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Haloperidol, but not olanzapine, may affect expression of *PER1 and CRY1* genes in human glioblastoma cell line

Łukasz Mokros[a], Michał Seweryn Karbownik[b], Katarzyna Nowakowska-Domagała[c], Janusz Szemraj[d], Łukasz Wieteska[d], Karol Woźniak[e], Andrzej Witusik[f], Adam Antczak[g] and Tadeusz Pietras[a]

[a]Department of Clinical Pharmacology, Medical University of Łódź, Łódź, Poland; [b]Department of Pharmacology and Toxicology, Medical University of Łódź, Łódź, Poland; [c]Cognitive Sciences Department, Institute of Psychology, University of Łódź, Łódź, Poland; [d]Department of Medical Biochemistry, Medical University of Łódź, Łódź, Poland; [e]Pabianice Mental Health Centre, Pabianice Medical Centre, Pabianice, Poland; [f]Department of Psychology, Faculty of Social Sciences, Piotrków Trybunalski Division, Jan Kochanowski Memorial University of Humanities and Sciences, Piotrków Trybunalski, Poland; [g]Department of General and Oncological Pulmonology, Medical University of Łódź, Barlicki Memorial Hospital, Łódź, Poland

ABSTRACT

Background: There is barely any evidence of antipsychotic drugs affecting the molecular clockwork in human, yet it is suggested that clock genes are associated with dopaminergic transmission, i.e. the main target of this therapeutics. We decided to verify if haloperidol and olanzapine affect expression of *CLOCK, BMAL1, PER1* and *CRY1* in a human central nervous system cell line model. **Methods**: U-87MG human glioblastoma cell line was used as an experimental model. The cells were incubated with or without haloperidol and olanzapine in the concentration of 5 and 20 μM for 24 h. Real-time quantitative polymerase chain reaction with the ΔC_T analysis was used to examine the effect of haloperidol and olanzapine on the mRNA expression of the genes. **Results**: At 5 μM, haloperidol decreased expression of *CRY1* almost 20-fold. There was nearly a 1.5-fold increase in expression of *PER1*. Considering the 20 μM haloperidol concentration and both olanzapine concentrations, no other statistically significant effect was observed. **Conclusions**: At certain concentration, haloperidol seems to affect expression of particular clock genes in a human central nervous system cell line model, yet mechanism underlying this phenomenon remains elusive.

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Background

Since the discovery of the first circadian rhythm gene in mammals, the molecular bases of biological clockwork have been studied intensively. Entrained by the central oscillator located in the suprachiasmatic nucleus and responsive to light, almost every cell holds its own, peripheral oscillator which runs a period of approximately 24 h (Liu & Chu 2013).

CONTACT Łukasz Mokros lukaszmokros@gmail.com

The main component of the clock gene network is a cell-autonomous and auto-regulatory transcription–translation feedback loop. It comprises heterodimer CLOCK:BMAL1 (circadian locomotor output cycles kaput, brain and muscle ARNTL-like protein 1, respectively). It is a transcriptional complex enhancing Period1,2,3 (PER) and Cryptochrome1,2 (CRY) transcription. PER and CRY proteins accumulate during the day and inhibit their own expression by interaction with CLOCK and BMAL1 back in the cell nucleus (Gustafson & Partch 2015).

The genes mentioned above also regulate the transcription of the so-called clock-controlled genes. In consequence, certain components of cell metabolism change throughout the day (Duffield et al. 2002).

As the clock gene network is present in every cell, a wider aspect of circadian rhythmicity is studied. It emphasizes its role in pathogenesis of certain somatic diseases. Those include arterial hypertension, obesity, hyperlipidaemia and diabetes (Sherman et al. 2012). Also, there is evidence that clock genes are associated with behaviour and mood as well. Their role in mental disorders seems particularly interesting (Kishi et al. 2009).

Circadian rhythm disruption is a component of psychotic disorders (Monti et al. 2013). The links between clock gene polymorphisms and schizophrenia was established (Mansour et al. 2009; Zhang et al. 2011). Furthermore, the *CRY1* gene is located near the possible schizophrenia-susceptibility locus (Peng et al. 2007).

There is almost no evidence of antipsychotic drugs (APDs) affecting the molecular clockwork in human. Yet, clock genes may be associated with dopaminergic transmission, the main pharmacological target of APDs (Stahl 2013).

Some APDs were already proven to alter circadian rhythms in animal models (MacDonald & Meck 2005; Buhusi & Meck 2007). Haloperidol and olanzapine represent first- and second-generation APDs, respectively. We decided to verify whether they affect expression of molecular clockwork key components, i.e. *CLOCK, BMAL1, PER1* and *CRY1,* in a human central nervous system cell line model.

Materials and methods

Cell cultures

The human U-87MG glioblastoma cell lines (HTB-14; ATCC; Rockville, MD, USA), were grown in 60-mm Petri dishes (NUNC) in Advanced Minimum Essential Medium (Invitrogen, Carlsbad, CA, USA). They were supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), 2 mM L-glutamine (Sigma-Aldrich, St. Louis, MO, USA) and an antibiotic solution (100 U/mL penicillin and 100 µg/mL streptomicin, Invitrogen). The cells underwent incubation at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. For subcultures, the cells were harvested in 0.25% trypsin-EDTA solution (Invitrogen) three times a week and inoculated at a density of 3.0×10^5 per dish.

Cell viability assay

Cell viability was measured by assessing the reduction rate thiazolyl blue tetrazolium bromide (MTT; Sigma-Aldrich) to formazan derivative through cellular mitochondrial dehydrogenases. Haloperidol and olanzapine (Sigma-Aldrich) were dissolved separately in dimethyl sulfoxide (DMSO, Sligma-Aldrich) to produce 10 mM stock solution. U-87MG cells were inoculated in quadruplicate at a density of 1.0×10^4 cells per well in 96-well plates (Nunc, Roskilde, Denmark) and grown in standard culture conditions for twenty-four hours. Subsequently,

Table 1. Primer sequences of investigated genes for RT-qPCR.

Gene	5′-3′ primer sequences	
	Forward	Reverse
CLOCK	TCGACAGGACTGGAAACCTAC	GCTTCCATCTGTCATGATTGC
BMAL1	CCACCAATCCATACACAGAAGC	TCCCTCGGTCACATCCTACG
PER1	GTCCGTCTTCTGCCGTATCA	GTCACATACGGGGTTAGGCG
CRY1	TTGGAAAGGAACGAGACGCA	GCGGTTGTCCACCATTGAGT
GAPDH	AGCCACATCGCTCAGACA	GCCCAATACGACCAAATCC

Notes: CLOCK – circadian locomotor output cycles kaput; BMAL1 – brain and muscle ARNTL-like protein 1; PER – Period; CRY – Cryptochrome; GAPDH –glyceraldehyde 3-phosphate dehydrogenase.

the culture medium was replaced with fresh serum-containing medium and the cells were exposed to 5 μM and 20 μM haloperidol, same concentrations of olanzapine and along with relevant controls incubated for the next twenty-four hours. After incubation, MTT was added at a final concentration of 1.0 mg/mL and the cells were incubated for 2 h at 37 °C. Then, the supernatants were aspirated. Formazan crystals were solubilized in DMSO and absorbance, directly proportional to the number of viable cells, was measured at 560 nm using GloMax-Multi Detection System (Promega, Madison, WI, USA). The results were expressed as relative absorbance, i.e. $[(A_{560}$ of experimental wells)/$(A_{560}$ of control wells)$] \times 100\%$.

Real-time quantitive polymerase chain reation (RT-qPCR)

All of the assay procedures were performed according to the basic principles described by Nolan et al. (2006).

The cell were exposed to tested APDs as described above. cDNA obtained from four cell cultures was subjected in triplicate to the reaction with Brilliant II SYBR Green QPCR Master Mix Kit (Stratagene, CA, USA). The sequences of gene-specific primers investigated are presented in Table 1 (Sigma-Aldrich). The pre-selected temperature of primer annealing was 60 °C and primer annealing time set to 20 s. An Agilent Technologies Stratagene Mx3005P thermocycler was used for the PCR reactions. For each sample, the C_T value (threshold cycle) was calculated with Mx-Pro software. The threshold cycle (C_T) values of the investigated genes were compared to that of *GAPDH* (glyceraldehyde 3-phosphate dehydrogenase), a reference gene, and ΔC_T values were determined, where $\Delta C_T = C_{T,\text{target gene}} - C_{T,\text{GAPDH}}$.

Data analysis

The changes in expression of the investigated genes were assessed by means of the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen 2001). Statistical analysis included analysis of variance with Dunnett *post hoc* test. $p < 0.05$ was considered statistically significant. Normal distribution of the parameters was verified with Shapiro–Wilk test. The analysis was performed using STATISTICA 12 PL (StatSoft, Inc., Tulsa, OK, USA).

Results

Effect of haloperidol and olanzapine on the growth of U-87MG glioblastoma cells

No statistically significant differences were found between the viability of the cells treated with the tested APDs at a concentration of 5 μM and the relevant control (haloperidol relative

Table 2. Mean $2^{-\Delta\Delta C_T}$ values representing the effect of two concentrations (5 and 20 μM) of haloperidol and olanzapine on fold change in expression of the investigated clock genes in glioblastoma cell cultures.

	Haloperidol		Olanzapine	
	5 μM	20 μM	5 μM	20 μM
CLOCK	1.49 (1.04–2.13)	0.41 (0.13–1.31)	1.27 (0.76–2.09)	0.53 (0.13–2.16)
BMAL1	1.32 (1.02–1.71)	0.64 (0.40–1.04)	1.01 (0.60–1.7)	0.58 (0.24–1.4)
PER1	1.36 (1.23–1.51)*	0.50 (0.21–1.23)	1.04 (0.85–1.29)	0.80 (0.52–1.23)
CRY1	0.05 (0.01–0.82)**	0.54 (0.24–1.2)	0.81 (0.45–1.46)	0.55 (0.26–1.15)

Note: CLOCK – circadian locomotor output cycles kaput; BMAL1 – brain and muscle ARNTL-like protein 1; PER1 – Period 1; CRY1 – Cryptochrome 1. Presented with normalized values, defined as $2^{-\Delta\Delta C_T \pm SD}$ (Livak & Schmittgen 2001).
*$p<0.05$; **$p<0.001$.

absorbance 96.7 ± 1.4%, olanzapine relative absorbance 102.1 ± 5.0%, $p > 0.05$). Statistically significant differences were found between the viability of the cells treated with the tested APDs at a concentration of 20 μM the relevant control (haloperidol relative absorbance 86.8 ± 5.5%, $p < 0.01$, olanzapine relative absorbance 91.5 ± 1.8%, $p < 0.05$.

Effect of haloperidol on the mRNA expression

In cells exposed to 5 μM haloperidol expression of *CRY1* was decreased almost 20-fold ($p < 0.001$). There was nearly a 1.5-fold increase in expression of *PER1* ($p < 0.05$). No effect on CLOCK and *BMAL1* expression was observed ($p > 0.05$). Haloperidol concentration of 20 μM had a different impact, as no change in *CLOCK, BMAL1*, *PER1* and *CRY1* expression was noted ($p > 0.05$).

Effect of olanzapine on the mRNA expression

Both 5 and 20 μM olanzapine concentrations did not present a statistically significant effect on expression of *CLOCK, BMAL1, PER1 and CRY1* ($p > 0.05$).

Detailed results are presented in Table 2.

Discussion

Our findings suggest that haloperidol may affect expression of *CRY1* and *PER1*. The presented results are of certain value since they are consistent with previous findings. Haloperidol was proven to influence biological clock of rats, shifting the period of the rhythm (MacDonald & Meck 2005). At molecular level, there is evidence that haloperidol decreases expression of *mPer1* in the hippocampus, prefrontal and cerebral cortex, paraventricular nucleus of the hypothalamus and other areas of mouse brain (Coogan et al. 2011). This seems contradictory to our results, but two aspects should be considered. The D2-receptor agonists were proven to have an inhibitory effect on expression of mPer1 and mClock, which supports the thesis that we may expect an opposite action from APDs, i.e. D2-receptor antagonists. But, it should be remembered that in modulation of clock gene expression, due to the transcription–translation feedback loop, the shift of phase and period may be of greater importance than the temporal change in mRNA levels (Gallego & Virshup 2007). Besides, the effect of haloperidol on mPer1 expression patterns has been proven to vary, depending on the time of the day the exposition started (Viyoch et al. 2005).

The molecular mechanism underlying the effect of haloperidol on clock gene expression remains elusive. One possibility is that haloperidol increases phosphorylation of cAMP-responsive element-binding protein (CREB) via certain pathways beggining with blocking of D2 receptors and CREB is a transcription factor for *mPer1* and many other genes (Viyoch et al. 2005).

The observed difference between the investigated APDs in the impact on clock gene expression may be explained by more complex receptor-binding profile of olanzapine. Also, haloperidol, especially in low concentration, blocks D2 dopamine receptor. Olanzapine inhibits e.g. 5HT2A, 5HT2C serotonin receptors, H1 histamine receptor and several other serotonin and dopamine receptors more potently than it does D2 receptor (Stahl 2013). It may appear that the blockade of the receptors other than D2 inhibits its effect on expression of the clock genes. It is also possible that the hereby shown action of haloperidol may be due to currently unknown mechanisms.

Haloperidol and olanzapine reach similar blood plasma levels, which makes a rationale for comparison of effects of the same dosage on the cell cultures. We explain the choice of two concentrations (5 and 20 μM) by the fact that both haloperidol and olanzapine reach much higher levels in brain tissue than in plasma. Also, those levels exceed the therapeutic plasma range. Those concentrations correlate with each other, which is also important information (Aravagiri et al. 1999; Kornhuber et al. 1999). Still, it should be remembered that the therapeutic reference is different for haloperidol and olanzapine (Hiemke et al. 2011).

The difference of effect on clock gene expression between the two tested concentrations raises questions. Interestingly, 5 μM haloperidol affects the expression of *PER1* and *CRY1*, while no such effect was observed for 20 μM. Since the mechanisms underlying the presented relation are unclear and the lack of evidence is not the evidence of absence, we consider the effect dose-related.

Despite the statistically significant change in cell viability induced by haloperidol and olanzapine, the effect is not marked since the relative absorbance is still around 100%.

We recognize that human glioblastoma cells are not an *in vitro* model in cellular oscillators research. Rat-1 fibroblasts are preferred as they present features similar to the suprachiasmatic nucleus (Rosbash 1998). Yet, the advantage of our study is that it shows a possible influence of haloperidol on *PER1* and *CRY1* in human cells. Moreover, glioblastoma cells may be considered a human brain tissue model. Glial cell also takes part in neurotransmission, which gives rationale to use them as a CNS model (Köles et al. 2015). Yet, it should be remembered that it is a neoplasm and does not comprise neurons.

The effect of APDs on clock genes and circadian rhythm is particularly interesting. It is proven that the sleep–wake cycle among patients with schizophrenia is disrupted (Monti et al. 2013). Apart from being a potential therapeutic target in schizophrenia, it is unclear whether the molecular clockwork is affected by the currently used antipsychotic agents.

The influence of clock genes on the lipid and carbohydrate metabolism in the cells and the whole organisms may also be of interest in the context of schizophrenia and its treatment. Metabolic syndrome is a serious side effect of antipsychotic drugs, although the mechanisms underlying this phenomenon remain unclear (De Hert et al. 2006). Also, despite the evidence of a link between the biological clock and development of insulin resistance, the influence of APDs on circadian regulation of metabolism-related gene expression has not been elucidated (Dashti et al. 2014).

We find that further research on association between biological clockwork and antipsychotic drugs is still required. Those may include a wider spectrum of clock genes and APDs. Also, other cellular models and animal models should be considered.

Abbreviations

CLOCK	circadian locomotor output cycles kaput
BMAL1	brain and muscle ARNTL-like protein 1
PER	Period
CRY	Cryptochrome
APDs	antipsychotic drugs
CREB	cAMP-responsive element-binding protein
GAPDH	glyceraldehyde 3-phosphate dehydrogenase

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