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Deposited on: 02 February 2015

Title: Subanaesthetic ketamine treatment promotes abnormal interactions between neural subsystems and alters the properties of functional brain networks

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Running Title: acute ketamine treatment disrupts brain networks

Abstract

Acute treatment with subanaesthetic ketamine, a non-competitive NMDA receptor antagonist, is widely utilized as a translational model for schizophrenia. However, how acute NMDA receptor blockade impacts on brain functioning at a systems level, to elicit translationally relevant symptomatology and behavioural deficits, has not yet been determined. Here, for the first time, we apply established and recently validated topological measures from network science to brain imaging data gained from ketamine treated mice to elucidate how acute NMDA receptor blockade impacts on the properties of functional brain networks. We show that the effects of acute ketamine treatment on the global properties of these networks are divergent from those widely reported in schizophrenia. Where acute NMDA receptor blockade promotes hyperconnectivity in functional brain networks, pronounced dysconnectivity is found in schizophrenia. We also show that acute ketamine treatment increases the connectivity and importance of prefrontal and thalamic brain regions in brain networks, a finding also divergent to alterations seen in schizophrenia. In addition, we characterise how ketamine impacts on bipartite functional interactions between neural subsystems. A key feature includes the enhancement of PFC-neuromodulatory subsystem connectivity in ketamine treated animals, a finding consistent with the known effects of ketamine on PFC neurotransmitter levels. Overall, our data suggest that, at a systems level, acute ketamine-induced alterations in brain network connectivity do not parallel those seen in chronic schizophrenia. Hence, the mechanisms through which acute ketamine treatment induces translationally relevant symptomatology may differ from those in chronic schizophrenia. Future effort should therefore be dedicated to resolving the conflicting observations between this putative translational model and schizophrenia.

1. Introduction

Schizophrenia is a common, chronic psychiatric disorder characterised by positive symptoms (e.g. hallucinations), negative symptoms (e.g. blunted affect) and cognitive deficits (e.g. executive functioning and memory). The disorder involves dysfunction in a number of brain regions including the prefrontal and temporal cortices, the hippocampus and thalamic regions. Recent data from functional brain imaging studies in schizophrenia has advanced our understanding of how functional interactions between distinct neural subsystems, such as the prefrontal cortex (PFC) and hippocampus ([HP], Meyer-Lindenberg *et al.*, 2005; Benetti *et al.*, 2009) and between other discrete cortical regions (Kim *et al.*, 2005) are disrupted in the disorder. However, given that these studies often involve *a priori* selection of the functional interaction of interest, the data may over-emphasise the relative importance of these particular interactions while missing others that are biologically important. Therefore, there has been much interest in taking the alternative approach of characterising disrupted functional interactions between brain regions in the context of complex brain networks, which may provide added insight to those interactions disrupted in schizophrenia (Lynall *et al.*, 2010; Micheloyannis *et al.*, 2006; Bassett *et al.*, 2008; Liu *et al.*, 2008). More recently these approaches have also been applied to further understand how risk factors for psychiatric disorder impact on structural brain networks (Li *et al.*, 2012) and their application to preclinical brain imaging data with translational relevance to the disorder has started to emerge (Bifone *et al.*, 2010; Dawson *et al.*, 2012a).

There is compelling evidence to support a role for altered glutamate system functioning, and N-methyl-D-aspartic acid (NMDA) receptor hypofunction in particular, in schizophrenia. For example, recent *in vivo* ^{1}H magnetic resonance spectroscopy (^{1}H-MRS) data support altered glutamate and glutamine levels in the brains of schizophrenia patients (de la Fuente-Sandoval *et al.*, 2011; Marsman *et al.*, 2013; Natsubori *et al.*, 2013). Furthermore, both acute and repeated exposure to NMDA receptor antagonists can induce schizophrenia-like symptoms in humans (Cosgrove *et al.*, 1991; Krystal *et al.*, 1994) and acute treatment with

the NMDA receptor antagonist ketamine exacerbates the symptoms of schizophrenia patients (Lahti *et al.*, 2001; Malhotra *et al.*, 1997). Due to these observations acute, subanaesthetic ketamine treatment has become one of the most widely utilised pharmacological manipulations in both humans (Krystal *et al.*, 1994; Morgan *et al.*, 2004; D'Souza *et al.*, 2012) and animals (Nikiforuk *et al.*, 2010; Pitsikas *et al.*, 2008; Roberts *et al.*, 2010; Skoblenick and Everling, 2012) to model translationally relevant behavioural deficits relevant to schizophrenia. For example, in rodents the subanaesthetic dose of ketamine used in this study (30mg.kg^{-1}) has been shown to induce deficits in the startle response (as measured by pre-pulse inhibition), hyperlocomotion and deficits in working memory that have translational relevance to schizophrenia (Galci *et al.*, 2008; Irifune *et al.*, 1991; Kos *et al.*, 2006; Miyamoto *et al.*, 2000; Verma and Moghaddom *et al.*, 1996; Yang *et al.*, 2010). In addition many studies, in both humans (Langsjo *et al.*, 2004; Vollenweider *et al.*, 1997a; 1997b) and animals (Chih-Liang *et al.*, 2011; Dawson *et al.*, 2013) have been dedicated to elucidating the impact of acute, subanaesthetic ketamine treatment on regional brain functioning, with PFC hypermetabolism (hyperfrontality) most consistently reported. The paradox of this ketamine-induced hyperfrontality given the hypofrontality characteristic of long-term schizophrenia (Hill *et al.*, 2004) has not been resolved. Surprisingly, despite the widespread use of ketamine treatment as a translational model there is a relative paucity of data on how acute ketamine treatment impacts on the functional interactions between brain regions and the neural subsystems known to be dysfunctional in schizophrenia. Indeed, only recently have efforts begun to elucidate how acute, subanaesthetic doses of ketamine impact on functional connectivity between select brain regions (Niesters *et al.*, 2012; Dawson *et al.*, 2013). Furthermore, to our knowledge, no study has considered the impact of subanaesthetic ketamine treatment on functional brain network structure or on functional interactions between neural subsytems within the context of complex brain networks. Here, for the first time, we apply topological measures based on the algorithms of network science to functional 2-deoxyglucose (2-DG) autoradiographic brain imaging data to quantitatively define how acute ketamine treatment impacts on functional brain network connectivity on a global, regional and neural subsystem scale. Given that recent studies have

begun to quantify brain network alterations in schizophrenia, the recent transition of these algorithms to preclinical brain imaging data, and the great interest in the application of subanaesthetic ketamine treatment as a translational model for the disorder, it is particularly timely to characterise altered brain network structure in this model. Here, we test the hypothesis that acute NMDA receptor hypofunction models the systems level alterations seen in functional brain networks in schizophrenia and gain new, quantitative insight into the true nature of disrupted brain functioning in this translational model through the application of this analytical approach.

2. Materials and Methods

All experiments were completed using male C57BL/6J mice (aged 8-9 weeks) group housed (5-6 animals per cage) under standard conditions (21°C, 45-65% humidity, 12-h dark/light cycle [lights on 0600h]). All experimental manipulations were carried out at least 1 week after entry into the facility and all experiments were carried out under the Animals (Scientific Procedures) Act 1986. Access to food was restricted for 4 to 5 hours prior to the semi-quantitative ^{14}C-2-deoxyglucose imaging protocol to obviate the potential influence of ketamine treatment on plasma glucose levels.

2.1 Semi-quantitative ^{14}C-2-deoxyglucose Autoradiographic Imaging

Measurement of local cerebral glucose utilisation (LCGU) was initiated 1 minute after the treatment of mice with 30mg.kg^{-1} ketamine (Sigma-Aldrich, UK; in 2ml.kg^{-1} saline, interperitoneally [*i.p.*], n = 9) or physiological saline (1.25 mls.kg^{-1} , *i.p.*, n = 9) in accordance with previously published protocols (Dawson *et al.*, 2011; 2013). Mice were injected with 4.625 MBq.kg^{-1} of [^{14}C]-2-deoxyglucose (Perkin-Elmer, UK) at a steady rate over a 10 second period before being returned to their home cage. At exactly 45 minutes after isotope injection animals were decapitated and a terminal blood sample was collected, by torso inversion, in heparinised weigh boats. The timing of this imaging protocol ensures that the alterations in brain functioning detected align with the maximal behavioural effects of this dose of ketamine (Miyamoto *et al.*, 2000; Yang *et al.*, 2010). The brain was rapidly dissected out intact then frozen in isopentane (-40°C) and stored at -80°C until sectioning. Blood samples were centrifuged to separate the plasma and aliquots were removed for the determination of plasma glucose (10μl) and ^{14}C (20μl) concentrations by semi-automated glucose oxidase assay (Beckman Glucose Analyser) and liquid scintillation analysis (Packard), respectively.

Frozen brains were sectioned (20μm) in the coronal plane in a cryostat (-20°C). A series of three consecutive sections were retained from every 60μm, thaw mounted onto slide covers

and rapidly dried on a hot plate (70°C). Autoradiograms were generated by apposing these sections, together with precalibrated ^{14}C-standards (40-1069 nCi/g tissue equivalents: Amersham International, UK) to X-ray film (Kodak, SB-5) for 5 days. Autoradiographic images were analysed by a computer-based image analysis system (MCID/M5+). The local isotope concentration for each brain region of interest (RoI) was derived from the optical density (O.D.) of autoradiographic images relative to that of the coexposed ^{14}C standards. Measurements were taken from 66 anatomically distinct brain regions defined with reference to a stereotaxic mouse brain atlas (Paxinos and Franklin, 2001). The rate of metabolism, LCGU, in each RoI was determined as the ratio of ^{14}C present in that region relative to the average ^{14}C concentration in the whole brain of the same animal, and from hereon in will be referred to as the ^{14}C-2-DG uptake ratio. Whole brain average ^{14}C levels were determined from the average ^{14}C concentration across all sections in which a RoI was measured. Ketamine induced alterations in overt LCGU were analysed using Student's t-test and significance was set at $p<0.05$, and anatomically discrete brain regions were assumed to represent independent variables (as previously discussed in McCulloch *et al*., 1982).

2.2 Inter-regional correlations and functional brain networks

The inter-regional Pearson's correlation coefficient was used as the metric of the functional association between brain regions generated from the ^{14}C-2-DG uptake ratios for each RoI across all animals within the same experimental group (i.e. either control or ketamine-treated). These correlations were Fisher *z*-transformed to give the correlation data a more normal distribution. This resulted in a pair of {66 x 66} correlation matrices, each within-group matrix representing the specific association strength between each of the 2,145 possible pairs of regions. From each correlation matrix (*R)* we derived a binary adjacency matrix (*A)* where the functional connection between two regions ($a^{i,j}$ element) was zero if the Pearson's correlation coefficient was lower than the defined threshold (p|i,j|<*T*) and unity if the coefficient was greater or equal to the defined threshold (p|i,j|≥*T*). The adjacency matrix can also be represented as an undirected graph *G*, where a line or edge

represents the functional interaction between two brain regions (nodes) if the correlation coefficient exceeds the defined threshold value.

2.3 Network Analysis

Network architecture was characterised at the global, divisional and regional scales. Global network architecture was quantified in terms of the mean degree (<k>), average pathlength (L_p) and mean clustering co-efficient (C_p) of the whole brain network, as previously described in Dawson *et al.*, 2012a and outlined in Section 2.4 below. Alterations in divisional architecture were determined in terms of altered functional clustering, identified in the brain networks through application of the generalized singular value decomposition (GSVD) algorithm (Dawson *et al.*, 2012a; Xiao *et al.*, 2011), that provides a data driven approach to identifying clustering difference between the two experimental matrices. Regional properties were defined in terms of degree (k_i), betweeness (B_c) and closeness (C_c) centrality. Global and regional metrics were determined on the binary adjacency matrices generated over a range of correlation thresholds (Pearson's r, T = 0.3 to 0.4, Fisher's z, T = 0.310 to 0.424) that were selected on the basis that the maximum threshold utilised yielded fully connected networks in each treatment group and was similar to that utilised in previous reports (Dawson *et al.*, 2012; Liu *et al.*, 2008; Micheloyannis *et al.*, 2006).

2.4 Global Network Architecture

Here we provide brief, formal definitions of the global network metrics determined in this study.

The degree of a node (*k)* is simply the number of edges that connect that node to the network, so highly connected nodes have a high degree. The mean degree (<k>, equation [1]) is the average number of edges across all nodes. A sparse network therefore has a low mean degree**.**

$$< K >= \frac{1}{N} \sum_{i \in G} k_i.$$

(1)

The minimum path length between two nodes in a graph ($L_{i,j}$) is the smallest number of edges that must be traversed to make a connection between them. If two nodes are immediate neighbours, directly connected by a single edge, then $L_{i,j}$ = 1. The average path length (L_p, equation [2]), or average $L_{i,j}$ across all possible pairs, is the average number of steps along the shortest paths across the network. This provides a measure of global network efficiency, where networks with a low average path length are more efficient.

$$L_p = \frac{1}{2N(N-1)} \sum_{i>j} L_{ij}.$$

(2)

The clustering coefficient of node i (C_i) is the ratio of the number of edges between neighbours of node i relative to the maximum possible number of connections between them. It provides an indication of how well connected the neighbourhood of a node is. The mean clustering coefficient (C_p, equation [3]) is the average clustering coefficient of all of the nodes in the network, which provides a measure of the local density or cliquishness of the network. A high mean clustering coefficient suggests high clustering and so efficient local information transfer.

$$C_p = \frac{1}{N} \sum_{i \in G} C_i.$$

(3)

The significance of ketamine-induced alterations in the global properties of 2-DG functional brain networks was determined by comparison of the real difference in each measure to that of networks generated from 1,000 to 5,000 random permutations of the raw 2-DG data at each correlation threshold (11,000 to 55,000 random permutations in total). Significance

was set at p<0.05 and was determined from the average p-value across the entire correlation threshold range analysed.

2.5 Regional centrality and Hub region identification

In this study we consider node centrality as determined by degree (k_i), betweeness (B_c) and closeness (C_c). Degree centrality (k_i) simply measures the number of nodal connections. Betweeness centrality (B_c, equation [4]) is based upon how many short paths go through a given node and for the ith node in a graph (G) is defined by:

$$B_c(i) = \sum_{\substack{s \neq i \neq t \in G \\ s \neq t}} \frac{\sigma_{st}(i)}{\sigma_{st}}.$$

(4)

Here σ_{st} denotes the number of shortest paths from node s to node t, and $\sigma_{st}(i)$ denotes the number of these that involve node i.

Closeness centrality (C_c, equation 5) is based upon the mean geodesic distance of a node to all other reachable nodes in the network, given by:

$$C_c(i) = \frac{1}{\sum_{t \in G \setminus i} d_G(i,t)}.$$

(5)

Here dG(i,t) denotes the geodesic between nodes i and t.

A brain region that is considered to be an important hub region in the network has a high degree, betweenness or closeness centrality. In this study a brain region was defined as a hub in the brain networks of ketamine-treated or control (saline-treated) animals if, for any centrality measure, the regional centrality measure in the real network relative to that of calibrated random Erdös-Rényi graphs (1,000 random graphs at each correlation threshold, 11,000 random graphs in total), was z>1.96. The z-score for each centrality measure was calculated as illustrated in equation 6 for the case of betweenness centrality (B_c)

$$ZB_c(i) = \frac{B_c^{real} - B_c^{random}}{SD_{(B_c^{random})}}.$$

(6)

The significance of ketamine-induced alterations in regional centrality was determined by comparison of the z-score difference in regional centrality in the real brain networks with that in networks generated from 11,000 random permutations of the raw 2-DG data (1,000 random permutations at each correlation threshold). Significance was set at $p<0.05$ with *post-hoc* Bonferroni correction applied to adjust for multiple comparisons on the basis of comparing 66 RoI.

2.6 Divisional architecture; bipartite neural subsystem interactions

Differences in the divisional architecture of the functional brain networks between experimental groups was characterised through the application of a recently developed and validated algorithm for cluster identification based upon generalized singular value decomposition (GSVD). The functionality of this algorithm has previously been proposed, justified and validated through its application to both synthetic and real biological data sets (Xiao *et al.*, 2011), and has previously been applied to preclinical brain imaging data (Dawson *et al.*, 2012a). In the context of this study the aim of applying this algorithm to the data was to quantitatively define the functional interactions between two defined neural subsystems (bipartite interactions) that are present in one of the experimental groups but not the other. The ability of the algorithm to elucidate mutually exclusive clusters in two matrices has previously been validated through its application to synthetic data sets (Xiao *et al.*, 2011).

In essence this algorithm allows the re-ordering of two square, symmetric, real-valued matrices *A* and *B* with the aim of discovering a new node (brain region) ordering that reveals clusters of nodes that exhibiting strong functional connectivity (mutual weights) in one network but not the other. In the case of our data, matrix *A* and matrix *B* are comprised of the real-value inter-regional correlation coefficients (Pearson's coefficient Fisher transformed) for the control and ketamine treatment group's respectively. These matrices

are identical to those used for the generation of the binary adjacency matrices used to characterise the global and regional characteristics of the functional brain networks in these experimental groups. Once the matrices have been reordered through this iterative algorithm we assessed whether the variance of the node set for a given pair of defined neural subsystems (defined neural subsystems are shown in Figure 4) in the reordered matrix was significantly lower than expected by chance. To determine this the variance of the nodes for the two neural subsystems of interest in the real GSVD reordered networks was compared with that of a set of nodes equal in size to that of the defined neural subsystem in matrices derived from 10,000 random permutations of the raw data. The p-values gained from this permutation analysis were corrected by post-hoc Bonferroni-Holm correction to adjust for multiple comparisons (110 neural subsystem comparisons made in the control and ketamine treated data sets [55 in each experimental group]). Significance was set at $p<0.05$.

3. Results

3.1 Ketamine-induced alterations in overt cerebral metabolism

Acute ketamine treatment produced both significant increases and decreases in cerebral metabolism on a region-dependent basis, consistent with previous reports (Dawson *et al*., 2013; Duncan *et al*., 1999). Significant overt alterations in cerebral metabolism were identified in 32 of the 66 regions analyzed (Supplementary Table S1[A-D]); Select regions shown in Figure 1). In particular, acute ketamine treatment induced hyperfrontality, an increased rate of cerebral metabolism in subfields of the PFC, an effect that was evident in all subfields of the prelimbic cortex (anterior prelimbic [PrL] and medial prelimbic (layers 1, 2 and 3 [mPrL1, mPrL2, mPrL3]). In addition, significant hypermetabolism was found in the entorhinal cortex (EC) and subfields of the hippocampus (molecular layer [ML] and subiculum [Sub]), the nucleus accumbens core (NacC), substantia nigra pars compacta (SNC) and the mamillary body (MB). By contrast, acute ketamine treatment induced significant thalamic hypometabolism (anteroventral [AVthal], mediodorsal [MDthal], ventrolateral [VLthal] and dorsal reticular [dRT]), along with hypometabolism in the raphé (dorsal [DR], median [MR] and paramedian [PMR]), tegmental (dorsal [DTg] and ventral [VTg]) and habenula (medial [mHab] and lateral [lHab]) nuclei. In addition, ketamine-treatment induced significant hypometabolism in the locus coeruleus (LC), the diagonal band of broca (horizontal [HDB] and vertical [VDB]), medial geniculate (MG), inferior colliculus (IC), the CA3 subfield of the hippocampus and in the retrosplenial cortex (RSC).

3.2 Ketamine-induced alterations in global functional brain network topology

Acute ketamine treatment resulted in a functional brain network with increased connectivity, as evidenced by the significant increase in mean degree (<k>, P = 0.0490), the average number of functional connections each brain region (node) has in the network, across the entire correlation threshold range (Figure 2[A]). However, while the number of

connections in the brain network was significantly increased by ketamine treatment this did not significantly impact on average path length (L_p, Figure 2[B], P = 0.207), across the observed correlation threshold range. A low average path length is indicative of a network organised for efficient information transfer on a global scale. The observation that ketamine does not alter L_p, thus does not alter the efficiency of information transfer across the network, is surprising given the increased number of functional connections (mean degree, Figure 2[A]) seen in the brain networks of these animals, but suggests that the efficiency of information transfer across the network on a global scale is not modified by acute ketamine treatment. By contrast acute ketamine treatment induced a significant increase in the mean clustering coefficient (C_p, Figure 2[C], P = 0.0333), a measure of local connectivity or cliquishness in the brain network, across the entire correlation threshold range analysed. This indicates that acute ketamine treatment induces a topological reorganisation of the functional brain network resulting in increased efficiency for localised information transfer. When brain networks were considered at the same cost, in essence comparing the global properties of the functional brain networks when they have the same number of functional connections (by allowing weaker functional connections in control animals to be considered as connected) there was no significant difference in the average path length (L_p, Figure 1[D], P = 0.0987) or the mean clustering coefficient (C_p, Figure 1[E], P = 0.1448) of the brain networks. This suggests that the enhanced efficacy of functional connections between brain regions, as indicated by a significantly increased mean degree (<k>), that results from ketamine treatment underlies the increased mean clustering coefficient (C_p) seen in the brain networks of these animals.

3.3 Ketamine induced alterations in regional functional connectivity

In the functional brain networks of control (vehicle treated) animal's 4 of the 66 brain regions analysed were identified as important hubs by centrality analysis (Figure 3, Table 1) including the retrosplenial cortex (RSC), nucleus accumbens shell (NacS), interpeduncular nucleus (IP) and mammillary body (MB). In the functional brain network of ketamine treated

animals an increased number of brain regions (14 out of 66) were identified as important hubs. This included two subfields of the prefrontal cortex (lateral orbital [LO], medial prelimbic cortex, layer 3 [mPrL3]), 4 thalamic nuclei (anteromedial thalamic nucleus [AMthal], mediodorsal thalamus [MDthal], ventromedial thalamus [VMthal] and the centrolateral thalamus [CLthal]) and 2 amygdala nuclei (medial amygdala [meA], basolateral amygdala [BLA]). There was little overlap in the identity of brain regions identified as important hubs in the functional brain network of ketamine treated and control animals, with only the nucleus accumbens shell (NacS) being identified as an important hub in the functional brain network of both experimental groups. This suggests that the organization of the functional brain network is markedly altered by ketamine treatment. Indeed, a statistically significant increase in regional centrality was found for 2 PFC subfields (mPrL3, LO), 3 thalamic nuclei (MDthal, VMthal, CLthal), 2 amygdala nuclei (MeA, BLA) and the medial habenula (mHab, Table 1). Therefore, these brain regions show significantly increased connectivity and importance in the brain networks of ketamine treated animals. By contrast, ketamine treatment induced a significant reduction in regional centrality for the retrosplenial cortex (RSC) and mammillary body (MB), suggesting that these hub brain regions in the brain network of control animals were less important and showed significantly reduced connectivity in the brain networks of ketamine treated animals (Table 1).

3.4 Ketamine induced alterations in bipartite neural subsystem interactions

GSVD analysis, allowing for the identification of bipartite neural subsystem interactions, revealed that in control animals the thalamus was significantly functionally coupled to both the hippocampus (P = 0.043) and the neuromodulatory nuclei (P = 0.011, Figure 4). The functional interactions between these neural subsytems was not significant in ketamine-treated animals. Rather, GSVD analysis revealed a significant interaction between the neuromodulatory nuclei and the prefrontal cortex (P = 0.043) and other cortical regions (cortex subsystem, P = 0.022) in ketamine treated animals that was not significantly present in controls. In addition, a significant functional interaction between the thalamus and cortex

subsystems (P = 0.040) was found in ketamine treated animals that was not present in the brain network of control animals.

4. Discussion

Through the application of established and recently validated network science algorithms we have gained important new insight into how acute NMDA receptor blockade impacts on brain functioning at a systems levels. Importantly, ketamine induced alterations in the global properties of functional brain networks (supporting enhanced connectivity) do not parallel those seen in schizophrenia patients (Micheloyannis *et al*., 2006; Liu *et al*., 2008; Lynall *et al*., 2010), that overall support widespread functional dysconnectivity in the disorder. In particular our data support a ketamine induced enhancement of prefrontal, thalamic and amygdala regional functional connectivity, as determined by centrality analysis (Table 1, Figure 3). Furthermore, our analysis of ketamine induced alterations in bipartite neural subsystem interactions suggests that the connectivity of these, and other, neural subsystems is profoundly altered as a result of acute ketamine treatment.

Functional brain networks in chronic schizophrenia characteristically have fewer connections (reduced mean degree <k>), a decreased efficiency for global information transfer (increased average pathlength, L_p) and decreased local connectivity (decreased mean clustering, C_p [Micheloyannis *et al*., 2006; Liu *et al*., 2008; Lynall *et al*., 2010]). By contrast, we have shown that acute ketamine treatment results in a functional brain network with increased connectivity, as evidenced by an increased number of functional connections (Figure 2(A), increased mean degree, <k>), and increased local connectivity (Figure 2(C), increased mean clustering coefficient, C_p). While the functional connectivity measure utilised in our study is derived from regional metabolic activity across an extended time period, rather than in real time as derived in human brain imaging studies, the parallels between the network connectivity alterations detected using our approach in mice and that reported in recent human brain imaging studies suggests that this is a valid approach. For example, the increased functional connectivity found in brain networks of mice treated with ketamine in our study parallels the increased functional connectivity found in the brain of healthy humans treated with subanaesthetic ketamine (Driesen *et al*., 2013). These data suggest

that, at a systems level, the mechanisms through which acute ketamine treatment induces schizophrenia-like symptoms may be profoundly divergent from those that contribute to these symptoms in the disorder. This suggestion is also consistent with the alterations we have identified at the level of regional connectivity, as determined through centrality analysis, which supports both enhanced PFC and thalamic functional connectivity. Therefore, our data suggest that the disruption of PFC-dependent cognitive processes by subanaestetic ketamine treatment (Krystal *et al*., 2000; Nikiforuk *et al*., 2010; Moghaddam *et al*., 1997) results not from PFC functional dysconnectivity, but may result from abnormally increased PFC functional connectivity, a suggestion consistent with our previous observation that ketamine enhances functional connectivity between subfields of the PFC (Dawson et al., 2013). Decreased PFC functional connectivity is widely reported in schizophrenia (Deserno *et al*., 2012; Honey *et al*., 2005; Spence *et al*., 2000; Zhou *et al*., 2007), although some studies have identified regionally selective increases in PFC connectivity in the disorder (Schlösser *et al*., 2003; Klingner *et al*., 2013). Overall our data suggest that rather than impeding the capability of the PFC to receive, integrate and output relevant information to other neural subsystems, through PFC dysconnectivity as seen in schizophrenia, acute ketamine treatment may decreases the signal-to-noise ratio of information received by the PFC, by the promotion of an abnormally increased functional connectivity of this neural subsystem. This suggested mechanism is not only consistent with recent human brain imaging data showing that subanaesthetic ketamine treatment increases global brain functional connectivity (Driesen *et al*., 2013), but is also consistent with emerging electophysiological data supporting a reduced signal-to-noise after ketamine treatment in the hippocampal CA3 subfield (Saunders *et al*., 2011) and of gamma oscillations in the somatosensory thalamocortical system (Kulikova *et al.,* 2012) of rodents. Our data suggest that similar mechanisms may occur in the PFC, where ketamine is known to directly increase the amplitude of gamma oscillations through the blockade of local NMDA receptors (McNally *et al*., 2011) and where the NMDA receptor antagonist MK-801 has been shown to disrupt the firing pattern of PFC neurons (Jackson *et al*., 2004). Interestingly, our recent data show that prolonged NMDA receptor hypofunction, as induced by repeated subchronic phencyclidine

treatment in rats, is capable of inducing the functional network dysconnectivity characteristic of chronic schizophrenia (Dawson *et al*., 2012a). This suggests that plasticity dependent modifications in brain circuitry that result from prolonged but not acute NMDA receptor hypofunction, such as decreased expression of parvalbumin in GABAergic neurones (Cochran *et al*., 2003) and altered neurotransmitter receptor expression (Steward *et al*., 2004), are essential in order to recapitulate the alterations in functional brain networks seen in chronic schizophrenia. Moreover, sustained NMDA receptor hypofunction induces the hypofrontality characteristic of the disorder (Cochran *et al*., 2003; Dawson *et al*., 2012a; 2012b), whereas acute ketamine treatment induces a contrasting hyperfrontality, a paradox that is yet to be resolved given the schizophrenia-like symptoms induced by acute ketamine treatment. It may be the case that ketamine induced alterations in functional brain network structure have the greatest translational relevance to those symptoms present during acute psychotic episodes rather than those that remain present in chronic schizophrenia patients treated with antipsychotics. That is to say that ketamine induced alterations in functional brain networks may more accurately model the *state*-dependent alterations in functional brain connectivity that are present during an acute psychotic episode, rather than the *trait* alterations in functional brain network connectivity evident in chronic schizophrenia, which may be masked during acute psychosis. The suggestion that decreased brain network connectivity may be a trait of brain networks in schizophrenia is supported by recent evidence showing that individuals with an increased risk for the disorder show altered brain network properties characteristic of schizophrenia (Li *et al*., 2012), again supporting network dysconnectivity. However, as the properties of functional brain networks during acute psychosis are yet to be characterised, the possibility that state dependent alterations in brain network connectivity during acute psychosis may mask those evident as a trait of the disorder remains speculative. The characterisation of functional brain network connectivity in schizophrenic individuals during their first episode of acute psychosis and subsequently when stabilised by antipsychotic treatment would be an essential step in testing this hypothesis, and also in further resolving the true translational relevance of subanaethetic ketamine treatment. The suggestion that subanaesthetic ketamine treatment may have

greater translational relevance to at least some of the cognitive deficit seen in the disorder that are evident during the early stages of the disease but not in chronic schizophrenia is supported by recent data (Hauser *et al.*, 2011; Moore *et al.*, 2013).

Our data also support thalamic hyperconnectivity in the brain networks of animals treated with ketamine. This is consistent with our previous observation, that acute ketamine treatment enhances connectivity between the thalamic nuclei (Dawson *et al.*, 2013). This contrasts with the thalamocortical dysconnectivity widely reported in schizophrenia, which is particularly evident in terms of PFC-thalamic dysconnectivity (Marenco *et al.*, 2012; Welsh *et al.*, 2010; Zhou *et al.*, 2007; Woodward *et al.*, 2012), although this is not always found (Klingner *et al.*, 2013; Schl sser *et al.*, 2003). In addition, there are examples of enhanced thalamic connectivity to other cortical subfields in schizophrenia, such as to motor and somatosensory areas (Woodward *et al.*, 2012) and to the insular cortex (Corradi-Dell'Acqua *et al.*, 2012). Interestingly, we did find evidence for enhanced thalamic connectivity to cortical regions (Figure 4C), with the exception of the PFC, following ketamine treatment in this study. Our previous data, and data from other groups, suggest that the direct actions of ketamine in the reticular thalamus (RT) may be a key mechanism through which ketamine promotes the abnormal connectivity of other thalamic regions, in part through the disinhibition of thalamocortical projections (Dawson *et al.*, 2013; Zhang et al., 2009). Given that the cognitive and functional roles of specific thalamic nuclei and their cortical connections is poorly defined, with the exception of the PFC-thalamic connection, the potential contribution of these alterations to ketamine's ability to model schizophrenia certainly warrants further systematic investigation. Clearly, our data suggest a disconnect between many of the systems level alterations induced by acute ketamine treatment and those seen in the brain of schizophrenia patients and thus these must be more clearly resolved in order to more rigorously define the specific translational value of this model. Understanding the plasticity alterations that occur in the brain as a result of repeated NMDA receptor blockade may be particularly important in elucidating the contribution of this mechanism to the alterations in brain network connectivity seen in schizophrenia.

For the first time we have shown that bipartite functional interactions between distinct neural subsystems are dramatically altered as a result of acute NMDA receptor blockade. In particular the functional connectivity of the PFC, thalamus, hippocampus and neuromodulatory neural subsystems are significantly altered by ketamine treatment. The ketamine induced alterations we have shown in the bipartite connectivity of these neural subsystems, as determined by GSVD analysis, parallel those we have previously reported using regional functional connectivity analysis (Dawson *et al.*, 2013), that included a ketamine induced enhancement of functional connectivity between selected neuromodulatory nuclei (dorsal raphé and locus coeruleus) and the PFC. As the neuromodulatory nuclei defined in this study included both the raphé (dorsal [DR] and medial [MR]), sources of serotonergic innervation to the PFC, and the locus coeruleus (LC), a primary source of noradrenergic innervation to the PFC, the enhanced connectivity between these neural subsystems is consistent with the known ability of acute ketamine to profoundly enhance both serotonin (Lindefors *et al.*, 1997; Amargos-Bosch *et al.*, 2006) and noradrenaline levels in the PFC (Kubota *et al.*, 1999). The altered connectivity of these neuromodulatory neurotransmitter systems may directly contribute to the disruption of PFC dependent processes by acute NMDA receptor blockade, a suggestion supported by the observation that the application of antagonist for the receptors of these neuromodulatory systems can attenuate the disruptive effects of NMDA receptor antagonists on PFC dependent cognitive processes (Metzer *et al.*, 2011; Mirjana *et al.*, 2004), PFC physiology (Jentsch *et al.*, 1988) and other translationally relevant behaviours (Bakshi *et al.*, 1997; Stuchlik *et al.*, 2009). Overall, our data suggest that the connectivity of the neuromodulatory subsystem to the PFC is profoundly altered by acute ketamine treatment and is likely to contributes to ketamine's disruptive effects on PFC dependent cognitive processes. In contrast to the PFC, we found that the bipartite functional connectivity of the thalamus to the neuromodulatory subsystem and hippocampus was decreased, whereas thalamic connectivity to the cortical subsystems was increased by acute ketamine treatment. The direct inhibition of thalamic GABAergic neurons, consistent with the decreased metabolic demand of these nuclei (Figure 1, as 2-deoxyglucose imaging largely reflects the metabolic

demands of localised synapses), by NMDA receptor blockade (Zhang *et al.*, 2009) may underlie the functional dysconnectivity of this neural subsystem from that of the neuromodulatory nuclei. This in turn would lead to the disinhibition of thalamic glutamatergic projections to the cortex and other neural subsystems (Sharp *et al.*, 2001), a suggestion consistent with the enhanced bipartite thalamus-cortex connectivity also seen in ketamine treated animals.

Overall, our data suggest that the systems level alterations induced by subanaesthetic ketamine treatment are distinct from those seen in chronic schizophrenia. These, along with other data, suggest that plasticity dependent alterations in brain circuitry that result from chronic but not acute NMDA receptor blockade most accurately reflect the systems level differences in brain network functioning seen in schizophrenia. Characterising these plasticity mechanisms may provide key insight into the transition from the acute psychotic to the chronic disease state in schizophrenia, and could provide opportunities for the development of novel treatment interventions. In addition, these data provide dramatic new insight into the altered brain functioning, and the altered interactions between defined neural subsystems, that result from acute NMDA receptor blockade.

5. Funding and Disclosure

6. Acknowledgements

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8. Legends

8.1 Tables

Table 1. Altered hub brain regions in the functional brain networks of control and ketamine-treated mice. Data shown as *z*-score for each centrality measure (degree, betweenness and closeness) for each hub brain region in the brain networks of control and ketamine treated animals. Values in bold denote centrality measures in which a given brain region reaches the defined criteria (z-score >1.96 relative to 11,000 calibrated random Erd s-Rényi graphs across the entire correlation threshold range [T = 0.3 to 0.4]) to be considered as a hub. **denotes p<0.01 significant difference in a given centrality measure between ketamine treated and control animals (determined by comparison of the difference in real brain networks to that in 11,000 random permutations of the data across the entire correlation threshold range (T = 0.3 to 0.4) with Bonferroni *post-hoc* correction for multiple comparisons). Full data are shown in the supplemental material Tables S2(A-D).

8.2 Figures

Figure 1. Ketamine induced alterations in overt local cerebral glucose utilization (LCGU). Data shown as mean ± SEM. *denotes p<0.05, **denotes p<0.01 and ***denotes p<0.001 significant difference from control (saline treated) animals (t-test). Full data for all brain regions analysed are shown in Supplemental Table S1(A-D).
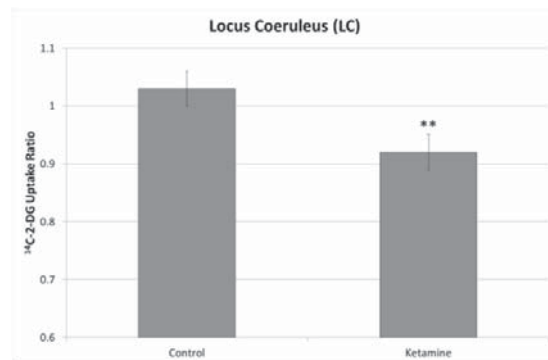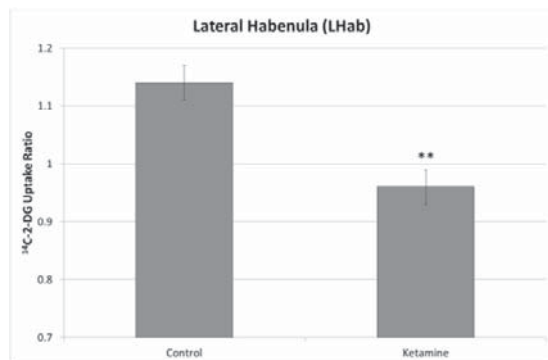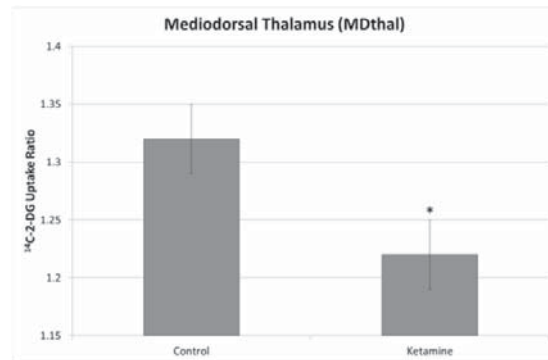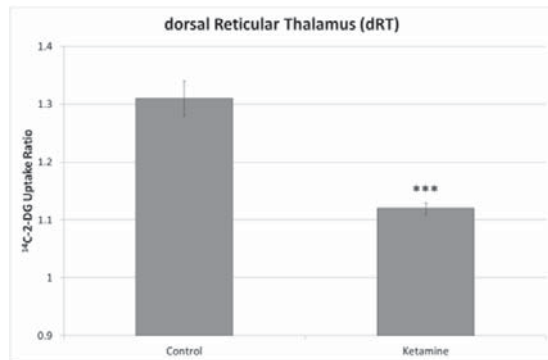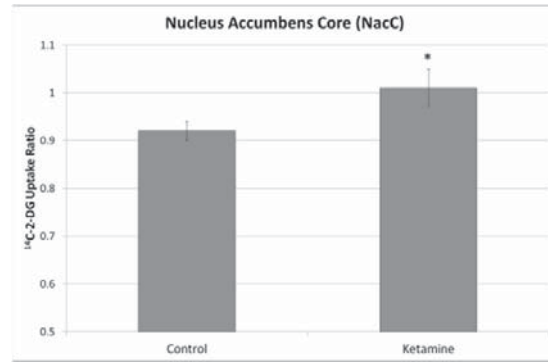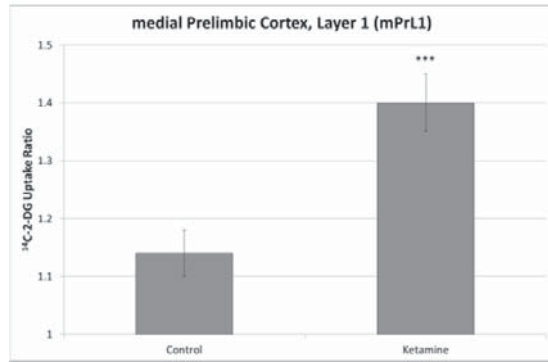
Figure 2. Ketamine induced alterations in global functional brain network topography. Acute Ketamine treatment (30 mg.kg^{-1}) resulted in functional brain networks that displayed a significantly increased (A) mean degree (P = 0.0490), had a similar (B) average path length (P = 0.207) but a significantly increased (C) mean clustering coefficient (P = 0.0333) in comparison to networks in control animals. When the functional brain networks of each experimental group were compared at the same cost there was no significant difference between the two groups in terms of the (D) average path length (P = 0.0987) or mean
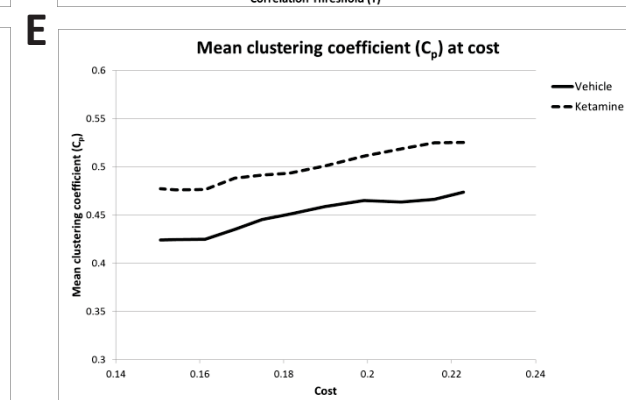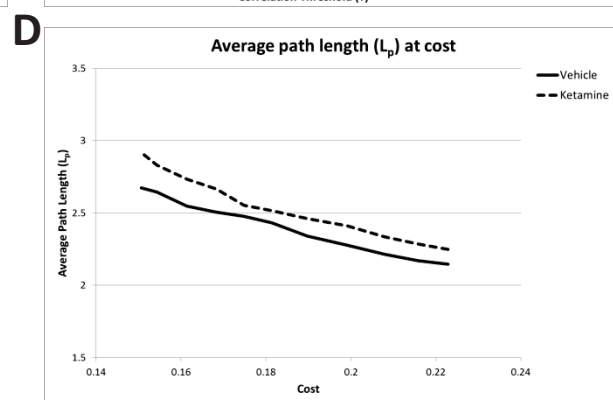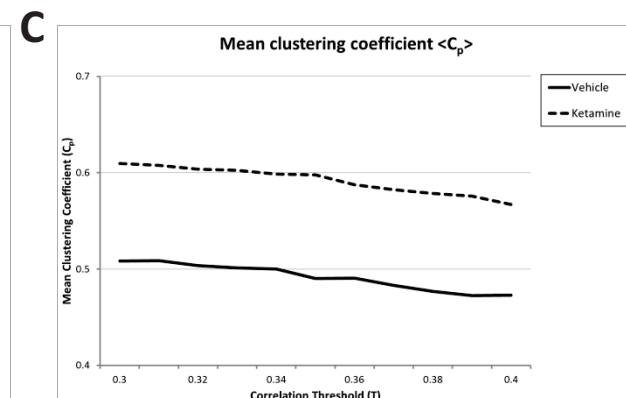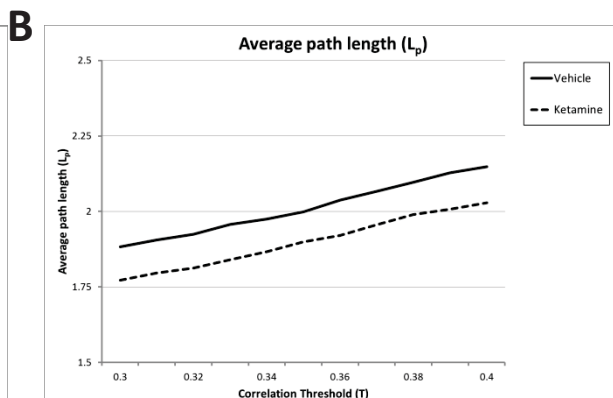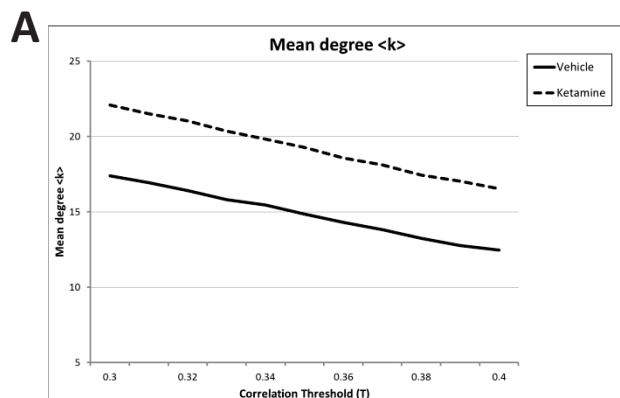
clustering coefficient (P = 0.1448). The significance of ketamine induced alterations in global network measures was analyzed by comparison to that in networks generated from 5,000 (A,B,C) or 1,000 (D,E) random permutations of the raw experimental data. Significance was set at p<0.05.
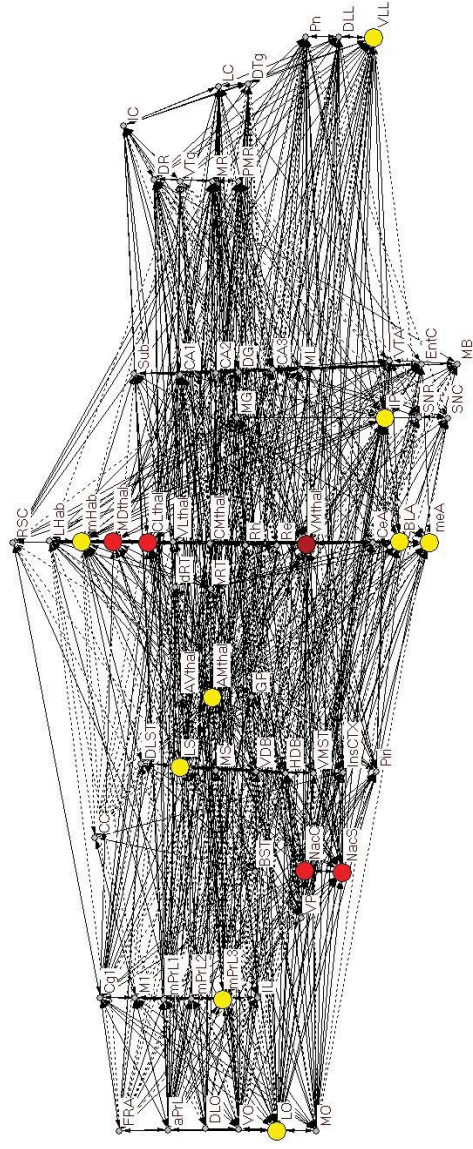
Figure 3. Hub brain regions in the functional brain networks of control and ketamine treated mice. Graph representations of the 2-DG brain networks in control and ketamine treated mice. Brain networks are shown at the 0.4 correlation threshold (T = 0.4). Solid edges between nodes represent a positive correlation in cerebral metabolism between brain regions, whereas broken connections (edges) denotes a negative correlation in cerebral metabolism between two brain regions. If regional centrality, for any centrality measure, surpassed the z > 1.96 threshold, calculated in comparison with 11,000 calibrated random Erdős-Rényi graphs, across the entire correlation threshold range (T = 0.3 to 0.4), then that region was considered to be a hub in the functional brain network. Large nodes are those that are defined as important hubs in the network. Node colour denotes the centrality measure in which a brain region was considered to be an important hub in the brain network. These graphs were generated using Pajek software (http://pajek.imfm.si/doku.php?id=download).

Figure 4. Ketamine induced alterations in bipartite neural subsystem interactions. (A) Heatmaps showing the clustering of neural subsystems in control and ketamine treated animals brain network matrices following GSVD reordering. Warm colours (red/orange) represent high/positive functional correlations between brain regions and cold colours (blue/green) represent low/negative correlations between brain regions. The Control reordered matrix identified clusters of brain regions present in the control group not present in ketamine treated animals, and the ketamine ordered matrix shows clusters present in ketamine treated animals not present in controls. (B) Brain region lists showing the order of brain regions in the original (alphabetical) and GSVD reordered matrices for ketamine treated and control animals. (C) Summary diagram of significant neural subsystem bipartite

interactions seen in the GSVD reordered matrices of control but not ketamine treated animals (defined in blue) and those seen in ketamine treated but not control animals (defined in red). Values indicate the significance level of the given bipartite neural subsystem interaction, determined by comparison of the joint variance of each bipartite neural subsystem in the real GSVD reordered matrices as compared to that seen in 10,000 random permutations of the real data. These p-values were adjusted *post-hoc* by Bonferroni-Holm correction for multiple (110) comparisons. Significance is set at $p < 0.05$. Full data are shown in the Supplementary Material (Tables S3[A-B]).

medial Prelimbic Cortex, Layer 1 (mPrL1)

Nucleus Accumbens Core (NacC)

dorsal Reticular Thalamus (dRT)

Mediodorsal Thalamus (MDthal)

Lateral Habenula (LHab)

Locus Coeruleus (LC)

A Mean degree <k>

B Average path length (L$_p$)

C Mean clustering coefficient <C$_p$>

D Average path length (L$_p$) at cost

E Mean clustering coefficient (C$_p$) at cost

Control

Ketamine

Degree
Betweenness
Closeness
2 measures
3 measures

Brain Region	Degree Control	Degree Ketamine	Betweenness Control	Betweenness Ketamine	Closeness Control	Closeness Ketamine
Prefrontal Cortex						
medial Prelimbic Cortex, Layer 3 (mPrL3)	0.02	**2.03****	-0.63	0.41	-2.09	0.48
Lateral Orbital Cortex (LO)	-1.17	**2.61****	-0.48	1.04	-3.38	**1.87**
Cortex						
Retrosplenial Cortex (RSC)	**2.38**	-0.53**	0.39	-0.17	1.21	-1.22
Thalamic Nuclei						
Mediodorsal Thalamus (MDthal)	0.23	**3.25****	-1.18	1.00	-2.01	**3.23**
Anteromedial Thalamus (AMthal)	0.52	**2.00**	-0.01	-0.11	-0.73	0.95
Ventromedial Thalamus (VMthal)	1.33	**4.50****	0.44	**2.67****	0.45	**5.22**
Centrolateral Thalamus (CLthal)	0.49	**3.45****	-1.05	0.99	-1.83	**3.57**
Mesolimbic System						
Nucleus Accumbens Core (NacC)	1.37	**2.85**	0.62	0.16	0.15	**2.50**
Nucleus Accumbens Shell (NacS)	**2.54**	**2.99**	0.83	1.48	1.21	**3.14**
Amygdala						
Medial Amygdala (MeA)	-2.79	**2.17****	-1.90	1.47	-6.75	1.62
Basolateral Amygdala (BLA)	-0.54	**1.95****	-0.23	0.25	-1.99	1.49
Septum/Diagonal Band of Broca						
Lateral Septum (LS)	-1.94	**1.42****	-0.58	**2.87****	-4.15	0.51
Mutlimodal						
Medial Habenula (mHab)	-2.09	**2.16****	-0.97	0.06	-4.35	0.95
Mamillary Body (MB)	**2.20**	-2.37**	1.41	-1.95	0.88	-4.65
Interpeduncular Nucleus (IP)	**3.24**	**2.35**	1.42	0.62	1.43	1.60
Venterolateral Lemniscus (VLL)	1.67	**2.59**	-0.64	1.16	-0.57	**1.82**