Supplemental Data file for Dieset et al., *NOTCH4* Gene Expression is Upregulated in Bipolar Disorder.

Methods

mRNA analyses

Total RNA was isolated from whole blood using the Tempus 12-Port Isolation kit (Applied Biosystems; Ambion, Austin, TX, USA) and quantified using the ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). The tubes were stored at –80oC until reverse transcription was performed, using a High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA) with ~0.5 µg total RNA in 96-well polymerase chain reaction plates (Applied Biosystems) with 80 samples on each plate. To minimize the impact of different efficiency of the reverse transcription or subsequent real-time polymerase chain reaction, the different study populations were randomly represented on each plate. Quantification of mRNA was performed with the q polymerase chain reaction Master Mix for SYBR Green I (Applied Biosystems) in 20 µL duplicate reactions on a ABI Prism 7500 (Applied Biosystems) using the 2(-Delta Delta C[T]) method with the average of four pools of cDNA, that were included on each plate in the reverse transcription reaction and followed the samples in the real time polymerase chain reaction, as reference. The CV% for the average of these calibrators between all the plates analyzed was <10%. For real-time reverse transcription –polymerase chain reaction, sequence specific mRNA specific (primer spanning exon-exon junction) oligonucleotide primers for the full-length *NOTCH4* (forward primer: TCTCCGGCACCCGATGT, reverse primer: TCAAAGCCTGGGAGACACTTG) were designed using Primer Express software version 3.0 (Applied Biosystems). Data were normalized to ß-actin (forward primer: AGGCACCAGGGCGTGAT, reverse primer: TCGTCCCAGTTGGTGACGAT).

Isolation of cellular subpopulations for *NOTCH4* mRNA expression analysis

Peripheral blood mononuclear cells were obtained from EDTA-anticoagulated blood by

Isopaque-Ficoll (Lymphoprep; Axis-Shield, Oslo, Norway) gradient centrifugation. Further

separation of CD14$^+$ monocytes (positive selection by antiCD14-labeled magnetic beads;

Dynal, Oslo, Norway) and CD3$^+$ T-cells (antiCD3-labeld magnetic beads; Dynal, Oslo,

Norway) was performed as described elsewhere (1). Erythrocytes were collected, mixed in a

hypotonic saline (0.45% NaCl) to return them to an isoosmotic condition, applied once more

to a Polymorphprep gradient and finally mixed in hypotonic saline. Erythrocytes were when

centrifuged at 250g for 10 minutes, the plastic tube was punctured by a cannula and ¾ of the

sedimented erythrocytes (bottom) were allowed to drop out (2).

For purified platelets, citrated platelet-rich plasma was mixed with

Dynabeads (Dynal, Oslo, Norway) to remove any potential contamination of other

blood cells from the citrated platelet-rich plasma suspension. One-fourth volume of acid-

citrate-dextrose was added to citrated platelet-rich plasma   prior to centrifugation at 1500g

for 7 minutes at 22°C, and the pellet was resuspended in RNA extraction buffer. RNA from

the cell pellets was isolated from cells by RNeasy Mini kit (Qiagen)**.**

Statistical analyses

One-way analyses of covariance (ANCOVA): As we had little prior literature for guidance,

covariates entered in the model were selected based on an exploratory approach using

separate regression analysis with *NOTCH4* mRNA as dependent variable and age, gender,

smoking, diagnosis, different types of medication (lithium, antidepressants, mood stabilizers

and antipsychotics), cannabis use and alcohol consumption the past two weeks as independent

variables. As the NOTCH4 protein is known to play a role in the immune system, we also

adjusted for C-reactive protein levels. In addition, we controlled for creatinine, s-cortisol, alanine aminotransferase and hemoglobin levels. As PANSS positive sum score for evaluation of psychotic symptoms, Young Mania Rating Scale score for evaluation of mania symptoms, Inventory of Depressive Symptoms score for evaluation of depressive symptoms are illness specific and not relevant for the healthy control group, they were tested in a stratified model for the three diagnostic groups. Furthermore, we investigated a potential relationship between *NOTCH4* expression and number of previous affective episodes, psychotic episodes and number of previous hospital admissions (Table S2). The variables that turned out as significant contributors in the initial regression analyses were entered as covariates in the final model where we investigated the variance in *NOTCH4* mRNA levels across the different diagnostic groups using ANCOVA. All values were corrected for multiple tests using a Bonferroni correction for 16 tests; i.e. leaving a $p<0.05/16<0.003$ as statistically significant. SNP-expression analyses including interaction tests were performed with linear regression, also implemented in PLINK, assuming an additive model. The residuals from the initial analysis were entered as the quantitative trait in all further analyses. All groups were used for the interaction analysis. Linkage disequilibrium blocks were defined by confidence bounds on the normalized measure of allelic association (D) (3).

Table ST1: *NOTCH4* and medication

	Types of medication										ANOVA F(df)	P	Post hoc Tukey
ANTI-PSYCHOTICS	No AP (n=372)	Typical AP[1] (n=23)	Clozapine (n=5)	Apiprazol (n=40)	Risperidone (n=29)	Quetiapine (n=75)	Amisulpride (n=6)	Ziprazidone (n=20)	Olanzapine (n=113)	Sertindole (n=4)			
NOTCH4	1.84 (2.19)	2.01 (1.86)	1.52 (1.51)	2.00 (1.36)	1.62 (2.03)	1.92 (2.73)	1.54 (1.21)	1.46 (1.11)	1.46 (1.40)	2.20 (2.83)	1.0 (9,679)	0.43	n.s
ANTI-DEPRESSANTS		No AD (n=545)	Escitalopram (n=66)	Venlafaxine (n=30)	Fluoxetine (n=6)	Paroxetine (n=4)	Setraline (n=16)	Tetracyclic[2] (n=19)	TCA [3] (n=2)	Buprion[4] (n=1)			
NOTCH4		1.74 (1.73)	2.13 (4.17)	1.94 (1.64)	0.91 (0.61)	2.3 (1.27)	1.87 (1.25)	1.60 (1.10)	0.96 (0.78)	1.40	0.40 (7,680)	0.90	n.s
ANTI-EPILEPTICS						No AE (n=568)	Lamotrigine (n=66)	Clonazepam (n=11)	Valproic Acid (n=37)	Other AE (n=7)			
NOTCH4						1.67 (1.69)	2.85 (4.12)	1.11 (0.48)	1.75 (1.59)	1.30 (0.99)	2.33 (4,684)	0.51	No AE <Lamo-trigine (p=0.03)
LITHIUM									No Lithium (n=662)	Lithium (n=26)			
NOTCH4									1.42 (1.34)	1.79 (2.10)	1.01 (1,686)	0.32	n.s

Medication was confirmed by serum concentration. ANOVA analyses of *NOTCH4* according to medication are performed with log transformed values. The presentations of data on mean (SD) levels of *NOTCH4* in the table are presented as not log transformed for the readers benefit. AP=antipsychotics, AD=antidepressants, AE=antiepileptics. [1]Typical AP=Typical Antipsychotics: Zuclopenthixol (n=5), Flupenthixol (n=1), Chlorpromazine (n=1), Levomepromazine (n=1), Perphenazine (n=8), Chlorprothixine (n=6), [2]Tetracyclic= tetracyclic antidepressants: Mirtazepine (n=14), Mianserine (n=6), [3]TCA=tricyclic antidepressants: Clomipramine (n=1), Amitriptyline (n=1), [4] Due to n=1 in the Buprion group, post hoc test was done without this case, [5]Other Antiepileptics: Garbapentin (n=2), Carbamazeoine (n=3), Topiramate (n=2)

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Table ST2: Linear regression model investigating the relationship between *NOTCH4* and cumulative daily dosage of medication and clinical characteristics

	B	S.E	df	t	p
DDD[1]					
Antipsychotics	-0.060	0.18	315	-0.91	0.37
Antidepressants	0.000	0.07	136	0.31	0.76
Antiepileptics	-0.100	0.17	118	-0.98	0.33
Number of episodes[2]					
Depressive	-0.001	0.003	433	-0.31	0.74
Mania	0.005	0.009	456	0.49	0.62
Hypomania	-0.001	0.001	450	-0.64	0.52
Psychosis	-0.001	0.008	431	0.18	0.85
Hospital admissions[3]					
	-0.004	0.004	461	-1.02	0.31
PANSS					
Positive Scale	-0.152	0.201	474	-0.75	0.45
Negative Scale	-0.301	0.161	474	-1.87	0.06
YMRS					
	-0.023	0.076	438	-0.31	0.76
IDS					
	0.036	0.079	378	0.30	0.65

Abbreviations: DDD=defined daily dosage, PANNS=Positive and Negative Syndrome Scale, YMRS=Young Mania Rating Scale, IDS=Inventory of Depressive Symptoms. [1]DDD is calculated in accordance with the guidelines from the World Health Organization Collaborating Center for Drug Statistics Methodology (hhtp://www.whocc.no/atcdd). [2]Episodes were defined according to the SCID-I manual. Information regarding previous episodes was obtained from medical records and from the patient. [3]Hospital admissions were defined as previous admissions to psychiatric acute or intermediate wards. Analyses are performed with log transformed data

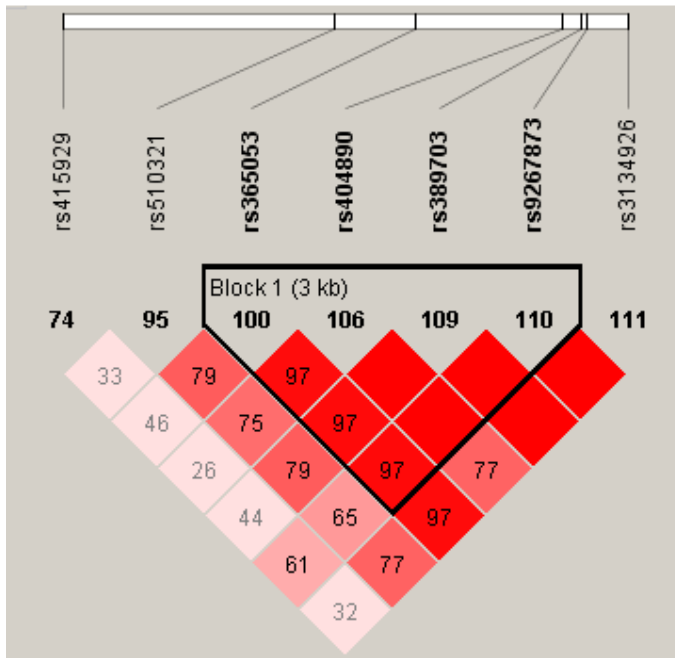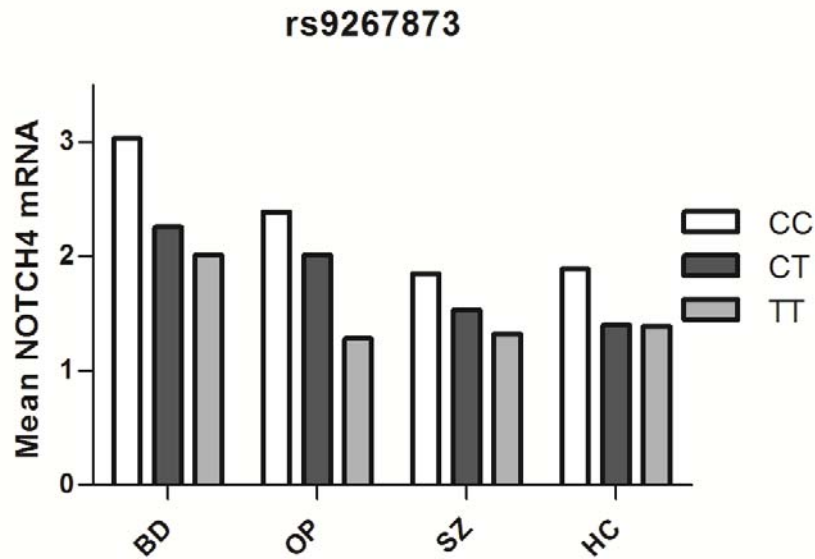**Figure SF1.** Haploview LD plot of associated SNPs

Figure SF2. Direction of allelic effect for rs9267873 displayed for all groups.

Abbreviations: BD=bipolar disorder, OP=other psychosis, SZ=schizophrenia, HC=healthy controls.

Overall effect: $F_{(2,458)}=5.4$, $p≤0.005$, post hoc Tukey: CC>TT

Unadjusted means (±SD) of *NOTCH4* expression according to alleles. BD: CC=3.03(3.98), CT=2.26 (2.31), TT=2.01(2.19), OP: CC=2.39(1.72), CT=2.01(1.57), TT=1.28(0.64), SZ: CC=1.85(1.81), CT=1.53(1.23), TT=1.32(1.04), HC: CC=1.89(1.56), CT=1.40(1.21), TT=1.39(1.44).

Reference List

(1) Aukrust P, Aandahl EM, Skalhegg BS, Nordoy I, Hansson V, Tasken K, Froland SS, Muller F: Increased activation of protein kinase A type I contributes to the T cell deficiency in common variable immunodeficiency. J.Immunol. 15-1-1999; 162:1178-1185.

(2) Kabanova S, Kleinbongard P, Volkmer J, Andree B, Kelm M, Jax TW: Gene expression analysis of human red blood cells. Int.J.Med.Sci. 2009; 6:156-159.

(3) Gabriel SB, Schaffner SF, Nguyen H, Moore JM, Roy J, Blumenstiel B, Higgins J, DeFelice M, Lochner A, Faggart M, Liu-Cordero SN, Rotimi C, Adeyemo A, Cooper R, Ward R, Lander ES, Daly MJ, Altshuler D: The structure of haplotype blocks in the human genome. Science 21-6-2002; 296:2225-2229.