Several lines of evidence suggest that programmed cell death not only plays an important role in developmental processes of neural tissue but may also contribute to the loss of neurons observed in CNS structures of aging brain and in patients suffering from affective disorders as well as other CNS diseases, e.g., schizophrenia, neurodegenerative diseases, panic disorder. This process may underlie a common pathophysiological mechanism shared by several mental disease processes and provide some insight into the high rates of comorbidity that exist between neuropsychiatric disorders. It is assumed that the mechanism of action of numerous drugs modulating CNS function is associated with their protective and anti-apoptotic properties. It is supposed that anti-apoptotic effects are involved in the therapeutic activity of antidepressants. The aim of the present study was to evaluate anti-apoptotic effect of desipramine, fluoxetine and tranylcypromine in OGD of cortical neurons. Cell cultures were exposed to OGD (3% of O2) and one of the studied drugs at a concentration of 0.1, 1 or 10 µM for 6, 12 or 24 h. The drugs positively influenced cell viability estimated by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay but their effect declined during incubation longer than 6 h. Only fluoxetine in all applied concentrations increased the number of cells with the fragmented nuclei visualized by Hoechst staining after 24-h incubation. Moreover, fluoxetine stronger than desipramine and tranylcypromine stimulated Bcl-2 (B cell lymphoma protein 2) expression evaluated by Western blotting in 6-h and 24-h experiments. Fluoxetine (0.1 or 1 µM) and tranylcypromine (0.1 µM) in time dependent manner induced the positive effect on pGSK3β(Ser9) expression. Our results indicate that fluoxetine more efficiently than desipramine or tranylcypromine prevented OGD-induced apoptosis in the primary neuronal culture.

Keywords: antidepressant drugs, apoptosis, glycogen synthase kinase 3β, oxygen-glucose deprivation, primary neuronal culture

* Corresponding author: e-mail: apude1309@yahoo.com; phone/fax: +48 32 2523 835
entists has been focused on importance of canonic Wnt/β-catenine pathway in CNS pathology. Nowadays, it is known that protein components of this pathway and kinases involved in the regulation of its activity play an important role in Alzheimer’s disease, schizophrenia and bipolar affective disorders (10, 11). Glycogen synthase kinase 3 (GSK3) seems to be an interesting component of canonic Wnt (Wingless+Int-1)/β-catenine signaling pathway. It has been found that phosphorylated GSK3β(Ser9) via β-catenine enhances transcription of several genes involved in processes of cell proliferation, differentiation, synaptic plasticity and apoptosis (12-14). Discovery of the lithium influence on GSK3 activity and its anti-depressant, mood improving and neuroprotective effects encouraged intensive studies on the participation of GSK3 in the mechanism of action of drugs affecting brain function (15, 16). The fact that GSK3 inhibition might regulate processes of neurogenesis and synaptic plasticity gave foundation to the idea that this kinase might be an important element of molecular mechanism of action of antidepressants (14, 17, 18). The observation that antidepressant drugs do not ameliorate disease symptoms immediately but usually after fourteen or more days from the onset of therapy may provide a rationale for such conviction. On the other hand, it is known that GSK3 also contributes to apoptosis (13). In fact, there are several data indicating that antidepressants target GSK3 signaling pathways and this ultimately promotes neurogenesis and their anti-apoptotic activity.

This study was designed to examine the effect of desipramine (a tricyclic antidepressant), fluoxetine (a representative of selective serotonin reuptake inhibitors) and tranylcypromine (a non-selective irreversible inhibitor of monoamine oxidase) on cell viability, apoptosis (Hoechst staining and Bcl-2 expression) and the level of pGSK3β(Ser9) in primary culture of cortical neurons subjected to combined oxygen-glucose deprivation. Expression/activity of both studied proteins - Bcl-2 and pGSK3β(Ser9) is regulated via the same pro-survival PI3K/Akt (phosphatidylinositol 3 kinase/Protein kinase B) pathway.

Combined oxygen-glucose deprivation (OGD) is widely used as an in vitro model of ischemic/hypoxic insult. Parallel withdrawal of glucose and trophic factors from culture medium and decreased oxygen concentration induces alterations in cultured cell viability, development of apoptosis and disturbances of different intracellular signaling pathways. This model is considered to be useful for assessment of cytoprotective/anti-apoptotic potency of different drugs or chemicals (19) and that is why it was used in current study. Another rationale for testing anti-depressant drug in this model is also fact that depression might develop in the aftermath of ischemic stroke episodes in some patients (20). To the best of our knowledge, there are only a few studies on the effect of antidepressants on OGD-induced apoptosis in primary cortical neuronal cultures and even when this model was applied, the studies were focused on reoxygenation events (21, 22). The aim of this study was to examine what is the influence of the chosen antidepressants on some markers of apoptosis in neurons exposed to OGD insult.

MATERIALS AND METHODS

Cell culture

Primary cultures of cortical neurons were prepared from Wistar rat embryos at day 18th of pregnancy according to the method of Toborek et al. (23). After dissection, brain tissue was first mechanically disrupted and then digested with a medium containing 1 mL of 10 × concentrated 0.05% trypsin-EDTA (ethylenediaminetetraacetic acid) solution (Sigma, USA) and 9 mL of DMEM (Dulbecco’s modified Eagles medium) (Invitrogen, USA). After a short incubation at 37°C, the enzyme was inactivated with an equal volume of 10% FBS (fetal bovine serum) (Invitrogen, USA) containing DMEM (DMEM-FBS10) and centrifuged for 5 min at 1000 rpm (225 × g). Supernatant was discarded and cells were next dissociated using DNAse I (Roche, Switzerland) solution (40 µg/mL of DMEM-FBS10). The cortical neurons were plated at a density of 2 million cells/1 mL of DMEM-FBS10 into 96 well plates for viability measurement or on 60 mm polyethyleneimine (Fluka, USA) coated culture dishes (p60) (Nunc, Denmark or Becton Dickinson, USA) for other experiments (100 µL/well and 2 mL of cell suspension/dish, respectively). On the next day after seeding, culture medium was changed to Neurobasal medium with B27 supplement, L-glutamine (2 mM), gentamicin (100 µg/mL) and fungizone (2.5 µg/mL) (all from Invitrogen, USA). The medium was then exchanged every third day. Neuronal cultures were maintained in an atmosphere of 5% CO2 and 95% relative humidity at 37°C (NUAIR CO2 Incubator, USA). To prevent growth of glial cells, fluordeoxyuridine (54 µM) and uridine (14 µM) mixture (Sigma, USA) was added to medium for 24 h. Some cultures were incubated with neuron-specific anti-MAP2 (microtubul associated protein 2) antibody or glia-specific anti-GFAP (glial fibrillary acidic protein) antibody.
(Sigma, USA) and stained with secondary Texas Red or FITC (fluorescein isothiocyanate) conjugated antibodies (Santa Cruz Biotech., USA) to confirm purity of cell cultures. Above 90% of cell population were MAP2 positive. Experiments were performed on 9th – 12th day after plating.

**Treatment of neuronal cultures**

Neuronal cell culture standard medium (Neurobasal) was replaced with glucose free DMEM containing examined antidepressant drugs and incubated for 6, 12 or 24 h in the ischemia simulating conditions (New Brunswick Scientific CO2 Incubator Galaxy 48R, USA). The OGD model is based on restricted oxygen concentration (3% O2, 92% N2, 5% CO2) and glucose and growth supplement (B27) withdrawal from culture medium containing mannitol instead to maintain adequate medium osmolarity (319 mOsm) (24). Cells were treated with the following antidepressants: desipramine [3-(10,11-dihydro-5H dibenzo[b,f]-azepin-5-yl)-N-methylpropan-1-amine hydrochloride] (Sigma, USA), fluoxetine [(RS)-N-methyl-3-phenyl-3-[4-(trifluromethyl)phenoxy]propan-1-amine hydrochloride] (Polfa, Poland) and tranylcypromine [(±)-trans-2-phenylcyclopropan-1-amine] (Sigma, USA) at a concentration of 0.1, 1 and 10 µM for 6, 12 or 24 h. The concentrations of antidepressant drugs in this study were inside the clinically useful range. The brain concentration of antidepressant drugs vary from 5 to 25 µM after systemic administration of pharmacologically effective doses to animals and humans (25-27). Control neuronal cultures were maintained in normoxic condition or OGD condition only and not exposed to the antidepresant drugs.

The study was approved by the local ethics committee for animal experiments of the Medical University of Silesia.

**Cell viability**

To estimate general cell viability 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyletetrazolium bromide (MTT) reduction assay was performed. The cells ability to convert MTT indicates mitochondrial integrity and activity, which in turn reflects cell viability. MTT (Sigma, USA) dissolved in PBS (phosphate buffered saline) was added to the culture medium 3 h before the end of experiment at a final concentration of 0.25 mg/mL. Medium was then removed and cells were lysed with MSO (methyl sulfoxide) (Sigma, USA) to allow formazan crystals to dissolve. Absorbance at the wavelength of 590 nm was read on a Multiscan RC microplate reader (Labsystems, Finland). Influence of antidepressant drugs on neuronal cultures was first tested under normoxic conditions. Results are presented as the means absorbance + SD of three independent experiments.

**Assay for nuclear apoptosis**

Hoechst dye 33342 (Sigma, USA) was used to stain nuclei of cortical neurons. After 24 h of incubation at experimental conditions, cell cultures growing on 60 mm dishes were rinsed with PBS and fixed in methanol for 15 min at RT (room temperature), washed again with PBS and incubated with Hoechst dye (1 µg/mL) for 20 min at RT in the dark. Analysis of cell nuclei was conducted under a fluorescence microscope (Nikon, Japan). Twenty-four hours exposure time was appointed to show explicit effect of antidepressant drugs on chromatin structure.

Apoptotic nuclei were identified as nuclei with chromatin fragmentation or condensation. The percent of apoptotic nuclei (apoptotic versus total number of nuclei) was counted at 40x magnification on at least six randomly selected fields, containing about 100 cells each. The results are expressed as the means ± SD of three independent cell cultures.

**Preparation of cell lysates**

Cells were harvested and lysed in ice-cold lysis buffer pH = 7.4 containing 50 mM Tris [tris(hydroxymethyl)aminomethane], proteases and phosphatases inhibitors (aprotinin, leupeptin, pepstatin A, PMSF (phenylmethylsulfonyl fluoride) and sodium orthovanadate, 150 mM NaCl, 1 mM EDTA, Igepal and 1% SDS (sodium dodecyl sulfate) (all reagents from Sigma, USA). Disrupted cells suspensions from 2 or 3 p60 dishes were combined together as one sample. Samples were then centrifuged (12 000 ◊ g, 15 min, 4°C). Supernatants were collected and stored at -20°C until electrophoresis was performed. Protein concentration was determined using Bradford reagent (Sigma, USA).

**Western blotting**

Equal amounts of total protein (25-50 µg) were loaded into 10% SDS-polyacrylamide gel (SDS-PAGE) wells and electrophoresis was performed using 25 mM Tris-glycine-SDS buffer (pH = 8.3) at 100 V (all reagents from Sigma, USA). The resolved proteins were transferred from the gel onto nitrocellulose membrane (Bio-Rad Laboratories Ltd., UK) in 48 mM Tris-glycine-SDS methanol containing (20% v/v) buffer (pH = 8.3) at 100 V for 1 h.

Transfer quality was confirmed by staining of the membranes with Ponceau S (Sigma, USA). Membranes were then washed with TBST (Tris buffered saline – Tween 20) buffer (20 mM Tris, 0.5
M NaCl, 0.5% Tween 20) to remove the dye and incubated with 5% non-fat milk in TBST for 1 h at RT on orbital shaker to prevent non-specific binding of antibodies. The blots were incubated overnight at 4°C with specific primary antibodies (concentration range 1 : 200 – 1 : 1000) raised against a protein of interest, e.g., Bcl-2 anti-apoptotic protein, glycogen synthase kinase 3β (GSK3β) and phosphorylated pGSK3β (Ser9) form (inactive) (all antibodies from Santa Cruz Biotech., USA).

On the next day, the membranes were rinsed 3 times (10 min each) in a copious amount of TBST and incubated with secondary antibody conjugated with horseradish peroxidase (HRP) for 1 h at RT (1 : 2000 in 5% milk/TBST solution). Membranes were rinsed in TBST as described previously and briefly (1 min) incubated with ECL (enhanced chemiluminescence) system reagents (GE Health Care, UK). Results were detected by exposing Kodak XAR-5 film (Sigma, USA) to luminescent signal. Protein bands intensity was assessed by densitometry measurement (Image J Software 1.42 q, NIH, USA). Results were expressed as percent of OGD control and reported as the means ± SEM of 3 independent cell cultures.

Statistical analysis of results

Statistical analysis of the data was performed using a one-way ANOVA followed by post-hoc Newman-Keuls test (GraphPad Prism 4.01 software Inc., USA). Statistical probability above 95% (p < 0.05) was considered significant.

RESULTS

Cell viability

Neuronal cell viability was not affected by the antidepressant drugs in the applied range of concentrations under normoxic conditions (data not shown). OGD exposure for 6, 12 or 24 h diminished neuronal viability measured by MTT assay by 33, 45 or 57%, respectively. During a 6-h exposure to OGD and the

![Figure 1](image-url)
Desipramine, fluoxetine and tranylcypromine have different effects on apoptosis... 1155

studied antidepressants, cell viability was increased in comparison to the OGD effect alone. This effect was induced by desipramine, fluoxetine and tranylcypromine at the lowest concentration of 0.1 µM (an increase by 32, 15 and 26%, respectively) as well as by fluoxetine at the concentration of 1 µM (by 13%) and by tranylcypromine at the highest concentration of 10 µM (by 22%) (Fig. 1A). After a 12-h exposure to OGD and desipramine or fluoxetine, neuronal viability was not changed in comparison to OGD alone but was increased after incubation with tranylcypromine at the concentration of 0.1 or 1 µM (about 14%) (data not shown). In the 24-h experiment, desipramine and tranylcypromine at both lower concentrations of 0.1 and 1 µM increased cell viability: desipramine about 17% and tranylcypromine by 17 or 23%, respectively. Also fluoxetine at these concentrations induced a tendency towards an increased cell viability (Fig. 1B).

Hoechst dye staining

In order to estimate the influence of the antidepressants studied on intensity of chromatin condensation and nuclei disintegration induced by OGD, cell cultures were exposed to OGD and antidepressants for 24 h. In OGD-exposed cultures, the apoptotic cell population increased markedly up to 11% in comparison to about 3% of such cells visible under normoxic conditions (Fig. 2B). In comparison to the effect of OGD alone, desipramine at the concentration of 0.1 µM and fluoxetine in all applied concentrations reduced the number of apoptotic cells by about 50%. Unexpectedly, tranylcypromine significantly enhanced DNA fragmentation at the concentration of 10 µM up to 14% but a tendency towards a reduced number of cells with apoptotic nuclei was observed when the drug was used at the lowest concentrations (Fig. 2A).
Expression of Bcl-2 protein
Under OGD conditions, a tendency towards an increased expression of Bcl-2 protein was noted in neuronal cultures exposed for 6 h to desipramine or fluoxetine. Only fluoxetine at a concentration of 0.1 µM produced significant effect - an increase by 18% (Fig. 3A).

The studied drugs did not affect Bcl-2 protein levels after 12 h of incubation (data not shown). The more pronounced positive tendency was observed after 24-h incubation with desipramine as well as fluoxetine in all concentrations and tranylcypromine at concentration of 0.1 µM. The significant increase by 32% was induced only by fluoxetine at the lowest concentration (Fig. 3B).

Expression of pGSK3β(Ser9) and pGSK3β(Ser9)/GSK3β ratio
In the result of a 12-h incubation under OGD conditions, desipramine did not affect the expression of pGSK3β(Ser9) with the exception of 1 µM concentration. Effect was not observed after standardization to GSK3β. The relative blot intensity was increased by 10% in comparison to its value estimated after a 6-h incubation with desipramine. Fluoxetine in a concentration-dependent manner, namely at 0.1 µM (by 27%), 1 µM (by 20%) and at 10 µM (by 15%) increased the expression of pGSK3β(Ser9). Similar effect was observed in the pGSK3β(Ser9)/GSK3β ratio. Tranylcypromine increased the expression of pGSK3β(Ser9) and a relative blot intensity only at a concentration of 0.1 µM (Fig. 4B and 5B).

In 24-h experiment, only tranylcypromine when applied at 0.1 µM decreased pGSK3β(Ser9) expression apparently but had no influence on the relative blot intensity (Fig. 4C and 5C).

DISCUSSION
The present study was performed on primary cortical neuronal cultures in the well-known oxy-
Desipramine, fluoxetine and tranylcypromine have different effects on apoptosis... 1157

gen-glucose deprivation model. To the best of our knowledge, the effect of antidepressants on OGD-induced apoptosis was mainly evaluated during re-oxygenation period which is considered as a phase more responsible for the ischemic toxic effects (7, 28, 29) but not immediately after OGD neurotoxic insult as it was in the present study.

As demonstrated by Hoechst staining, the most pronounced anti-apoptotic effect in cells exposed to OGD for 24 h was induced by fluoxetine at all applied concentrations. Desipramine to the same extent reduced the number of apoptotic cells only at the lowest applied concentration (0.1 µM). The results of the whole our study indicate that tranylcypromine is able to trigger or attenuate apoptosis in concentration-dependent manner. This drug induced the opposite effects when applied at the concentration of a 0.1 µM or at a 100-fold higher concentration - 10 µM. A positive tendency towards a decreased population of apoptotic cells we observed

Figure 4. pGSK3β(Ser9) expression after exposure to OGD and desipramine, fluoxetine or tranylcypromine applied at a concentration of 10, 1 and 0.1 µM. The results of pGSK3β(Ser9) level are expressed as the mean (n = 4) percent of blot integrated optical density measured in OGD only exposed cell cultures + SEM. (A) 6-h experiment (B) 12-h experiment (C) 24-h experiment. OGD = oxygen-glucose deprivation condition. Statistical significance (Newman-Keuls test after one-way ANOVA): * p < 0.001; # p < 0.01 vs. OGD. Representative blots of pGSK3β(Ser9) are attached
induced only a moderate positive effect on cell viability mainly observed after short, 6-h exposure to OGD. One may assume that discrepancy between the cell viability and Hoechst staining results is due to the reported by others inhibitory effect of fluoxetine and amitriptyline, for example, on mitochondrial respiratory chain enzymes (31).

In opposite, tranylcypromine was reported to have a positive influence on mitochondrial function

Figure 5. Total GSK3β expression after exposure to OGD and desipramine, fluoxetine or tranylcypromine applied at a concentration of 10, 1 and 0.1 μM. The results of GSK3β level are expressed as the mean (n = 4) percent of blot integrated optical density measured in OGD only exposed cell cultures + SEM. (A) 6-h experiment (B) 12-h experiment (C) 24-h experiment. OGD = oxygen-glucose deprivation condition. Statistical significance (Newman-Keuls test after one-way ANOVA): * p < 0.05 vs. OGD. Representative blots of GSK3β are attached.
Desipramine, fluoxetine and tranylcypromine have different effects on apoptosis... 1159

and in our study, a positive tendency towards an increased cell viability in cultures exposed to tranylcypromine at the lower concentrations was also observed (32). The effects of the studied drugs on mitochondria seems to be important because MTT assay used to evaluate cell viability is an indicator of mitochondrial function.

Moreover, our findings indicate that especially fluoxetine has positive influence on expression of Bcl-2. The observed up-regulation of Bcl-2 expression was parallel to anti-apoptotic effects (detected by Hoechst staining) of desipramine, fluoxetine and tranylcypromine at the lowest applied concentration. The obtained results are in line with the data from the other studies in which the anti-apoptotic effects of fluoxetine and desipramine were observed in studies conducted *in vitro* or in a few *in vivo* experiments (25, 33). Tranylcypromine was not as intensively studied as other MAO inhibitors in regard to its neuroprotective potential. Kosten et al. (6) reported an increased expression of the Bcl-2 and Bcl-XL (B-cell lymphoma-extra large) mRNA measured in

Figure 6. Relative blot intensity of pGSK3β(Ser9)/total GSK3β after exposure to OGD and desipramine, fluoxetine or tranylcypromine applied at a concentration of 10, 1 and 0.1 µM. The results are expressed as the percent of relative optical density calculated for OGD only exposed cell cultures. (A) 6-h experiment (B) 12-h experiment (C) 24-h experiment. OGD = oxygen-glucose deprivation condition.
different rat brain structures after tranylcypromine administration. In our study, the effect of tranylcypromine on Bcl-2 expression was time and concentration dependent. Parallel positive influence of tranylcypromine on cell viability and on Bcl-2 expression was found only in 24 h experiment.

Furthermore, the results obtained in this study reveal that the examined antidepressants display different impact on GSK3β phosphorylation. GSK3β plays a role as a pro-apoptotic agent. Activation of Wnt signaling increases GSK3 inactivation by serine phosphorylation in position 9 of the GSK3β isoform or position 21 of GSK3α, respectively. Hence, factors intensifying phosphorylation of GSK3β can prevent initiation of programmed cell death. Numerous papers concerning mood stabilizers or effects of SSRI have suggested that GSK3β inhibition produces both anti-apoptotic and neuroprotective effects through the influence on neurogenesis and synaptic plasticity and/or remodeling (14, 17, 18, 34).

In the applied OGD model, fluoxetine induced a more beneficial influence, namely, it preserved or increased pGSK3β(Ser9) level. These results supported the mentioned above anti-apoptotic effect of fluoxetine. However, we did not observe the parallel effect of desipramine on pGSK3β expression and percentage of apoptotic cells induced by OGD. Influence of tranylcypromine on GSK3β phosphorylation was not homogenous. Also in this experiment, the strongest positive effect was found when the drug was applied at the concentration of 0.1 µM for 6 or 12 h.

Until now, only several studies in the primary neuronal cultures or in the in vitro OGD model have been performed to investigate the influence of desipramine or fluoxetine used in the present study on GSK3β activity (28, 35).

Obtained results suggest that desipramine and tranylcypromine especially at concentration higher than 0.1 µM have an essential influence on some other mechanisms or signal transduction pathways but not Wnt/β-catenine signaling. To the best of our knowledge, the effect of neither desipramine nor tranylcypromine on OGD-induced apoptosis in the primary neuronal cultures has not been studied. Apart from PI3/Akt kinase and Wnt signaling pathways that regulate GSK3 activity, other mechanisms are considered to be involved in the anti-apoptotic drug effect in the OGD model. OGD-induced damage in PC12 (pheochromocytoma) cells, associated with a marked activation of ERK (extracellular signal regulated kinase), JNK (c-Jun N-terminal kinase) and p-38 MAPK (mitogen activated protein kinase) was prevented by exogenous neuronal growth factor mediated JNK inhibition (36). Tranycyromine has positive effect on MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) induced neurotoxicity (32) or enhanced CREB (cAMP responsive element binding transcription factor) mRNA level in hippocampus (37) - thus could possibly stimulate neurogenesis. There are some evidence indicating that antidepressants may enhance synthesis or release of neurotrophic factors from neurons. They presumably modify the signal transduction pathways associated with neurotrophic factor receptor stimulation. This may also explain their neuroprotective and/or anti-apoptotic effects (7, 12, 38).

To sum up, in the OGD experimental model, fluoxetine exerted stronger anti-apoptotic effects than desipramine or tranylcypromine in primary cortical neuronal culture under hypoxic insult as demonstrated by Hoechst staining, MTT cell viability test, Bcl-2 and pGSK3β(Ser9) expression level determination. It occurred that tranylcypromine is able to trigger or attenuate apoptosis in a concentration dependent manner. The obtained results suggest that the effect of the studied antidepressants on apoptosis induced by OGD in vitro only partly is mediated by GSK3β. Better recognition of neuroprotective activity of antidepressants is important because drugs with stronger such activity could be more preferable in the treatment of mood disturbances related especially to neurodegenerative diseases or in the therapy of post-stroke patients.

Acknowledgment

This study was supported by grant project NN 401003436 from the Ministry of Science and Higher Education in Warszawa, Poland.

REFERENCES

Desipramine, fluoxetine and tranylcypromine have different effects on apoptosis...

1161

34. Su H.Ch., Ma Ch.T., Yu B.Ch., Chien Y.Ch., Tsai Ch.Ch. et al.: Int. Immunopharmacol. 14, 150 (2012).

Received: 16. 08. 2014