Supplementary Online Content

Tao R, Cousijn H, Jaffe AE, et al. Expression of *ZNF804A* in human brain and alterations in schizophrenia, bipolar disorder, and major depressive disorder: a novel transcript fetally regulated by the psychosis risk variant rs1344706. *JAMA Psychiatry*. Published online August 27, 2014. doi:10.1001/jamapsychiatry.2014.1079.

This supplementary material has been provided by the authors to give readers additional information about their work.

eAppendix. Supplemental Methods

Western blotting. Protein was extracted from frontal cortex of adult and fetal human brains using RIPA buffer (Thermo-Fisher), with protease inhibitor (Roche, Welwyn Garden City, UK), fractionated using sodium dodecyl sulphate polyacrylamide gel electrophoresis using pre-cast gels (BIO-RAD Mini-Protein TGX Gels, any kD), and blotted onto polyvinyl difluoride membranes (Immobilon-P, Millipore, Watford, UK) overnight at 25 volts. The membranes were soaked in blocking buffer (5% milk in PBS with 0.1% Tween20) for 1 hour at room temperature and probed with a goat anti-human ZNF804A polyclonal antibody (D-14; Santa Cruz sc-241170) diluted 1:250 (0.8μg/ml), in blocking buffer, for 1 hour at room temperature. The D-14 antibody maps to an 'internal' epitope of human ZNF804A. Following three 15 minute washes in PBS containing 0.1% Tween20 (PBS-Tw), membranes were incubated for 30 minutes with donkey anti-goat secondary antibody horseradish peroxidase conjugate diluted to 1:25000 in blocking buffer, followed by a further three 15 minute washes in PBS-Tw. Antibody binding was visualised using ECL Plus Western Blotting Detection System and Hyperfilm ECL (GE Healthcare).

Several experimental controls were used. (1) The D-14 antibody was incubated together with its blocking peptide (D-14p, Santa Cruz, sc-241170P; final concentration 8μg/ml). (2) ZNF804A was expressed in HEK293 cells,[1] and in competent bacteria (KRX cells, Promega UK). Extracted protein from these cells was run alongside the human brain samples, with the transfected and untransfected cells serving as positive and negative controls respectively. (3) We used a second antibody, targeting a C-terminal epitope of mouse ZNF804A (P-13; Santa Cruz, sc-241173, diluted 1:200 [1μg/ml]). (4) After immunostaining and visualization of ZNF804A, membranes were rinsed in wash buffer and incubated with anti-β-actin antibody (Sigma-Aldrich, UK: A5316, clone AC74) at 1:100,000 as a loading control.

Immunohistochemistry
Free-floating sections were placed in 5ml tubes, washed in two changes of phosphate-buffered saline (PBS), then placed in antigen unmasking solution (Vector Laboratories, Peterborough UK; H-3300) which had been pre-heated to 90^{o}C for 30 minutes, then allowed to cool for 20 minutes. After washing in PBS for 3 x 15 minutes, sections were incubated at 4^{o}C on a rocking platform for 72 hours with the D-14 primary antibody diluted 1:1000 in 10% normal rabbit serum (NRS) in PBS with 0.3% Triton (PBS-T). Sections were rinsed in PBS-T for 3 x 15 minutes and incubated at room temperature for 1 hour in secondary rabbit anti-goat antibody, at 1:100 in PBS-T, with visualization using a Vectastain ABC Elite kit and diaminobenzidine following manufacturer's recommendations (Vector Laboratories). Finally, sections were rinsed, mounted onto 5% gelatinized slides and allowed to dry before being dehydrated and coverslipped. Some sections were counterstained with cresyl violet.

Slide-mounted sections were de-waxed, rehydrated and placed in 10% hydrogen peroxide for 30 minutes before applying the same immunohistochemical protocol, except that the primary antibody was used at 1:100 and incubated overnight. For the peptide block control experiment, the above protocol was repeated with the addition of 1:3 D-14p.

To investigate whether ZNF804A immunoreactivity was affected by schizophrenia or rs1344706, we used the free-floating sections of inferior parietal lobule (*eTable 2*). Adjacent sections were stained with cresyl violet to aid laminar identification. Given the findings (see Results), we focused our quantitative analyses on lamina III pyramidal neurons. Pilot studies showed no differences in ZNF804A immunoreactivity rostro-caudally across the inferior parietal lobule, and the studies were carried out on sections (three per subject, blind to group) randomly taken from the most rostral block. To estimate the density of ZNF804A-immunoreactive neurons, an Olympus BX50 microscope was used with the CASTgrid system, with a counting frame of 35,000μm² at x40 magnification, and a field sampler. Criteria for counting a cell were: that it was within lamina III, could be brought into focus, did not touch the lower or right border of the counting box, that there was clear immunostaining, and was pyramidal-shaped.[2] The cross-sectional area and staining intensity of ZNF804A-immunoreactive lamina III pyramidal neurons were measured by manually tracing around the perimeter (N=50 neurons per subject) using a Nikon Eclipse E600 microscope linked to an MCID Elite 7.0 image analysis system.[3] We also measured the depth of the grey matter, depth of lamina III, and the final section thickness (Z-axis); none of these indices differed between diagnostic or genotype groups (data not shown). Because of the small sample size, G carriers were grouped together and compared to TT homozygotes for statistical analysis.

eReferences

1. Girgenti MJ, LoTurco JJ, Maher BJ. ZNF804a regulates expression of the schizophrenia-associated genes PRSS16, COMT, PDE4B, and DRD2. PloS One 2012;**7**:e32404.
2. Gittins R, Harrison PJ. Neuronal density, size and shape in the human anterior cingulate cortex: a comparison of Nissl and NeuN staining. Brain Res Bull 2004;63(2); 55-60.
3. Law AJ, Harrison PJ. Pyramidal neuron sub-populations in the dorsolateral prefrontal cortex identified using anti-neurofilament antibodies SMI32, N200 and FNP7: Normative data, and a comparison in subjects with schizophrenia, bipolar disorder and major depression. J Psychiat Res 2003;37(6):487-499.

eTable 1. Subjects From Stanley Medical Research Institute, Used for ZNF804A Immunohistochemical Studies of Inferior Parietal Lobe

	Controls	Schizophrenia
Number	23	24
Hemisphere (L,R)	13,10	14,10
Age (y)	44.3 (2.0)	39.8 (2.2)
Smoker (Y,N,NK)	5,12,6	18,2,4
Age of onset (y)	-	19.0 (5.8)
Duration of illness (y)	-	20.8 (10.3)
Lifetime antipsychotic dose	-	79,002 (16,233)
Brain pH	6.67 (0.04)	6.53 (0.05)
Post mortem interval (h)	24.7 (2.3)	29.1 (2.4)
Suicides	0	6
Storage time (months)	119 (5)	117 (5)
rs1344706 (TT, G carriers)	6,17	10,14

Values are mean (SEM). All subjects are males.

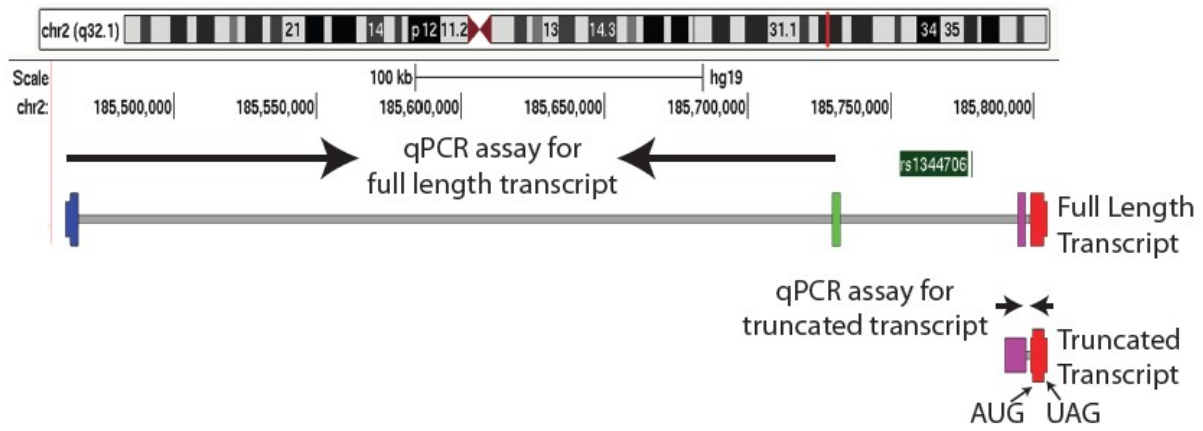
eTable 2. ZNF804A Immunoreactivity of Layer III Pyramidal Neurons in Inferior Parietal Lobe

	Diagnosis		rs1344706 genotype	
	Controls (N=23)	Schizophrenia (N=24)	AA (N=16)	C carriers (N=31)
Neuronal density (mm^{-2})	46.5 (2.6)	42.0 (3.0)	42.9 (3.7)	44.9 (2.3)
Neuronal size (μm^2)	249 (9)	242 (7)	254 (11.3)	241.1 (6.1)
Staining intensity[a]	0.25 (0.01)	0.26 (0.01)	0.26 (0.2)	0.25 (0.01)

Values are mean (SEM).
[a]Arbitrary optical density units.

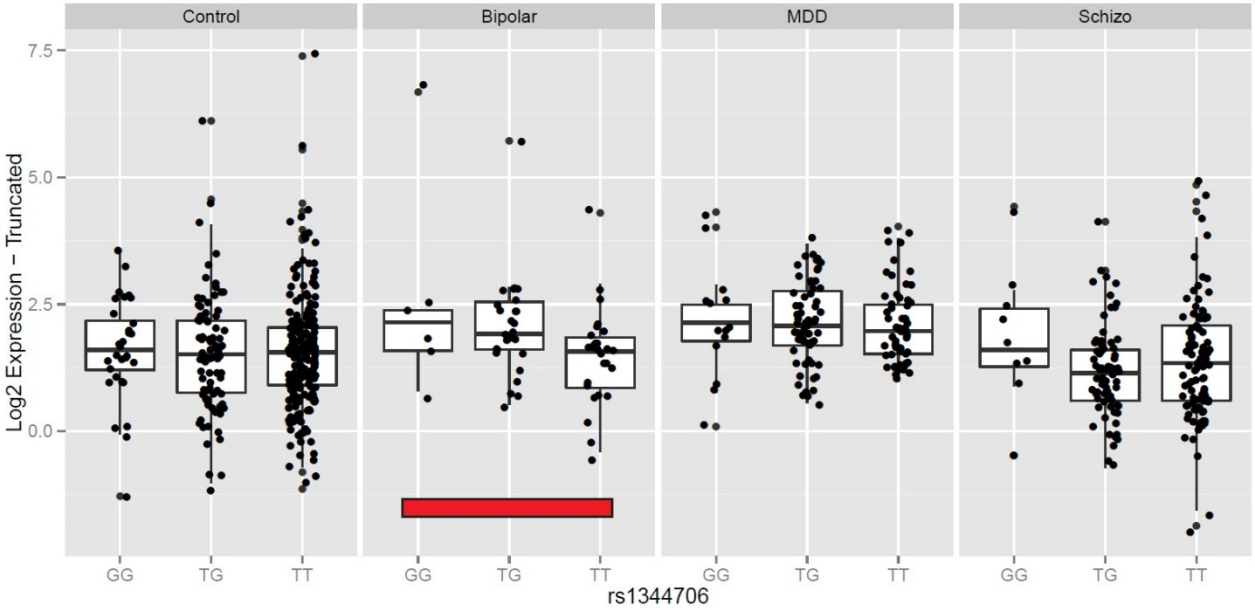
The post-mortem brain samples involved are from Stanley Medical Research Institute (eTable 1). There are no main effects of diagnosis or genotype, nor interactions between them, on any of the parameters.

eFigure 1. Schematic of *ZNF804A* Locus, Gene, and Primer Locations



Exons are colored (E1: blue, E2: green, E3: purple, E4: red), introns in grey. The truncated transcript, *ZNF804A*^{E3E4}, lacks exons 1 and 2, has a 5' extension of exon 3, and has a new start codon (AUG) in exon 4.

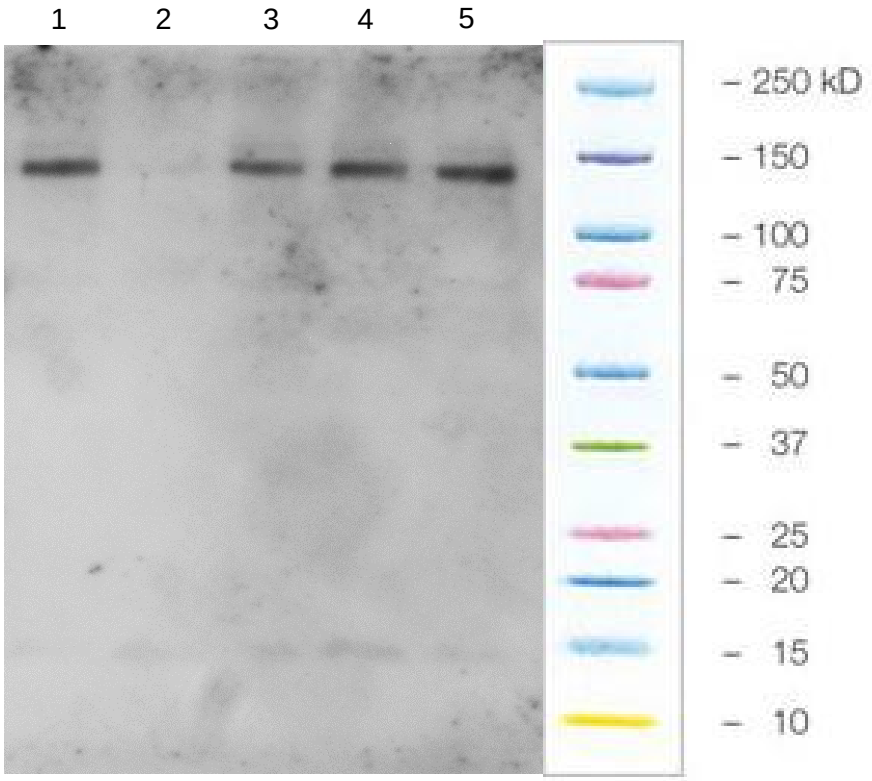
eFigure 2. *ZNF804*^{E3E4} mRNA According to Genotype in Each Diagnostic Group



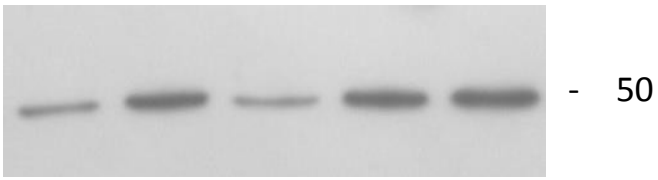
In bipolar disorder (Bipolar), risk TT homozygotes show lower expression than G carriers (p=0.002). MDD: major depressive disorder. Schizo: schizophrenia. The post mortem brain samples involved are from NIMH/Lieber Institute for Brain Development (Table 1).

eFigure 3. Full-Length Western Blot for ZNF804A With D-14 Antibody (A), and β-Actin Loading Control (B)

A



B



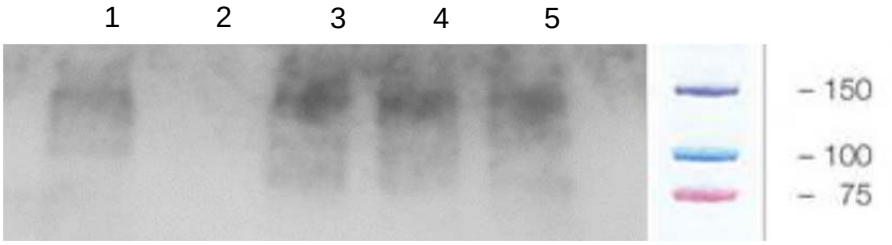
A: Full-length version of the ZNF804A Western blot shown in Fig. 4A. Lane 1: Transfected HEK293 cells. 2: Untransfected HEK293 cells. 3: Fetal cortex. 4, 5: Adult frontal cortex. Size markers shown on the right.

B: Blot probed for β-actin as loading control.

eFigure 4. Additional Western Blots

A. Western blot after blocking with D-14 peptide.

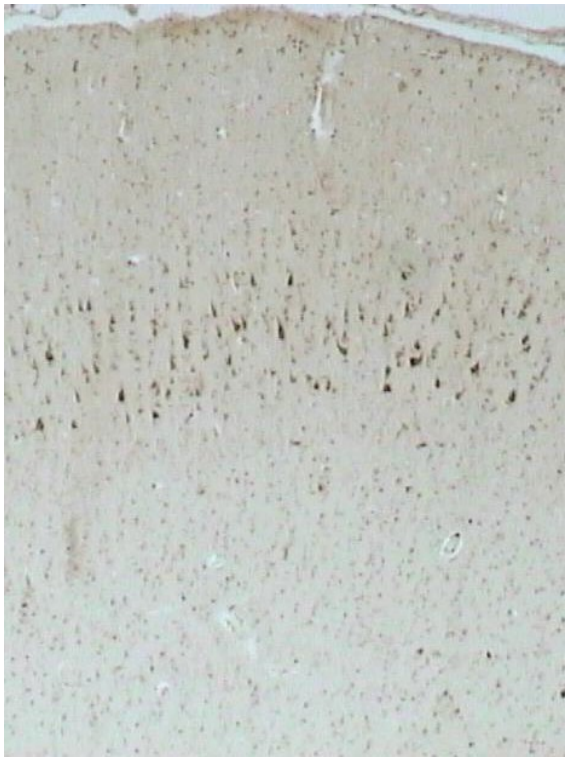
B. Western blot using mouse P-13 antibody.



Lanes as in eFigure 3. Note the similarly sized band (and lack of band in the untransfected cells in lane 2) as seen with the D-14 antibody. The greater smearing with P-13 may reflect mouse-human differences in the targeted epitope.

eFigure 5. Immunohistochemistry in Frontal Cortex With D-14 Antibody Without (A) or With (B) Preincubation With D-14 Peptide

A



B