

## **Title Page:**

# **Unravelling the GSK3 $\beta$ -related genotypic interaction network influencing hippocampal volume in recurrent major depressive disorder**

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**Short title:** GSK3 $\beta$  network and hippocampal volume in depression

**Key words:** major depressive disorder; hippocampus; glycogen synthase kinase 3 beta, network, endoplasmic reticulum stress, histone deacetylase modifications

**Word count:** Abstract: 243 / Article body: 3495

**Tables: 2 / Figure: 1**

**Supplementary Tables: 3 / Supplementary Figure: 0**

## Abstract

**Objective:** Glycogen synthase kinase 3 beta (GSK3 $\beta$ ) has been implicated in mood disorders. We reported associations between a GSK3 $\beta$  polymorphism and hippocampal volume in major depressive disorder (MDD). We then reported similar associations for a subset of GSK3 $\beta$ -regulated genes. We now investigate a comprehensive list of genes encoding proteins that directly interact with GSK3 $\beta$  to identify a genotypic network influencing hippocampal volume in MDD. **Methods:** We used discovery and replication MDD samples. Our gene list was generated from the NetworkKIN database. Hippocampal measures were derived using an optimized Freesurfer protocol. We identified interacting SNPs using the machine learning algorithm Random Forest and verified interactions using likelihood ratio tests (LRTs) between nested linear regression models. **Results:** The discovery sample revealed multiple two-SNP interactions with hippocampal volume. The replication sample revealed a replicable interaction (LRT  $p=0.0088$ , replication sample;  $p=0.017$ , discovery sample; Stouffer's combined  $p=0.0007$ ) between genes previously associated with endoplasmic reticulum stress, calcium regulation, and histone modifications. **Conclusions:** Our results provide genetic evidence supporting associations between hippocampal volume and MDD, which may reflect underlying cellular stress responses. Our findings are relevant to cognitive impairments in depression and we speculate that micro RNA 124 and peroxisome proliferator-activated receptor-gamma play important roles. Our study provides evidence of biological mechanisms that should be further explored in the search for disease modifying therapeutic targets for depression.

## **Text:**

### **Introduction**

Glycogen synthase kinase 3 beta (GSK3 $\beta$ ; OMIM 605004) is a uniquely pleiotropic protein kinase. It was originally identified for its function involving glycogen synthesis (Embi et al., 1980), but it is now recognized for playing multiple cellular roles in metabolism, transcription, apoptosis, neurogenesis, cell survival, neural differentiation, immune responses, neurotransmitter function, and synaptic plasticity (Grimes and Joep, 2001; Kim and Snider, 2011; Beurel et al., 2015; Gao et al., 2016).

GSK3 $\beta$  inhibition has been implicated as a biological mechanism of mood regulation (Li and Joep, 2010), mood stabilizers, antidepressants (Beaulieu, 2012), and treatment-resistant depression (Costemale-Lacoste et al., 2016). Behavioural studies have shown that GSK3 $\beta$  regulates depressive-like behaviours and memory function (Pardo et al., 2016), hippocampal plasticity in maternal separation models (Bian et al., 2015) and models of behavioural despair (Strekalova et al., 2016).

We previously identified associations between hippocampal volume and a functional GSK3 $\beta$  polymorphism (rs6438552) in MDD patients (Inkster et al., 2009). We subsequently reported brain structural associations with a subset of genes that biologically interact with GSK3 $\beta$  (Inkster et al., 2010).

We now examine a comprehensive list of genes that encode proteins that directly interact with GSK3 $\beta$ . Our aim is to identify a GSK3 $\beta$ -related genotypic interaction network influencing hippocampal volume in MDD patients using machine learning methods (Nicodemus et al., 2010a; Nicodemus et al., 2010b) applied to discovery and replication samples (Inkster et al., 2009; Cohen-Woods et al., 2009).

## **Subjects and Methods**

### *The Discovery Sample*

#### *MDD Patients*

The discovery sample included 145 patients with recurrent MDD described in detail elsewhere (Inkster et al., 2009; Inkster et al., 2010). In brief, MDD patients belonged to a cohort of 1022 recurrent MDD patients and 1000 healthy controls (Tozzi et al., 2008). The recruiting hospital obtained approval from the Research Ethical Board. Patients were primarily assessed at the Max Planck Institute of Psychiatry, Munich, Germany. Patients with bipolar disorder, mood incongruent psychotic symptoms, a lifetime history of drug use or diagnosis of drug dependency, depression secondary to alcohol or substance abuse or depression as consequence of medical illnesses or use of medications were not included in the study. Age and gender demographics are summarized in Table 1.

### *Structural Brain Imaging*

#### *MRI Acquisition*

High-resolution T1-weighted MRIs were acquired on a 1.5-T General Electric

scanner (Signa, later upgraded to Signa Excite; Waukesha, Wisconsin); inversion recovery prepared spoiled gradient echo recalled with a field of view of 22 x 22cm<sup>2</sup>, a matrix of 256 x 256, 124 sagittal slices, and a resulting voxel size of (1.2–1.4) 0.9 x 0.9mm<sup>3</sup> (time to repetition, 10.3ms; echo time, 3.4ms; flip angle 20°).

### *FreeSurfer*

We used FreeSurfer (<http://surfer.nmr.mgh.harvard.edu>) to create an optimized protocol to derive right hippocampal volume measures. The *recon-all* command was used to process each T1 image. This process entails removal of non-brain tissue using a hybrid watershed/surface deformation procedure, intensity normalization, automated transformation to the Talairach atlas and segmentation of the subcortical grey matter nuclei.

### *Image Quality Control*

The sample originally had N=193 patients. Previous quality control (QC) procedures reduced this number to N=145 (detailed in Inkster et al., 2009). In this study, FreeSurfer images were visually inspected to ensure accuracy of registration and segmentation procedures. The sample was reduced to N=141 (3 patients were removed with +/- 3 standard deviation (SD) and 1 with a missing value for the covariate, intracranial volume; ICV).

### *The Replication Sample*

#### *MDD Patients*

The replication sample included 77 recurrent MDD patients recruited at the Institute of Psychiatry, Psychology & Neuroscience, King's College London, UK. Patients

previously took part in genetic association studies (Cohen-Woods et al., 2009; Uher et al., 2008) and imaging genetics studies (Cole et al., 2011, Cole et al., 2013). The Bexley & Greenwich NHS Research Ethics Committee approved this study. Patients had experienced two or more depressive episodes of at least moderate severity, separated by at least two months of remission. Diagnosis was made using the Schedules for Clinical Assessment in Neuropsychiatry interview (Wing et al., 1990), according to DSM-IV criteria. Exclusions were made if the patient, or a first-degree relative, ever fulfilled criteria for mania, hypomania, schizophrenia or mood incongruent psychosis, had a diagnosis of any neurological disorder or other condition known to affect brain structure or function. Other exclusion criteria included: a lifetime diagnosis of alcohol or substance abuse, depression only secondary to medical illness or medication, a diagnosis of mania or psychosis in first- or second-degree relatives or a contraindication to MRI. Age and gender demographic details are described in Table 1.

### *Structural Brain Imaging*

#### *MRI Acquisition, FreeSurfer & Image Quality Control*

Magnetisation-Prepared Rapid Gradient Echo (MP-RAGE) T1-weighted scans were collected at the Institute of Psychiatry, King's College London, on a 1.5T Signa HDx system (General Electric, USA). Acquisition parameters were; echo time = 3.8ms, repetition time = 8.59ms, flip angle = 8°, field of view = 24cm x 24cm, slice thickness = 1.2mm, number of slices = 180, image matrix = 256 x 256. We used the same FreeSurfer protocol as described in the discovery sample.

#### *GSK3 $\beta$ Network Gene List*

Our *GSK3 $\beta$*  gene network list (Supplementary Table S1) was derived using the NetworkKIN algorithm from Linding et al., 2007; <http://networkin.lindinglab.org>, which integrates consensus substrate motifs (NetPhorest) with context modeling (STRING) for improving prediction of cellular kinase-substrate relations (Linding et al., 2007).

### *Genetic Data*

#### *The Discovery Sample*

Whole-genome scan genotypes were obtained following QC procedures described elsewhere (Tozzi et al., 2008; Muglia et al., 2010). In brief, genotypes were obtained using two channel signal intensity data, corresponding to the two alleles at each SNP that were evaluated using Beadstudio 3.1 (Illumina Inc., San Diego, California, USA). The initial genotype calls were generated using the cluster file. The whole-genome association analysis of the full sample of patients and controls produced a genomic control of  $\lambda = 1002$  (Muglia et al., 2010). The data were imputed as part of the Psychiatric Genomics Consortium MDD genome-wide association study to HapMap3 reference sequence using the CEU and TSI populations. Of the 271 genes in the network, we removed non-autosomal genes (N=9) and genes that contained no SNPs (N=10) (Supplementary Table 1). A total of 8846 SNPs were available in the 252 genes. Hard-called genotypes from dosage data were used in the interaction analyses, with dosage hard call threshold of 0.8 using PLINK v1.0.7. Missing genotypes (missingness range per individual = 1.3% - 3.5%) were imputed using median imputation, as the RF algorithm does not handle missing values. The Hardy Weinberg Equilibrium (HWE) threshold  $p$ -value was set to 0.001; none were removed. Before analysis with RF, SNPs were LD-pruned ( $r^2 = 0.25$ ) as strongly correlated predictors can influence the results of RF (Nicodemus et al., 2011,



Nicodemus et al., 2010c; Nicodemus and Malley, 2009), leaving a total of 1155 SNPs for analysis.

### *The Replication Sample*

Genotypes were derived from genome-wide microarray data described elsewhere (Lewis et al., 2010). DNA samples were genotyped using the Illumina HumanHap610-Quad BeadChips (Illumina Inc., San Diego, CA, USA) by the Centre National de Genotypage, Evry, France. Patients were excluded based on missingness per individual greater than 1% or abnormal heterozygosity. A single patient was excluded if pairs of patients showed greater than 2<sup>nd</sup> degree relatedness. SNPs were excluded if they showed a departure from HWE with  $p$ -value  $< 0.00001$ . Principal component analysis was performed using EIGENSTRAT (Price et al., 2006) after QC procedures. Imputation was performed after using LiftOver to map SNPs from hg18 to hg19 coordinates then Genotype Harmoniser (Deelen et al. 2014) was used to prepare the genotypes for imputation through alignment to the Haplotype Reference Consortium (HRC; McCarthy et al., 2016). Phasing and imputation was completed on the Michigan imputation server (Das et al., 2016) using the HRC reference panel version r1.1, phasing using Eagle v.2.3 (Loh et al., 2016) using the EUR population. Of the 77 individuals, the following were removed prior to analysis: 2 after failing imaging QC, 1 with inconsistent sex-versus-genotype data reported, 1 with non-European ancestry, and 4 after failing genotyping or imputation QC, leaving 69 for analysis.

### *Statistical Analyses*

Initial analysis of the discovery sample used standard single SNP linear regression

models on right hippocampal volume, controlling for age, sex, ICV and head coil upgrade. The discovery sample analysis was conducted using the machine learning algorithm Random Forest (RF; Breiman, 2001), which is designed for high-dimensional data sets and captures main effects of single predictors as well as complex interactions. This method has successfully identified validated epistasis in the context of IQ in psychosis and in schizophrenia case-control genomics data (Nicodemus et al., 2010a; Nicodemus et al., 2010b). To control for the effects of sex, age, ICV and imaging head coil upgrade in the Random Forest analysis, we regressed these variables of non-interest out on both sides of the equation (right hippocampal volume and SNPs) and used the residuals as input, as previously described (Zhao et al., 2012). For the RF analysis, the number of variables selected at each split of the tree (*mtry*) was set to 300 and the number of trees constructed per forest was 1000 (Figure 1). We used the permutation-based variable importance measure as a measure of association between SNPs and outcome. To obtain stable estimates of the variable importance measures, we re-ran RF on the same data 1000 times, changing the random number seed each time, and used the median of these variable importance values as the final set of variable importance measures (Nicodemus and Malley, 2009). We re-ran the RF algorithm on 1000 sets of data in the discovery sample where the outcome had been randomly permuted to obtain a null distribution of variable importance measures for each SNP (Nicodemus, 2011) to calculate an empirical *p*-value. The empirical *p*-value associated with RF variable importance measures was used to determine which SNPs would be tested for 2-way interaction using likelihood ratio tests (LRT) between nested linear regression models:

*Full Model: Right\_Hippocampal\_Volume ~  $\beta_1$  Age +  $\beta_2$  Sex +  $\beta_3$  ICV +  $\beta_4$*

*Head\_Coil\_Upgrade +  $\beta_5$  SNP<sub>i</sub> +  $\beta_6$  SNP<sub>j</sub> +  $\beta_7$  SNP<sub>i</sub> \* SNP<sub>j</sub>*

*Reduced Model: Right\_Hippocampal\_Volume ~  $\beta_1$  Age +  $\beta_2$  Sex +  $\beta_3$  ICV +  $\beta_4$*

*Head\_Coil\_Upgrade +  $\beta_5$  SNP<sub>i</sub> +  $\beta_6$  SNP<sub>j</sub>*

The 1000 null replicates were used to calculate an empirical experiment-wise  $p$ -value for the number of significant LRTs out of the 300 possible 2-way interactions from the reduced list provided by RF. Replication was attempted only for those models showing  $p$ -values < 0.05 uncorrected. For the replication sample analyses, linear regression models were used and LRTs between nested models tested the significance of the interaction, just as we had done above for the discovery sample. The replication sample model included sex, age 10 principal components to control for population stratification and ICV as covariates. Only 19 SNPs from the two-SNP interactions were available; of those, 5 two-SNP interaction pairs were found where both SNPs were available for analysis (rs12469994-rs2291862, rs12469994-rs939626, rs2291862-rs1052751, rs11780700-rs1052751 and rs939626-rs4387877).

## **Results**

### *Discovery Sample*

No single SNP was significantly associated with right hippocampal volume in the discovery sample (Supplementary Table S2). The most strongly associated single SNP was rs7364220, an intronic variant in the gene *PPARA* ( $p$ -value =  $6.36e^{-05}$ ; Bonferroni threshold =  $5.654e^{-06}$ ). Random Forest analysis revealed 15 SNPs with

empirical  $p$ -values  $< 0.05$  (Table 2) which were then subjected to all possible two-way interaction modelling using linear regression, controlling for age, sex, head coil upgrade and ICV, resulting in 105 tests in the discovery sample. Ten of the 15 of the RF-significant SNPs also had single SNP  $p$ -values  $< 0.05$ , uncorrected, and all but one SNP were found to participate in one to three two-SNP interactions using LRTs between nested models (Supplementary Table S3). Histone deacetylase 4 (*HDAC4*) had two SNPs participating in interactions. Although no two-SNP interaction LRT  $p$ -value passed correction for multiple testing (Bonferroni-corrected critical value = 0.00048), given 105 two-SNP interaction models the expected number of interactions with a LRT  $p$ -value  $< 0.05$  is 5.25; we observed 12 using our SNPs as identified as significant with RF. This excess of LRT  $p$ -values  $< 0.05$  was not due to SNPs in linkage disequilibrium as SNPs were LD-pruned prior to RF analysis. To obtain an experiment-wise null distribution of the number of interactions with a LRT  $p$ -value  $< 0.05$ , we re-ran all 105 two-SNP interactions on 1000 null replicates where the phenotype had been permuted without replacement, using the same model as in the analysis of the observed data. Twelve of the 1000 replicates showed  $\geq 12$  interactions with a LRT  $p$ -value  $< 0.05$  (empirical experiment-wise  $p$ -value = 0.012).

### *Replication Sample*

Five interaction models were taken forward for testing in the replication sample. One interaction model was replicated showing the same direction of effect. The model included *HDAC4* rs12469994 and *ITPR1* rs2291862, the most significant interaction in the original discovery sample. Individuals who carried more copies of minor alleles at both SNPs showed a significant decrease in hippocampal volume in both the discovery and replication samples (replication sample LRT  $p$ -value = 0.0088 and

discovery sample LRT  $p$ -value = 0.017). Combining  $p$ -values across the two independent samples using Stouffer's  $Z$  trend (which takes into account the individual  $p$ -values, the sample size and the direction of effect) led to a combined  $p$ -value = 0.0007 for the *HDAC4-ITPR1* interaction.

### *Healthy Control Sample Analysis*

The replicated interaction in the MDD samples between *HDAC4* and *ITPR1* was tested for interaction using N=147 healthy control participants available from the discovery sample (Inkster et al., 2009) using the same model and QC protocol. N=153 healthy control participants were originally available for analysis; three did not pass imaging QC, two had missing covariate or genotype values and one was excluded because their right hippocampal volume was greater than 3 SDs from the mean of controls. The LRT between nested models, testing for interaction effects, was not significant ( $p$ -value = 0.77). In addition, the main effects for both SNPs were also not significant in the full model or in the model with main effects and no interaction term (all  $p$ -values > 0.83).

## **Discussion**

Our study aimed to identify a *GSK3 $\beta$* -related genotypic interaction network influencing hippocampal volume in MDD using a comprehensive list of known proteins that bind to *GSK3 $\beta$* . Using two independent imaging genetics recurrent MDD data sets, we confirmed a significant genotypic interaction (with hippocampal volume) in genes linked to endoplasmic reticulum (ER) stress, calcium regulation and histone deacetylase modifications.

Our findings are important for several reasons. This is the first psychiatric imaging genetics study to systematically examine a comprehensive list of genes with direct biological GSK3 $\beta$  interactions. It is therefore the first examination of putative genotypic combinations amongst this network. We used a machine learning algorithm in the discovery sample that explicitly models both genetic main effects and interactions through creating recursively partitioned trees. Given that these genes physically interact in this biological network, we hypothesised that an epistatic effect may be present. We did not observe any single SNP effects that were significant after multiple testing, whereas we discovered and replicated a two-SNP interaction between *HDAC4-ITPR1* that was associated with decreased hippocampal volume among MDD patients carrying putative ‘risk’ alleles at both SNPs.

Inositol 1,4,5-triphosphate receptor, type 1 (*ITPR1*; OMIM 147265) is a calcium channel that regulates the release of calcium from the ER (Yamada et al., 1994). The ER contains the largest reservoir of calcium in the cell. It is also responsible for the correct folding of proteins prior to their delivery into the cytoplasm. When the ER system is stressed a large amount of calcium is released into the cytoplasm, which can lead to apoptosis. Our identification of *ITPR1* can be interpreted using the framework proposed by Gold et al., 2013, suggesting that impaired ER stress responses play a role in depression. Our study adds to the literature of genetic associations with ER stress and mood disorders (Kakiuchi et al., 2003; Kakiuchi et al., 2007; Hayashi et al., 2009; Grunebaum et al., 2009; Nevell et al., 2014), in particular, the discovery of an *ITPR1* gene variant that was amongst the most significant SNPs in a MDD genome-wide association study meta-analysis (Muglia et

al., 2010).

The unfolded protein response (UPR) system is a cellular defensive mechanism activated in response to ER-related protein misfolding. Timberlake and Dwivedi (2016) investigated the role of the UPR system in depression. The authors demonstrated hippocampal upregulation of two critical UPR markers (GRP78 and GRP94) in rats with learned helplessness. GRP78 and GRP94 are highly involved in apoptosis and inflammation. Evidence has implicated these processes in the etiology of depression (Joje et al., 2016; Mechawar and Savitz, 2016). Additional evidence showed that mood disorders may involve mechanisms related to ITPR, ER stress and GSK3 $\beta$  signaling albeit using an endothelial cell degeneration model in prefrontal cortical tissue (Kurauchi et al., 2016). Overall, supporting the efficiency of the ER stress response and UPR system may play a role in the treatment of mood disorders.

*HDAC4* was another gene identified in our study. HDAC4 regulates gene transcription by interacting with transcription factors, signal transduction molecules and HDAC3 to carry out many cellular functions, such as proliferation, differentiation, neuronal survival, and synaptic plasticity (Wu et al., 2016). Hobara et al. (2010) reported increased HDAC4 mRNA expression in patients with unipolar and bipolar depression. In addition, Sarkar et al. (2014) reported that viral-mediated hippocampal HDAC4 overexpression was associated with a significant increase in depression-like behaviour in a preclinical model.

Our findings may be relevant for developing future hypotheses involving cognitive

impairments in MDD, especially given prior evidence implicating GSK3 $\beta$  in cognition (O'Leary and Nolan, 2015). For example, the ER stress inhibitor, tauroursodeoxycholic acid (TUDCA), may alleviate dysfunction of cognition (Cai et al., 2015) and preclinical evidence has shown that ER stress-induced hippocampal apoptosis and cognitive impairments were inhibited by pretreatment with the ER stress inhibitor, salubrinal (Zhang et al., 2014; Ge et al., 2015). Salubrinal has been shown to have neuroprotective effects (Rubovitch et al., 2015), but it has not been tested in human clinical trials. HDAC4 also may play a role in cognitive function (Wu et al., 2016). The gold standard and commonly used mood stabilizers for the treatment of bipolar disorder, lithium and divalproate, have been implicated to have HDAC and GSK3 $\beta$  inhibitory effects. A study by Sharma and Taliyan (2014) showed that cognitive impairments in rats treated with a low dose combination treatment of lithium and divalproex showed improved spatial learning and memory.

Our findings have direct biological relevance to other molecular targets previously implicated in mood disorder pathophysiology, including non-coding microRNA precursor, miR-124 (Roy et al., 2017). Higuchi et al. (2016) found that miR-124-mediated regulation of HDAC4 and GSK3 $\beta$  hippocampal expression may have implications for chronic stress and depression. miR-124 has been identified as a biological mechanism underlying the effects of erythropoietin treatment, which may be relevant to mood disorder treatment, cognitive improvements and increased hippocampal volume (unpublished review by Inkster et al., 2017, Under Revision to be resubmitted to Editor of *Biological Psychiatry*). Another related molecular target is peroxisome proliferator-activated receptor  $\gamma$  (PPARG), supported by evidence that PPARG activation improves depressive-like behaviours (Gold et al., 2013), plays a



protective role against ER stress (Gold et al., 2013), and PPARG pro-survival activity is inhibited by HDAC4 activation (Yang et al., 2011).

Our study has several limitations. Although this work suggests a potential genetic network associated with brain changes in depression with *GSK3 $\beta$* , it does not differentiate between whether these MDD-specific genotype-dependent brain structural associations are related to the pathogenesis of MDD or occur as a consequence of disease expression. As there is evidence showing that neuroplastic or neurodegenerative processes cause structural brain changes with depression, stress, and pharmacotherapy, this impact of stress, depression, and medications may influence hippocampal morphology. We did not test whether these structural changes are specific to major depression. We restricted our analysis to the right hippocampus based on our previous findings (Inkster et al., 2009); however, future work could examine both hippocampi and relevant regions in temporal and prefrontal cortices. Both of the samples used in this study involved recurrent MDD patients. Therefore, we were unable to consider hypotheses related to early onset MDD or first episode MDD to delineate disease processes across time; for example, in first episode MDD patients the literature suggests there are no hippocampal volume deficits (Schmaal et al., 2016) and so it remains unknown how or whether our identified biological mechanisms would be involved. There are neuroimaging methodological differences for generating hippocampal volume measures between our current study (i.e., FreeSurfer software was used to measure the entire volume of the right hippocampus) that differ from our previous study (i.e., a SPM software-based brain-wide voxel-wise cluster-based method was used, which identified a cluster within the right hippocampus; Inkster et al., 2009).

In conclusion, our study provides genetic evidence supporting associations between hippocampal volume and recurrent MDD, suggesting that ER stress inhibition and HDAC4 modifications should be explored in the search for disease modifying therapeutic targets for depression. They also encourage additional drug classes and medications to be considered, and to encourage pharmacogenetic studies and clinical trials to be designed to assist with translating these scientific findings into clinical practice.

## **Acknowledgements, Ethics and Disclosures**

We would like to acknowledge all patients and control subjects who have participated in this study. We would also like to thank the staff at the Max Planck Institute of Psychiatry, Munich, Germany, who contributed to this study, and to colleagues at GSK, in particular Brandon Whitcher, Anil Rao, Khanum Ridler, Federica Tozzi, and Emilio Merlo-Pich for their contributions to the overall conduct of the study. KKN was supported by a University of Edinburgh Chancellor's Fellowship. GZ was supported by the W. Garfield Weston Doctoral Fellowship and the Canadian Institutes of Health Research Postdoctoral Fellowship and she is currently supported by the Centre for Addiction and Mental Health Tanenbaum Pharmacogenomics Fund. PMM gratefully acknowledges the generous support of the Edmond J Safra Foundation and Lily Safra and the Imperial College Healthcare Trust Biomedical Research Centre.

**Financial Disclosures:** GlaxoSmithKline & NIHR Biomedical Research Centre for Mental Health. Authors BI, TN, PM, and PMM were employees of *GlaxoSmithKline* when the original data was collected. All other authors have nothing to disclose.

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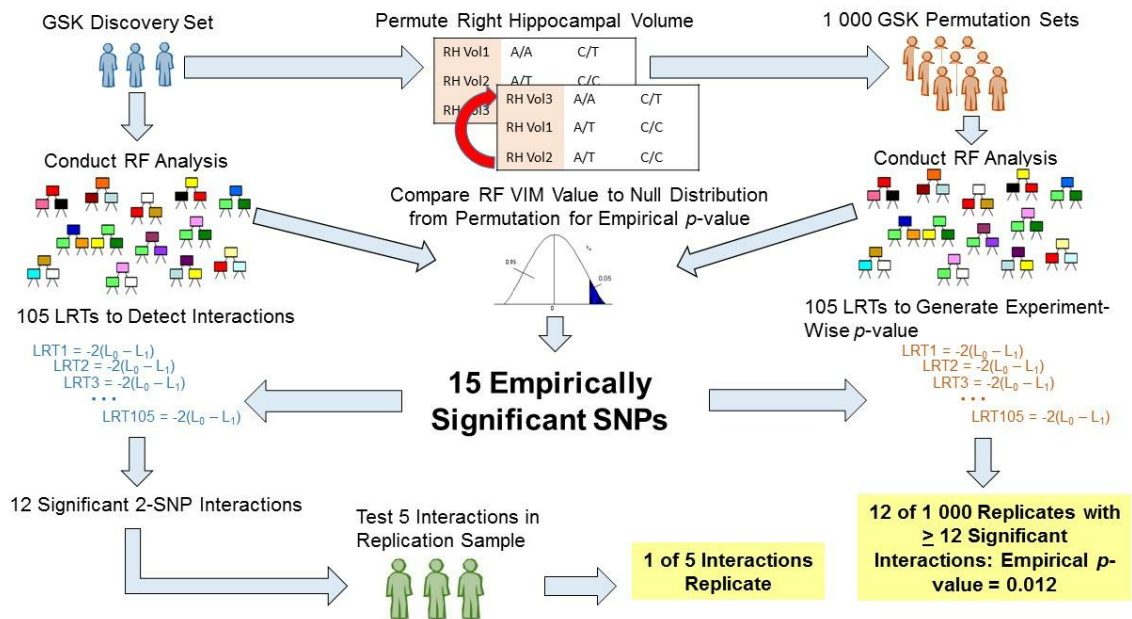
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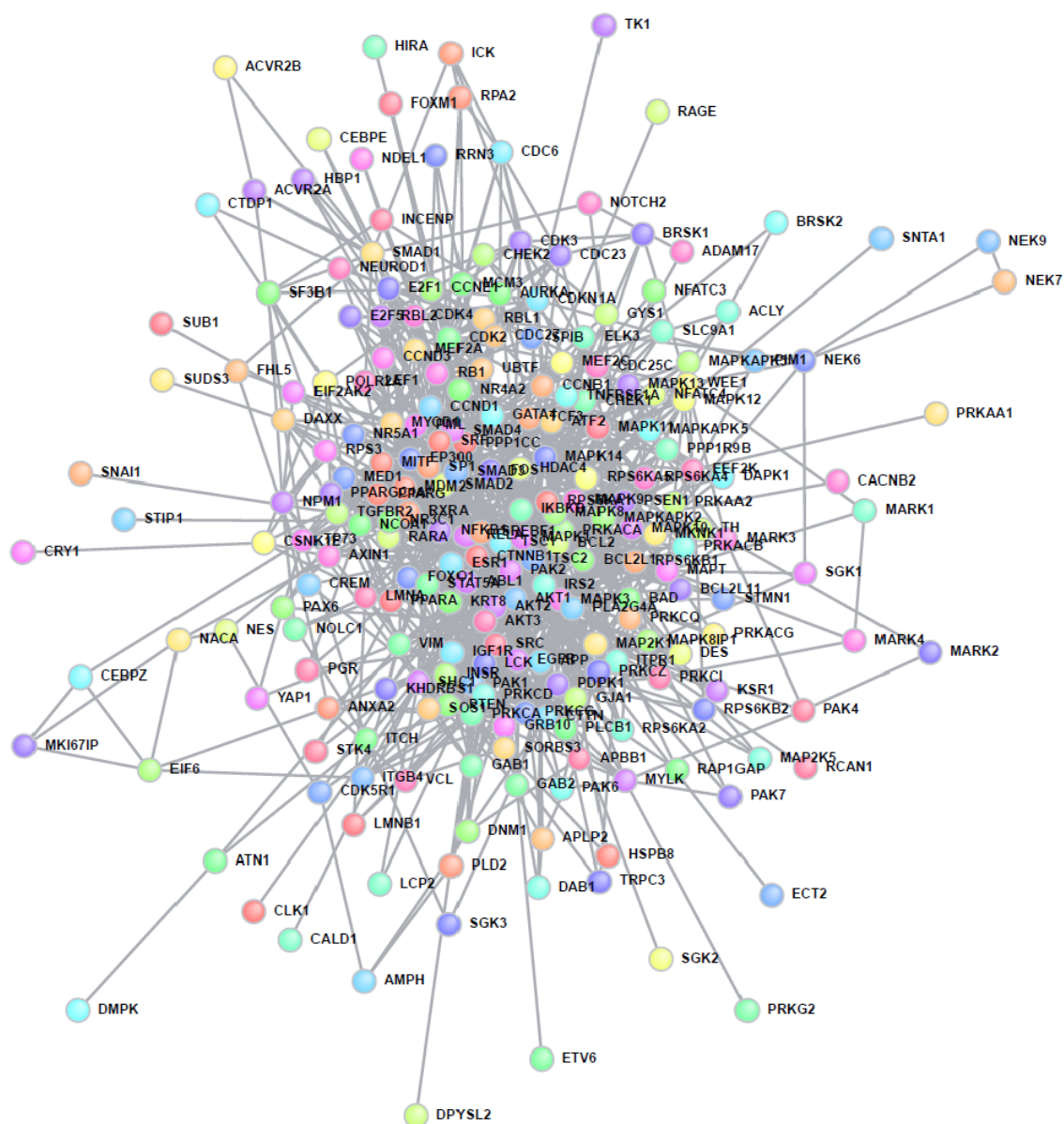
**Figure 1. Schema of Random Forest Analysis**

**Table 1.** Demographics for the discovery and replication imaging genetics samples

<b><u>Sample</u></b>	<b><u>Discovery</u></b>	<b><u>Replication</u></b>
N	141	69
Mean Right Hippocampal Volume (SD)	4150.8 (387.7)	3950.7 (473.8)
Female, N (%)	86 (61.0)	48 (69.6)
Mean Age (SD)	49.2 (13.3)	48.5 (8.1)
Mean ICV (SD)	1506774 (160739)	1473184 (235564)
MRI Coil Upgrade, N (%)	36 (25.5)	NA

**Table 2.** Random Forest-identified empirically significant SNPs associated with right hippocampal volume in MDD patients

<b>SNP</b>	<b>Single SNP <i>p</i>- value</b>	<b>RF Empirical <i>p</i>-value</b>	<b>N 2-SNP Interactions</b>	<b>Gene</b>	<b>Function</b>
rs4844550	7.53E-05	0.044	2	MAPKAPK2	upstream variant
rs3791424	0.0081	0.021	1	HDAC4	intron
rs12469994	0.003	0.014	2	HDAC4	intron
rs2291862	0.012	0.02	2	ITPR1	synonymous
rs13083813	0.14	0.017	2	TGFBR2	intron
rs3798290	0.058	0.016	1	FHL5	intron
rs4720279	0.0052	0.012	1	AMPH	intron
rs2058502	0.0044	0.003	2	EGFR	intron
rs11780700	0.034	0.034	2	C8orf44, SGK3	intron
rs11014511	0.0098	0.049	1	CACNB2	intron
rs939626	0.089	0.01	2	IGF1R	intron
rs1052751	0.21	0.004	3	PLD2	synonymous
rs11654719	0.0052	< 0.001	1	PRKCA	intron
rs2279103	0.18	0.031	0	CTDP1	missense
rs4387877	7.53E-05	0.002	2	PLCB1	intron



**Supplementary Figure 1 GSK3β Network Protein-Protein Interaction Plot**

**Supplementary Table S1. GSK3 $\beta$  Gene Network**

<b>Gene</b>	<b>Chromosome</b>	<b>Included In Analysis</b>
AKT3	1	Y
CDC42BPA	1	Y
DAB1	1	Y
JUN	1	N
KHDRBS1	1	Y
LCK	1	Y
LMNA	1	Y
MAPKAPK2	1	Y
MARK1	1	Y
MKNK1	1	Y
NEK7	1	Y
NES	1	Y
NOTCH2	1	Y
ORC1	1	Y
PLA2G4A	1	Y
PRKAA2	1	Y
PRKACB	1	Y
PRKCZ	1	Y
RAP1GAP	1	Y
RPA2	1	Y
RPS6KA1	1	Y
SHC1	1	Y
SLC9A1	1	Y
STMN1	1	Y
TP73	1	Y
ACVR2A	2	Y
ADAM17	2	Y
ANAPC1	2	N
ATF2	2	Y
BCL2L11	2	Y
CEBPZ	2	Y
CLK1	2	Y
DES	2	Y
EIF2AK2	2	Y
HDAC4	2	Y
IRS1	2	N
MKI67IP	2	Y
NCOA1	2	Y
NEUROD1	2	Y
NR4A2	2	Y
SF3B1	2	Y
SOS1	2	Y
ACVR2B	3	Y



CTNNB1	3	Y
ECT2	3	Y
ITPR1	3	Y
MAPKAPK3	3	Y
MITF	3	Y
MYLK	3	Y
NUP210	3	Y
PAK2	3	Y
PPARG	3	Y
PRKCD	3	Y
PRKCI	3	Y
TGFBR2	3	Y
GAB1	4	Y
HNRPD	4	N
LEF1	4	Y
MAPK10	4	Y
NFKB1	4	Y
PPARGC1A	4	Y
PRKG2	4	Y
SMAD1	4	Y
TRPC3	4	Y
CCNB1	5	Y
CDC23	5	Y
CDC25C	5	Y
DPYSL3	5	Y
LCP2	5	Y
LMNB1	5	Y
MAPK9	5	Y
MEF2C	5	Y
NPM1	5	Y
NR3C1	5	Y
PRKAA1	5	Y
SUB1	5	Y
CCND3	6	Y
CDKN1A	6	Y
DAXX	6	Y
ESR1	6	Y
FHL5	6	Y
GJA1	6	Y
ICK	6	Y
MAPK13	6	Y
MAPK14	6	Y
MCM3	6	Y
PIM1	6	Y
RPS6KA2	6	Y
SGK1	6	Y

SRF	6	Y
TTK	6	Y
AMPH	7	Y
CALD1	7	Y
CHRM2	7	Y
EGFR	7	Y
GRB10	7	Y
HBP1	7	Y
DPYSL2	8	Y
E2F5	8	Y
GATA4	8	Y
IKBKB	8	Y
MCM4	8	N
PLEC	8	Y
SGK3	8	Y
SORBS3	8	Y
STK3	8	Y
ABL1	9	Y
DAPK1	9	Y
DNM1	9	Y
NEK6	9	Y
NR5A1	9	Y
PRKACG	9	Y
RXRA	9	Y
TSC1	9	Y
CACNB2	10	Y
CAMK1D	10	Y
CDK1	10	Y
CREM	10	Y
MAPK8	10	Y
NOLC1	10	Y
PRKCQ	10	Y
PTEN	10	Y
VCL	10	Y
VIM	10	Y
APBB1	11	Y
APLP2	11	Y
BAD	11	Y
BRSK2	11	Y
CCND1	11	Y
CHEK1	11	Y
CTTN	11	Y
GAB2	11	Y
INCENP	11	Y
MAPK8IP1	11	Y
MARK2	11	Y

MYOD1	11	Y
PAK1	11	Y
PAX6	11	Y
PGR	11	Y
RELA	11	Y
RPS3	11	Y
RPS6KA4	11	Y
RPS6KB2	11	Y
SNF1LK2	11	Y
STIP1	11	Y
TH	11	Y
WEE1	11	Y
YAP1	11	Y
ATN1	12	Y
CDK2	12	Y
CDK4	12	Y
CRY1	12	Y
ELK3	12	Y
ETV6	12	Y
FOXM1	12	Y
HSPB8	12	Y
KRT8	12	Y
MAPKAPK5	12	Y
MDM2	12	Y
NACA	12	Y
NUAK1	12	Y
PPP1CC	12	Y
SP1	12	Y
SUDS3	12	Y
TNFRSF1A	12	Y
FOXO1	13	Y
IRS2	13	Y
RB1	13	Y
AKT1	14	Y
CEBPE	14	Y
FOS	14	Y
MARK3	14	Y
NEK9	14	Y
NFATC4	14	Y
PSEN1	14	Y
PTGER2	14	Y
RAGE	14	Y
RPS6KA5	14	Y
ANXA2	15	Y
IGF1R	15	Y
MAP2K1	15	Y

MAP2K5	15	Y
MEF2A	15	Y
PAK6	15	Y
PML	15	Y
SMAD3	15	Y
AXIN1	16	Y
EEF2K	16	Y
MAPK3	16	Y
NFATC3	16	Y
PDPK1	16	Y
RBL2	16	Y
RRN3	16	Y
TSC2	16	Y
ACLY	17	Y
CDC27	17	Y
CDC6	17	Y
CDK3	17	Y
CDK5R1	17	Y
ITGB4	17	Y
KIAA1303	17	N
KSR1	17	Y
MAPT	17	Y
MED1	17	Y
NDEL1	17	Y
PLD2	17	Y
POLR2A	17	Y
PPP1R9B	17	Y
PRKCA	17	Y
RARA	17	Y
RPS6KB1	17	Y
SREBF1	17	Y
STAT5A	17	Y
TK1	17	Y
UBTF	17	Y
BCL2	18	Y
CTDP1	18	Y
SMAD2	18	Y
SMAD4	18	Y
AKT2	19	Y
BRSK1	19	Y
CCNE1	19	Y
DMPK	19	Y
FPR1	19	Y
GYS1	19	Y
INSR	19	Y
MARK4	19	Y

PAK4	19	Y
PRKACA	19	Y
PRKCG	19	Y
SIRT2	19	N
SPIB	19	Y
TCF3	19	Y
ZFP36	19	N
AURKA	20	Y
BCL2L1	20	Y
CEBPB	20	N
E2F1	20	Y
EIF6	20	Y
ITCH	20	Y
MYBL2	20	N
NSFL1C	20	Y
PAK7	20	Y
PLCB1	20	Y
RBL1	20	Y
SGK2	20	Y
SNAI1	20	Y
SNTA1	20	Y
SRC	20	Y
STK4	20	Y
APP	21	Y
RCAN1	21	Y
SNF1LK	21	Y
CHEK2	22	Y
CSNK1E	22	Y
EP300	22	Y
HIRA	22	Y
TAB1	22	Y
MAPK1	22	Y
MAPK11	22	Y
MAPK12	22	Y
PPARA	22	Y
DCX	X	N
FOXO4	X	N
GJB1	X	N
IRAK1	X	N
PAK3	X	N
RPS6KA3	X	N
RPS6KA6	X	N
SYN1	X	N
UBE2A	X	N

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**Supplementary Table S2. Top 10 Single SNP Results for SNPs in the GSK3 $\beta$  Gene Network**

CHR	SNP	BP	A1	A2	FRQ	INFO	BETA	SE	P
22	rs7364220	44963648	A	G	0.7837	1.0168	-172.73	41.8396	6.36E-05
20	rs4387877	8605680	A	G	0.5624	1.0679	-137.64	33.6995	7.53E-05
20	rs6055995	8610222	A	G	0.5709	1.0591	-136.23	34.0241	1.02E-04
20	rs1033566	8607242	A	G	0.6773	1.0752	-140.84	35.6568	1.25E-04
20	rs6077404	8600060	A	T	0.6768	1.0507	-142.38	36.0548	1.26E-04
20	rs2076639	8587474	A	C	0.3191	1.0031	146.263	37.0622	1.27E-04
20	rs4347922	8595462	A	G	0.4304	1.0144	137.343	34.8517	1.30E-04
20	rs1022596	8596450	T	C	0.3233	1.0442	141.939	36.1891	1.39E-04
22	rs8138102	44970416	A	G	0.7583	0.9671	-158.84	41.8505	2.22E-04
20	rs6055987	8595972	T	C	0.4889	0.8336	-137.05	38.2856	4.79E-04

**Supplementary Table S3. Significant Two-SNP LRT Results for SNPs Identified by Random Forest**

<b>SNP 1</b>	<b>Gene 1</b>	<b>SNP 2</b>	<b>Gene 2</b>	<b>LRT <i>p</i>-value</b>
rs4844550	MAPKAPK2	rs11014511	CACNB2	0.019
rs4844550	MAPKAPK2	rs1052751	PLD2	0.029
rs3791424	HDAC4	rs3798290	FHL5	0.038
rs12469994	HDAC4	rs2291862	ITPR1	0.0088
rs12469994	HDAC4	rs939626	IGF1R	0.032
rs2291862	ITPR1	rs1052751	PLD2	0.044
rs13083813	TGFBR2	rs11780700	SGK3	0.035
rs13083813	TGFBR2	rs11654719	PRKCA	0.02
rs4720279	AMPH	rs2058502	EGFR	0.049
rs2058502	EGFR	rs4387877	PLCB1	0.029
rs11780700	SGK3	rs1052751	PLD2	0.011
rs939626	IGF1R	rs4387877	PLCB1	0.033