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Effects of dexamipexole on brain mitochondrial conductances and cellular bioenergetic efficiency

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Abstract

Cellular stress or injury can result in mitochondrial dysfunction, which has been linked to many chronic neurological disorders including amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD). Stressed and dysfunctional mitochondria exhibit an increase in large conductance mitochondrial membrane currents and a decrease in bioenergetic efficiency. Inefficient energy production puts cells, and particularly neurons, at risk of death when energy demands exceed cellular energy production. Here we show that the candidate ALS drug dexamipexole (DEX; KNS-760704; ((6R)-4,5,6,7-tetrahydro-N6-propyl-2,6-benzothiazole-diamine) and cyclosporine A (CSA) inhibited increases in ion conductance in whole rat brain-derived mitochondria induced by calcium or treatment with a proteasome inhibitor, although only CSA inhibited calcium-induced permeability transition in liver-derived mitochondria. In several cell lines, including cortical neurons in culture, DEX significantly decreased oxygen consumption while maintaining or increasing production of adenosine triphosphate (ATP). DEX also normalized the metabolic profile of injured cells and was protective against the cytotoxic effects of proteasome inhibition. These data indicate that DEX increases the efficiency of oxidative phosphorylation, possibly by inhibition of a CSA-sensitive mitochondrial conductance.

Keywords

leak conductance; mitochondria; bioenergetics; neural metabolism; patch clamp electrophysiology

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CONFLICT OF INTEREST STATEMENT Steven I. Dworetzky, Jamie E. Mangold, Armando P. Signore, and Ulrike DeMarco are employees of Knopp Biosciences, LLC, and all own equity in the company. Valentin K. Gribkoff and Damon R. Demady are former employees of Knopp Neurosciences; Damon R. Demady has a continuing equity interest in the company. Elizabeth A. Jonas is the recipient of a research grant from Knopp Neurosciences, but otherwise has no financial interest in the sponsoring company or in the drug dexamipexole. Laura Bonnani, Marco Onofri, Astrid Thomas, Silvio Sacchetti, Kambiz N. Alavian, Ping Zhang, Hongmei Li, Panah Nabili, Emma Lazrove and Peter J.S. Smith have no financial interest in Knopp Neuroscience or in the drug dexamipexole.

1. Introduction

Mitochondrial dysfunction has been implicated in ALS and other chronic neurodegenerative disorders, including changes in the structural integrity of mitochondria, disruption of energy metabolism and abnormal calcium (Ca^{2+}) buffering (Beal, 2007; Dodson and Guo, 2007; Jordan et al., 2003; Kawamata and Manfredi, 2010; Pedrini et al., 2010; Perry et al., 2010; Sasaki and Iwata, 1996; Swerdlow et al., 1998). This suggests that when critical proportions of mitochondria in specific neuronal populations become dysfunctional, neurons are put at risk. Necessary functions are compromised and death may result if ATP synthesis cannot match cellular energy requirements (Hinerfeld et al., 2004; Nicholls, 2008). In this study we present evidence of enhancement of the efficiency of oxidative phosphorylation by the candidate ALS drug dextramipexole (DEX; KNS-760704; (6R)-4,5,6,7-tetrahydro-N6-propyl-2,6-benzothiazole-diamine) (Gribkoff and Bozik, 2008). DEX is a neuroprotective drug previously suggested to slow ALS disease progression in an open label clinical trial (Wang et al., 2008a). It is safe and well-tolerated at doses that should produce pharmacodynamically effective concentrations in the CNS (Bozik et al.), and it demonstrates unprecedented preliminary evidence of drug activity, including reduction of functional decline and decreased mortality in a recent Phase 2 study in subjects with ALS (Bozik, 2009).

DEX is the non-dopaminergic R(+) enantiomer of the dopamine agonist and Parkinson's disease therapeutic pramipexole (Mirapex®; (6S)-4,5,6,7-tetrahydro-N6-propyl-2,6-benzothiazole-diamine) (Gribkoff and Bozik, 2008). Pramipexole is neuroprotective by a non-dopaminergic mechanism in *in vitro* and *in vivo* models of cell death and neurodegenerative diseases (NDDs), but only at higher concentrations (10 μM) than would be tolerated in humans (Abramova et al., 2002; Ferrari-Toninelli et al., 2010; Iravani et al., 2006; Le et al., 2000). DEX is equally protective and is tolerated at clinical doses which allow its use as a neuroprotective drug (Bozik et al.). Both enantiomers are believed to exert their neuroprotective potential at the level of the mitochondrion (Danzeisen et al., 2006; Ferrari-Toninelli et al., 2010), but their mitochondrial effects have not been well characterized..

Mitochondria produce adenosine triphosphate (ATP) by oxidative phosphorylation. Membrane currents in mitochondria can reduce the efficiency of this process by uncoupling the electron transport system from oxidative phosphorylation. Mitochondrial membrane ion channels participate in the initiation of cell death in conditions such as neuronal trauma and hypoxia/ischemia (Bernardi and Rasola, 2007; Bonanni et al., 2006; Dejean et al., 2005; Jonas, 2009; Miyawaki et al., 2008), and may play a major role in controlling the daily metabolic health of cells by governing the efficiency of oxidative phosphorylation (Lemasters, 2007; Mannella and Kinnally, 2008; Nowikovsky et al., 2009). We have described recently that the anti-apoptotic protein Bcl-xL regulates mitochondrial membrane potential by decreasing an inner membrane leak conductance associated with the ATP synthase (Alavian, 2011; Chen, 2011). We find that these Bcl-xL-associated changes in membrane properties directly increase bioenergetic efficiency of neurons and enhance cell survival (Alavian, 2011). In dysfunctional neuronal mitochondria aberrant membrane currents may contribute to the pathology associated with chronic NDDs such as ALS (Nicholls, 2008). Recently, pramipexole was shown to inhibit membrane currents induced by high calcium (Ca^{2+}) concentrations, a mitochondrial stressor, in rat liver mitoplasts (Sayeed et al., 2006). In the current study we found that DEX and cyclosporine A (CSA) inhibited stress-induced membrane currents in whole brain-derived mitochondria. Profiling the bioenergetics of neurons and other cultured cells, DEX decreased oxygen consumption rates while maintaining or enhancing ATP levels; therefore inhibition of a pathological

mitochondrial conductance may produce the observed enhancements of bioenergetic efficiency that underlies the cellular protective effects of DEX.

2. RESULTS

2.1 PSI pre-treatment of rats produced currents in isolated brain mitochondria that were inhibited by DEX and CSA

To model changes in aberrant protein accumulation that may lead to chronic neuronal mitochondrial dysfunction we exposed rats *in vivo* to a proteasome inhibitor (carbobenzoxyl-Ile-Glu(O-t-butyl)-Ala-leucinal; PSI) (McNaught et al., 2004). We previously developed a technique to patch clamp the outer membrane of mitochondria inside living neurons (Jonas et al., 1997). These techniques, used here, were later modified to record from the outer membrane of isolated whole mitochondria (Bonanni et al., 2006; Miyawaki et al., 2008), and are similar to techniques described by others (Kinnally et al., 1987; Kinnally et al., 2006; Lohret et al., 1997; Pavlov et al., 2001). Studies of ischemic injured neurons have shown increased levels of intermediate- (200-750 pS) and large- (>750 pS) conductance channel activity in whole-organelle recordings of isolated mitochondria (Bonanni et al., 2006; Miyawaki et al., 2008). Therefore patch clamp recordings were made from whole mitochondria isolated from a subcortical brain fraction after repeated dosing of rats with the proteasome inhibitor PSI (PSI-mitochondria, see Experimental Procedure). Recordings of PSI-mitochondria incubated in the absence of added Ca^{2+} exhibited intermediate- and large-conductance channel activity at a significantly higher frequency than recordings from control mitochondria (Fig. 1A).

DEX inhibited intermediate- and large-conductance channel activity of PSI-mitochondria; the inhibition was concentration-dependent and reversible (Fig. 1B,1C,1D). The decrease in the mean open probability (NP_o) of discrete single channel ion currents had an estimated EC_{50} of 98nM (Fig. 1E). The full effect of the drug could take up to several minutes, and washout followed an approximately equal time course, although in some cases the onset and washout were quite rapid. The concentration-response curve (CRC) was shallow, with a Hill slope <1; full inhibition of PSI-induced aberrant currents (EC_{100}) required DEX concentrations >10 μM , which we surmise may have consequences for the potency of the drug in cellular systems (see below).

Similar to pramipexole (Sayeed et al., 2006), DEX may inhibit the pore-forming protein complex known as the mitochondrial permeability transition pore (mPTP) (Bernardi, 1992; Sayeed et al., 2006). The mPTP participates in the initiation of some forms of cell death, although its roles in long term changes in mitochondrial function are likely to be different from its roles in more acute phenomena (Crompton, 1999; Nicholls and Chalmers, 2004). Addition of Ca^{2+} to isolated mitochondria activates the mPTP (see below); cyclosporine A (CSA) specifically inhibits it (Szabo and Zoratti, 1991; Szabo et al., 1992). CSA effectively blocked channel activity in PSI-mitochondria and significantly decreased the peak membrane conductance (Fig. 2A). Currents sensitive to DEX were equally sensitive to CSA after DEX washout. DEX-sensitive currents in PSI-mitochondria therefore share features with the mPTP, such as inhibition by CSA.

2.2 DEX inhibited outer membrane ion channel conductances in $[\text{Ca}^{2+}]$ -treated brain-derived mitochondria

To determine if DEX also modulates mitochondrial ion channel activity during Ca^{2+} -induced mitochondrial stress, an initiator of mPTP activity, isolated control rat brain mitochondria were challenged with high $[\text{Ca}^{2+}]$ or with high $[\text{Ca}^{2+}]$ followed by DEX. Addition of 100 μM Ca^{2+} resulted in an increase in intermediate- and large-conductance

currents recorded under whole-organelle patch clamp. DEX (2 or 20 μ M, in 100 μ M Ca²⁺ (Fig. 2B), effectively decreased these Ca²⁺-induced mitochondrial membrane currents. These data demonstrate that DEX also potently inhibited Ca²⁺-induced ion conductance in mitochondria.

2.3 DEX did not inhibit Ca²⁺-induced mitochondrial transition in liver-derived mitochondria

Unlike a previous report using pramipexole (Sayeed et al., 2006), we found no effect of DEX (or pramipexole) on optically recorded permeability transition in liver mitochondria, following addition of high (50-100 μ M) external Ca²⁺. In these experiments, known inhibitors of permeability transition, including CSA and lithium chloride (LiCl) (Azzolin et al.; Shalbuyeva et al., 2007), were effective at blocking Ca²⁺-induced mitochondrial permeability transition (Fig. 2C). In brain mitochondria, therefore, DEX-sensitive currents may have another role apart from permeability transition in mitochondrial function, such as the regulation of cellular metabolism.

2.4 DEX enhanced metabolic efficiency of cultured cells

We had recently reported that the anti-apoptotic protein Bcl-xL regulates inner membrane properties and cellular bioenergetics (Alavian, 2011; Chen, 2011). We therefore undertook to determine if DEX altered metabolic function in neurons and other cell types. Unlike CSA, DEX has no known cellular effects other than its mitochondrial modulation, and is nontoxic even at high concentrations *in vitro*. Consistent with a role in metabolism, DEX (10 μ M) increased ATP levels in cultured hippocampal neurons (DIV 14) by 11% compared to vehicle-treated cultures (Fig. 3A). Under basal conditions, mitochondrial oxygen uptake contributes both to the production of ATP by oxidative phosphorylation and to counteracting inner membrane 'leak' which decreases coupling between the electron transport system and ATP production (Andrews et al., 2005; Brand, 2005). To determine if oxygen uptake was altered by DEX, oxygen flux was measured from single hippocampal neurons using a self-referencing oxygen-sensitive electrode (Alavian, 2011; Gleichmann et al., 2009; Land et al., 1999) (Fig. 3B, 3C, 3D). Single neuron measurements target the cell of interest, and self-referencing electrodes improve the signal:noise ratio compared to fluorescence-based techniques (Gerencser et al., 2009). The average oxygen flux of control neurons was stable over 5 min. Acute application of 10 μ M DEX lowered average oxygen flux by ~16% to a new stable level after 1-5 min (Fig. 3C, 3D). These data, in conjunction with the increased ATP levels in identically treated neurons, suggest that DEX-treated neurons couple ATP production more efficiently to oxygen uptake.

To further study the effects of DEX on neurons, oxygen consumption rate (OCR) was studied in digitonin-permeabilized primary cortical neurons (9 DIV) using a Seahorse® multi-well flux analyzer. When permeabilized neurons were incubated in control medium containing the complex I inhibitor rotenone (Fig. 3Ei), acute application of DEX (30 μ M) did not affect the already low levels of basal respiration (Fig. 3Eii). In control cells, addition of the complex II substrate succinate produced a significant increase in OCR (Fig. 3Eiii); following a pulse of ADP (see Methods), OCR was further increased (Fig. 3Eiv). In contrast, however, in DEX-treated cells, the increase in OCR produced by succinate and following the ADP pulse was less than in control cells (Fig. 3Eiii, 3Eiv), consistent with the single neuron oxygen recordings. In other experiments, when DEX was applied to digitonin-treated cortical neurons 1 hr. prior to the onset of recording, cells likewise had lower OCR during succinate incubation, and significantly lower OCR following an ADP pulse, relative to controls (Fig. 3F). To control for possible changes in cell number, mitochondrial biomass, or mitochondrial integrity after DEX treatment, measurements of the initial slope of ATP synthesis following an ADP pulse, maximal ATP levels, and citrate synthase activity were studied and were found to be unchanged (Fig. 3G, 3H, 3I).

Incubation of SH-SY5Y neuroblastoma cells in DEX (1-100 μ M; 24 hr), also significantly increased ATP levels relative to control values, with maximal group increases of ~18% (Fig. 4). DEX (30 μ M) also modestly decreased basal OCR in these cells. To determine whether the changes in ATP or oxygen consumption were related to an increase in glycolysis, SH-SY5Y cells were incubated in galactose, a sugar that exclusively requires mitochondrial (rather than glycolytic) metabolism to produce ATP. Cells treated with DEX in galactose-containing medium had similar DEX-induced increases in ATP levels, suggesting that the effects of DEX on cellular ATP levels were mediated via effects on mitochondrial metabolism (Fig. 4).

To determine if the protective properties of DEX on PSI-induced mitochondrial membrane currents were linked to protective effects on metabolism, we used two cell lines to examine the effects of PSI and DEX on bioenergetics and cell survival. To measure effects on bioenergetics in the absence of cell death, a CRC for cell death by PSI was established in C2C12 cells (Fig. 5A), and a PSI concentration that was near the inflection point of the killing curve (30nM) was used to stress but not kill C2C12 myoblasts. Under these conditions, neither ATP levels nor cell viability (Fig. 5B) were significantly affected in any group (PSI, DEX or the combination, relative to control). In PSI, however, although C2C12 cells did not display a significant change in OCR, they did display a very significant increase in ECAR (Fig. 5C), indicating a higher relative level of glycolytic activity to mitochondrial metabolism following low-level PSI treatment. In contrast, cells incubated only in DEX displayed a significant decrease in OCR, which was not accompanied by a change in ECAR (Fig. 5C), indicative of an increase in basal mitochondrial efficiency of DEX-treated cells. Co-incubation with DEX and PSI resulted in a profile identical to DEX alone (Fig. 5C), suggesting that DEX blocked the PSI-induced bioenergetic changes. This normalization of respiratory profiles may be protective under conditions where cell survival is at risk. When SH-SY5Y cells were exposed to lethal levels of PSI, pretreatment with DEX significantly reduced cell death (Fig. 6).

3. DISCUSSION

In the current study we found that DEX and CSA potently and effectively inhibited ion channel activity evoked by Ca^{2+} or PSI pre-treatment in brain-derived mitochondria. We have previously shown that intermediate- and large-conductance channel activity is present at higher frequency in mitochondria isolated from affected brain regions of rodents exposed *in vivo* to global ischemic injury (Bonanni et al., 2006; Miyawaki et al., 2008). These previous works suggested that the ischemia-induced channel is comprised of a complex of proteins including an inner membrane-localized divalent-sensitive component (possibly the mPTP) and outer membrane proteins including a pro-apoptotic version of Bcl-xL, and the voltage-dependent anion channel (VDAC). Therefore the activity inhibited by DEX in our studies may reflect an ion conductance associated with both the inner and outer mitochondrial membranes. The current work does not localize the site(s) of action of DEX to any particular membrane or component, although effects of DEX on bioenergetic properties of the cells, as in our studies with Bcl-xL (Alavian, 2011; Chen, 2011), suggest at least a partial inner membrane localization of the DEX effects.

Increases or unchanged ATP levels were observed in neurons and other cells after exposure to DEX, while basal OCR levels were significantly reduced. In addition, DEX normalized ECAR levels and lowered OCR in PSI-treated C2C12 cells, suggesting that it ameliorated injury-induced mitochondrial stress. The results of experiments in which DEX was administered in the setting of PSI exposure suggest that DEX restored a 'normal' bioenergetic phenotype in compromised cells. The timeframe of most chronic NDDs is long,

and small changes in mitochondrial bioenergetic modulation may have meaningful effects over the course of the years during which neurons are at risk.

While both CSA and DEX similarly inhibited conductance in these preparations from brain-derived mitochondria at submaximal concentrations, only CSA was able to block transition measured optically in liver mitochondria. This suggests either that CSA and DEX have differential effects, at least in liver mitochondria, or it may simply mean that there is a difference in the degree to which these agents can maximally inhibit the ion channel complex (not examined here for CSA). Nevertheless, we did not observe any consistent effect of either DEX or pramipexole on mitochondrial transition in liver mitochondria.

DEX inhibited PSI-induced cell death in a concentration-dependent manner when tested in SH-SY5Y neuroblastoma cells. In our study, as in previous studies (Abramova et al., 2002; Cassarino et al., 1998; Danzeisen et al., 2006), effective cytoprotective concentrations of DEX (or pramipexole in most previous studies), and concentrations required for significant effects on metabolic efficiency in cells, were significantly higher than the EC₅₀ for mitochondrial current inhibition. Because of the shallow slopes of this CRC, concentrations of the drug producing complete elimination of these aberrant currents (an EC₁₀₀), correspond to 10-30 μM or even higher concentrations, and this range may be required for enhanced bioenergetic efficiency and significant cytoprotection.

Mitochondrial dysfunction has long been implicated in the pathogenesis of neurodegenerative disease (Baron et al., 2007; Beal, 2007; Dupuis et al., 2004). Proteasomal dysfunction is a potential mechanism for accumulation of undegraded proteins in mitochondria (Naiki and Nagai, 2009), and this has been suggested as one of many potentially stressful events coupled to the onset of neurodegeneration in diseases with diverse clinical presentation including ALS, Alzheimer's disease, and PD (Bandopadhyay and de Belleruche, 2010; Hanger et al., 2009; Shaw and Valentine, 2007). We have demonstrated that mitochondrial stress induced by either PSI or high Ca²⁺ resulted in increased membrane current in mitochondrial membranes, and produced a shift in the bioenergetic profile of cultured cells. This membrane current likely constitutes a leak conductance of the inner mitochondrial membrane which results in a reduction in bioenergetic efficiency. If this inefficiency cannot be countered by sufficient compensatory increases in metabolism of available substrate and OCR, or by shifting to glycolysis, it may stress energy supplies, resulting in increased risk of cellular and particularly neuronal death when energy demand exceeds supply. These data suggest that DEX reduces this risk by inhibiting aberrant mitochondrial leak conductance, resulting in neuroprotection and, quite possibly, therapeutic benefit in NDDs. Recently, in an initial carefully controlled Phase 2 study in subjects with ALS, DEX-treated subjects exhibited a lower rate of decline in measures of motor function, and suffered a lower level of mortality over the course of the study (Cudkowicz et al., 2011). These initial clinical results suggest that the cellular effects of DEX have important consequences in ALS. The results from the current study represent a first step in understanding the specific mechanism by which DEX exerts its neuroprotective actions.

4. EXPERIMENTAL PROCEDURES

4.1 Isolation of Brain-derived Mitochondria

Standard techniques were adapted for isolating brain-derived mitochondria (Brown et al., 2004). Mitochondria were stored in isolation buffer (IB: 250mM sucrose, 20mM HEPES, pH 7.2, 1mM EDTA, and 0.5% BSA) at -80 °C. For mitochondria treated with a proteasome inhibitor, male Sprague-Dawley rats (age 6 weeks, ~200 gm, 3-6 rats/group/condition, at least 2 different preparations for each experimental paradigm) were injected

with the ubiquitin proteasome inhibitor Z-Ile-Glu(OtBu)-Ala-Leu-al (PSI; Peptides International Inc, Kentucky, USA; s.c. every other day for 1 or 2 weeks; 6.0mg/kg PSI in DMSO, or with DMSO, 200 μ L/rat) (McNaught et al., 2004), and mitochondria isolated and stored as above.

4.2 Dexpramipexole

DEX ((6R)-4,5,6,7-tetrahydro-N6-propyl-2,6-benzothiazole-diamine) was prepared by contract with Albany Molecular Research Inc. (AMRI), and was determined to have chemical purity >99.99%, and enantiomeric (R+) purity relative to pramipexole of >99.95%.

4.3 Electrophysiological recording from rat brain-derived mitochondria

Patch-clamp recordings were made from frozen de-energized mitochondria in intracellular solution (120mM potassium chloride, 8mM NaCl, 0.5mM EGTA, 10mM HEPES, pH adjusted to 7.3) at room temperature (22–25°C). Pipettes (80–100M Ω) were filled with the same solution; recordings were made using a Heka 8 amplifier with V_m held at positive voltages up to + 180 mV. Data were recorded at 20 kHz and filtered at 500–1000 Hz. DEX (Knopp Neurosciences, Pittsburgh, PA; purity established to be >99.95%) was prepared as a 10mM aqueous stock and diluted in intracellular recording solution; cyclosporine A (CSA; Sigma, St. Louis, MO) was prepared as an 8mM stock solution in EtOH and diluted in buffer. Reagents were rapidly perfused into the recording chamber. Peak membrane conductance was measured as the peak amount of current (pA) from zero, converted to pS by assuming a linear I-V relationship. Discrete channel conductances were sorted by activity (% occurrence of a conductance level per unit time measured using pCLAMP 10 software, Molecular Devices, Sunnyvale, CA); levels defined as closed (no current), small (<200pS), intermediate (>200pS and <750pS) and large (>750pS) (Bonanni et al., 2006). Mean open time (NPO; open time of discrete single channels times the apparent number of single channels in the patch) was used to assess drug effect.

4.4 Preparation and Maintenance of Cultured Cells

Primary cultures of cortical or hippocampal neurons were prepared as described previously (Li et al., 2008; Wang et al., 2008b), plated at 8×10^5 cells/well for Seahorse XF24 experiments and grown in Neurobasal A supplemented with B27, 1mM glutamine and penicillin/streptomycin for 4 days *in vitro* (DIV). On DIV 7, media without antibiotics was substituted. The cultures were used on DIV 9–10.

Undifferentiated SH-SY5Y human neuroblastoma cells (ATCC, Manassas, VA) were maintained in humidified 5% CO in 1:1 Ham's F12 nutrient mixture with Glutamax® 2 and Minimal Essential Medium with L-glutamine (MEM-Alpha), 10% FBS, and 1% penicillin/streptomycin (Gibco® Invitrogen Corp., Carlsbad, CA).

The C2C12 mouse myoblast cell line (ATCC, Manassas, VA) was cultured in Dulbecco's Modified Eagle Medium (DMEM; Gibco®; Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS), 2mM GlutaMax® and penicillin/streptomycin. Cells were maintained in 10% CO₂ humidified at 37°C.

4.5 Cell Viability and Cellular ATP quantitation

Cellular viability was measured using CellTiter-Blue® (Promega Corp., Madison, WI) in black-walled optical imaging multiwell plates, and cellular ATP was measured using the CellTiter-Glo® (Promega Corp., Madison, WI) luminescence assay in opaque-walled multiwell plates according to the manufacturer's protocol.

4.6 Cell culture oxygen consumption experiments

Oxygen uptake was measured from single hippocampal neurons grown in culture (DIV 14-17) (Li et al., 2008). A 2-4 μm (tip diameter) oxygen-sensing electrode oscillated 10 μm close-to and away-from the cell every 3 sec. The difference in current detected at the two positions was translated into oxygen flux (Gleichmann et al., 2009; Land et al., 1999). DEX (10 μM) or control solution was added after a 5 min. baseline measurement, and flux was measured for >5 min. post-treatment. In these cells ATP was measured by a luciferase assay (details?).

In other experiments OCR and ECAR were measured with a Seahorse® XF24 Flux Analyzer (Seahorse Bioscience, Billerica, MA). For PSI experiments on C2C12 cells, basal values were assessed in non-buffered DMEM Assay Medium without glucose, glutamine or pyruvate, and supplemented with 25mM glucose, 6mM Glutamax®, and 1mM sodium pyruvate.

For primary cortical neurons the assay had 2 modifications: 1) MAS1 buffer (70mM sucrose, 220mM mannitol, 5mM KH_2PO_4 , 5mM MgCl_2 , 2mM HEPES, 1mM EGTA, 0.2% FA-free BSA; pH 7.2; Sigma-Aldrich, St. Louis, MO) was used instead of Assay Media, and 2) 10x concentrated digitonin (10 $\mu\text{g/mL}$), rotenone (100nM) and DEX (30 μM) or control (equivalent volume of water) were added to the cells 45 minutes prior to assay. Succinate and ADP stocks were diluted to 10x concentrations in pre-warmed XF MAS buffer, pH 7.2. Using a Plate Prep Station (Seahorse Bioscience, Billerica, MA), cells were washed with 1 mL buffer and were brought to 607 μL with buffer. DEX or water (68 μL ; 10x concentrated) was added to the wells, and cells incubated at 37°C in a non- CO_2 incubator for 45 minutes to 1 hour.

Raw basal OCR and ECAR values were normalized as a percent of the mean control value for each individual experiment to minimize the between-plate variability. Normalized data from experiments were expressed as mean $\pm$ SEM. The number of measurements (n) is equal to the number of wells at each condition, but in every case all conditions were averaged across multiple plates and multiple cell preparations.

4.7 Statistical analyses and Curve Fitting

For comparisons involving 2 groups, paired or unpaired Student's t-tests (2-tailed) were used. In all figures, *= $p < 0.05$, **= $p < 0.01$, and ***= $p < 0.001$ to denote significance level, and exact p values are provided in the figure legends. For more than 2 groups, one-way or 2-way analyses of variance (ANOVA), or 2-factor MANOVA, were performed; in the case of a significant F-test, the p value is provided in the figure legend, and pre-planned *post hoc* comparisons (Bonferroni-corrected t-tests or Tukey's HSD) were performed and significance levels displayed in figures and exact p values provided as described above. Where possible, p values for tests are presented to 2 significant digits. All statistical analyses were performed using GraphPad Prism 5, InStat (GraphPad Software, La Jolla, CA) or SPSS (IBM Corporation, Somers, NY).

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Abbreviations

CSA	cyclosporine A
DEX	dexpramipexole, (6R)-4,5,6,7-tetrahydro-N6-propyl-2,6-benzothiazole-diamine
ECAR	extracellular acidification rate
OCR	oxygen consumption rate
PSI	Z-Ile-Glu(OtBu)-Ala-Leu-al

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FIGURE 1.

DEX inhibited PSI-induced currents in brain-derived mitochondria. A. Histograms show the % total time that patches displayed specified conductance levels in all recorded traces (n=190 recordings from a total of 10 PSI-exposed sub-cortical mitochondria, n=83 recordings from a total of 9 vehicle (DMSO)-exposed sub-cortical mitochondria). Conductance level frequencies for PSI-dosed mitochondria compared with the comparable level in non-PSI-dosed mitochondria, using 2-tailed unpaired t-tests; p=0.0005 for closed PSI-mitochondria compared to closed control, p=0.0426 for intermediate conductance PSI-mitochondria compared to intermediate control, p=0.0192 for large PSI-mitochondrial conductance compared to large control. In all figures, the specific analyses performed and unadjusted or adjusted p values connoting significance are presented in the figure legend. The general level of significance obtained when comparing 2 groups, including pre-planned *post hoc* comparisons, is indicated on any figure by the number of asterisks above or below the mean value; *= $p < 0.05$, **= $p < 0.01$, ***= $p < 0.0001$.—B. Example of a continuous patch clamp recording from a PSI-mitochondrion before and after bath application of DEX. C. Group data showing peak conductance recorded from mitochondria isolated from subcortex of PSI-injected rats (n=15 mitochondria, except for wash, where n=6 mitochondria). 2-tailed paired t-test, p=0.0352. The wash was not included in the analysis. D. Intermediate-conductance (~500pS) channels recorded from PSI mitochondria before and after DEX and after wash at the indicated concentrations. Holding potential = + 80 mV. Note that in this example many closures reveal sub-conductance states. Sample recordings were obtained at steady-state for each condition. E. Mean inhibitory effect of different concentrations of DEX on NP_o, recorded in brain-derived PSI-mitochondria (n=5 mitochondria for all except 20μM, where n=9). One-way ANOVA, p=.000014; pre-planned *post hoc* comparisons, Bonferroni corrected t-tests, p=0.0455 for 200nM DEX, p=0.00038 for 2μM DEX, p=0.0005 for 20μM DEX; EC₅₀=98nM by logistic fit; Hill slope, nH<1.

FIGURE 2.

CSA and DEX have similar effects on brain mitochondrial currents, but not on permeability transition in liver mitochondria. A. (*Left*) Bar graph of mean level of inhibition of peak conductance by 1 μ M CSA in recordings from PSI-mitochondria (n=7 mitochondria); p=0.0003, paired t-test. (*Right*) Bar graph of the mean inhibition of peak conductance by 200nM DEX in PSI-mitochondria, wash (>5 min.) and 1.0 μ M CSA (n=3 mitochondria). B. (*Left*) Bar chart of the mean effect of 100 μ M Ca²⁺ on peak membrane conductance (in pS) (n=14 mitochondria); p=0.0092, 2-tailed paired t-test. (*Right*) Bar chart of the mean inhibition of peak conductance (in pS) by 20 μ M DEX in the continued presence of Ca²⁺ (n=7 mitochondria); p=0.0094, 2-tailed paired t-test; n=4 for wash; the wash was not included in the analyses. C. Optical absorbance of fresh, respiring rat liver mitochondria (measured respiratory control ratio >5) before and during Ca²⁺-induced permeability transition, and its amelioration by LiCl and CSA, but not DEX. Each point represents the mean $\pm$ SEM for the group at each time point; n=12 wells for each condition.

FIGURE 3.

DEX modulation of cellular bioenergetics. A. Effect of DEX (10 μ M, 12 hr. incubation prior to measurements) on ATP levels, measured in a luciferase assay in cultured hippocampal neurons (n=21 wells each condition, 2 independent cultures, p=0.0024, unpaired t-test). B. Image of an oxygen-sensitive electrode placed in position to record oxygen uptake by a single cultured hippocampal neuron. Shown are the recording and self-referencing positions of the electrode. Scale bar with arrow represents 10 μ m; measurement of oxygen level in proximity of neurons occurred ~1-2 μ m from cell surface and the reference point was 10-12 μ m away from cell surface. C. Single self-referencing oxygen electrode recording from a cultured hippocampal neuron after addition of DEX (10 μ M) or an equivalent volume of water to the bath. D. Histogram of group data (mean $\pm$ SEM) for recordings as in C before and after addition of DEX (10 μ M; n=14 neurons) or an equivalent volume of water (n=6 neurons). Unpaired t-test, p = 0.0001. E. Effect of DEX on OCR on rat cortical cultures exposed to digitonin(10 μ g/ml) and rotenone (100nM) (45 min.) followed by succinate and ADP injection. Oxygen consumption rate (OCR) was measured by a Seahorse® flux analyzer. OCR at baseline (i.), 30 μ M DEX (n=10 wells) or vehicle injection (n=10 wells) (ii.), 10mM succinate (iii.), 1mM ADP (iv.) and 10 μ M antimycin A (v.). 10 wells/treatment. F. Group histograms illustrating the effect of DEX on OCR under the indicated conditions relative to control values $\pm$ SEM. For succinate, p=0.1211 (2-tailed t-test). For ADP injection, p=0.0001 (2-tailed t-test). Primary rat cortical cultures, from several separate isolations and multiple independent plates, were pretreated with DEX (30 μ M; n=95 wells) or control medium (n=94 wells) for 1 hr., then incubated with digitonin (10 μ g/ml), rotenone (100nM), and DEX (30 μ M) prior to OCR measurements. G. Initial slope of ATP production following ADP addition in isolated cortical neurons in absence and presence of 30 μ M DEX. Third bar indicates ATP level after antimycin A (AMA) addition at end of experiment. H. Maximal level of ATP production for experiment shown in G. I. Citrate synthase levels in absence and presence of 30 μ M DEX after the flux experiments in F (n=17 wells/treatment arm).

FIGURE 4.

Dexpramipexole enhances mitochondrial metabolism in cells grown in a medium where glucose is replaced with galactose. SH-SY5Y neuroblastoma cells were exposed to the indicated concentrations of dexpramipexole for 24 hr. before measurement of ATP levels by a luciferin-luciferase assay. Data shown represent mean $\pm$ SEM. Statistical analyses: 1-Way-ANOVA, $p=0.0001$ for both normal and galactose medium; pre-planned *post hoc* comparisons (Bonferroni corrected t-tests) for normal medium and galactose medium, respectively, $p=0.0068$ and $p=0.0018$ for 1 μM , $p=0.0013$ and $p=0.0039$ for 3 μM , $p=0.0001$ (galactose) for 10 μM , $p=0.0009$ and $p=0.0001$ for 30 μM , $p=0.0129$ (galactose) for 100 μM dexpramipexole; $n=14-17$ wells/concentration for each substrate).

FIGURE 5.

DEX altered bioenergetic parameters and ATP production in the C2C12 myoblast cell line. A. Cellular viability of C2C12 cells after 18 hr. exposure to PSI at indicated concentrations. Data are presented as a percentage of the vehicle-treated control $\pm$ SEM (n=10 wells at each concentration). A sub-lethal concentration of PSI, 30nM, was used in subsequent experiments to stress C2C12 cells. B. (*Left*) ATP levels (%control) after 18 hr exposure to indicated agents. (2-way-ANOVA; p=0.1763; n=18 wells for each group). (*Right*) cell viability after 18 hr exposure to indicated agents (2-way-ANOVA, p=0.2824). Data are presented as a percentage of the vehicle-treated control $\pm$ SEM (n = 14-17 wells for each group; for all conditions multiple plates and cell platings contributed to all data obtained). C. (*Left*) Oxygen consumption rate (OCR) and (*Right*) extracellular acidification rate (ECAR) of cells exposed to DEX (30 μ M) and/or PSI (30nM), or no drug (control) for 18 hr. (control, black bars, n=27; DEX, n=24; PSI, n=12, from 5 independent multiwell plates, from multiple cell platings). (*p=0.0477; **p=0.0002, blue bars). ***p=0.00015, gray bar; (n=9, OCAR ***p=0.0009, red bars). Data are expressed as a percentage of the corresponding control OCR or ECAR value, and p values represent results of pre-planned *post hoc* comparisons (Tukey HSD) following one-way ANOVA (p=0.001). Data shown represent mean $\pm$ SEM.

FIGURE 6.

Exposure of SH-SY5Y cells to high concentrations of PSI compromises cell viability. Cells were pre-treated with DEX for 24 hr. prior to 24 hr. exposure to PSI at indicated concentrations. Pre-exposure to dexamipexole (DEX) significantly reduced the PSI-mediated cell death (2-factor MANOVA, DEX+150 nM PSI vs. 150 nM PSI, $p=0.001$, DEX+650 nM PSI vs. 650 nM PSI, $p=0.001$; pre-planned *post hoc* Bonferroni-corrected t-tests, 150 nM PSI vs. 150 nM PSI + 30 μ M DEX, $p=0.031$; 150 nM PSI + 100 μ M DEX, $p=0.001$; 650 nM PSI + 100 μ M DEX, $p=0.001$; all other comparisons were not statistically significant). Bar graphs represent normalized mean $\pm$ SEM; $n=41$ -98 wells/condition.