Hyperforin and Miquelianin from St. John's Wort Attenuate Gene Expression in Neuronal Cells After Dexamethasone-Induced Stress

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ABSTRACT

Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis plays an important part in the development of depressive symptoms. In this study, the effects of a commercial St. John's wort extract (STW3-VI), hyperforin, miquelianin, and the selective serotonin reuptake inhibitor citalopram on the expression of genes relevant to HPA axis function were investigated in human neuronal cells. SH-SY5Y cells were treated with STW3-VI (20 µg/mL), hyperforin (1 µM), miquelianin (10 µM), or citalopram (10 µM) in the presence of the glucocorticoid receptor agonist dexamethasone (DEX, 10 µM) for 6 h and 48 h, respectively. Quantitative real-time polymerase chain reaction was used to determine the expression of FKBP5 (FK506 binding protein 51), CREB (cAMP responsive element binding protein), GRIK4 (glutamate ionotropic receptor kainate type subunit 4), VEGF (vascular endothelial growth factor), NET (norepinephrine transporter), and ARRB (β-arrestins), promising biomarkers of antidepressant therapy. Using DEX to mimic stress conditions, it was shown that the gene expression pattern of FKBP5, CREB, GRIK4, VEGF, NET, and ARRB2 in SH-SY5Y cells is time- and treatment-dependent. Most pronounced effects were observed for FKBP5: after 6 h of co-incubation, only STW3-VI could reverse the DEX-induced increase in FKBP5 expression, and after 48 h, citalopram, miquelianin, and hyperforin also reversed the glucocorticoid-induced increase in FKBP5 mRNA expression. The effects observed on FKBP5, CREB, GRIK4, VEGF, NET, and ARRB2 are in good correlation with published data, suggesting that this *in vitro* model could be used to screen the responsiveness of antidepressants under stress conditions.

Introduction

The flowering herb of *Hypericum perforatum* L. (Clusiaceae), commonly referred to as St. John's wort (SJW), is used in many countries for the treatment of mild to moderate forms of depression (for review see [1]). Several bioactive compounds have been detected in the plant extract, and the pharmacological activity has been reviewed previously [1]. It has been documented that the antidepressant activity of *Hypericum* extracts can be attributed to the phloroglucinol derivative hyperforin, to the naphthodianthrone hypericin, and to several flavonoids. Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis plays an important role in the pathogenesis of depression [2]. It has been shown that chronic exposure to environmental stress contributes to the development of depression. The HPA axis, as one of the key endocrine adaptors against stressors, is one of the major systems involved in physiological stress response.

The effects of SJW and single compounds on the expression of genes that are potentially involved in the regulation of stress-induced aberrations of HPA axis function have been reported earlier by our group using *in vivo* models [3, 4]. To further investigate the action mechanism(s) of SJW (commercial preparation STW3-VI) and its active compounds hyperforin and miquelianin, we established an *in vitro* stress model using the human neuroblastoma cell

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line SH-SY5Y since they are widely employed in neuropharmacology and are considered a suitable model for investigating the cellular and molecular basis of depression [5]. Using these cells, we assessed the expression of genes using real-time qRT-PCR with and without treatment of dexamethasone (DEX). Cells were treated with the glucocorticoid receptor (GR) agonist DEX to mimic stress conditions [5]. The selected panel of genes comprises receptors and regulators of neuronal function, which had been previously used in other cell types to investigate effects of neurotropic substances [6,7]. Glucocorticoids affect cellular and molecular events in brains by modulating the expression of many genes during stress. It was therefore of interest to investigate the effects of STW3-VI, hyperforin, miquelianin (▸ **Fig. 1**), and the selective serotonin-reuptake inhibitor (SSRI) citalopram on gene expression alone and in combination with DEX. Quantitative real-time PCR was used to determine the expression of FKBP5 (FK506 binding protein 51), CREB (cAMP responsive element binding protein), GRIK4 (glutamate ionotropic receptor kainate type subunit 4), VEGF (vascular endothelial growth factor), NET (norepinephrine transporter), and ARRB (β-arrestins), which have been shown to be meaningful biomarkers in the treatment response for depression [8].

Results

DEX alone or in combination with the different substances did not affect cell viability in SH-SY5Y cells at 6 h or 48 h of co-incubation (▸ **Fig. 2 A–E**). The time points were chosen based on the study of Donnici et al. [9], who showed time-dependent differences in the expression of BDNF (brain-derived neuropathic factor) after treatment of various antidepressant.

For the initial co-incubation experiments, DEX was used in concentration of 1–100 μM, as reported in the literature [10]. The effects on cell viability are shown in ▸ **Fig. 2 A, B**. The ATP assay revealed that DEX at concentrations of 1, 10, and 100 μM given for 6 and 48 h (▸ **Fig. 2 A, B**) was not toxic for SH-SY5Y cells. Cell viability slightly decreased after 48 h for 100 μM DEX (82% viability).

To evaluate whether the citalopram, hyperforin, miquelianin, or STW3-VI had any toxic effects, the viability of SH-SY5Y cells after exposure to the various compounds for 6 or 48 h alone (▸ **Fig. 2 C, D**) or in combination with DEX (10 μM) (▸ **Fig. 2 E, F**) was determined using the ATP assay. As shown in ▸ **Fig. 2 C**, citalopram (0.1–10 μM), miquelianin (0.1–10 μM), hyperforin (0.1–1 μM), or STW3-VI (10–50 μg/mL) did not induce any significant changes in cell viability after 6 h. However, after 48 h of exposure, STW3-VI at a concentration of 50 μg/mL significantly reduced cells viability as shown in ▸ **Fig. 2 D**. Based on these experiments, for the following co-incubation treatments with DEX, citalopram and miquelianin were used in concentrations of 10 μM, hyperforin in 1 μM, and STW3-VI in 20 μg/mL, respectively.

As shown in ▸ **Fig. 3**, FKBP5 was upregulated after 6 h of DEX treatment (1.4-fold) (▸ **Fig. 3 A**), but an even stronger increase in mRNA expression was observed after 48 h (2.0-fold) (▸ **Fig. 3 B**). While after 6 h of co-incubation only STW3-VI could reverse the DEX-induced increase in FKBP5 expression, after 48 h citalopram, miquelianin and hyperforin abolished the glucocorticoid-induced increase in FKBP5 mRNA expression.
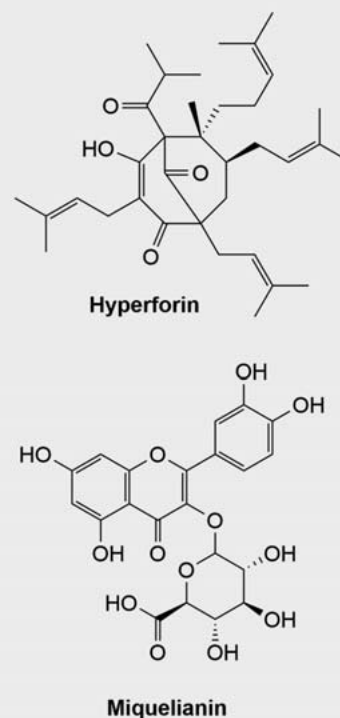

▸ **Fig. 1** Chemical structures of hyperforin and miquelianin.

In the present study, a decrease in the gene expression of CREB was observed after 6 h and 48 h of co-incubation with DEX; however, the effect level remained the same. After 6 h, only miquelianin, hyperforin, and STW3-VI abolished the DEX stress-induced decrease in CREB expression. Co-incubation of cells with DEX and citalopram, miquelianin, hyperforin, or STW3-VI for 48 h showed that only hyperforin and the *Hypericum* extract could increase CREB expression by 1.6- and 1.3-fold, respectively.

We found differing effects of citalopram, hyperforin, miquelianin, and STW3-VI on GRIK4 expression in SH-SY5Y cells. Time-dependent effects were observed after co-incubation with DEX. After 6 h, GRIK4 expression was markedly decreased after DEX treatment (0.58-fold), while co-incubation of DEX with citalopram, miquelianin, hyperforin, and STW3-VI increased GRIK4 mRNA expression by 1.4–1.6-fold, respectively (▸ **Fig. 3 A**). However, after 48 h, DEX-treated cells still showed a decrease in GRIK4 expression, but the effect was less pronounced than after 6 h (▸ **Fig. 3 B**). The treatment effect with the various test compounds also seemed to be time-dependent since after 48 h we did not observe a difference in fold expression compared to the DEX-treated control. Only in cells that were cotreated with DEX and hyperforin did a marginal increase in GRIK4 mRNA expression occur (▸ **Fig. 3 B**).

In the present study, time- and treatment-dependent effects have been observed for VEGF mRNA expression in SH-SY5Y cells. VEGF mRNA expression was decreased when cells were exposed to DEX, the effect being time-dependent. The DEX-induced de-

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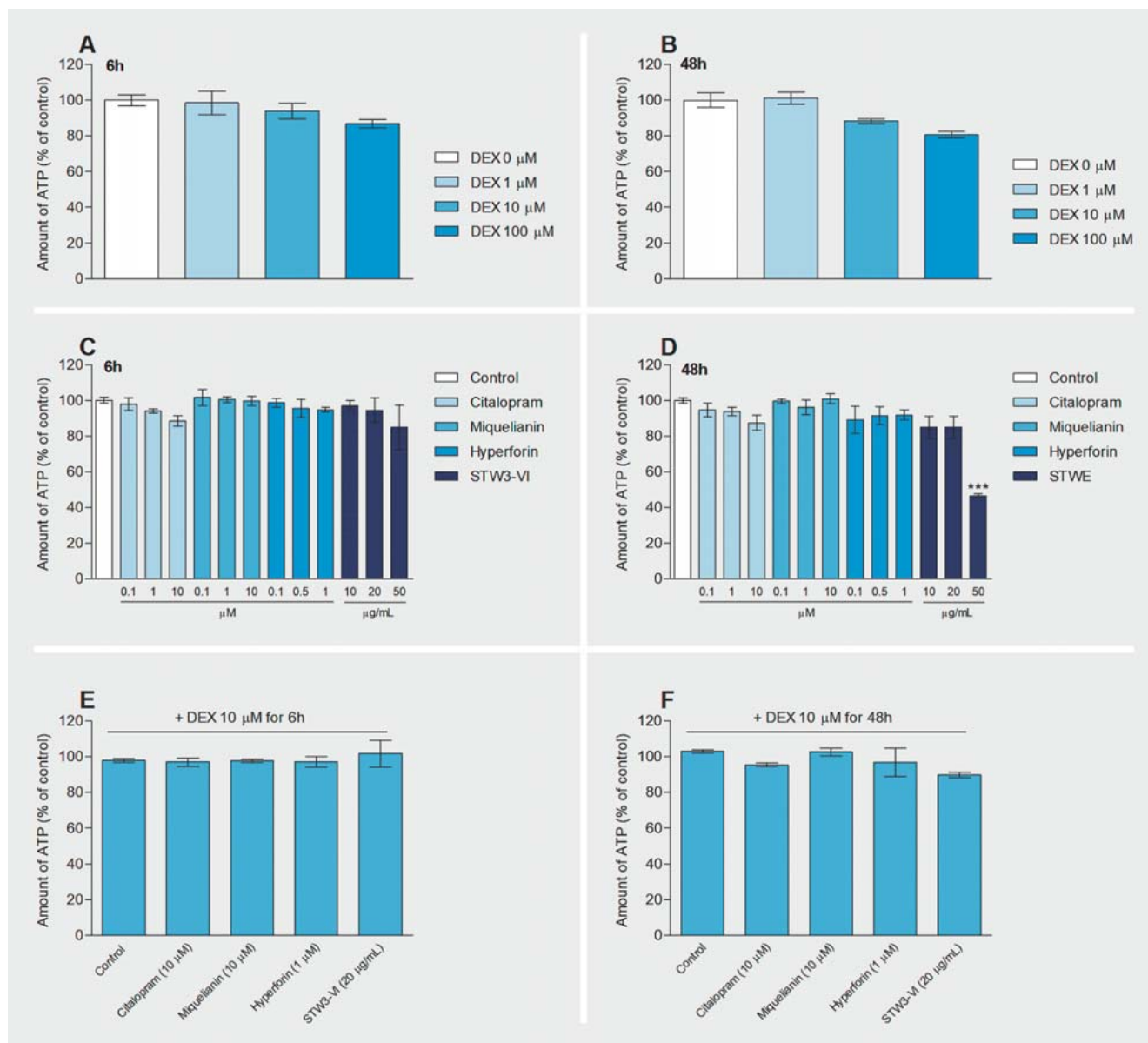
▶ **Fig. 2** **A, B** Effect of DEX (1, 10, and 100 µM) on cell viability of SH-SY5Y cells (ATP assay). The cells were cultured with DEX for 6 and 48 h. **C, D** The effect of citalopram (0.1–10 µM), miquelianin (0.1–10 µM), hyperforin (0.1–1 µM), and STW3-VI (10–50 µg/mL) after 6 and 48 h on cell viability in SH-SY5Y cells. **E, F** The cells were cotreated with citalopram, miquelianin, hyperforin, and STW3-VI for 6 and 48 h, after which the ATP assay was performed. Experiments were performed in triplicate and results are shown as mean ± SEM from three independent experiments (n = 9). *** p < 0.001 vs. control treated cells.

crease was more pronounced after 48 h (0.54-fold) than after 6 h (0.82-fold) (▶ **Fig. 3 A, B**).

While after 6 h of co-incubation with DEX citalopram, miquelianin, hyperforin, and STW3-VI increased VEGF mRNA expression by up to 1.5-fold (hyperforin), after 48 h, only STW3-VI showed an increase in VEGF mRNA expression.

NET expression was increased after 6 h (1.35-fold) and 48 h (1.25-fold) after treatment of the cells with DEX (▶ **Fig. 3 A, B**). The stress-induced increase in NET mRNA expression was attenuated by antidepressant treatment at both time points as displayed in ▶ **Fig. 3 A, B**.

Treatment with DEX, mimicking stress conditions, caused a marked decrease in the expression of ARRB2. The effect was time-dependent since it was more pronounced after 6 h (0.39-fold) of incubation compared to 48 h (0.69-fold). However, the drug treatment effect also was time-dependent. After 6 h of co-incubation with DEX, only hyperforin diminished the stress-induced effect (0.96-fold), while after 48 h hyperforin (1.39-fold) and STW3-VI (1.43-fold) could reverse the effect of DEX.

To confirm the results in mRNA expression levels of FKBP5 and VEGF, ELISA assays were carried out. ELISA for FKPB5 and VEGF production were performed only after 48 h since the gene expres-

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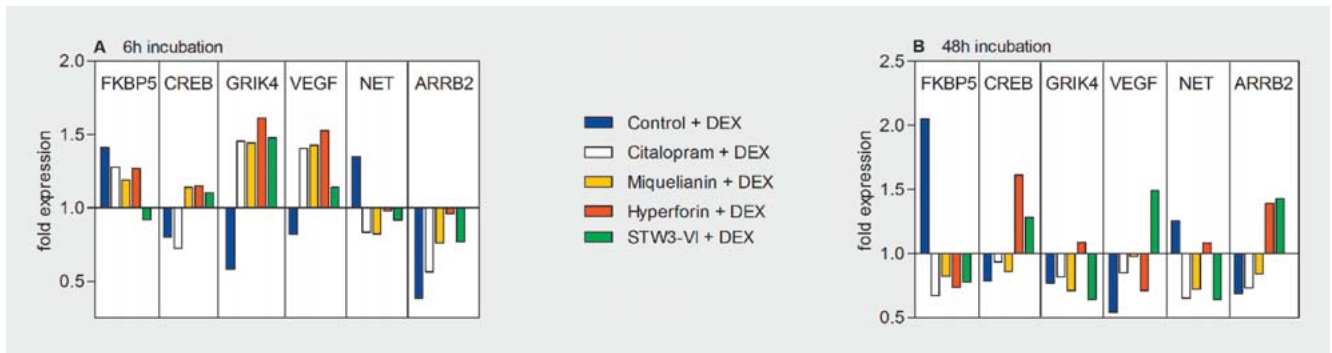
► **Fig. 3** Fold changes occurring in FKBP5, CREB, GRIK4, VEGF, NET, and ARRB2 expression after (**A**) 6 h co-incubation with DEX (10 µM) or (**B**) 48 h co-incubation with DEX (10 µM). Citalopram and miquelianin were used in a concentration of 10 µM, hyperforin in a concentration of 1 µM, and STW3-VI at 20 µg/mL. Data represent the mean of three independent experiments. The bars represent an increased (> 1) or decreased (< 1) change in comparison to the unstressed control (for DEX) or to the DEX-treated control (for treatments). The absolute numbers and corresponding confidence intervals are presented in ► **Table 1**.

► **Table 1** Fold expressions of FKBP5, CREB, GRIJ4, VEGF, NET, and ARRB2 after 6 h co-incubation with DEX (10 µM) or 48 h co-incubation with DEX (10 µM). Citalopram (10 µM), miquelianin (10 µM), (1 µM), and STW3-VI (20 µg/mL) were co-incubated with DEX for the designated time intervals. The numbers represent an increased (> 1) or decreased (< 1) change in comparison to the unstressed control (for DEX) or to the DEX-treated control (for treatments). Data are shown as mean and confidence interval of three independent experiments.

Symbol	Control + DEX	Citalopram + DEX	Miquelianin + DEX	Hyperforin + DEX	STW3-VI +DEX
6 h + DEX					
FKBP5	1.41 (1.28–1.55)	1.28 (1.23–1.31)	1.19 (1.08–1.30)	1.27 (1.15–1.31)	0.92 (0.88–0.95)
CREB	0.80 (0.78–0.81)	0.72 (0.59–0.88)	1.14 (1.08–1.20)	1.15 (1.04–1.27)	1.10 (1.08–1.21)
GRIK4	0.58 (0.50–0.67)	1.45 (1.23–1.70)	1.44 (1.22–1.70)	1.61 (1.55–1.68)	1.48 (1.15–1.89)
VEGF	0.82 (0.76–0.87)	1.40 (1.14–1.72)	1.43 (1.28–1.59)	1.53 (1.46–1.59)	1.14 (1.05–1.23)
NET	1.35 (1.31–1.39)	0.84 (0.76–0.92)	0.82 (0.74–0.91)	0.98 (0.97–0.99)	0.92 (0.86–0.97)
ARRB2	0.39 (0.37–0.40)	0.57 (0.56–0.58)	0.76 (0.67–0.86)	0.96 (0.95–0.97)	0.77 (0.67–0.87)
48 h + DEX					
FKBP5	2.05 (1.82–2.31)	0.67 (0.56–0.80)	0.82 (0.77–0.87)	0.74 (0.65–0.83)	0.78 (0.69–0.87)
CREB	0.78 (0.74–0.82)	0.94 (0.84–1.03)	0.86 (0.79–0.94)	1.61 (1.53–1.84)	1.28 (0.17–1.39)
GRIK4	0.77 (0.70–0.84)	0.82 (0.77–0.86)	0.71 (0.63–0.80)	1.09 (0.80–1.47)	0.64 (0.49–0.83)
VEGF	0.54 (0.49–0.60)	0.85 (0.82–0.86)	0.98 (0.91–1.04)	0.71 (0.62–0.80)	1.49 (1.23–1.80)
NET	1.25 (1.20–1.30)	0.65 (0.63–0.67)	0.72 (0.63–0.82)	1.08 (1.07–1.09)	0.64 (0.52–0.76)
ARRB2	0.69 (0.59–0.79)	0.73 (0.71–0.75)	0.84 (0.80–0.88)	1.39 (1.35–1.42)	1.43 (1.42–1.66)

sion experiments revealed that the effect is more pronounced after a longer incubation time. As shown in ► **Fig. 4A**, FKPB5 production after treatment with DEX (10 µM) was increased after 48 h compared to the untreated control group. Co-incubation with citalopram (10 µM), miquelianin (10 µM), hyperforin (1 µM), and STW3-VI (20 µg/mL) diminished the glucocorticoid-induced increase in FKBP5. Addition of DEX (10 µM) decreased VEGF secretion (► **Fig. 4B**) after a 48-h incubation. In the presence of citalopram (10 µM), miquelianin (10 µM), and STW3-VI (20 µg/mL), the DEX-induced decrease in VEGF could be partly reversed. However, cotreatment of DEX with hyperforin caused no changes in VEGF secretion after 48 h.

Discussion

Our current study provides new insights on the molecular mechanism of SJW and two of its active compounds. To the best of our knowledge, no data are available yet on the effect of SJW, miquelianin, and hyperforin on the expression of genes that have been identified as promising biomarkers of antidepressant therapy [8]. We could demonstrate in our study that the gene expression pattern of FKBP5, CREB, GRIK4, VEGF, NET, and ARRB2 in SH-SY5Y neuronal cells is time- and treatment-dependent. It is also shown that hyperforin and miquelianin, two compounds that have been shown to possess antidepressant activities, exert different effects on various genes.

▶ **Fig. 4** Effects of citalopram (10 µM), miquelianin (10 µM), hyperforin (1 µM), and STW3-VI (20 µg/mL) on FKBP5 (**A**) and VEGF levels (**B**) after 48 h of DEX (10 µM) induced stress. FKBP5 and VEGF secretion were quantified by commercial ELISA kits. Results are expressed as mean ± SEM (n = 3). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. DEX control group; ## $p < 0.01$ and ### $p < 0.001$ vs. untreated control group.

It is well known that dysregulation of the HPA axis plays an important part in the development and maintenance of depressive symptoms [2]. The GR is a cytosolic transcription factor activated by the stress hormone cortisol [11]. The GR translocates to the cell nucleus after cortisol binding. The translocation of GR is a complex process and involves diverse molecular chaperones and cochaperones including the FK506 binding protein 51 (FKBP5) [12]. FKBP5 regulates cortisol levels by attenuating the negative feedback of glucocorticoids on the HPA axis [12]. Upon activation of GR, FKBP5 mRNA is upregulated. Interestingly, several FKBP5 genetic variants have been identified, which have been linked with various stressors (e.g., psychosocial stress) and diverse phenotypes ranging from depression, psychosis, or post-traumatic stress disorders to cardiovascular disease or cancer, supporting the hypothesis that FKBP5 is a stress-sensitive biomarker [13]. Increased FKBP5 mRNA and protein expression have been reported in postmortem frontal cortex from individuals suffering from major depression [14]. These findings in humans were also found in animal models. Guidotti et al. [15], for example, found an increased expression of FKBP5 after chronic mild stress in rodents, which was normalized by chronic antidepressant treatment.

In the present study, we could show that not only the SSRI citalopram but also the SJW extract (STW3-VI) and its active compounds miquelianin and hyperforin were able to abolish the stress-induced increase in FKBP5 mRNA expression. Effects of STW3-VI on HPA axis regulation have been shown earlier by our group [3], and the present mechanistic *in vitro* data are in good correlation with our previous findings, confirming an involvement of STW3-VI in HPA axis regulation.

CREB1 is a member of the leucine zipper family of DNA binding proteins and is under the inhibitory control of several kinases including GSK3A (glycogen synthase kinase 3 alpha) [16]. Several studies have focused on the role of CREB1 in antidepressant response mechanism. CREB1 is upregulated by chronic antidepressant treatment, and increasing CREB levels in rodent models result in antidepressant-like behaviors. Patients taking an antidepressant at the time of death showed an increased level of CREB [17], whereas those who were not medicated at the time of death showed decreased levels of CREB in temporal cortex. In a recent study, Lee et al. [18] showed that CREB phosphorylation increased

time-dependently in SH-SY5Y cells after 20 µM tianeptine (SSRI) treatment, although the mRNA expression of CREB remained unchanged. In another study by Zheng et al. [19], the transcript abundance of CREB in PC12 cells was elevated upon hyperoside treatment. The authors conclude that the possible cellular mechanisms of the hyperoside antidepressant-like effect is related to an elevation of the expression of CREB. Miquelianin and hyperoside are structurally related and only differ in the substituent in position 3 of the aglycone quercetin (hyperoside = quercetin-3-O-galactoside; miquelianin = quercetin-3-O-glucuronide). In our present study, we used miquelianin (quercetin-3-O-glucuronide) representing the group of flavonoids in SJW because it has been shown in earlier studies that this compound can cross the blood-brain barrier [20]. Miquelianin abolished the DEX-induced decrease in CREB, but the effect was time-dependent, being more evident after 6 h than after 48 h.

Our data are also in good correlation with the study of Gibon et al. [21], who demonstrated that a 72-h hyperforin treatment enhanced the expression of CREB in cultured cortical neurons. In our experiments, only STW3-VI and hyperforin could counteract the DEX-induced decrease of CREB gene expression in SH-SY5Y cells after 48 h. In a previous study, Trofimiuk et al. [22] found that an SJW extract increased the levels of pCREB in the aged rat's hippocampus, suggesting an increase of CREB levels contributes to the mechanism of action of this medicinal plant. Comparable to hyperforin, STW3-VI counteracted the DEX-induced decrease in CREB mRNA levels in the current study, although the effect was less pronounced than for the phloroglucinol derivative. Our data further suggest that the activity of STW3-VI cannot be explained by hyperforin and miquelianin alone, since both compounds had partially opposite effects of CREB gene expression. The SSRI citalopram had no effects after 6 h and could only slightly counteract the stress-induced decrease in CREB mRNA expression after 48 h. It is possible that the effect of citalopram on CREB mRNA expression is time-dependent; in the study of Lee et al. [18], CREB phosphorylation increased time-dependently after tianeptine treatment, being more evident after 72 h of incubation than after 24 h.

Glutamate receptors are traditionally divided into the ionotropic, ion channel-associated AMPA, NMDA and kainate receptors 5 (GRIK1–5), and the G protein-coupled metabotropic recep-

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tors [23]. In the last decade, the glutamatergic theory of depression has gained growing interest, as many studies have documented the important role played by the glutamatergic system in the pathophysiology of depression and mechanism of action of antidepressants [24]. Glutamate levels were increased in patients with major depressive disorder by proton magnetic resonance spectroscopy [25], suggesting enhanced glutamatergic transmission. Ionotropic glutamate receptors, such as the GRIK4-encoded kainic acid-type subunit, are responsible for fast-acting neurotransmission. A role for ionotropic glutamate receptors in antidepressant action has been supported by studies in rodents in which chronic treatment with antidepressants, including citalopram, resulted in a region-specific reduction in binding activity of N-methyl-D-aspartate receptors and N-methyl-D-aspartate receptor subunits [26]. Interestingly, decreased expression of the highly related GRIK1 and/or GRIK5 genes has been observed in postmortem brains of patients with major depression and bipolar disorder [27,28]. In neurons, the expression of GRIK4 receptors is downregulated by chronic mild stress and restored with fluoxetine [29]. In our present experiments, GRIK mRNA expression in SH-SY5Y cells was downregulated after DEX treatment, with the effect being more pronounced after 6 h than after 48 h of co-incubation. The treatment effect with various antidepressants also seemed to be time-dependent. After 6 h, citalopram, miquelianin, hyperforin, and STW3-VI could counteract the stress-induced decrease in GRIK4 expression. However, after 48 h, only hyperforin showed a minor effect while all other treatment remained on the DEX control level. To our knowledge, this is the first study demonstrating that natural compounds can reverse a stress-induced decrease in GRIK4 mRNA expression.

VEGF is an important signaling protein involved in vasculogenesis and angiogenesis [30] but is also expressed in the brain and has neuroprotective and neurogenic effects [31]. Several studies demonstrate that VEGF is induced by various classes of antidepressants, such as specific serotonin and norepinephrine reuptake inhibitors that increase VEGF mRNA and protein expression in several types of brain cells [31]. Furthermore, chronic stress exposure decreased [32] and antidepressant administration increased hippocampal VEGF [31]. Interestingly, only a few number of studies exist reporting an increase in VEGF expression after treatment with natural products, mostly in the context of wound healing [33,34]. In a previous study, Tassone et al. [35] demonstrated that VEGF expression increased more than three-fold in DAOY medulloblastoma cells when the cells were treated with hyperforin, which is comparable to our present findings. To our knowledge, the current study is the first study demonstrating a decrease in VEGF mRNA expression in neuroblastoma cells after incubation with DEX. The effect was time-dependent, being more evident after 48 h. However, the antidepressant treatment effect was also time-dependent. After 6 h of co-incubation with DEX, citalopram, miquelianin, and hyperforin reversed the stress-induced decrease in VEGF expression while the SJW extract had only moderate effects. A different picture occurred after 48 h, where STW3-VI was the only treatment, which significantly counteracted the DEX effect. However, the VEGF effects determined by real-time PCR were only partially confirmed by the ELISA assay: a possible explanation is the strict specificity of the immuno-assay, which detects only the 165-isoform of VEGF. The primers used for real-time PCR also detected the most common RNA isoforms.

NET is responsible for the reuptake of norepinephrine and is mainly located on presynaptic terminals of noradrenergic nerves throughout central and peripheral nervous systems [36]. NET is a key protein in the regulation of noradrenergic transmission, and therefore, its expression and function might be affected during stress. Studies reporting the effects of acute stress on NET expression have shown inconsistent results and mRNA levels of NET or NET binding sites in the locus coeruleus of rats and mice were reduced [37], unchanged [38], or increased [39] by acute stress. Although these studies are controversial and incomplete, they suggest that NET can be affected by stress. Glucocorticoids are the key hormones released during stress and therefore may play an essential role in the pathophysiological regulation of NET. So far, there are very few reports regarding the possible regulatory effects of stress hormones on NET expression in vitro. Sun et al. [40] used SK-N-BE(2)C cells and PC12 cells, two cell lines that naturally express NET proteins, to assess the regulatory effect of corticosterone on NET expression. The authors demonstrated that there is a closer association between this hormone and the in vitro expression and function of NET. The results of Sun et al. [40] after 48 h are in line with the findings of Zha et al. [41], who showed that exposure of neuroblastoma SK-N-BE(2)M17 cells to DEX significantly increased NET mRNA and protein levels in a time- and dose-dependent manner. In our experiments, NET mRNA levels were increased after DEX stress after 6 and 48 h, the effect being more pronounced after 6 h. In an earlier study, Zavosh et al. [42] used the human neuroblastoma cell line SH-SY5Y to estimate NET mRNA expression following incubation with desipramine for 24 or 72 h. The authors could show that mRNA levels of NET were decreased by desipramine, with somewhat different time courses, showing a reliable decrease of NET mRNA at 72 h. We also could demonstrate that antidepressant treatment decreased NET in a time-dependent manner, since in our study the reduction in NET mRNA levels after antidepressant treatment was more evident after 48 h of co-incubation with DEX.

ARRB are proteins that are important for regulating signal transduction at G protein-coupled receptors [43], and findings in various research models support a major role for ARRB in the pathophysiology of mood disorders as well as in the mechanism of action of antidepressants [44]. It has been shown that ARRB protein and mRNA levels in mononuclear leucocytes of untreated patients diagnosed with a major depressive episode were significantly lower than those of healthy subjects [45]. Interestingly, the low ARRB levels were increased by antidepressant treatment [45]. It was shown previously that DEX enhanced the expression of ARRB1 and suppressed the expression of ARRB2 in human lung adenocarcinoma cells (A549) [46].

In our present study, we found time-dependent effects on ARRB2 mRNA expression in SH-SY5Y cells after DEX-induced stress, the downregulation being more evident after 6 h. However, a treatment effect was visible only after 48 h and only for hyperforin and STW3-VI. To our knowledge, this is the first study reporting an effect of natural compounds on the expression of ARRB.

In conclusion, in an *in vitro* model of stress we analyzed for the first time a panel of genes comprising receptors and regulators of neuronal function, which have been associated with antidepressant response in previous studies. Using the glucocorticoid-receptor agonist DEX to mimic stress conditions, we were able to show the responsiveness of the selected genes under these *in vitro* conditions. Pronounced effects were observed 48 h after DEX treatment. The effects observed on FKBP5, CREB, GRIK4, VEGF, NET, and ARRB2 were in good correlation with published data, suggesting that this *in vitro* model can be used to screen the responsiveness of antidepressants under stress conditions. Similarities as well as differences between the standard antidepressant citalopram and hyperforin, miquelianin, and STW3-VI were detected. While both citalopram and STW3-VI reduced the expression of FKBP5, NET, and GRIK4 after 48 h, they exerted opposite effects on CREB, VEGF, and ARRB2, citalopram reducing and STW3-VI increasing the expression of those genes. Interestingly, the effects of STW3-VI could not be explained by hyperforin or miquelianin alone, suggesting that STW3-VI appears to have several active components that interact differently with various molecular targets. It is important to mention that this study was performed in cells; therefore, direct extrapolation to brain effects should thus be taken with due reservation. However, bearing in mind this limitation, neuronal cell cultures can provide a convenient and reliable tool to unravel new mechanisms of antidepressant action that need to be further verified in more complex experimental models.

Material and Methods

Materials

Accutase solution, DEX (≥ 97%), DMSO, DMEM, horse serum, hyperforin (dicyclohexylammonium) salt (≥ 98%), and citalopram hydrobromide (≥ 98%) were purchased from Sigma-Aldrich. Miquelianin (≥ 95%) was obtained from Extrasynthese. CellTiter-Glo Luminescent Cell Viability Assay, M–MLV reverse transcriptase, and M–MLV RT (5X) buffer were ordered from Promega. Fetal bovine serum (FBS), GlutaMA (100X), Dulbecco's phosphate-buffered saline (DPBS) without Ca^{2+}/Mg^{2+} and Penicillin/Streptomycin were purchased from Gibco. dNTP Mix (20 mM of each) was obtained from Solis Biodyne. TaqMan Gene Expression Assay probes were bought from Thermo Fisher Scientific. FastStart TaqMan Probe Master Mix (2X) was bought from Roche Diagnostics.

SJW extract (STW3-VI) was kindly provided by Steigerwald Arzneimittelwerk GmbH (STW3-VI, DER = 3–6 : 1, extraction solvent 80% EtOH, containing 0.16% hypericin, 1.2% hyperforin, and 8% total flavonoids calculated as rutin; Lot Nr. 0100228602). The extract (voucher specimen # STWE-IPT2016) was stored at – 80°C.

Cell culture

The human neuroblastoma cell line SH-SY5Y was a gift of Prof. A. Eckert (Psychiatric University Clinics, Basel, Switzerland). Cells were cultured in DMEM supplemented with 10% (v/v) FBS, 5% (v/v) horse serum, 1% (v/v) GlutaMAX, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in a humidified atmosphere of 5% CO_2. At 70–90% confluence, the cells were subcultured by dis-

association with Accutase a split ratio of 1 : 3–1 : 4. For experiments, cells of passage number 23–25 were used.

Test substances

Test substances were dissolved in DMSO (citalopram hydrobromide 10 mM, miquelianin 10 mM, hyperforin dicyclohexylammonium salt 10 mM, DEX 10 mM, STW3-VI 200 mg/mL) and stored in aliquots at – 80°C. For experiments, test substances were diluted with DMEM supplemented with 1% (v/v) FBS, 1% (v/v) GlutaMAX, 100 U/mL penicillin, and 100 µg/mL streptomycin to the final assay concentrations (citalopram and miquelianin 10 µM, hyperforin 1 µM, and STW3-VI 20 µg/mL). The final DMSO concentration did not exceed 0.2%.

ATP assay

To determine cell viability, ATP levels were determined using the CellTiter-Glo luminescent assay. One hundred microliters of the SH-SY5Y cell suspension was seeded into 96-well plates (Corning) at a density of 25 000 cells/well, followed by an incubation period of 24 h (37°C, 5% CO_2). Cells were treated with test substances in the presence or absence of DEX (1–100 µM) for 6 h and 48 h, respectively. After the desired incubation time, 100 µL CellTiter-Glo reagent was added to each well and shaken for 2 min on an orbital shaker at 200 rpm (Edmund Bühler GmbH). After 10 min, 100 µL of each well was transferred to a white 96-well plate (Greiner Bio-One) and luminescence was measured at 470 nm (SpectraMax L, Molecular Devices).

Real-time qRT-PCR

SH-SY5Y cells were seeded into 12-well plates (Corning) at a density of 250,000 cells/well, followed by an incubation period of 24 h (37°C, 5% CO_2). Cells were treated with test substances in the presence or absence of DEX (10 µM) for 6 h and 48 h, respectively. After the desired incubation time, cells were washed with DPBS and total RNA was extracted using the RNeasy Mini Kit according to the manufacturer's instructions. After extraction, RNA was treated with RNase-free DNase. Reverse transcription was performed using the Biometra T3000 thermocycler. Five microliters RNA was incubated for 5 min at 70°C with 0.24 µg oligo-dT primers (Qiagen), followed by incubation for 1 h at 37°C with 200 U of M–MLV reverse transcriptase, 1× M–MLV reverse transcriptase buffer, and 2 mM of each dNTP.

Real-time qPCR was performed using a Rotor-Gene Q PCR cycler (Qiagen) under the following conditions: 10 min at 95°C for initial denaturation, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C. Fluorescence was measured at 510 nm. qPCR reactions consisted of 3 µL cDNA, or water as nontemplate control, 7.5 µL FastStart TaqMan Probe Master Mix (2X), 0.75 µL of TaqMan Gene Expression Assay probes, and 3.75 µL of nuclease-free water.

Primer and TaqMan probe sets for FKBP5 (Hs01561006_m1), CREB1 (Hs00231713_m1), GRIK4 (Hs00205979_m1), VEGF (Hs00900055_m1), ARRB2 (Hs01034132_m1), and *β*-actin (Hs01034132_m1) were obtained from Thermo Fisher.

All values were normalized to the expression of the reference gene *β*-actin.

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ELISA assays

SH-SY5Y cells were seeded into 12-well plates (Corning) at a density of 250 000 cells/well, followed by an incubation period of 24 h (37 °C, 5 % CO_2). Cells were treated with test substances in the presence of DEX (10 µM) for 48 h. Test substances were additionally diluted in DMEM supplemented with 1 % FBS and 1 % charcoal stripped FBS, respectively. Human VEGF ELISA (Thermo Fisher Scientific) and FKBP5 ELISA (LifeSPan Biosciences) were performed according to the manufacturer's instructions. Optical density was measured at 450 nm (microplate reader Spectramax M2e, Molecular Devices). All ELISA assays were performed in triplicate on three independent days (n = 3).

Statistical analysis

ATP and ELISA experiments were performed in triplicates; each experiment was repeated at least three times (on different days). Data are shown as mean ± standard error of the mean (SEM).

Further statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test using the software package GraphPad Prism (version 5.0, GraphPad Software Inc.). In all cases, differences were considered significant if $p < 0.05$.

PCR experiments were executed in duplicates, a sample and one technical replicate; all experiments were repeated at least four times (on different days). To determine relative expression values, the $-\Delta\Delta Ct$ method (Applied Biosystems) was used, where triplicate threshold cycle (Ct) values for each sample were averaged and subtracted from those derived from the housekeeping gene actin. The Ct difference for a calibrator sample was subtracted from those of the test samples, and the resulting $-\Delta\Delta Ct$ values were raised to a power of two to determine normalized relative expression ($2^{-\Delta\Delta Ct}$ = fold expression). Based on this calculation, the corresponding control groups for the 6 h and 48 h incubation time points were set to 1. Changes in fold expression were then calculated either relative to the untreated control cells for DEX effects or relative to the DEX treated cells for antidepressant treatment effects and were defined as unchanged (= 1), increased (> 1), or decreased (< 1). All data are expressed as mean and confidence interval, and all numbers are shown in ▶ **Table 1**. Data presentation was performed according to Chittur et al. [47].

Conflict of Interest

H. A. A., C. K., and V. B. designed the study. S. V. and A. W. conducted the research and analyzed the data, and V. B., C. K., and H. A. A. wrote the manuscript. C. K. and H. A. A. are employees of Bayer Consumer Health Division, Steigerwald Arzneimittelwerk GmbH. V. B. had full access to all data in this study.

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