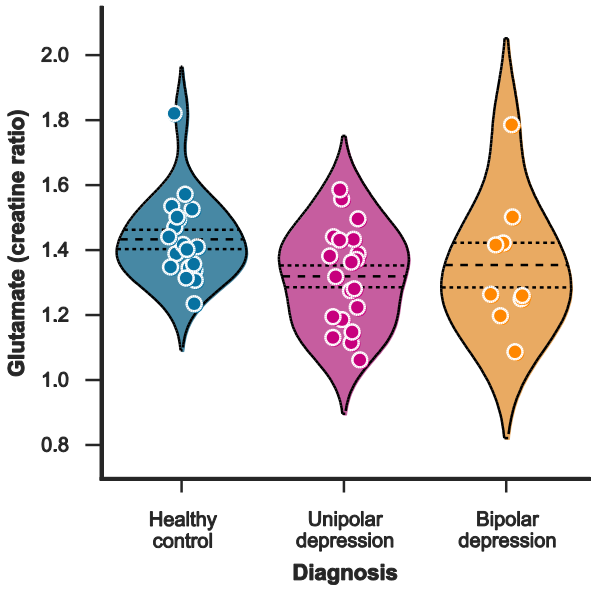
Highlights

- Glutamate has been implicated in the pathology and treatment of both unipolar and bipolar depression
- We quantified glutamate levels in the anterior cingulate cortex of patients with unmedicated unipolar and bipolar depression
- Glutamate levels were similarly reduced in both patient groups relative to healthy individuals
- A reduction in glutamatergic neurotransmission may explain the efficacy of ketamine in treating both unipolar and bipolar depression

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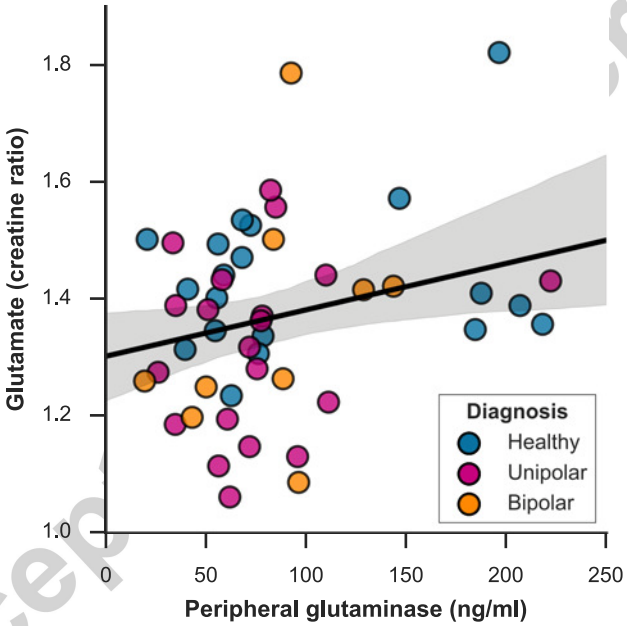


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