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Dimebon (Latrepirdine) Enhances Mitochondrial Function and Protects Neuronal Cells from Death

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Abstract. Dimebon, a drug currently being evaluated in multiple Phase III Alzheimer's disease trials, has previously been shown to have effects on isolated mitochondria at μM concentrations. Here the effects of nM concentrations of Dimebon on mitochondrial function were investigated both in primary mouse cortical neurons and human neuroblastoma cells (SH-SY5Y). Under non-stress conditions nM concentrations of Dimebon increased succinate dehydrogenase activity (MTT-assay), mitochondrial membrane potential ($\Delta\Psi$m), and cellular ATP levels. Dimebon treatment had no effect on mitochondria DNA content, implying that mitochondrial biogenesis was not induced. Under stress conditions, mitochondria in Dimebon-treated neurons showed increased resistance to elevated intracellular calcium concentrations, thus, maintaining their $\Delta\Psi$m throughout the experiment, in contrast to control neurons, which rapidly lost their $\Delta\Psi$m. Moreover, we show that serum-starved differentiated SH-SY5Y cells treated with Dimebon had an increased survival rate as compared to untreated cells. In conclusion, these data demonstrate that Dimebon enhances mitochondrial function both in the absence and presence of stress and Dimebon-treated cells are partially protected to maintain cell viability.

Keywords: Alzheimer's disease, Dimebon (latrepirdine), mitochondria, neuronal cells

INTRODUCTION

Alzheimer's disease (AD) is a complex disease and several hypotheses have been proposed to explain its pathogenesis. It is generally believed that amyloid-β peptide is important in the development of the hallmarks of the disease, including synapse loss, neurodegeneration, amyloid plaques, and neurofibrillary tangles [1]. In addition to these disease hallmarks, a consistent pattern of changes in mitochondrial morphology and function is detected in AD at an early stage. For example, there is a decrease in the number of mitochondria in vulnerable neurons accompanied by increase in mitochondrial DNA (mtDNA) and protein in cytoplasm and vacuoles, indicating a greater turnover of mitochondria by autophagy or decreased proteolytic turnover [2]; brain glucose metabolism is decreased [3]; and the activities of both tricarboxylic acid (TCA) cycle enzymes [4] and cytochrome c oxidase (COX) are reduced [5–8]. Recently it was also shown that the levels of mitochondrial fission/fusion proteins are altered and that mitochondria are redistributed away from axons in pyramidal neurons of AD brain [9,10]. These

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abnormalities may be related to synapse loss and/or synapse dysfunction, which are the most robust correlate of AD-associated cognitive deficits [11]. Besides the loss of synapses, the mitochondrial dysfunction may also result in neuronal cell apoptosis that is a common characteristic in brains of patients suffering from neurodegenerative diseases [12–16]. Thus, therapeutic approaches targeting mitochondrial dysfunction hold great promise in neurodegenerative diseases [17–20]. Examples of such drugs are mitochondria-targeted antioxidants and Dimebon as recently reviewed by Moreira and colleagues [21].

Dimebon was originally approved in the former Soviet Union as a non-selective antihistamine for skin allergy and allergic rhinitis [22] but was withdrawn from the market with the advent of more selective treatments. Dimebon attracted renewed interest due to findings suggesting a neuroprotective effect [23–25]. In a Phase II AD trial, Dimebon treatment was associated with benefits on cognition, global function, activities of daily living, and behavior [26]. Dimebon exhibits a rich pharmacological profile and binds to histamine-, adrenergic-, dopamine-, and serotonin-receptors [25, 27]. It is known to be a weak inhibitor of acetylcholinesterase (IC_{50} = 8–42 μM) [24], N-methyl-D-aspartate (NMDA) receptors (IC_{50} = 10 μM) [25, 28], and voltage-gated calcium channels (IC_{50} = 50 μM) [25,29]. In addition, μM concentrations of Dimebon have previously been shown to protect against neuronal cell death induced by $A\beta_{25-35}$ [24] and to modulate the mitochondrial permeability transition pore (10–200 μM) [20]. To date, the precise mechanisms whereby Dimebon exerts its pro-cognitive and neuroprotective effects are unknown.

In the present study, we investigated Dimebon's mode of action in primary mouse cortical neuronal cells (MCN) and human neuroblastoma SH-SY5Y cells. We report that in these cell models, nM concentrations of Dimebon improve mitochondrial function on aspects such as mitochondrial membrane potential ($\Delta\Psi$m) and ATP synthesis. No effect was detected on the mtDNA copy number, implying a constant mitochondrial biogenesis. Moreover, we show that serum-starved differentiated SH-SY5Y cells treated with Dimebon had an increased survival rate as compared to untreated cells. The plasma concentration of rats administered 5mg/kg Dimebon is 100 nM, while the brain concentration is 10-times higher [30]. The plasma concentration in humans taking 3×20 mg Dimebon/day is 10–15 nM, with an estimated brain concentration of 100 nM. Thus, the nM range used here resembles the calculated brain concentration in patients receiving Dimebon in the Phase II AD trial [26]. Previous studies have shown that the cognition-enhancing effect of Dimebon is not associated with acetylcholinesterase inhibition or NMDA receptor binding [30]. From our studies we hypothesize that Dimebon enhances and stabilizes mitochondrial function.

MATERIALS AND METHODS

Reagents

Dimebon (latrepirdine) (2,8-Dimethyl-5-[2-(6-methylpyridin-3-yl)ethyl]-2,3,4,5-etrahydro-1H-pyrido[4, 3-b]indole dihydrochloride) was obtained from Medivation Inc. (San Francisco, CA). Brain-derived neurotrophic factor (BDNF), retinoic acid (RA), ionomycin (IM), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), trypan blue, and thapsigargin (TG) were purchased from Sigma-Aldrich (St. Louis, MO). Unless otherwise noted, all growth medium and supplementary serum or reagents were purchased from Invitrogen Corporation (Eugene, OR, USA).

Primary mouse cortical neuron cell preparation and culture

Primary cultures of MCN were prepared as described previously [31]. Approval for these experiments was received from the Animal Ethics Committee of South Stockholm, Sweden. Neocortices of 16- or 17-days-old mouse embryos were dissociated and plated on poly-D-lysine-coated plates at a density of 200,000 cells/ml in Neurobasal medium supplemented with B27 (Invitrogen Corporation). Cells were then maintained up to 7 days in a humidified incubator with 5% CO_2 at 37°C.

Differentiation of SH-SY5Y cells

SH-SY5Y human neuroblastoma cells were obtained from the American Type Culture Collection (ATCC, Manassa, VA, USA). Cells were grown in Dulbecco modified Eagle's medium/Nutrient Mixture Ham's F12 (1:1) (DMEM/F12) supplemented with 10% FCS. Cells were maintained at 37°C at saturated humidity in an atmosphere of 5% CO_2 in air. Cell culture medium was replaced every two days and the cultures were used up to the 15th passage. For differentiation, all-trans RA was added to the medium to a final concentra-

tion of 10 μM. After incubation with RA for 5 days, cells were suspended and separated with disassociation buffer (GIBCO^{TM} Cell dissociation Buffer enzyme free Hanks' based, Invitrogen), followed by reseeding on growth factor reduced Matrigel^{TM} matrix (BD Biosciences, Two Oak Park, Bedford, MA, USA) coated 35 mm glass-bottom dishes or other surfaces. Reseeded cells were allowed to attach 24 h in 10% FCS supplemented medium with RA. The medium was then replaced by serum-free DMEM/F12 containing either BDNF (25 ng/ml), Dimebon, or vehicle. For quantification of surviving cells, cells were detached with dissociation buffer and harvested in growth medium. Harvested cells were stained with 0.4% trypan blue (Invitrogen), and living cells were counted in a counting chamber.

Measurement of $\Delta\Psi m$ by confocal microscopy

MCN were seeded onto 35 mm glass bottom culture dishes (MatTek Corporation, Ashland, USA) coated with poly-D-lysine (Sigma-Aldrich). The neurons were grown for approximately one week before the experiments started. After Dimebon (0.1, 1, 10, or 100 nM) or vehicle treatment for 24 h, cells were stained with 500 nM DAPI (4-6-diamidino-2-phenylindole) (Vector Laboratories, Burlingame, CA) and 5 nM tetramethylrhodamine methyl ester (TMRM) (Invitrogen Corporation, Calsbad, CA) for 1 h. Live cell imaging was performed using an inverted microscope Axiovert 200M (Carl Zeiss MicroImaging GmbH, Jena, Germany) connected to a LSM510 META confocal unit (Carl Zeiss MicroImaging GmbH, Jena, Germany). TMRM (5 nM) and Dimebon/vehicle were present during the acquisition of all images. Four hundred times magnification, and with no change in settings, were used during all runs. The acquired images were analyzed using LSM510 software program. The mitochondria uncoupler, CCCP (Sigma-Aldrich), was used as a negative control for TMRM. Neurons were exposed to 50 μM CCCP for 10 min; and the decrease in $\Delta\Psi$m was monitored.

Calcium stress was introduced to Dimebon/vehicle pretreated (24 h) neurons by subjecting cells to a mixture of ionomycin and thapsigargin. The neurons were exposed to two different concentrations of the mixture, either 5 μM/0.5 μM or 10 μM/1 μM ionomycin and thapsigargin respectively in Locke buffer (134 mM NaCl, 5 mM KCl, 4 mM $NaHCO_3$, 5 mM Hepes (pH 7.2), 2.3 mM $CaCl_2$, and 2.5 mM glucose). Dimebon (10 nM)/vehicle was present during the acquisition of all images. Pictures were taken 0, 2.5, 5, 7.5, and 10 min after the initiation of calcium stress.

ATP-assay

Primary MCN (E16-17) were seeded in 24 well plates coated with poly-D-lysine (Sigma-Aldrich). After Dimebon (0.1, 1, 10, or 100 nM) or vehicle treatment for 24, 48, or 72 h, the neurons were washed in PBS and lysed in ATP-lysis buffer (91 mM K_2HPO_4, 9 mM KH_2PO_4 (pH 7.8), 2 mM EDTA, 1 mM DTT, and 1% Triton X-100). After 200 μl lysis buffer was added per well, the plate was placed in -20°C for 1 h. The ATP concentration was determined using Adenosine 5'-triphosphate (ATP) Bioluminescent Assay Kit (Sigma-Aldrich) according to the manufacturer's instructions. The ATP levels were normalized to the total protein amount. Triplicates were performed within each ATP experiment.

MTT assay

MTT reduction assay was performed according to methods described by Mosmann [32]. In brief, SH-SY5Y cells were grown to about 70% confluence and MCN were grown for four days before starting the experiment. MTT was dissolved in PBS at 5 mg/ml. At the end of the experiment, cell medium was substituted with Phenol-red free DMEM containing 0.5 mg/ml MTT. After either 1.5 h (SH-SY5Y cells) or 4 h (MCN) incubation at 37°C, the MTT formazan products were solubilized in DMSO. Absorbance was measured at 570 nm (reference at 620 nm) with a microplate reader.

For recovery assay, MCN were stressed with ionomycin/thapsigargin in Locke buffer, with or without Dimebon pretreatment for 24 h. Stressed cells were further grown in normal growth medium, with or without Dimebon, for 48 h before MTT analysis of cell viability.

Apoptosis analysis

SH-SY5Y cells were differentiated using RA for 5 days, followed by serum withdrawal and addition of Dimebon or BDNF for the indicated time-points. Cells were harvested with dissociation buffer and washed once with PBS. Cells were then either subjected to 0.2% trypan blue staining in order to count living cells or to staining with FITC-labeled recombinant Annexin V and Propidium iodide (PI) (Vybrant^{TM}, Apoptosis Assay Kit, Invitrogen, Eugene, OR). Annexin V/PI stained cells were analyzed on a FACScan flow cytometer (BD Biosciences, San Jose, CA). Cell debris was excluded by scatter gating (forward versus side).

Quantification of mtDNA copy number

Total DNA (genomic and mitochondrial) was extracted from MCN by using DNeasy blood and tissue extraction kit (Qiagen, Hilden, Germany). During the extraction procedure, DNA was treated with RNase A according to the protocol. 10 ng total DNA per reaction was used in quantitative real-time PCR. Taqman technology was used to quantify the mitochondrial genes cytochrome C oxidase (mtCOX1) and cytochrome b (mtCYTB); and genomic glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Thermo Scientific and Applied Biosystems) in 7900HT Sequence Detector System (Applied Biosystems). Final concentration of primers and probes was 900 and 250 nM, respectively. The final volume of PCR-reaction was 13 μl, including Gene Expression PCR Master Mix 2X (Applied Biosystems). The sequences for primers and probes have been previously described [33]. The copy number of mtDNA was quantified by calculating the ratio of mitochondrial genes CYTB and COX1 to the genomic gene GAPDH, according to Strum et al. [33].

Statistics

Statistical analyses were performed using the Student's *t*test or Kruskal Wallis one-way ANOVA by ranks followed by the Conover Post Hoc test to determine individual differences in the treatment groups as compared to vehicle-treated group. Values are expressed as mean $\pm$ SD or in box plots (the box represents the 25th and the 75th percentile, in which the line within the box indicates the median and the end of the whiskers represents minimum and maximum values). At least three independent experiments were performed. *P*values of < 0.05 were considered to be significant.

RESULTS

Dimebon enhanced $\Delta\Psi m$ in primary MCN and SH-SY5Y cells

$\Delta\Psi$m is a key indicator of mitochondrial function and cell viability. TMRM was used throughout this study to monitor the alternation of $\Delta\Psi$m. As a lipophilic cation, TMRM is reported to accumulate in mitochondria in proportion to $\Delta\Psi$m, with minimal phototoxicity and low photobleaching [34–36]. In our staining protocol using 5 nM TMRM, staining reaches a plateau in about 30–45 min. MCN treated with 0.1, 1, or 10 nM Dimebon for 24 h showed an equally spread, enhanced staining intensity of TMRM in whole cells, indicating increased $\Delta\Psi$m (Fig. 1A and B). Elongation of Dimebon treatment to 72 h also showed an increase in $\Delta\Psi$m (Fig. 1C). Similar results were obtained in SH-SY5Y cells treated with Dimebon for 24 h (Fig. 1D and E), showing that, at as low concentration as 0.1 nM, Dimebon caused around 40% increase of TMRM staining. In response to mitochondrial depolarization caused by the addition of the mitochondrial uncoupler carbonyl cyanide m-chlorophenylhydrazone (CCCP, 50 μM) to MCN, the $\Delta\Psi$m was rapidly dissipated, thus confirming the sensitivity and specificity of TMRM staining (Fig. 1F).

Dimebon increased mitochondrial ATP levels in primary MCN and differentiated SH-SY5Y cells

Based on the results that Dimebon increased the $\Delta\Psi$m, we further studied mitochondrial function by measuring ATP in MCN pretreated with Dimebon (0.1, 1, or 10 nM) or vehicle for 24, 48, or 72 h. While 24 h and 48 h treatment did not show significant difference in ATP levels (data not shown), cells treated for 72 h with Dimebon (1 and 10 nM) contained about 30–50% more ATP as compared to control cells (Fig. 2A). In addition, the ATP levels were increased in serum deprived differentiated SH-SY5Y (D-SH-SY5Y) cells treated with Dimebon (0.1, 1, or 10 nM) for 24 h (Fig. 2B). These data demonstrate that Dimebon treatment promotes ATP production both under non-stressed and stressed conditions.

Dimebon does not affect mtDNA copy numbers

It is apparent that a larger number of mitochondria would result in increased TMRM staining and produce higher amounts of ATP. Thus we investigated the effect of Dimebon on mitochondrial biogenesis by quantification of mtDNA copy numbers. The mtDNA copy number of two mitochondrial genes, mtCOX1 and mtCYTB, was measured by quantitative real-time PCR and compared to the nuclear gene GAPDH. Neither mtCOX1 nor mtCYTB DNA showed significant increase in copy numbers in Dimebon-treated primary MCN, suggesting that Dimebon did not promote mitochondrial biogenesis (Fig. 3). Therefore, we propose that Dimebon enhances the function of existing mitochondria by increasing their $\Delta\Psi$m (hyperpolarization) and elevating ATP production.

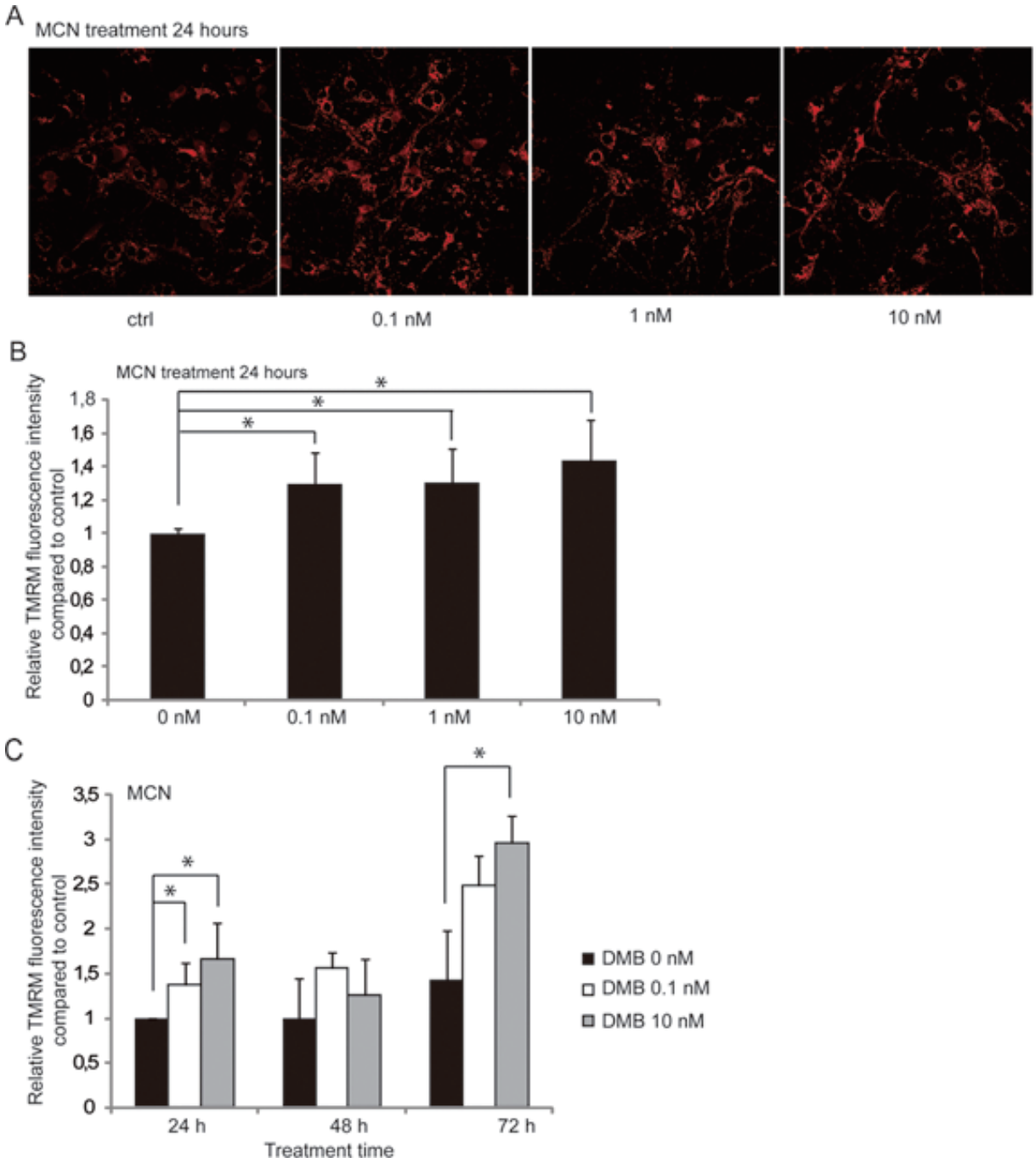
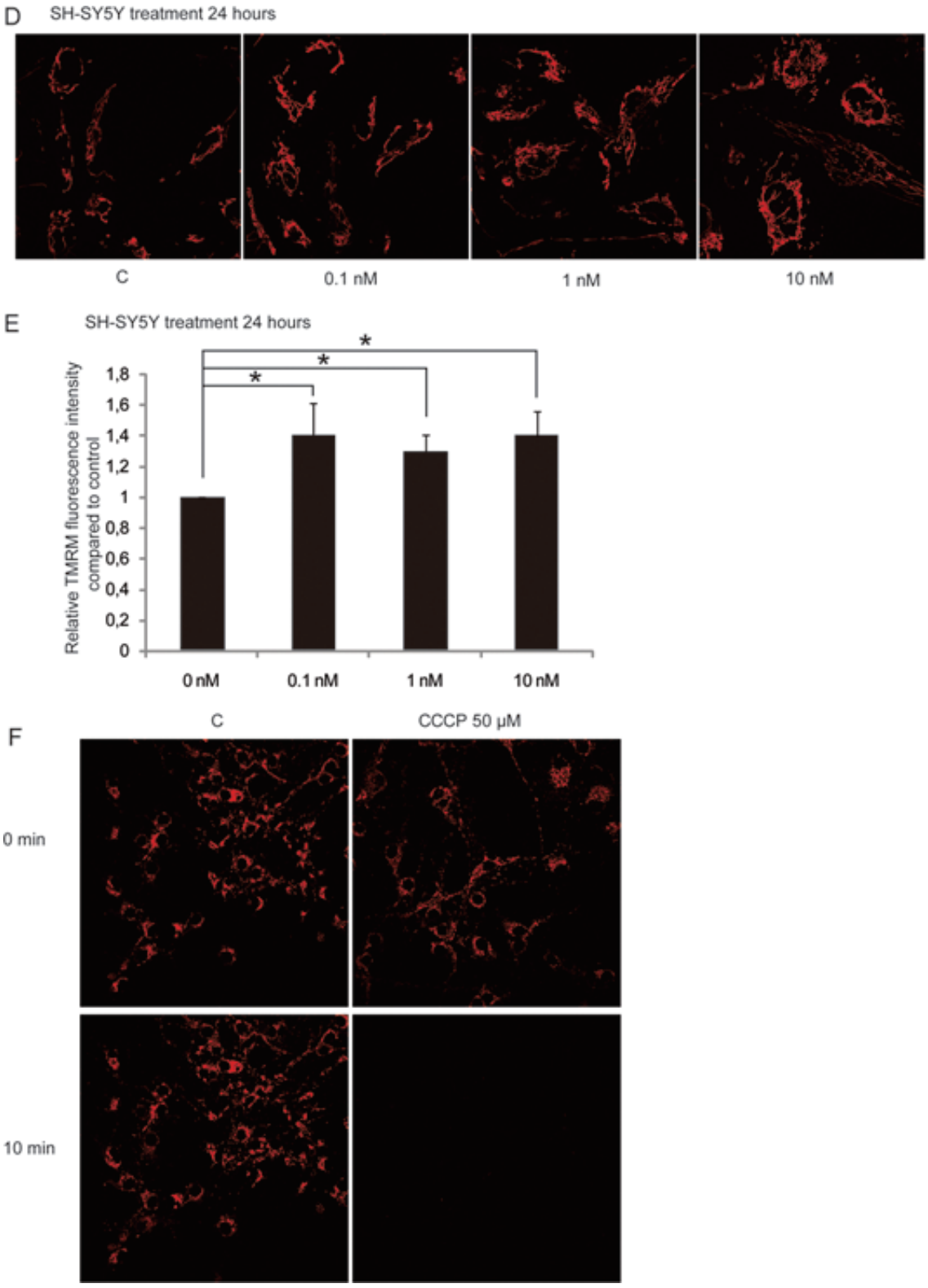


Fig. 1. Analysis of mitochondria membrane potential ($\Delta\Psi$m) by TMRM staining of Dimebon- pretreated primary mouse cortical neurons (MCN) and SH-SY5Y cells. MCN were cultured for four days and SH-SY5Y cells were grown to a confluence of 70% before starting Dimebon (DMB) treatment (0.1, 1, or 10 nM). After cultivation for 24 h, cells were stained with TMRM (5 nM) in growth medium for 1 h at 37°C, and then analyzed by confocal microscopy. A, D) Representative images of MCN and SH-SY5Y cells pretreated with indicated Dimebon concentrations. B, E) Quantification of mean TMRM staining intensity showing relative intensity compared to untreated control (mean $\pm$ SD, of three independent experiments, from six representative areas). C) Quantification of TMRM staining intensity in MCN cells pretreated with Dimebon for 24, 48, or 72 h. Data was analyzed using Kruskal Wallis one-way ANOVA by ranks and Conover Post Hoc test (mean $\pm$ SD, *significance at $P < 0.05$, $N = 4$). F) Representative images of MCN cells treated with 50 μM CCCP for 10 min.



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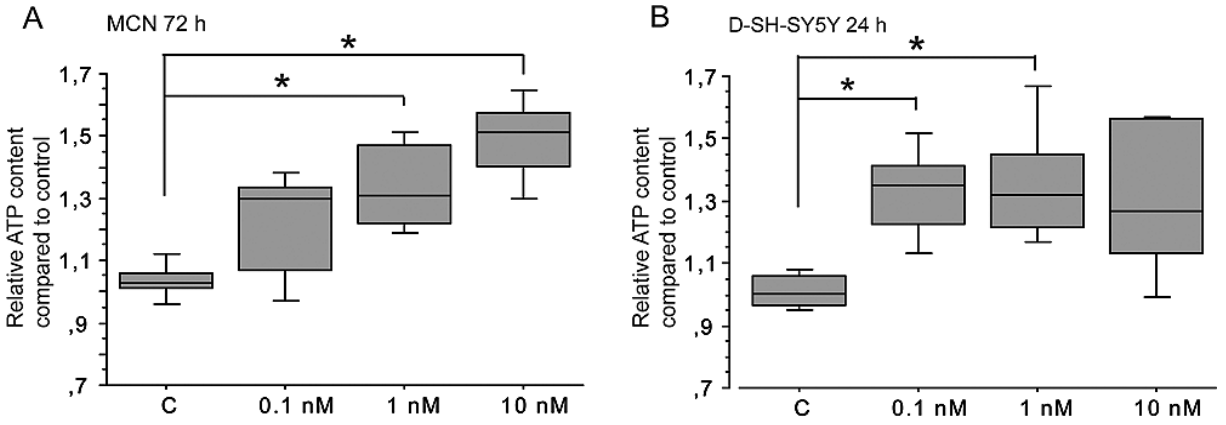


Fig. 2. Measurement of ATP concentration in Dimebon pretreated MCN and serum deprived differentiated SH-SY5Y cells. A) MCN pretreated with Dimebon (0.1, 1, or 10 nM) or vehicle for 72 h. B) SH-SY5Y cells were differentiated for five days with RA (10 μM), reseeded on Matrigel^{TM} coated plates. After attachment, cells were subjected to serum-free media supplemented with Dimebon (0.1, 1, or 10 nM) and incubated for 24 h before subjected to ATP concentration measurement. Triplicates were performed within each ATP experiment and normalized to protein amount. ATP concentration relative to untreated cells is shown by box plots (the box represents the 25th and the 75th percentile, in which the line within the box indicates the median and the end of the whiskers represents minimum and maximum values). Data was analyzed using Kruskal Wallis one-way ANOVA by ranks and Conover Post Hoc test (*significant at $P < 0.05$, $N = 5$).

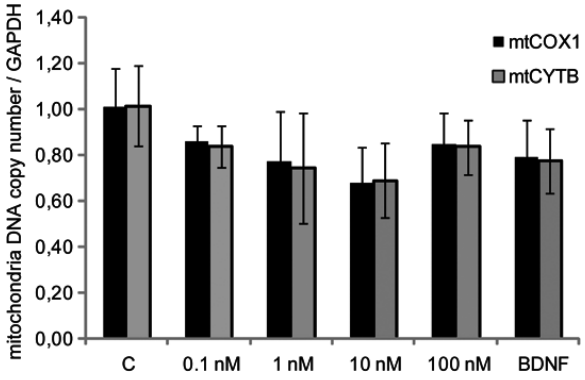


Fig. 3. Quantification of mtDNA copy number by quantitative real-time PCR. MCN were treated with Dimebon for 5 days with refreshment of medium and Dimebon every 2-3 days. The number of mitochondria was quantified by calculating the ratio of the mitochondrial genes cytochrome C oxidase 1 (mtCOX1) and cytochrome b (mtCYTB) to the genomic gene GAPDH (mean $\pm$ SD, $N = 3$).

Dimebon stabilized $\Delta\Psi m$ in calcium stressed primary MCN

Next, we decided to investigate the effect of Dimebon in cells under calcium stress induced by a combination of ionomycin and thapsigargin. The calcium ionophore, ionomycin, induces elevation of intracellular calcium, depending on intracellular and extracellular calcium sources [37]. Thapsigargin, a sarco-endoplasmic reticulum (ER) calcium-ATPase (SERCA) inhibitor, raises the cytosolic calcium concentration by blocking ER calcium uptake. As an extracellular source of calcium, a Locke buffer (described in Material and Methods) containing 2.3 mM $CaCl_2$ was used. MCN pretreated with either Dimebon (10 nM) or vehicle for 24 h were subjected to a mixture of ionomycin and thapsigargin in Locke buffer at the indicated concentrations. Dimebon/vehicle was present during the acquisition of all images. Pictures were taken 0, 2.5, 5, 7.5, and 10 min after starting the exposure. Persistent TMRM fluorescence in control cells indicated stable staining during the experiment and DAPI staining confirmed that a constant focus was kept during the acquisition of images (Fig. 4A). Stress with 5 μM ionomycin/0.5 μM thapsigargin induced depolarization of $\Delta\Psi m$ starting after 7.5 min in vehicle pretreated cells (Fig. 4B); while in cells pretreated with Dimebon, only a small decrease in TMRM staining was detected after 10 min. A stronger stress with 10 μM ionomycin/1 μM thapsigargin prompted similar depolarization of $\Delta\Psi m$ in both Dimebon and vehicle pretreated cells (Fig. 4D). Figure 4D shows relative changes of $\Delta\Psi m$ as calculated from the quantification of three independent experiments. In each, six representative image areas were analyzed.

Dimebon enhanced MTT reduction in both primary MCN and differentiated SH-SY5Y cells

Reduction of MTT in isolated cells and tissues is regarded as an indicator of "cell redox activity" [38,39]. The reaction is attributed mainly to mitochondrial enzymes and electron carriers [38,40,41], although MTT is also reduced by a number of non-mitochondrial enzymes in other cellular compartments [42]. MCN was

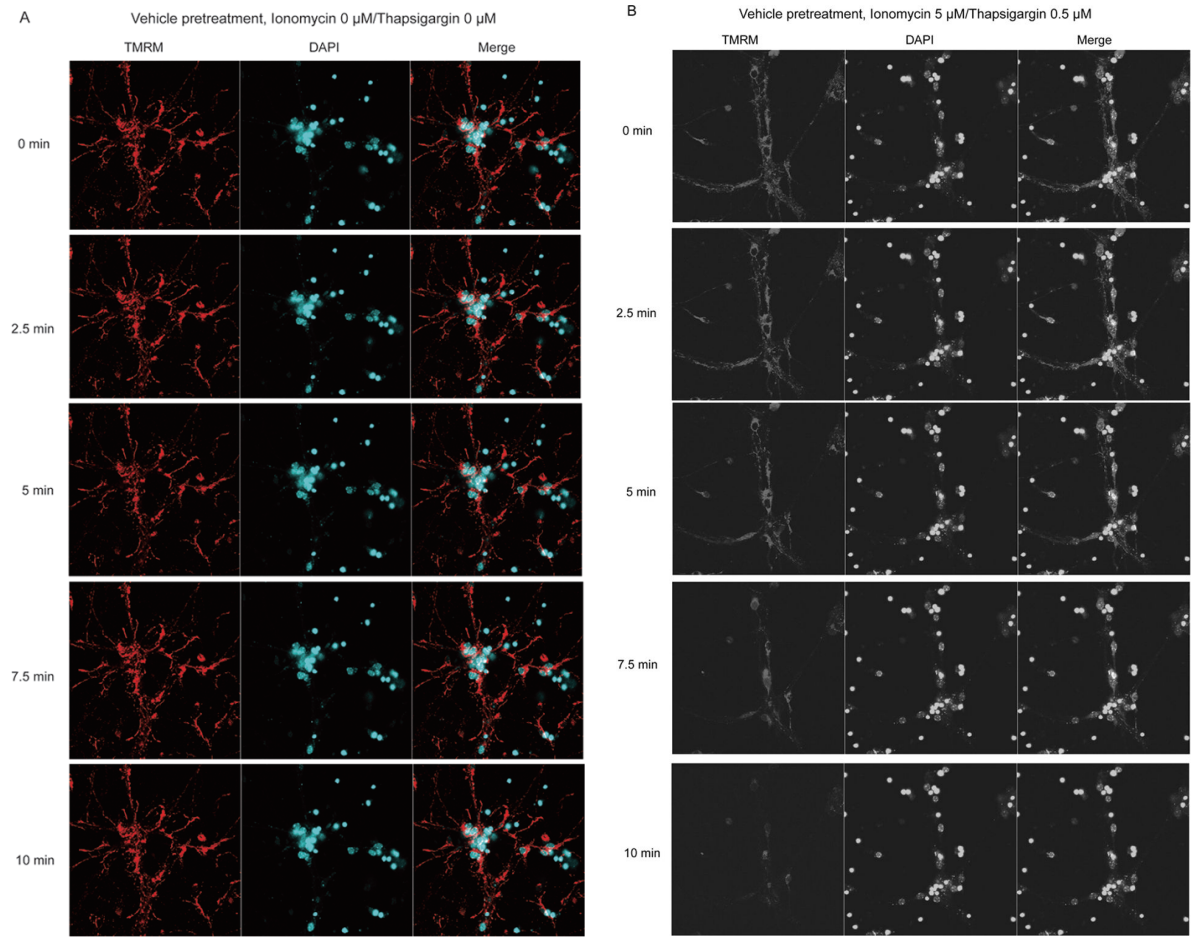
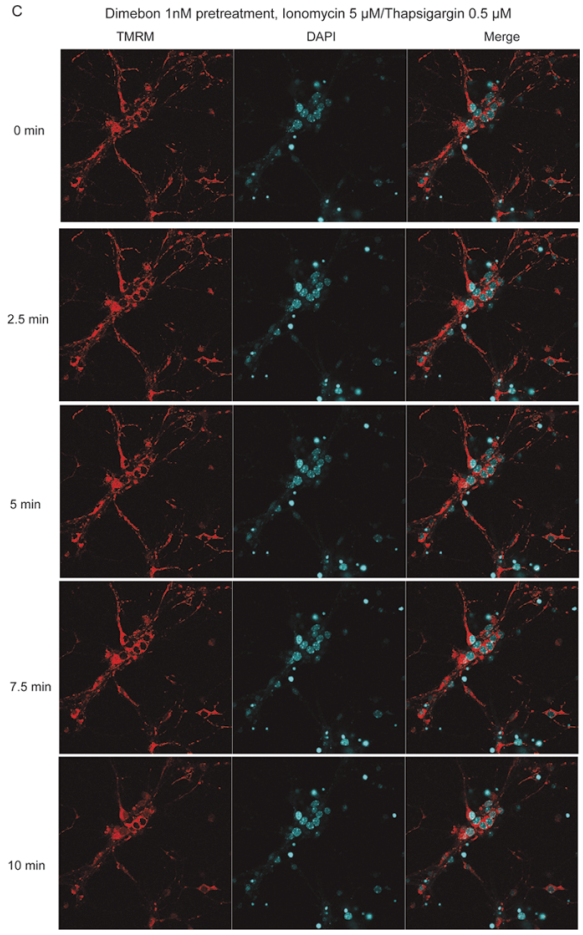


Fig. 4. Measurement of $\Delta\Psi$m in calcium ionophore stressed MCN with confocal microscopy. Neurons were pretreated for 24 h with either 1 nM Dimebon (DMB) or vehicle. One hour before stress, cells were stained with TMRM (5 nM) and DAPI (500 nM). After wash with PBS, cells were exposed to a mixture of ionomycin/thapsigargin in Locke buffer in the presence of Dimebon or vehicle. A time serial images were taken at 0, 2.5, 5, 7.5, and 10 min to monitor the $\Delta\Psi$m during calcium stress. DAPI staining confirmed constant level of focus. A) Representative images showed vehicle-treated cells without exposure to stress and B) when exposed to 5 μM ionomycin/0.5 μM thapsigargin. C) Representative images showed Dimebon pretreated cells exposed to 5 μM ionomycin/0.5 μM thapsigargin. D) Quantitative analysis of fluorescence intensity obtained from three independent experiments. Five representative areas were measured in each sample. Data was analyzed using student's t-test (mean $\pm$ SD, *significant at $P < 0.05$ relative to vehicle treated cells at the same exposure time).

pretreated with Dimebon (0.1, 1, 10, or 100 nM) for 24 h. Neurons treated with 100 nM showed moderate enhancement in MTT reduction, suggesting induced mitochondrial activity by Dimebon (Fig. 5A). In all other experiments in the present study, 0.1–10 nM Dimebon showed significant effects and therefore the 100 nM concentration was not used except in the MTT assay as presented in Fig. 5A. The MTT assay can also be used to detect cell viability. To investigate if Dimebon could protect cells from calcium-induced cell death, MCN were either pre-treated with Dimebon (10 nM) 24 h before addition of ionomycin/thapsigargin (5 min) or treated with Dimebon (10 nM) immediately after the 5 min stress-period. The MTT assay was performed 48 h after stress withdrawal. Interestingly, only cells pretreated with Dimebon showed significant protection from stress, while addition of Dimebon after stress did not protect against decreased MTT reduction and loss of cell viability (Fig. 5B). The ability of Dimebon pretreated cells to keep their $\Delta\Psi$m and MTT-reducing capacity during moderate ionomycin/thapsigargin stress again points toward an effect of Dimebon on mitochondrial function.

When RA-differentiated SH-SY5Y cells were subjected to serum deprivation for 5 days with, or without, the addition of Dimebon (0.1–10 nM) or BDNF (25ng/ml), cells treated with Dimebon displayed enhanced cell viability as detected by the MTT assay, al-



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though far less enhanced than the protective effect of BDNF (Fig. 5C).

Dimebon inhibited serum withdrawal-induced apoptosis in retinoic acid-differentiated SH-SY5Y human neuroblastoma cells

The enhancement of cell viability by Dimebon to RA-differentiated SH-SY5Y cells raised the question of whether Dimebon can inhibit apoptosis induced by serum-deprivation. The effects of RA in SH-SY5Y are well documented. These include an attenuation of proliferation rate and extension of neuritic processes [43]. RA treatment induces the expression of TrkB in SH-SY5Y cells, making them responsive to BDNF [44]. Subsequent BDNF treatment enhances the differentiating effect of RA [45] and promotes short-term survival of RA-differentiated SH-SY5Y cells [46]. When RA-treated cells are grown in a serum-free medium, cell

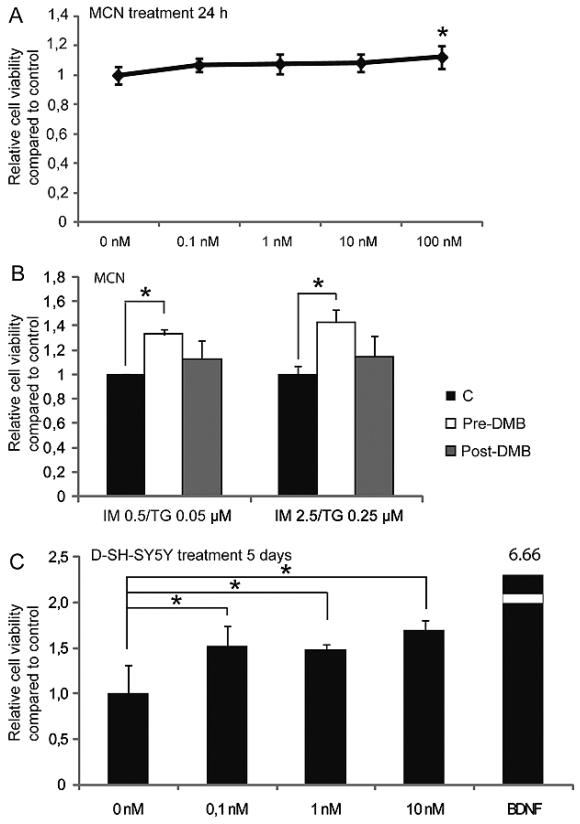

Fig. 5. Analysis cellular succinate dehydrogenase activity of Dimebon (DMB) treated MCN and differentiated SH-SY5Y cells. A) MTT assay of MCN pretreated with Dimebon at indicated concentration for 24 h (mean $\pm$ SD, *significance at $P < 0.05$ by Kruskal Wallis one-way ANOVA by ranks and Conover Post Hoc test, $N = 4$). B) MTT assay of MCN treated with Dimebon either before (pre-DMB) or after (post-DMB) stress. Pretreatment was carried out for 24 h with 10 nM Dimebon and post-treatment 48 h with 10 nM Dimebon. After pretreatment with Dimebon or vehicle cells were exposed to ionomycin (IM)/thapsigargin (TG) (0.5/0.05 μM, or 2.5/0.25 μM) stress for 5 min in Locke buffer. Cells were then allowed to grow in normal medium for another 48 h with (pre-DMB and post-DMB) or without Dimebon 10 nM before MTT measurement. Data was analyzed using Kruskal Wallis one-way ANOVA by ranks and Conover Post Hoc test (mean $\pm$ SD, $N = 4$). C) MTT assay of serum deprived differentiated SH-SY5Y cells. SH-SY5Y cells were differentiated with 10 μM retinoic acid for 5 days, reseeded to Matrigel^{TM} coated 24-well plates and incubated for 24 h for attachment. Cells were then incubated in serum-free medium supplemented with Dimebon (0.1, 1, 10 nM) for 5 days before measurement of MTT assay. BDNF (25 ng/ml) was used as positive control. Data was analyzed using Kruskal Wallis one-way ANOVA by ranks and Conover Post Hoc test (mean $\pm$ SD, *significant at $P < 0.05$ relative to vehicle treated cells, $N = 3$).

survival is dependent on the presence of BDNF. Removal of this neurotrophin induces S-phase entry and apoptotic cell death [47]. In order to study the effect of Dimebon on apoptosis, SH-SY5Y cells were differ-

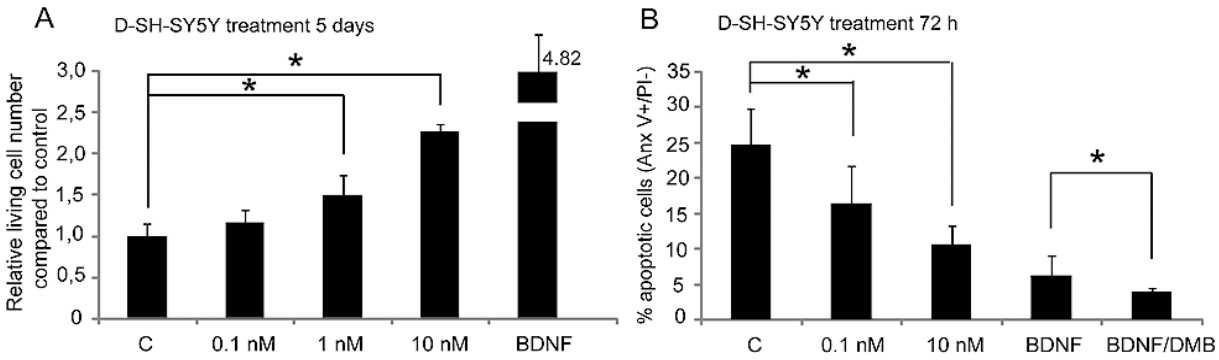

Fig. 6. Analysis of the capacity of Dimebon to inhibit apoptosis in differentiated SH-SY5Y cells (D-SH-SY5Y). A) Surviving rate of Dimebon treated D-SH-SY5Y cells. SH-SY5Y cells were differentiated for five days with RA (10 μM), reseeded on Matrigel^{TM} coated plates. After attachment, cells were subjected to serum-free media supplemented with Dimebon or BDNF (25 ng/ml) and incubated for 5 days. Cell samples were harvested and stained with trypan blue. Living cells were counted in counting chamber (mean $\pm$ SD, *significant at $P < 0.05$ by Kruskal Wallis one-way ANOVA by ranks and Conover Post Hoc test, $N = 4$). B) FACS analysis of apoptotic cells. Six-days RA-differentiated cells were subjected directly to serum free-media supplemented with Dimebon or BDNF (5 ng/ml), or mixture of BDNF (5 ng/ml) and Dimebon (10 nM). Cells were incubated for 72 h and harvested and stained for Annexin V/PI, thereafter subjected to FACS analysis (mean $\pm$ SD, *significant at $P < 0.05$). Dimebon treatments were compared to vehicle treatment using Kruskal Wallis one-way ANOVA by ranks and Conover Post Hoc test. BDNF and BDNF/DMB treatment was compared using Student's t-test (*significant at $P < 0.05$, $N = 7$).

entiated with 10 μM RA for 5 days. After being reseeded on Matrigel^{TM} coated plates and grown for another 5 days in the presence of Dimebon (0.1–10 nM) or BDNF (25 ng/ml), cells were harvested and living cells were counted. Dimebon significantly increased the number of living cells counted after trypan blue staining (Fig. 6A). The experiment was repeated at 72 h with similar outcome (data not shown). In another experiment, at day 5 of RA treatment, cells were directly subjected to serum withdrawal for 72 h with the addition of either Dimebon (0.1 or 10 nM) or low concentration of BDNF (5 ng/ml), or a combination of BDNF (5 ng/ml) and Dimebon (10 nM). Cell death was analyzed by flow cytometry of annexin V/PI stained cells. The number of apoptotic cells (annexin V+/PI−) decreased significantly in the presence of Dimebon (Fig. 6B). Interestingly, a combination of BDNF and Dimebon exhibited an additive effect of apoptosis inhibition (Fig. 6B).

DISCUSSION

This study is the first to report that nM concentrations of Dimebon improve mitochondrial function both under stress and non-stress conditions. We investigated mitochondrial function by monitoring $\Delta\Psi$m, ATP levels, mtDNA copy number, cellular succinate dehydrogenase activity, and cell apoptosis upon Dimebon treatment in MCN and differentiated SH-SY5Y cells. Previous findings demonstrating effects on mitochondria at μM concentrations may be due to different investigation models and measures [24]. In the present study, a 24 h treatment with 0.1–10 nM Dimebon significantly increased $\Delta\Psi$m (TMRM staining) in both MCN and SH-SY5Y cells. After 72 h treatment, the effect on $\Delta\Psi$m was even more pronounced when measured in MCN. The hyperpolarization of $\Delta\Psi$m was accompanied by increased cellular ATP levels at 72 h. Increased $\Delta\Psi$m and ATP levels could potentially reflect mitochondrial biogenesis induced by Dimebon. However, we found that the mtDNA copy number was not increased in MCN exposed for Dimebon up to 5 days. These data indicates that mitochondrial biogenesis is not induced by Dimebon under these conditions and that the increase in $\Delta\Psi$m and ATP levels instead reflects actual effects of Dimebon on mitochondrial function. Increased ATP production is normally linked to a decrease in $\Delta\Psi$m since the energy in the $\Delta\Psi$m is used when protons flux back into the mitochondrial matrix via the F_1ATPase. In the case of uncoupling (with CCCP for example; see Fig. 1F) protons are carried into the matrix over the inner membrane; thus, the $\Delta\Psi$m is lost and ATP cannot be produced. One possible mechanism is that Dimebon may act as an anti-uncoupling agent decreasing proton leakage over the inner membrane, resulting in enhanced $\Delta\Psi$m and ATP production. An example of a known anti-uncoupling agent is albumin, which binds natural uncouplers like free fatty acids [48]. In a study by Ward and colleagues, it was shown that hyperpolarization of $\Delta\Psi$m is associated with increased glucose uptake, NADPH availability, ATP levels, and survival responses during excitotoxic

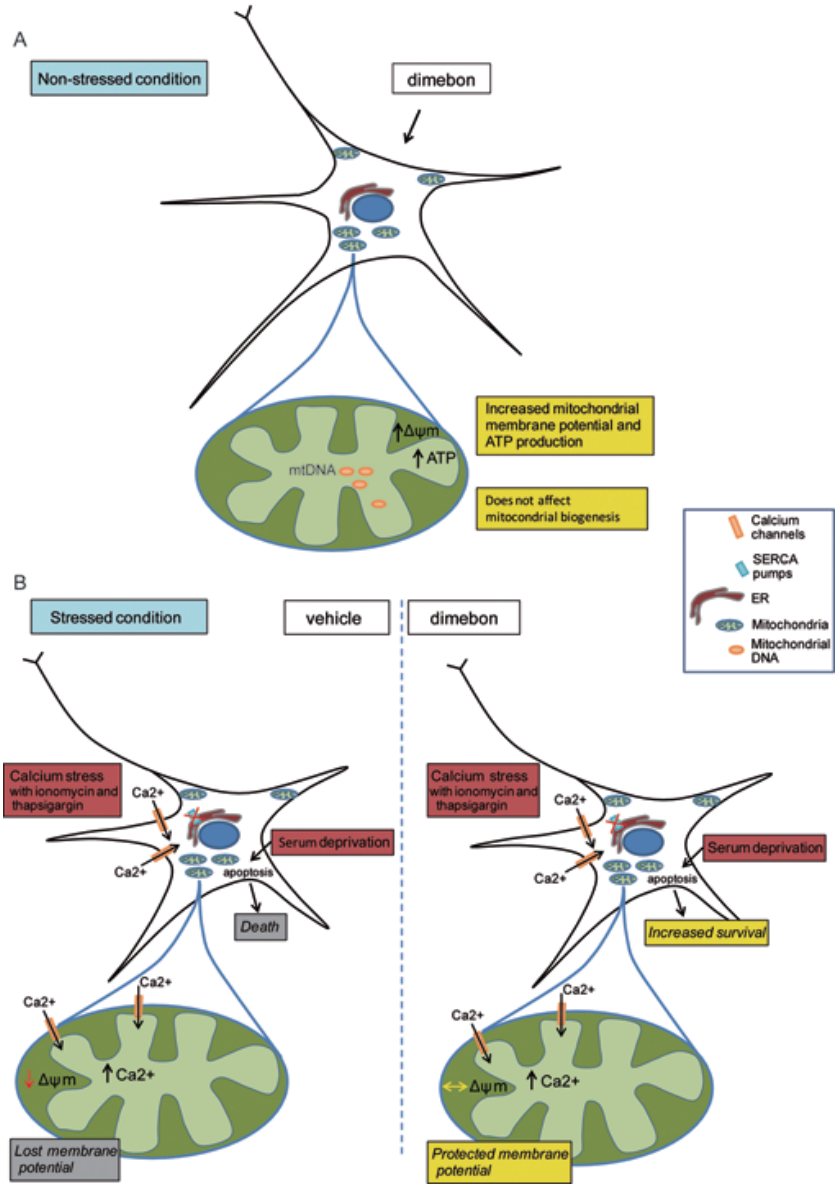

Fig. 7. Illustration of the effect of Dimebon on mitochondria functions. A) Non-stressed conditions: Dimebon increases mitochondria membrane potential ($\Delta\Psi$m) and ATP production in MCN and in differentiated SH-SY5Y cells. Dimebon does not affect mtDNA copy number suggesting an improvement of the function of existing mitochondria. B) Stressed conditions: Dimebon protects MCN and differentiated SH-SY5Y cells from calcium ionophore stress (ionomycin and thapsigargin) by strengthening the capacity of the mitochondria to cope with elevated intracellular calcium concentrations. Cells treated with Dimebon maintained their $\Delta\Psi$m while control neurons rapidly lost their $\Delta\Psi$m. During serum deprivation, Dimebon treatment increases the neuronal survival rate as compared to vehicle treated neurons.

injury. Neurons that displayed a more pronounced hyperpolarization of $\Delta\Psi$m after glutamate treatment survived longer [36]. It appears that hyperpolarization of $\Delta\Psi$m accompanied by elevated ATP production may be neuroprotective.

The data from non-stressed cells showing effects of Dimebon treatment on mitochondrial function were supported by experiments performed under stress conditions. Primary MCN pre-treated with 1 nM Dimebon for 24 h, then exposed to 5 μM ionomycin and 0.5 μM thapsigargin, kept their $\Delta\Psi$m during the 10 min incubation period. In contrast, primary MCN exposed to 5 μM ionomycin and 0.5 μM thapsigargin, in the absence of Dimebon, were depolarized and rapidly lost their

$\Delta\Psi$m. The combination of ionomycin (which brings in calcium from the outside of the cell) and thapsigargin (which blocks ER-calcium uptake) puts pressure on mitochondria to buffer cytosolic calcium. Obviously, Dimebon-treated cells resist this type of stress better than control cells; and Dimebon treatment may directly or indirectly keep the mitochondria in a polarized state. Interestingly, this protective effect appeared only under mild stress. When cells were subjected to severe calcium stress (ionomycin 10 μM/thapsigargin 1 μM), Dimebon treatment showed no protection from loss of $\Delta\Psi$m (Fig. 4D), implying that the capacity of mitochondria to buffer calcium can be strengthened by Dimebon but only to a certain extent. It was previously reported that Dimebon binds to L-type calcium channels and can block potential-dependent calcium entry into neurons [29]. This mode of action of Dimebon was therefore suggested to be neuroprotective. In our model system, we used the ionophore, ionomycin, to induce elevation of intracellular calcium concentrations in a calcium receptor independent manner. Therefore, the effect of Dimebon on $\Delta\Psi$m detected here is probably due to mechanisms other than blockage of plasma membrane calcium channels. Our data also show that Dimebon protects against loss of cell viability, assessed by the MTT assay, when added 24 h before low doses of ionomycin/thapsigargin (Fig. 5B). However, when Dimebon was added after the exposure to ionomycin/thapsigargin, no protection was detected. Thus, the results of this recovery assay suggest that Dimebon exerts its protective effect before and during stress; and that Dimebon cannot rescue cells once the cell death program has been triggered.

The neuroprotective effect of Dimebon was further investigated in RA-differentiated SH-SY5Y cells. In this model, apoptosis was induced by serum withdrawal [47]. The presence of Dimebon during a five-day treatment in serum-free medium markedly increased the cell viability assessed by the MTT assay or trypan blue staining, although the degree of protection was even higher in BDNF treated cells. The number of apoptotic cells detected by FACS analysis of AnnexinV/propidium iodide stained cells was lower in Dimebon treated cells (72 h). Interestingly, the combination of Dimebon and BDNF reduced the number of apoptotic cells as compared to BDNF alone, suggesting a synergistic effect of the two compounds. Higher ATP production could be noticed after 24 h of Dimebon treatment in serum-withdrawal stressed cells confirming the effect of Dimebon on mitochondrial function obtained in primary MCN cells.

In conclusion, the present study demonstrated that Dimebon affects mitochondrial function both in the absence and presence of stress, and that Dimebon-treated cells are partially protected to maintain cell viability (Fig. 7). Since no changes in the copy number of mtDNA were detected, it is suggested that Dimebon improves the function of existing mitochondria. Mitochondria are abundant in synaptic terminals and crucial for synaptic function, therefore Dimebon could potentially help to maintain neuronal communication.

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