ORIGINAL ARTICLE

RNA expression profiling in depressed patients suggests retinoid-related orphan receptor alpha as a biomarker for antidepressant response

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Response to antidepressant treatment is highly variable with some patients responding within a few weeks, whereas others have to wait for months until the onset of clinical effects. Gene expression profiling may be a tool to identify markers of antidepressant treatment response and new potential drug targets. In a first step, we selected 12 male, age- and severity-matched pairs of remitters and nonresponders, and analyzed expression profiles in peripheral blood at admission and after 2 and 5 weeks of treatment using Illumina expression arrays. We identified 127 transcripts significantly associated with treatment response with a minimal P -value of 9.41×10^{-4} (false discovery rate-corrected). Analysis of selected transcripts in an independent replication sample of 142 depressed inpatients confirmed that lower expression of retinoid-related orphan receptor alpha (*RORa*, $P = 6.23 \times 10^{-4}$), germinal center expressed transcript 2 (*GCET2*, $P = 2.08 \times 10^{-2}$) and chitinase 3-like protein 2 (*CHI3L2*, $P = 4.45 \times 10^{-2}$) on admission were associated with beneficial treatment response. In addition, leukocyte-specific protein 1 (*LSP1*) significantly decreased after 5 weeks of treatment in responders ($P = 2.91 \times 10^{-2}$). Additional genetic, *in vivo* stress responsivity data and murine gene expression findings corroborate our finding of *RORa* as a transcriptional marker of antidepressant response. In summary, using a genome-wide transcriptomics approach and subsequent validation studies, we identified several transcripts including the circadian gene transcript *RORa* that may serve as biomarkers indicating antidepressant treatment response.

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INTRODUCTION

Response to antidepressant treatment is highly variable with 30–40% patients showing insufficient improvement even after administration of a sequence of different antidepressants.^{1,2} Currently available antidepressant drugs act almost exclusively as enhancers of monoaminergic neurotransmission. Thus, the identification of biomarkers that help preselecting patients that will respond to a specific treatment is from the clinical perspective as important as the discovery of new potential drug targets that ultimately may lead to more specific treatment options.

Genotyping of single-nucleotide polymorphisms (SNPs) did not deliver robust antidepressant response predictors that entered clinical routine.³ The use of high-throughput technologies such as gene expression profiling to identify biomarkers for antidepressant response is a relatively new approach. Apart from a first study in six elderly depressed patients showing various nominal associations,⁴ Mamdani *et al.*⁵ found Interferon regulatory factor 7 to be upregulated in citalopram responders of an outpatient depression sample ($N = 62$). Another microarray study of the same group⁶ with 77 patients identified *SMAD7* and *SIGLECP3* as potential predictors of citalopram response. Using a selection of 14 putative candidates emerging from a case-control mRNA and miRNA scan, Belzeaux *et al.*⁷ found a set of four possible predictors of antidepressant treatment response in 16 depressed patients (*PPT1*, *TNF*, *IL1b* and *HIST1H1e*). Although pointing to some known

and some new possible candidates as biomarkers for antidepressant response, all previous studies lack sufficient replications. In addition, particularly mRNA analysis and transcriptomics are subject to technical variability that may explain some of the inconsistencies of previous findings.^{8,9}

Here, we present the results of a genome-wide pharmacotranscriptomics study using highly selected male depressed inpatients matched by age and depression severity at study inclusion. They were selected according to clinical extremes with respect to their response to antidepressants within a 5-week treatment period. Transcripts identified as significantly regulated in this analysis were subsequently analyzed in an independent replication sample of 142 depressed inpatients. In addition, we analyzed a promising candidate gene from the discovery round, retinoid-related orphan receptor alpha (*RORa*), and performed *in vivo* stress responsivity assays and a mouse model of stress-evoked gene expression.

MATERIALS AND METHODS

Sample description and study design

Twenty-four male depressed inpatients participating in the Munich Antidepressant Response Signature project² were selected as 12 age- and depression severity-matched pairs. Briefly, in the Munich Antidepressant Response Signature study, at least moderately depressed inpatients were followed during their hospital stay and received a doctor's choice

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antidepressant medication mimicking the everyday clinical situation. We assessed psychopathology with the 21-item HAMD (Hamilton Depression Rating Scale)¹⁰ and obtained blood samples within 5 days after hospital admission, and after 2 and 5 weeks of antidepressant treatment in 12 patients defined as remitters (HAMD < 10 after 5 weeks; comparable to the HAMD-cutoff of 7 frequently used for 17-item HAMD Rating Scale;¹¹ mean: 4.8 ± 2.2 s.d.) and 12 nonresponders (HAMD improvement < 25% from baseline after 5 weeks compared with admission; mean: 18.9 ± 4.3 s.d.). The patients were treated with different types of antidepressants. Nevertheless, there was no significant difference between the compared groups within the first 5 weeks of treatment. One hundred and forty-two additional unipolar depressed inpatients (80 responders, 62 nonresponders after 5 weeks) of the Munich Antidepressant Response Signature study with available RNA sampling served as a replication and underwent the same assessment protocol as the discovery sample.

The study was approved by the Ethics Committees of the Medical Faculties at the Ludwig Maximilians University, Munich, Germany. Written informed consent was obtained from all subjects, and the study was carried out in accordance with the latest revision of the Declaration of Helsinki.

RNA preparation and gene expression analysis

Blood was drawn between 0800 and 0900 h; 10 h before blood collection, eating, drinking (except water), caffeine and nicotine intake were not allowed. Whole blood was collected in PAXgene RNA Blood tubes and isolated using PAXgene Blood RNA Kits (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was quantified by UV absorption using the Nanophotometer (Implen, Munich, Germany) and quality was determined on the Agilent Bioanalyzer (mean RIN = 7.64 ± 0.82 (s.d.); Agilent, Santa Clara, CA, USA). Two hundred nanograms of total RNA was reverse transcribed and converted to cDNA and subsequently biotin labeled using the Ambion TotalPrep Amplification Kit (Ambion, Austin, TX, USA). Seven hundred and fifty nanograms of cDNA was hybridized to Illumina HT-12 v3.0 arrays (Illumina, San Diego, California, USA) and incubated overnight at 55 °C according to the manufacturer's protocol. To reduce batch effects, complete probe sets of matched pairs were run in the same array slot. Arrays were washed, stained with Cy3 labeled streptavidin, dried and scanned on the Illumina BeadScan confocal laser scanner according to the manufacturer's protocol. The generated data were analyzed using the Illumina Beadstudio program and loaded into R for downstream statistical analysis.¹² Principal validity of the chip expression data was confirmed by technical replication of selected transcripts using quantitative real-time PCR (qPCR). qPCR assays were designed using the Roche universal probe library (<http://qpcr.probefinder.com/organism.jsp>).

For gene expression analysis in the replication sample, selected transcripts (Supplementary Table S1) were analyzed using qPCR. Total RNA was reverse transcribed to cDNA using random primers and the Superscript II reverse transcriptase (Invitrogen, Darmstadt, Germany) for qPCR run on the Roche 480 LightCycler (Roche Applied Science, Mannheim, Germany). All the samples were run in duplicates and duplicates discordant in crossing points by more than 0.4 cycles were excluded from the analysis. *RORa*, *GCET2*, *CHI3L2*, *LSP1*, *SMARCC2*, *VPS13A*, *RECQL* and *RAP1GAP* were chosen for RT-PCR validation from the microarray experiment. We applied the standard curve method for relative quantification using *PLA*, *TBP*, *GUSB* and *HPRT* as housekeeping genes reaching a high level of interclass correlation (interclass correlation coefficient range 0.789–0.996).

Combined dexamethasone/corticotropin-releasing hormone test

The combined dexamethasone suppression/corticotropin-releasing hormone challenge (dexamethasone/CRH) test was available in 80 patients at admission (6.9 ± 2.4 (s.d.) days after admission) and in 39 patients after 6 weeks of treatment (46.9 ± 3.3 (s.d.) days after admission). The test was performed as described in detail previously.¹³ Briefly, the patient received 1.5 mg dexamethasone orally at 1800 h the day before the test, and the following day, adrenocorticotrophic hormone (ACTH) and cortisol plasma levels were determined at 1500 h, 1530 h, 1545 h, 1600 h and 1615 h. An intravenous bolus of 100 µg of human CRH (Ferring, Kiel, Germany) was given at 1502 h. A radioimmunoassay kit was applied for the measurement of plasma cortisol concentrations (CT Cortisol RIA, DRG Diagnostics, Marburg, Germany). Plasma ACTH concentrations were assessed by an immunometric assay without extraction (cobas ECLIA, Roche Diagnostics, Rotkreuz, Switzerland). For the analysis of hormonal tests, we correlated

RNA expression levels at admission and after 5 weeks with ACTH and cortisol at basal (that is, before CRH application) and after CRH application (that is, maximal concentration; A_{basal} , A_{max} , C_{basal} , C_{max} , respectively) using age and sex as covariates.

Animal experiments

Blood samples and brains were collected from adult male mice of the 'stress reactivity' mouse model (details in S2).¹⁴ To exclude phenotypic extremes, 16 mice of the 'intermediate reactivity' line were used. The animals were killed under unstressed conditions and circadian trough levels of plasma corticosterone were determined by radioimmunoassay as described previously.¹⁴ Hippocampi were quickly dissected and analyzed for *RORa* expression using qPCR as described before.^{15,16} At the time of sampling, all mice were about 4 months of age and single housed at least 2 weeks before the experiment. Animal husbandry was performed under standard laboratory conditions.

Genotyping for association analysis

Genome-wide genotypes were available from a different project (Illumina 100 K, Illumina 300 K, Illumina 610 K and Illumina OmniExpress chips).¹⁷ All genotypes underwent a stringent quality control (removal of SNPs with callrates < 98%, MAF < 5%, deviation from HWE (P -value < 10^{-5}), removal of IDs with callrates < 98%). Genotypes for rs12912233 were not available for all individuals and were imputed using impute v2 (http://mathgen.stats.ox.ac.uk/impute/impute_v2.html). The imputation info score was 0.97 indicating a good imputation quality.

Statistical analysis

Microarray expression data were transformed and normalized using variance stabilizing normalization.¹⁸ For differential expression analysis, significantly regulated genes were ranked using an empirical Bayes method implemented in R.¹⁹ A total of 48 770 annotated gene probes were detected. A total of 4826 genes were not expressed in any sample (no signal above background) and were excluded from the subsequent analysis. We performed an analysis of variance of contrasting gene expression of remitters and nonresponders at admission, after 2 and 5 weeks of treatment. We corrected for multiple testing using the false discovery rate (FDR).²⁰

Group differences in demographic and baseline clinical data were compared using Pearson χ^2 test in case of qualitative data and with two-sided t -tests (if not otherwise specified) for independent samples in case of quantitative data. GLM was applied for the analysis of gene expression in the replication experiment with response and remission at week 5 as dependent variables and age and sex as covariates. These statistical analyses were conducted with SPSS for Windows (version 18.0, SPSS, Chicago, IL, USA). PLINK (<http://pngu.mgh.harvard.edu/~purcell/plink/>) was used for genetic association studies.

RESULTS

Clinical characteristics

Twelve remitting and 12 nonresponding male patients matched by age and baseline depression severity did not differ with respect to number of previous episodes, age of disease onset, treatment resistance (at admission), psychotic symptoms and body mass index as well as psychotropic and other medication during the study period (Table 1).

Whole-genome gene expression

Using a combined contrast analysis of variance (comparing remitters and nonresponders at admission, after 2 and after 5 weeks), we found 127 genes having adjusted (FDR) P -values < 0.05 (Supplementary Table S3). The lowest P -values were reached for transcripts of the Rap1 GTPase-activating protein (*RAP1GAP*, $P = 2.14 \times 10^{-8}$, $P_{\text{FDR}} = 9.41 \times 10^{-4}$), germinal center expressed transcript 2 (*GCET2*, $P = 6.73 \times 10^{-8}$, $P_{\text{FDR}} = 1.13 \times 10^{-3}$) and chitinase 3-like protein 2 (*CHI3L2*, $P = 7.69 \times 10^{-8}$, $P_{\text{FDR}} = 1.13 \times 10^{-3}$) with lower expression levels in remitters compared with nonresponders in each case.

Table 1. Sample characteristics

	N	All (N = 12)		Non-responder (N = 12)		Remitter (N = 12)		Value	df	P ^a
		N/mean	(%/s.d.)	N/mean	(%/s.d.)	N/mean	(%/s.d.)			
<i>Baseline and disease characteristics</i>										
Age, mean (s.d.)	24	44.9	13.5	45.5	15.2	44.3	12.3	0.21	22	0.8382
Duration of hospital stay (weeks), mean (s.d.)	24	12.1	6.1	16.6	5.4	7.6	2.0	5.32	22	0.0000
Number of previous episodes, mean (s.d.)	21	4.4	7.4	7.0	9.6	1.6	1.6	1.75	19	0.0964
Age at disease onset, mean (s.d.)	24	29.8	15.2	28.3	17.0	31.3	13.7	-0.48	22	0.6389
HAMD at admission, mean (s.d.)	24	28.8	6.1	28.3	5.6	29.3	6.7	-0.36	22	0.7206
HAMD at discharge, mean (s.d.)	23	6.5	5.4	9.3	5.8	3.5	2.8	2.96	21	0.0074
HAMD at week 5, mean (s.d.)	22	12.5	8.0	18.9	4.3	4.8	2.2	9.43	20	0.0000
BMI at admission, mean (s.d.)	23	25.7	4.6	26.7	5.6	24.8	3.4	0.97	21	0.3441
HAMA at admission, mean (s.d.)	24	26.7	9.5	27.5	9.1	25.8	10.2	0.42	22	0.6775
Psychotic depression, No. (%)	24	3	12.5	1	8.3	2	16.7	0.38	1	> 0.999
Treatment resistance, No. (%)	24	2	8.3	2	16.7	0	0.0	2.18	1	0.4780
<i>Antidepressants (within 5 weeks), no. of patients (%)</i>										
Tricyclics	24	8	33.3	5	41.8	3	25.0	0.75	1	0.6670
SSRI	24	7	29.2	3	25.0	4	33.3	0.20	1	> 0.999
SNRI	24	10	41.7	7	58.3	3	25.0	2.74	1	0.2140
NaSSA (= Mirtazapine)	24	9	37.5	4	33.3	5	41.7	0.18	1	> 0.999
NARI	24	0		0		0				
MAOI	24	1	4.2	0	0.0	1	8.3	1.04	1	> 0.999
<i>Others</i>										
Bupropion	24	4	16.7	4	33.3	0	0.0	4.80	1	0.0930
Trazodon	24	3	12.5	3	25.0	0	0.0	3.43	1	0.2170
Buspiron	24	1	4.2	1	8.3	0	0.0	1.04	1	> 0.999
<i>Psychotropics, others (within 5 weeks), no. of patients (%)</i>										
Antipsychotics	24	15	62.5	9	75.0	6	50.0	1.60	1	0.4000
Mood stabilizers	24	14	58.3	6	50.0	8	66.7	0.69	1	0.4080
Lithium	24	3	12.5	2	16.7	1	8.3	0.38	1	> 0.999
Benzodiazepines	24	13	54.2	7	58.3	6	50.0	0.17	1	0.6820
<i>Internal medication (within 5 weeks), no. of patients (%)</i>										
Thyroid medication	24	4	16.7	3	25.0	1	8.3	1.20	1	0.5900
Analgetics	24	6	25.0	2	16.7	4	33.3	0.89	1	0.6400
Cardiovascular medication	24	8	33.3	6	50.0	2	16.7	3.00	1	0.1930
Antibiotics	24	1	4.2	0	0.0	1	8.3	1.04	1	> 0.999

Abbreviations: BMI, body mass index; HAMA, Hamilton Anxiety Rating Scale; HAMD, Hamilton Depression Rating Scale. For some variables, there are missing data and *N* does not equal number of total patients. Percentages are based on available data. Bold values signify $P < 0.05$. ^aTwo-sided *t*-test and Chi-square, respectively, comparing nonresponders and remitters; Fisher's exact test used when cell count was < 5 .

Comparing gene expression after 2 or 5 weeks of treatment with admission within remitters and nonresponders, respectively, lowest *P*-values were observed for SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily c, member 2 (*SMARCC2*, $P = 5.91 \times 10^{-8}$, $P_{FDR} = 2.42 \times 10^{-3}$) and leukocyte-specific protein 1 (*LSP1*, $P = 1.49 \times 10^{-7}$, $P_{FDR} = 2.42 \times 10^{-3}$; Supplementary Table S4). *RORa* recently implicated in depression and posttraumatic stress disorder^{21,22} ranked 12th in the combined contrast ($P = 2.48 \times 10^{-6}$, $P_{FDR} = 9.06 \times 10^{-3}$) and showed up in additional single contrasts (Supplementary Table S4).

Replication of selected transcripts in 142 patients

One hundred and forty-two additional unipolar depressed inpatients served as a replication sample. Eighty responders and 62 nonresponders after 5 weeks did not differ in baseline characteristics including age, sex, baseline depression severity, age of disease onset, body mass index and psychotic symptoms (Supplementary Table S5). For qPCR, we included the most significant associated transcripts of the whole-genome expression experiment of the combined (*RAP1GAP*, *GCET2*, *CHIL2*) and single

contrasts (*SMARCC2*, *LSP1*). In the replication experiment, we combined this hypothesis-free replication approach with a candidate-gene approach based on biological plausibility: we included *RORa*, because it was the highest associated gene in the combined contrast rank list with a previously described association with depression and stress-related disorders.^{21,22} To proof the robustness of the chip experiment, we randomly selected two further transcripts with sufficient average expression levels and fold changes from the top 120 hits, vacuolar protein sorting 13 homolog A (*VPS13A*) and RecQ protein-like (DNA helicase Q1-like; *RECQL*), to be included in the replication experiment.

Using a GLM with age and sex as covariates, we confirmed association of *RORa* ($P = 6.23 \times 10^{-4}$), *GCET2* ($P = 2.08 \times 10^{-3}$) and *CHIL2* ($P = 4.45 \times 10^{-2}$) with lower expression levels in responders compared with nonresponders at admission in each case, which is the same direction of effect as in the chip experiment (Table 2). In addition, *LSP1* significantly decreased after 5 weeks of treatment in responders and remitters ($P = 2.91 \times 10^{-2}$, $P = 4.71 \times 10^{-3}$, respectively). Comparing responders and nonresponders, expression of *RORa* was lower in responders also after 5 weeks of

Table 2. Replication sample (N = 142)

		Nonresponders ^a		Responders ^a		p ^b	Non-remitters ^a		Remitters ^a		p ^b
		Mean	s.d.	Mean	s.d.		Mean	s.d.	Mean	s.d.	
<i>RORα</i>	Admission	1.10	0.35	0.93	0.25	0.0006	1.05	0.32	0.92	0.26	0.0105
	After 2 weeks (% change)	19.79	33.92	25.42	45.79	0.5163	20.24	36.84	28.93	48.94	0.3127
	After 5 weeks (% change)	3.25	38.95	4.66	41.11	0.9689	1.03	35.13	9.87	48.03	0.2749
<i>GCET2</i>	Admission	0.95	0.32	0.78	0.28	0.0021	0.90	0.32	0.76	0.26	0.0162
	After 2 weeks (% change)	12.35	30.63	23.14	54.58	0.2825	13.89	34.47	28.45	63.62	0.1409
	After 5 weeks (% change)	1.24	34.10	6.68	41.04	0.5768	1.11	32.79	10.44	46.50	0.2536
<i>CHI3L2</i>	Admission	1.33	0.74	1.11	0.62	0.0446	1.35	0.76	1.18	0.67	0.0968
	After 2 weeks (% change)	13.73	33.00	11.71	32.54	0.7466	11.24	34.77	15.41	27.69	0.5049
	After 5 weeks (% change)	10.58	30.38	14.90	37.99	0.6186	10.18	31.86	18.45	39.67	0.2471
<i>LSP1</i>	Admission	0.76	0.39	0.82	0.27	0.6231	0.75	0.33	0.86	0.30	0.0427
	After 2 weeks (% change)	10.31	33.27	15.97	42.13	0.2446	15.67	38.53	9.04	38.72	0.5172
	After 5 weeks (% change)	12.98	41.09	-1.95	34.48	0.0291	11.27	40.47	-8.28	29.42	0.0047
<i>SMARCC2</i>	Admission	0.87	0.32	0.90	0.20	0.5490	0.86	0.27	0.92	0.23	0.2106
	After 2 weeks (% change)	7.59	25.73	9.56	27.22	0.5133	9.67	24.90	6.68	29.86	0.6903
	After 5 weeks (% change)	2.66	29.74	-2.25	21.38	0.3179	2.19	27.06	-4.52	21.48	0.1619
<i>VPS13A</i>	Admission	1.09	0.22	1.02	0.21	0.0919	1.07	0.21	1.00	0.22	0.0636
	After 2 weeks (% change)	5.94	20.96	8.11	32.24	0.7332	4.30	22.63	13.32	36.31	0.1094
	After 5 weeks (% change)	0.42	26.24	2.08	28.96	0.8927	-0.80	24.51	5.51	32.93	0.2800
<i>RECQL</i>	Admission	1.25	0.33	1.18	0.30	0.1336	1.24	0.32	1.17	0.30	0.1725
	After 2 weeks (% change)	13.94	36.48	17.72	37.76	0.5302	13.84	34.70	20.93	41.87	0.3016
	After 5 weeks (% change)	3.03	33.97	4.59	36.14	0.3016	2.98	34.56	5.69	36.39	0.8388
<i>RAP1GAP</i>	Admission	1.12	2.06	1.56	7.54	0.7471	0.92	1.74	2.24	9.70	0.2255
	After 2 weeks (% change)	115.49	220.90	153.86	418.30	0.3219	156.49	353.31	97.11	335.46	0.5409
	After 5 weeks (% change)	157.89	314.37	86.77	165.87	0.1227	148.46	273.69	59.11	160.18	0.0596

Abbreviations: *RORα*, retinoid-related orphan receptor alpha; *GCET2*, germinal center expressed transcript 2; *CHI3L2*, chitinase 3-like protein 2; *LSP1*, leukocyte-specific protein 1; *SMARCC2*, SWI/SNF-related, matrix-associated, actin-dependent regulator of chromatin, subfamily c, member 2; *VPS13A*, vacuolar protein sorting 13 homolog A; *RECQL*, RecQ protein-like; *RAP1GAP*, Rap1 GTPase-activating protein. Bold values signify $P < 0.05$. ^aAfter 5 weeks. ^bGLM with age and sex as covariates.

treatment ($P = 3.29 \times 10^{-3}$), but did not reach significance after 2 weeks ($P = 1.18 \times 10^{-2}$; Figure 1).

Applying the conservative Bonferroni-based correction for multiple testing in our replication experiment ($\alpha = 6.25 \times 10^{-3}$), *RORα* and *GCET2* remained significantly different at baseline, and *LSP1* after 5 weeks. Given the suspected impact of *RORα* in psychiatric disorders, we focused on this gene in our further validation experiments.

RORα expression, sleep, diurnal depressive symptoms and hypothalamic–pituitary–adrenal axis

As *RORα* is a so-called clock gene involved in circadian rhythm generation, we further tested whether the expression of *RORα* was related to presence of sleep disturbances or diurnal variations of depressive symptoms at admission using the respective items on the HAMD scale (4, 5, 6 and 18, respectively). We could not find a correlation of these variables with *RORα* expression ($P = 5.56 \times 10^{-2}$, $P = 8.35 \times 10^{-2}$, $P = 3.46 \times 10^{-2}$, $P = 7.35 \times 10^{-2}$, respectively; partial correlation analysis with age and sex as covariates).

As functional *RORα* knockout mice show an altered ACTH and corticosterone response to stress,²³ we further tested the hypothesis whether *RORα* expression has any impact on the regulation of the hypothalamic–pituitary–adrenal-axis in the combined dexamethasone/CRH test. We found significant negative correlations (after controlling for age and sex) with *RORα* expression for $A_{\max}$ (-231 ; $P = 4.49 \times 10^{-2}$; $N = 80$) at admission and for C_{bas} (-0.366 ; $P = 2.58 \times 10^{-2}$; $N = 39$) after 6 weeks (Figure 2). No significant correlation was found with HAMD values at the time of testing (that is, at admission and at week 6; data not shown).

Figure 1. Relative *RORα* expression in antidepressant responders and nonresponders. Note that *RORα* expression appears to be elevated in nonresponders throughout the observation period reaching statistical significance at admission and after 5 weeks ($P = 6.23 \times 10^{-4}$, $P = 3.29 \times 10^{-3}$, respectively; GLM with age and sex as covariates). ** P -value < 0.01 ; *** P -value < 0.001 . Error bars are s.e.m. *RORα*, retinoid-related orphan receptor alpha.

Further investigating the negative correlation of *RORα* and hypothalamic–pituitary–adrenal axis activity observed in our patients, we quantified the expression of *RORα* in the hippocampus of mice. Testing for a correlation of hippocampal *RORα*

Figure 2. ACTH (a) and cortisol (b) response to the dex/CRH test at admission (left) and after 6 weeks (right), depending on the *RORa* expression level (upper vs lower 50% of expression values). Error bars are s.e.m. ACTH, adrenocorticotrophic hormone; dex/CRH, dexamethasone/corticotropin-releasing hormone; *RORa*, retinoid-related orphan receptor alpha.

expression in intermediate reactivity animals of the 'stress reactivity' mouse model¹⁴ with plasma corticosterone concentrations under basal conditions, we indeed detected a significant negative correlation ($r = -0.534$; $P = 3.31 \times 10^{-2}$; $N = 16$).

Association of *RORa* mRNA with *RORa* polymorphisms (SNPs)

We tested the association of *RORa* expression with two recently described *RORa* SNPs implicated in the traits depression (rs12912233)²¹ and antidepressant response (rs809736),²⁴ respectively. Using linear regression, we found a nominal association of *RORa* mRNA expression change after 5 weeks with the rs809736 genotype ($\beta = 11.98 \pm 5.61$ (s.e.m.); $P = 3.46 \times 10^{-2}$; genotype distribution: 85 (AA), 50 (AG) and 7 (GG); with the G allele being

associated with lower *RORa* mRNA expression), but not with rs12912233 ($\beta = -8.5160 \pm 5.15$ (s.e.m.); $P = 1.01 \times 10^{-1}$).

Interrogating the publicly available pharmacogenetic data set of the PCG project (<http://www.broadinstitute.org/mpg/ricopili/>; PhaCoGe Quan12 (Feb. 2013)), no association of *RORa* rs809736 with antidepressant response was found ($OR = 1.01$; $P = 1.53 \times 10^{-1}$), whereas data for rs12912233 were not available.

DISCUSSION

We identified several gene transcripts emerging from an unbiased whole-genome expression approach that were differentially expressed in antidepressant remitters and nonresponders, and that replicated in a representative clinical sample.

Using whole-genome expression arrays for the identification of antidepressant predictors so far produced conflicting results.^{4–7} The fact that we were able to replicate our findings from a relatively small discovery sample may be related to the carefully selected and severity-matched clinical extremes of remitters and nonresponders in the microarray experiment.⁸ Three of the five top ranked transcripts from the microarray experiment (*GCET2*, *CHI3L2* and *LSP1*) could be replicated in a sample of 142 patients with the same direction of effect. *CHI3L2* has recently been reported to activate the MAPK pathway through phosphorylation of ERK1/2 in glial tumor cells.^{25,26} *GCET2* and *LSP1* are expressed in leukocytes and have a role as markers in B-cell lymphoma (*GCET2*)²⁷ and cellular transmigration (*LSP1*),^{28,29} respectively. Interestingly, in this context, long-term treatment (≥ 12 weeks) with selective serotonin-reuptake inhibitors modulates B-cell proliferation.^{30,31}

RORa, the most significantly associated transcript in our replication study, has very recently gained attention as a new candidate in stress-related disorders, especially depression. The clock gene *RORa* is a transcription factor belonging to the steroid hormone receptor superfamily and has been implicated in response to cellular stress, cellular differentiation and proliferation, as well as circadian rhythm generation in different tissues.^{32,33} *RORa*-responsive genes include genes involved in calcium second messenger signaling and glutamatergic signaling pathways,³³ as well as the control of metabolic pathways.^{34,35} *RORa* is rhythmically expressed via E-box-binding of CLOCK-BMAL heterodimers. *RORa* then drives *Bmal1* expression as a part of the so-called stabilizing loop of the molecular clock of the hypothalamic suprachiasmatic nucleus.^{36,37}

For a long time, it has been speculated that an altered molecular clock is involved in the pathophysiology of depression as many patients show diurnal variations of depressive symptoms and disrupted sleep.^{38,39} Lithium, used as a treatment of mania and as prevention of bipolar disorder, inhibits glycogen synthase kinase 3b that, in turn, regulates protein stability of components of the molecular clock.^{40,41} Under treatment with lithium, increased amplitudes of clock gene oscillations have been observed on the cellular and tissue level,⁴² as well as increased circadian period length on the behavioral level.^{42,43} Intriguingly, McCarthy et al.⁴⁴ showed in fibroblasts of bipolar patients and healthy controls that circadian period lengthening induced by lithium is dependent on the *RORa* rs1292233 genotype.

Rs12912233 was recently found to be associated with trait depression in a genome-wide association study in a Caucasian epidemiologic sample.²¹ Another intronic *RORa* SNP (rs809736) ranked third in the genome-wide association study with antidepressant response to citalopram in the STAR*D sample.²⁴ *RORa* polymorphisms have further been implicated in posttraumatic stress disorder²² and autism spectrum disorder.^{45,46} We found rs809736 to be nominally associated with *RORa* expression in the present sample with the same direction of effect, that is, the allele associated with response in STAR*D was associated with lower *RORa* expression in the present study. Nevertheless, rs809736 was not replicated in the large PGC pharmacogenetic sample, hence, the nominal significant association of rs809736 with *RORa* gene expression in our cohort needs further replication.

Functional *RORa* knockout Staggerer mice (*RORa* sg/sf) show an enhanced ACTH and corticosterone response to novelty stress and a flattened corticosterone circadian rhythm.²³ Consistent with this observation, we found in our patients a negative correlation of *RORa* expression with basal and stimulated stress hormones in the dexamethasone/CRH test, an effect that was independent of the depressive states on the hypothalamic–pituitary–adrenal-axis. As a validation of this observation and extension to central pathophysiology, we found a negative correlation of hippocampal *RORa* expression with plasma corticosterone levels in intermediate reactivity mice. Nevertheless, *RORa* expression in our patients was

lower in responders compared with nonresponders in the microarray as well as in the replication study which is partly counter-intuitive regarding the negative correlation with the hypothalamic–pituitary–adrenal-axis.^{2,47} The physiological meaning of this finding in depression remains unclear, but may point to adaptive changes in nonresponders. The idea of adaptive processes may be indirectly supported by the recent finding of Ikeda et al.⁴⁸ who showed that *RORa* upregulates expression of dopamine D3 receptor (*DRD3*) in mice ventral striatum. This interpretation is in line with restored dopaminergic signaling that has been implicated in recovery from depression.⁴⁹

The present study harbors several limitations. The study did not follow a strict hypothesis-free design. Although we chose an unbiased approach in the discovery sample, not only were the most significant hits included in the replication experiment, but also the ‘promising candidate’ *RORa* that was then subject for further validation in functional experiments as it turned out to be the most significantly associated transcript in the replication study. Thus, our results can only be interpreted cautiously, and a further replication of *RORa* is crucial, as is the validation of the other transcripts, *GCET2*, *CHI3L2* and *LSP1*.

RNA expression analysis is challenging in numerous methodological aspects that can significantly impact on quality and reproducibility of our data.⁸ Although we cannot exclude environmental factors having affected gene expression more than antidepressant response in our patients, we undertook several measures to reduce biological variability accompanied with this method, for example, standardized pre-analytic conditions, consideration of batch effects, technical replication with qPCR and the use of four housekeeping genes for normalization of expression values yielding a high interclass reliability. By carefully selecting our patients, we further tried to reduce biological variability emerging from the subjects themselves. This approach allowed us to observe slight, but robust expression differences of *RORa* that were comparable to gene expression changes observed in similar studies⁵⁰ and independent of effects of sleep disturbances or circadian symptom patterns that have recently been reported to affect *RORa* expression in a transcriptomics study.⁵¹ Further, although gender was included as a covariate in each analysis, the effect observed for *RORa* may be primarily mediated by male patients as the discovery sample included males only. Indeed, the association of *RORa* expression (at admission) with response was stronger when analyzing males only ($N=75$; $P=3.17 \times 10^{-4}$ vs $P=6.23 \times 10^{-4}$), but lost significance in women ($N=67$; $P=2.19 \times 10^{-2}$). This observation may be related to the reduced statistical power in this subanalysis, but needs further attention in future studies.

The current study does not have a placebo arm. Thus placebo-related and real pharmacological effects cannot be separated. Different dosages between responders and nonresponders may have affected gene expression levels. Nevertheless, analyzing our data in this respect for the most frequently prescribed antidepressants in our study (venlafaxine; escitalopram; citalopram; mirtazapine; amitriptyline, including amitriptylin oxide) at week 5, we could not find a significant difference in prescribed dosages between responders and nonresponders (data not shown). In addition, only a part of our patients ($N=101$) was treated with only one class of antidepressant agent and medication switches within the 5-week observation period have not been analyzed specifically. On the other hand, all available antidepressants act as monoaminergic enhancers and effects of antidepressants are not specific, particularly after prolonged administration. The current study design was well suited to identify possible new targets that make some patients respond more easily to antidepressant drugs than others, independent of the drug given. Due to the naturalistic design of the present study, our results are possibly more generalizable and more relevant for clinical practice because

most patients are treated with more than one psychotropic agent drug.^{2,52,53}

In summary, in this study, we identified several transcripts that have the potential to serve as biomarkers for antidepressant response. Among these biomarker candidates, the circadian gene transcript *RORα* may deserve special attention as a candidate conveying disposition for depression and as a predictor of antidepressant response.

CONFLICT OF INTEREST

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REFERENCES

- Rush AJ, Trivedi MH, Wisniewski SR, Nierenberg AA, Stewart JW, Warden D et al. Acute and longer-term outcomes in depressed outpatients requiring one or several treatment steps: a STAR*D report. *Am J Psychiatry* 2006; **163**: 1905–1917.
- Hennings JM, Owashi T, Binder EB, Horstmann S, Menke A, Kloiber S et al. Clinical characteristics and treatment outcome in a representative sample of depressed inpatients - findings from the Munich Antidepressant Response Signature (MARS) project. *J Psychiatr Res* 2009; **43**: 215–229.
- GENDEP Investigators, MARS Investigators, STAR*D Investigators. Common genetic variation and antidepressant efficacy in major depressive disorder: a meta-analysis of three genome-wide pharmacogenetic studies. *Am J Psychiatry* 2013; **170**: 207–217.
- Kálmán J, Palotás A, Juhász A, Rimanóczy A, Hugyecz M, Kovács Z et al. Impact of venlafaxine on gene expression profile in lymphocytes of the elderly with major depression—evolution of antidepressants and the role of the 'neuro-immune' system. *Neurochem Res* 2005; **30**: 1429–1438.
- Mamdani F, Berlim MT, Beaulieu M-M, Labbe A, Merette C, Turecki G. Gene expression biomarkers of response to citalopram treatment in major depressive disorder. *Transl Psychiatry* 2011; **1**: e13.
- Mamdani F, Berlim MT, Beaulieu M-M, Turecki G. Pharmacogenomic predictors of citalopram treatment outcome in major depressive disorder. *World J Biol Psychiatry* 2013; **15**: 135–144.
- Belzeaux R, Bergon A, Jeanjean V, Loriod B, Formisano-Tréziny C, Verrier L et al. Responder and nonresponder patients exhibit different peripheral transcriptional signatures during major depressive episode. *Transl Psychiatry* 2012; **2**: e185.
- Menke A. Gene expression: biomarker of antidepressant therapy? *Int Rev Psychiatry* 2013; **25**: 579–591.
- Mitchell AC, Mirnics K. Gene expression profiling of the brain: pondering facts and fiction. *Neurobiol Dis* 2012; **45**: 3–7.
- Hamilton SP. A rating scale for depression. *J Neurol Neurosurg Psychiatr* 1960; **23**: 56–62.
- Rush AJ, Kraemer HC, Sackeim HA, Fava M, Trivedi MH, Frank E et al. Report by the ACNP Task Force on response and remission in major depressive disorder. *Neuropsychopharmacology* 2006; **31**: 1841–1853.
- Dunning MJ, Smith ML, Ritchie ME, Tavaré S. beadarray: R classes and methods for Illumina bead-based data. *Bioinformatics* 2007; **23**: 2183–2184.
- Menke A, Klengel T, Rubel J, Brückl T, Pfister H, Lucae S et al. Genetic variation in FKBP5 associated with the extent of stress hormone dysregulation in major depression. *Genes Brain Behav* 2013; **12**: 289–296.
- Touma C, Bunck M, Glasl L, Nussbaumer M, Palme R, Stein H et al. Mice selected for high versus low stress reactivity: a new animal model for affective disorders. *Psychoneuroendocrinology* 2008; **33**: 839–862.
- Knapman A, Heinzmann J-M, Hellweg R, Holsboer F, Landgraf R, Touma C. Increased stress reactivity is associated with cognitive deficits and decreased

- hippocampal brain-derived neurotrophic factor in a mouse model of affective disorders. *J Psychiatr Res* 2010; **44**: 566–575.
- Kohli MA, Lucae S, Saemann PG, Schmidt MV, Demirkan A, Hek K et al. The neuronal transporter gene SLC6A15 confers risk to major depression. *Neuron* 2011; **70**: 252–265.
- Ising M, Lucae S, Binder EB, Bettecken T, Uhr M, Ripke S et al. A genome-wide association study points to multiple loci that predict antidepressant drug treatment outcome in depression. *Arch Gen Psychiatry* 2009; **66**: 966–975.
- Huber W, von Heydebreck A, Sülthmann H, Poustka A, Vingron M. Variance stabilization applied to microarray data calibration and to the quantification of differential expression. *Bioinformatics* 2002; **18**(Suppl 1): S96–S104.
- Smyth GK. Linear models and empirical bayes methods for assessing differential expression in microarray experiments. *Stat Appl Genet Mol Biol* 2004; **3**: Article 3.
- Benjamini Y, Hochberg Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Statist Soc B* 1995; **57**: 289–300.
- Terracciano A, Tanaka T, Sutin AR, Sanna S, Deiana B, Lai S et al. Genome-wide association scan of trait depression. *Biol Psychiatry* 2010; **68**: 811–817.
- Logue MW, Baldwin C, Guffanti G, Melista E, Wolf EJ, Reardon AF et al. A genome-wide association study of post-traumatic stress disorder identifies the retinoid-related orphan receptor alpha (RORA) gene as a significant risk locus. *Mol Psychiatry* 2013; **18**: 937–942.
- Frédéric F, Chianale C, Oliver C, Mariani J. Enhanced endocrine response to novel environment stress and lack of corticosterone circadian rhythm in staggerer (Rora sg/sg) mutant mice. *J Neurosci Res* 2006; **83**: 1525–1532.
- Garriock HA, Kraft JB, Shyn SI, Peters EJ, Yokoyama JS, Jenkins GD et al. A genome-wide association study of citalopram response in major depressive disorder. *Biol Psychiatry* 2010; **67**: 133–138.
- Areshkov PO, Avdieiev SS, Balyńska OV, Leroith D, Kavsans VM. Two closely related human members of chitinase-like family, CHI3L1 and CHI3L2, activate ERK1/2 in 293 and U373 cells but have the different influence on cell proliferation. *Int J Biol Sci* 2012; **8**: 39–48.
- Areshkov PA, Kavsans VM. Chitinase 3-like protein 2 (CHI3L2, YKL-39) activates phosphorylation of extracellular signal-regulated kinases ERK1/ERK2 in human embryonic kidney (HEK293) and human glioblastoma (U87 MG) cells. *Tsitol Genet* 2010; **44**: 3–9.
- Gualco G, Bacchi LM, Domeny-Duarte P, Natkunam Y, Bacchi CE. The contribution of HGAL/GCET2 in immunohistological algorithms: a comparative study in 424 cases of nodal diffuse large B-cell lymphoma. *Mod Pathol* 2012; **25**: 1439–1445.
- Lei X, Hossain M, Qadri SM, Liu L. Different microvascular permeability responses elicited by the CXC chemokines MIP-2 and KC during leukocyte recruitment: role of LSP1. *Biochem Biophys Res Commun* 2012; **423**: 484–489.
- Liu L, Cara DC, Kaur J, Raharjo E, Mullaly SC, Jongstra-Bilen J et al. LSP1 is an endothelial gatekeeper of leukocyte transendothelial migration. *J Exp Med* 2005; **201**: 409–418.
- Edgar VA, Cremaschi GA, Sterin-Borda L, Genaro AM. Altered expression of autonomic neurotransmitter receptors and proliferative responses in lymphocytes from a chronic mild stress model of depression: effects of fluoxetine. *Brain Behav Immun* 2002; **16**: 333–350.
- Hernandez ME, Martinez-Fong D, Perez-Tapia M, Estrada-Garcia I, Estrada-Parra S, Pavón L. Evaluation of the effect of selective serotonin-reuptake inhibitors on lymphocyte subsets in patients with a major depressive disorder. *Eur Neuropsychopharmacol* 2010; **20**: 88–95.
- Jetten AM. Recent advances in the mechanisms of action and physiological functions of the retinoid-related orphan receptors (RORs). *Curr Drug Targets Inflamm Allergy* 2004; **3**: 395–412.
- Gold DA, Baek SH, Schork NJ, Rose DW, Larsen DD, Sachs BD et al. RORα coordinates reciprocal signaling in cerebellar development through sonic hedgehog and calcium-dependent pathways. *Neuron* 2003; **40**: 1119–1131.
- Kang HS, Okamoto K, Takeda Y, Beak JY, Gerrish K, Bortner CD et al. Transcriptional profiling reveals a role for RORα in regulating gene expression in obesity-associated inflammation and hepatic steatosis. *Physiol Genomics* 2011; **43**: 818–828.
- Bass J, Takahashi JS. Circadian integration of metabolism and energetics. *Science* 2010; **330**: 1349–1354.
- Sato TK, Panda S, Miraglia LJ, Reyes TM, Rudic RD, McNamara P et al. A functional genomics strategy reveals Rora as a component of the mammalian circadian clock. *Neuron* 2004; **43**: 527–537.
- Emery P, Reppert SM. A rhythmic Ror. *Neuron* 2004; **43**: 443–446.
- Albrecht U. Circadian clocks and mood-related behaviors. *Handb Exp Pharmacol* 2013; **227**: 227–239.
- Li JZ, Bunney BG, Meng F, Hagenauer MH, Walsh DM, Vawter MP et al. Circadian patterns of gene expression in the human brain and disruption in major depressive disorder. *Proc Natl Acad Sci USA* 2013; **110**: 9950–9955.

- 40 Yin L, Wang J, Klein PS, Lazar MA. Nuclear receptor Rev-erb α is a critical lithium-sensitive component of the circadian clock. *Science* 2006; **311**: 1002–1005.
- 41 Klein PS, Melton DA. A molecular mechanism for the effect of lithium on development. *Proc Natl Acad Sci USA* 1996; **93**: 8455–8459.
- 42 Li J, Lu W-Q, Beesley S, Loudon ASI, Meng Q-J. Lithium impacts on the amplitude and period of the molecular circadian clockwork. *PLoS ONE* 2012; **7**: e33292.
- 43 Kripke DF, Judd LL, Hubbard B, Janowsky DS, Huey LY. The effect of lithium carbonate on the circadian rhythm of sleep in normal human subjects. *Biol Psychiatry* 1979; **14**: 545–548.
- 44 McCarthy MJ, Wei H, Marnoy Z, Darvish RM, McPhie DL, Cohen BM *et al*. Genetic and clinical factors predict lithium's effects on PER2 gene expression rhythms in cells from bipolar disorder patients. *Transl Psychiatry* 2013; **3**: e318.
- 45 Nguyen A, Rauch TA, Pfeifer GP, Hu VW. Global methylation profiling of lymphoblastoid cell lines reveals epigenetic contributions to autism spectrum disorders and a novel autism candidate gene, RORA, whose protein product is reduced in autistic brain. *FASEB J* 2010; **24**: 3036–3051.
- 46 Sarachana T, Hu VW. Genome-wide identification of transcriptional targets of RORA reveals direct regulation of multiple genes associated with autism spectrum disorder. *Mol Autism* 2013; **4**: 14.
- 47 Ising M, Horstmann S, Kloiber S, Lucae S, Binder EB, Kern N *et al*. Combined dexamethasone/corticotropin releasing hormone test predicts treatment response in major depression - a potential biomarker? *Biol Psychiatry* 2007; **62**: 47–54.
- 48 Ikeda E, Matsunaga N, Kakimoto K, Hamamura K, Hayashi A, Koyanagi S *et al*. Molecular mechanism regulating 24-hour rhythm of dopamine D3 receptor expression in mouse ventral striatum. *Mol Pharmacol* 2013; **83**: 959–967.
- 49 Price JL, Drevets WC. Neural circuits underlying the pathophysiology of mood disorders. *Trends Cogn Sci* 2012; **16**: 61–71.
- 50 Cattaneo A, Gennarelli M, Uher R, Breen G, Farmer A, Aitchison KJ *et al*. Candidate genes expression profile associated with antidepressants response in the GENDEP study: differentiating between baseline 'predictors' and longitudinal 'targets'. *Neuropsychopharmacology* 2013; **38**: 377–385.
- 51 Möller-Levet CS, Archer SN, Bucca G, Laing EE, Slak A, Kabiljo R *et al*. Effects of insufficient sleep on circadian rhythmicity and expression amplitude of the human blood transcriptome. *Proc Natl Acad Sci USA* 2013; **110**: E1132–E1141.
- 52 Keks NA, Burrows GD, Copolov DL, Newton R, Paoletti N, Schweitzer I *et al*. Beyond the evidence: is there a place for antidepressant combinations in the pharmacotherapy of depression? *Med J Aust* 2007; **186**: 142–144.
- 53 Connolly KR, Thase ME. If at first you don't succeed: a review of the evidence for antidepressant augmentation, combination and switching strategies. *Drugs* 2011; **71**: 43–64.

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