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MOLECULAR AND SYNAPTIC MECHANISMS

Human cellular differences in cAMP - CREB signaling correlate with light-dependent melatonin suppression and bipolar disorder

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Abstract

Various lines of evidence suggest a mechanistic role for altered cAMP-CREB (cAMP response element - binding protein) signaling in depressive and affective disorders. However, the establishment and validation of human inter-individual differences in this and other major signaling pathways has proven difficult. Here, we describe a novel lentiviral methodology to investigate signaling variation over long periods of time directly in human primary fibroblasts. On a cellular level, this method showed surprisingly large inter-individual differences in three major signaling pathways in human subjects that nevertheless correlated with cellular measures of genome-wide transcription and drug toxicity. We next validated this method by establishing a likely role for cAMP-mediated signaling in a human neuroendocrine response to light – the light-dependent suppression of the circadian hormone melatonin – that shows wide inter-individual differences of unknown origin *in vivo*. Finally, we show an overall greater magnitude of cellular CREB signaling in individuals with bipolar disorder, suggesting a possible role for this signaling pathway in susceptibility to mental disease. Overall, our results suggest that genetic differences in major signaling pathways can be reliably detected with sensitive viral-based reporter profiling, and that these differences can be conserved across tissues and be predictive of physiology and disease susceptibility.

Introduction

Discrete mutations in various signaling cascades have been associated with familial instances of many diseases, including bipolar disorder (Smoller & Gardner-Schuster, 2007), a debilitating disease affecting 2.4% of the world's population (Merikangas *et al.*, 2011). However, identifying mechanistically relevant underlying differences in signaling cascade function in sporadic instances of this disease has been more problematic and less obvious due to inaccessibility of human brain tissue. Helping to resolve this issue, one of the most interesting findings on signal transduction in the last decade was that only a few major signal transduction cascades are responsible for this sophisticated intracellular communication, each conserved among many different cell types. Although the initial sensor or receptor and the downstream targets can vary among cell types according to local epigenetic modifications and the availability of coregulatory factors, the 'cytoplasmic part in the middle' is the same. For this reason, numerous investigators have begun to collect peripheral tissues (especially easy-to-obtain fibroblasts or immortalised lymphoblastoid cells) from both controls and patients suffering from affective and depressive disorders, and have characterised signaling pathway function therein by analysing gene expression or protein abundance and post-translational modifications (Eykelenboom *et al.*, 2012; Bamne *et al.*, 2013). Such studies have shifted the focus away from direct alterations in monoamine concentration as key factors in the etiology of these diseases, and towards the possibility that changes in pathways parallel to these neurotransmitters could cause global changes in plasticity affecting susceptibility to disease (Dwivedi & Pandey, 2008; Chuang *et al.*, 2013).

Two such pathways are the cAMP-CREB pathway and mitogen-activated protein kinases (MAPKs), which play well-documented roles in many aspects of neuronal signal transduction and brain plasticity (Lonze & Ginty, 2002; Wiegert & Bading, 2011). Several branches of these pathways exist, each mediated by different discretely encoded factors; however, each results in an increase in the

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level of the second messenger cAMP produced by the membrane-bound enzyme adenyl cyclase, and/or the phosphorylation and activation of common downstream transcription factors. A third such pathway is the circadian clock, which regulates diurnal behavior. Bipolar disorder is tightly correlated with disruptions in circadian rhythmicity (McCarthy & Welsh, 2012; Jagannath *et al.*, 2013) and major depressive disorder (Li *et al.*, 2013), even when patients are not acutely ill (Jones *et al.*, 2005). Moreover, a disruption in the circadian gene *Clock* in mice results in a mania-like phenotype (Roybal *et al.*, 2007), and two drugs used in the treatment of bipolar disorder, lithium and agomelatine, have possible targets within the circadian clock mechanism (Yin *et al.*, 2006; Srinivasan *et al.*, 2012a). Although they are mechanistically distinct, these pathways functionally connect at multiple levels. For example, cAMP signaling has been shown to be an integral part of the circadian clock mechanism in the brain (O'Neill *et al.*, 2008), and the circadian clock controls the activity of several branches of the MAPK pathway (Goldsmith & Bell-Pedersen, 2013).

In this study, we used a novel, peripheral cell-based methodology to investigate whether augmentation of cAMP–CREB signaling is a feature of bipolar disorder, and whether the same signaling changes also correlate with reduced light-dependent suppression of the circadian hormone melatonin. Our method represents a simple and robust way to use integrating viruses to profile pathway function in human peripheral cells that could be widely applicable to neurological disease.

Materials and methods

Human subjects and ethical statement

The protocols used with human subjects in this study were undertaken with the understanding and written consent of each subject. The study complied with the World Medical Association Declaration of Helsinki, and was approved by the ethical committees of the Karolinska Institute (Sweden, 04-273/1, supplements 2006/637-32 and 2009-06-12) and the University Medical Center of Groningen (The Netherlands).

The 10 initial control subjects (Cohort 1, recruited in Sweden) of Caucasian origin (equally gender-balanced, aged 45 ± 11 years) were prescreened by use of the Structured Clinical Interview for DSM-IV (Pincus *et al.*, 1996), and were free of mental and somatic disease (Asp *et al.*, 2011). The subsequent cohort of 10 patients with bipolar disorder type I (Cohort 2, recruited in Sweden), who were screened with the Structured Clinical Interview for DSM-IV prior to inclusion in the study, included 40% females and 60% males, with an average age of 40.6 ± 6.8 years. All bipolar subjects included in this study were medically treated and showed a depressive episode during the time of the skin biopsy (Table S7). The cohort used for testing light-dependent melatonin suppression (Cohort 3, recruited in The Netherlands) consisted of 14 healthy unmedicated males (mean age ± standard deviation, 21.9 ± 2.0 years).

Tissue isolation and culture

As described previously (Brown *et al.*, 2005), human primary fibroblasts of all subjects used in the study were isolated from two skin biopsies by 4–7 h of digestion of the tissues in 2 mL of Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum, 1% penicillin/streptomycin, 1% gentamicin, and 0.325 WU/mL Liberase Blendzyme3. After the isolation, human primary fibroblasts were kept in DMEM/high-glucose medium supplemented with 20% fetal bovine serum, 1% penicillin/streptomycin, 1% gentamicin, and 2.5 μg/mL amphotericin B. Antifungal amphotericin B was left out of the growing medium after 1 week.

Vector production

The cell-based reporter system used for the measurement of the transcriptional activation of CREB, ELK1 and CHOP signaling features a reporter construct coding for firefly luciferase downstream of a synthetic promoter that is joined to five tandem repeats of GAL4 binding elements. A transcriptional activator construct containing coding sequences for the activation domains of CREB, ELK1 or CHOP fused with the DNA-binding domain of yeast transactivator GAL4 is separately expressed from the cytomegalovirus (CMV) promoter. Lentiviral derivatives of this reporter system were produced by restriction cloning of the coding sequences of activator plasmids (pFA2–CREB, pFA2–ELK1, and pFA2–CHOP) and reporter plasmids (pFR–Luc) into the pENTR4 gateway entry vector (Invitrogen) between the *att*L1 and *att*L2 sites. Ultimately, the Gateway LR Clonase II reaction was used to recombine and transfer inserts from entry vectors into a lentiviral destination vector containing either a hygromycin (pLDEST–CREB–Hygro, pLDEST–ELK1–Hygro, pLDEST–CHOP–Hygro) or a puromycin (pLDEST–FRLuc–Puro) selection marker. In addition to the reporter and activator constructs, a third virus consisting of a secreted alkaline phosphatase gene under control of the CMV promoter was included. All viruses were produced and concentrated as previously described (Cepko, 2001).

Bioluminescence measurement of the transcriptional activation of different signaling pathways

Semiconfluent plates ($2.5–5 \times 10^5$ cells in 35-mm dishes) of primary fibroblasts initially transduced and selected for the lentiviral luciferase reporter construct carrying puromycin resistance (pLDEST–FRLuc–Puro) were additionally cotransfected with the activator (pLDEST–CREB–Hygro, pLDEST–ELK1–Hygro, or pLDEST–CHOP–Hygro) and normalising [pLDEST–CMV–secreted alkaline phosphatase (SEAP)–Hygro] lentivirus vectors without selection. Prior to bioluminescence measurement of the transcriptional activation, aliquots of the medium taken from the fully confluent samples were used to detect levels of secreted alkaline phosphatase with the Phospha-Light Secreted Alkaline Phosphatase Reporter Gene Assay System (Cat. No. T1015). DMEM/high-glucose medium, without phenol red, 10% KnockOut Serum Replacement, 1% penicillin/streptomycin, 1% gentamicin and 0.1 mM luciferin was used to measure basal levels of light emission over a period of 20–30 min. Then, a pathway-specific drug stimulus was added to the tested samples [5 μM forskolin for CREB, 100 ng/mL phorbol 12-myristate 13-acetate (PMA) for ELK1, and 10 ng/mL methyl methanesulfonate (MMS) for CHOP], and the signal was recorded over a period of 24–48 h.

Data analysis and normalisation

A single experiment included four sample replicates from two biopsies. Each sample was normalised by dividing the activator activity (maximal photon count value of CREB, ELK1 and CHOP induction) by the control reporter activity (photon counts corresponding to SEAP values). Replicate samples were then averaged, and the same calculation was performed for all tests. Results are expressed as average ± standard error.

Protein purification and western blot analysis

Proteins from uninduced and induced human primary fibroblast samples (for CREB, subjects 1 and 10; for ELK1, subjects 5 and 10; for CHOP, subjects 7 and 8) were extracted at the time of the maximal transcriptional activation (CREB, after 5 h; ELK1, after 8 h; CHOP, after 24 h) according to the previously described NaCl–urea–NP-40 protocol (Lavery & Schibler, 1993). Twenty-five micrograms of protein was resolved by 10% sodium dodecylsulfate polyacrylamide gel electrophoresis, transferred to a Protran Nitrocellulose membrane, rinsed in phosphate-buffered saline with 0.1% Tween, and incubated for 1 h at room temperature with either primary polyclonal antibody against phospho-CREB (1 : 50 dilution; Cell Signaling Technology Europe, Leiden, The Netherlands; #9191S) or polyclonal antibody against phospho-ELK1 (1 : 50 dilution; Cell Signaling Technology; #9181). IRDye 680 goat anti-rabbit IgG secondary antibody was used at a dilution of 1 : 10 000 for 1 h at room temperature. Washed and dried membranes were used to detect and analyse the signal intensities with the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA). Anti-actin clone C4 monoclonal antibody (Cat. No. MAB1501) was used as a loading control for each immunoblot.

RNA isolation and whole genome expression analysis

Total RNA from uninduced and induced fibroblast samples (for CREB, subjects 1 and 10; for ELK1, subjects 5 and 10; for CHOP, subjects 7 and 8) were extracted as described by Xie and Rothblum (1991). RNA quality and quantity were determined with an Agilent 2100 Bioanalyser (Santa Clara, CA, USA). Single-color gene expression microarray analysis was performed with the Human Whole Genome OneArray (Phalanx Biotech Group, Belmont, CA, USA). For each sample, three technical replicates were performed. The data from all microarrays was passed to Rosetta Biosoftware (Seattle, WA, USA) for analysis, and a single average expression value for each gene was returned. For each subject, induced values were divided by uninduced values, and then ordered by fold induction. Relative fold inductions were calculated by dividing fold induction for a given gene and subject by the same value for another subject. To calculate values for an overall pathway, a list of pathway-specific genes was taken from references (Zhang *et al.*, 2005; Boros *et al.*, 2009) and from the NEXTBIO Y platform for CHOP. Within this set, the average fold induction of the 50 most induced genes was then used as a measure of the overall induction of the pathway.

Viability determination

In vitro sensitivity of the human primary fibroblasts from CHOP extreme subjects (subjects 7 and 8) to an alkylating agent, MMS, was assessed with the Trypan Blue exclusion test of cell viability (Strober, 2001). Cells (1.5×10^4) were seeded into 48-well microculture plates and kept for an additional day in the incubator at 37 °C in 5% CO_2. On day 3, 100 ng/mL MMS was added to the microculture plates, and the viability of fibroblast cells was determined over the next 4 days. Measurement for each sample was performed in six replicates, and untreated cells were used as a control.

Measurement of melatonin suppression

The study consisted of two nights in the laboratory, separated by a minimum of 1 week and a maximum of 2 weeks. All subjects stayed in individual rooms. After 2 h of dim light (< 5 lux) exposure, subjects were randomly assigned to one of two conditions: dim light or bright light [full-spectrum Philips TL-D36W/830 (AE Eindhoven, The Netherlands), mounted on the ceiling, horizontal 627 ± 16 lux, vertical 164 ± 33 lux] from 23:00 to 07:00 h. During the second night in the laboratory, subjects were exposed to the alternative light condition. Subjects stayed awake during the whole night, performing computer exercises. The screen was covered with a color filter to block blue light transmission (E-colour +, 105 Orange; Rosco Laboratories, London, UK). Subjects received hourly snacks of 100 Kilocalories and 130 mL of water. Each hour (from 21:30 to 06:30 h), cotton swabs (Salivettes; Sarstedt B.V., Etten-Leur, The Netherlands) were used to collect saliva samples for melatonin analysis, which was performed by radioimmunoassay (RK-DSM; Bühlmann Laboratories AG, Alere Health BV, The Netherlands). Melatonin suppression was determined from 23:30 to 06:30 h by subtracting the ratio of the area under the curve (AUC) in full-spectrum light and the AUC in dim light from 1, and expressing the result as a percentage (Method 1; Fig. 3A). In a second approach (Method 2; Fig. 3B), the maximal suppression in response to full-spectrum light, disregarding phase, was used. These approaches are conservative in not assuming similar phasing of melatonin production in the dim and full-spectrum conditions. Starting from lights-on or from dim light melatonin onset, defined according to Lewy (1983) (if a subject's dim light melatonin onset was after lights-on), the ratio of the minimal melatonin value measured in the full-spectrum light condition and the maximum melatonin value in the dim light condition was subtracted from 1, and the result was expressed as a percentage.

Results

A lentiviral system to report signaling pathway function in primary cells

We have developed a lentiviral reporter system that allows the profiling of transcriptional activation of major signal transduction pathways in primary fibroblasts. This system consists of a set of three vesicular stomatitis virus glycoprotein-pseudotyped lentiviral vectors. (i) A specific activator virus contains the human CMV immediate early promoter driving expression of a GAL4 DNA-binding domain fused with a pathway-specific transcriptional activator [CREB, ELK1, CHOP, c-Jun, nuclear factor of activated T cells (NFAT), etc.]. These activators are phosphorylated, and are thereby potentiated by endogenous proteins of their cognitive cascades within the cell. (ii) A reporter virus carries a synthetic minimal promoter with five tandem repeats of the yeast GAL4 binding site that controls the expression of the firefly luciferase gene. The reporter is bound and activated only by its respective activator protein described above. Such activator–reporter pairs have long been used to report differences in signaling cascade function (Hexdall & Zheng, 2001). (iii) A normalising control virus contains the SEAP gene under the control of the CMV promoter (Fig. 1A). Co-transduction of mammalian cells with these lentiviruses allows the specific activator-dependent luciferase reporter signal to be expressed as a ratio relative to the activator-insensitive SEAP reporter signal, thereby controlling for pathway-independent effects such as infection efficiency and toxicity.

In total, reporters were designed for eight major signal transduction pathways: CREB, ELK1, CHOP, nuclear factor-κB (NF-κB), p53, c-jun, NFAT, and serum response element. Each of these was transduced into primary human skin fibroblasts, and these fibroblasts were then treated with a pharmacological agent known to activate the pertinent pathway. Bioluminescence was then measured for 24 h with real-time bioluminometry in the presence of luciferin. Our ini-

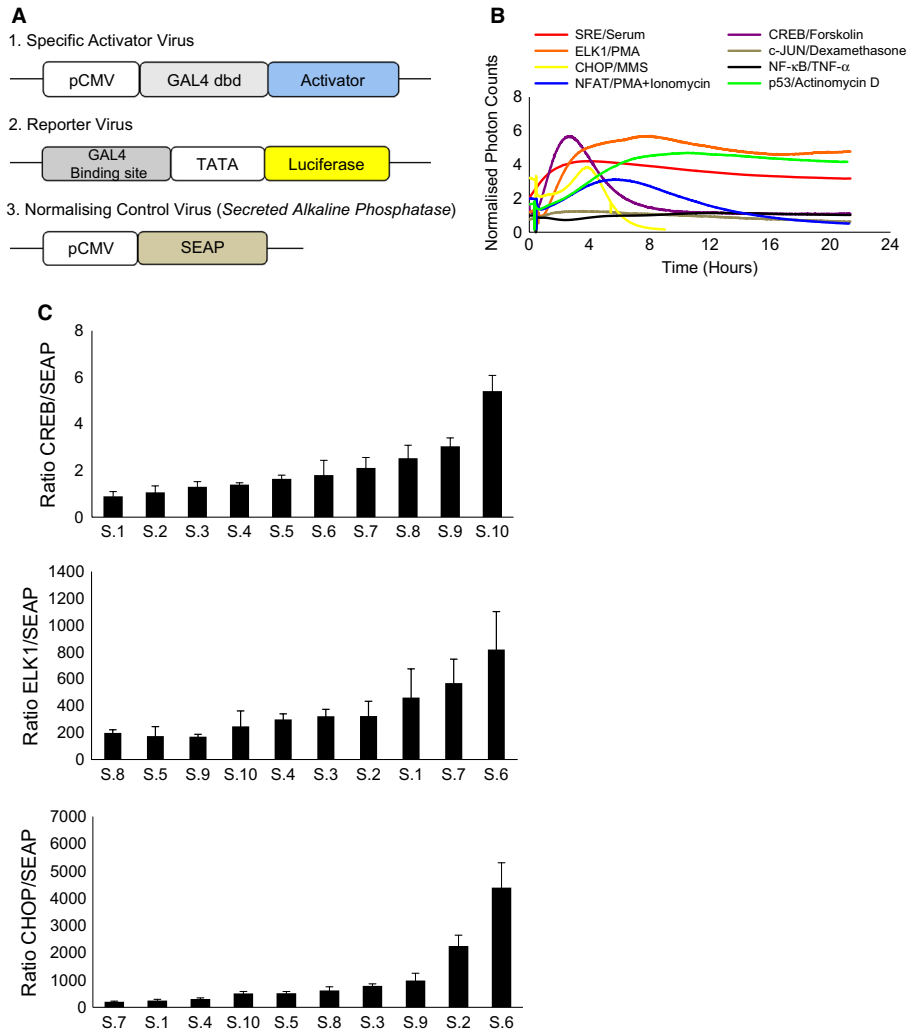

FIG. 1. Human primary fibroblasts from different individuals show widely varying induction of signal transduction pathways. (A) Lentiviral cell-based reporter system. 1: The specific activator virus expresses a chimeric pathway-responsive transcription factor. pCMV, CMV early promoter; GAL4 dbd, teast GAL4 DNA-binding domain; activator, transcriptional activation domain from the transcription factor responding to a given signal transduction pathway. 2: The reporter virus produces luciferase in response to the chimeric activator. TATA, minimal TATA box-containing synthetic promoter. 3: The normalising virus produces a constant amount of alkaline phosphatase over time. (B) Representative bioluminescence profiles of a human primary fibroblast line infected with pathway-specific reporters, and then treated at time 0 with pathway-specific activating compounds. *y*-axis: raw bioluminescence signal divided by average baseline. Drugs used for activation: for CREB, forskolin (5 μM); for ELK1, PMA (100 ng/mL); for c-Jun, dexamethasone (5 μM); for CHOP, MMS (10 ng/mL); for NFAT, PMA/calcimycin (100 ng/mL); for nuclear factor-κB (NF-κB), human tumor necrosis factor-α (TNF-α, 50 ng/mL); for SRE, fetal bovine serum (20%); and for p53, actinomycin D (5 nM). (C) Fibroblasts from 10 subjects were transduced with pathway-specific reporters for CREB, ELK1, or CHOP (from top to bottom), and then identically stimulated with 5 μM forskolin, 100 ng/mL PMA, or 10 ng/mL MMS. Peak activation for each subject is plotted as a ratio to constantly expressed SEAP in the same cells. Data are plotted as average ± standard error from four replicates in two separate measurements. SRE, for serum response element.

tial test for these chosen signal transduction pathways showed interesting individual kinetic patterns of pathway-specific transcriptional activation in response to specific drug stimuli (Fig. 1B). For our further analyses, we decided to concentrate upon three well-known signal transduction pathways: CREB, which is essential for cAMP-dependent G-protein-coupled signaling from a wide variety of receptors; ELK1, a MAPK that is important in cell growth signaling; and CHOP, another branch of the MAPK pathway that is responsive to endoplasmic reticulum stress and is involved in DNA damage, growth arrest, and apoptosis (McAlpine, 2007). Each pathway showed a dose-dependent response to an inducing drug: for CREB, the diterpene forskolin, which is commonly used to increase the levels of cAMP (Montminy & Bilezikjian, 1987) and CREB without increasing those of ELK1 or CHOP (Seternes *et al.*, 1999); for ELK1, PMA, a structural analog of diacylglycerol that is able to activate protein kinase C (Myers *et al.*, 1985); and for CHOP, MMS, an alkylating agent (Fig. S1). For most pathways, these reporters were robust to changes in infection efficiency, viral titer, and cell confluence, passage, and health (Fig. S2A–C). However, where predicted, reporters also showed covariation with cellular features, e.g. dependence of cell division status and senescence on the ELK1 pathway (Fig. S2C). Responses were uniform across different biopsies from the same individual (Fig. S2D).

Signaling pathway amplitude varies widely in fibroblasts from different individuals

To investigate inter-individual differences in the amplitude of induction of CREB, ELK1 and CHOP pathways in different human individuals, duplicate dermal punch biopsies were taken from 10 healthy control individuals, and isolated primary skin fibroblasts from these biopsies were transduced and measured as above. In

addition to pathway-specific kinetic patterns, we found surprisingly large inter-individual differences in the peak expression of these tested pathways. We found nearly 10-fold differences in CREB induction between the most extreme subjects in response to identical 5 μM concentrations of forskolin [coefficient of variation (CoV), 41% of mean expression], 5-fold differences in ELK1 induction in response to 100 ng/mL PMA (CoV, 57% of mean expression), and 20-fold differences in CHOP induction in response to 10 ng/mL MMS (CoV, 120% of mean expression) (Fig. 1C). The rank order of subjects showing high or low pathway expression varied among the three pathways, thereby arguing against systematic bias (Spearman correlation: CREB/ELK1, $\rho = -0.418$, $P = 0.229$; ELK1/CHOP, $\rho = -0.0181$, $P = 0.960$; CREB/CHOP, $\rho = 0.0303$, $P = 0.934$). Furthermore, the observed differences were well preserved across three different concentrations (1×, 5×, 25×) of the lentivirus used for the experiment for each pathway (Fig. S3).

Reporter-based variations in pathway signaling are mirrored by genome-wide variation in transcription and in cellular physiology

The pathway-specific inter-individual differences that were observed were larger than what other studies have shown for microarray-based gene expression (Choy *et al.*, 2008). Therefore, we next compared the results from viral pathway profiling with those from corresponding experiments performed with microarray-based transcriptome analysis of the same cells harvested at the time of maximal observed induction for each pathway. In fact, simple pathway analysis with existing tools (KEGG and GENEGO) not only did not show major differences among subjects in these pathways, but did not even correctly predict these pathways as principal targets of the inducing drugs that were used, probably because of extensive crosstalk (Tables S4–S6). Next, using published lists of human genes activated by each pathway (as identified by chromatin expression and microarray analyses by others) (Zhang *et al.*, 2005; Boros *et al.*, 2009), we filtered the whole transcriptome information to consider only 'true' transcriptional targets in fibroblasts, based on the convergence of these chromatin immunoprecipitation and transcriptome datasets. In this filtered dataset, notable differences were found in the gene expression profiles of CREB, ELK1 and CHOP target genes between subjects identified as 'high inducers' and 'low inducers' for each pathway, for the 50 most highly activated genes in each subject (Fig. 2; individual genes from Fig. 2 are detailed in Tables S1–S3). For example, when we investigated the expression profile of CREB target genes, we found that forskolin evoked an average 4-fold difference in activation of these 50 most-induced CREB target genes between the 'high' and 'low' subjects (Fig. 2A, inset). The same differences between maximum and minimum induction were found when other pharmacologically relevant agonists were employed (Fig. S4A).

The same strategy for analysing the data was used for ELK1 and CHOP target genes (Zhang *et al.*, 2005; Boros *et al.*, 2009). This approach revealed a 3-fold difference and 2.5-fold difference in transcription from promoters known to be activated by the ELK1 and CHOP pathways, respectively (Fig. 2B and C, inset). No difference was seen in the average expression of the full chip gene set (Fig. 2D).

Where suitable antibodies could be obtained, pathway-specific differences also corresponded with inter-individual variations in the levels of the endogenous transcription factors: levels of both phosphorylated CREB and phosphorylated ELK1 differed among these individuals (Fig. 2E and F). For CHOP, where no suitable antibody existed, we instead measured a defined cellular cytotoxicity response induced by this pathway. CHOP, a small nuclear protein also known as DNA damage gene 153 (GADD153), induces growth arrest in response to various extracellular stresses, such as ultraviolet radiation, endoplasmic reticulum stress, and alkylating reagents (Wang *et al.*, 1998). Therefore, we tested cytotoxicity caused by MMS, an alkylating agent that induces phosphorylation of CHOP and DNA damage (Fornace *et al.*, 1988). As predicted, the cells corresponding to the subject with the highest CHOP expression level showed decreased cellular viability after MMS treatment as compared with the cells corresponding to the subject with the lowest CHOP expression level (Fig. 2G; untreated control cells in Fig. S4B). Therefore, at a cellular level, the large and robust changes in pathway function found with viral profiling among different healthy individuals mirror smaller and harder to detect changes in both post-translational modifications of proteins and transcriptional changes of genes regulated by these pathways, making the method potentially useful for diagnostic purposes.

Inter-individual differences in cellular pathway signaling correspond to physiological differences in the human melatonin response

Our results point to unsuspected variability in the amplitude of CREB-dependent signaling – as well as in other pathways – among primary fibroblasts from different human subjects. To test the relevance of this variability to human neurophysiology, we attempted to apply the technology that we developed to a well-known problem: the neuroendocrine response to light of the pineal circadian hormone melatonin. This hormone has long attracted interest for its roles in the circadian system (Lewy *et al.*, 2006; Pevet & Challet, 2011), as a sleep-promoting agent (Srinivasan *et al.*, 2012b), and more recently for its oncostatic properties (Cutando *et al.*, 2012). Moreover, receptors for this hormone are targets of the drug agomelatine, which is used to treat mood disorders, including bipolar disorder (Srinivasan *et al.*, 2012a). Melatonin is secreted in a circadian fashion, with a night-time peak at the approximate time of sleep onset, but its production is nevertheless acutely suppressed by night-time light. The amount of melatonin produced in different individuals varies greatly, probably because of differences in the size and calcification of the pineal gland that produces it (Kunz *et al.*, 1999; Nolte *et al.*, 2009). In addition, however, the degree of melatonin suppression by light varies remarkably among individuals, independently from the aforementioned parameters (Higuchi *et al.*, 2008).

Rodent models suggest that melatonin secretion is dependent on the cAMP–CREB signaling pathway (Simonneaux & Ribelayga, 2003). Therefore, one might predict that the inter-individual differences in drug-induced CREB signaling shown in Figs 1 and 2 would impact on the degree of night-time suppression of melatonin: the higher the CREB signaling, and therefore the drive to synthesise melatonin, the lower would be the ability of night-time light to suppress it. To test this prediction, 14 additional subjects were recruited at the University of Groningen. Fibroblasts were cultivated from each, and infected with CREB reporter. After induction of adenyl cyclase with the drug forskolin, the maximal amount of reporter expression was quantified. In the same subjects, the degree of melatonin suppression in response to a full night of full-spectrum light (164 ± 33 lux) was determined. An inverse correlation was observed between the degree of melatonin suppression and the magnitude of CREB signaling in fibroblasts (Fig. 3A and B), reflecting the dependence of melatonin synthesis on the adenyl cyclase–cAMP–CREB pathway in humans (see Discussion). Our results show that one portion of the variation in light-dependent melatonin suppression in humans can likely be attributed to inter-individual variation in this signaling pathway.

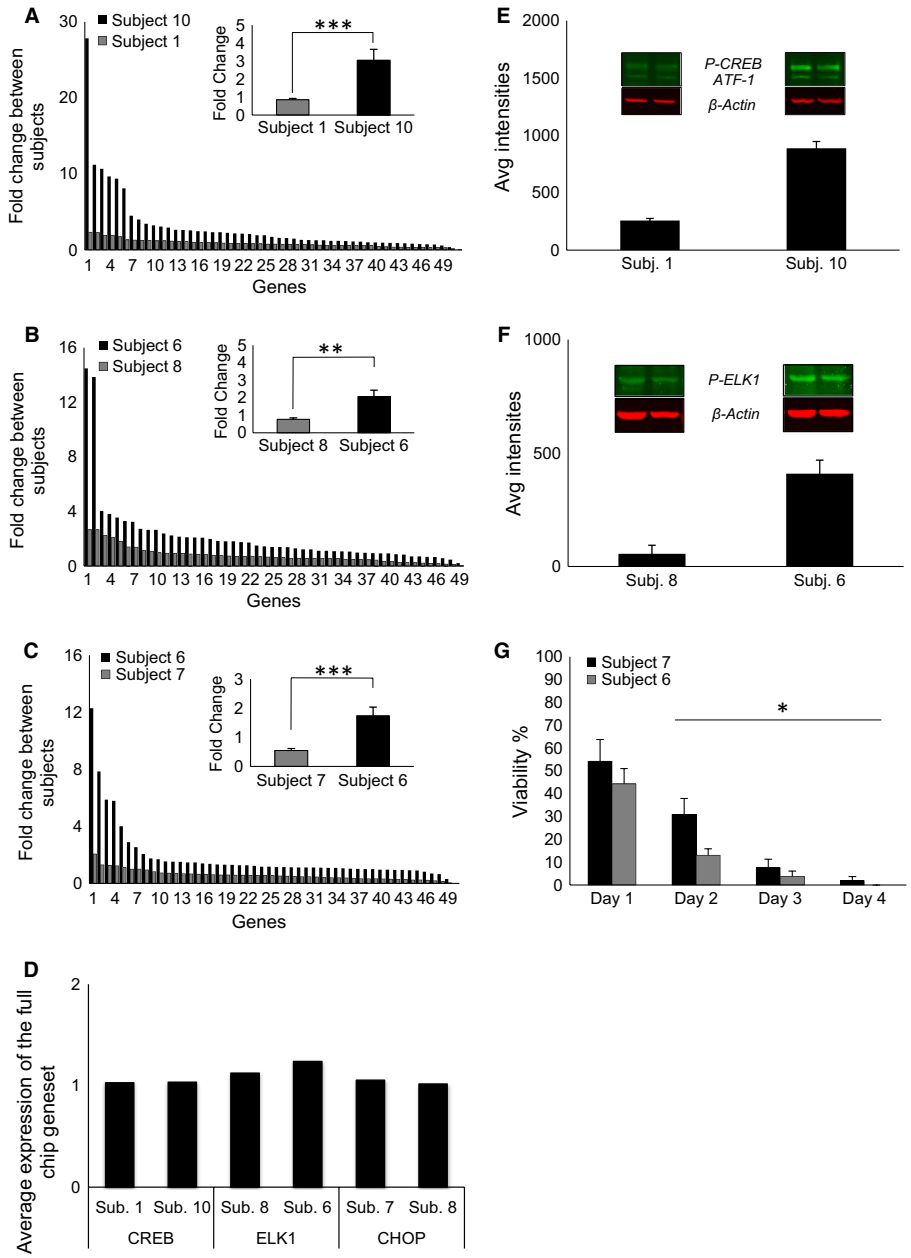

FIG. 2. Microarray-based analysis confirms transcriptome-wide differences in pathway-specific gene targets among fibroblasts from different subjects. Whole transcriptome analysis was performed on cells from the indicated subjects, treated as in Fig. 1 and harvested at the time of maximal expression for each pathway. (A) CREB. (B) ELK1. (C) CHOP. The fold-difference in activation between the subject with maximal activation and the subject with minimal activation is plotted for the 50 most activated target genes (with drug vs. baseline) for each pathway, with comparison of either the 50 most activated target genes for the subject with maximal activation (black bars) or for the the subject with minimal activation (gray bars); these genes are listed in Tables S1–S3. Inset: average fold-differences in induction of all pathway-specific genes (with drug vs. baseline, ± standard deviation). For each case, single-dye gene expression microarray analysis was performed in biological duplicates and three technical replicates. (D) Average fold-differences in induction of all expressed genes (with drug vs. baseline). (E) Western blot against phospho-CREB examined in whole cell extracts from cells treated identically to those in A. β-Actin was used as a loading control. Top panel: representative raw blots. Bottom panel: quantification. (F) Western blot against phospho-ELK1, from cells treated identically to those in B. (G) Viability of human primary fibroblasts from the subjects in C (measured by Trypan Blue staining) after treatment with toxic concentrations of MMS (100 ng/mL) for 1–4 days. (Student's *t*-test, $P = 0.04$; $N = 6$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

Viral profiling shows higher induced CREB signaling among individuals with bipolar disorder

Finally, to demonstrate the applicability of viral profiling to mental disease, we recruited 10 additional subjects who were age-matched and sex-matched to our initial controls, but suffered from bipolar disorder. Fibroblasts from each subject were infected with reporters for CREB signaling, which has been previously implicated in this disease (Dwivedi & Pandey, 2008). CREB induction in response to forskolin stimulation of adenyl cyclase was then measured in these fibroblasts, both separately and again in the presence of the previous controls. Surprisingly, a 3.3-fold increase in induction ($P < 0.001$) was observed in fibroblasts from bipolar patients as compared with simultaneously measured controls, with very little overlap between these groups (Fig. 4). This result not only confirms previous literature suggesting changes in cAMP–CREB signaling in bipolar

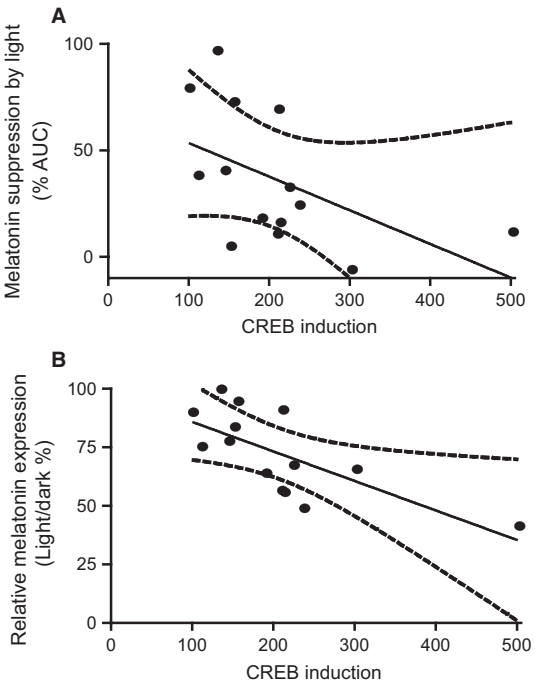

FIG. 3. CREB expression in fibroblasts predicts melatonin suppression in human subjects: correlation between percentage melatonin suppression under full-spectrum light exposure at night-time in human subjects and CREB induction in response to forskolin in fibroblasts from the same subjects. (A) Melatonin suppression *in vivo* vs. CREB induction *in vitro*. *x*-axis: CREB induction, plotted as baseline-subtracted SEAP-normalised arbitrary units. *y*-axis: AUC of melatonin levels in the full-spectrum light condition, divided by AUC of melatonin in the dim light condition (Spearman $\rho = -0.666$, $P = 0.009$; additional regression line with confidence intervals depicted for clarity, Pearson $r = -0.5078$, $P = 0.06$). (B) Relative melatonin expression *in vivo* vs. CREB induction *in vitro*. *x*-axis: CREB induction as in A. *y*-axis: minimal melatonin value in the full-spectrum light condition divided by the maximal melatonin value in the dim light condition (Spearman $\rho = -0.688$, $P = 0.007$; additional regression line with confidence intervals depicted for clarity, Pearson $r = -0.705$, $P = 0.004$).

disorder, but also suggests that a simple fibroblast-based assay could have predictive value.

Discussion

In this study, a suite of lentiviruses containing optimised luciferase-based signal transduction reporters was used to investigate human inter-individual differences in cellular signaling and their influence on neuroendocrine physiology and bipolar disorder. By virally profiling signaling differences in primary human cells, we were able to demonstrate and validate robust inter-individual differences missed by conventional transcriptomics. We then used this information to predict a likely role for variations in cAMP–CREB-dependent signaling in the human neuroendocrine response of the hormone melatonin to light, as well as in susceptibility to bipolar depression. Our results suggest that variation in the cAMP–CREB pathway, which is important for brain processes underlying both mood disorders and circadian clock function, could provide a mechanistic link between circadian clock disruption and bipolar disorder. In addition, the method that we present could provide a simple assay for signaling variation both in bipolar disorder and in other diseases, as well as a more powerful platform for genome-wide association studies seeking novel expression quantitative trait loci.

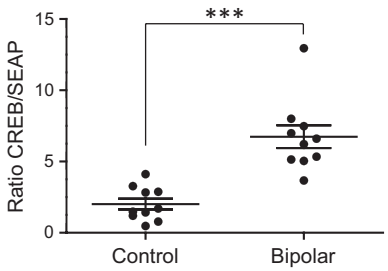

FIG. 4. Greater pharmacological induction of CREB in fibroblasts from subjects with bipolar disorder. Relative CREB induction in response to forskolin in fibroblasts from control subjects and from patients suffering from bipolar depression (three biological replicates, four technical replicates of each sample, $P < 0.001$, Student's *t*-test). ***$P < 0.001$.

Viral approaches to measure signaling in human primary cells

Because of the difficulty in accessing human brain tissue, peripheral cells have long been used in psychiatry as substitutes. However, a robust and reliable methodology for detecting subtle differences in signaling cascade function – of the sort that might be expected from the common regulatory variation found in genome-wide association studies – is lacking. By utilising optimised low-noise viral reporters integrated throughout the genome to report dynamically on human signaling pathway function at the amplified ends of major transduction cascades, the method presented here represents a novel way to detect inter-individual differences in cascade amplitude that would be widely applicable in high-throughput studies. Previously, we have used similar technology to show that circadian clock properties in fibroblasts predict daily behavior (Brown *et al.*, 2005): people whose fibroblasts show shorter periods are more likely to be 'larks', and those with long periods are more likely to be 'owls' (Brown *et al.*, 2008).

Transcriptomic, proteomic and metabolomics methods have been used successfully to achieve similar goals in the context of signaling pathway expression, genome-wide association, and drug development (Martoglio *et al.*, 2000; Evans & McLeod, 2003; Clayton *et al.*, 2006). However, a major problem with such technologies is that effect sizes are typically quite small, probably because most genes possess regulatory elements that bind a host of different factors. For example, Zhang *et al.* (2005) used both chromatin immunoprecipitation and microarray approaches to identify CREB target genes in different human tissues. The authors identified 4084 putative CREB target genes. Nevertheless, only 100 (i.e. 2%) were activated in response to forskolin, a strong stimulant of cAMP signaling. Similarly, the validation of hits in genome-wide analyses involving expression quantitative trait loci has been relatively poor (Choy *et al.*, 2008). In this study, we used GAL4-based chimeric transcription factors to create a series of reporters that represent clean, optimised promoters to detect activation of a single pathway. These reporters were delivered into unmodified human primary cells via lentiviral vectors, autonormalised to eliminate differences in transduction efficiency, and they integrated at thousands of different locations within the genome in different cells. For this reason, context-specific interference was greatly reduced, and we found surprisingly high inter-individual variations in pathway expression among even young, healthy individuals. In addition, 'crosstalk' between pathways resulting from the presence of multiple regulatory elements in the same promoter region is greatly reduced. It is interesting to note that this methodology can complement existing genome-wide technologies by uncovering variation that would otherwise be missed with conventional microarray-based detection

technologies as primary tools, especially with small numbers of subjects: given the primary data only, a popular pathway-based analysis platform (GENEGO) was unable to unearth the pathway-specific differences that we documented by a variety of methods above (Tables S4–S6). Importantly, in all three instances that we examined, the effects that we show could be verified both by filtering transcriptomic datasets to consider only known regulatory targets published by others, and by direct molecular assays (western blot, cellular toxicity, etc.).

Prediction of human physiology and disease

Our laboratory has used viral profiling of circadian gene expression in fibroblasts as a predictor of human circadian behavior in daily life and in the laboratory (Brown *et al.*, 2005, 2008; Pagani *et al.*, 2010). Since then, measurement of circadian properties in human fibroblasts has become an important tool in psychiatric research (Nomura *et al.*, 2008; Yang *et al.*, 2009; Johansson *et al.*, 2011; Bamne *et al.*, 2013). Therefore, it was logical to suppose that viral profiling of psychiatrically relevant signaling pathways might be important in neurophysiology and disease. To test the predictive power of such strategies, we applied our technology to discover the molecular sources of variation in the human neuroendocrine response to light, and then in bipolar disorder, in both of which an involvement of cAMP–CREB signaling has been suggested.

First, we show here that CREB induction in fibroblasts correlates with suppression of melatonin by light in the same subjects. Looking back at what is known about this pathway, such a correlation makes mechanistic sense. At the molecular level, the circadian secretion of mammalian melatonin begins with a noradrenergic promoting signal to the pineal gland (Stoschitzky *et al.*, 1999; Scheer *et al.*, 2012), and subsequent activation of the cAMP signaling pathway (Vanecek *et al.*, 1985). This cascade modulates the level of the rate-limiting aralkamine-*N*-acetyltransferase enzyme (Tamotsu *et al.*, 1995; Baler *et al.*, 1997; Ackermann *et al.*, 2006). Nocturnal light results in inhibition of this cascade ('melatonin suppression') (Klein & Weller, 1972). Therefore, inter-individual differences in the amplitude of adrenergic signaling via adenyl cyclase and cAMP would affect the rate of melatonin synthesis, and thus inversely the degree of melatonin suppression by light that blocks this synthesis, as we observed. As other factors, such as pineal size and calcification, also affect melatonin production (Kunz *et al.*, 1999; Nolte *et al.*, 2009), this fibroblast assay will not substitute directly for assays of light-dependent melatonin suppression *in vivo*; however, the mechanistic predictions of the correlation that we observe are clear.

Second, we demonstrate that pharmacologically stimulated cAMP–CREB signaling is significantly elevated in fibroblasts from individuals with bipolar disorder. Given the considerable role played by cAMP–CREB signaling in the enhancement of synaptic plasticity (Kandel, 2012) and in crosstalk across neurotransmitter systems (Blier, 2001; Svenningsson *et al.*, 2003), a role for this signaling in susceptibility to bipolar disorder would be easy to rationalise, and has been supported in diverse genome-wide association studies (Mühleisen *et al.*, 2014; Xu *et al.*, 2014). Nevertheless, bipolar disorder has been associated with an elevation of CREB–cAMP-dependent signaling in both lymphocytes and human brain tissue in some studies, but not in others (Dwivedi & Pandey, 2008). Consistent with our work, a further recent study (Alda *et al.*, 2013) suggested that phosphorylated CREB levels are already elevated in lymphoblasts from bipolar patients and family members as compared with controls. However, they showed no further increase upon forskolin treatment *in vitro*, as compared with 24% in controls. In comparison, the robust stimulation that we observed probably reflects the low inherent noise of the viral reporters, as well as their placement at the amplified ends of the signal transduction cascade. Moreover, an *in vitro* cell-based assay such as ours provides a buffer to effects of medication that might be measured in direct assays of lymphocytes, brain, or spinal fluid. For example, lithium has been shown to enhance the association between CREB and its coactivator regulated transcription coactivator (CRTC) (Heinrich *et al.*, 2013), and such an effect is unlikely in a cellular assay conducted *in vitro* after weeks in culture.

Knowing that inter-individual differences in cAMP-CREB signaling affect both the human endocrine response to light and susceptibility to bipolar disorder would make it a common neurological signaling pathway that could help explain the observed relationship between this disorder, circadian behavior, and light exposure. Given the role of this signaling pathway in synaptic plasticity (Dwivedi & Pandey, 2008), as a part of the circadian clock mechanism (O'Neill *et al.*, 2008), and in the regulation of melatonin synthesis (Vanecek *et al.*, 1985), such a link is quite plausible. In addition, such a link could help to furnish therapeutically useful endophenotypes of bipolar disorder and its treatment. For example, chronotherapeutics have been shown to be effective in the treatment of bipolar disorder in approximately half of treated patients (Benedetti, 2012), and lithium affects the expression of the clock gene *Per2* in only a subset of patients, possibly because of specific genetic and clinical factors (McCarthy *et al.*, 2013).

More broadly, understanding the origins of pathway variations in fibroblasts could provide a powerful tool for understanding the roles of diverse pathways in a variety of hard-to-access disorders. Although signaling pathways in general are highly conserved, the question of how well inter-individual differences in gene expression are conserved from one tissue to another in humans remains a matter of controversy (Emilsson *et al.*, 2008; Dimas *et al.*, 2009). Recent studies have also highlighted the fact that skin fibroblasts can even be de-differentiated and re-differentiated into different cell types such as cardiomyocytes and neurons to predict disease phenotypes, albeit with high variability among different isolates (Grskovic *et al.*, 2011; Robinton & Daley, 2012). Our data show that the inherent conservation of basic signaling pathways can allow the simple fibroblasts from which these stem cells are derived to predict already complex behavioral responses without de-differentiation/re-differentiation, and provide a simple and reproducible tool for measuring such responses. Understanding how human genetic variation influences cellular signal transduction, and in turn how it influences physiology, is a crucial step towards personalised medicine.

Supporting Information

Additional supporting information can be found in the online version of this article:

Fig. S1. Response of CREB, ELK1 and CHOP pathway profiling reporters is dose-responsive.

Fig. S2. Pathway profile values are robust to experimental variation.

Fig. S3. Intersubject differences in pathway expression are conserved regardless of differences in viral titer, cellular confluence, and passage number.

Fig. S4. Reporter - based variations in measured signaling pathways are robust.

Table S1. Fold-activation of CREB target genes by forskolin.

Table S2. Fold-activation of ELK1 target genes by PMA.

Table S3. Fold-activation of CHOP target genes by MMS.

Table S4. GENEGO pathway analysis of the differences between sub-

ject 1 and subject 10 in response to forskolin treatment.
Table S5. GENEGO pathway analysis of the differences between subject 6 and subject 8 in response to PMA treatment.
Table S6. GENEGO pathway analysis of the differences between subject 7 and subject 6 in response to MMS treatment.
Table S7. Cohort of 10 patients with bipolar disorder type I [Cohort 2/recruited in Sweden/bipolar patients (Cohort 2/SW/BIPO)].

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Abbreviations

AUC, area under the curve; CMV, cytomegalovirus; CoV, coefficient of variation; CREB, cAMP response element-binding protein; DMEM, Dulbecco's modified Eagle's medium; MAPK, mitogen-activated protein kinase; MMS, methyl methanesulfonate; NFAT, nuclear factor of activated T cells; PMA, phorbol 12-myristate 13-acetate; SEAP, secreted alkaline phosphatase.

Conflicts of interest

The authors declare no conflicts of interest.

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