

# Cabergoline protects dopaminergic neurons against rotenone-induced cell death in primary mesencephalic cell culture

Jörn Meinel<sup>1,2\*</sup>, Khaled Radad<sup>3\*</sup>, Wolf-Dieter Rausch<sup>4</sup>, Heinz Reichmann<sup>2</sup>, Gabriele Gille<sup>2</sup>

<sup>1</sup>Department of Pathology, Technical University, Dresden, Germany, <sup>2</sup>Department of Neurology, Technical University, Dresden, Germany, <sup>3</sup>Department of Pathology, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt, <sup>4</sup>Institute of Medical Biochemistry, Department of Biomedical Sciences, University for Veterinary Medicine, Vienna, Austria

\*Authors contributed equally to the publication. They share the first authorship.

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## Abstract

*In the present study, primary mesencephalic cell cultures prepared from embryonic mouse mesencephala were used to investigate the neuroprotective effect of cabergoline, an ergoline D<sub>2</sub> receptor agonist, against the pesticide and neurotoxin rotenone relevant to Parkinson disease (PD). Treatment of cultures with cabergoline alone significantly increased the number of tyrosine hydroxylase immunoreactive (THir) neurons and reduced the release of lactate dehydrogenase (LDH) into the culture medium compared to untreated controls. Against rotenone toxicity, cabergoline significantly rescued degenerating THir neurons, reduced the release of LDH into the culture medium and improved the morphology of surviving THir neurons. The neuroprotective effects afforded by cabergoline were independent of dopaminergic stimulation as blocking of dopamine receptors by the dopamine receptor antagonist sulpiride did not prevent them. Furthermore, rotenone-induced formation of reactive oxygen species (ROS) was significantly reduced by cabergoline. Although cabergoline increased the glutathione (GSH) content in the culture, the protective effect for dopaminergic neurons seemed not to be predominantly mediated by increasing GSH, as depletion of GSH by L-buthionine-(S,R)-sulfoximine (BSO), a GSH biosynthesis inhibitor, did not prevent cabergoline-mediated neuroprotection of THir neurons in rotenone-treated cultures. Moreover, cabergoline significantly increased the ATP/protein ratio in primary mesencephalic cell cultures when added alone or prior to rotenone treatment. These results indicate a neuroprotective effect of cabergoline for dopaminergic neurons against rotenone toxicity. This effect was independent of dopamine receptor stimulation and was at least partially mediated by reducing ROS production and increasing the ATP/protein ratio.*

**Key words:** cabergoline, dopamine agonist, rotenone, dopaminergic cell culture, neuroprotection, Parkinson's disease.

## Introduction

Parkinson disease (PD) as the second most common neurodegenerative disease affects up to 10 million people worldwide [8]. Its specific symptomatology results primarily from progressive degeneration

of dopaminergic neurons in the substantia nigra pars compacta (SNpc) and subsequent depletion in striatal dopamine levels [3]. Thus dopamine denervation leads to the classic motor symptoms of PD, most notably tremor, rigidity, bradykinesia and postural instability [30].

## Communicating author:

Ass. Prof. Gabriele Gille, Department of Neurology, Technical University, Fetscherstr. 74, 01307 Dresden, Germany, e-mail: Gabriele.Gille@uniklinikum-dresden.de

Since the 1960s, dopamine replacement therapy using levodopa has been the most effective choice for treatment of PD. However, as the disease progresses, the medication becomes increasingly inadequate in controlling motor complications such as dyskinesia, wearing off and on-off motor fluctuation [31]. In addition to these disabling side effects, there is also serious concern about possible toxic actions of levodopa on the remaining dopaminergic neurons [13].

Dopamine receptor agonists are clinically indicated in PD to postpone the onset of levodopa therapy, and to delay and minimize levodopa-related motor complications [20]. They exert their antiparkinsonian effects through direct activation of dopamine receptors mimicking the endogenous neurotransmitter dopamine [21]. Unlike levodopa, dopamine receptor agonists are not metabolized by an oxidative pathway and so do not lead to the cytotoxic free radical formation that is associated with the metabolism of dopamine [25]. Besides providing symptomatic relief of PD, data from *in vitro* and *in vivo* studies indicated potential neuroprotective effects of some dopamine agonists [24].

Cabergoline is an ergot-derived dopamine agonist with high affinity for the dopamine D<sub>2</sub> receptor, but also possesses significant affinity for the D<sub>3</sub> and D<sub>4</sub> receptor. It has the longest half-life among related dopamine agonists [15]. Although physicians will nowadays avoid prescribing cabergoline as first-line therapy, since it has been shown to cause valvular heart disease in a subset of patients [34], there is still a considerable amount of patients treated with the agonist. Cabergoline was reported to elicit neuroprotection in *in vitro* and *in vivo* models of neurodegeneration. In this context, Ohta *et al.* found that cabergoline increased concentrations of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell line-derived neurotrophic factor (GDNF) in primary cultured mouse astrocytes [19]. Miglio *et al.* reported that cabergoline protected SH-SY5Y cells from ischemia-induced cell death [16]. Yoshioka *et al.* reported that cabergoline normalized the dopamine turnover in the striatum of 6-OHDA-treated mice [32]. Finally, in cultures of cortical neurons cabergoline prevented H<sub>2</sub>O<sub>2</sub>-induced neuronal cell death by reducing excitotoxicity [18].

To date, there are no investigations exploring the direct neuroprotective effect of cabergoline towards dopaminergic neurons. Accordingly, in the current

study primary mesencephalic cell cultures were used to investigate the potential neuroprotective effects of cabergoline for dopaminergic neurons in a rotenone toxicity model.

## Material and methods

### Preparation of primary mesencephalic cell culture

Mice (C57Bl/6 mice from Charles River Wiga, Sulzfeld, Germany) were cared for and handled in accordance with the guidelines of the European Union Council (86/609/EU) for the use of laboratory animals. The killing of mice for scientific purposes was officially approved by the Landesdirektion Dresden (State Directorate)/Free State of Saxony. Primary mesencephalic cell cultures were prepared according to Gille *et al.* [6] with some modifications. Cultures were not sex-segregated. In brief, embryonic mouse mesencephala were dissected on the 14<sup>th</sup> day of gestation and cut into small pieces in a drop of Dulbecco's Phosphate Buffered Saline (DPBS; Invitrogen, Germany), 2 ml of 0.2% trypsin solution (Invitrogen, Germany) and 2 ml of 0.02% DNase I solution (Roche, Germany) were added, and the tissue was subsequently incubated in a water bath at 37°C for 7 min. Then, 2 ml of trypsin inhibitor (0.25 mg/ml) (Invitrogen, Germany) were added, the tissue was centrifuged at 100 g for 4 min and the supernatant was aspirated. The tissue pellet was triturated 2-3 times with a fire-polished Pasteur pipette in 3 ml of Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen, Germany) containing 0.02% DNase I. Dissociated cells were plated in 4-well culture dishes (NUNC, Germany) at a density of 257,000 cells/cm<sup>2</sup> in DMEM supplemented with 4 mM glutamine (Sigma, Germany), 10 mM HEPES buffer (Sigma, Germany), 30 mM glucose (Sigma, Germany), 100 IU/ml penicillin, 0.1 mg/ml streptomycin (Roche, Germany) and 10% heat-inactivated Fetal Calf Serum (FCS; Sigma, Germany). When 8-well glass chamber slides (BD Biosciences, Germany) were used for fluorescence microscopy, cells were plated at a density of 365,600 viable cells/cm<sup>2</sup>. Two thirds of medium was changed on the 1<sup>st</sup> day *in vitro* (DIV). On the 3<sup>rd</sup> DIV the medium was changed completely. On the 5<sup>th</sup> DIV half of the medium was replaced by serum-free DMEM containing 2% B-27 supplement (Invitrogen, Germany). Serum-free supplemented DMEM was used for feeding from the 6<sup>th</sup> DIV and was subsequently replaced every 2<sup>nd</sup> day.

### Treatment of cultures with cabergoline

A 4 mM stock solution of cabergoline (kindly provided by Pfizer) was prepared and further diluted in ethanol and DMEM. Each final cabergoline concentration used for treatment contained 0.25% ethanol. To investigate the effect of cabergoline on the survival of dopaminergic neurons, cultures were treated with cabergoline (0.001, 0.01, 0.1, 1, 10  $\mu$ M) from the 6<sup>th</sup> DIV for eight consecutive days. During the treatment period, culture medium containing cabergoline was changed every two days. On the 14<sup>th</sup> DIV cultures were fixed and stained.

### Treatment of cultures with cabergoline and rotenone

To investigate the neuroprotective effect of cabergoline against rotenone (Sigma, Germany) toxicity, cultures were treated with cabergoline (0.001, 0.01, 0.1, 1, 10  $\mu$ M) on the 6<sup>th</sup> DIV for 6 consecutive days. During the treatment period, culture medium containing cabergoline was changed every two days. On the 10<sup>th</sup> DIV rotenone (80 or 100 nM) was concomitantly added to the cultures for 48 h. On the 12<sup>th</sup> DIV cultures were fixed and stained.

### Treatment of cultures with sulpiride, cabergoline and rotenone

Sulpiride (Tocris, UK), a dopamine D<sub>2</sub>/D<sub>3</sub> receptor antagonist, was diluted to 10 mM in ethanol. On the 8<sup>th</sup> DIV, culture medium was completely removed and cultures were incubated with 40  $\mu$ M sulpiride in 375  $\mu$ l of fresh culture medium for 10 min. After 10 min, 375  $\mu$ l of fresh medium containing different concentrations of cabergoline (0.001, 0.02, 2  $\mu$ M) were additionally added to the cultures, so that the final concentration of sulpiride was 20  $\mu$ M and that of cabergoline 0.0005, 0.01 and 1  $\mu$ M, respectively. On the 9<sup>th</sup> DIV, the culture medium was replaced with medium containing 80 nM rotenone and the cultures were grown to the 11<sup>th</sup> DIV.

### Identification of tyrosine hydroxylase immunoreactive neurons

Dopaminergic neurons were identified immunocytochemically by tyrosine hydroxylase staining. Cultures were rinsed carefully with phosphate buffered saline (PBS, pH 7.2) at the end of each treatment, placed on ice and fixed for 30 s in Accustain (Sigma,

Germany) pre-cooled to 4°C. After washing with PBS, cells were permeabilized with 0.4% Triton X-100 (Fluka, Switzerland) for 15 min at room temperature. Then, 30% H<sub>2</sub>O<sub>2</sub> (Merck, Germany) was diluted with 50% methanol (Merck, Germany) to 1% and added for 15 min to the cultures in order to saturate endogenous peroxidases. Cultures were washed 3 times with PBS and incubated with 5% horse serum (Vectastain ABC Elite kit; Vector Laboratories, USA) for 60 min to block nonspecific binding sites. To determine the number of tyrosine hydroxylase immunoreactive (THir) neurons in cultures, cells were sequentially incubated with anti-TH primary antibody (Chemicon, UK) overnight at 4°C, biotinylated secondary antibody (Vectastain ABC Elite kit) and avidin-biotin-horseradish peroxidase complex (Vectastain ABC Elite kit) for 90 min each at room temperature and washed with PBS between stages. The reaction product was developed with a peroxidase substrate kit (Vector VIP purple; Vector Laboratories, USA). Stained cells were counted with an inverted microscope (Axiovert 35, Carl Zeiss AG, Germany) in 20 randomly selected fields per well at 100x magnification (1.302 mm<sup>2</sup>/field). The average number of THir cells in controls on the 12<sup>th</sup> DIV was 1000-1200 cells/well.

### Measurement of lactate dehydrogenase activity

Cellular injury was quantitatively assessed by measuring the activity of LDH released from damaged cells into the culture medium. Lactate dehydrogenase (LDH) activity was measured with the Cytotoxicity Detection Kit (Roche, Germany) according to the manufacturer's instructions. Briefly, LDH catalyses the conversion of lactate to pyruvate with a concomitant reduction of NAD<sup>+</sup> to NADH + H<sup>+</sup>. The protons of NADH + H<sup>+</sup> are transferred to the yellow tetrazolium salt 2-[4-iodophenyl]-3-[4-nitrophenyl]-5-phenyltetrazolium chloride (INT) by diaphorase, resulting in red formazan, which was measured spectrophotometrically (ELISA-reader Tecan Sunrise, Germany) at 490 nm with a reference at 688 nm. Supplemented medium was used as a blank and subtracted as background.

### Measurement of reactive oxygen species with CM-H<sub>2</sub>DCFDA fluorescent dye

To investigate the effect of cabergoline on reactive oxygen species (ROS) production following rote-

none treatment, cultures were treated with 0.01  $\mu\text{M}$  cabergoline from the 8<sup>th</sup> to the 9<sup>th</sup> DIV and 80 nM rotenone on the 9<sup>th</sup> DIV. Production of ROS was detected using 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate acetyl ester (CM-H<sub>2</sub>DCFDA; Molecular Probes, Invitrogen, Germany). Stock solutions of CM-H<sub>2</sub>DCFDA (1 mM) were prepared in dimethylsulfoxide (DMSO) and further diluted in PBS. Cultures were loaded with a final concentration of 0.75  $\mu\text{M}$  CM-H<sub>2</sub>DCFDA for 15 min, washed and further incubated in colorless M 199 (GIBCO, Germany) with low autofluorescence. CM-H<sub>2</sub>DCFDA is believed to diffuse passively into cells, where its acetate groups are cleaved by intracellular esterases and subsequent oxidation by ROS yields a green fluorescent adduct that is trapped inside the cells. Images were taken with a computer-driven digital camera (Leica DC350 FX, Wetzlar, Germany) on an inverted microscope (Leica DM IRE2 HC FLUO, Wetzlar, Germany) equipped with an incubator and temperature control for live cell experiments. The green fluorescence was visualized with the MS 2 filter [excitation 430–510 Band Pass (BP)/emission 482–562 Band Pass (BP)]. Visualization of nuclei was performed with Hoechst 33342 with the DAPI filter [excitation 340–380 Band Pass (BP)/emission 425 Long Pass]. The fluorescence intensity of ROS was always normalized to the density of nuclei representing cellular density. Semi-quantitative image analysis was performed densitometrically with Adobe Photoshop (Munich, Germany).

### Glutathione measurement

To investigate the effect of cabergoline on glutathione (GSH) synthesis in cultured cells, cultures were treated with cabergoline (0.001, 0.01, 0.1, 1, 10  $\mu\text{M}$ ) on the 8<sup>th</sup> DIV for 24 h. On the 9<sup>th</sup> DIV, GSH concentrations were determined using a GSH detection kit (Chemicon International, Temecula, CA, USA) following the protocol of the manufacturer. Cultures were grown in 4-well culture dishes as usual and two wells of lysed cells were pooled for measurement. After centrifugation the supernatant was transferred into a 96-well plate (flat bottom, black) and monochlorobimane (MCB), a dye with high affinity for GSH that fluoresces in its bound state, was added. The fluorescence intensity was analyzed using a Tecan Genios plate reader with excitation at 380 nm and emission at 460 nm. To investigate whether increasing GSH concentrations played a role in cab-

ergoline's neuroprotective effect against rotenone, cultures were treated with 10  $\mu\text{M}$  buthionine sulfoximine (BSO; Sigma, Steinheim, Germany), a GSH biosynthesis inhibitor, on the 8<sup>th</sup> DIV for 2 h. Then, cabergoline (0.0005, 0.01, 1  $\mu\text{M}$ ) was additionally added to the cultures for 24 h and rotenone (80 nM) on the 9<sup>th</sup> DIV for 48 h. On the 11<sup>th</sup> DIV, cultures were fixed and stained immunohistochemically against TH antibody.

### ATP measurement

The total ATP content of individual wells was determined with the CellTiter-Glo Luminescent Cell Viability Assay (Promega, WI, USA) according to the manufacturer's instructions. The test principle is based on the ATP-dependent mono-oxygenation of luciferin to oxyluciferin by firefly luciferase leading to a luminescent signal that is directly proportional to the quantity of ATP. The luminescence signal was measured with a microplate reader (TECAN GENios, Crailsheim, Germany) and a run with PBS was subtracted as background.

ATP was measured in cultures that were treated either with cabergoline or cabergoline and rotenone, respectively. In the former case cabergoline (0.0005, 0.01, 1  $\mu\text{M}$ ) was added on the 8<sup>th</sup> DIV for 24 h. Then, cultures were kept in fresh medium from the 9<sup>th</sup> to the 11<sup>th</sup> DIV. Alternatively, cabergoline (0.0005, 0.01, 1  $\mu\text{M}$ ) was added on the 8<sup>th</sup> DIV for 24 h and rotenone (80 nM) on the 9<sup>th</sup> DIV for 2 consecutive days. The measured ATP values were correlated with the protein contents in the treated cultures.

### Protein measurement

Cellular protein was measured using the BCA protein assay reagent kit (Pierce, Rockford, IL, USA). Briefly, the assay is based on the reduction of Cu<sup>2+</sup> to Cu<sup>1+</sup> by protein in an alkaline medium (biuret reaction) and the formation of a red-violet chelate complex of 2 molecules of bicinchoninic acid (BCA) with one Cu<sup>1+</sup> ion. The photometrically measured absorbance of the formed complex at 562 nm is directly proportional to the total protein concentration ( $\mu\text{g}/\text{ml}$ ). A run with PBS instead of a sample was used as a blank. Absorbance was measured with a Tecan Sunrise absorbance reader (Crailsheim, Germany).

### Statistics

Data were expressed as mean values  $\pm$  standard error of the mean (SEM). Statistical differences were

determined using the Mann-Whitney  $U$ -test or Kruskal-Wallis ( $H$ ) test followed by the  $\chi^2$  test. Differences with  $p < 0.05$  were regarded as statistically significant.

## Results

### Cabergoline increased the survival of tyrosine hydroxylase immunoreactive neurons in primary mesencephalic cell culture and protected against rotenone-induced cell death

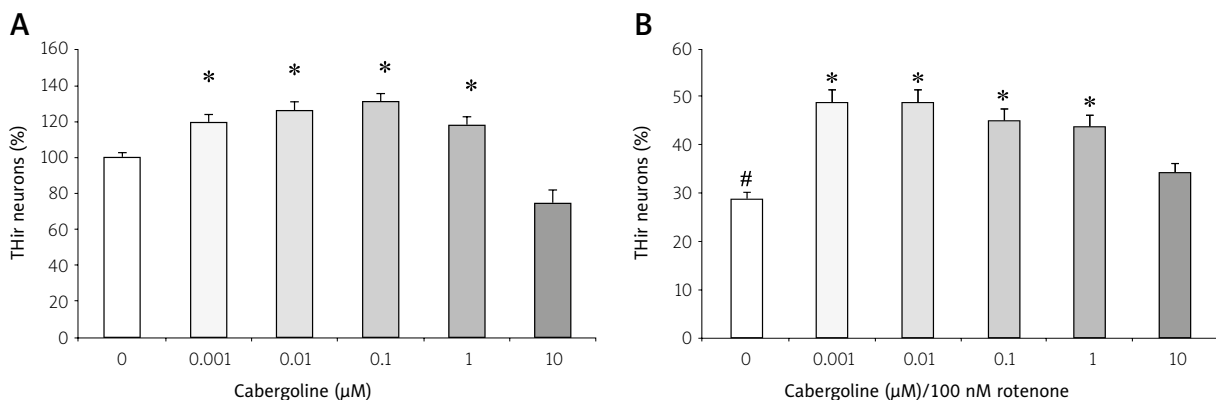
Treatment of cultures with cabergoline on the 6<sup>th</sup> DIV for 8 consecutive days significantly increased the number of THir neurons by up to 31% (0.1  $\mu$ M cabergoline) (Fig. 1A). On the other hand, at an unphysiological high concentration of 10  $\mu$ M, cabergoline reduced the number of THir neurons by 26% (Fig. 1A).

Treatment of cultures with 100 nM rotenone on the 10<sup>th</sup> DIV for 48 h reduced the number of THir neurons by 71% compared to control cultures (Fig. 1B). Surviving neurons after rotenone treatment showed fewer, shortened and dysmorphic neurites (Fig. 2C,D) compared to control cells (Fig. 2A,B). Treatment with cabergoline from the 6<sup>th</sup> DIV for 6 consecutive days

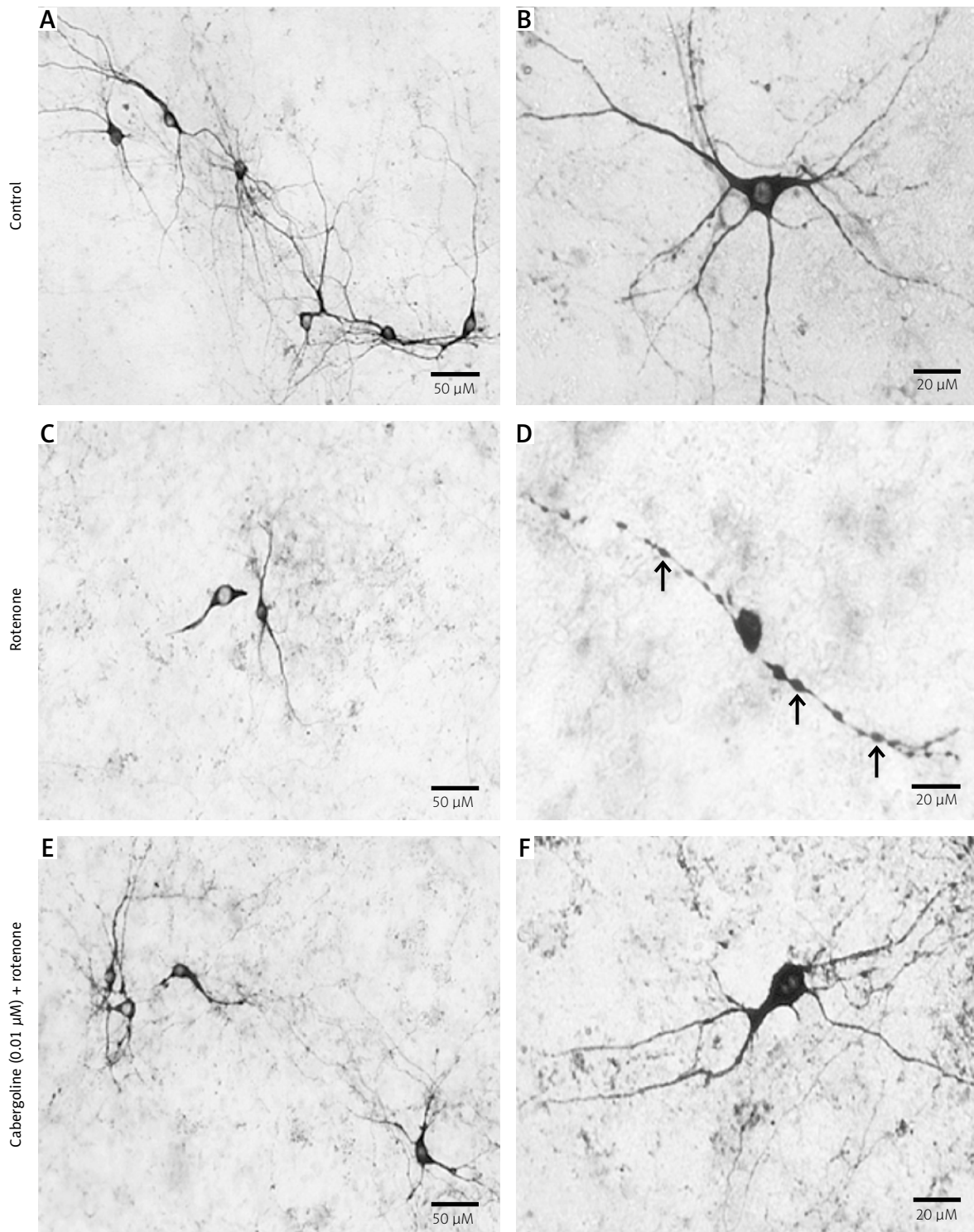
rescued THir neurons from degeneration when rotenone (100 nM) was concomitantly added on the 10<sup>th</sup> DIV for 48 h. The number of THir neurons was increased significantly by up to 20% (0.001 and 0.01  $\mu$ M cabergoline) (Fig. 1B). Moreover, the morphologic deteriorations induced by rotenone were also ameliorated by cabergoline treatment (Fig. 2E,F).

### Cabergoline reduced lactate dehydrogenase release in primary mesencephalic cell culture

Cabergoline significantly reduced the release of LDH when mesencephalic cell cultures were treated with cabergoline alone or together with rotenone. Treatment with cabergoline from the 6<sup>th</sup> DIV for six consecutive days significantly reduced the release of LDH into the culture medium by 66-50% at concentrations of 0.001-10  $\mu$ M as measured on the 12<sup>th</sup> DIV (Fig. 3). When control cultures were incubated with 80 nM rotenone on the 10<sup>th</sup> DIV for 48 h, the release of LDH was increased by 73% compared to untreated cultures (Fig. 3). Concomitant addition of rotenone on the 10<sup>th</sup> DIV for 48 h during cabergoline treatment revealed that cabergoline significantly reduced the release of LDH by 60-36% at concentra-

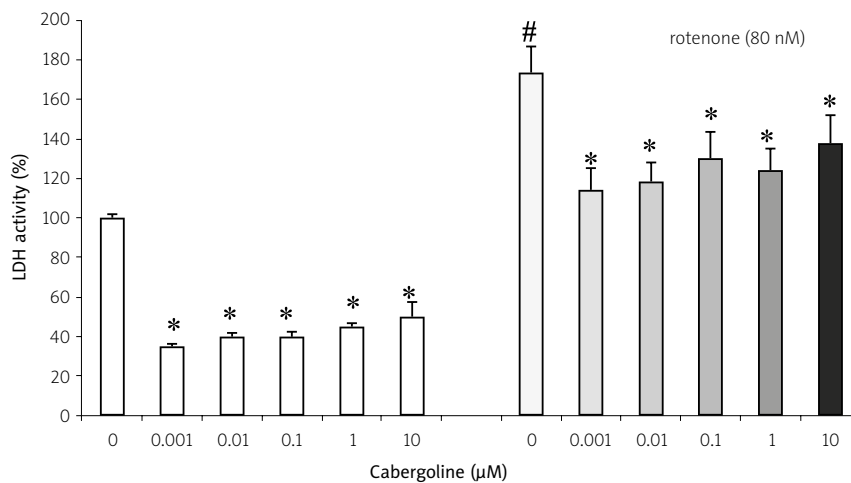


**Fig. 1. A)** Effect of cabergoline treatment on the number of tyrosine hydroxylase immunoreactive (THir) neurons. One hundred percent corresponds to the total number of THir neurons after 14 days *in vitro* (DIV) in untreated control cultures. Values represent the mean  $\pm$  SEM of three independent experiments with four wells in each treatment. In each well 10 randomly selected fields were counted for tyrosine hydroxylase (TH) immunocytochemistry. \* $p < 0.0001$ , significance compared with untreated controls using Kruskal-Wallis test followed by  $\chi^2$  test. **B)** Effect of cabergoline on the survival of rotenone-treated THir neurons. One hundred percent corresponds to the total number of THir neurons after 12 DIV in untreated control culture. Values represent the mean  $\pm$  SEM of three independent experiments with four wells in each treatment. In each well 10 randomly selected fields were counted for TH immunocytochemistry. # $p < 0.0001$ , significance compared with untreated controls using Mann-Whitney test; \* $p < 0.001$ , significance compared with rotenone-treated control cultures using Kruskal-Wallis test followed by  $\chi^2$  test.



**Fig. 2.** Representative micrographs of tyrosine hydroxylase immunoreactive (THir) neurons in primary mesencephalic cell cultures after 12 days *in vitro* (DIV). **A, B**) Untreated control cultures show intact THir neurons with long and branched processes. **C, D**) Surviving neurons after rotenone exposure appear few in number with shortened and dysmorphic neurites with bead-like structures (arrows). **E, F**) Cabergoline improves the number and morphology of THir neurons compared to rotenone-treated cultures.





**Fig. 3.** Release of lactate dehydrogenase (LDH) in primary mesencephalic cell cultures. One hundred percent corresponds to the amount of LDH in the culture medium after 12 days *in vitro* (DIV). Values represent the mean  $\pm$  SEM of three independent experiments with four wells in each treatment. # $p < 0.0001$ , significance compared with untreated controls using Mann-Whitney test; \* $p < 0.001$ , significance compared with untreated and rotenone-treated control cultures using Kruskal-Wallis test followed by  $\chi^2$  test.

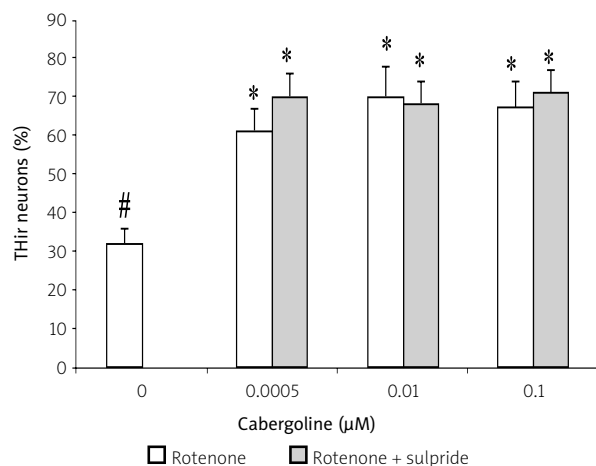
tions of 0.001-10  $\mu\text{M}$ , compared to cultures treated with rotenone only (Fig. 3).

### The protective effect of cabergoline against rotenone was not dopamine receptor-dependent

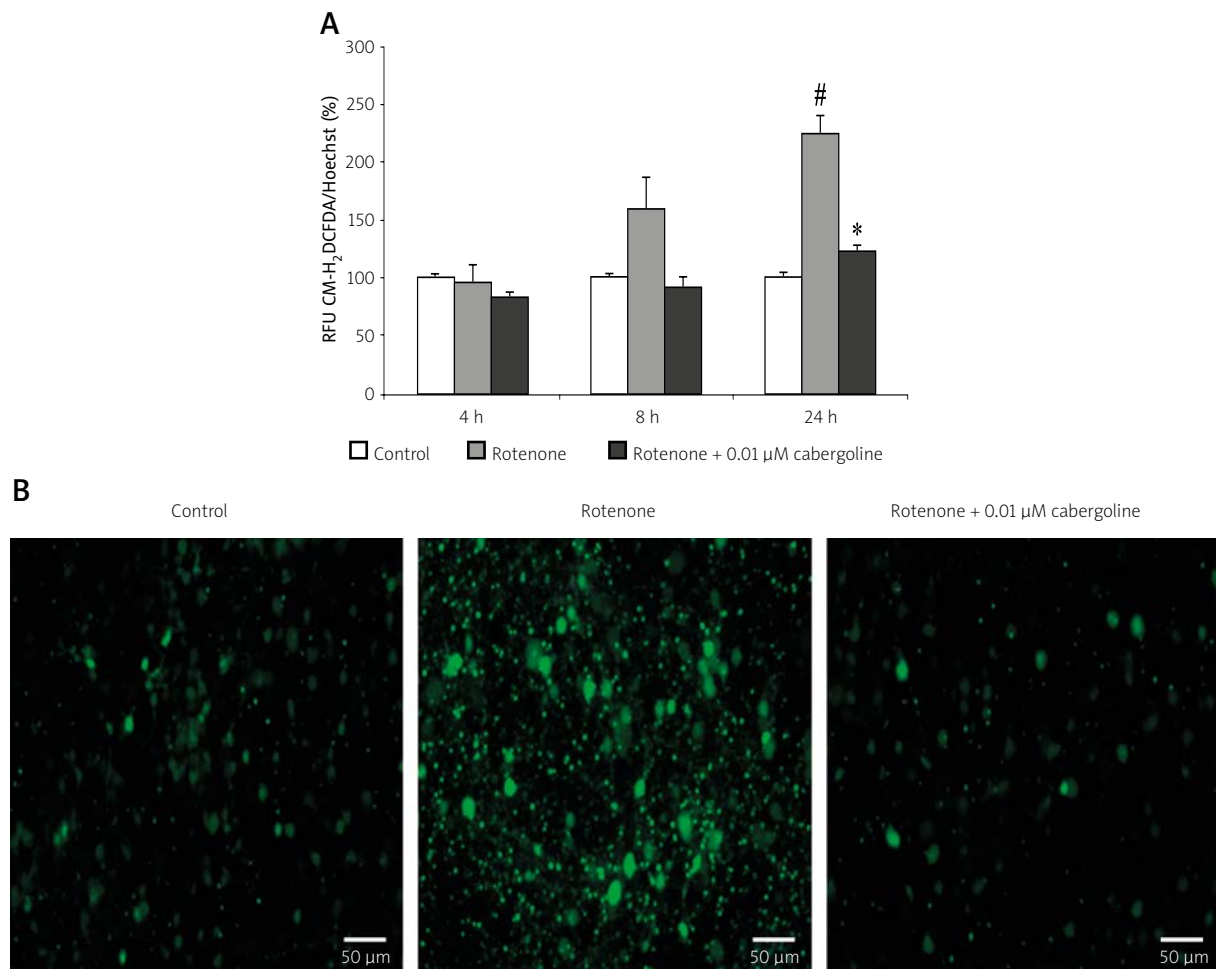
The dopamine receptor antagonist sulpiride did not antagonize the neuroprotection afforded by cabergoline in rotenone-treated cultures. When cultures were treated with cabergoline together with sulpiride on the 8<sup>th</sup> DIV for 24 h and treatment with 80 nM rotenone was started on the 9<sup>th</sup> DIV for 48 h, no reduction in the protective effect of cabergoline on the number of THir neurons could be detected (Fig. 4). Treatment with sulpiride alone had no effect on the number of dopaminergic neurons (data not shown).

### Cabergoline inhibited the production of reactive oxygen species in primary mesencephalic cell culture

Since the production of ROS is a central mechanism of rotenone toxicity, it was tested whether cabergoline could counteract the effect of rotenone-induced ROS production. The treatment of cultures with 80 nM rotenone on the 9<sup>th</sup> DIV significantly elevated overall ROS production by 224% after 24 h as measured by CM-H<sub>2</sub>DCFDA fluorescence compared to untreated control cultures (Fig. 5A). Pre-treatment with cabergoline (0.01  $\mu\text{M}$ ) significantly reduced



**Fig. 4.** Effect of different concentrations of cabergoline with and without sulpiride on the survival of rotenone-treated tyrosine hydroxylase immunoreactive (THir) neurons. One hundred percent corresponds to the total number of dopaminergic neurons after 11 days *in vitro* (DIV) in untreated control cultures. Values represent the mean  $\pm$  SEM of three independent experiments with four wells in each treatment. In each well 10 randomly selected fields were counted for tyrosine hydroxylase (TH) immunocytochemistry. # $p < 0.0001$ , significance compared with untreated controls using Mann-Whitney test; \* $p < 0.001$ , significance compared with rotenone-treated control cultures using Kruskal-Wallis test followed by  $\chi^2$  test.



**Fig. 5. A)** Measurement of reactive oxygen species (ROS) production in primary mesencephalic cell cultures using CM-H<sub>2</sub>DCFDA fluorescence staining. One hundred percent corresponds to the fluorescence intensity after 10 days *in vitro* (DIV) in untreated control cultures. Values represent the mean ± SEM of four independent experiments. Fluorescence intensity was determined densitometrically from a total of 80 randomly selected micrographs (20 photos in each experiment). <sup>#</sup>*p* < 0.0001, significance compared with untreated controls using Mann-Whitney test; <sup>\*</sup>*p* < 0.0001, significance compared with rotenone-treated control cultures. **B)** Representative micrographs of primary mesencephalic cell cultures after staining with CM-H<sub>2</sub>DCFDA fluorescence dye on the 10<sup>th</sup> DIV.

rotenone-induced ROS production by about 100% when added on the 8<sup>th</sup> DIV for 24 h (Fig. 5A). Different fluorescence intensities could be detected microscopically in control, rotenone-treated and rotenone-cabergoline-treated cultures (Fig. 5B).

### Cabergoline increased the content of glutathione in primary mesencephalic cell culture

The antioxidant GSH is essential for the cellular detoxification of reactive oxygen species in brain

cells. Therefore, it was investigated whether cabergoline might confer protection for dopaminergic neurons against rotenone toxicity by increasing the production of GSH. Treating the cultures with cabergoline from the 8<sup>th</sup> DIV for 24 h stimulated the content of GSH by up to 35% (0.01 μM cabergoline) when measured on the 9<sup>th</sup> DIV (Fig. 6A). When cultures were treated with the GSH synthesis inhibitor BSO on the 8<sup>th</sup> DIV for 24 h, GSH was significantly reduced to 44% (from 2.57 μmol/mg protein to 1.13 μmol/mg protein) of control values (data not



shown). However, when 80 nM rotenone was added to the GSH depleted cultures from the 9<sup>th</sup> to the 11<sup>th</sup> DIV, the protective effect for dopaminergic neurons afforded by cabergoline was not prevented (Fig. 6B), indicating that GSH increase is not decisive in cabergoline-induced neuroprotection.

### Cabergoline significantly increased the content of ATP in primary mesencephalic cell culture

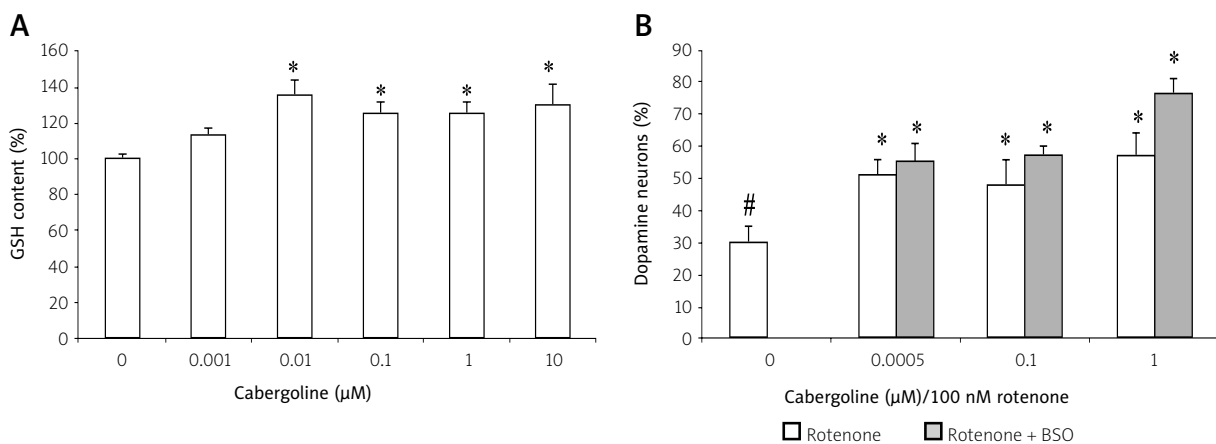
As an inhibitor of the mitochondrial respiratory chain, rotenone is known to reduce the intracellular content of ATP. It was therefore studied whether cabergoline influenced the content of ATP in the culture with and without rotenone treatment. The treatment of cultures with cabergoline from the 8<sup>th</sup> DIV for 24 h significantly increased the content of ATP by 37% at 0.0005  $\mu$ M compared to control cultures when measured on the 11<sup>th</sup> DIV (Fig. 7). Addition of 80 nM rotenone significantly reduced the ATP content by 53% when added on the 9<sup>th</sup> DIV for 48 h (Fig. 7). On the other hand, pretreatment of cultures with cabergoline significantly increased

the ATP content by 33 and 28% at concentrations of 0.0005 and 0.01  $\mu$ M, respectively, compared to rotenone-treated control cultures (Fig. 7).

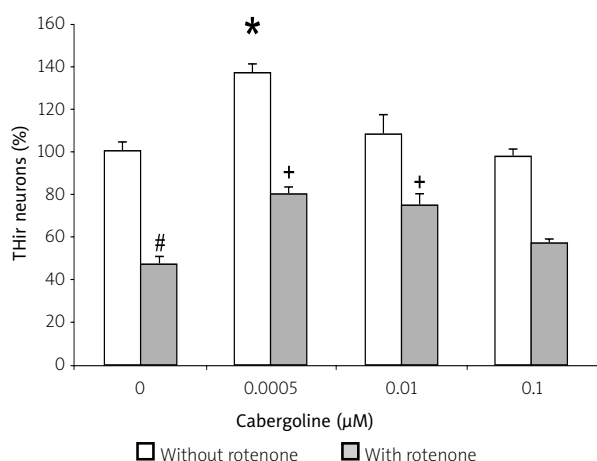
### Discussion

In the present study, cabergoline was found to promote the survival of THir neurons when added to primary mesencephalic cell cultures (Fig. 1A). Such a stimulatory effect is not exhibited by all ergoline dopamine agonists, since it was for example demonstrated for lisuride [6], but not for pergolide [5] or  $\alpha$ -dihydroergocryptine [4] when analogously used under the same treatment conditions. Similarly to these ergoline agonists, cabergoline reduced the number of dopamine neurons at an unphysiological high concentration (10  $\mu$ M).

Remarkably, cabergoline was able to protect THir neurons against the potent mitochondrial complex I inhibitor rotenone (Fig. 1B). The pesticide is well known to increase the risk for PD during long-term use [27]. In the tested concentration range, cabergoline rescued a significant number of THir neurons and improved their morphology under rotenone



**Fig. 6. A)** Effect of cabergoline on glutathione (GSH) synthesis in primary mesencephalic cell cultures. One hundred percent corresponds to GSH content after 9 days *in vitro* (DIV) in untreated control cultures and amounts to 2.57  $\mu$ mol/mg protein. Values represent the mean  $\pm$  SEM of three independent experiments with four wells in each treatment. \* $p < 0.001$ , significance compared with untreated control cultures using Kruskal-Wallis test followed by  $\chi^2$  test. **B)** Effect of cabergoline with and without L-buthionine-(S,R)-sulfoximine (BSO) on the survival of rotenone-treated tyrosine hydroxylase immunoreactive (THir) neurons. One hundred percent corresponds to the total number of dopaminergic neurons after 11 DIV in untreated control culture. Values represent the mean  $\pm$  SEM of three independent experiments with four wells in each treatment. In each well, 10 randomly selected fields were counted for tyrosine hydroxylase (TH) immunocytochemistry. # $p < 0.0001$ , significance compared with untreated controls using Mann-Whitney test; \* $p < 0.05$ , significance compared with rotenone-treated control cultures using Kruskal-Wallis test followed by  $\chi^2$  test.



**Fig. 7.** Effect of cabergoline on ATP content in primary mesencephalic cell cultures. One hundred percent corresponds to the ATP content after 11 days *in vitro* (DIV) in untreated control cultures and amounts to 3.5 nmol/mg protein. Values represent the mean  $\pm$  SEM of three independent experiments with four wells in each treatment. \* $p < 0.01$ , significance compared with untreated control cultures using Kruskal-Wallis test followed by  $\chi^2$  test; # $p < 0.0001$ , significance compared with untreated control cultures using Mann-Whitney test; + $p < 0.01$ , significance compared with rotenone-treated control cultures using Kruskal-Wallis test followed by  $\chi^2$  test.

treatment (Fig. 2). Protection against rotenone toxicity by dopamine agonists has been only sparsely reported in the literature. In mice treated with rotenone the non-ergoline agonist pramipexole saved dopaminergic neurons in the substantia nigra from degeneration [11]. Ropinirole, also belonging to the non-ergoline agonists, prevented rotenone-induced apoptosis in the dopaminergic cell line SH-SY5Y via caspase- and JNK-dependent pathways [2]. Recently, we detected protective properties of the non-ergoline dopamine agonist rotigotine for dopaminergic neurons in primary mesencephalic culture [22]. To our knowledge, this is the first report of neuroprotective properties of an ergot agonist against rotenone toxicity for dopaminergic neurons.

Neuroprotection by cabergoline has been reported previously in different cellular and animal models of neurodegeneration. For instance, Lombardi *et al.* [14] and Miglio *et al.* [16] reported that cabergoline protected SH-SY5Y human neuroblastoma cells against tert-butylhydroperoxide (t-BOOH)- and ischemia-

induced cell death, respectively. *In vivo*, Yoshioka *et al.* [32] found that cabergoline reduced the cell death of striatal dopaminergic neurons provoked by 6-hydroxydopamine (6-OHDA) in mice. In our study we found an overall protective effect of cabergoline on the primary mesencephalic cell culture. Cabergoline significantly reduced the release of LDH when applied either alone or together with rotenone (Fig. 3). Cabergoline was similarly reported to reduce LDH release induced by ischemia in SH-SY5Y cells [16].

Neuroprotective effects exerted by dopamine agonists are often mediated via stimulation of dopamine receptors. For instance, blocking of dopamine D<sub>2</sub> receptors by the dopamine receptor antagonist sulpiride prevented dopaminergic neuroprotection by lisuride and  $\alpha$ -dihydroergocryptine against glutamate and dopamine, respectively [4,17]. Sulpiride also abolished the protection of dopaminergic neurons by ropinirole against 6-OHDA [9]. In SHSY-5Y cells expressing D<sub>2</sub> receptors the protective effect of cabergoline against paraquat toxicity was partially reduced by inhibition of the D<sub>2</sub> receptor with clozapine [1], and recently it was shown that in cortical neurons cabergoline prevented cell death induced by H<sub>2</sub>O<sub>2</sub> through a D<sub>2</sub> receptor-mediated mechanism [18]. In contrast, the protection of THir neurons against rotenone toxicity by cabergoline appears independent of dopamine receptor stimulation. The pretreatment of cultures with sulpiride did not attenuate the rescuing effect of cabergoline for THir neurons in primary mesencephalic cell culture (Fig. 4). Since sulpiride has almost equal affinity to dopamine D<sub>2</sub> and D<sub>3</sub> receptors (Ki~15 nM for the dopamine D<sub>2</sub> receptor and Ki~13 nM for the dopamine D<sub>3</sub> receptor) [26], stimulation of the D<sub>3</sub> receptor is not decisive for the neuroprotective effect of cabergoline either. In this respect cabergoline resembles the non-ergot dopamine agonist pramipexole, which has been shown to protect SHSY-5Y cells against apoptotic cell death induced by rotenone or 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>) independent of dopamine receptor stimulation [7]. In contrast, neuroprotective effects against oxidative stress in rat mesencephalic cultures provided by the ergot agonist bromocriptine depended on dopamine receptor stimulation and the synthesis of radical scavenging proteins [23].

Recently, it was shown that male dopaminergic neurons were slightly more sensitive towards rotenone, and estrogen exerted a moderate protective

effect towards both genders [28]. Therefore, it might be of interest in future experiments to investigate the neuroprotective efficacy of cabergoline towards male and female dopaminergic neurons.

Oxidative stress is believed to contribute to the degeneration of dopaminergic neurons in PD [29]. We therefore tested whether cabergoline was able to reduce rotenone-induced oxidative stress in the cultures. Pre-treatment of the cultures with cabergoline was found to reduce overall ROS production elicited by subsequent rotenone addition as measured by CM-H<sub>2</sub>DCFDA fluorescence (Fig. 5). Similarly, cabergoline was reported to relieve oxidative stress in different experimental models of PD and in PD patients. For example, Chau *et al.* reported that cabergoline diminished dihydroethidium (DHE) oxidation in SHSY-5Y cells exposed concomitantly to paraquat [1]. Since DHE is predominantly a superoxide indicator, we detected that cabergoline is also able to reduce general oxidative stress induced by rotenone even when the culture is only pre-incubated with the agonist. Isobe *et al.* found that cabergoline scavenged peroxynitrite induced by levodopa in PD patients [12]. Although cabergoline increased GSH synthesis in mesencephalic cell cultures, its neuroprotective effect against rotenone toxicity was not dependent on GSH synthesis, as pre-treatment of mesencephalic cell cultures with the GSH biosynthesis inhibitor BSO did not prevent protection of dopaminergic neurons afforded by cabergoline (Fig. 6). In contrast, Chau *et al.* postulated that the increased GSH level induced by cabergoline in SHSY-5Y cells may at least partially explain the protection against paraquat [1].

Depletion of ATP was reported to be among the major consequences of complex I inhibition in different *in vitro* and *in vivo* PD models. For instance, Im *et al.* [10] and Zaitone *et al.* [33] found that rotenone reduced ATP levels in PC12 cells and Sprague-Dawley rats, respectively. In accordance with these results, treatment of primary mesencephalic cell culture with rotenone resulted in a significant decrease in the ATP/protein ratio compared to untreated cultures (Fig. 7). On the other hand, cabergoline not only significantly increased the ATP content in the culture, but even attenuated rotenone-induced ATP depletion, which might contribute to cabergoline's neuroprotective effect. To our knowledge, this is the first report demonstrating protection against rotenone-induced ATP loss by a dopamine agonist.

These results imply that cabergoline might exert its neuroprotective properties also at and via the mitochondrial level.

## Conclusions

Cabergoline protected dopaminergic neurons against rotenone-induced cell death in primary mesencephalic cell culture. This effect was independent of dopaminergic stimulation and did not predominantly depend on increased GSH production. Cabergoline could reduce excessive ROS formation and restore the ATP/protein ratio. Moreover, its beneficial effect for the whole culture was detected by reduced LDH release. These results undermine the neuroprotective potential of dopamine agonists and demonstrate that ergot agonists may exert their protective action independently of dopamine receptor stimulation.

## Disclosure

The authors report no conflict of interest.

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