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Orexins Protect Neuronal Cell Cultures Against Hypoxic Stress: an Involvement of Akt Signaling

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Abstract Orexins A and B are peptides produced mainly by hypothalamic neurons that project to numerous brain structures. We have previously demonstrated that rat cortical neurons express both types of orexin receptors, and their activation by orexins initiates different intracellular signals. The present study aimed to determine the effect of orexins on the Akt kinase activation in the rat neuronal cultures and the significance of that response in neurons subjected to hypoxic stress. We report the first evidence that orexins A and B stimulated Akt in cortical neurons in a concentration- and time-dependent manner. Orexin B more potently than orexin A increased Akt phosphorylation, but the maximal effect of both peptides on the kinase activation was very similar. Next, cultured cortical neurons were challenged with cobalt chloride, an inducer of reactive oxygen species and hypoxia-mediated signaling pathways. Under conditions of chemical hypoxia, orexins potently increased neuronal viability and protected cortical neurons against oxidative stress. Our results also indicate that Akt kinase plays an important role in the pro-survival effects of orexins in neurons, which implies a possible mechanism of the orexin-induced neuroprotection.

Keywords Orexin · Akt kinase · Neuroprotection · Chemical hypoxia

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Abbrevations

MAPK	Mitogen-activated protein kinase
OX_1R	Type 1 orexin receptor
OX_2R	Type 2 orexin receptor
PA	Phosphatidic acid
PLC	Phospholipase C

Introduction

Orexins are neuropeptides discovered simultaneously in 1998 by two independent research groups, one of which named them orexins (A and B) and the other hypocretins (hypocretin-1 and hypocretin-2) (de Lecea et al. 1998; Sakurai et al. 1998). Orexins A (33 amino acids) and B (28 amino acids) are proteolytically processed from a common precursor peptide, preproorexin (Alvarez and Sutcliffe 2002). Both neuropeptides interact with G protein-coupled receptors, types 1 (OX_1R) and 2 orexin receptor (OX_2R), which display different affinity for orexins (Voisin et al. 2003). On the basis of binding and Ca^{2+} elevation studies in recombinant expression systems, it has been established that orexin A couples to OX_1R with a 5- to 100-fold higher affinity than orexin B, whereas both peptides have similar affinities to OX_2R (Ammoun et al. 2003; Sakurai et al. 1998). OXRs are widely distributed in the central nervous system and peripheral tissues. Interestingly, orexin-containing neuronal cell bodies, although localized only in lateral hypothalamic area and nearby regions, project to numerous brain structures and are regulated by many neurotransmitter systems (Kukkonen et al. 2002; Kukkonen 2012; Nambu et al. 1999; Peyron et al. 1998). This anatomical architecture of orexin neurons and the wide distribution of orexin receptors appear to be essential for multiple functions of these neuropeptides. Orexins were originally described to play a key role in the control of feeding

and sleep–wake cycle, with an emphasis on their contribution to the pathogenesis of narcolepsy, but since their discovery many other physiological functions of the peptides have been well-documented, i.e., regulation of motivated behaviors, such as arousal, reward-seeking and drug addiction, metabolic rate and thermogenesis, autonomic or endocrine processes (Aston-Jones et al. 2010; Berthoud et al. 2005; Chemelli et al. 1999; Mieda et al. 2004; Randeva et al. 2001; Sakurai et al. 1998; Spinazzi et al. 2006; Thompson and Borgland 2011; Zhang et al. 2009). Multitude of physiological actions controlled by orexins originates from highly diverse cellular responses to the orexin receptors stimulation. It has been demonstrated that stimulation of orexin receptors may trigger activation of classical phospholipase C (PLC) cascade (PLC-IP_3/DAG) with a subsequent robust increase in intracellular Ca^{2+} concentration in recombinant cell lines and native systems (Johansson et al. 2007; Lund et al. 2000; Mazzocchi et al. 2001; Randeva et al. 2001). Other orexin-activated signaling pathways include adenylyl cyclase (AC)/cyclic AMP, phospholipase D/phosphatidic acid (PA), phospholipase A_2/arachidonic acid release and mitogen-activated protein kinase (MAPK)/stress-activated protein kinase (Holmqvist et al. 2005; Magga et al. 2006; Tang et al. 2008; Urbańska et al. 2012; Woldan-Tambor et al. 2011; Zhu et al. 2003). Despite a large number of orexins-mediated signaling pathways described in the literature, a role of these neuropeptides in activation of Akt kinase is at present largely unknown. Therefore, the aim of our work was to study the effect of orexins on the Akt kinase activation in primary neuronal cell cultures.

It has been demonstrated that, depending on the activated signal cascade, orexins can affect different cellular processes, such as neuronal excitation, cell growth, plasticity, death or survival (Ammoun et al. 2006; Holmqvist et al. 2005; Kukkonen 2012; Ramanjaneya et al. 2009; Rouet-Benzineb et al. 2004; Tang et al. 2008). Recent studies in a model of cerebral ischemia in rats indicated a new role of orexin A as a neuroprotective factor. The peptide was shown to attenuate ischemia–reperfusion injury by reducing the number of apoptotic cells (Kitamura et al. 2010; Yuan et al. 2011). As ischemia is a restriction in blood supply to the tissue, causing a shortage of oxygen and glucose needed for cellular metabolism, in the present study we also tested neuroprotective potential of both orexins in neuronal cultures derived from rat cerebral cortex subjected to hypoxic stress induced by cobalt chloride. Cobaltous ions mimic hypoxia conditions by activation of hypoxia-mediated signaling pathways and generation of reactive oxygen species (ROS) (Chandel et al. 1998; Vengellur and LaPres 2004). Finally, as Akt kinase is well known for its ability to control cellular survival and apoptosis, we checked whether this kinase is involved in neuroprotective effects of orexins in cells subjected to chemical hypoxia.

Materials and Methods

Animals and Neuronal Cell Culture

Experiments were performed on primary neuronal cell cultures prepared from Wistar rat embryos on day 17 of gestation. Animal procedures were in a strict accordance with the Polish governmental regulations concerning experiments on animals (Dz.U.05.33.289), and the experimental protocol was approved by the Local Ethical Commission for Experimentation on Animals.

Primary neuronal cell cultures were prepared according to the method of Brewer (1995), as previously described in detail (Nowak et al. 2007; Urbańska et al. 2012). Briefly, the rat cerebral cortex was isolated from fetal brain, incubated for 15 min in trypsin/EDTA (0.05 %) at 37 °C followed with a trituration in a solution of DNase I (0.05 mg/ml) and fetal bovine serum (20 %) in Ca^{2+}- and Mg^{2+}-free PBS. The cells were maintained in Neurobasal medium supplemented with B27 (2 %), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Seventy-two hours after plating, the cellular proliferation was stopped by adding to the medium the solution of 1-β-D-arabinofuranosylcytosine (5 μM). The glial content in neuronal cultures, analyzed by using antibody against glial fibrillary acidic protein, was estimated to be 6–10 % of the total cell population (Urbańska et al. 2012). Cells were cultured as monolayer on poly-L-ornithine coated multi-well plates or 60 mm culture dishes for 7–8 days before experiments at 37 °C in a humidified atmosphere with 5 % CO_2.

Protein Extraction and Western Blot Analysis

Whole cell extracts were prepared from neuronal cultures treated with orexins to activate Akt. Briefly, following treatments cells were washed in ice-cold PBS and removed from the surface by scraping in cell lysis buffer (M-PER Mammalian Protein Extraction Reagent, Thermo Scientific, Rockford, IL, USA) with protease and phosphatase inhibitors (Halt Protease and Phosphatase Inhibitor Cocktail, Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions. Cell extracts were collected and stored at −70 °C until protein concentration was measured by the Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, USA). Electrophoresis and blotting was performed using NuPAge Novex system with the XCell SureLock Mini-Cell and the Novex Semi-Dry Blotter (Invitrogen, Paisley, UK) according to the manufacturer's instruction. Denatured samples with an equal amount of protein per lane (20 μg) were separated on gradient gels (4–12 % Bis-Tris gel), electrotransferred on nitrocellulose membrane (Hybond C extra, Amersham Biosciences, Buckinghamshire, UK) and blocked in 5 % (*w*/*v*) bovine serum albumin in TBST (0.1 % Tween-20 in Tris Buffered Saline,

TBS), for 1 h at room temperature. After 4 °C overnight incubation with primary antibodies (phospho^{Ser473}-Akt, 1:1,000, Cell Signaling Technology, Danvers, MA, USA) membranes were washed several times in TBST, and then incubated in TBST-milk with horse radish peroxidase-conjugated secondary antibodies (anti-rabbit IgG, 1:2,000, Dako, Glostrup, Denmark) for 1 h at room temperature. Peroxidase enzymatic activity was detected using Super Signal West Pico Chemiluminescent Substrate (Thermo Scientific, Rockford, IL, USA) and visualized on G-box iChemi XT4 system utilizing GeneSys automatic control software (Syngene, Cambridge, UK). Then, membranes were stripped in Restore Western Blot Stripping Buffer (Thermo Scientific, Rockford, IL, USA) for 10 min at room temperature and after extensive washing reprobed with total Akt antibody (1:1,000, Cell Signaling Technology, Danvers, MA, USA). The same secondary antibody and detection system was used as described above. The phospho-specific signal was correlated to the total Akt.

Cell Viability Assay

Cell viability and mitochondrial function were measured by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) reduction to MTT formazan by cellular mitochondrial dehydrogenases. Following exposure to orexin A, orexin B, and cobalt chloride, neuronal cells were washed in PBS before the addition of MTT (0.5 mg/ml) and incubated for 3 h at 37 °C. Formazan crystals were solubilized in dimethyl sulfoxide (100 %) and absorbance, proportional to the number of viable cells, was measured at 570 nm using a microplate reader (EnVision 2103, Perkin Elmer, Turku, Finland).

Reduced-MitoTracker Orange Staining

We monitored oxidative stress in rat neuronal cultures by utilizing a dye that is internalized within cells and does not fluoresce until its reduced moieties are oxidized. MitoTracker Orange CM-H_2TMRos (Invitrogen, Paisley, UK) was successfully used to study ROS generation in neurons (Ibarretxe et al. 2006; Kweon et al. 2001; Lampe et al. 2010). After 24-h incubation of rat neuronal cultures with orexins A and B in the presence or absence of cobalt chloride (100 μM), culture medium was removed and the cells were stained with CM-H_2TMRos according to the manufacturer's instruction. Finally, the cells were washed with PBS, counterstained with 2 μg/ml Hoechst 33342 for 20 min, and subjected for analysis by high content reader ArrayScan VTI HCS Reader (Thermo Scientific, Rockford, IL, USA) equipped with ×10 objective. Routinely, images of 20 fields/well were acquired and 5000 cells/well were analyzed with Cell Health Profiling Bioapplication V3 software (Cellomics BioApplications, Cellomics, Thermo Scientific, Rockford, IL, USA). Oxidative stress level in rat cortical neurons was calculated on the basis of mean cellular fluorescence and expressed as a percentage of the respective control.

Chemicals

Orexins A and B were from NeoMPS (Strasbourg, France). Poly-*L*-ornithine, DNase I, trypsin, glutamine, penicillin, streptomycin, 1-β-D-arabinofuranosylcytosine, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT), dimethyl sulfoxide, cobalt chloride, TBS, Tween 20, albumin from bovine serum, non-fat dried bovine milk were from Sigma-Aldrich (Poznan, Poland). 10-DEBC (10-[4′-(*N*,*N*-diethylamino)butyl]-2-chlorophenoxazine hydrochloride) and GSK690693 (4-[2-(4-amino-1,2,5-oxadiazol-3-yl)-1-ethyl-7-[(3*S*)-3-piperidinylmethoxy)-1*H*-imidazo[4,5-c]pyridin-4-yl]-2-methyl-3-butyn-2-ol) were from Tocris Bioscience (Bristol, UK). Neurobasal medium, B27, fetal bovine serum, CM-H_2TMRos, NuPAGE MES SDS Running Buffer, NuPAGE Transfer Buffer were from Invitrogen (Paisley, UK).

Data Analysis

Data are expressed as mean±standard error of the mean (SEM) values and were analyzed for statistical significance by one-way ANOVA followed by post hoc Student–Newman–Keul's test, using GraphPad Prism 5 (GraphPad, San Diego, CA, USA).

Results

Orexins Stimulate the Akt kinase Activity in Rat Cortical Neurons

Orexins A and B evoked phosphorylation of Akt kinase in cultured rat cortical neurons. The kinetics of Akt activation by orexins was analyzed by Western blot, using an antibody that detects endogenous levels of Akt when phosphorylated (activated) at Ser^{473}. Under resting conditions the activity of Akt in neuronal cells was very low, but upon stimulation of the cells with 0.1 μM orexins A and B it increased significantly (Fig. 1, top). The maximal phosphorylation of Akt (approximately 500 % above the control values) was observed after 1 h of stimulation by orexins and decreased toward a plateau between 6–24 h. After 24 h stimulation of cortical neurons with orexins, the phospho-Akt immunoreactivity still remained higher than the basal level (114 and 76 % above control for orexins A and B, respectively) (Fig. 1, bottom). The profile of action of both peptides was very similar (Fig. 1, bottom).

A dose-dependence study showed that orexins, incubated with neuronal cells for 1 h, were able to activate Akt kinase at all concentrations tested (0.0001–1 μM) (Fig. 2). Interestingly,

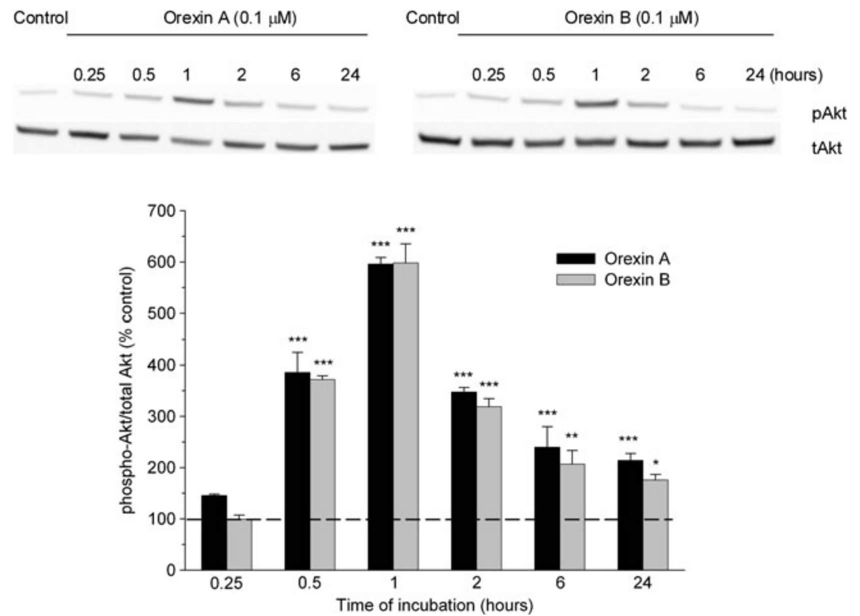

Fig. 1 Effects of orexins A and B on the Akt activation in primary neuronal cultures from rat cerebral cortex. Cells were incubated with the peptides (0.1 μM) for the indicated time periods and analyzed by immunoblot against pAkt (Ser^{473}). Top panel shows results of representative experiments. Data are presented as mean±SEM of 4–7 values per group and expressed as a percentage of the respective control. *Asterisks* indicate statistically significant difference from control; **P*<0.05; ****P*<0.001

orexin B more potently (EC_{50}=1.8 nM) than orexin A (EC_{50}=7 nM) stimulated Akt phosphorylation but, as described above, the maximal effect of both peptides on the Akt activation was very similar (Fig. 2, bottom).

Orexins Protect Neurons from the Cobalt Chloride-Induced Toxicity

Cultured cortical neurons were challenged with 100 μM cobalt chloride and cell viability and mitochondrial function were measured by MTT test. Incubation of cortical neurons with cobalt chloride for 48 h resulted in a potent, about 50 %, reduction of cell viability as compared with control values (taken as 100 %). Parallel incubation of neurons with orexins (0.0001–1 μM) attenuated the toxic effect of cobalt chloride (Fig. 3). Under conditions of chemical hypoxia, orexin A induced a statistically significant (at 0.01–1 μM concentration range) increase of neuronal cell viability, with the highest response, up to 71 % of control values, observed at 0.1 μM (Fig. 3, top). Orexin B more potently than orexin A enhanced cell viability by 80 % of control values at 0.01 μM and protected neurons at a wider range of concentrations (0.0001–1 μM) (Fig. 3, bottom).

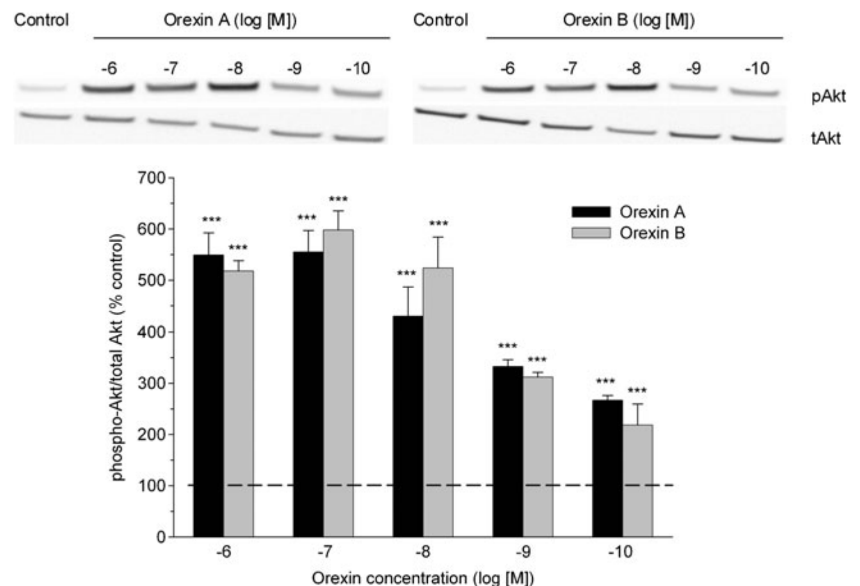

Fig. 2 Concentration-dependent effects of orexins A and B on the Akt activation in primary rat neuronal cultures. Cells were incubated with the peptides (0.0001–1 μM) for 1 h and analyzed by immunoblot against pAkt (Ser^{473}). *Top*, results of representative experiments. Data are presented as mean±SEM of 4–7 values per group and expressed as a percentage of the respective control. *Asterisks* indicate statistically significant difference from control; **P*<0.05; ****P*<0.001

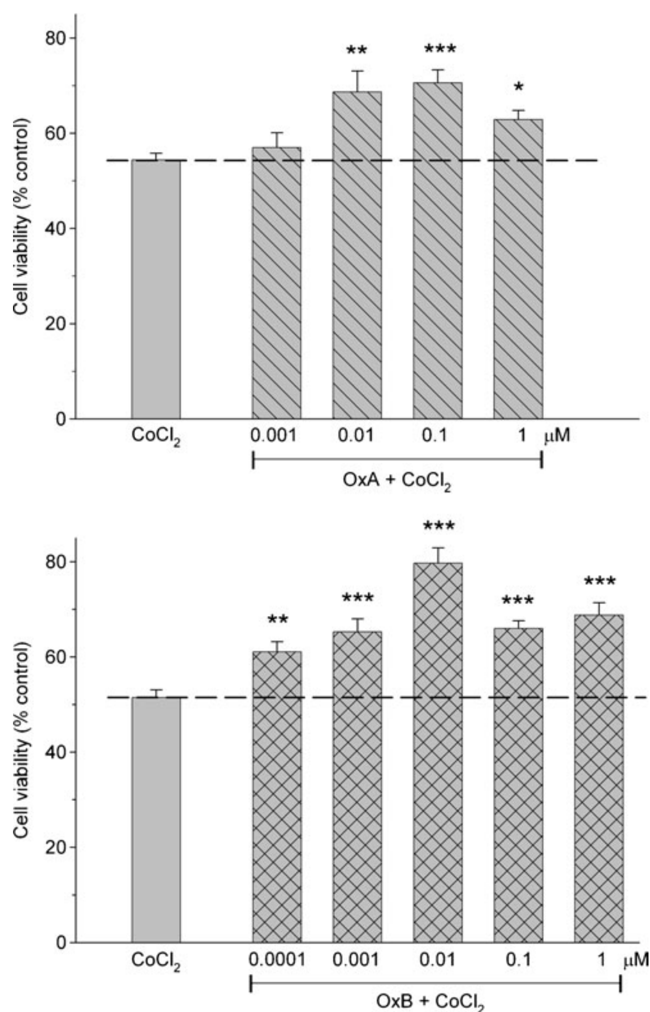

Fig. 3 Protective effects of orexins A and B in rat neuronal cultures challenged with cobalt chloride (100 μM). Cells were incubated with the peptides (0.0001–1 μM) in parallel with cobalt chloride for 48 h and cell viability was analyzed by MTT test. Data are mean±SEM of 6–24 values per group and expressed as a percentage of the respective control. *Asterisks* indicate statistically significant difference from cobalt chloride; $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$

Orexins Protect Cortical Neurons from the Cobalt Chloride-Induced Oxidative Stress

Mitochondria act as O_2 sensors by increasing the generation of ROS during hypoxia, and cobalt chloride mimics this response by inducing ROS generation (Chandel et al. 1998; Stenger et al. 2011). To confirm that orexins protect cortical neurons from the cobalt chloride toxicity, we measured oxidative stress level in rat cortical neurons using CM-H_2TMRos, a ROS-sensitive probe, in the presence or absence of cobalt chloride (100 μM), treated with orexins at concentrations at which we observed the highest peptides responses, i.e., 0.1 μM for orexin A and 0.01 μM for orexin B. Both peptides diminished the cobalt chloride-induced ROS generation to the basal level. In the absence of cobalt chloride, orexins did not alter cellular oxidative stress status (Fig. 4).

Akt Kinase Is Involved in Neuroprotection by Orexins Against the Cobalt Chloride Toxicity

To test whether activation of Akt kinase is involved in the neuroprotective effect of orexins, we measured neuronal viability in the presence of Akt kinase inhibitors, 10-DEBC hydrochloride (2.5 μM) and GSK690693 (1 μM). Control experiments performed in the absence of orexins revealed that these Akt inhibitors had no effect on the cobalt chloride toxicity in cortical neurons. When orexins were added together with Akt inhibitors to cortical neurons cultured under hypoxic conditions, the neuroprotective effect of peptides was completely abolished (Fig. 5). To test possible toxic effects of these inhibitors, neuronal cell cultures were treated with 10-DEBC hydrochloride and GSK690693, at concentrations used in experiments, and no toxicity was observed (data not shown).

Discussion

In the present work, we focused on the effect of orexins on Akt kinase stimulation in primary rat neuronal cultures and the significance of the orexin-induced Akt activation in neurons subjected to hypoxic stress. We have previously demonstrated that rat cortical neurons express both types of orexin receptors and their activation by orexins initiated different signaling pathways (Urbańska et al. 2012; Zawilska et al. 2013).

Despite of many literature reports indicating an ability of orexins to activate various intracellular signals, there are only a few findings demonstrating the effect of orexins on the Akt

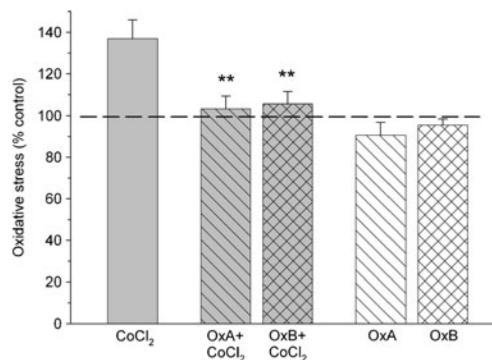

Fig. 4 Effects of orexins A and B on oxidative stress level in neuronal cultures from rat cerebral cortex. Neurons were treated with orexins A (0.1 μM) and B (0.01 μM) for 24 h in the presence or absence of cobalt chloride (100 μM) and stained with CM-H_2TMRos. Data are mean± SEM of 5–12 replicates per group and expressed as a percentage of the respective control. *Asterisks* indicate statistically significant difference from control; $^{**}P<0.01$

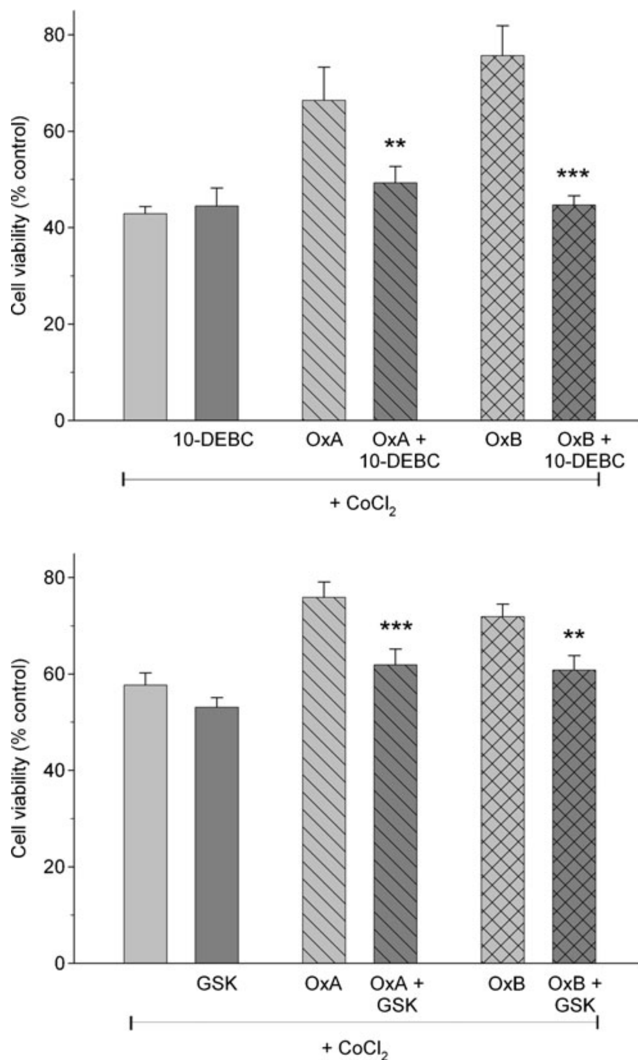

Fig. 5 Effects of Akt kinase inhibitors, 10-DEBC hydrochloride (2.5 μM) and GSK690693 (1 μM), on orexins A- (0.1 μM) and B-stimulated (0.01 μM) cell viability in rat neuronal cultures challenged with cobalt chloride (100 μM). Data are mean±SEM of 6–19 values per group and expressed as a percentage of the respective control. *Asterisks* indicate statistically significant difference from orexins A and B, respectively; $**P<0.01$; $***P<0.001$

signaling. For example, orexin A was shown to inhibit proglucagon gene expression through activation of Akt kinase in clonal pancreatic A cells (Göncz et al. 2007) and stimulated glucose uptake in primary rat adipocytes in a phosphatidylinositide 3-kinase-dependent manner (Skrzypski et al. 2011). The results obtained in the present study provide the first evidence that orexins A and B were able to potently induce Akt phosphorylation in rat primary cortical neurons. Both peptides acted at the wide range of concentrations and the calculated EC_{50} values were in low nanomolar range (~2–7 nM), suggesting that the Akt stimulation is of physiological relevance. The orexins-mediated Akt response was rather slow as there was no statistically significant increase of Akt activity during the first 15 min of incubation. The most pronounced response was observed after one hour of incubation with the peptides. Interestingly, elevated phospho-Akt immunoreactivity was also observed after 24 h, suggesting an involvement of Akt signaling in long-term cellular effects.

In different types of neuronal cells, the Akt signaling plays a crucial role in mediating survival signals (Brunet et al. 2001). This kinase also plays an important role in neurogenesis, axon establishment, and elongation (Diez et al. 2012). Altered Akt function has been associated to many pathologies, such as Alzheimer's disease (Liao and Xu 2009), Huntington disease (Warby et al. 2009; Zala et al. 2008), schizophrenia (Emamian et al. 2004), spinocerebellar ataxia type I (Chen et al. 2003), or autism (Kwon et al. 2006). Studies performed on recombinant cell lines have shown that orexins are capable of inducing both death- and survival-promoting signaling through activation of the classical MAPK pathways (Ammoun et al. 2006; Tang et al. 2008). The essential role of Akt signaling in neuronal survival and development prompted us to check whether this kinase mediates the orexin-initiated survival of cortical neurons against the cobalt-induced hypoxic toxicity. According to the literature, exposure to cobalt promotes a response similar to hypoxia by activating hypoxia-mediated signaling pathways aberrantly under normoxia and potently augmenting ROS generation in mitochondria (Chandel et al. 1998; Vengellur and LaPres 2004). Prolonged chemical hypoxia can also induce genes involved in cell death (Vengellur and LaPres 2004). In our study, we observed about 50 % reduction of neuronal cells survival after 48-h incubation with cobalt chloride at 100 μM. Orexins, added to hypoxic neuronal cultures, potently attenuated cobalt toxicity. In this context, it is worth to note that our previous findings in cortical neurons cultures showed the stimulatory effect of orexins A and B on neuronal survival under normoxia with a parallel attenuation of caspase-3 activity, indicating a new role of orexins as potential neuroprotective factors (Sokołowska et al. 2012). Recently, Butterick et al. (2012) have demonstrated that orexins protected immortalized hypothalamic neurons against H_2O_2-induced toxicity by decreasing lipid peroxidative stress and caspase-dependent apoptosis. In the light of these findings orexins seem to be neuroprotective against oxidative stress by modulation of anti-apoptotic mechanisms. Although the knowledge of the orexin-evoked neuroprotection is evolving, there are still many questions concerning factors mediating neuronal survival. Results from the present study point at an important role of Akt as one of the possible mediators of the pro-survival effects of orexins in neurons. Two Akt inhibitors, 10-DEBC hydrochloride and GSK690693, entirely suppressed the orexins A- and B-induced protection of cortical neurons from cobalt toxicity. Thus, the role of Akt in this process seems to be undeniable.

The question remains how the orexin-activated Akt induces the anti-apoptotic machinery. A series of studies with

the use of survival factors have demonstrated that Akt directly phosphorylates a major class of transcription factors—the Forkhead box transcription factor, class O and inhibits their ability to induce the expression of death genes (Brunet et al. 1999). Akt was also reported to promote cell survival by inhibiting the activity of p53 or inducing the expression of survival genes by activating two transcription factors, cAMP-responsive element binding protein, and nuclear factor κB (Du and Montminy 1998; Ozes et al. 1999; Yamaguchi et al. 2001). In addition to its effects on transcription, Akt phosphorylates BAD (Bcl-2 family member) inhibiting BAD pro-apoptotic functions (Datta et al. 1997). It is also suggested that Akt may promote survival indirectly by affecting cellular metabolism. In particular, Akt prevents the depletion of metabolites by increasing ATP or glucose levels (Brunet et al. 2001). Thus, it seems possible that orexins, the well-known factors influencing metabolism, arousal, and energy expenditure, may regulate energy balance towards cell survival with the Akt contribution to this process. Understanding the molecular mechanisms of orexin-promoted neuroprotection could be addressed in future studies to design new therapies of brain pathologies.

In summary, we demonstrated the first evidence that both orexins A and B potently stimulated Akt activation in primary neuronal cultures from rat cerebral cortex. We also showed that orexins protected cortical neurons against cobalt-induced oxidative stress and pointed at the important role of Akt as the mediator of pro-survival effects of orexins in neurons.

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Conflict of Interest The authors state no conflict of interest.

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