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ORIGINAL PAPER

# Retinoic acid as target for local pharmacokinetic interaction with modafinil in neural cells

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**Abstract** While the biological importance of the cytochrome P450 system in the liver is well established, much less is known about its role in the brain and drug interactions at the level of brain cells have hardly been investigated. Here, we show that modafinil, a well-known inducer of hepatic CYP enzymes, also increases CYP3A4 expression in human-derived neuron-like SH-SY5Y cells. Upregulation of CYP3A4 by modafinil was associated with increased retinoic acid (RA) degradation, which could be blocked by specific CYP3A4 inhibitor erythromycin. In turn, reduced RA levels in culture medium during modafinil treatment resulted in decreased neuronal differentiation of SH-SY5Y cells as assessed by intracellular neurotransmitter concentrations and proliferative activity. Again, this differentiation-impeding effect of modafinil on SH-SY5Y cells was antagonized by erythromycin. Similarly, modafinil treatment of the murine GL261 glioma cell line resulted in increased proliferative activity. This was associated with upregulation of RA-degrading CYP26A1 in GL261 cells. Taken together, our results indicate that psychopharmacological agents such as modafinil may directly act on CYP enzymes in neural tissue. These kinds of drug effects may become highly relevant especially in the context of biomolecules such as RA whose local metabolism in brain is under tight spatial and temporal control.

**Keywords** Modafinil · Retinoic acid · Cytochrome P450 · Differentiation · Pharmacokinetics

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## Introduction

Drug effects on cytochrome P450 enzymes in the liver are a common research subject in neuro- and psychopharmacology. By contrast, the importance of CYP enzymes in adult neural tissue has only rarely been studied and pharmacological interactions occurring locally within the brain have largely been ignored. However, drug effects on cytochrome P450 isozymes in the central nervous system may become highly relevant when molecules are concerned whose metabolism is regulated locally [25]. Retinoic acid (RA), the bioactive derivative of vitamin A, falls into this category. It subserves a critical role in embryonic development as a morphogen and exhibits pleiotropic

effects in adult organ homeostasis, not least in the brain [6, 18]. RA potently induces neuronal differentiation of precursor cells and promotes adult neurogenesis in the dentate gyrus [13, 18]. RA metabolism is also tightly linked to the regulation of synaptic plasticity [2]. Furthermore, RA exhibits potent anti-inflammatory properties, e.g. by inhibiting microglia activation [8, 9]. Importantly, subtle regulation of RA signaling in brain is determined by tight spatio-temporal control of local RA synthesis and catabolism, also by 'xenobiotic metabolizing' enzymes [1, 35, 40].

Modafinil is a potent inducer of the cytochrome P450 isoenzyme 3A4 in human liver microsomes [33]. CYP3A4 is involved in the hepatic degradation of RA [27]. Despite evidence for expression of cytochrome P450 isotypes in the central nervous system [10, 21, 34], the RA catabolic pathway in the mammalian brain, especially with respect to pharmacokinetic interactions, has so far received little attention. Since modafinil has previously been shown to accelerate the degradation of RA via induction of CYP3A4 in the liver, we hypothesized that an analogous interaction might also operate locally in neural tissue. Using a human-derived cell culture system, we here show that modafinil induces neuronal CYP3A4 and reduces the amount of RA in culture medium. Quite similarly, GL261 mouse glioma cells treated with modafinil show upregulation of cytochrome P450 26A1, an enzyme known to rapidly metabolize RA [20]. When cultured in the presence of RA, modafinil-treated cells showed increased proliferative activity relative to vehicle-treated cells. Taken together, these results highlight the potential of modafinil to influence local RA metabolism in brain.

## Methods

### Cell culture and sample preparation

Human SH-SY5Y neuroblastoma cells (Geweberesourcenzentrum Braunschweig, Germany) were seeded at an initial density of $8 \times 10^4$ cells/cm$^2$ and cultured as described in detail previously [15, 16]. Briefly, cells were grown in minimum essential medium containing Earle's salts, 100 units/ml penicillin, 100 µg/ml streptomycin, 10 % fetal bovine serum (Biochrom, Berlin, Germany) and the respective test substances.

Stably transfected eGFP-GL261 murine glioblastoma cells (kindly provided by Helmut Kettenmann, Max Delbrück Center for Molecular Medicine, Berlin-Buch, Germany) were used [12, 26] and cultivated in DMEM containing 10 % fetal bovine serum, 1 % sodium pyruvate, 100 units/ml penicillin and 100 µg/ml streptomycin (all from Biochrom AG, Berlin, Germany). Cells were seeded in either 12-well plates at an initial density of $1 \times 10^4$/cm$^2$ or T75 cell culture flasks at an initial density of $6.6 \times 10^3$/cm$^2$.

All-trans RA, modafinil and erythromycin (all from Sigma Aldrich, Taufkirchen, Germany) were dissolved in DMSO. RA was applied at final concentrations of 10 or 1 µM, respectively, as indicated. Modafinil was applied at a final concentration of 40 µg/ml. Erythromycin (Sigma) was applied at 10 µg/ml (13.6 µM; IC50 for CYP3A4 inhibition ~100 nM). DMSO was added as vehicle control with final DMSO concentrations being equal for all conditions, never exceeding a maximum concentration of 0.1 % (v/v).

Cell culture supernatants were collected in duplicates and RA extracted by adding 1 ml of ice-cold acetonitrile (ACN) to 0.5 ml of supernatant. After 15 min, the precipitated protein was spun down at 6,000×*g* for 15 min at 0 °C. The upper organic phase of the sample was kept for subsequent HPLC-based RA quantification. Due to the photosensitivity of retinoids, all stocks and samples containing dissolved retinoids were protected from UV irradiation and processed either in dark glass vials or under yellow light [7].

### Immunohistochemistry

Immunohistochemistry followed the peroxidase method as described previously with minor modifications [23]. Briefly, cells were cultured on poly-L-lysine-coated glass coverslips. Cells were fixed by immersion in 4 % paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.4). Cells were stained with anti-CYP3A4 antibody (1:200; Abcam), anti-neurofilament antibody (1:200; Sigma), anti-NeuN antibody (1:200; Sigma) and the respective biotinylated secondary antibodies (all 1:250; Jackson Immuno-Research-Laboratories, West Grove, PA). All samples were developed with ABC Elite Reagent (Vector Laboratories, Burlingame, CA, USA) and nickel-enhanced (0.04 %) diaminobenzidine (DAB; Sigma) as chromogen. All microscopy was performed using an inverted Carl-Zeiss microscope (Carl Zeiss, Jena, Germany).

### Immunoblotting

SDS-PAGE and Western blotting were performed as described in detail elsewhere [16, 39]. In brief, cells were lysed with ice-cold mammalian protein extraction reagent (Pierce Biotechnology, Rockford, IL, USA) and cellular debris was precipitated by centrifugation at 25,000×*g* and 4 °C for 25 min. Protein concentration was determined using BCA assay (Pierce Biotechnology). Equal amounts of protein were loaded on sodium dodecyl sulfate polyacrylamide gels (10–20 %) and blotted onto PVDF membranes (Millipore, Schwalbach, Germany). Blots were

probed with a specific antibody directed against the human cytochrome 3A4-isozyme family (1:500; Abcam, Cambridge, UK) and co-probed using a pre-labeled anti-$\beta$-actin antibody (HRP-labeled, 1:60.000; Sigma). Densitometric quantification was performed using the LAS 3000 imaging system and Aida image analysis software, version 4.1, which features an automatic exposure control to avoid potential saturation effects during image capture (Raytest, Straubenhardt, Germany).

### HPLC analyses

Levels of dopamine and of 3,4-dihydroxyphenylacetic acid (DOPAC) were determined in cellular homogenates as described previously [24, 38, 39]. Briefly, cell pellets were homogenized in 0.1 M perchloric acid and briefly lysed by ultrasound (10 s). Cellular debris and precipitated protein were removed by centrifugation at 15,000$\times g$ for 20 min at 4 °C and the clear supernatant was separated on a Waters Nova-Pak® C18, 60 Å, 4 µm, 3.9 × 150 mm column (Waters Corporation, Milford, MA, USA) by isocratic elution at 0.45 ml/min using a sodium acetate buffer with 2 % methanol and 1 % triethylamine, pH adjusted to 3.9 by addition of glacial acetic acid. Separation was followed by electrochemical detection using a Model 5011A High Sensitivity Dual Electrode Analytical Cell in a Coulochem II detector system (ESA Coulochem, Bedford, MA, USA).

#### *Retinoic acid*

HPLC analysis was conducted using an Agilent HPLC array with a binary pump for high pressure gradient elution from a C18 reversed phase-column (33 mm ProntoSIL™ AQ phase-column, 120 Å, 3 µm, 4.6 × 33 mm) and a high sensitivity diode array detector (Agilent Technologies, Santa Clara, CA, USA). Since the three major geometric RA isomers are equally subject to cellular enzymatic degradation, an HPLC protocol was developed for a rapid and simultaneous elution of all three RA isomers, resulting in a reliably quantifiable, single RA peak. Gradient elution was performed using two buffers (A, B), with buffer A containing 50 mM sodium acetate and 5 % acetonitrile (ACN), pH 5.75, buffer B containing 10 % HPLC-grade water and 90 % ACN. Gradient settings were (in % of buffer B): 0 min 35 %, 8 min 100 %, 10 min 100 %, 10.1 min 35 %. Column temperature was kept constant at 55 °C. For quantification of total RA levels in cell culture supernatants, authentic RA standards were prepared in ACN at 0.1, 1 and 10 ng/µl. Retinoid quantification was conducted by measuring UV absorbance at 340 nm. Peak identity was confirmed by analysis of the characteristic spectrum and peak purity was constantly monitored by online spectral analysis. Recovery rates of retinoids are subject to large variation, depending in particular on the nature of the materials in contact with RA (e.g. plasticware versus glassware; silanization) and the composition of the solutions used in the experiments [22]. Therefore, recovery rates were experimentally optimized and found to be maximal using plastic cell culture dishes and aqueous solutions (e.g. cell culture medium) containing 10 % FCS. Using 10 % FCS, average recovery was found to be 95.5 ± 4.5 % ($n = 6$) after 48 h incubation at 37 °C, indicating long-term stability of RA in the presence of 10 % FCS. Moreover, early-eluting oxidation products with characteristic retinoid spectra were found only in the presence of viable cells.

### Statistical analyses

Values are given as means ± SEM from at least three independent experiments. HPLC measurements were performed in duplicate for each sample. All numerical analyses were performed using the statistical software PASW Statistics 18, Release 18.0.0.0 (IBM Corporation, Somers, NY, USA). Differences between group means were analyzed by $t$ test or by one-way ANOVA followed by Tukey's post hoc where appropriate. $p < 0.05$ was considered statistically significant.

## Results

### Neuronal differentiation and CYP3A4 expression in human SH-SY5Y cells

The human SH-SY5Y neuroblastoma cell model has been described in detail previously [30]. Neuronal differentiation in response to RA exposure (10 µM for 48 h) was confirmed by the expression of neuronal markers neurofilament (NF) and NeuN (Fig. 1a, b). Additionally, RA-differentiated cells were probed for the expression of human CYP3A4, which has previously been shown to play a role in the degradation of RA in the liver [27]. Interestingly, all cells in culture showed strong CYP3A4 immunostaining (Fig. 1c).

Upon neuronal differentiation, SH-SY5Y cells exhibit catecholaminergic properties [32]. In particular, intracellular levels of dopamine and of dopamine metabolite DOPAC reflect neuronal differentiation. Dopamine and DOPAC concentrations were measured in homogenates of undifferentiated (Undiff) as well as of RA-differentiated (1 µM for 7 days) cells. RA-differentiated cells were either co-treated with vehicle (VEH), modafinil (MOD) or modafinil + CYP3A4 inhibitor erythromycin (MOD + ERY). Neuronal differentiation led to significantly increased

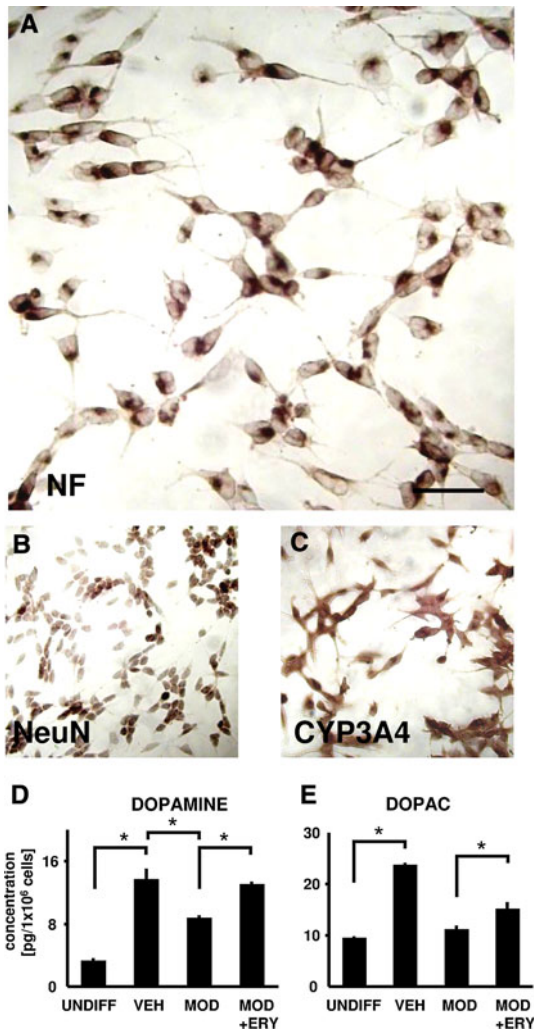


**Fig. 1** Differentiating human SH-SY5Y cells express cytochrome P450 oxidase CYP3A4 along with neuronal markers and mature into a catecholaminergic phenotype. **a**–**c** 48 h-differentiated SH-SY5Y cells grown on glass coverslips were immunostained with neuronal markers neurofilament (**a**) and NeuN (**b**). Along with expression of neuronal markers, cells also showed strong CYP3A4 staining (**c**). **d**, **e** Intracellular levels of dopamine and of dopamine metabolite 3,4-dihydroxyphenylacetic acid (DOPAC) were determined in undifferentiated (Undiff) SH-SY5Y neuroblastoma cells as well as in 7-day RA-differentiated cells. RA-differentiated cells were either co-treated with vehicle (VEH), modafinil (MOD) or a combination of modafinil and erythromycin (MOD + ERY). Neuronal differentiation resulted in significantly increased intracellular dopamine and DOPAC levels. Modafinil significantly reduced this effect of differentiation with RA. Simultaneous administration of CYP3A4 inhibitor erythromycin and of modafinil largely attenuated the effects of modafinil and rescued the prodopaminergic development of the model system. *Scale bar* (in **a**) 100 μm in (**a**), 215 μm in (**b**, **c**). *$p < 0.05$ (one-way ANOVA followed by Tukey's post hoc test)

intracellular levels of dopamine and DOPAC in the vehicle-treated group. However, coadministration of modafinil during differentiation significantly reduced dopamine and DOPAC levels as compared to the vehicle group. Interestingly, combined treatment with modafinil and erythromycin (MOD + ERY) attenuated the modafinil-induced decrease in intracellular dopamine and DOPAC concentrations (Fig. 1d, e).

## Modafinil induces CYP3A4 expression in human-derived neuron-like cells and CYP26A1 in a mouse glioblastoma cell line

Modafinil has previously been shown to induce CYP3A4 activity in human liver microsomes [33]. Here, we investigated the effect of modafinil on the expression of key P450 cytochrome enzymes involved in RA degradation both in differentiated human neuron-like cells as well as in a murine glioblastoma cell line. Continuous treatment of 5-day RA-differentiated SH-SY5Y cells with modafinil resulted in increased expression of CYP3A4, which was readily apparent by immunohistochemistry (Fig. 2a, b). Western blot analysis confirmed upregulation of CYP3A4 with a single immunoreactive band at the expected molecular weight of ~57 kDa (Fig. 2c, d). While 5-day modafinil treatment of GL261 cells did not affect CYP3A4 protein levels, it significantly increased CYP26A1 expression, which is regarded as the major mediator of endogenous RA clearance [20, 36] (Fig. 2e, f).

## Modafinil accelerates RA degradation and increases the proliferative activity of SH-SY5Y and GL261 cells

Again, human SH-SY5Y neuroblastoma cells were RA differentiated for 5 days in the presence or absence of test substances modafinil and erythromycin. After 5 days, cell culture medium was changed with the initial medium containing 10 μM RA and test substances. After 48 h of further incubation, supernatants were collected and immediately processed for HPLC determination of RA (Fig. 3a, b). In vehicle-treated controls, a moderate decrease in RA concentration from 10 to 6 μM was observed, indicating the capacity of neuronally differentiated SH-SY5Y cells to degrade RA in vitro. Modafinil treatment strongly increased RA degradation. Importantly, coadministration of CYP3A4 inhibitor erythromycin and modafinil abrogated modafinil's strong inducing effect on RA degradation (Fig. 3b).

RA possesses potent pro-differentiating and antiproliferative properties. We therefore studied the effect of modafinil on the number of cultured SH-SY5Y neuroblastoma and GL261 glioblastoma cells. SH-SY5Y cells were chronically treated with test substances for 7 days under differentiation-favoring conditions (1 μM RA). Cell densities were quantified after 7 days. While modafinil treatment of SH-SY5Y cells resulted in significantly increased cellular densities, co-treatment with erythromycin counteracted this effect, resulting in reduced cellular densities at the end of the treatment period (Fig. 3c). Similarly,

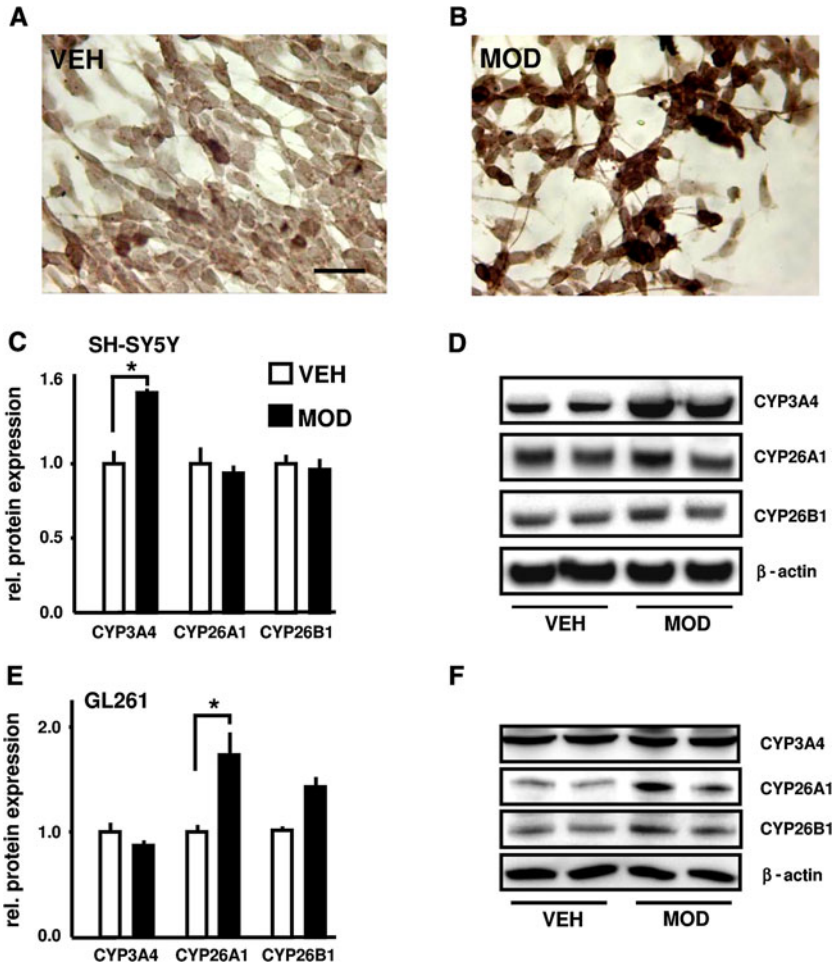


**Fig. 2** Modafinil treatment increases the expression of cytochrome P450 enzymes involved in oxidative RA catabolism. **a**, **b** SH-SY5Y cells were differentiated with RA (10 μM) for 5 days. Concomitant treatment with modafinil conferred increased CYP3A4 immunoreactivity. **c**, **d** Accordingly, Western blot analysis yielded significant upregulation of CYP3A4 expression in modafinil-treated SH-SY5Y cells. **e**, **f** 5-day modafinil treatment of murine GL261 glioblastoma cells did not affect CYP3A4 protein levels. However, modafinil significantly increased expression of CYP26A1 in these cells. Protein expression was normalized to $\beta$-actin expression in each sample and is expressed relative to vehicle-treated cells ($n = 3$ independent experiments per condition). $^*p < 0.05$ ($t$ test). *Scale bar* (in **a**) 100 μm in (**a**) and (**b**)

modafinil treatment of GL261 glioblastoma cells in the presence of 10 μM RA resulted in significantly increased proliferation of GL261 cells after 3 and 5 days (Fig. 3d).

## Discussion

Modafinil is a wake-promoting agent which is FDA-approved for the treatment of excessive sleepiness associated with narcolepsy, obstructive sleep apnea syndrome, and shift work sleep disorder. Modafinil has been suggested as a treatment for a number of psychiatric disorders, most notably attention-deficit/hyperactivity disorder, depression and stimulant dependence [3, 28]. Due to its analeptic properties, modafinil is also widely prescribed off-label for fatigue associated with neurological or medical conditions, e.g. multiple sclerosis, brain tumors, or cancer-related fatigue in general [5, 19, 31, 37]. The precise mechanisms through which modafinil confers heightened wakefulness remain to be firmly established.

Drug effects on cytochrome P450 enzymes in the liver are well established. By contrast, expression of CYP enzymes in brain cells has only scarcely been investigated and local pharmacokinetic interactions within brain cells have largely been ignored. However, especially in the case of molecules whose local metabolism is under tight spatial and temporal control, drug effects on cytochrome P450 isozymes in the brain may have significant consequences. RA is such a biomolecule, acting as a morphogen during brain development and promoting neuroplasticity by regulating neurogenesis and synaptic homeostasis during adulthood [2, 18]. Importantly, to be useful as a transcriptional regulator, the conversion of vitamin A into RA occurs locally, close to the site of ultimate RA bioactivity [29]. Therefore, any substance interfering with local neuronal or glial RA metabolism may potentially affect downstream cellular pathways directly in the brain.

Modafinil has previously been identified as an inducer of the cytochrome P450 system in human liver microsomes [33]. The findings reported here demonstrate for the first time the presence of neuronal CYP3A4 in differentiating SH-SY5Y cells. The SH-SY5Y neuroblastoma cell line is widely used as an in vitro model to study biochemical and functional properties of neurons. Importantly, it is a human-derived cell line [4, 14]. Administration of modafinil during RA-mediated differentiation resulted in strong

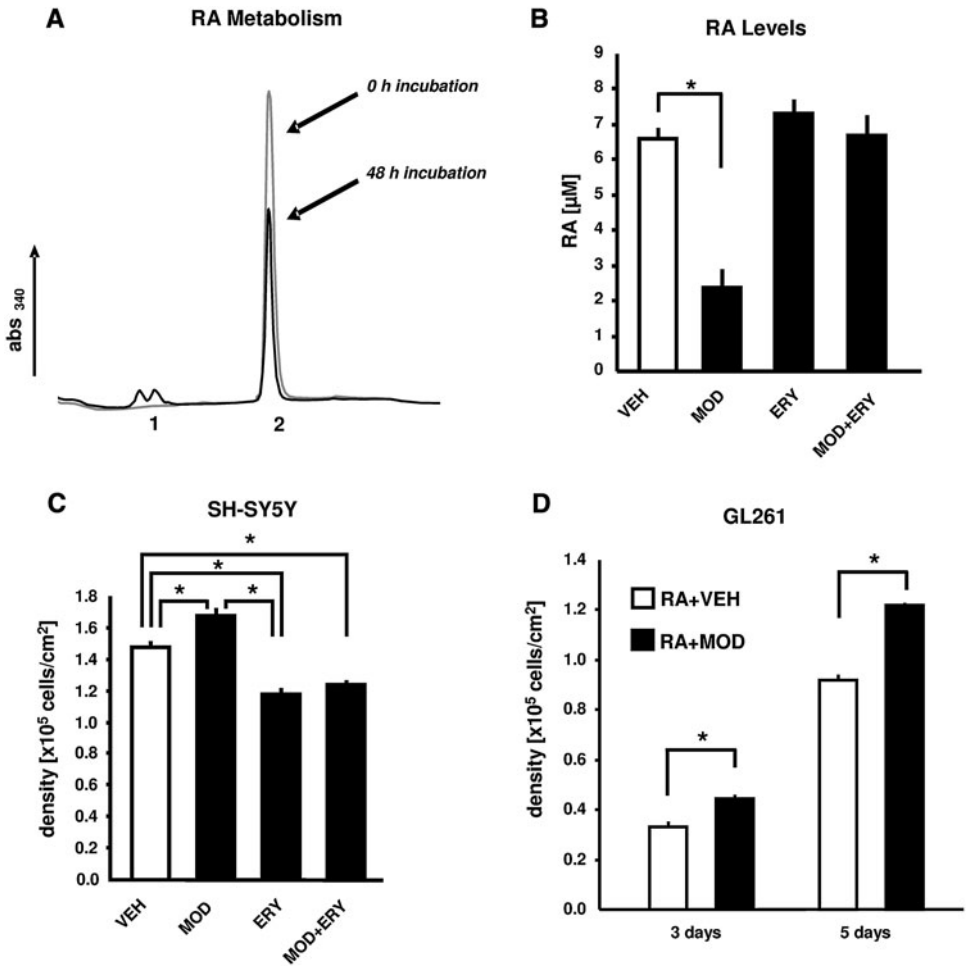


**Fig. 3** Modafinil induces cell proliferation by promoting RA degradation. **a** Superimposed, representative reverse phase HPLC chromatographs of RA-containing cell culture supernatants demonstrating the metabolic activity of human SH-SY5Y neuroblastoma cells. Relative absorption is shown for a wavelength of 340 nm. Note that after 48 h incubation, *peak 1* emerges while *peak 2* is decreased. *Peak 1* represents oxidized RA whereas *peak 2* represents unmodified RA isomers. **b** SH-SY5Y cells were RA differentiated for 5 days in the presence or absence of test substances modafinil (40 µM) and/or erythromycin (10 µM). Then, cell culture medium was changed with the initial medium again containing 10 µM RA and test substances. After 48 h, supernatants were collected and RA concentrations measured by HPLC relative to authentic standards. **c** SH-SY5Y cells were differentiated for 7 days in the presence of 1 µM RA and test substances as described in the text. **d** GL261 mouse glioblastoma cells were cultured in the presence of RA (10 µM) and modafinil. Again, modafinil treatment conferred increased proliferation as compared to vehicle-treated cultures. $N = 3$ independent experiments per condition. $^*p < 0.05$ (one-way ANOVA followed by Tukey's post hoc test)

upregulation of CYP3A4 protein expression in SH-SY5Y cells. For further functional analyses, we also used specific CYP3A4 inhibitor erythromycin [17]. While erythromycin-mediated inhibition of neuronal CYP3A4 in vehicle and modafinil-treated cells resulted in RA levels similar to those of the vehicle group after a 48 h metabolization period, the induction of CYP3A4 in the modafinil group led to significantly lower RA levels in culture medium. These findings show that upon induction, CYP3A4 plays a significant role in RA catabolism in this model system. However, another significant portion of RA metabolism appears to be independent of CYP3A4 in SH-SY5Y cells, not being inhibitable by erythromycin and thus probably occurring through other cytochrome isozymes such as the RA-oxidizing CYP26 family and other P450-dependent enzymes [1, 27, 40].

Our findings regarding neurotransmitter levels confirm the important role of RA in inducing a differentiated dopaminergic phenotype in SH-SY5Y cells. While modafinil reduced dopaminergic differentiation as assessed by intracellular dopamine and DOPAC concentrations, coadministration of erythromycin attenuated the differentiation-impeding action of modafinil. Taken together, these results therefore indicate that modafinil decreases neuronal differentiation of SH-SY5Y cells by upregulation of CYP3A4 and increased RA degradation.

Interestingly, modafinil exerted a similar effect in another cell line investigated, namely in murine GL261 glioblastoma cells. Proliferation of GL261 cells in the presence of RA was early and significantly increased by modafinil treatment. This finding fits well with studies demonstrating strong growth inhibition of glioma cells by RA [11]. Interspecies differences in the patterns of induction of different CYP enzymes are well established. In the case of murine GL261 cells, the effect of modafinil was not attributable to upregulation of CYP3A4. Instead, Western blotting revealed upregulation of other RA-metabolizing CYP enzymes. Modafinil thus increased RA degradation and exerted pro-proliferative effects in both cell lines investigated.

In conclusion, the kinds of drug effects described here may be of relevance to the treatment of patients with diverse brain diseases, especially when neuroinflammation or disturbances in cell proliferation/differentiation are involved in the underlying pathology. The collective findings of this investigation also provide proof-of-principle evidence for effects of modafinil on local RA metabolism in brain cells leading to functional consequences.

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