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EXPRESSION OF p21-ACTIVATED KINASES 1 AND 3 IS ALTERED IN THE BRAIN OF SUBJECTS WITH DEPRESSION

BEATA FUCHSOVA,[a]* ANABEL ALVAREZ JULIÁ,[a] HOORIYAH S. RIZAVI,[b] ALBERTO CARLOS FRASCH[a] AND GHANSHYAM N. PANDEY[b]

[a] *Instituto de Investigaciones Biotecnológicas, CONICET-UNSAM, 1650 San Martin, Argentina*

[b] *Department of Psychiatry, University of Illinois at Chicago, Chicago, IL 60612, USA*

Abstract—The p21-activated kinases (PAKs) of group I are the main effectors for the small Rho GTPases, critically involved in neurodevelopment, plasticity and maturation of the nervous system. Moreover, the neuronal complexity controlled by PAK1/PAK3 signaling determines the postnatal brain size and synaptic properties. Stress induces alterations at the level of structural and functional synaptic plasticity accompanied by reductions in size and activity of the hippocampus and the prefrontal cortex (PFC). These abnormalities are likely to contribute to the pathology of depression and, in part, reflect impaired cytoskeleton remodeling pointing to the role of Rho GTPase signaling. Thus, the present study assessed the expression of the group I PAKs and their activators in the brain of depressed subjects. Using quantitative polymerase chain reaction (qPCR), mRNA levels and coexpression of the group I PAKs: *PAK1*, *PAK2*, and *PAK3* as well as of their activators: *RAC1*, *CDC42* and *ARHGEF7* were examined in postmortem samples from the PFC ($n = 25$) and the hippocampus ($n = 23$) of subjects with depression and compared to control subjects (PFC $n = 24$; hippocampus $n = 21$). Results demonstrated that mRNA levels of *PAK1* and *PAK3*, are significantly reduced in the brain of depressed subjects, with *PAK1* being reduced in the PFC and *PAK3* in the hippocampus. No differences were observed for the ubiquitously expressed *PAK2*. Following analysis of gene coexpression demonstrated disruption of coordinated gene expression in the brain of subjects with depression. Abnormalities in mRNA expression of *PAK1* and *PAK3* as well as their altered coexpression patterns were detected in the brain of subjects with depression.

Key words: postmortem, mRNA expression, coexpression analysis, p21-activated kinase, qPCR.

INTRODUCTION

It has been widely accepted that stress induces alterations at the level of structural and functional synaptic plasticity that are likely to contribute to the pathology of depression (Pittenger and Duman, 2008; Christoffel et al., 2011). For example, reductions in size and activity of the hippocampus and the prefrontal cortex (PFC) in different types of depression have been reported. The neuropathological correlates of these abnormalities include reductions in synapses or synaptic proteins, elevations in neuronal density, and reductions in neuronal size and neuropil (Rajkowska et al., 1999; Stockmeier et al., 2004; Drevets et al., 2008; Pittenger and Duman, 2008; Kang et al., 2012). Accordingly, shortening and reduced complexity of dendritic trees as well as reductions in dendritic spines and synapses has been reported also in the PFC and in the hippocampus in animal models of chronic stress (McKittrick et al., 2000; Radley et al., 2004; Drevets et al., 2008; Pittenger and Duman, 2008).

The intracellular mechanisms promoting these changes and their relevance to behavioral outcomes are poorly understood. Nevertheless, emerging evidence suggests that these structural abnormalities, in part, reflect impaired cytoskeleton remodeling and point to the role of Rho GTPase signaling as a central contributor.

The p21-activated kinases (PAKs), a family of serine/threonine protein kinases, are the main effectors for the small Rho GTPases of the RAC1 and CDC42 family. The three kinases PAK1, PAK2 and PAK3 constitute together PAKs of group I (Dan et al., 2001). It has been shown that group I PAKs have different tissue distributions: PAK1 is highly expressed in the brain and spleen, PAK2 is ubiquitously expressed and PAK3 is mainly expressed in the brain (Manser et al., 1995; Teo et al., 1995; Rousseau et al., 2003). PAKs serve as key regulators of cytoskeleton dynamics and cell motility, cell cycle progression, as well as of death and survival events (Kreis and Barnier, 2009; Rane and Minden, 2014). Recent studies on neurons indicate that PAKs are important for neurite outgrowth, neuronal migration, spine morphology, and synaptic and behavioral plasticity (Daniels et al., 1998; Hayashi et al., 2002; Boda et al., 2004, 2008; Hayashi et al., 2004; Marler et al., 2005; Zhang et al., 2005; Sakakibara and Horwitz, 2006; Cobos et al., 2007; Hayashi et al., 2007; Jacobs et al., 2007; Kreis et al., 2007; Causeret et al., 2009). More importantly, it has been shown that the postnatal brain size and synaptic properties are determined by the neuronal

*Corresponding author. Address: Instituto de Investigaciones Biotecnológicas, Universidad Nacional de General San Martin, Av. 25 de Mayo y Francia, Edificio IIB, 1650 San Martin, Buenos Aires, Argentina. Fax: +54-11-4006-1500x2125.
E-mail address: beata@iibintech.com.ar (B. Fuchsova).

Abbreviations: ANCOVA, analysis of covariance; PAK, p21-activated kinase; PFC, prefrontal cortex; PMI, postmortem interval; qPCR, quantitative polymerase chain reaction; RT, reverse transcription.

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complexity controlled by PAK1 and PAK3 signaling. Double knockout mice lacking both PAK1 and PAK3 exhibit reduced brain size that is accompanied by minimal changes in total cell count, due to a significant increase in cell density. Moreover, double knockout neurons have smaller soma, with markedly simplified dendritic arbors/axons, and reduced synapse density (Huang et al., 2011).

Due to the critical involvement of PAK signaling in neuronal physiology, dysregulation of group I PAKs in the brain often leads to neuronal development disorders as well as neurodegenerative pathologies such as Alzheimer's disease, mental retardation (with PAK3 being specifically involved), and Huntington's disease (Ma et al., 2012). On the other hand, in rats prone to depression, PAK1 was found to be downregulated in the frontal cortex when compared to animals resistant to depression (Nakatani et al., 2007).

Since the expression levels and activity of these neuroplasticity-related kinases are influenced by various factors under pathological conditions associated with neurodevelopment, neuroplasticity and maturation of the nervous system, we hypothesized that PAK signaling could be altered also in the brain of depressed subjects. Therefore, in the present study we examined the expression of PAKs of group I: *PAK1*, *PAK2*, and *PAK3* as well as the expression of their regulators *RAC1*, *CDC42* and *ARHGEF7*, known regulators of synaptic structure, in the hippocampus ($n = 23$) and the PFC ($n = 25$) of subjects with depression. Gene products that function together in common signaling cascades are expected to show greater similarities in their expression patterns than random sets of gene products (Vidal et al., 2011). Thus, correlation analyses were also conducted to determine if networks of co-expressed genes exist in control subjects and whether these networks are disrupted in the brain of subjects with depression.

EXPERIMENTAL PROCEDURES

Subjects

Brain tissues were collected by the Brain Collection Program of the Maryland Psychiatric Research Center, Baltimore. The same cohort has been used previously in various studies published by our group (Dwivedi et al., 2008; Dwivedi et al., 2009, 2010; Pandey et al., 2014; Fuchsova et al., 2015). All the tissues from neuropsychiatric controls and depressed subjects were screened for macro- and microscopic neuropathological abnormalities by board-certified neuropathologists at the brain collection program. The presence of Alzheimer's disease, infarcts, demyelinating diseases, or heterotopia (or clinical history of these disorders) disqualified subjects from the study. Brain samples were free of human immunodeficiency virus antibodies. Toxicology screening for alcohol and screening for antidepressant/psychoactive drugs taken prior to death were performed in blood/urine of each subject. Psychiatric diagnoses in depressed and control subjects were performed using the Diagnostic Evaluation After Death (Salzman et al., 1983) and the Structured Clinical Interview for DSM-IV (Spitzer et al., 1995). All subjects in the depression group died by suicide. The present studies were performed in the PFC in Brodmann area 9 in 25 depressed subjects and 24 nonpsychiatric control subjects. The PFC was defined as the gray matter from the most anterior 1-cm coronal slice of the cortex and was further dissected according to the Brodmann atlas. White matter was removed as much as possible, but there was still some white matter left. Hippocampi were available for 23 depressed subjects and 21 nonpsychiatric controls. This study was approved by the institutional review board of the University of Illinois at Chicago, and written informed consent was obtained from next-of-kin for each subject. Detailed demographic characteristics of subjects are provided in Table 1.

RNA isolation and reverse transcription (RT)

We used the Trizol® reagent (Invitrogen, Carlsbad, California) to extract total RNA from 100 mg of tissue according to the manufacturer's instructions. Nano Drop®ND-1000 (NanoDrop Technologies, Montchanin, Delaware) was used to determine RNA yield and purity by absorbance ratios A260/A280 and A260/A230. All the samples had OD ratios close to two indicating absence of contaminants. To assess the quality of RNA, we used Agilent Bioanalyzer 2100. All samples showed RNA integrity number (RIN) above 6.6 and 28S/18S ratios > 1.2. Random hexamers (2.5 μM) (Invitrogen) were employed to synthesize first-strand cDNA from 1 μg of total RNA with MMLV-reverse transcriptase (Invitrogen) according to manufacturer's directions.

Oligonucleotide primers

We used Primer Express 3.0 software (Applied Biosystems, Foster City, California) to design primers for the amplification of human *PAK1*, *PAK2*, *PAK3*, *RAC1*, *ARHGEF7*, *CDC42* and internal reference genes (*GAPDH*, *CYC1*, and *PPIA*). In all cases primers were designed to amplify all known transcript variants for each gene. Only in case of *RAC1*, an additional set of primers was designed to distinguish the amplification of *RAC1B*, a longer transcript variant of *RAC1* (NM_018890.3) that includes the alternatively spliced 57 bp region (exon 3b). Primer sequence, full gene name, gene ID, function, and chromosomal localization are listed in Table 2 (reference genes) and Table 3 (target genes).

Quantitative polymerase chain reaction (qPCR)

Levels of mRNA were quantified by conducting qPCRs with SYBR®Select Master Mix (Applied Biosystems) according to the manufacturer's directions. Stratagene Mx3005P equipped with MxPro software (Stratagene, La Jolla, CA, USA) was used to perform measurements. Plate setup always included no-RT control, no-template control and three different inter-run calibrators for each gene assay. All reactions were done in duplicate. A heat dissociation protocol to verify primer specificity was included after the final cycle of each PCR reaction. All pairs of primers showed a single peak corresponding to the melting temperature (Tm) of expected PCR product

Table 1. Demographic characteristics of subjects with depression and normal control subjects

		Age (years)	Race	Gender	PMI (hours)	Brain pH	Cause of death	Psychotropic drugs (at the time of death)	Psychiatric diagnosis
Normal control subjects[a]									
1	CONTROL	22	Black	Male	19	6.9	GSW	None	Normal
2	CONTROL	42	White	Female	23	7.2	Pneumonia	None	Normal
3	CONTROL	37	Black	Male	5	7.1	ASCVD	None	Normal
4	CONTROL	31	Black	Male	8	7.2	GSW	None	Normal
5	CONTROL	46	Black	Male	9	7.1	Multiple injuries	None	Normal
6	CONTROL	33	White	Male	15	7.0	GSW	None	Normal
7	CONTROL	48	White	Male	26	6.9	ASCVD	None	Normal
8	CONTROL	40	White	Female	7	7.0	ASCVD	None	Normal
9	CONTROL	23	Black	Male	15	6.8	GSW	None	Normal
10	CONTROL	38	Black	Male	16	6.9	Lung Sarcoidosis	None	Normal
11	CONTROL	65	Black	Female	23	6.9	ASCVD	None	Normal
12	CONTROL	52	White	Male	30	7.3	ASCVD	None	Normal
13	CONTROL	35	White	Male	24	6.9	Crush injury to abdomen and chest	None	Normal
14	CONTROL	37	White	Male	24	7.0	ASCVD	None	Normal
15	CONTROL	45	White	Male	22	7.3	ASCVD	None	Normal
16	CONTROL	26	White	Male	12	6.9	Arrhythmia	None	Normal
17	CONTROL	72	White	Female	23	6.9	MVA	None	Normal
18	CONTROL	42	White	Female	23	6.9	Mitral valve prolapse	None	Normal
19	CONTROL	47	White	Male	10	7.0	ASCVD	None	Normal
20	CONTROL	31	White	Male	16	7.2	MVA	None	Normal
21	CONTROL	60	White	Male	15	7.1	Accidental drowning	None	Normal
22	CONTROL	28	White	Male	13	6.8	Electrocution	None	Normal
23	CONTROL	45	White	Female	16	6.9	Cardiac arrhythmia	None	Normal
24	CONTROL	62	White	Male	19	7.0	Cardiac arrest	None	Normal
Depressed subjects[b]									
1	SUICIDE	27	White	Male	24	7.0	GSW	None	MDD, Ethanol abuse
2	SUICIDE	44	White	Female	11	7.2	Drug OD	Nortriptyline	MDD, Ethanol abuse
3	SUICIDE	36	White	Female	10	7.1	GSW	None	MDD
4	SUICIDE	24	White	Male	7	7.1	GSW	Ethanol	MDD
5	SUICIDE	43	White	Male	12	7.0	Drug OD	None	MDD, Polysubstance Abuse
6	SUICIDE	53	White	Male	23	6.9	Jump from height	None	MDD
7	SUICIDE	41	White	Female	27	7.1	Drug OD	Amitriptyline, Desipramine, Nortriptyline, Ethanol	MDD, Ethanol abuse
8	SUICIDE	22	Black	Female	16	7.3	Drug OD	None	MDD
9	SUICIDE	46	White	Female	21	6.9	Drug OD	Amitriptyline, Desipramine, Ethanol	MDD
10	SUICIDE	36	White	Female	18	7.2	GSW	None	MDD
11	SUICIDE	38	White	Male	24	7.0	Drug OD, Ethanol intoxication	Ethanol	MDD, Ethanol abuse
12	SUICIDE	46	White	Female	16	6.8	Drug OD	Nortriptyline	MDD, Panic disorder with agoraphobia
13	SUICIDE	23	White	Male	12	7.0	Hanging	Paroxetine	MDD
14	SUICIDE	30	White	Male	17	7.1	Hanging	Venlafaxine	MDD
15	SUICIDE	44	White	Female	30	7.2	Drug OD, Ethanol intoxication	Fluoxetine, Ethanol	MDD, Ethanol abuse, Opioid abuse
16	SUICIDE	74	White	Female	27	7.0	Venlafaxine OD	Venlafaxine, Ethanol	MDD, Ethanol abuse
17	SUICIDE	25	White	Male	14	6.8	Hanging	Ethanol	MDD
18	SUICIDE	23	Black	Male	23	6.9	Hanging	None	MDD
19	SUICIDE	63	White	Male	19	6.9	Drug OD, Ethanol intoxication	Ethanol	MDD
20	SUICIDE	67	White	Male	22	7.0	GSW	Fluoxetine, Venlafaxine	MDD
21	SUICIDE	40	White	Female	20	7.0	Drug OD	Xanax	MDD
22	SUICIDE	53	White	Male	26	7.1	Stabbing	Sertraline	MDD

(*continued on next page*)

Table 1 (*continued*)

		Age (years)	Race	Gender	PMI (hours)	Brain pH	Cause of death	Psychotropic drugs (at the time of death)	Psychiatric diagnosis
23	SUICIDE	68	White	Female	26	6.8	GSW	Amitriptyline	Bipolar Disorder
24	SUICIDE	31	White	Female	31	6.8	Hanging	Trazadone, Ethanol	Bipolar Disorder, Bulimia, OCD
25	SUICIDE	51	White	Female	28	6.9	Amitriptyline OD	Amitriptyline, Ethanol	Bipolar Disorder, Ethanol abuse

Abbreviations: ASCVD, atherosclerotic cardiovascular disease; CO, carbon monoxide; DKA, diabetic ketoacidosis; GSW, gunshot wound; MDD, major depressive disorder; MVA, motor vehicle accident; OCD, obsessive–compulsive disorder; OD, overdose; PMI, postmortem interval.

[a] Mean ± SD age is 41.96 ± 13.19 years; PMI, 17.21 ± 6.65 h; and brain pH 7.01 ± 0.15.

[b] Mean ± SD age is 41.92 ± 15.07 years; PMI, 20.16 ± 6.68 h; and brain pH 7.00 ± 0.14.

(Table 4). Reproducibility of RT reaction and inter-run reproducibility of qPCR was evaluated as described in our previous work (Fuchsova et al., 2015). We employed the relative standard curve method as described in the "Applied Biosystems Guide to Performing Relative Quantitation of Gene Expression Using Real-Time Quantitative PCR" to determine relative expression levels and report them as fold change (FC) to control. To determine the linear range and PCR reaction efficiency of each gene assay we build standard curves using 10-fold serial dilutions of cDNA derived from all subjects. We report $r^2 \geqslant 0.988$ and efficiencies between 90% and 110% for all gene assays (Table 4). qBasePLUS software (Biogazelle, Zwijnaarde, Belgium) was employed to analyze raw expression data (C_T values). For further statistical analysis we used values normalized to the normalization factor calculated as a geometric mean of the expression of three reference genes (Vandesompele et al., 2002).

Determination of reference targets for qPCR data normalization

The algorithm geNorm of qBasePLUS software was used to evaluate expression stability of reference genes used for qPCR data normalization (Vandesompele et al., 2002) as described in our previous work (Fuchsova et al., 2015). The reference targets *PPIA*, *CYC1*, and *GAPDH* were determined as the most stably expressed genes and were found adequate for the optimal normalization of data in our experimental setting (Table 5, see the Table 2 for full gene name, accession number, function, chromosomal localization, and primer sequences). Fig. 1 shows that control and depressed subjects did not differ with respect to *GAPDH*, *CYC1* and *PPIA* mRNA expression levels. The geometric mean of *GAPDH*, *CYC1*, and *PPIA* was further used for normalization in all relative quantitation assays.

Statistical analysis

Analysis of covariance (ANCOVA) was used to compare the groups of controls and depressed subjects. Data analysis was done independently for each brain region and gene of interest adjusting the effects of age, postmortem interval (PMI), and brain pH. Bonferroni correction was applied for multiple comparisons to maintain alpha at 0.05 to adjust the type I error rates. Statistical outliers were identified by the ROUT method with the value *Q* set to 1% in order to control the false discovery rate. We report results as individual values and group mean ± SD. For coexpression analysis of gene expression levels, correlation analyses were conducted to calculate pairwise Spearman correlation coefficients along with its 95% confidence intervals, and *p* value (two-tailed) on normalized mRNA expression levels. Cross-correlations were performed between the expression levels of the six different genes for the depression group and for the control group independently in each brain area. This resulted in fifteen separate comparisons for each group in each area. For multiple comparisons, Spearman correlation values with Bonferroni correction to maintain alpha at 0.05 were used. To determine differences in the frequency of significant correlations between groups, Chi-square analyses were performed. GraphPad Prism software was used to do statistical analysis and graphs.

RESULTS

Demographic characteristics

Detailed demography of studied subjects is reported in Table 1. The age range of control and depressed subjects was between 22 and 74 years without significant differences between depressed subjects and their matched controls ($p = 0.9925$, unpaired *t*-test, two-tailed). The PMI was between 5 and 31 h and did not differ statistically between two groups ($p = 0.1279$, unpaired *t*-test, two-tailed). The mean brain pH ± SD was 7.01 ± 0.15 in control group and 7.00 ± 0.14 in depressed group and did not significantly differ between groups ($p = 0.9163$, unpaired *t*-test, two-tailed). In the PFC, the mean RIN ± SD was 7.21 ± 0.56 in controls and 7.23 ± 0.61 in subjects with depression. The mean RIN ± SD in the hippocampus was 7.16 ± 0.72 in controls and 7.45 ± 0.83 in subjects with depression. In both the PFC and the hippocampus, the RIN values did not differ between control group and depressed subjects ($p = 0.7$ and $p = 0.37$; respectively, unpaired *t*-test, two-tailed).

PAK1 expression is downregulated in the PFC of subjects with depression

To evaluate if alteration in the expression of *PAK1* is associated with pathophysiology of human depression, *PAK1* mRNA level was assessed in the entire cohort using qPCR. Fig. 2A, B shows mRNA levels of *PAK1* in

Table 2. Primer sequences for reference genes

Symbol	Entrez Gene ID	Name	Function	Forward primer	Reverse primer	Location
GAPDH	2597	Glyceraldehyde-3-phosphate dehydrogenase, transcript variant 1	Catalysis of oxidative phosphorylation of glyceraldehyde-3-phosphate in the presence of NAD	GAAGGTGAAGGTCGGAGTC	GAAGATGGTGATGGGATTTC	12p13
PPIA	5478	Peptidylprolyl isomerase A (cyclophilin A)	Peptidyl-prolylcis–transisomerase	GGCAAATGCTGGACCCAACACA	TGCTGGTCTTGCCATTCCTGGA	7p13
CYC1	1537	Cytochrome c-1	Electron transporter	CCAGATAGCCAAGGATGTGTGC	GACTGACCACTTGTGCCGCTTT	8q24.3

the PFC (Fig. 2A) and in the hippocampus (Fig. 2B) of depressed subjects and matched non-psychiatric controls. In the hippocampus, no significant differences were observed between groups. On the other hand, *F*-test of ANCOVA showed that in the PFC, the mean *PAK1* mRNA level was significantly decreased in the depressed group ($F = 23.87$, $^{**}p < 0.01$ after Bonferroni correction, FC = 0.87). The age, PMI and brain pH had no significant effects on mRNA expressions of *PAK1*.

Altered expression of PAK3 in the hippocampus of subjects with depression

We next examined whether the expression of two other members of group I PAKs, *PAK2* and *PAK3*, is altered in the brain of subjects with depression. *PAK2* and *PAK3* mRNA levels were measured by qPCR in the PFC (Fig. 2A) and in the hippocampus (Fig. 2B) of subjects with depression and matched non-psychiatric controls. In the PFC, we did not detect any differences between groups for *PAK3*. In contrast, *F*-test of ANCOVA revealed that in the hippocampus, the mean *PAK3* mRNA level was significantly reduced in the depressed subjects ($F = 17.27$, $^{**}p < 0.01$ after Bonferroni correction, FC = 0.78). The age, PMI and brain pH had no significant effects on mRNA expression of *PAK3*. Expression of *PAK2* mRNA did not differ in neither of the tissues.

Expression of RAC1, CDC42, and ARHGEF7 in the postmortem brain of subjects with depression

We next assessed if the expression of the genes encoding PAKs regulators: the cell division cycle 42 (CDC42), the Ras-related C3 botulinum toxin substrate (RAC1) and ARHGEF7 (also known as betaPIX) is altered in the postmortem brain of subjects with depression. Fig. 3 shows mRNA levels of *RAC1*, *CDC42*, and *ARHGEF7* in the PFC (Fig. 3A) and in the hippocampus (Fig. 3B) of depressed subjects and matched control individuals.

No significant changes were observed for *RAC1*, *CDC42*, and *ARHGEF7* in neither of the tissues. Furthermore, we evaluated separately the expression of *RAC1B*, a constitutively active splice variant of *RAC1*, whose increased expression has been found to contribute to neurodegeneration seen in Alzheimer's disease (Perez et al., 2012). Similarly to the previous cases, the expression of *RAC1B* mRNA did not differ in neither of the tissues.

Coordinated expression

To assess interrelationships in the expression levels of *PAK1*, *PAK2*, *PAK3*, *RAC1*, *CDC42*, and *ARHGEF7*, cross-correlation analyses were conducted in the control group and in the depressed subjects group independently for each brain region. Normalized mRNA expression levels of all studied genes have been correlated to each other and Spearman correlation coefficients and *p* values were calculated.

Table 3. Primer sequences for target genes

Symbol	Entrez Gene ID	Name	Forward primer	Reverse primer	Location
PAK1	5058	p21 protein (Cdc42/Rac)-activated kinase 1	GTGAAGGCTGTGTCTGAGACTC	GGAAGTGGTTCAATCACAGACCG	11q13-q14
PAK2	5062	p21 protein (Cdc42/Rac)-activated kinase 2	CGACTCCAACACAGTGAAGCAG	TCACTACTGCGGGTGCTTCTGT	3q29
PAK3	5063	p21 protein (Cdc42/Rac)-activated kinase 3	CGCTGTCTTGAGATGGATGTGG	CAGTCTTAGCGGCTGCTGTTCT	Xq23
RAC1	5879	ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1) isoforms Rac1 and Rac1b	AGTGCTCGGCGCTCACA	CGGATCGCTTCGTCAAACA	7p22
RAC1B	5879	ras-related C3 botulinum toxin substrate 1 (rho family, small GTP binding protein Rac1) isoform Rac1b	CGGTGAATCTGGGCTTATGGGA	GGAGGTTATATCCTTACCGTACG	7p22
CDC42	998	Cell division cycle 42	TGACAGATTACGACCGCTGAGTT	GGAGTCTTTGGACAGTGGTGAG	1p36.1
ARHGEF7	8874	Rho guanine nucleotide exchange factor 7	CGCAAACCTGAACGGAAGCCTT	GTTTTGGCGCTGGTGCAGTAAG	13q34

Table 4. Efficiency of qPCR

Assay	Threshold (dRn)	RSq (dRn)	Slope (dRn)	Efficiency (%)	Tm (°C)
Reference genes					
GAPDH	0.2	0.992	−3.552	91.2	83.6
PPIA	0.2	0.997	−3.353	98.7	84.4
CYC1	0.2	0.988	−3.455	94.7	86.8
Target genes					
PAK1	0.2	0.988	−3.566	90.7	83.8
PAK2	0.2	0.991	−3.351	98.8	81.8
PAK3	0.2	0.997	−3.103	110	82.8
RAC1	0.2	0.995	−3.178	106.4	83.2
RAC1b	0.2	0.967	−3.147	107.9	80.4
ARHGEF7	0.2	0.995	−3.333	99.5	83.25
CDC42	0.2	0.99	−3.426	95.8	79.8

Efficiency data for reference genes and target genes. C_T values obtained from 10-fold serial dilutions of cDNA were plotted against dilution factors and the reaction efficiency was calculated using qBasePLUS software.
RSq, r-squared; Rn, normalized reporter signal; dRn, delta Rn (Rn minus the baseline); Tm, melting temperature.

Table 5. Reference target stability values (*M* and CV)

Reference target	*M*	CV
PFC		
CYC1	0.4716	0.1812
GAPDH	0.6866	0.2651
PPIA	0.4709	0.2495
Average	0.5430	0.2319
Hippocampus		
CYC1	0.5128	0.2437
GAPDH	0.5878	0.2331
PPIA	0.4601	0.1525
Average	0.5202	0.2098

Reference target stability values (*M* and CV) determined for the combination of the reference targets *GAPDH*, *CYC1*, and *PPIA* in human brain samples from the PFC and hippocampus. Expression stability of reference genes in human brain samples was calculated by geNorm algorithm using qBasePLUS software (Biogazelle) as described in our previous publication (Fuchsova et al., 2015). GeNorm analysis was performed on the gene expression data from PFC and hippocampal postmortem samples obtained from all depressed subjects and matched nonpsychiatric controls. The gene stability measure *M* is defined as the average pairwise variation of a particular gene with all other control genes (Vandesompele et al., 2002). Genes with lowest values have the most stable expression (optimal geNorm $M \leqslant 0.5$). To assess that the genes with the lowest *M* values have indeed the most stable expression, the gene-specific variation of each control gene is determined as the variation coefficient (CV) of the expression levels after normalization. Mean CV values equal or lower than 0.2 are typically observed for stably expressed reference genes in relatively homogenous sample panels. Normalization to a single control gene can lead to erroneous normalization. For this reason, in our previous publication the optimum number of three reference genes required for adequate data normalization in this experimental situation was calculated using geNorm (Fuchsova et al., 2015). Thus, in our experiments in human postmortem PFC and hippocampal samples, the optimal normalization factor was calculated as the geometric mean of reference targets *PPIA*, *CYC1*, and *GAPDH*.

Fig. 4A shows correlation matrices with Spearman correlation coefficients and statistically significant Spearman correlation *p* values indicated by asterisks. In the control PFC, four of 15 possible correlations were statistically significant. Within the hippocampus of control individuals, two of 15 correlations were statistically significant and these were the same two positive correlations as detected in the control PFC (correlations between *PAK2* and *RAC1*, and *RAC1* and *ARHGEF7*). In the PFC in of subjects with depression, the number of significant correlations declined to one, which in fact was a new positive correlation between *PAK1* and *PAK3*. The difference in the frequency of significant correlations between groups was statistically significant (chi-square = 6, $^{*}p = 0.0143$).

In the hippocampus, only one significant correlation evident also in controls was detected in the brain of subjects with depression. The frequency of significant correlations between the depressed group and the control group in the hippocampus did not statistically differ (chi-square = 1.034, $p = 0.3091$).

Fig. 4B depicts the networks of the gene coexpression patterns in the control and depressed groups to illustrate

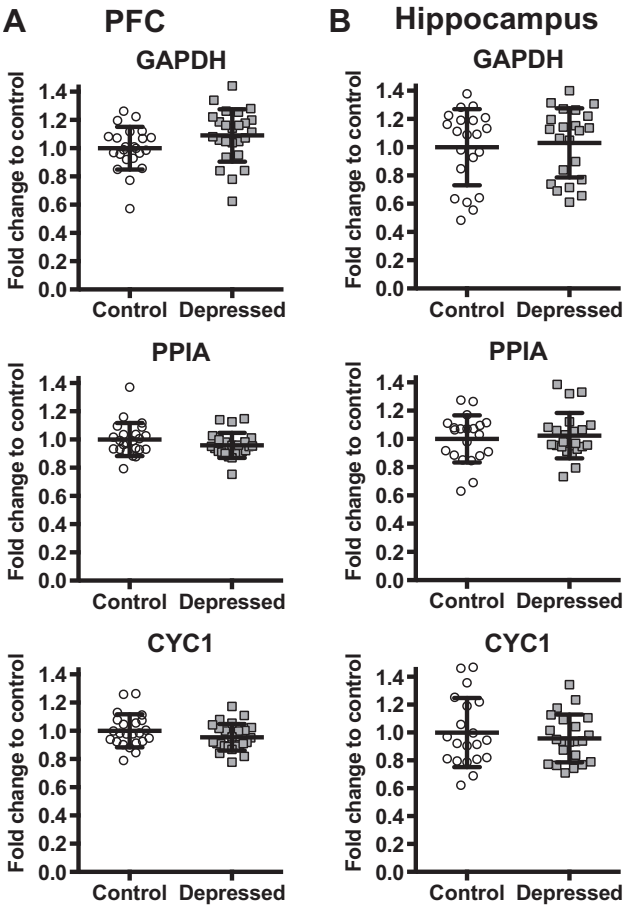

Fig. 1. Expression levels of reference genes *GAPDH*, *PPIA* and *CYC1* in the PFC (A) and in the hippocampus (B) of non-psychiatric controls (Control: PFC $n = 23$ for *GAPDH*, *PPIA* and *CYC1*; Hippocampus $n = 21$ for *GAPDH*, *PPIA* and $n = 20$ for *CYC1*) and depressed subjects (Depressed: PFC $n = 25$ for *GAPDH*, *CYC1* and $n = 24$ for *PPIA*; Hippocampus $n = 22$ for *GAPDH*, *PPIA* and *CYC1*). Results are expressed as fold change in mRNA levels. Values are fold change ± SD. No significant differences between the depressed group and the control group were determined for any of the genes.

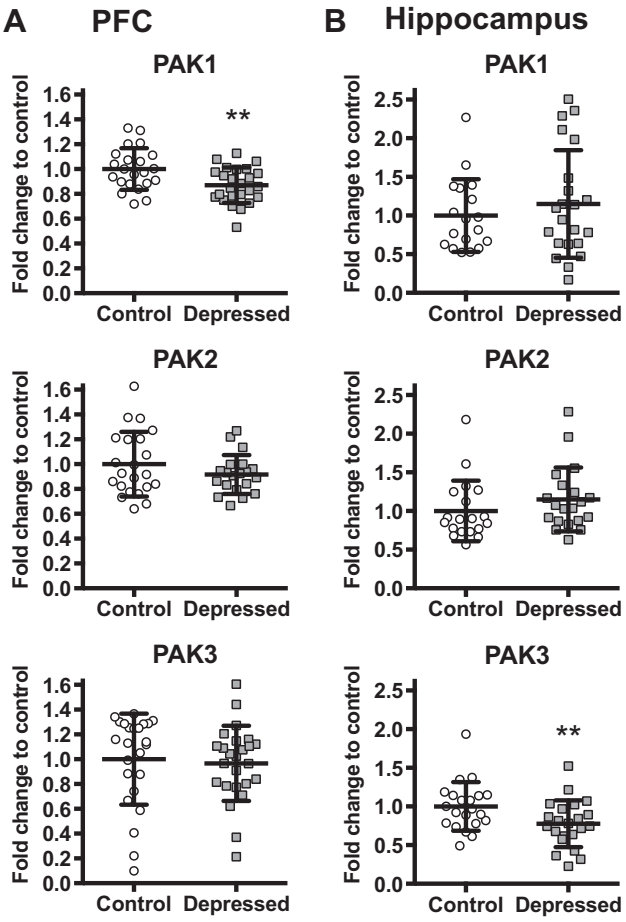

Fig. 2. mRNA levels of *PAK1*, *PAK2*, and *PAK3* in the PFC (A) and in the hippocampus (B) of non-psychiatric controls (Control: PFC $n = 22$ for *PAK1*, *PAK2*, and $n = 24$ for *PAK3*; Hippocampus $n = 18$ for *PAK1*, $n = 19$ for *PAK2*, and $n = 21$ for *PAK3*) and depressed subjects (Depressed: PFC $n = 25$ for *PAK1*, *PAK3*, and $n = 20$ for *PAK2*; Hippocampus $n = 22$ for *PAK1*, *PAK3*, and $n = 20$ for *PAK2*) normalized to the geometric mean of the three reference targets (*GAPDH*, *CYC1*, *PPIA)*. Results are expressed as fold change in mRNA levels. Values are fold change ± SD. Significant differences between the subjects with depression and the control group were determined for *PAK1* ($F = 23.87$, $^{**}p < 0.01$) in the PFC and *PAK3* ($F = 17.27$, $^{**}p < 0.01$) in the hippocampal tissue. Significant effects are marked by asterisks showing Bonferroni corrected significance thresholds.

our findings. Significant positive or negative correlation between two genes is shown with a full or dashed line respectively. In addition, genes showing differential expression between depressed subjects and their non-psychiatric matched controls (namely *PAK1* in the PFC and *PAK3* in the hippocampus) are shaded in gray. In both tissues of the control group, correlated genes connect suggesting that they function in common signaling cascades. If the pathway is affected by disease, this type of analysis is expected to reveal changes in the coordinated gene expression. Indeed, in the depressed group, this coordinated gene expression is slightly altered in the hippocampus while it almost disappears in the PFC, where the only correlation detected was a new correlation between *PAK3* and *PAK1*.

Effect of psychotropic drugs, ethanol and gender

Since several of the subjects in the depressed group were on antidepressant medication at the time of death and/or suffered from comorbid substance or ethanol abuse, we examined if the presence of psychotropic drugs or ethanol had any influence on the mRNA expression or on the coordinated gene expression of studied genes. For this purpose, depressed subjects were separated in three groups: (1) the depressed group free of any psychotropic medication (seven subjects), (2) the group taking different types of antidepressants (14 subjects), and (3) the group with ethanol consumption (four subjects).

When we compared the mRNA expression, we did not find any significant effect of antidepressants or ethanol on the expression of any of the studied genes in the hippocampus and on the expression of *PAK1*, *PAK3*, *RAC1*, *RAC1B*, *CDC42*, and *ARHGEF7* in the PFC. On the other hand, *F*-test of ANCOVA determined a

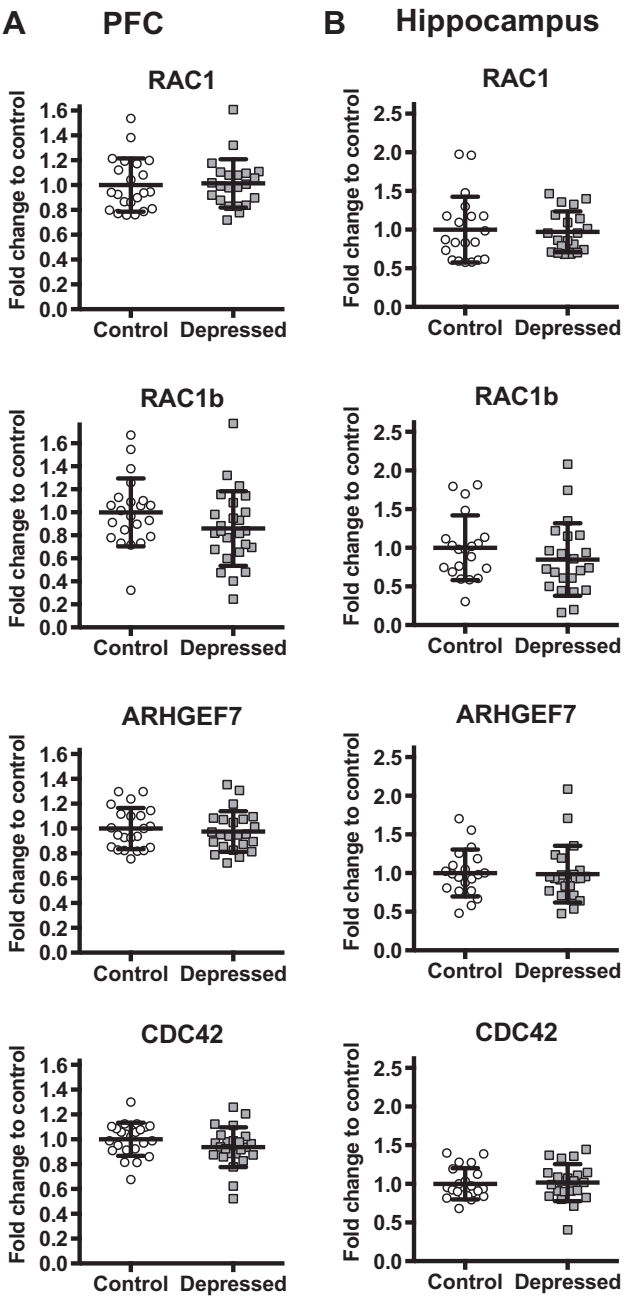

Fig. 3. mRNA levels of *RAC1*, *RAC1B*, *CDC42*, and *ARHGEF7* in the PFC (A) and in the hippocampus (B) of non-psychiatric controls (Control: PFC $n = 22$ for *RAC1*, *RAC1B*, and *ARHGEF7*, $n = 23$ for *CDC42*; Hippocampus $n = 20$ for *RAC1*, *RAC1B*, and *ARHGEF7*, $n = 21$ for *CDC42*) and depressed subjects (Depressed: PFC $n = 22$ for *RAC1*, $n = 25$ for *RAC1B*, $n = 23$ for *ARHGEF7*, and $n = 24$ for *CDC42*; Hippocampus $n = 20$ for *RAC1*, and $n = 22$ for *RAC1B*, *ARHGEF7*, and *CDC42*) normalized to the geometric mean of the three reference targets (*GAPDH*, *CYC1*, *PPIA*). Results are expressed as fold change in mRNA levels. Values are fold change ± SD. No significant differences between the depressed group and the control group were determined for any of the genes.

statistically significant effect of drugs and ethanol on the expression of *PAK2* in the PFC ($F = 3.98$, $p = 0.0449$). However, subsequent post hoc tests for multiple comparisons using the Bonferroni correction revealed no significant differences among the three groups. Moreover, ANCOVA on each of the three groups compared to control group did not reveal any significant differences in the *PAK2* expression in the PFC. We conclude that the differences in the *PAK1* and *PAK3* expression in the PFC and the hippocampus between the depressed group and normal controls do not appear to be related to the presence of psychotropic drugs and ethanol.

To evaluate potential effects of antidepressants and ethanol on coordinated gene expressions correlation analyses were performed. Spearman correlation coefficients and p values with Bonferroni correction for multiple comparisons were calculated for all genes on normalized mRNA expression levels independently in each brain region. No significant correlations were detected in any of the three groups. Consequently, no differences were detected between subjects with depression who were free of any psychotropic medication and those who had been taking antidepressants or ethanol. This would suggest that the presence of antidepressants or ethanol might not have a significant influence on coordinated gene expressions. However, this observation is limited by the small n of analyzed samples (seven in the group 1, 14 in the group 2, and four in the group 3).

To assess the effect of gender on gene expression we analyzed the data by a two-way ANOVA independently for each brain region with diagnosis and gender as categorical independent variables adjusting the effects of age, PMI, and brain pH. In the PFC, a gender effect with tendency toward significance was detected for *PAK3* ($F = 4.04$, $p = 0.0510$) but no significant gender by diagnosis interaction was revealed ($F = 0.72$, $p = 0.3999$). In accordance, ANCOVA on males and females separately did not reveal any significant differences in the *PAK3* expression in the PFC of depressed male or female subjects when compared to control males or females. No gender effect on *PAK3* expression was detected in the hippocampus. On the other hand, in the hippocampus, a significant gender effect was detected for *PAK1* ($F = 5.19$, $^{*}p = 0.0294$). Here, also a significant gender by diagnosis interaction was revealed ($F = 4.47$, $^{*}p = 0.0422$). ANCOVA on males and females separately revealed a significant difference in the *PAK1* expression in the hippocampus of depressed males when compared to control males ($F = 16.48$, $^{**}p < 0.01$ after Bonferroni correction, FC = 1.56). No differences were detected in depressed females (Fig. 5). No gender effect on *PAK1* expression was detected in the PFC.

DISCUSSION

The highly conserved PAK family of serine/threonine kinases participates in the regulation of cytoskeletal dynamics, and therefore is implicated in a wide range of physiological and pathological processes (Kreis and Barnier, 2009; Ma et al., 2012; Ba et al., 2013; Rane and Minden, 2014). In the present study, we show that in the brain of subjects with depression mRNA levels of two members of group I PAK predominantly expressed

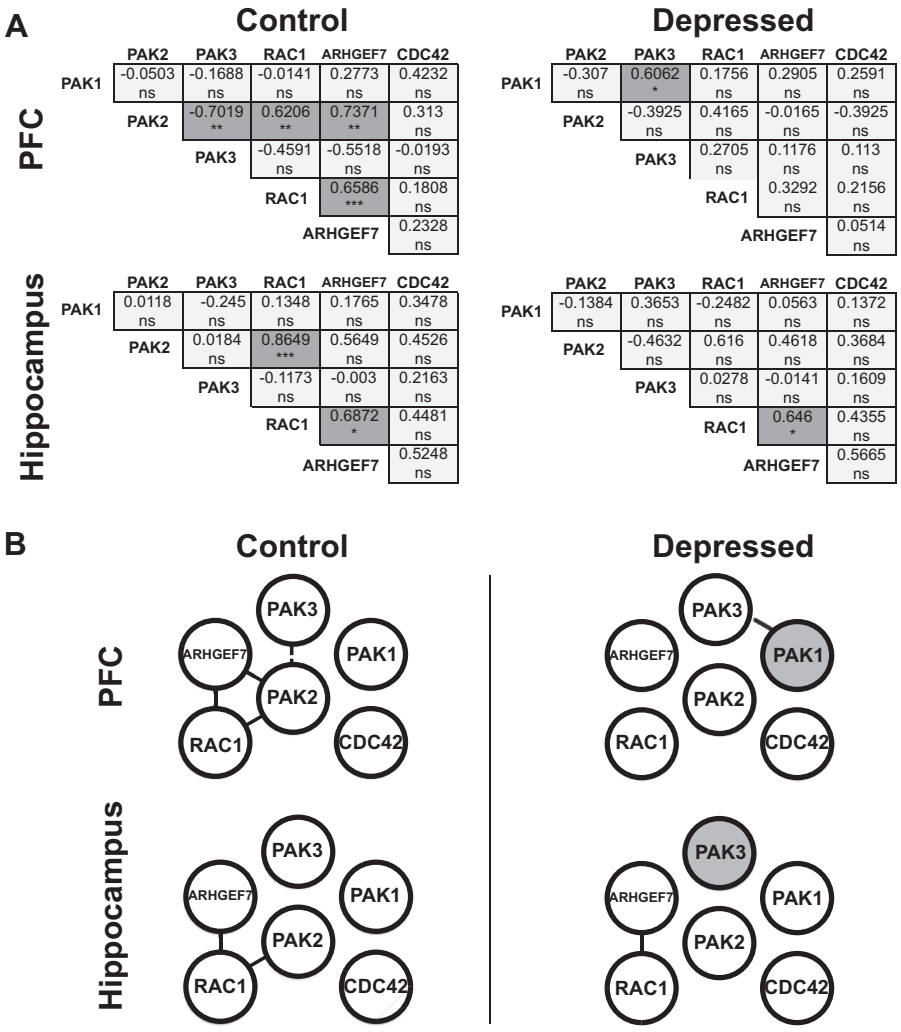

Fig. 4. Coordinated expression is altered in the brain of subjects with depression. (A) Correlation analysis of mRNA expression levels in the PFC and in the hippocampus. In the correlation matrices all pairwise Spearman correlation coefficients calculated on normalized mRNA expression levels are shown. Cross-correlations were performed between the expression level of the six different genes for the depression group and for the control group independently in each brain area. This resulted in fifteen separate comparisons for each group in each area. Statistically significant Spearman correlation p values (two-tailed) are indicated by asterisks (Bonferroni corrected significance threshold, after correction: $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, ns = no significant; dark gray indicates a statistically significant correlation, light gray indicates a non significant correlation). In the PFC and in the hippocampus of depressed subjects, significant correlations appear less frequently than in the control PFC and the control hippocampus. In the PFC, decrease in the frequency of significant correlations between controls and depressed subjects is statistically significant (chi-square = 6, $^{*}p = 0.0143$). No statistical difference is detected in the hippocampus (chi-square = 1.034, $p = 0.3091$). (B) Network of the gene coexpression pattern in the PFC and in the hippocampus in control and depressed group. Gene pairs that show a significant correlation are indicated: a positive significant correlation (full line); a negative significant correlation (dashed line). Dark circles indicate differentially expressed genes. Differences between control and depressed groups are observed in both tissues.

in neurons, *PAK1* and *PAK3*, are significantly diminished in the PFC and in the hippocampus respectively. Moreover, a significant gender effect was detected for *PAK1* expression in the hippocampus, where a significant difference was detected in depressed males but not females. No differences were observed for the ubiquitously expressed *PAK2*.

PAK3 is the only group I member whose expression is relatively restricted to neurons whereas *PAK1* is highly expressed in the nervous system but also in other tissues (Manser et al., 1995; Teo et al., 1995; Rousseau et al., 2003). Changes in their activity have been detected in pathology of some cognitive and neurodegenerative disorders (Ma et al., 2012). For example, X-linked mental retardation can be caused by mutations in *PAK3* that disrupt actin dynamics in dendritic spines (Allen et al., 1998). On the other hand, in Alzheimer's disease, both *PAK1* and *PAK3* are significantly decreased in the hippocampus and *PAK3* also in the temporal cortex (Zhao et al., 2006). Moreover, PAK1 is involved in the pathology of Huntington's disease (Luo et al., 2008). The observed dysregulation of *PAK1* and *PAK3* in the brain of subjects with depression in the present study suggests that disruption in the signaling of both PAKs might also play a role in the pathology of human depression.

Both PAK1 and PAK3 have been shown to be critically involved in biological mechanisms associated with neurodevelopment, neuroplasticity and maturation of the

nervous system such as neurite outgrowth, spine morphogenesis and maintenance, and synapse formation (Rashid et al., 2001; Hayashi et al., 2002, 2007; Boda et al., 2004; Bryan et al., 2004; Zhang et al., 2005; Cobos et al., 2007; Kreis et al., 2007; Dubos et al., 2012). They both share important homologies with regard to structure and possibly also functions (Hofmann et al., 2004). For example, in spine morphogenesis, PAK1 and PAK3 share distinct but also compensatory roles and their activity may overlap allowing the compensation of the PAK3 deficit by PAK1 (Boda et al., 2008). Furthermore, some PAK functions may depend on a crosstalk between PAK1 and PAK3 during the activation process since PAK3 proteins were shown to form heterodimers with PAK1 (Combeau et al., 2012).

Knockout of either PAK1 or PAK3 alone does not cause a robust morphological phenotype in mice (Meng et al., 2005; Asrar et al., 2009; Kelly and Chernoff, 2012). On the other hand, double PAK1 and PAK3 knockout are severely impaired in postnatal brain growth. Reduced brain size is observed due to simplified neuronal dendrites/axons, neurons with smaller soma and significant increase in cell density without global neuron loss. As a consequence, synapse development and function is impaired in such a way that these mice exhibit reduced synapse density, defects in dendritic spines, LTD, LTP, as well as learning and memory deficits, hyperactivity and increased anxiety (Huang et al., 2011).

Remarkably, in depressed patients structural and functional alterations have been observed in the PFC and hippocampus, where reduced volume is inversely correlated with length of illness, time of treatment, and severity of depression (Drevets et al., 2008; MacQueen and Frodl, 2011). In patients, characterization of the cellular determinants underlying these changes is limited. Nevertheless, postmortem studies demonstrate decreased neuronal cell body size, atrophy of dendritic processes, as well as decreased synapse number (Rajkowska et al., 1999; Stockmeier et al., 2004; Drevets et al., 2008; Pittenger and Duman, 2008; Kang et al., 2012). Thus, we hypothesize that neuronal size, complexity and synaptic properties regulated by PAK1/PAK3 dependent signaling may be a key factor responsible for volumetric abnormalities of hippocampus and PFC in depression resulting in altered connectivity of these regions.

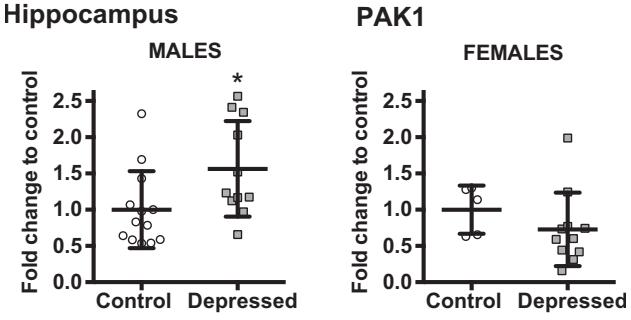

Fig. 5. Effect of gender on mRNA levels of *PAK1* in the hippocampus of non-psychiatric controls (Control: Males $n = 13$; Females $n = 5$) and depressed subjects (Depressed: Males $n = 11$; Females $n = 11$) normalized to the geometric mean of the three reference targets (*GAPDH*, *CYC1*, *PPIA*). Results are expressed as fold change in mRNA levels. Values are fold change ± SD. Significant difference between the subjects with depression and the control group was determined in the *PAK1* expression in the hippocampual tissue of depressed males when compared to control males ($F = 16.48$, $^{**}p < 0.01$). No differences were detected in depressed females. Significant effects are marked by asterisks showing Bonferroni corrected significance thresholds.

Regulation of PAK activity is a complex process that apart from the homo/heterodimer formation and phosphorylation events is also dependent on activators. In particular, the small GTPases RAC1 and CDC42 play a central role in the activation of PAK1 and PAK3 (Manser et al., 1994). Here, we observed that neither *RAC1* nor *CDC42* expression is altered in the hippocampus or in the PFC of subjects with depression. Similarly, we did not observe any significant changes in neither of the tissues for *RAC1B*, a constitutively active splice variant of *RAC1*, whose increased expression has been found to contribute to neurodegeneration seen in Alzheimer's disease (Perez et al., 2012).

A number of other signaling proteins can also play key roles in PAK activation. In fact, PAK1 can even lie upstream to RAC, by binding to the ARHGEF7 (also known as betaPIX for PAK-interacting exchange factor), which is a guanine nucleotide exchange factor (GEF) for RAC (Manser et al., 1998; Obermeier et al., 1998; Rane and Minden, 2014). Evidence shows that the GIT1/ARHGEF7/PAK complex plays a crucial role in neuronal signaling during neurite outgrowth and spine morphogenesis (Obermeier et al., 1998; Zhang et al., 2005). Alterations in this complex has been suggested to associate with decreased neuronal connectivity and cognitive defects such as those seen in nonsyndromic mental retardation (Zhang et al., 2005). Furthermore, GIT1/ARHGEF7/RAC1/PAK pathway has been also recently shown to be required for $GABA_A$ receptor synaptic stability and inhibitory neurotransmission (Smith et al., 2014). In the present work, when *ARHGEF7* mRNA expression was measured in the hippocampus or in the PFC of subjects with depression, we did not observed any alterations. The same was true for the mRNA expression of *GIT1* assessed in our previous study (Fuchsova et al., 2015).

Our observations are in line with findings by Golden et al. (2013)). In their recent work, transcriptional profiling for Rho GTPase–related genes revealed that *RAC1* is transcriptionally downregulated through an epigenetic mechanism in the NAc of subjects with depression and after chronic social defeat stress in animal model of depression-like behaviors. However, they did not observe downregulation of *RAC1* in other brain structures in susceptible mice. The expression levels of neither *PAK1* nor *ARHGEF7* were affected in the NAc by social defeat (Golden et al., 2013). We suggest that observed differences in the present work reflect region-specific effects of depression in agreement with rodent studies that demonstrate reductions in dendrite complexity and spine density in the hippocampus and PFC but increases in the basolateral amygdala and NAc (Duman and Duman, 2015). We hypothesize that depression is associated with

a shift toward synaptic instability through region-specific dysregulation of different Rho GTPase–related mechanisms.

Depression may lead to the downregulation of *PAK1* and *PAK3* through different mechanisms. Epigenetic mechanisms could be a possible explanation since both genes have been identified as targets of posttranscriptional regulation by microRNAs. *PAK1* and *PAK3* are validated targets of miR-320 and miR-335 respectively in humans (Tavazoie et al., 2008; Helwak et al., 2013). miR-320 and miR-335 were found to be differentially expressed in blood of depressed patients (Camkurt et al., 2015; Li et al., 2015). Whether these microRNAs regulate *PAK1/PAK3* expression in the hippocampus and PFC remains to be established. Another possible mechanism could involve Erk5 activation. In a recent study, Komaravolu et al. showed that endothelial Erk5 activation decreased *PAK1* mRNA levels and that this process is mediated by the Krüppel-like transcription factor KLF2 (Komaravolu et al., 2015). It would be interesting to evaluate association of Erk5/KLF2 with depression.

Gene products functioning along in common signaling pathways are supposed to display higher coordination in their expression levels than random sets of gene products (Vidal et al., 2011). In accordance, in our investigation we detected significant correlations between *PAK2* and *RAC1*, and *ARHGEF7* and *RAC1* in the control hippocampus. In the control PFC, we detect the same positive significant correlations. Moreover, a positive correlation between *ARHGEF7* and *PAK1* and a negative correlation between *PAK3* and *PAK2* are observed indicating that co-regulation of some of the studied gene products exists in the hippocampus and the PFC of humans.

However, the coordination we observe is disrupted or reorganized in subjects with depression. In the PFC, the only significant correlation is a new positive correlation between *PAK3* and *PAK1*. In the hippocampus, one correlation between the expression levels of *ARHGEF7* and *RAC1* detected also in controls remains significant. Different authors have provided important insights into the pathways disbalanced in psychiatric disease using the analysis of gene coexpression networks (Merali et al., 2004; Poulter et al., 2010; Zhurov et al., 2012; Gaiteri et al., 2014). Our observations fit well with the conclusions that pathological mechanisms resulting in depression affect the coordination of gene expression in the brain (Merali et al., 2004; Gaiteri et al., 2010, 2014; Poulter et al., 2010; Zhurov et al., 2012). The observed changes may reflect loss of coordinated transcriptional response or may result from other regulatory processes such as altered mRNA stability or microRNA actions.

Since PAK3 and PAK1 signaling are linked together, and PAK1 and PAK3 share distinct but also compensatory roles, it is tempting to speculate that as a consequence of their differential expression as well as altered coexpression in the brain of subjects with depression, their function in neurite outgrowth, spine morphogenesis and synaptic plasticity would be affected with consequences for neuronal complexity and connectivity. This would fit with the observations that dendritic complexity and synapse density are reduced in depression (Drevets et al., 2008; Kang et al., 2012; Duman and Duman, 2015). Observed differences in the expression of *PAK1* and *PAK3* between control and subjects with depression in the present work are low. Nevertheless, major depressive disorder and other neuropsychiatric disorders are complex diseases implicating a large number of genes. Each of them confers a small and incremental risk that potentially converges in dysregulated biological pathways, cellular functions, local circuit changes, and, eventually scales up to brain region pathophysiology (Belmaker and Agam, 2008). We suppose that the collective effect of the changes in the balance between mRNA levels as a result of altered expression as well as altered coexpression of these genes in a genetically complex disease such as depression can lead to alterations in the molecular interactions of cellular pathways, affect neuronal connectivity, and, thus, contribute to the behavioral symptoms of depression.

One of the limitations of the present study is that the samples originate from suicide victims. Thus, the present observations cannot be automatically generalized to depression without suicidal intent, even though all subjects that had died by suicide had a history of depression (depressed phase in case of bipolar disorder). Moreover, we cannot say that suicide itself was without effect (van Heeringen, 2001). Gene expression patterns associated with suicidality have been reported (Sequeira et al., 2007, 2009), although some overlap exists with those associated with major depression. Furthermore, additional studies to evaluate the effect on the protein levels of studied genes are required. Nevertheless, despite these limitations, we think that the reported changes in mRNA levels and disruption of the coordinated gene expression of the PAK1/PAK3 signaling signify an important contribution to the efforts of identifying potential candidates associated with mental illness.

Since PAKs are common downstream substrates of a number of signaling pathways, including those mediated by the Rho/Ras GTPases, neurotrophins, and glutamate receptors (Kreis and Barnier, 2009), and many molecules involved in these pathways have been shown to be important for activity-dependent neuronal morphogenesis *in vitro* (Kreis and Barnier, 2009), it is possible that the PAK1/PAK3-dependent actin reorganization may represent a converged mechanism dysregulated in the pathology of depression.

CONCLUSION

Abnormalities in the *PAK1* and *PAK3* mRNA levels as well as their altered coexpression patterns were observed in the postmortem brain of subjects with depression. We hypothesize that dysregulated PAK1/PAK3 dependent signaling may be a key factor responsible for volumetric abnormalities observed in the hippocampus and in the PFC in depression resulting in altered connectivity of these regions.

CONFLICT OF INTEREST

The authors report no biomedical financial interests or potential conflicts of interest. Dr. Pandey reports grants from USA: National Institute of Health, during the conduct of the study.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: BF. Performed the experiments: BF AAJ HR. Analyzed the data: BF AAJ. Interpretation of data: BF AAJ ACF GNP. Wrote the paper: BF.

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