

## ORIGINAL ARTICLE

Calcium-dependent intracellular signal pathways in primary cultured adipocytes and *ANK3* gene variation in patients with bipolar disorder and healthy controlsA Hayashi<sup>1,3</sup>, K Le Gal<sup>1,3</sup>, K Södersten<sup>2</sup>, D Vizlin-Hodzic<sup>1</sup>, H Ågren<sup>2</sup> and K Funa<sup>1</sup>

Bipolar disorder (BD) is a chronic psychiatric disorder of public health importance affecting > 1% of the Swedish population. Despite progress, patients still suffer from chronic mood switches with potential severe consequences. Thus, early detection, diagnosis and initiation of correct treatment are critical. Cultured adipocytes from 35 patients with BD and 38 healthy controls were analysed using signal pathway reporter assays, that is, protein kinase C (PKC), protein kinase A (PKA), mitogen-activated protein kinases (extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK)), Myc, Wnt and p53. The levels of activated target transcriptional factors were measured in adipocytes before and after stimulation with lithium and escitalopram. Variations were analysed in the loci of 25 different single-nucleotide polymorphisms (SNPs). Activation of intracellular signals in several pathways analysed were significantly higher in patients than in healthy controls upon drug stimulation, especially with escitalopram stimulation of PKC, JNK and Myc, as well as lithium-stimulated PKC, whereas no meaningful difference was observed before stimulation. Univariate analyses of contingency tables for 80 categorical SNP results versus diagnoses showed a significant link with the *ANK3* gene (rs10761482; likelihood ratio  $\chi^2=4.63$ ;  $P=0.031$ ). In a multivariate ordinal logistic fit for diagnosis, a backward stepwise procedure selected *ANK3* as the remaining significant predictor. Comparison of the escitalopram-stimulated PKC activity and the *ANK3* genotype showed them to add their share of the diagnostic variance, with no interaction (15% of variance explained,  $P < 0.002$ ). The study is cross-sectional with no longitudinal follow-up. Cohorts are relatively small with no medication-free patients, and there are no 'ill patient' controls. It takes 3 to 4 weeks of culture to expand adipocytes that may change epigenetic profiles but remove the possibility of medication effects. Abnormalities in the reactivity of intracellular signal pathways to stimulation and the *ANK3* genotype may be associated with pathogenesis of BD. Algorithms using biological patterns such as pathway reactivity together with structural genetic SNP data may provide opportunities for earlier detection and effective treatment of BD.

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## INTRODUCTION

Almost 1% of the population in the world is affected by bipolar disorder (BD), a mood disorder characterised by switches between depression and hypomanic/manic episodes. Key signs and symptoms manifest not only as mood-related phenomena, but also as more general disturbances in circadian rhythms.<sup>1,2</sup> Clinically, the overall suicide risk in patients with BD is high, with ~15% of patients taking their own lives.<sup>3</sup> Despite syndrome remission, the premorbid functional level rarely fully recovers.<sup>4,5</sup> BD has been shown to comprise neurodegenerative features in which relapses gradually lead to deterioration of cognitive functions, underlining the importance of early detection in order to prevent an otherwise negative prognosis.<sup>6</sup>

Evidence for heritability of BD has been recognised in family and monozygotic twin studies, but causal genes have not been identified.<sup>7,8</sup> Several single-nucleotide polymorphisms (SNP) have been linked to susceptibility for BD by genome-wide association studies.<sup>9</sup> However, it is still unclear how and to what

extent different combinations of SNPs affect the susceptibility for BD.

The treatment strategy for BD has focussed mainly on lithium, valproate, lamotrigine and quetiapine—thus, drugs not primarily targeting noradrenaline or serotonin synaptic reuptake mechanisms. Nevertheless, imbalanced neurotransmitter function has been posited to be important in the underlying pathophysiology. However, data support the idea that there exists other endogenous, as well as exogenous factors triggering BD. Pro- and anti-inflammatory cytokines may play a role, and selective serotonin reuptake inhibitors help decrease interleukins and cortisol, at least in major depressive disorder.<sup>10</sup> Decreased neurogenesis or increased apoptosis is also thought to be involved in the pathophysiology of this disease.<sup>11</sup> Neurotrophic factors are clearly involved—meta-analyses and a large cohort study have reported significantly lower brain-derived neurotrophic factor (BDNF) levels in major depressive disorder patients compared with healthy controls,<sup>12</sup> and BDNF has been shown to possess antidepressant

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effects in animals, effects appearing to be mediated by the mitogen-activated protein (MAP) pathway.<sup>13</sup>

Furthermore, serum concentrations of mature BDNF and the ratio between mature BDNF and proBDNF are significantly higher in BD patients than in controls.<sup>14</sup> Cyclic adenosine monophosphate (cAMP)-dependent protein kinase A (PKA) and protein kinase C (PKC) appear to be potential therapeutic regulatory candidates in mood disorders, although their specific role in BD has yet to be elucidated.<sup>15–18</sup> Abnormalities in Ca<sup>2+</sup> signalling have been reported.<sup>19</sup> Interestingly, lithium-responsive BD patients had longer telomeres than those not responding well, irrespective of age.<sup>20</sup> These reports do support the notion that several pathways are affected in BD.

To study postsynaptic signalling pathways we established adipocyte-derived cell cultures from abdominal fat samples from patients with BD and healthy controls. These adipocytes were exposed to several drugs of interest. We selected escitalopram, which effects neurones in more ways than only inhibiting serotonin synaptic reuptake, and lithium, being the mood stabiliser of choice in BD.

The aims of the study were (1) to test whether a simple drug test shows different patterns in reactivity of postsynaptic signalling pathways in primary cultured adipocytes between patients with BD and healthy controls, and (2) to elucidate whether an algorithm using biological patterns such as pathway reactivity together with structural genetic SNP data could be used for clinical predictions.

## SUBJECTS AND METHODS

### Bipolar patients

A total of 35 mood-stabilised Caucasian research patients (12 males and 23 females) were recruited from the Bipolar Outpatient Department at the Psychiatric Clinic, Sahlgrenska University Hospital in Gothenburg, Sweden. Mean age was 38±10 years. To validate their diagnoses they were interviewed with the structured psychiatric interview, Mini-International Neuropsychiatric Interview (M.I.N.I.), version 6,<sup>21</sup> in an authorised Swedish translation, yielding DSM-IV (Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition) diagnoses. Subdiagnoses were bipolar I (*n* = 11), bipolar II (*n* = 7) and bipolar not otherwise specified or spectrum (*n* = 17) diagnoses. The patients with bipolar I diagnoses all had suffered from psychotic symptoms at least once during a previous depressive or manic episode. All patients were above 18 years of age. Mean age was 40.2±10.3 years. Table 1 shows the subdiagnostic composition.

The Regional Research Ethics Board in Gothenburg approved the study (172-08).

### Healthy controls

As controls, 38 psychiatrically healthy Caucasian individuals (18 men and 20 women) were used. They were interviewed and presented a nondiagnostic result on M.I.N.I. version 6. All controls were above 18 years of age. Mean age was 30±12 years.

Before commencement of the study and signing the written consent, all subjects were provided with verbal and written information about the study and about potential risks and benefits of study participation.

### Assessment of clinical variables

Age, height, weight, sagittal abdominal diameter and waist circumference were measured, and the body mass index was calculated. Age, age at first diagnosis, the latency before diagnosis, years with diagnosis, number of suicide attempts and numbers of depressive, manic and mixed episodes were noted. For dimensional assessments, the Montgomery-Åsberg Depression Rating Scale (MADRS)<sup>22</sup> and Young Mania Rating Scale (YMRS)<sup>23</sup> were used to assess mood states, and Mini-Mental State Examination (MMSE)<sup>24</sup> for cognitive impairment. Disability was assessed by Global Assessment of Functioning (GAF).<sup>25</sup> To assess addiction, Alcohol Use Disorder Test (AUDIT)<sup>26</sup> and the Drug Use Disorder Test (DUDIT)<sup>27</sup> were used. When needed, supplementary information was collected from

**Table 1.** Subdiagnostic composition of patients

	Males	Females
Bipolar I, psychotic	6	5
Bipolar I, nonpsychotic	0	0
Bipolar II	3	4
Bipolar not otherwise specified (NOS)	3	14
Healthy controls	18	20

**Table 2.** Description of measured clinical variables

Arithmetic means (±s.d.)	Patients	Healthy controls
<i>n</i>	35	38
Age (yrs)	38±10	30±12
Height (cm)	173±20	174±8
Weight (kg)	83±18	71±12
Sagittal diameter (cm)	19.4±3.5	16.3±2.5
Waist circumference (cm)	93.3±14.6	79±11.1
BMI	25.5±4.8	23.5±3.3
Age at first symptoms	18±6.5	
Age at diagnosis	30.6±8.9	
Diagnostic latency	12.5±8.3	
Yrs with diagnosis	7.4±8.3	
No. of suicide attempts	1±2.5	
No. of depressive episodes	12±14	
No. of manic episodes	7.6±8.2	
No. of mixed episodes	0.125±0.3	
AUDIT	4.1±3.8	5.6±3.3
DUDIT	1.3±3.4	0.6±1.3
GAF	71±14	89±4
YMRS	0.8±2.2	0.2±0.6
MADRS	9.1±8.2	1.3±2.5
MMSE	28.5±1.7	29.6±0.6

Abbreviations: AUDIT, Alcohol Use Disorder Test; BMI, body mass index; DUDIT, Drug Use Disorder Test; GAF, Global Assessment of Functioning; MADRS, Montgomery-Åsberg Depression Rating Scale; MMSE, Mini-Mental State Examination; YMRS, Young Mania Rating Scale.

medical records. Healthy controls were assessed by the AUDIT, DUDIT, GAF, YMRS, MADRS and MMSE. All clinical measurements are listed in Table 2.

Individuals with active metabolic disorders and/or alcohol/drug addiction were excluded. Before sampling, the participants underwent a series of biochemical and somatic tests, as well as routine diagnostic screening and clinical estimates.

### Adipocyte isolation and primary cell culture establishment

Abdominal adipose tissue (2–3 ml) was isolated by liposuction and transported to the laboratory within 30 min. The tissue was washed several times with Hanks' balanced salt solution (Life Technologies, Carlsbad, CA, USA) to remove any remaining blood residues. The tissue was mechanically disrupted by using sterile action scissors, followed by enzymatic digestion in buffer consisting of 150 mM collagenase I (Sigma-Aldrich, St Louis, MO, USA), bovine serum albumin and phosphate buffer saline. The tissue was incubated for 1 h at 37 °C. The reaction was stopped by adding Dulbecco's modified Eagle's medium (Lonza, Basel, Switzerland) supplemented with 20% fetal bovine serum, 2 mM glutamine and 1% of penicillin/streptomycin, and the dissociated tissue was filtered through a 100 µm Nylon mesh cloth (Cell strainer, 100 mm; BD Biosciences, San José, CA, USA). The isolated adipocytes were grown as described above at 37 °C and 5% CO<sub>2</sub>. The initial primary cell culture is referred to 'passage 0.' Cell medium was changed every 2 to 3 days, and cells were passaged every 5–7 days, depending on the cell density. Adipocytes were harvested between passages four and six in order to perform the experiments.

### Luciferase assay

In order to analyse possible difference in various signalling pathways, we compared the degree of activation of their target transcription factors as read-out by using luciferase assays. The analyses were performed under three different conditions, that is, untreated cells and cells treated with escitalopram or with lithium. The intracellular signalling molecules, such as kinases that are activated by the drugs, phosphorylate the target transcription factors. Then, they bind the consensus binding sites of different promoters, inducing the expression of the reporter luciferase, used as read-out for the activity. The promoter reporter constructs have different consensus binding sites for transcription factors, that is, cAMP response element-binding protein (CREB; downstream of PKA), nuclear factor of activated T-cells (NFAT; PKC), Elk/SRF (extracellular signal-regulated kinase (ERK)), activation protein 1 (c-Jun N-terminal kinase (JNK)), MycMax (Myc), TCF/LEF (Wnt) and p53/p63/p73 (p53), that were compared with positive and negative controls. The experiment was performed according to the vendor's instructions. Briefly, cells were reverse transfected at 80% confluence in a 24-well plate with Cignal™ Reporter Assays DNA-based reporter for each pathway (Qiagen, Valencia, CA, USA) by using FuGENE HD transfection reagent (Promega, Madison, WI, USA) in a 0.4:6 ratio (plasmid to reagent). The culture medium used upon transfection was Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 2 mM glutamine without antibiotics. After 24 h, the medium was replaced with fresh one with lithium (5 mM, Sigma-Aldrich) or escitalopram (5 µM, H Lundbeck A/S Co, Valby, Denmark) or medium alone for the nonstimulated cells. On the fourth day, 72 h after transfection, the expression of luciferase was read on a Victor X light luminometer (Perkin-Elmer, Waltham, MA, USA) with the use of the Dual-Luciferase® Reporter Assay System (Promega).

### Genotyping

DNA extraction from blood samples was done by using DNeasy Blood and Tissue kit (Qiagen). Genes to be analysed were selected from earlier findings reported in recent genetic studies on bipolar disorder and schizophrenia.<sup>28,29</sup> Twenty-five selected genes (see Table 5 for chromosome locations and SNP numbers) were analysed in 41 patients and 39 controls with TaqMan SNP genotyping assay from Applied Biosystems (ABI, Foster City, CA, USA) on ABI StepOne plus thermocycler using TaqMan Genotyping Master Mix according to the manufacturer's instructions.

### Statistical analysis of the results

To analyse the results obtained by quantitative PCR analysis (see Supplementary Information), relative expression values of *protein kinase A catalytic subunit-α* (PRKACA), *protein kinase A regulatory subunit, type Ia* (PRKAR1A) and *protein kinase A regulatory subunit, type IIa* (PRKAR2A) and *protein kinase C-α* (PRKCA) were acquired by normalising the Cq values of each gene of interest to the geometric average of the reference genes *ACTB*, *GAPDH* and *TBP*. As two different reverse transcription reactions were tested for each clinical sample, the arithmetic average of these was considered the final mean relative expression value for each sample assayed.

IBM SPSS Statistics 20 (Armonk, NY, USA) and SAS JMP (Cary, NC, USA) software were used for analyses, where  $P < 0.05$  was established as statistically significant.

## RESULTS

Screening differences in drug response of intracellular signalling pathways by using adipocytes from patients and controls.

First, 10 inflammation pathways were screened, that is, nuclear factor-κB (NF-κB), PKC, cAMP/PKA, type-I interferon, interferon-γ, MAP/ERK, MAP/JNK, transforming growth factor-β, CCAAT-enhancer-binding proteins and glucocorticoid receptor. As drugs of interest, escitalopram, lithium, certain neurotransmitters (5-hydroxytryptamine, L-glutamine and dopamine) and dexamethasone were tested. The purpose was to compare differences of signal responses between cells derived from patients and healthy controls, even though adipocytes probably lack certain receptors and transporters that neuronal cells would express. Dopamine also yielded some discriminative responses, but less

frequently than the pathways mentioned above. We also tested other pathways not examined in the context of pathology of depression, such as Notch, Wnt, HRE, p53 and Myc, that are involved in development, stress, apoptosis and proliferation.

After several tests on two control cell lines established from volunteers, we decided to evaluate the following seven clearly responsive pathways – PKC, cAMP/PKA, MAP/ERK, MAP/JNK, Wnt, Myc and p53. We screened 15 patients and 15 controls, and after repeated screening of drugs in these pathways, we chose the selective serotonin reuptake inhibitor escitalopram and the mood stabiliser lithium. Both elicited clear responses in several of 10 immune pathways, although selective serotonin reuptake inhibitors are not routinely prescribed to bipolar patients. These results suggested increased activities in BD patient cells as compared with controls, especially after drug stimulations.

### Gene expression of PKA and PKC

Previous studies have shown an increased level of basal PKA activity in BD lymphoblasts.<sup>30,31</sup> Both untreated unipolar and bipolar patients with psychotic depression have significantly lower levels of platelet regulatory type I and higher levels of catalytic subunits of PKA than controls, whereas the levels of regulatory type II are higher only in psychotic unipolar patients.<sup>32</sup> In contrast, other reports claim no difference in PKA basal levels between bipolars and controls, but a significant increase in cAMP-stimulated PKA activity in platelets.<sup>33</sup>

A majority of previous results on the basal mRNA expression of PKA and PKC indicate no significant difference in bipolar patients and controls. However, as it is not fully known if PKA or PKC signalling is affected at the mRNA level of their regulatory and catalytic subunits, we examined their levels by quantitative PCR analysis on randomly selected samples from both groups. A description of the method is described in the Supplementary Information. The basal relative expression of each gene of interest was obtained by quantitative PCR analysis for *PRKACA*, *protein kinase A catalytic subunit-β* (*PRKACB*), *PRKAR1A* and *PRKAR2A*, as well as for *PRKCA*. They were analysed for both patient and control groups (14 randomly selected subjects in each) with error bars set at a 95% confidence interval. Independent-samples *t* tests were run giving *P*-values of 0.072 for *PRKACA*, 0.123 for *PRKAR1A*, 0.151 for *PRKAR2A* and 0.121 for *PRKCA*, revealing no significant differences at transcriptional level in drug-naive samples. Non-parametric Kruskal–Wallis tests also yielded nonsignificant findings.

### PKC and PKA signalling pathways

As shown in Table 3, the basal activities of the PKC pathway are nonsignificantly higher in the bipolar group compared with the controls ( $P = 0.10$  to  $0.12$ ). However, the difference becomes highly significant after stimulation with escitalopram ( $P = 0.00052$ ) or lithium ( $P = 0.004$ ). The PKA pathway shows diagnostic significant differences similar to those of PKC, but they are weaker.

### JNK and ERK signalling pathways

Table 3 shows that the basal activities of the JNK pathway are higher in the bipolar group compared with the controls ( $P = 0.048$  to  $0.28$ ) that becomes highly significant after stimulation with escitalopram ( $P = 0.00034$ ) or, less so, with lithium ( $P = 0.047$ ). Weaker but still significant differences for ERK activation were found after escitalopram ( $P = 0.034$ ), but no difference was found by lithium stimulation.

### Wnt, Myc and p53 signalling pathways

Lithium, serotonin and dopamine all affect glycogen synthase kinase 3 (GSK-3) phosphorylation, a Wnt target.<sup>34–36</sup> C-myc is known as a proto-oncogene associated with cell proliferation,

**Table 3.** Univariate comparisons of intracellular signalling pathways in bipolar patients and healthy controls, showing significant differences in activities stimulated by escitalopram and lithium

Pathways	Diagnosis			Escitalopram			Lithium				
	Basal level	P	Escitalopram stimulated	Esc/Basal	P	Basal level	P	Lithium stimulated	P	Li/Basal	P
PKC pathway (NFAT)	Bipolars (n = 35) Controls (n = 35)	1.90 ± 0.55 1.70 ± 0.49	0.10	2.54 ± 0.63 2.01 ± 0.63	1.42 ± 0.50 1.20 ± 0.26	0.00052	1.92 ± 0.59 1.72 ± 0.52	0.12	1.88 ± 0.65 1.47 ± 0.52	0.99 ± 0.27 0.85 ± 0.13	0.011
PKA pathway (CREB)	Bipolars (n = 35) Controls (n = 35)	1.91 ± 0.60 1.76 ± 0.53	0.27	2.49 ± 0.87 2.03 ± 0.61	1.32 ± 0.36 1.20 ± 0.40	0.011	1.91 ± 0.68 1.77 ± 0.55	0.35	1.88 ± 0.81 1.57 ± 0.49	0.99 ± 0.30 0.88 ± 0.14	0.044
JNK pathway	Bipolars (n = 35) Controls (n = 35)	2.57 ± 0.62 2.31 ± 0.48	0.048	2.35 ± 0.56 1.95 ± 0.29	0.94 ± 0.22 0.89 ± 0.28	0.00034	2.55 ± 0.61 2.40 ± 0.55	0.28	2.65 ± 0.67 2.35 ± 0.59	1.04 ± 0.14 0.98 ± 0.09	0.025
ERK pathway	Bipolars (n = 35) Controls (n = 35)	2.10 ± 0.57 1.94 ± 0.53	0.21	2.55 ± 0.73 2.18 ± 0.75	1.24 ± 0.30 1.11 ± 0.18	0.034	2.11 ± 0.57 1.95 ± 0.57	0.21	2.10 ± 0.63 1.88 ± 0.67	1.00 ± 0.23 0.96 ± 0.19	0.40
myc pathway	Bipolars (n = 35) Controls (n = 35)	2.04 ± 0.57 1.86 ± 0.43	0.13	2.64 ± 0.73 2.18 ± 0.61	1.41 ± 0.78 1.18 ± 0.19	0.0055	2.06 ± 0.59 1.84 ± 0.45	0.085	2.09 ± 0.63 1.80 ± 0.51	1.03 ± 0.17 0.98 ± 0.15	0.13
p53 pathway	Bipolars (n = 33) Controls (n = 35)	2.56 ± 0.62 2.24 ± 0.64	0.90	2.76 ± 0.78 2.51 ± 0.76	1.24 ± 0.21 1.13 ± 0.16	0.16	2.26 ± 0.61 2.27 ± 0.65	0.95	2.22 ± 0.73 2.32 ± 0.59	0.98 ± 0.19 1.04 ± 0.18	0.17
Wnt pathway	Bipolars (n = 25) Controls (n = 30)	1.75 ± 0.60 1.92 ± 0.62	0.30	2.54 ± 0.78 2.16 ± 0.74	1.53 ± 0.43 1.16 ± 0.25	0.00064	1.76 ± 0.59 1.94 ± 0.27	0.27	1.71 ± 0.80 1.72 ± 0.68	0.93 ± 0.29 0.87 ± 0.13	0.34

Abbreviations: CREB, cAMP response element-binding protein; ERK, extracellular signal-regulated kinase; Esc, escitalopram; JNK, c-Jun N-terminal kinase; Li, lithium; NFAT, nuclear factor of activated T-cells; PKA, protein kinase A; PKC, protein kinase C. Pathway data are raw activity measures (± s.d.), normalised by <sub>10</sub>log transformations and expressed as logged counts per second. Bipolar and control adipocytes were transfected with CREB or NFAT reporter plasmids, and stimulated 24 h after with escitalopram (5 µM) or lithium (5 mM). Luminescence was measured 48 h after stimulation with Victor X light luminometer (Perkin-Elmer) and Dual-Luciferase<sup>®</sup> Reporter Assay System (Promega). P-values refer to univariate comparisons between bipolar patients and controls using two-tailed t-tests. Raw data were <sub>10</sub>log transformed. P values less than 0.05 are marked in bold.

but it is also known as a pro-apoptotic molecule in certain conditions.<sup>37,38</sup> In addition, Myc is a target of Wnt and stabilises β-catenin.<sup>39</sup> p53 has been considered to act negatively on neuronal development, promoting neurodegeneration,<sup>40</sup> probably by its repressive functions on cell cycle and survival. Lithium has been shown to possess survival effects by antagonising p53.<sup>41</sup>

Table 3 shows that in the Myc pathway, a significantly higher response was seen in the patient group after escitalopram ( $P=0.0055$ ) and lithium ( $P=0.033$ ). In the p53 pathway, patients show a significantly higher level only in the escitalopram/basal ratio ( $P=0.013$ ). There is also higher response in the Wnt escitalopram/basal ratio ( $P<0.0006$ ). However, p53 and Wnt pathways did not render any significant differences after lithium stimulation.

Figure 1a illustrates the significant differences in escitalopram-stimulated activities of PKC, JNK and Myc between bipolar patients and healthy controls.

Multivariate comparisons of seven pathways predicting diagnosis Log transformed values of PKC, PKA, JNK, ERK, Myc, p53 and Wnt were used as independent variables in nominal logistic fits, predicting the diagnostic dichotomy. Sex was used as a covariate. Table 4a shows results for basal values, after stimulation of escitalopram and the ratios between stimulated and basal activities. Table 4b shows the same for lithium.

The best predictions were obtained with the stimulated/basal ratio (block 3 in Table 4a and b). For the escitalopram/basal data, Myc was the strongest individual predictor ( $P=0.0084$ ), and for the lithium data three individual predictors were significant: PKC ( $P=0.014$ ), JNK ( $P=0.0045$ ) and p53 ( $P=0.030$ ). Maximal variance explained (overall  $R^2$ ) was 39% for the escitalopram/basal data set.

The power to predict diagnosis from these equations was high, with a specificity of 81% and a sensitivity of 85% for the ratios between escitalopram-stimulation/basal level ( $\chi^2$  test,  $P<0.0001$ ).

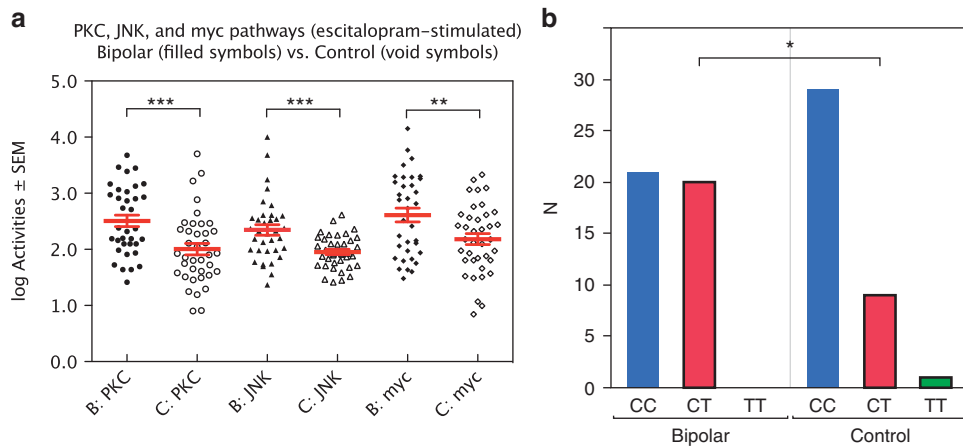
Genetic analyses of 25 SNPs, selected for relevance

Selection of the 25 SNPs to be included in our statistical analysis was driven by published hypotheses concerning relevance to bipolar and other severe psychiatric disorders. Univariate  $\chi^2$  analyses of the 80 individuals with contingency tables for categorical SNP results (2 to 3 levels) versus diagnosis (2 levels) are shown in Table 5. The strongest and only significant link to diagnosis was seen with ANK3 (location 10: 62085337; rs10761482; likelihood ratio  $\chi^2=4.63$ ;  $P=0.031$ ). Figure 1b shows the sample distribution of the ANK3 gene (SNPs CC, CT and TT), split for diagnosis. Rare homozygotic alleles were assigned to the heterozygotic variety before statistical calculations (for example, TT was assigned to CT, if TT was represented by only one to three individuals—the minor allele frequency being < 5%).

Table 6 shows a multivariate, ordinal logistic fit for diagnosis, using in a backward stepwise selection procedure, starting with four genes with univariate  $P<0.20$ . The SNPs that showed obvious linkage disequilibrium were omitted from statistical analysis. These genes explained 9% of the diagnostic variation with a diagnostic specificity of 74% and sensitivity of 71% ( $\chi^2=14.47$ ,  $P<0.0001$ ). In the final backward step, only ANK3 remained significant.

Comparing the powers of PKC activity and ANK3 alleles to predict diagnosis

Two independent variables—the quantitative escitalopram-stimulated PKC activity and the qualitative information on ANK3 alleles—were compared in predicting the dependent bipolar | control dichotomy (Table 7). PKC turned out significant ( $P=0.0005$ ) and ANK3 was at trend level ( $P=0.073$ ), overall



**Figure 1.** (a) Escitalopram-stimulated protein kinase C (PKC), c-Jun N-terminal kinase (JNK) and myc activities vs diagnosis. (b) *ANK3* gene (rs10761482) vs diagnosis. \*\*\* $P < 0.001$ ; \*\* $P < 0.01$ ; \* $P < 0.05$ .

**Table 4.** Multivariate nominal logistic fits for diagnosis (bipolars vs controls), using seven pathways plus sex as independent variables, demonstrating diagnostic power

	$\chi^2$	P		$\chi^2$	P		$\chi^2$	P
<i>(a) Stimulation with escitalopram</i>								
PKC basal	6.50	0.090	PKC+esc	8.99	<b>0.030</b>	PKC esc/basal	2.87	0.41
PKA basal	10.52	<b>0.015</b>	PKA+esc	2.62	0.45	PKA esc/basal	4.68	0.20
JNK basal	7.27	0.064	JNK+esc	7.22	0.065	JNK esc/basal	2.43	0.49
ERK basal	0.83	0.84	ERK+esc	8.23	<b>0.041</b>	ERK esc/basal	13.23	<b>0.0042</b>
Myc basal	1.95	0.58	Myc+esc	0.48	0.92	Myc esc/basal	5.84	0.12
p53 basal	1.66	0.64	p53+esc	3.70	0.30	p53 esc/basal	1.51	0.68
Wnt basal	8.02	<b>0.046</b>	Wnt+esc	3.42	0.33	Wnt esc/basal	5.61	0.13
Sex	2.80	0.42	Sex	4.26	0.24	Sex	1.18	0.76
Overall $R^2$	0.26		Overall $R^2$	0.31		Overall $R^2$	0.39	
n	58		n	58		n	52	
P=	0.071		P=	<b>0.018</b>		P=	<b>0.0023</b>	
<i>(b) Stimulation with lithium</i>								
PKC basal	7.92	<b>0.048</b>	PKC+Li	13.44	<b>0.0038</b>	PKC Li/basal	13.45	<b>0.0038</b>
PKA basal	10.38	<b>0.016</b>	PKA+Li	4.54	0.21	PKA Li/basal	12.49	<b>0.0059</b>
JNK basal	8.26	<b>0.041</b>	JNK+Li	15.15	<b>0.0017</b>	JNK Li/basal	6.61	0.085
ERK basal	2.62	0.45	ERK +Li	2.37	0.50	ERK Li/basal	4.86	0.18
Myc basal	1.25	0.74	Myc+Li	1.41	0.70	Myc Li/basal	8.77	<b>0.033</b>
p53 basal	2.54	0.49	p53+Li	12.17	<b>0.0068</b>	p53 Li/basal	6.72	0.081
Wnt basal	8.89	<b>0.031</b>	Wnt+Li	13.63	<b>0.0035</b>	Wnt Li/basal	4.10	0.25
Gender	4.34	0.23	Gender	9.08	<b>0.028</b>	Gender	7.91	<b>0.048</b>
Overall $R^2$	0.26		Overall $R^2$	0.32		Overall $R^2$	0.34	
n	59		n	59		n	59	
P=	0.070		P=	<b>0.0076</b>		P=	<b>0.0040</b>	

Abbreviations: ERK, extracellular signal-regulated kinase; esc, escitalopram; JNK, c-Jun N-terminal kinase; Li, lithium; PKA, protein kinase A; PKC, protein kinase C. Raw data were  $_{10}\log$  transformed before division. Numbers in bold denote significant probabilities ( $P < 0.05$ ).

$R^2=0.15$ ,  $P=0.0016$ . An interactive term was clearly nonsignificant, suggesting the contributions of the PKC pathway and the *ANK3* gene to be additive, not synergistic. Diagnostic specificity was 70% and sensitivity 67%.

Predicting bipolar heredity and psychotic episode(s) in patients' psychiatric history

Table 7 also demonstrates presence of bipolar heredity in first-degree relatives to be predictable from the PKC and *ANK3* data, but at a somewhat weaker level than that for diagnosis. Prediction of psychotic qualities in any previous manic or depressive episode proved not possible.

## DISCUSSION

The relevance of intracellular signalling cascades in mood disorders has become accepted during the past decade, and ever more molecules have been discovered. In this report, escitalopram and lithium were used to stimulate patient-derived primary cultured adipocytes in order to compare their responses in a battery of different signalling pathways.

### PKC and PKA

There is evidence that selective serotonin reuptake inhibitors activate presynaptic 5-HT<sub>3</sub> receptors, increasing Ca<sup>2+</sup> concentration, that activates sigma-1 receptors and efflux of Ca<sup>2+</sup> from the

**Table 5.** Univariate comparisons of contingency tables of 25 selected SNPs

Gene	Chr location	SNP variant	Psychiatric phenotypes <sup>a</sup>	Alleles	Likelihood ratio $\chi^2$	d.f.	P
ANK3	10:61839831	rs9804190	BD	CC; CT/TT	0.49	1	0.49
ANK3	10:62085337	rs10761482	SZ	CC; CT/TT	4.63	1	<b>0.031</b>
ANK3	10:62179812	rs10994336	BD, MDD	CC; CT	0.01	1	0.91
ANK3	10:62279124	rs10994397	BD, ASD, ADHD	CC; CT	0.03	1	0.88
BDNF-AS	11:27679916	rs6265	BD	CC; CT	0.30	1	0.58
C11orf80	11:66551002	rs10896135	BD	CC/CG; GG	0.80	1	0.37
CACNA1C <sup>b</sup>	12:2345295	rs1006737	BD, MDD, SZ	AA/AG; GG	0.81	1	0.37
CACNA1C <sup>b</sup>	12:2349584	rs4765905	SZ	CC/CG; GG	0.81	1	0.37
CACNA1C	12:2419896	rs4765913	BD	AA/AT; TT	0.08	1	0.78
CACNA1C <sup>b</sup>	12:2420377	rs4765914		CC; CT/TT	1.12	1	0.29
CACNA1C <sup>b</sup>	12:2420526	rs10774037		AA; AG/GG	1.05	1	0.31
CACNB3	12:49218171	rs2078615	BD	AA; AG; GG	2.41	2	0.30
CACNB3	12:49219138	rs11168751		CC; CG/GG	2.05	1	0.15
COMT	22:19951271	rs4680		AA; AG; GG	0.03	2	0.98
COMT	22:19956781	rs165599		AA; AG; GG	0.51	2	0.78
DHH	12:49479968	rs7296288		AA; AC; CC	0.91	2	0.64
HSP4	2:65758525	rs3845817	BD	CC; CT; TT	2.95	2	0.23
ITIH3	3:52835354	rs736408	BD	CC; CT; TT	1.65	2	0.44
ITIH4	3:52852538	rs4687657		GG; GT/TT	0.44	1	0.51
ITIH4	3:52855229	rs2239547	SZ	CC/CT; TT	0.44	1	0.51
MIR137HG	1:98502934	rs1625579	BD, MDD, SZ, ADHD	GG/GT; TT	1.98	1	0.16
SYNE1	6:152790573	rs9371601	BD, MDD, SZ, ADHD	GG; GT; TT	0.19	2	0.91
SYNE1	6:152791474	rs7747960	BD	AA/AC; CC	1.02	1	0.31
TENM4	11:79077193	rs12576775	BD, MDD, SZ	AA; AG/GG	2.39	1	0.12
TENM4	11:79123883	rs2175420		CC; CT/TT	0.01	1	0.93

Abbreviations: ADHD, attention deficit hyperactivity disorder; ASD, autism spectrum disorder; BD, bipolar disorder; Chr, chromosome; MDD, major depressive disorder; SNP, single-nucleotide polymorphism; SZ, schizophrenia. Genes marked with grey ( $P < 0.20$ ) were selected for multivariate analysis. Comparisons between bipolar diagnoses vs controls ( $N = 80$ ). <sup>a</sup>Information from Ensembl Genome Browser (www.ensembl.org). <sup>b</sup>Identical distributions of alleles because of linkage disequilibrium. Numbers in bold denote significant probabilities ( $P < 0.05$ ).

**Table 6.** Multivariate stepward logistic regressions with genes as independent variables, predicting diagnosis ( $N = 80$ )

Gene	Chr	SNP	$\chi^2$	P	$\chi^2$	P
<i>Multivariate logistic regression:</i>						
ANK3	10	rs10761482	4.05	<b>0.044</b>	4.63	<b>0.031</b>
CACNB3	12	rs11168751	1.89	0.17	—	—
MIR137HG	1	rs1625579	1.34	0.25	—	—
TENM4	11	rs12576775	2.04	0.15	—	—
<i>Overall statistics:</i>						
		$R^2$	0.094		0.042	
		$n$	80		80	
		$P$	0.034		0.031	
<i>Classification power:</i>						
		$\chi^2$	14.47		3.63	
		$P$	<b>&lt; 0.0001</b>		0.056	
		Specificity	74%		58%	
		Sensitivity	71%		67%	

Abbreviations: Chr, chromosome; SNP, single-nucleotide polymorphism. Adding sex as an independent variable produced a nonsignificant weight ( $P = 0.35$ ;  $P = 0.29$ ). Numbers in bold denote significant probabilities ( $P < 0.05$ ).

**Table 7.** Nominal logistic fits for three dependent variables (diagnosis, heredity and psychotic ever)

Dependent variables →	Bipolar/control		Bipolar heredity (YIN)		Psychotic ever (YIN)	
Independent variables ↓	$\chi^2$	P	$\chi^2$	P	$\chi^2$	P
logPKC_esc	12.24	<b>0.0005</b>	7.62	<b>0.0058</b>	2.19	0.14
ANK3 (CC, CT)	3.21	0.073	4.30	<b>0.038</b>	2.04	0.15
logPKC × ANK3	1.19	0.28	0.04	0.84	0.07	0.79
Overall $R^2$	0.15		0.14		0.05	
$n$	73		73		71	
$P =$	<b>0.0016</b>		<b>0.0067</b>		0.21	
Classification $\chi^2$	8.48		8.24		0.00	
$P =$	<b>0.0036</b>		<b>0.0041</b>		0.98	
Sensitivity	70%		70%		40%	
Specificity	67%		79%		71%	

Independent variables: escitalopram-stimulated protein kinase C activity (logPKC\_esc) and the ANK3 (rs10761482) genotype. Simple effects are additive; no significant interactions were detected. Numbers in bold denote significant probabilities ( $P < 0.05$ ).

endoplasmic reticulum. This increases intrasynaptosomal  $Ca^{2+}$ , resulting in activation of PKC.<sup>42</sup> Here, escitalopram was shown to also activate postsynaptic signals.

In contrast, lithium inhibits inositol phosphate hydrolysis and PKC. Increased PKC activity in manic subjects was reported by Friedman *et al.*<sup>43</sup> in 1993, whose work showed higher activity in membrane-bound PKC in platelets derived during mania. When stimulated with lithium for some weeks, both cytosolic and

membrane activity decreased. When PKC activity was analysed in post-mortem BD patient brains, cytosolic PKC $\alpha$  and membrane-bound PKC $\gamma$  and PKC $\zeta$  activities were elevated, whereas PKC $\epsilon$  was diminished.<sup>16</sup> Activation of PKC was shown to impair the cognitive function of the prefrontal cortex in rat.<sup>44</sup>

These findings suggested a strong relationship between increased PKC activity and mania; subsequently, considerable effort has been put into finding suitable PKC inhibitors. Lithium, valproate and tamoxifen have proven therapeutic values,

decreasing PKC activity.<sup>17,43,45</sup> It might be difficult to compare *in vivo* and *in vitro* data, but our results showed that the basal activity between cells of patients and controls did not differ. After stimulation with escitalopram or lithium, significantly higher responses were detected in patient-derived cells.

PKA signalling is involved in  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor trafficking by phosphorylating the GluR1 subunit at the S845 site that has currently been shown to be of importance in psychiatric disorders.<sup>45</sup> Chang *et al.*<sup>46</sup> analysed the mRNA expression of PKA in post-mortem brains, finding no differences in expression of PKA RII $\beta$  and Ca subunits between patients and controls. We found no differences in the mRNA levels either. Following chronic antidepressant treatments, CREB becomes phosphorylated by PKA but more markedly by a nuclear calmodulin-II (CaMII) kinase and increases the expression of ERK1/2.<sup>47</sup> Calcium influx activates CaMII kinase and phosphorylates CREB.<sup>48</sup> In our univariate comparisons of PKA, we found significantly higher responses in patients than in controls after drug stimulation, but no difference was seen in basal activity. In agreement, our PCR results showed no difference at the mRNA levels of both the regulatory and catalytic subunits of PKA and PKC, comparing patients and controls in drug-deprived conditions. We did not perform the PCR analysis in other pathways as we focus on intracellular signalling molecules that are often activated by protein modification.

Our study adds to previous reports of differences in PKA and PKC activity in patients suffering from BD.<sup>49,50</sup> Lithium and valproic acid treatments reduce PKC activation and receptor-G protein coupling in platelets of bipolar manic patients.<sup>50</sup> The luciferase reporter assay showed that these pathways undergo clear changes in activity *in vitro* after 48 h upon stimulation with lithium or escitalopram, but no differences could be seen at basal level. These responses were stronger in patients, supporting the idea of increase in PKA, CaMKII (both target CREB) as well as PKC signalling in this disorder. However, the methods used in other publications have been diverse as for used cell types as well as the status of the patients. Our method focusses on transcription factors, the most downstream targets next to mRNA and induced proteins.

#### ERK and JNK

Both ERK and JNK reporter assays showed higher activities in BD patients. Particularly for JNK, a significant difference was noted even at the basal level, stronger after stimulation with both escitalopram and lithium. However, for ERK only escitalopram-stimulated activities yielded significance, and only in univariate comparisons. JNK is activated by various conditions, such as oxidative, mitochondrial and endoplasmic reticulum stress. Independent transcriptome studies on the frontal cortex of BD patients demonstrated upregulation of immune response genes<sup>51,52</sup> and this has also been described in patients with schizophrenia.<sup>53</sup>

Furthermore, decreases of mitochondrial mRNA and proteins important for certain functions, that is, oxidative phosphorylation, have been found in the prefrontal cortex from patients with BD.<sup>54–56</sup> Oxidative phosphorylation regulates adenosine triphosphate (ATP) levels and affects ATP-dependent ion pumps maintaining the membrane potential in mitochondria. Failure leads to apoptotic cell death.<sup>57</sup> This decreased mitochondrial function might contribute to the response seen in Myc and p53 pathways.

#### p53

Our results on p53 are less clear, as the escitalopram-stimulated/basal ratio was significantly higher in patients in the univariate analysis, but the lithium-stimulated/basal ratio was underlined in the multivariate analysis.

The p53 pathway has been discussed in the context of schizophrenia, where an association of pro72arg variation with abnormal myelin integrity in the frontal lobe has been reported.<sup>58</sup> Furthermore, the p53 genomic locus is in proximity with Lis-1.<sup>59</sup> A p53 family member, p73, colocalises with reelin in developing human cortex,<sup>60</sup> and deletion of 31 bp of reelin causes lissencephaly in sheep.<sup>61</sup> These relatively new pathways active in BD need to be further investigated. Our results suggest that there is a difference in the sensitivity to drugs stimulating intracellular signalling molecules. Lithium has been recognised to exert its neuroprotective effects<sup>62,63</sup> especially through induction of Bcl-2, possibly via upregulation of BDNF,<sup>64,65</sup> as well as inhibition of GSK-3,<sup>34</sup> calpain<sup>66,67</sup> and Ca<sup>2+</sup> influx. Escitalopram has not been reported to possess such activities, and this might explain the fact that it did not affect Myc or p53 pathways.

Long-term lithium treatment decreases p53 and its downstream Bax, whereas anti-apoptotic Bcl-2 is increased; lithium also prevents a glutamate-induced increase of p53 and Bax in cerebellar granule cells.<sup>41</sup> However, in our analysis (where lithium was added only for 48 h) it might have induced stress to more vulnerable patients' cells, leading to stabilisation of p53. Consequently, pro-apoptotic BH<sub>3</sub> molecules, induced by p53, bind anti-apoptotic Bcls on the mitochondrial membrane, leading to the release of cytochrome c from mitochondria.<sup>68</sup> Decreased ATP production will render mitochondria dysfunctional, leading to decreased energy metabolism in brain that is associated with BD.<sup>69</sup>

It has been shown that both c-Myc and p53 are upregulated by intrastriatal infusion of the *N*-methyl-D-aspartate receptor agonist quinolinic acid via NF- $\kappa$ B activation, leading to DNA fragmentation of striatal cells.<sup>70</sup> In our study, it is not obvious whether the induction of p53 and c-Myc leads to apoptosis, as we did not see any morphological characteristics of apoptosis. This can only be examined by biochemical methods.

#### Wnt

The escitalopram-stimulated/basal Wnt activity was clearly higher in our patients, both uni- and multivariately.

Wnt has earlier been reported to be dysregulated in monozygotic twins discordant for bipolar disorder.<sup>71</sup> Not only the Wnt canonical pathway targets  $\beta$ -catenin, but also some noncanonical pathways.<sup>72,73</sup> Recently,  $\beta$ -catenin was found to bind the promoters for many genes (being known candidates for schizophrenia, autism spectrum disorder and BD), including CACNA1B, NRNG, SNAP29, FGFR1, PCDH9 and others being identified in published genome-wide association studies.<sup>74</sup> The Wnt/Ca<sup>2+</sup> signal activates the transcription factors NF- $\kappa$ B, NFAT and CREB, through calcineurin, CamKII and PKC.<sup>73</sup> Some of the other non-canonical Wnt pathways, that is, planar cell polarity, JNK, Ror, GSK3MT, aPKC, RYK and mTOR, are shown to activate or interfere with Ca<sup>2+</sup> signalling. Ca<sup>2+</sup> influx through the L-type voltage-activated channels activates CREB and MEF-2. Thus, Ca<sup>2+</sup>-dependent signalling could be activated by several pathways.

#### ANK3

Of the 25 genes analysed by us in a hypothesis-driven endeavour, only the ANK3 gene (rs10761482) yielded significance in both univariate and multivariate comparisons. Two other genes were at trend significance when studied univariately. Our sample is small, and no conclusions can be reached as for the biological impact of the genes chosen. Nevertheless, the ANK3 gene turned out to carry a diagnostic value.

The genome-wide association studies and meta-analyses have identified ANK3 and CACNA1C (*alpha 1C subunit of the L-type voltage-activated channels*) as the most notable risk genes for BD.<sup>75–77</sup> ANK3 encodes Ankyrin-G isoforms that anchor membrane

protein complexes, such as Na<sup>+</sup>-gated channels, to the cytoskeleton at the axonal initial segment and nodes of Ranvier to facilitate action potential firing and the synapse targeting process at the axonal initial segment.<sup>78–81</sup> *ANK3* polymorphisms have also been associated with decreased integrity of white matter.<sup>82</sup> We could not find significant associations for rs10994336 or rs984190 as in previously reported genome-wide association studies.<sup>75,76</sup>

In the present study with the small number of samples, *ANK3* rs10761482 showed a significant association with BD. Previously, this SNP has been reported to be associated with schizophrenia in a large European sample.<sup>83</sup> However, a German study on rs10761482, including 400 BD and 920 schizophrenia samples, showed an association with BD but not with schizophrenia.<sup>84</sup> In a recent study, cognitive functional effects of two risk *ANK3* variants (rs10761482 and rs10994336) were examined on 7 neurocognitive domains in 49 Japanese BD patients and 633 controls. The risk C-allele of rs10761482, but not of rs10994336 (linkage disequilibrium with 10994397), was significantly associated with worse performance.<sup>85</sup> In another Canadian study, the G-allele of rs1938526 was found to be associated with cognitive impairment on 6 domains and cortical thinning in 173 patients with first-episode psychosis.<sup>86</sup> As such endophenotypes are shared between schizophrenia and BD, more studies are needed in order to distinguish these entities.

#### State or trait?

By logistic regression, we compared the classification power of escitalopram-stimulated activity of the PKC pathway and the *ANK3* gene in predicting diagnosis, presence of bipolar heredity and whether individuals had suffered from any psychotic episode in their lives.

First, an interaction term was clearly nonsignificant, suggesting that the contributions of these two independent variables are additive, not synergistic. Second, the diagnostic dichotomy was better predicted than the presence of bipolar heredity in a first-degree relative, even though the heritability of BD is very high. We have evidence that quantitative clinical measures such as age, body size, MADRS, YMRA and GAF are not related to any of the variables measured in this study (not shown).

This offers some support that the signalling deviations detected are more trait than state like. Only a longitudinal study may bring clarity.

#### Limitations

The study is cross-sectional with no longitudinal follow-up. Cohorts are relatively small with no medication-free patients, and there are no 'ill patient' controls. It takes 3 to 4 weeks to expand adipocytes that may change epigenetic profiles. However, adipocytes are easier to handle compared with lymphoblastoid cells immortalised by transfection of exogenous genes. Another issue might be that we do not target neuronal cells, which should be the proper target, although BD manifests with diverse symptoms in the whole body. Yet, our analysis resulted in clear differences between the responses to drugs between BD and controls.

#### CONCLUSION

To our knowledge, this is the first time postsynaptic signalling analyses have been performed on adipocytes, an easily accessible source of cells. We report several instances of significantly increased activity in BD of calcium-dependent postsynaptic signals, such as escitalopram- and lithium-stimulated PKC, JNK and myc activities, as well as a genetic allelic disproportion of the *ANK3* gene. Using multivariate logistic equations, correct

classification of bipolar patients and healthy controls was possible, with high degrees of specificity and sensitivity.

More studies are needed to ascertain these results, and to do so we are in the process of creating induced pluripotent stem cell lines from adipocytes and inducing neurones and astrocytes. In order to confirm these findings and study signalling activities in a more native environment, we will use neural progenitors and generated neuronal cells from induced pluripotent stem cells from patient and control adipocytes.

#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

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