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The differential effects of neuroleptic drugs and PACAP on the expression of *BDNF* mRNA and protein in a human glioblastoma cell line

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It has been suggested that, in addition to modulation of monoaminergic neurotransmission, antipsychotic drugs can also affect expression of neurotrophic factors in the brain. The present study was aimed to examine the effects of the first generation neuroleptic drug (FGA; haloperidol) and second generation neuroleptic drugs (SGAs; olanzapine and amisulpride) on expression and level of brain-derived neurotrophic factor (BDNF) in astrocyte-like T98G glioblastoma cell line. Effects of these drugs were compared to the action of PACAP38, a neuropeptide with well known BDNF-mediated neuroprotective effects. The tested neuroleptics differentially regulated the mRNA expression and protein level of BDNF depending on the concentration and incubation time. Using rtPCR technique, we demonstrate that, from the three tested neuroleptics, both haloperidol as well as olanzapine at 5 μM concentration (but not at 20 μM) increased *BDNF* mRNA expression with a similar efficacy after a 72 h incubation. In order to confirm the observed changes in the mRNA expression of *BDNF*, a protein expression assay was performed. The exposure of cells only to 5 μM olanzapine for 72 h increased BDNF concentration in the culture medium by 29%. Additionally, PACAP significantly up-regulated BDNF mRNA expression in T98G cells and the obtained results correlated positively with the increased production of *BDNF* protein, by 22% above control. The results of the paper show that olanzapine, similarly to exogenous PACAP38, increased *BDNF* mRNA expression and protein release, which can contribute to its neuroprotective mechanism of action in the cells of nonneuronal origin. The results of the present paper confirm the findings that BDNF may represent the key target for olanzapine and PACAP.

Key words: BDNF, schizophrenia, neuroleptic drugs, neuropeptides, PACAP

INTRODUCTION

Schizophrenia is a common neuropsychiatric disorder affecting 0.5-1% of the general population worldwide. The disease is characterized by a profound disruption in cognition and emotion, affecting the most fundamental human attributes: language, thought, perception, affect, and the sense of self (Cohen et al. 2015, Tsapakis et al. 2015). The pathophysiology of schizophrenia is still not clear; it includes complex genetic and environmental interactions that impair neurodevelopmental processes. It has been suggested that disruptions in neurotrophin function, which play important roles in neurodevelopment and neuroprotection, may have an impact on etiology of the disorder. One neurotrophin whose signaling is altered in a schizophrenic patient is brain-derived neurotrophic factor (BDNF) (Ratajczak et al. 2015). BDNF is the most abundant neurotrophin in the brain involved in the development of the nervous system (Bramham and Messaoudi 2005, Paczkowska et al. 2015), and it has been shown that BDNF increases neuronal survival by protecting adult neurons from ischemic (Beck et al. 1994), glutamatergic (Mattson et al. 1995), and hypoglycemic insults (Mattson et al. 1993). Studies show that BDNF is abnormally regulated in animal models of schizophrenia and is reduced in the plasma, CSF and postmortem brains of patients with schizophrenia (Durany and Thome, 2004, Tan et al. 2005, Pillai et al. 2010). Therefore, the up-regulation of the level of the neurotrophic factor like BDNF might be a new approach to treat such psychiatric states as schizophrenia.

Current antipsychotic drugs improve the symptoms of schizophrenia through complex mechanisms that are not completely understood (de Bartolomeis et al. 2015, Dold et al. 2015, Kusumi et al. 2015). It is known that they act through the modulation of monoaminergic neurotransmission, primarily involving the do-

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paminergic pathways (Seeman 2002). Recent studies have suggested that the action of neuroleptic drugs is mediated by their regulatory influence, not only upon small-molecule neurotransmitters, but also via neurotrophic factors. Several studies have reported the differential effects of antipsychotics on BDNF levels in both schizophrenia subjects and animal models of schizophrenia (Angelucci et al. 2005, Angelucci et al. 2000, Chlan-Fourney et al. 2002, Parikh et al. 2004, Pandya et al. 2013). These differences might be related to the different therapeutic effects observed for the first generation neuroleptic drugs (FGAs) and second generation antipsychotic drugs (SGAs) in patients with schizophrenia. However, besides neurons, little information is present on the modulation of BDNF by neuroleptics at the mRNA and protein levels in other cell types of CNS. Glial cells are able to store and release BDNF (Hutchinson et al. 2009), and it has been suggested that glial dysfunction may contribute to the pathophysiology of schizophrenia (Bernstein et al. 2015, Steffek et al. 2008).

Therefore, the aim of the current paper is to compare the effect of haloperidol (FGAs), olanzapine and amisulpride (SGAs) on the expression of *BDNF* mRNA and protein from astrocyte-like model T98G glioblastoma cells. The study uses the T98G human cell line, which shares many features with primary astrocytes; it has been widely used in astrocyte research, successfully and in our research group (Avila Rodriguez et al. 2014, de Joannon et al. 2000, Cabezas et al. 2015). Additionally, the pituitary adenylate cyclase-activating polypeptide (PACAP) was used as a reference. It is a known pleiotropic neuropeptide with strong BDNF-mediated neuroprotective potential (Frechilla et el. 2001, Jozwiak-Bebenista et al. 2015). For the purposes of comparison, the effect of exogenous PACAP on the expression of *BDNF* mRNA and secretion were also evaluated in this cell model.

MATERIALS AND METHODS

Cell Cultures

The T98G glioma cell line was purchased from the American Type Culture Collection (ATCC; Rockville, MD, USA). The cells were cultured in 25-mL flasks in a medium composed of Advanced MEM supplemented with 10% fetal bovine serum, 2 mM glutamine and a penicillin-streptomycin solution, in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. For subcultures, cells were harvested every third day in trypsin-EDTA (0.25% trypsin, 1 mM EDTA) solution. For the gene expression assay, T98G cells were plated onto six-well plates (Nunc) at a density of 3 x 10^5 cells per well. For the BDNF enzyme-linked immunosorbent assay (ELISA), the cells were seeded onto 24-well plates, at a density of 2.5 x 10^5 cells per well. The following substances were used: fetal bovine serum, penicillin-streptomycin solution (5,000 units/ml penicillin and 5,000 g/ml streptomycin sulphate in normal saline), phosphate buffered saline (PBS; pH 7.4) and trypsin-EDTA; all were purchased from Invitrogen (Carlsbad, CA, USA). Advanced MEM was obtained from Gibco (Paisley, Scotland, UK).

Drug and Peptide Treatments

T98G cells were treated with the neuroleptic drugs haloperidole, olanzapine, amisulpride (Sigma Chemical Co., St. Louis, Mo., USA), and with the peptide PACAP38 (H-8430, Bachem) and incubated for 24 and 72 hours. All the neuroleptic drugs were initially dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, Mo., USA) followed by a preparation of working concentrations in an appropriate medium. Control samples were treated with medium incorporating DMSO in amounts corresponding to the concentration of the used drugs. As PACAP38 was dissolved in water, the control group consisted of just the medium without the peptide.

Total RNA extraction and cDNA generation

Total RNA extraction was carried out using the RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. RNA content was measured using a PicoDrop spectrophotometer (Picodrop Limited). The quality of RNA samples was analyzed by measuring the absorption ratio at 260/280 nm. The purified total RNA was immediately used for cDNA synthesis or stored at -80^{0}C.

cDNA was generated with QuantiTect Reverse Transcription Kit (Qiagen) according to the manufacturer's protocol. Samples of 1 µg of total RNA were used as starting material, and reverse transcription was performed in the conditions optimized for use with the kit (42°C for 30 minutes, 95°C for 3 minutes). The cDNA samples were kept frozen at -20°C.

Real-Time PCR

Gene expression was measured using the TaqMan kit. Briefly, the reactions were performed in 10 µl amounts including 50 ng cDNA, 5 µl KAPA PROBE FAST qPCR Kit Master Mix ABI Prism (Kapa Biosystems) and 0.5 µl appropriate TaqMan Gene Expression Assay (20x). Specific pre-made TaqMan assays were used in

this study: brain-derived neurotrophic factor (BDNF, Hs02718934_s1) and beta actin (ACTB, Hs01060665_g1) as the endogenous control. TaqMan PCR assays were performed on a 7900HT Fast Real-Time PCR System (Applied Biosystems) in FastGene Fast 96-well PCR plates (Nippon Genetics Europe GmbH). The following thermal cycling specifications were performed: 20 s at 95°C and 40 cycles each for 3 s at 95°C and 30 s at 60°C. Real-time PCR data was analyzed using the $2^{-\Delta\Delta Ct}$ method (Schmittgen and Livak 2008).

ELISA assay

BDNF protein levels in the cell-conditioned media were determined using a BDNF ELISA kit, according to the manufacturer`s instructions (SunRed Biotechnology Company, Baoshan District, Shanghai). Data is given as pg/ml protein.

Data analysis

Analyses were conducted using Statistica 13.1 Software (StatSoft, Tulsa, Okla., USA). Data was expressed as mean ± standard error of the mean (SEM) values. The variables were analyzed using two-way analysis of variance (ANOVA) for repeated measures (time of incubation) followed by a *post hoc* Student-Newman-Keul's test. These analyses made it possible to evaluate statistically significant alterations in mRNA and protein level of BDNF as a function of treatment, and time of incubation. If necessary, the data was transformed to fulfill assumptions of linearity and homogeneity of variance. A two-tailed p-value less than 0.05 was regarded as statistically significant and was used for these statistical analyses.

RESULTS

Effects of neuroleptics on *BDNF* expression in the T98G cell line

Three antipsychotic drugs with different mechanisms of action were chosen: FGA, represented by haloperidol, and two SGAs, represented by olanzapine and amisulpride. The astrocyte-like model T98G glioblastoma cells were incubated with 5 and 20 μM haloperidol or olanzapine and with 20 and 100 μM amisulpride for 24 or 72 hours. After the incubation period, the total RNA was extracted and subjected to gene expression studies. The mRNA expression of *BDNF* was differentially regulated by the tested neuroleptics in T98G glioblastoma cells.

The results of the 24-hour and 72-hour incubation with three different interventions (5 μM haloperidol, 5 μM olanzapine and 100 μM amisulpride) were subjected to two-way ANOVA analysis. The statistical analysis revealed that time of incubation (24 *vs.* 72 hours) had a significant impact on the final outcome (F=16.111; df=1; p=0.00045). A significant relationship was also found between time of incubation and type of intervention used (F=5.868; df=3; p =0.00336): the *BDNF* expression was significantly higher at 72 hours than 24 hours following haloperidol (p=0.01849) and olanzapine treatment (p=0.00213). *Post hoc* analysis demonstrated also that after 72-hour incubation, both 5 μM of haloperidol (p=0.02784) and olanzapine (p=0.03119) significantly up-regulated *BDNF* mRNA expression to 1.48 and 1.54 of the expression status, respectively; however, 100 μM amisulpride was not found to have any effect on *BDNF* expression after 24-hour and 72-hour incubation (Fig. 1A).

No statistically significant changes in *BDNF* expression were observed for 20 μM haloperidol, olanzapine or amisulpride. However, after 72-hour incubation, 20 μM amisul-

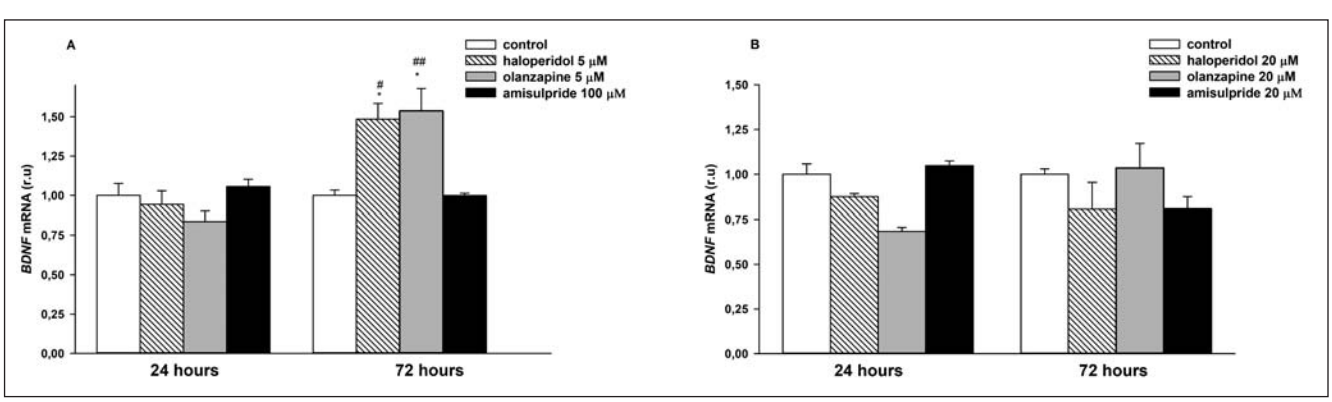

Fig. 1. The effects of haloperidol (5 and 20 μM), olanzapine (5 and 20 μM) and amisulpride (20 and 100 μM) on *BDNF* expression in the T98G cell line. Cells were incubated with neuroleptic drugs for 24 hours or 72 hours and subjected to gene expression studies (A and B). Control cells were treated with medium. 1 μg of total RNA isolated from cells was reverse transcribed and 50 ng of cDNA was used for the real-time PCR analysis. All real-time PCR reactions were performed in duplicate, using the ACTB gene as an endogenous normalization factor. Results are expressed as relative units (r.u.) – a number resulting from the normalization procedure; values are given as mean ± SEM (n=4-8). Statistical differences are shown as * at p<0.05 *vs.* control and # at p<0.05 *vs.* 24 hours; ## at p<0.005 *vs.* 24 hours (Two-way ANOVA interaction: p<0.005).

pride mildly decreased *BDNF* expression to 0.81 of the original status, similarly to 20 μM haloperidol. This observed effect was not maintained by 100 μM of amisulpride and the results were not statistically significant (Fig. 1B).

Effect of PACAP38 on *BDNF* expression in the T98G cell line

The effect of exogenous PACAP38 on *BDNF* gene expression was also evaluated in T98G cells treated with 1 μM PACAP38 for 24 hours or 72 hours.

Overall type of intervention (i.e., control *vs.* PACAP38) (F=2336.422; df=1; p<0.00001) and time of incubation (i.e., 24 *vs.* 72 hours) (F=22.472; df=1; p=0.00032) were found to have a significant impact on the final outcome. A significant relationship was also found between time of incubation and type of intervention used (F=22.133; df=1; p=0.00034). Detailed *post hoc* comparisons show that PACAP38 up-regulated *BDNF* mRNA expression in T98G cells significantly for both incubation times. The strongest stimulatory effect of PACAP38 was reported after 24-hour incubation with peptide: a four-fold up-regulation of *BDNF* mRNA expression was observed compared to the control group (p=0.00013). A 72-hour incubation with PACAP38 maintained neurotrophin levels at twice those of the control group (p=0.00013). The *BDNF* mRNA expression in the PACAP38 group significantly differed between 24 and 72 hours (p=0.00018) (Fig. 2).

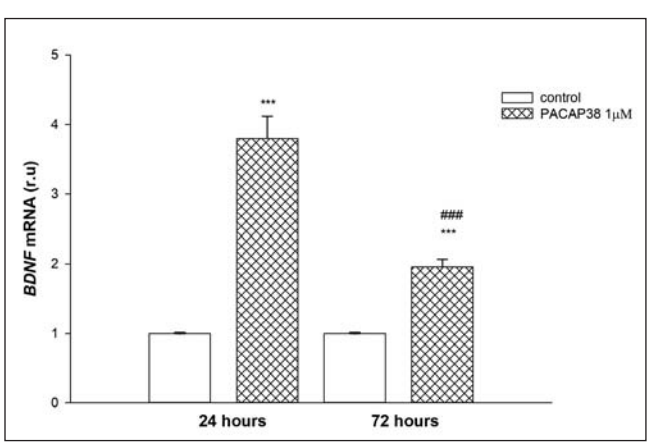

Fig. 2. The effect of PACAP38 on *BDNF* expression in the T98G cell line. Cells were incubated with PACAP38 for 24 hours or 72 hours and subjected to gene expression studies. Control cells were treated with medium. 1 μg of total RNA isolated from cells was reverse transcribed and 50 ng of cDNA was used for the real-time PCR analysis. All real-time PCR reactions were performed in duplicate, using ACTB gene as an endogenous normalization factor. Results are expressed as relative units (r.u.) – a number resulting from the normalization procedure; values are given as mean ± SEM (n=8). Statistical differences are shown as *** at p<0.0005 *vs.* control and ### at p<0.0005 *vs.* 24 hours (Two-way ANOVA interaction: p<0.0005).

Effects of neuroleptics on BDNF release from the T98G cell line

The effects of haloperidol and olanzapine on the total extracellular BDNF levels in T98G cells was determined by ELISA assay. The cells were treated for 24 and 72 hours with haloperidol and olanzapine, both at 5 μM (Fig. 3A) and at 20 μM (Fig. 3B). The results were analyzed using two-way ANOVA, which confirmed that time of incubation had a significant impact on the final outcome at lower doses of haloperidol and olanzapine (F=223.596; df=1; p<0.000001). The type of intervention was also found to have a significant impact on BDNF content (F=5.553; df=2; p=0.02686). Detailed *post hoc* comparisons revealed that treatment with 5 μM olanzapine (p=0.02368), but not 5 μM haloperidol (p=0.29709), significantly increased BDNF release, irrespective of time of incubation. The concentration of BDNF was approximately 815 pg/ml in the control group; this value increased to 1051 pg/ml in the presence of olanzapine and to 920 pg/ml in haloperidol (Fig. 3A). As the effect of interaction was found to be insignificant (F=2.647; df=2; p=0.12473) no further comparisons were performed.

After 24-hour and 72-hour treatment with 20 μM of haloperidol and olanzapine, no statistically significant differences in BDNF release were observed (Fig. 3B).

Effect of PACAP38 on BDNF release from the T98G cell line

Because the strongest stimulatory effect on BDNF mRNA expression was reported with 1 μM PACAP 38, its effect on BDNF release was measured at different time periods: after 6, 12, 24, 48 and 72 hours (Fig. 4). Two-way ANOVA confirmed that time of incubation had a significant impact on the final outcome (F=52.98; df=4; p<0.000001). A significant relationship was found between time of incubation and type of intervention used (F=10.59; df=4; p=0.000006). *Post hoc* analysis found that the amount of BDNF released in the presence of PACAP38 was significantly elevated compared to the appropriate control group, particularly at the 72-hour time point (804 pg/ml *vs.* 982 pg/ml) (p=0.000150). Interestingly, PACAP38 treatment was not found to have any effect on BDNF release after 12, 24 or 48 hours. The BDNF release in the PACAP38 group was also significantly greater at 72 hours than at 24 hours (p=0.00025) or than 48 hours (p=0.00014) (Fig. 4).

In this set of experiments, the BDNF content in the control group decreased over time; however, no such effect was observed with the experiments conducted with neuroleptic drugs.

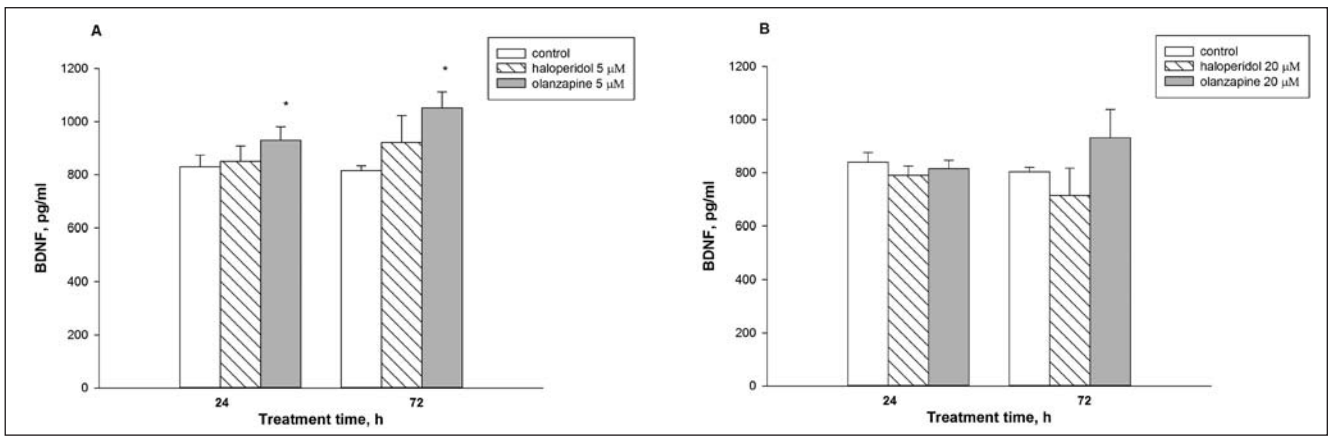

Fig. 3. The effects of haloperidol and olanzapine on the BDNF content of conditioned media from cultured T98G cells. Cells were treated with haloperidol and olanzapine, 5 (A) and 20 (B) μM, for 24 and 72 hours. BDNF protein levels in cell-conditioned media were detected using a BDNF ELISA kit. Results are expressed as mean ± SEM (n=4-12). Statistical differences are shown as * at $p<0.05$ compared with the respective control value independently on time of incubation (Two-way ANOVA intervention: $p<0.05$).

Relationship between mRNA and protein expression of BDNF

A positive correlation was observed between olanzapine-induced changes in expression of BDNF at the mRNA and protein levels (R=0.6906; $p=0.013$) (Fig. 5A). While similar changes were also observed for PACAP38 (R=-0.9553; $p<0.0001$) (Fig. 5B), the observed correlation is negative because the *BDNF* mRNA expression was lower after 72 hours than after 24 hours of PACAP38 treatment.

DISCUSSION

As Shao et al. (2006) report that antipsychotics increase glial cell line-derived neurotrophic factor (GDNF) secretion from rat C6 glioma cells, a trophic factor for dopaminergic neurons, the present paper examines the *in vitro* effects of neuroleptics on the expression of *BDNF* mRNA and protein release in a T98G human glioblastoma cell line, an astrocyte-like model. Three antipsychotic drugs with different mechanisms of action were chosen for the present study: one FGA, represented by haloperidol (a dopamine D_2 – receptor antagonist), and two SGAs, represented by olanzapine (a serotonin 5-HT_{2A} – receptor antagonist and a weak dopamine D_2 – receptor antagonist), and amisulpride (an atypical neuroleptic drug which exhibits poor affinity for the serotonin 5-HT_{2A} – receptor). The T98G cells were exposed to 5 and 20 μM concentrations of haloperidol and olanzapine or 20 and 100 μM amisulpride and incubated for 24 or 72 hours. The choice of such concentrations was based on literature data (Lu and Dwyer, 2005, Shin and Song, 2014) and our own previous cell viability measurements (Jóźwiak-Bębenista and Kowalczyk, 2017). Although these concentrations may seem high compared to the therapeutic plasma levels, they are similar to the concentrations of antipsychotic drugs (1–25 μM) that cause GDNF release from C6 cells (Shao at al, 2006) and to the concentrations of antidepressants (1–10 μM) that alter *PACAP* mRNA receptors in rat primary cortical neurons (Reichenstein et al. 2008). As gene expression varies to a great degree over the time course of exposure to chemicals, a range of incubation times were chosen. Two incubation periods were chosen to demonstrate mRNA and protein changes: The first was 24 hours, intended to replicate short-term treatment with drugs, which are only the initial event in cells, while a longer period of 72 hours, representing a long-term treatment period limited by *in vitro* studies, was intended to represent the delayed effect of

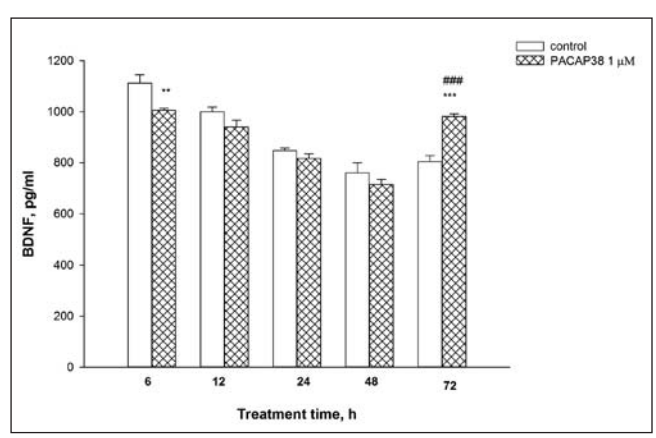

Fig. 4. The effect of PACAP38 on the BDNF content of conditioned media from cultured T98G cells at various times of treatment. Cells were treated with PACAP 38, 1 μM, for 6, 12, 24, 48 and 72 hours. BDNF protein levels in cell-conditioned media were detected using a BDNF ELISA kit. Results are expressed as mean ± SEM (n=6). Statistical differences are shown as ** at $p<0.005$ and *** at $p<0.0005$ compared with respective control value and as ### at $p<0.0005$ *vs.* 24 hours or 48 hours (Two-way ANOVA interaction: $p<0.0005$).

neuroleptic drugs. The T98G human cell line was used as an example of glial cells in the present *in vitro* study because of their functional similarity to normal astrocytes (Cabezas et al. 2015, Zabłocka et al. 2015). Studies show that glioblastomas express BDNF, and so may represent the stages of normal astrocyte lineage (Xie et al. 2008).

In our study, the tested antipsychotics differentially regulated the mRNA expression of *BDNF* depending on the concentration and incubation time. After the short-term treatment (24 hours) neither haloperidol nor olanzapine at a concentration of 5 μM exhibited any effect on the mRNA expression of *BDNF*. However, the chronic (72-hour) treatment with the neuroleptics up-regulated the levels of *BDNF* mRNA. After a 72-hour incubation period, at a concentration of 5 μM, haloperidol increased *BDNF* mRNA expression to 1.48 of the expression status while olanzapine increased it to 1.54. Our findings indicate that the tested neuroleptic drugs exert a potentially neuroprotective effect by increasing *BDNF* required time and repeating doses of drugs to develop.

The results achieved in studies on the effects of neuroleptics cannot be easily transferred from *in vitro* to *in vivo,* as the maximum duration of cell treatment is limited to 72 to 96 hours and a typical treatment regimen with antipsychotics lasts from 10 to 50 years. Nevertheless, it is possible that the effect observed in the present study in the cells exposed to neuroleptic drugs for 72 hours may be related to the delayed effect observed in the animal and clinical studies. It seems to be consistent with findings that the mechanisms by which neuroleptics produce their therapeutic effects, seen after a few weeks of continuous treatment, must involve the chronic regulation of a number of varied factors, like the appearance of neurotrophic factors following the immediate receptor blockade (Chlan-Fourney et al. 2002). It is possible that antipsychotics improve the symptoms of schizophrenia by altering the neuroplasticity of the affected neuronal pathways via the long-term regulation of genes involved in the remodeling and survival of adult neurons, for instance by increasing *BDNF* expression. The above findings correlate with pre-clinical studies on BDNF levels in rodents: Chronic (19-day) but not acute (45-minute) antipsychotic administration altered the levels of hippocampal *BDNF* mRNA in male Wistar rats significantly (Chlan-Fourney et al. 2002).

The next set of experiments examined whether the observed up-regulation of *BDNF* mRNA expression by haloperidol and olanzapine corresponded with the modification of protein level. It was found that both haloperidol and olanzapine at 5 μM concentration increased *BDNF* mRNA expression with similar efficacy after 72-hour incubation. Exposure of T98G cells to 5 μM haloperidol or olanzapine for 72 hours increased BDNF concentration in the culture medium by 13% and 29%, respectively. However, the up-regulation of *BDNF* mRNA level by olanzapine alone corresponded with the significantly increased production of BDNF protein detected by ELISA. The observed differences in BDNF level might be related to the different therapeutic effects of FGAs and SGAs in patients with schizophrenia. Our results correlate with previous *in vivo* studies showing that SGAs including olanzapine, clozapine, quetiapine and ziprasidone up-regulated BDNF levels, whereas haloperidol (FGA) down-regulates or does not affect BDNF levels (Bai et al. 2003, Park et al. 2006, 2009). Therefore, it can be supposed that the increased BDNF level caused by olanzapine may play a role in the neuroprotective mechanism of action of the tested drug in glial cells. BDNF, as the essential factor for the development of a number of glial properties, plays a key role in neuronal plasticity. Disturbances of the

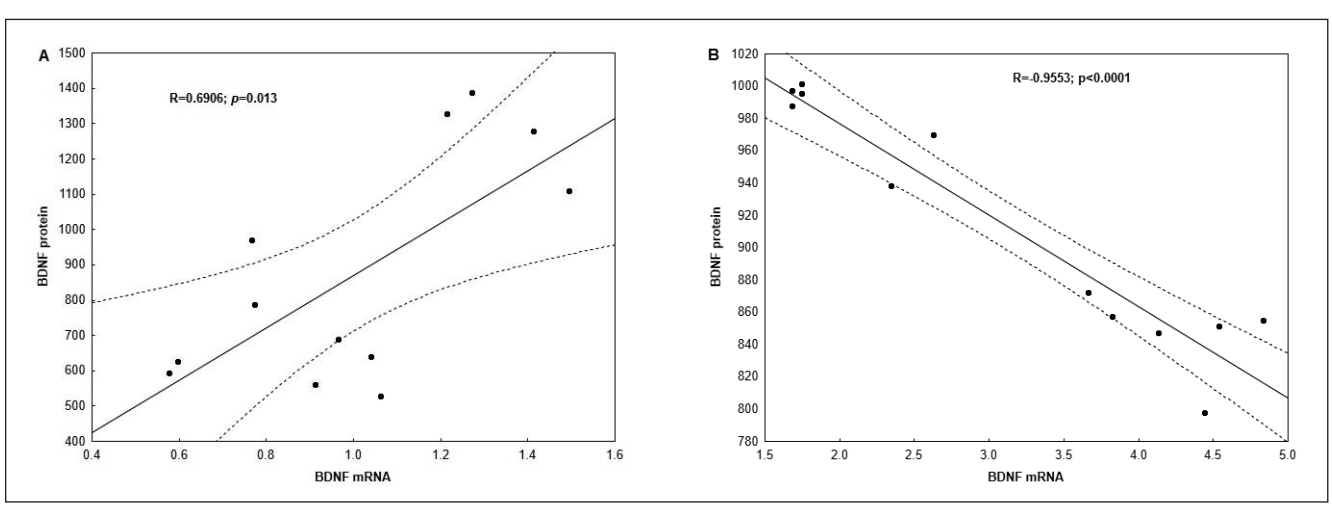

Fig. 5. Relationship between olanzapine and PACAP38-induced changes in mRNA and protein expression of BDNF in T98G cells in the 72-hour exposure period (A and B). The points represent the mRNA and protein gene expression data for olanzapine (5 μM) and PACAP38 (1μM), as well as the controls (dashed line*:* 95% confidence interval).

regulatory neurotrophic factor in glial cells could represent a major cause for neurological and psychiatric disorders. Therefore, the positive modulation of BDNF signaling may promote the normal state of astrocytes and support the protection and function of neuronal networks.

Surprisingly, haloperidol and olanzapine were not found to have analogous effects on *BDNF* mRNA level at a concentration of 20 μM. This higher concentration was found to have the opposite effect to lower concentration (5 μM) and caused a reduction of *BDNF* mRNA expression (especially after 24 hours of incubation). This demonstrates how the concentration of the drug affects the systems in the cell and shows how the secretion of BDNF is complicated and unclear. It is impossible to draw any firm conclusions about the clinical effects of haloperidol and olanzapine from our *in vitro* data because the doses of the neuroleptics we used were generally higher than those normally found in the brain tissue. Studies concerning antidepressants suggest that the negative effect on *BDNF* expression correlates with the simultaneous neurogenic and apoptotic processes of antidepressant drugs (Reichenstein et al. 2008). However, the concentration of neuroleptics used in this study was chosen on the basis of cell viability measurements: It was found that 20 μM was the highest concentration of haloperidol and olanzapine that did not exert any cytotoxic effect toward cells; this value was as high as 100 μM for amisulpride.

Some antidepressant drugs are known to induce both a bi-phasic and time-dependent change in *BDNF* gene expression (Coppell et al. 2006). This may also be the case with neuroleptics or, more likely, the effect of neuroleptics depends on their concentrations, and the lower concentrations of drugs used in the present study were effective in up-regulating *BDNF* mRNA expression and protein. There are findings from dose-response *in vivo* studies which suggest that lower doses of SGAs would upregulate hippocampal *BDNF* mRNA expression while higher doses would downregulate it (Chlan-Fourney et al. 2002). One explanation of this phenomenon may be the report by Kotani et al. (2006) that blockade or disruption of the cholinergic system down-regulates BDNF. Olanzapine, an SGA, is a selective 5-HT_{2A} and D2 receptor antagonist; however, as with all such drugs, it loses its selectivity at higher concentrations and can demonstrate the same effects as FGAs, which, besides the intended blockade of dopamine receptors, can also induce undesirable side effects by blocking cholinergic, adrenergic and histaminergic receptors.

The second type of SGA tested in the present study was amisulpride. Similarly to 20 μM haloperidol, amisulpride mildly decreased *BDNF* expression to 0.81 of expression levels following a 72-hour incubation at 20 μM. The observed effect wasn`t maintained by 100 μM of amisulpride and, therefore, the production of BDNF protein was not identified by ELISA. It has been shown that *BDNF* transcription is up-regulated by neuroleptic drugs which block 5-HT2A receptors in CNS (Vaidya et al., 1997). An example of this drug is olanzapine. Amisulpride exhibits poor affinity for the serotonin 5-HT_{2A} – receptor, and so probably does not exhibit a similar effect on BDNF as olanzapine. In turn, other *in vitro* studies on SH-SY5Y cells found amisulpride (1, 10, 100 μM) to dose-dependently increase the expression of the neuroprotective protein BDNF (Park et al. 2011). However, these results are not consistent with those of *in vivo* studies which found no significant differences were revealed in BDNF serum levels in 47 patients with schizophrenia after six weeks of amisulpride treatment, compared to their levels at study entry. The interesting fact is that serum BDNF was significantly increased in the subgroup receiving olanzapine compared to those treated with haloperidol, risperidone or amisulpride (Rizos et al. 2010). It is likely that the effect of given neuroleptics on BDNF expression may depend on the selectivity and/or pharmacodynamic profile of the drug.

Another aspect of the presented work was to compare the neuroprotective effect of neuroleptics with PACAP38, a peptide whose neuroprotective activity is known and well documented. Numerous findings provided by a number of research centers suggest that endogenous and exogenous PACAP, as well as its synthetic derivatives, demonstrate considerable neuroprotective and anti-inflammatory potential, suggesting the possibility of their use as new therapeutic strategies for the treatment of neurological insults and psychiatric diseases, such as stroke (Chen et al. 2006), schizophrenia (Matsuzaki and Tohyama 2008) and depression (Hashimoto et al. 2010). The mechanism of the neuroprotective action of PACAP is complex, but studies indicate that is mediated, at least partly, by preventing the suppressed expression of BDNF (Frechilla et al. 2001, Ogata et al. 2015).

In the present study, PACAP38 significantly up-regulated *BDNF* mRNA expression in T98G cells. The strongest stimulatory effect of PACAP38, a four-fold up-regulation of *BDNF* mRNA expression compared to the control group was observed after 24-hour incubation. A 72-hour incubation with PACAP38 resulted in levels of neurotrophin elevated two-fold compared to the control group (Fig. 2). The elevated levels of *BDNF* mRNA after 72-hour incubation with peptide was significantly lower than the effect observed after 24 hours. It is possible that the repetitive prolonged treatment of cultured cells with PACAP38 may lead to the adaptive changes in *BDNF* expression following 72-hour treatment. Georg and Fahrenkrug (2000) found PACAP38 to have a similar effect on endogenous VIP gene transcription in the human neuroblastoma cell line, with the maximal effect on VIP expression achieved following six-hour incubation, followed by a steep decline after 24 hours from the initial treatment. The upregulation of *BDNF* transcript levels measured 24 hours after exposure to PACAP38 may reflect the inhibition of the degradation of existing *BDNF*

transcripts while the delayed long-term up-regulated *BDNF* mRNA levels may represent the activation of transcriptional mechanisms involving cAMP-responsive element-binding protein (CREB) phosphorylation and the subsequent production of new *BDNF* transcripts. In order to confirm the observed changes in the mRNA expression of *BDNF*, a protein expression assay was performed. The results were coherent with those of the mRNA, and there was correlation between PACAP-induced changes in the mRNA and protein expression of BDNF. BDNF release from T98G cells was observed after 72 hours of PACAP38 treatment (Fig. 4).

In summary, of all the neuroleptic drugs tested in the present study, only olanzapine, similarly to exogenous PACAP38, increased the level of BDNF, which can contribute to its neuroprotective mechanism of action. Our findings offer a new insight into the mechanism of action of antipsychotics (neuroprotective) and confirm previous findings that BDNF may represent the key target for PACAP and SGAs - olanzapine.

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