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Neurodegenerative effects of azithromycin in differentiated PC12 cells

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ABSTRACT

Azithromycin is a widely used macrolide antibiotic with sustained and high tissue penetration and intracellular accumulation. While short-term exposure to low-dose azithromycin is usually well tolerated, prolonged treatment can lead to unwanted neurological effects like paresthesia and hearing loss. However, the mechanism causing neurodegeneration is still unknown. Here, we show that even low therapeutically relevant azithromycin concentrations like 1 µg/ml decreased cell viability by 15% and induced neurite loss of 47% after 96 h in differentiated PC12 cells, which are a well-established model system for neuronal cells. When higher concentrations were used, the drug-induced effects occurred earlier and were more pronounced. Thereby, azithromycin altered tropomyosin-related kinase A (TrkA) signaling and attenuated protein kinase B (Akt) activity, which subsequently induced autophagy. Simultaneously, the antibiotic impaired lysosomal functions by blocking the autophagic flux, and this concurrence reduced cell viability. In good agreement with reversible effects observed in patients, PC12 cells could completely recover if azithromycin was removed after 24 h. In addition, the detrimental effects of azithromycin were limited to differentiated cells, as confirmed in the human neuronal model cell line SH-SY5Y. Thus, azithromycin alters cell surface receptor signaling and autophagy in neuronal cells, but does not automatically induce irreversible damage when used in low concentrations and for a short time.

1. Introduction

Azithromycin is a second generation macrolide, which inhibits bacterial protein synthesis. It is frequently used and has an overall good safety profile, but can also cause unexplained neurological side effects like paresthesia after prolonged exposure (Taylor et al., 2003) and hearing loss after short-term treatment (Ress and Gross, 2000). Due to its large volume of distribution, it achieves high tissue concentrations with elevated intracellular accumulation (Foulds et al., 1990; Gladue et al., 1989). Azithromycin is mostly located in acidic organelles like lysosomes, where it can change the pH and alter lysosomal functions (Renna et al., 2011). Its dicationic structure also enables azithromycin to bind to phospholipids and reduce membrane fluidity, which can affect cell surface receptor mobility (Tyteca et al., 2003). Interaction with phospholipids and lysosomal accumulation finally interfere with endocytosis and autophagy, which are both essential for cellular metabolism and survival.

Macroautophagy (usually referred to merely as autophagy) is the major type of autophagy and responsible for the degradation of cytoplasmic components and ubiquitinated proteins (Ghavami et al., 2014). It can be induced during starvation, but especially quiescent cells like neurons depend on it under nutrient-rich conditions for a balanced turnover of cytoplasmic compounds (Komatsu et al., 2007; Maday and Holzbaur, 2016). Equally important for adult neurons is the functionality of neurotrophin-induced signaling cascades (Skaper, 2008). For instance, the nerve growth factor (NGF) receptor tropomyosin-related kinase A (TrkA) is essential for neurons and also for differentiated PC12 cells (Greene, 1978), as it activates mitogen-activated protein kinases (MAPKs) and phosphatidylinositol 3-kinase (PI3K) and thereby regulates differentiation and survival (Chen et al., 2006; Zhang et al., 2000).

Regarding the dependence of neuronal cells on neurotrophin-mediated signaling and basal autophagy, it is surprising that short-term treatment with azithromycin has rather few neurological side effects (Jaruratanasirikul et al., 1996). Long-term exposure can cause paresthesia (Taylor et al., 2003) and hearing loss (Nicholson et al., 2015; Tseng et al., 1997; Wallace et al., 1994). Symptoms are mostly mild to moderate and reversible. In individual cases, short-term and low-dose treatment can lead to ototoxicity, either reversible (Mamikoglu and Mamikoglu, 2001) or irreversible (Mick and Westerberg, 2007; Ress and Gross, 2000), but the mechanism for neuronal damage is still unclear.

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In the present study, differentiated PC12 cells were utilised as a widely-used and very well-characterised model system for neuronal cells. They were derived from a rat pheochromocytoma and resemble immature rat adrenal chromaffin cells under normal culture conditions (Greene and Tischler, 1976). When stimulated with NGF, PC12 cells extend neurites and adopt neuron-like characteristics that makes them a comparable model for peripheral neurons like sympathetic neurons, sciatic nerves and dorsal root ganglia (Adler, 2006; Fischer et al., 2001; Zhang et al., 2015; Zhu et al., 2014). We examined short-term effects of therapeutically relevant doses of azithromycin on differentiated PC12 cells. Azithromycin induced neurite loss and impaired cellular metabolic activity in a concentration- and time-dependent manner. NGF signaling and autophagy regulation were altered, but the effects could be reversed after removing azithromycin from the medium.

2. Materials and methods

2.1. Cell culture

Rat pheochromocytoma PC12 cells [obtained directly from the German Collection of Microorganisms and Cell Cultures (DSMZ); Braunschweig, Germany] were cultured on collagen-coated plates in RPMI 1640 medium (PAA; Cölbe, Germany) supplemented with 5% fetal bovine serum (FBS; Biochrom; Berlin, Germany) and 10% horse serum (Biochrom) at 37 °C and 5% CO_2. For differentiation, PC12 cells were plated at low density (0.2×10^6 cells per 35-mm plate; 1.8×10^6 cells per 10-cm plate) and kept in serum-containing medium for 24 h. To synchronise the cell cycle and facilitate neurite outgrowth (Rudkin et al., 1989), cells were cultured in medium supplemented with 0.5% FBS and 1% penicillin/streptomycin for 72 h. Subsequently, PC12 cells were differentiated in low serum medium (0.5% FBS) supplemented with NGF (50 ng/ml; Alomone labs; Jerusalem, Israel) for seven days. Fresh NGF was added every second day with the medium change.

Human SH-SY5Y neuroblastoma cells (obtained directly from the DSMZ) were cultured on 10-cm plates in Dulbecco's MEM medium (PAA) supplemented with 20% FBS (Biochrom) at 37 °C and 5% CO_2. For differentiation, SH-SY5Y cells were grown on 96-well plates (0.01×10^6 cells) and kept in serum-containing medium for 24 h. To slow down proliferation, cells were cultured in medium supplemented with 0.5% FBS and 1% penicillin/streptomycin for 72 h. Subsequently, SH-SY5Y cells were differentiated in low serum medium (0.5% FBS) supplemented with NGF (50 ng/ml; Alomone labs) for three days. In experiments where retinoic acid (5 µM, Sigma-Aldrich, Taufkirchen, Germany) was applied in addition to NGF, cells were treated with retinoic acid for three days before adding NGF for three more days.

Azithromycin dihydrate was purchased from Sigma-Aldrich, dissolved according to the manufacturer's instructions and added to the cells in different concentrations as indicated. The inhibitors GW-441756 (1 µM, Santa Cruz, Heidelberg, Germany) or wortmannin (500 nM; Merck Millipore; Darmstadt, Germany) were applied to the cells for 48 h. GW-441756 is an inhibitor of the NGF receptor TrkA (Wood et al., 2004), while wortmannin irreversibly inhibits PI3K, which is an upstream activator of protein kinase B (Akt) (Okada et al., 1994).

2.2. Measurement of intact cells

Electronic cell counting analyses were performed using a CASY® TT cell counter (Roche Applied Sciences; Mannheim, Germany), which can determine the total cell number, the proportion of cells with intact cell membrane and the mean volume of the cells. Cells were washed with phosphate-buffered saline (PBS), incubated with passage EDTA (0.5 mM EDTA in PBS) for 2 min and detached from the plate after adding further 5 ml of medium. Subsequently, cells were centrifuged (1,000 x g for 10 min) and resuspended in 1 ml of medium. An aliquot of the cell suspension was mixed with CASYton® buffer, and the proportion of cells with intact membranes was determined according to the manufacturer's instructions.

2.3. Metabolic activity assay

Cells were grown and differentiated on a 96-well-plate (2×10^6 cells per well). To determine metabolic activity as a standard indicator of cell viability, 10 µl of water-soluble tetrazolium-1 (WST-1) reagent (Roche Applied Sciences; Mannheim, Germany) were added to each well. The cells were incubated for 2 h at 37 °C and 5% CO_2. The plate was placed into a Tecan infinite M200 reader (Tecan; Crailsheim, Germany) and shaken thoroughly for 1 min. Absorbance was measured at 450 nm with a reference wavelength of 600 nm.

2.4. Whole cell extracts

If not indicated otherwise, chemicals were purchased from Sigma-Aldrich (Munich; Germany) or Carl Roth (Karlsruhe, Germany). Whole cell lysates were generated from subconfluent cells. Before harvesting, cells were washed with phosphate-buffered saline (PBS). For whole cell extracts, cells were resuspended in denaturing lysis buffer [20 mM Tris, pH 7.4; 2% sodium dodecyl sulfate (SDS); 1% phosphatase inhibitor and 1% protease inhibitor (both from Roche)], incubated at 95 °C for 5 min, briefly sonicated, and centrifuged to remove insoluble material (15,000x g for 15 min; 4 °C). Protein extracts were stored at −80 °C.

2.5. Extracts of membrane and cytoplasmic proteins

For the analysis of membrane proteins, two different methods were applied. The “Cell Surface Protein Isolation Kit” (Thermo Fisher Scientific; Dreieich, Germany) was used according to the manufacturer's instructions. Briefly, cell surface proteins were biotinylated by washing the cells with ice-cold PBS and incubating the cells with a biotin solution for 30 min at 4 °C. After quenching the reaction, cells were harvested and lysed for 30 min on ice. The lysate was centrifuged (10,000×g for 2 min; 4 °C). To isolate the labelled proteins, the supernatant was transferred to a NeutrAvidin agarose column. After a 60-min incubation at room temperature, the bound proteins were washed and finally eluted with sample buffer.

To verify the results obtained by isolating cell surface proteins and to additionally prepare cytoplasmic proteins, the “Subcellular Protein Fractionation Kit” (Thermo Fisher Scientific) was used according to the manufacturer's instructions. Here, cells were harvested in ice-cold PBS. Cytoplasmic proteins were extracted by adding cytoplasmic extraction buffer to the cell pellet and incubating the tube for 10 min on ice. After centrifugation (500×g for 5 min; 4 °C), the supernatant (cytoplasmic proteins) was transferred to a new tube and stored at 4 °C. Membrane extraction buffer was added to the pellet for 10 min on ice. After a subsequent centrifugation step (3000×g for 5 min; 4 °C), the membrane proteins were contained in the supernatant. In order to verify the purity of membrane proteins, their extracts were immunoblotted for (the absence of) β-actin.

2.6. Immunoprecipitation

For immunoprecipitation (IP), native cell lysates were prepared from differentiated cells. Before harvesting, cells were washed with PBS. Subsequently, cells were resuspended in native lysis buffer [20 mM Tris, pH 7.6; 250 mM NaCl; 3 mM EDTA; 3 mM EGTA; 0.5% Nonidet™ P-40; 1% β-glycerolphosphate; 1% phosphatase inhibitor; 1% protease inhibitor (both from Roche)], incubated on ice for 30 min and centrifuged to remove insoluble material (15,000×g for 15 min; 4 °C). One hundred and fifty micrograms of total protein were used for IP with the ImmunoCruz™ reagents according to the manufacturer's instructions (Santa Cruz). All steps were performed at 4 °C if not indicated otherwise. The samples were precleared for 30 min

with the appropriate preclearing matrix. The antibody-IP matrix complex was formed by incubation of 1 µg primary antibody with 50 µl of the appropriate IP matrix in 500 µl of PBS for 1 h. The antibody-IP matrix complex was washed twice with cold PBS. Subsequently, the precleared samples were mixed with the antibody-IP matrix complex and incubated overnight. The negative control was prepared by incubation with the antibody-IP matrix complex with native lysis buffer only. After centrifugation, the pellets containing immobilised proteins with IP matrix complexes were washed three times with PBS. The immobilised proteins were dissociated with 5 x reducing electrophoresis buffer (312.5 mM Tris, pH 6.8; 10% SDS; 10% 2-mercaptoethanol; 50% glycerol) in 20 µl ultra-pure water at 95 °C for 3 min. The supernatants from the dissociation were quantitatively loaded on 12% SDS-polyacrylamide gels for Western blotting (see Section 2.7.). The positive control contained 10 µg of native protein extracts.

2.7. Western blots

As a general rule, 20 µg of protein extract were separated on 10–15% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride transfer membranes (Millipore, Schwalbach, Germany). The membranes were blocked with 4% non-fat dry milk in Tris-buffered saline with Tween-20 (TBST; 0.1 M Tris, pH 7.5; 0.15 M sodium chloride; 0.1% Tween-20) and incubated with the primary antibodies according to the manufacturer's recommendations. After three washing steps with TBST, the membranes were incubated with the corresponding horseradish peroxidase-conjugated secondary antibody for 30 min. All Western blots were developed using the enhanced chemiluminescence (ECL) system and Hyperfilm ECL (GE Healthcare; Munich, Germany). Between the stainings against phosphorylated kinases and total kinase protein, or between stainings against autophagy-related proteins and β-actin for normalisation, blots were stripped in 2% SDS, 62.5 mM Tris and 100 mM 2-mercaptoethanol for 30 min at 50 °C, washed with TBST and blocked again. To normalise for the protein content of each lane and to confirm equal loading, all membranes were finally stained with Ponceau S. For densitometrical and statistical analysis, only films generating subsaturating levels of intensity were selected. The bands were quantified using the build-in gel analyzer of Image J v1.4 (National Institutes of Health, Bethesda, MD, USA). Antibodies against the following targets were purchased from the indicated sources and used according to the manufacturer's instructions: mouse IgG (Antibody Registry ID at http://antibodyregistry.org: AB_772209, GE Healthcare); Rab7 (AB_609910, Sigma-Aldrich); β-actin (AB_476744), Akt (AB_10699016), cleaved caspase-3 (AB_331441), extracellular signal-regulated kinase-1/2 (ERK1/2, AB_10695746), microtubule-associated protein 1 A/1B-light chain-3 phosphatidylethanolamine conjugate (LC3-II), p62 (AB_10624872), phospho-Akt (AB_916024), phospho-ERK1/2 (AB_331646) and rabbit IgG (AB_10697506) (New England Biolabs; Frankfurt/Main, Germany); caspase-3 (AB_637828), cathepsin B (AB_2261223), cathepsin D (AB_10991327), p75 (AB_784824) and TrkA (AB_632556) (Santa Cruz Biotechnology; Heidelberg, Germany).

2.8. LysoSensor staining

Cells were grown and differentiated on a collagen-coated 8-well chamber slide. Azithromycin was used in the concentrations indicated and left on the cells for 48 h. Bafilomycin (50 nM; Santa Cruz Biotechnology) was added to the medium for the 2 h directly before the experiment and was removed prior to staining. The cells were incubated with LysoSensor Green DND-189 (1 µM; Molecular Probes/Thermo Fisher Scientific) for 10 min at 37 °C. Subsequently, the staining solution was removed, fresh PBS was added and the cell were analysed by using an inverted fluorescence microscope (Leica DM IL; Leica Microsystems; Wetzlar, Germany) with a CC12 camera and the corresponding analysis software Cell^F (Olympus; Hamburg, Germany).

2.9. Neurite counting

The percentage of cells with neurites longer than 1.5 diameters of the cell body was manually counted in at least three fields of view per sample (bright field, 20x magnification). Five separate experiments were performed for each azithromycin concentration.

2.10. Statistical analyses

All data were obtained from at least three (usually four or five) independent sets of experiments. All quantitative data represent the mean ± standard deviation of these independent experimental replications. Statistical analyses were performed with the GraphPrism software (Graphpad Software, La Jolla, CA, USA) using one-way analyses of variance with repeated measurements.

3. Results

3.1. Neurite loss and cell viability after treatment with azithromycin

PC12 cells were grown and differentiated on collagen-coated 8-well chamber slides and treated with different concentrations of azithromycin, which were chosen on the basis of previously published plasma concentrations in patients (Matzneller et al., 2013; Rodvold et al., 2003). Oral application of 500 mg azithromycin once daily has been reported to result in maximum plasma concentrations of 0.5 µg/ml (Matzneller et al., 2013). Since concentrations in brain tissue can be at least ten times higher (Araujo et al., 1991), azithromycin was used in a range from 0.5 µg/ml to 5 µg/ml. After 24 h, higher concentrations of azithromycin (2 µg/ml or 5 µg/ml) significantly reduced the proportion of neurite-bearing cells to 70%, whereas 0.5 µg/ml or 1 µg/ml did not (Fig. 1A). When the time of incubation with azithromycin-containing medium was extended up to 96 h, also 0.5 µg/ml or 1 µg/ml azithromycin significantly decreased the fraction of neurite-bearing cells to 65% or 53%, respectively (Fig. 1A). Neurite loss after application of 2 µg/ml or especially 5 µg/ml azithromycin for 96 h was even more pronounced with only 29% or 2% of the cells still showing neurites.

Since the azithromycin-treated cells did not simply retract their neurites, but also adopted a different cellular appearance with rounded cell bodies and less attachment to the coated dishes (data not shown), we determined their metabolic activities as a marker of cell viability using WST-1 assays. Only after 24 h, no significant decrease could be observed (Fig. 1B). A 48-h exposure to 5 µg/ml azithromycin reduced metabolic activity to 85% (Fig. 1B). Longer incubation times up to 96 h further decreased absorbance measured in WST-1 assays, with 1, 2 or 5 µg/ml azithromycin resulting in 85%, 66% or 65% metabolic activity, respectively (Fig. 1B). When cells were treated with 0.5 µg/ml azithromycin, no significant decrease in metabolic activity could be detected (data not shown).

As azithromycin penetrates phospholipid bilayers, directly interacts with phospholipids and changes membrane dynamics (Tyteca et al., 2003), we chose an additional approach to determine the proportion of cells with intact membranes by using a CASY® TT cell counter (Roche Applied Sciences). Already after 24 h, 2 µg/ml and 5 µg/ml azithromycin significantly reduced the proportion of cells with intact membranes to 80% (Fig. 1C). Extending the incubation times led to a significantly lower detection of completely intact cells, with a minimum of 58% or 54% after a 96-h application of 2 or 5 µg/ml azithromycin, respectively (Fig. 1C).

As the activation of caspase-3 is a hallmark of the induction of apoptotic cell death, we also examined the cleavage of caspase-3 in response to azithromycin. After 24 h, activation of cleaved caspase-3 was hardly visible (data not shown), but exposure up to 48 h

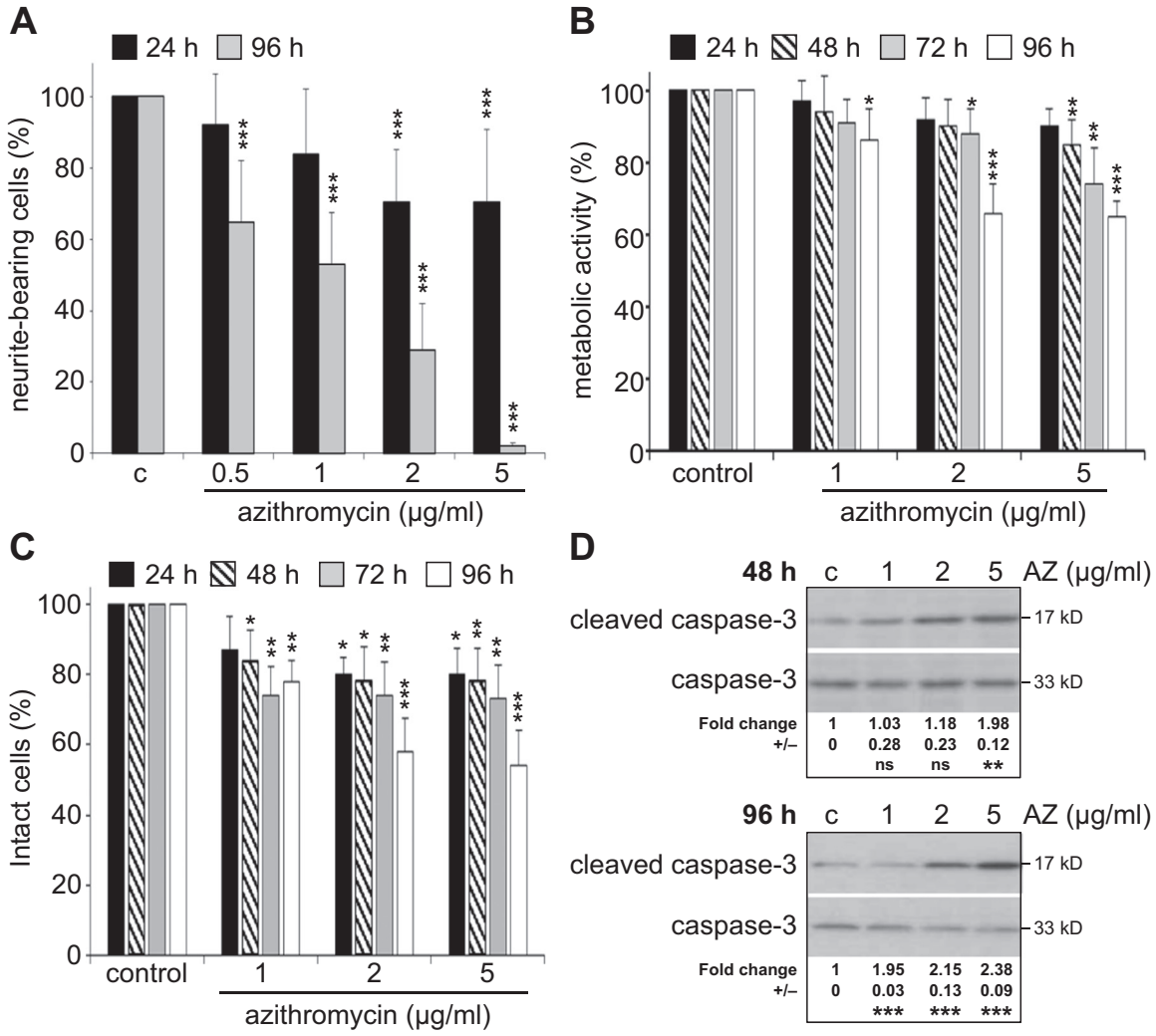

Fig. 1. Neurite loss and cell survival in response to azithromycin. A. Differentiated PC12 cells were treated with different concentrations of azithromycin for 24 h or up to 96 h. The proportion of neurite-bearing cells was decreased only by 2 or 5 µg/ml azithromycin after 24 h, whereas all concentrations significantly reduced the number of neurites after 96 h. Control =100%; ***, $P < 0.001$, **, $P < 0.01$ for control vs. azithromycin-treated cells. Data represent the mean ± standard deviation of five independent experiments. B and C. Effect of different azithromycin concentrations (1, 2 or 5 µg/ml) on metabolic activity (B, WST-1 assays) or the proportion of cells with intact membranes (C, electronic cell counts) of PC12 cells after 24, 48, 72 or 96 h. Control =100%; ***, $P < 0.001$, **, $P < 0.01$, *, $P < 0.05$ for control vs. azithromycin-treated cells. Data represent the mean ± standard deviation of five independent experiments. D. Azithromycin-mediated effect on caspase-3 cleavage and protein levels after 48 h or 96 h. Western blots of whole cell lysates of control or azithromycin-treated cells were performed using an antibody against cleaved caspase-3. Each blot was re-probed with an antibody against total caspase-3. Western blot data are representative for four independent experiments. AZ, azithromycin; c, control; kD, kilodalton.

significantly increased cleavage of caspase-3 1.98-fold in response to 5 µg/ml azithromycin (Fig. 1D). Incubation times of 96 h further enhanced the activation of caspase-3 1.95-fold, 2.15-fold or 2.38-fold for 1, 2 or 5 µg/ml azithromycin, respectively (Fig. 1D).

3.2. Distribution of NGF receptors

Due to the ability of azithromycin to change membrane dynamics (Tyteca et al., 2003) and the observed effects on membrane integrity, it seemed possible that the effects of membrane receptor signaling were also altered. In PC12 cells, NGF signals through two types of cell surface receptors: TrkA and the p75 neurotrophin receptor (Berg et al., 1991). Upon NGF binding, TrkA receptors form homodimers or associate with p75, resulting in the activation of TrkA's intrinsic tyrosine kinase activity (Kaplan et al., 1991). In response to azithromycin, TrkA distribution was distinctly changed, whereas the distribution of the second NGF receptor, p75, remained constant (Fig. 2A). Both 2 and 5 µg/ml of azithromycin significantly decreased the amount of TrkA in the cytoplasm by 25% or 37%, respectively, when compared to baseline levels normalized to the constantly expressed p75. Interestingly, the 110-kilodalton (kD) TrkA precursor and the mature 140-kD form were equally reduced (Fig. 2A). In the plasma membrane, only the mature 140-kD form of TrkA could be detected and was significantly increased by azithromycin (2.07-fold or 3.00-fold, respectively; Fig. 2A). When phosphorylation levels of TrkA were examined, the overall signal was low and only a tendency towards lower phosphorylation in the membrane fraction in response to azithromycin was observed (data not shown). To confirm the increased plasma membrane localisation of TrkA, we examined its association with Rab7, which is attached to cytoplasmic late endosomes (Hyttinen et al., 2013). After incubating the cells with azithromycin (2 µg/ml or 5 µg/ml) for 48 h, expression levels of Rab7 increased (Fig. 2B). In contrast, the cytoplasmic complex formation of TrkA and Rab7, as indicated by co-immunoprecipitation, decreased in response to azithromycin to 43% or 35% respectively (Fig. 2B). Taken together, treatment with azithromycin retained mature TrkA in the plasma membrane and reduced its cytoplasmic levels.

In order to investigate whether the inhibition of TrkA activity would affect the observed azithromycin effects on cell viability, differentiated PC12 cells were incubated with azithromycin (2 µg/ml) and/or the

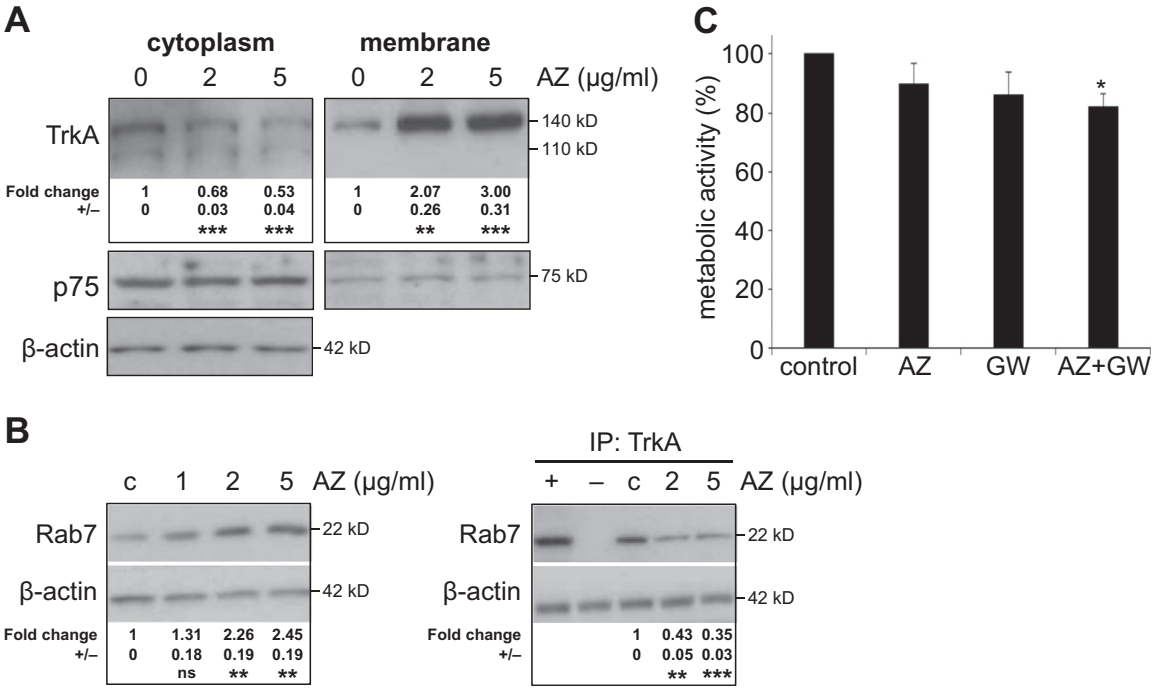

Fig. 2. Distribution of NGF receptors in response to azithromycin. **A.** Western blots of cytoplasmic or membrane extracts of control or azithromycin (AZ)-treated PC12 cells (2 or 5 µg/ml for 48 h) were performed using an antibody against TrkA. Each blot was re-probed with an anti-p75 antibody. Signals of TrkA were normalised to the levels of p75. Protein levels in the cytoplasm were also controlled by β-actin. Data represent the mean ± standard deviation of three independent experiments. kD, kilodalton. **B.** Western blots of whole cell lysates of control or azithromycin (AZ)-treated PC12 cells (2 or 5 µg/ml for 48 h) were performed using an antibody against Rab7. Each blot was re-probed with a β-actin antibody. Signals of Rab7 were normalised to the levels of β-actin. Co-immunoprecipitation (IP) using a TrkA antibody indicated that azithromycin decreased complex formation of TrkA and Rab7. Signals of TrkA-Rab7 were normalised to β-actin. The negative control was prepared by incubation of the antibody-IP matrix complex with native lysis buffer only. Twenty micrograms of lysate were used as a positive control. Data represent the mean ± standard deviation of three independent experiments. **C.** Metabolic activity of control cells and cells treated with azithromycin (AZ; 2 µg/ml) and/or GW-441756 (GW; 1 µM) after 48 h determined by WST-1 assays. Control =100%; *, $P < 0.05$ vs. control cells. Data represent the mean ± standard deviation of five independent experiments.

TrkA inhibitor GW-441756 (1 µM) for 48 h. Inhibition of TrkA decreased the metabolic activity to 86% (Fig. 2C), and the combination of both substances to 82% (Fig. 2C). These results suggested that the azithromycin-induced change in TrkA distribution (Fig. 2A, B) only partially contributed to the neurodegenerative effects of azithromycin.

3.3. Azithromycin and autophagic flux

As azithromycin can affect different processes during autophagy (Parnham et al., 2014) and as differentiated neurons are particularly sensitive to any disturbances of protein or organelle turnover (Komatsu et al., 2007), we also examined if the autophagic flux was altered by azithromycin. The membrane-bound protein LC3-II is a well-characterised marker for the induction of autophagy (Klionsky et al., 2012), while the accumulation of protein p62/sequestosome-1 served as a control for the inhibition of autophagic flux. Interestingly, azithromycin significantly increased the levels of LC3-II after 24 h and 48 h (Fig. 3A). Even low concentrations like 1 µg/ml azithromycin led to a 3.4-fold induction of LC3-II after 24 h (Fig. 3A). In contrast, p62 levels were significantly elevated only in response to 5 µg/ml azithromycin after 24 h. Within 48 h, however, a significant increase of p62 was detected when at least 1 µg/ml azithromycin was used. Thus, azithromycin generally induced autophagy in differentiated PC12 cells, but also inhibited the autophagic flux when used in higher concentration or after prolonged incubation.

To understand how azithromycin could concomitantly induce and inhibit autophagy, we first examined if the lysosomotropic behaviour of the drug contributed to one of the observed effects (Gladue et al., 1989). To test if azithromycin changed lysosomal pH in differentiated PC12 cells at the comparatively low concentrations used in this study, lysosomal function was studied using LysoSensor Green DND-189. LysoSensor dyes accumulate in acidic organelles due to protonation and exhibit an increase in fluorescence intensity upon acidification. Especially LysoSensor Green DND-189 is almost non-fluorescent except when inside acidic compartments. As a positive control for alkalization, we used bafilomycin A (50 nM, 2 h). After incubation with LysoSensor Green DND-189, the cells were analysed by fluorescence microscopy. Fluorescence of control cells was regarded as 100%. As expected, a 48-h treatment with 2 or 5 µg/ml azithromycin showed a dose-dependent decrease in the relative fluorescence compared to the untreated control cells to 68% or 56%, respectively, indicating a less acidic pH (Fig. 3B). The positive control bafilomycin A led to the strongest reduction of fluorescence (Fig. 3B).

As additional indicators of lysosomal involvement, we examined activation (cleavage) of cathepsin B and cathepsin D. The activity of cathepsins is optimal in acidic pH (Turk et al., 2012). We hypothesised that if azithromycin alkalised lysosomal pH at the concentrations used, the activation of cathepsins would be reduced. The amount of active (cleaved) cathepsin B was not changed by azithromycin (Fig. 3C). Cathepsin D was generally activated to a smaller extent that cathepsin B, but the corresponding low levels of cleaved cathepsin D were significantly decreased in a concentration-dependent manner by azithromycin (Fig. 3C). Taken together, lysosomal functions were significantly altered by azithromycin.

3.4. Regulation of ERK1/2, Akt and JNKs by azithromycin

So far, we had found that azithromycin impaired cell viability, changed TrkA distribution and affected autophagic flux in differentiated PC12 cells, but it was still unclear how these effects were connected. Therefore, we next examined kinases that are involved in NGF signaling, cell survival and the regulation of autophagy. Especially

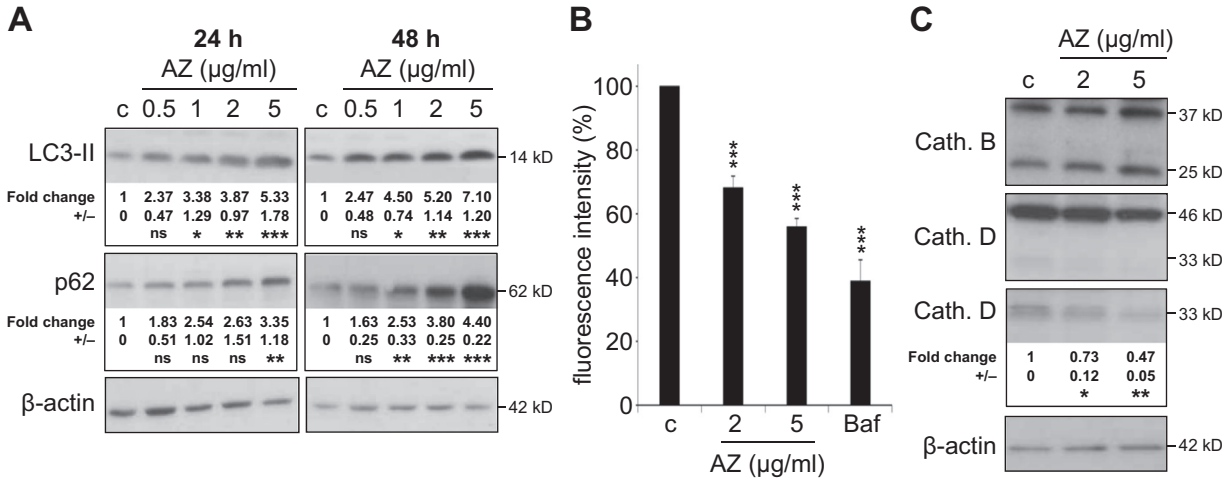

Fig. 3. Azithromycin-induced effects on autophagic markers after 24 h or 48 h. A. Whole cell extracts from control (c) or azithromycin (AZ)-treated PC12 cells were immunoblotted against LC3-II or p62 and re-probed with a β-actin antibody. LC3-II and p62 signals were normalised to β-actin. Control =100%; ***, $P < 0.001$, **, $P < 0.01$, *, $P < 0.05$ for control vs. azithromycin-treated cells. Data represent the mean ± standard deviation of four independent experiments. kD, kilodalton. B. Densitometric analysis of LysoSensor Green DND-189 staining. Untreated control PC12 cells or cells treated with azithromycin (2 or 5 µg/ml for 48 h) or bafilomycin A (50 nM for 2 h) were incubated with LysoSensor Green DND-189 (1 µM) for 10 min at 37 °C. Subsequently, fresh medium was added, and the cells were analysed by fluorescence microscopy. Control =100%; ***, $P < 0.001$ for control vs. azithromycin-treated cells. Data represent the mean ± standard deviation of four independent experiments. C. Cells were treated with 2 µg/ml or 5 µg/ml azithromycin for 48 h. Western blots from whole cell extracts were first incubated with antibodies against cathepsin B (37 kD full length, 25 kD cleaved) or cathepsin D (46 kD intermediate, 33 kD cleaved), which both recognise the cleaved and the full length protein. Subsequently, the membranes were stripped and re-probed with antibodies against β-actin. To better visualise the signal for activated cathepsin D, longer incubation times with ECL had to be chosen (lower panel). Signal intensities of the cleaved fragment of cathepsin D were normalised to the levels of the intermediate protein of cathepsin D. Control =100%; **, $P < 0.01$, *, $P < 0.05$ for control vs. azithromycin-treated cells. Data represent the mean ± standard deviation of four independent experiments. AZ, azithromycin; Baf, bafilomycin A; c, control; ns, not significant.

MAPKs and PI3K are activated in response to NGF and are important mediators of differentiation, survival (Obermeier et al., 1993) and autophagy (Sridharan et al., 2011). Azithromycin neither changed the phosphorylation of ERK1/2 nor its expression (Fig. 4A). In contrast, the activation of Akt was significantly and dose-dependently decreased by azithromycin after 24 and 48 h, while Akt protein levels were not affected (Fig. 4A). JNKs, which are often associated with cell death, were rarely activated by 2 or 5 µg/ml azithromycin (data not shown).

We hypothesized that if the reduction of Akt activity was essential for the effects of azithromycin, the inhibition of the PI3K/Akt pathway might cause similar effects. Therefore, metabolic activity in response to azithromycin (2 µg/ml) or the PI3K inhibitor wortmannin (500 nM) was compared after 48 h. Both substances led to a slight decrease in metabolic activity measured by WST-1 assays, but the effect of azithromycin was slightly more pronounced (Fig. 4B). When the cells were incubated with both substances simultaneously, the metabolic activity was significantly reduced (Fig. 4B). Interestingly, inhibition of PI3K/Akt by wortmannin increased phosphorylation of ERK1/2 (Fig. 4C), whereas azithromycin did not (Fig. 4A). This activation of the "survival kinases" ERK1/2 might account for a slightly higher metabolic activity in response to wortmannin compared to azithromycin. Thus, although the effects of PI3K/Akt inhibition and azithromycin on cell viability were similar, the underlying signal transduction was different. Still, the question remained whether the reduction of Akt activation was possibly linked to the disturbed TrkA signaling and altered autophagy. When TrkA was inhibited by GW-441756 (1 µM), Akt phosphorylation was decreased to a similar extent as with azithromycin (Fig. 4A and D). In response to wortmannin, LC3-II protein levels were elevated (Fig. 4E), but not as much as with azithromycin (Fig. 3A). Furthermore, p62 levels were reduced by wortmannin (Fig. 4E) and not increased as observed with azithromycin (Fig. 3A). When the cells were exposed to a combination of azithromycin and wortmannin, the increase of LC3-II was more pronounced than with each stimulus alone, whereas the azithromycin-induced increase and the wortmannin-induced decrease in p62 levels approximately balanced each other (Fig. 4F). Taken together, these results show that inhibition of NGF signaling attenuates Akt phosphorylation. The reduction of Akt activity, in turn, induces autophagy. Thus, Akt could be the mediator between receptor signals and autophagic flux.

3.5. Cell viability after removal of azithromycin

As azithromycin-induced side effects are usually reversible, we investigated the potential recovery of differentiated PC12 cells after transient exposure. To this end, the azithromycin-containing medium was replaced by normal differentiation medium after 24 h or 48 h, and the cells were cultured for an additional recovery period of 24 h or 48 h before performing WST-1 assays to measure cell viability via their metabolic activity. When cells were exposed to azithromycin for only 24 h, they completely recovered, and no significant decrease in cell viability was detected after another 24 h or 48 h (Fig. 5A). In contrast, when cells were exposed to 5 µg/ml azithromycin for 48 h, their metabolic activity was still reduced to 84% compared to control cells after a 24-h recovery period (Fig. 5B). After 48 h of recovery, their metabolic activity was already 90% compared control levels. Therefore, as indicated by previous experiments, azithromycin especially impairs viability when the cells are exposed to higher concentrations for longer periods, which is in good agreement with the observed clinical effects and validates the model system used in the present study.

3.6. Azithromycin-induced effects in naïve PC12 cells and SH-SY5Y cells

To assess if azithromycin mostly affected NGF-mediated signal transduction, proliferating (naïve) PC12 cells, which are independent of NGF signaling, were treated with 2 µM or 5 µM azithromycin. In contrast to differentiated cells (Fig. 1), metabolic activity of naïve PC12 cells was not significantly decreased after 24 h or 48 h (Fig. 6A). To confirm these findings in PC12 cells, an additional standard neuronal model cell system of human origin was used. SH-SY5Y cells are human neuroblastoma cells that are also suitable for studying NGF-mediated effects (Shan et al., 1990). Generally, the cells were treated with NGF (50 ng/ml) for three days before adding azithromycin (5 µg/ml). NGF significantly increased metabolic activity 1.34-fold. Since retinoic acid

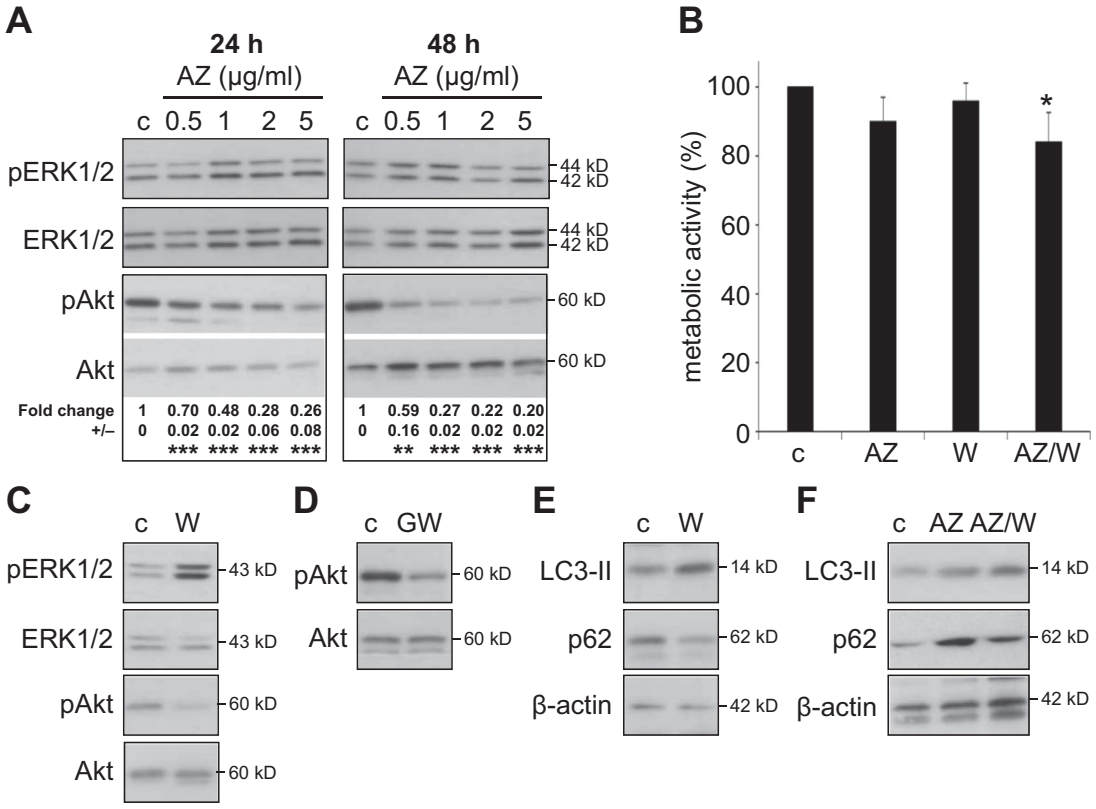

Fig. 4. Kinase activity and its influence on autophagy. A. Western blots of whole cell extracts of control (c) and azithromycin (AZ)-treated PC12 cells (0.5, 1, 2 or 5 µg/ml for 24 or 48 h) were performed using antibodies against the phosphorylated (p) forms of ERK1/2 or Akt. Each blot was re-probed with the corresponding total kinase antibodies. Signals of phosphorylated Akt were normalised to total Akt. Control =100%; ***, $P < 0.001$, **, $P < 0.01$ for control vs. azithromycin-treated cells. Data represent the mean ± standard deviation of four independent experiments. kD, kilodalton. B. Effect of azithromycin (AZ; 2 µg/ml), wortmannin (W; 500 nM) or both on metabolic activity, as determined by WST-1 assays after 48 h compared to untreated control cells (c). Control =100%; *, $P < 0.05$ for control vs. azithromycin- and/or wortmannin-treated cells. Data represent the mean ± standard deviation of five independent experiments. C. Western blots of whole cell extracts of control (c) or wortmannin-treated (W) cells (500 nM, 48 h) were performed using antibodies against phosphorylated forms of ERK1/2 and Akt. Each blot was re-probed with an antibody against ERK1/2 or Akt, respectively. D. Western blots of whole cell extracts of control (c) or GW-441756-treated cells (GW; 1 µM, 48 h) were performed using antibodies against LC3-II and p62. Each blot was re-probed with an antibody against β-actin. E. Western blots of whole cell extracts of control (c) or wortmannin-treated cells (W; 500 nM, 48 h) were performed using antibodies against LC3-II and p62. Each blot was re-probed with an antibody against β-actin. F. Western blots of whole cell extracts of control (c) cells or cells treated with azithromycin (AZ; 2 µg/ml) and/or wortmannin (W; 500 nM) for 48 h were performed using antibodies against LC3-II and p62. Each blot was re-probed with an antibody against β-actin. All Western blot data are representative for four independent experiments.

can enhance the expression of TrkA (Bogenmann et al., 1998), we treated SH-SY5Y cells with retinoic acid (5 µM) for three days before adding NGF, which further enhanced metabolic activity 2-fold (Fig. 6B). When so far untreated cells were incubated with 5 µg/ml azithromycin for 48 h, viability was not attenuated, while the NGF-induced increase of metabolic activity was completely inhibited by azithromycin (Fig. 6B). Moreover, metabolic activity was only enhanced 1.5-fold by retinoic acid and NGF when azithromycin was added (Fig. 6B). Taken together, the experiments show that especially NGF-mediated effects are attenuated by azithromycin in both rodent and human neuronal cell models.

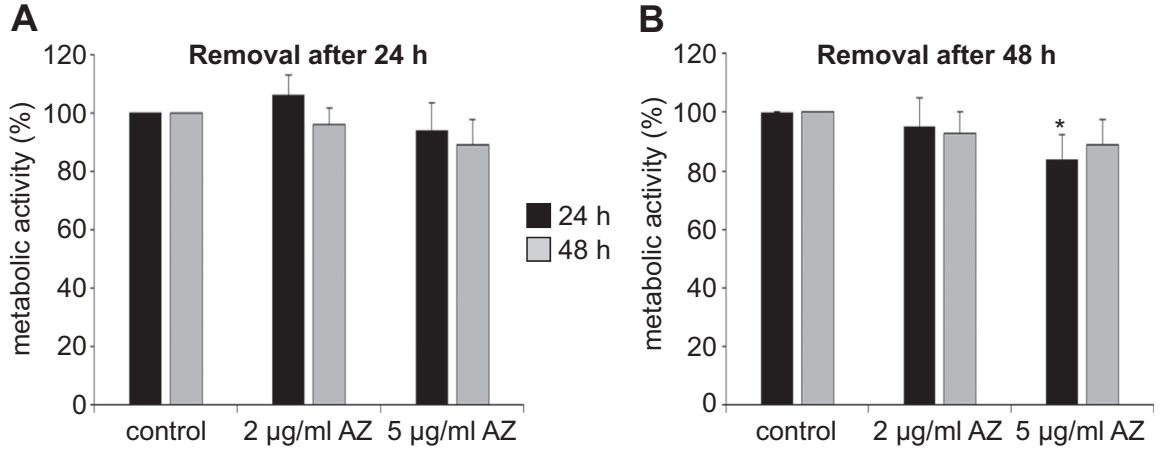

Fig. 5. Recovery of PC12 cells after the removal of azithromycin. Metabolic WST-1 assays of control or azithromycin (AZ)-treated cells (2 or 5 µg/ml). Azithromycin was removed from the cells after 24 h (A) or 48 h (B). Metabolic activity was measured after an additional recovery period of 24 h (black bars) or 48 h (grey bars). Control =100%; *, $P < 0.05$ for control vs. azithromycin-treated cells. Data represent the mean ± standard deviation of five independent experiments.

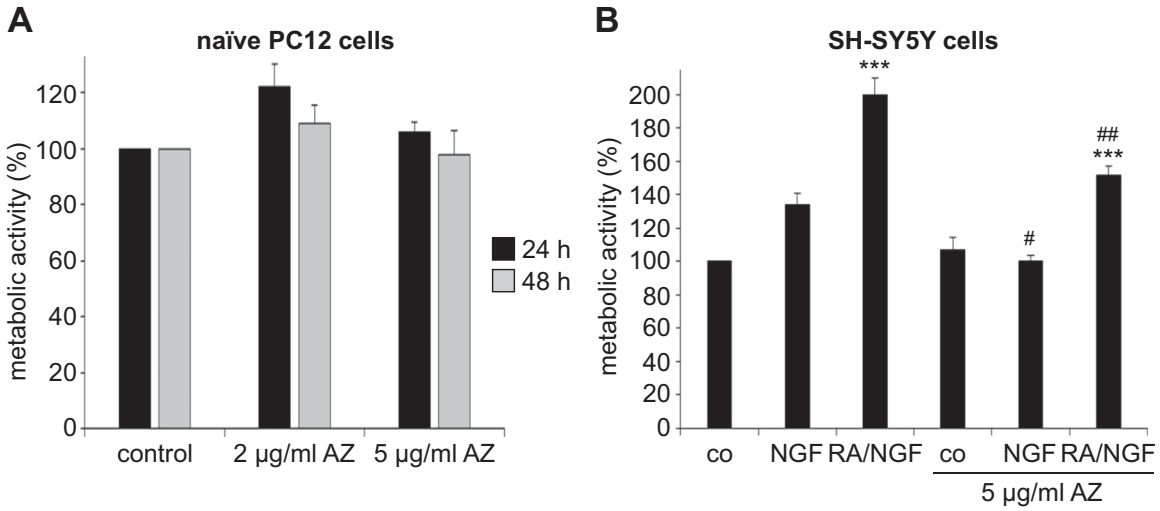

Fig. 6. Azithromycin-induced effects on naïve PC12 cells and SH-SY5Y cells. Metabolic WST-1 assays of control or azithromycin (AZ)-treated cells (2 or 5 µg/ml). A. Naïve PC12 cells were incubated with azithromycin for 24 h or 48 h before determining metabolic activity, which was not significantly affected. B. SH-SY5Y cells were treated with azithromycin (5 µM) for 48 h. The cells were either naïve (co) or differentiated with NGF (50 ng/ml) or with retinoic acid (5 µM) and NGF (50 ng/ml) (RA/NGF). Metabolic activity was attenuated in differentiated cells, but not in naïve cells. Control (co) =100%; ***, $P < 0.001$ for control vs. RA/NGF-treated cells; #, $P < 0.05$, ##, $P < 0.01$ for NGF- or RA/NGF- vs. NGF/azithromycin- or RA/NGF/azithromycin-treated cells. Data represent the mean ± standard deviation of five independent experiments.

4. Discussion

Azithromycin is a widely used macrolide antibiotic with an overall good safety profile, but unexplained neurological side effects after administration for five days or more. The aim of the present study was to analyze the short-term effects of azithromycin on differentiated PC12 cells, which are a well-characterised model system for neuronal cells. Incubation with azithromycin-containing medium led to neurite loss and to impaired cell viability in a concentration- and time-dependent manner. We found that azithromycin disturbed TrkA distribution, which attenuated Akt activity and thereby induced autophagy. Additionally, high macrolide concentrations also inhibited autophagy by affecting lysosomal function. This concurrence of inducing and inhibiting autophagy sensitised differentiated PC12 cells to cell death (Fig. 7). Still, the cells could completely recover, if azithromycin was removed from the medium after 24 h.

4.1. Neurite loss and cell survival

Although azithromycin is rarely neurotoxic after short-term administration (Jaruratanasirikul et al., 1996), it can cause paresthesia after prolonged exposure (Taylor et al., 2003) and hearing loss in response to a standard 5-day course of the antibiotic (Ress and Gross, 2000). Drug concentrations in plasma and in cerebrospinal fluid usually remain relatively low after oral and intravenous application (Jaruratanasirikul et al., 1996; Matzneller et al., 2013; Rodvold et al., 2003), but can be elevated in brain tissue (Araujo et al., 1991). When 500 mg azithromycin are applied once daily *per os* for three days, maximum plasma concentrations are observed in the range of 0.5 µg/ml (Matzneller et al., 2013), while the concentrations in brain tissue can be at least ten times higher (Araujo et al., 1991). Therefore, we used azithromycin in a range from 0.5 µg/ml to 5 µg/ml to assess possible harmful effects on neuronal cells.

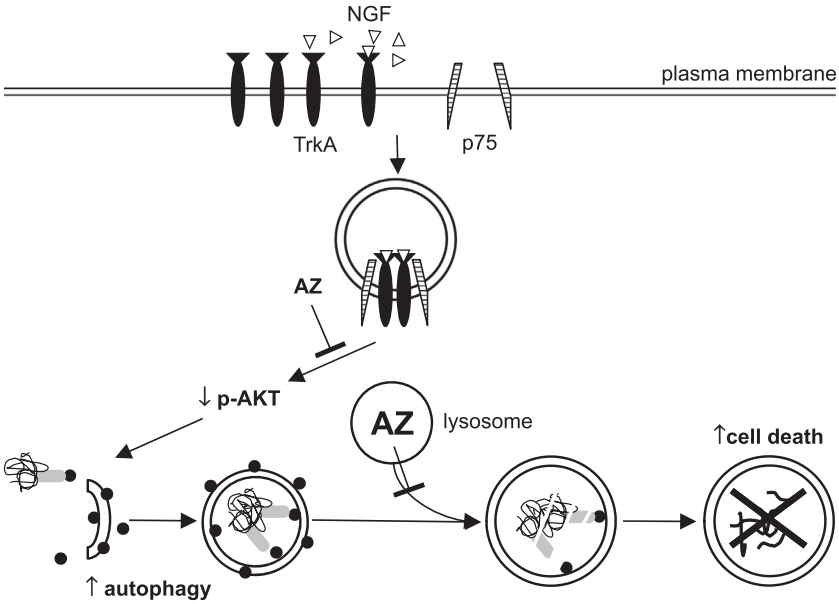

Fig. 7. Schematic model of azithromycin-mediated signaling in differentiated PC12 cells. Due to impaired TrkA signaling by azithromycin, Akt activity is attenuated, which induces autophagy. In higher concentrations, azithromycin additionally disturbs lysosomal function and thereby leads to the accumulation of autophagosomes. The concurrence of both effects increases neuronal cell death.

Treatment with azithromycin led to a significant decrease in neurite-bearing cells in a concentration- and time-dependent manner, which has also been observed with immune cells (Culic et al., 2002). While 0.5 μg/ml or 1 μg/ml azithromycin hardly had any effect after 24 h, higher concentrations and/or longer incubation times significantly reduced the number of neurites. So far, azithromycin had not been examined in the context of neurite stability. The question remains whether azithromycin directly interacts with the neuronal cytoskeleton, as differentiated PC12 cells did not simply retract their neurites in response to azithromycin, but clearly displayed a phenotype that was associated with decreased survival. Most likely, the observed neurite loss was one aspect of reduced viability, which is characteristic for this cell type (Greene, 1978). Similar to the reduction of neurite-bearing cells, cell viability particularly decreased after prolonged exposure to azithromycin or high concentrations like 2 μg/ml or 5 μg/ml. Although it is possible that even concentrations below 10 μg/ml induce apoptosis within 24 h (Stamatiou et al., 2010), we observed a decrease in intact cells and cell viability after 48 h. A clear activation of caspase-3 could be observed only in response to the highest dose of 5 μg/ml azithromycin after 48 h, whereas lower concentrations only led to an activation of caspase-3 after a 96-h incubation. This is also in line with our finding that removal of azithromycin after 24 h led to a complete regeneration of PC12 cells.

4.2. Signal transduction in response to azithromycin

Basal autophagy is an important process for protein turnover in quiescent cells like differentiated neurons (Komatsu et al., 2007). Depending on its concentration and the cell type, azithromycin can either induce or inhibit autophagy (Mukai et al., 2016; Renna et al., 2011; Stamatiou et al., 2009). The membrane-bound protein LC3-II, which is associated with autophagic vesicles, was examined as a well-characterised marker for the induction of autophagy (Klionsky et al., 2012), while the accumulation of protein p62/sequestosome-1, which binds to ubiquitinated proteins and shuttles them to the autophagosome, served as a control for the inhibition of autophagic flux.

In differentiated PC12 cells, a strong induction of LC3-II was observed in response to all azithromycin concentrations used, while the amount of p62 was only increased by high concentrations or longer incubation times. A similar regulation has been described in airway smooth muscle cells, where azithromycin induced autophagy with a concomitant reduction of cell viability (Stamatiou et al., 2009). A possible connection between phospholipid binding and induction of autophagy was discussed by the authors, but not experimentally shown (Stamatiou et al., 2009).

In the present study, we examined how azithromycin might induce autophagy by interfering with cell surface receptor distribution. After application of azithromycin, levels of the NGF receptor TrkA were considerably increased in the membrane fraction and reduced in the cytoplasm. It is known that azithromycin binds to phospholipids and thereby alters membrane fluidity and cellular functions that depend on cell surface receptor signaling (Tyteca et al., 2003). Decreased interaction between TrkA and Rab7 indicates impaired endocytosis. An additional effect of lysosomotropic drugs is an interference with protein processing in the trans-Golgi network (Basque et al., 2008), which could also be a reason for the reduced cytoplasmic TrkA levels apart from an impaired endocytosis.

Retaining TrkA in the membrane most likely disturbs receptor endocytosis and activation. The activity of TrkA, however, is important for survival signaling by ERK1/2 and Akt (Chen et al., 2006; Zhang et al., 2000). In the present study, Akt phosphorylation was inhibited by azithromycin. So far, only one related macrolide antibiotic has been shown to inhibit Akt phosphorylation (Kobayashi et al., 2013), but when the distribution and activation of TrkA are disturbed, Akt phosphorylation can also be impaired (Li et al., 2015). The reduced Akt activity, in turn, could be the connection between azithromycin and the induction of autophagy, since Akt suppresses autophagy (Hay and Sonenberg, 2004). Thus, altered TrkA signals attenuate Akt phosphorylation with subsequent induction of autophagy, which was also confirmed by treatment with wortmannin, an inhibitor of PI3K/Akt signaling. The latter effect might be confusing, as wortmannin is mainly thought to be an inhibitor of autophagy in other cell lines (Klionsky et al., 2012). However, in PC12 cells, wortmannin also enhances the activity of phospholipase D (Kanoh et al., 1992), which then compensates for the wortmannin-induced inhibition of the PI3K human vacuolar protein sorting 34 (hVps34) (Yoon et al., 2011).

The reduced phosphorylation of Akt observed in our study could also account for the decrease in cell viability, since neuronal survival can be regulated by TrkA-induced Akt signaling (Chen et al., 2006; Zhang et al., 2000). The recovery of PC12 cells after removal of the drug suggested that Akt was not the only mediator of cell viability. A potential contributor to the relatively high cell survival rates was the unchanged activity of ERK1/2, which was rather surprising because the phosphorylation of ERK1/2 can be inhibited by azithromycin (Imamura et al., 2004; Miyagawa et al., 2016; Yamada et al., 2014). Additionally, disturbed NGF receptor distribution should also lead to reduced ERK1/2 activity, since endocytosed TrkA sustains ERK1/2 phosphorylation (Zhang et al., 2000). However, apart from growth receptors, autophagic proteins can also activate ERK1/2, when autophagy is induced (Martinez-Lopez et al., 2013). In conclusion, attenuated TrkA signaling by azithromycin reduced Akt phosphorylation, which decreased cell viability, but also induced autophagy. The latter effect stabilised ERK1/2 phosphorylation and thereby enabled the cells to recover after removal of the azithromycin.

When higher concentrations of azithromycin like 5 μg/ml were used, differentiated PC12 cells were particularly sensitive towards the cell counting procedure and needed more time to recover. In addition to LC3-II levels, p62 levels also increased in response to 5 μg/ml azithromycin after 24 h, while concentrations of at least 1 μg/ml azithromycin increased p62 levels after 48 h. Similarly, protein levels of Rab7 were increased by azithromycin. An accumulation of Rab7 can occur when lysosomal recycling is impaired (Magalhaes et al., 2016). The staining pattern with LysoSensor Green DND-189 and the reduced levels of activated cathepsin D also suggested that high concentrations of azithromycin interfered with lysosomal functions, which resulted in an inhibition of autophagy. Similar effects were reported for immune cells (Renna et al., 2011). Still, our findings suggest that the simultaneous inhibition of Akt phosphorylation and lysosomal function in response to high azithromycin concentrations decreased cell viability in NGF-dependent cells. Only the combination of both effects induced and inhibited autophagy at the same time. A previous study drew similar conclusions for neuronal cells: an induction of autophagy could even protect cells, whereas a concomitant treatment with pharmacological inhibitors of autophagy induced apoptosis (Vucicevic et al., 2014). These results provide a good starting point for investigating the molecular basis of azithromycin side effects like paresthesia or hearing loss in primary cells and animal models. As autophagy seems to be important in drug-induced cell death of auditory cells (Youn et al., 2015), it is conceivable that azithromycin leads to ototoxicity by a comparable change of signal transduction. In addition, further studies are needed to investigate why azithromycin is neurotoxic in some patients and well tolerated in others.

5. Conclusions

The results of the present study demonstrate that azithromycin alters NGF receptor signaling in differentiated PC12 cells and thereby reduces Akt activity, which in turn induces autophagy. The higher the concentration of azithromycin, the more it reduces cell viability, as it additionally impairs lysosomal degradation. These short-term effects, however, are reversible, as shown by transient exposure experiments. Finally, our results closely mirror clinical findings and help to explain

the side effect profile of azithromycin.

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Ethical standards

The authors declare that all experiments complied with the current laws of Germany.

Conflict of interest

The authors declare no conflict of interest.

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