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Mitochondrial permeability transition following calcium overload. - Its role in neuronal cell death and potential as a pharmacological target.

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Mitochondrial permeability transition following calcium overload

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Abstract

There is currently no clinically available drug with neuroprotective properties to limit the evolving cell death following e.g. stroke or traumatic brain injury. The mitochondrial permeability transition (mPT) is a potential pathologic mechanism causing cell death in the CNS. As the name implies, the mPT is defined by a sudden increase in permeability of the inner mitochondrial membrane, whose normal impermeable state is fundamental for the bioenergetic function of mitochondria. The objective of the present study was to characterize the mPT phenomenon in isolated rodent brain mitochondria. Mitochondria serve an important role in cellular calcium homeostasis and buffer transient increases in calcium, but mitochondrial calcium overload is also the prime trigger for mPT. In the present studies, we found that brain mitochondria readily undergo changes attributable to mPT induction such as swelling, loss of membrane potential, uncoupling of oxidative phosphorylation and respiratory inhibition. The mitochondrial generation of reactive oxygen species (ROS) was also increased following mPT, and mitochondria became permeable to NAD(H).

Cyclosporin A (CsA) binds to the mitochondrial protein cyclophilin D (CypD) and can thereby inhibit mPT, an effect that is unrelated to its immunosuppressive action, and mPT in brain mitochondria was found to be highly sensitive to CsA inhibition. CsA has demonstrated prominent neuroprotective properties in several different animal models of neurological disease, and recent experiments subjecting mice lacking CypD to ischemia support the conclusion that the effect of CsA at least in this model is mediated by mPT inhibition. A library of CsA analogs was evaluated for mPT-inhibiting and ROS-reducing properties, and two newly developed non-immunosuppressive CsA analogs were found to be potent inhibitors of mPT already at nanomolar concentrations.

Mitochondria take up free calcium ions but retain them as inactive calcium phosphate complexes in order to prevent mPT. We find that increasing the conductance of potassium increases the pH of the matrix and this enhances the mitochondrial buffering of calcium, probably by increasing the complexation of calcium.

Drugs that inhibit mPT or indirectly prevent mPT by enhancing the mitochondrial complexation of calcium and thereby their resistance to calcium overload may therefore prove to be successful strategies in limiting accidental cell death in the CNS.

Key words: Brain, mitochondria, calcium, cell death, permeability transition, cyclosporin A, ischemia, traumatic brain injury, amyotrophic lateral sclerosis, potassium channels, free radicals

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To my family

Doubt is not a pleasant condition, but certainty is absurd
Voltaire

CONTENTS

ORIGINAL ARTICLES

This thesis is based on the following papers, which are referred to in the text by their respective Roman numerals (I-V).

I. Hansson M. J., Persson T., Friberg H., Keep M. F., Rees A., Wieloch T. and Elmér E. Powerful cyclosporin inhibition of calcium-induced permeability transition in brain mitochondria. *Brain Research* 2003, 960(1-2), 99-111.

II. Hansson M. J., Månsson R., Mattiasson G., Ohlsson J., Karlsson J., Keep M. F. and Elmér E. Brain-derived respiring mitochondria exhibit homogeneous, complete and cyclosporin-sensitive permeability transition. *J Neurochem* 2004, 89(3), 715-29.

III. Hansson M. J., Mattiasson G., Månsson R., Karlsson J., Keep M. F., Waldmeier P., Ruegg U. T., Dumont J. M., Besseghir K. and Elmér E. The nonimmunosuppressive cyclosporin analogs NIM811 and UNIL025 display nanomolar potencies on permeability transition in brain-derived mitochondria. *J Bioenerg Biomembr* 2004, 36(4), 407-13.

IV. Hansson M. J., Månsson R., Morota S., Uchino H., Kallur T., Sumi T., Ishii N., Shimazu M., Keep M. F., Jegorov A. and Elmér E. Calcium-induced generation of reactive oxygen species in brain mitochondria is mediated through permeability transition. (*manuscript*)

V. Hansson M. J., Teilum M., Morota S., Uchino H. and Elmér E. Intramitochondrial pH links modulation of potassium conductance with the mitochondrial permeability transition - implications for preconditioning and tolerance in cerebral ischemia. (*manuscript*)

LIST OF ABBREVIATIONS

5-HD, 5-hydroxydecanoate
AIF, apoptosis inducing factor
ALS, amyotrophic lateral sclerosis
AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
BBB, blood-brain barrier
BSA, bovine serum albumin
CA1, cornu ammonis 1 (anatomical field)
CaM, calmodulin
CCCP, carbonyl cyanide m-chlorophenylhydrazone
CsA, cyclosporin A
CypD, cyclophilin D
dATP, deoxyadenosine triphosphate
$\Delta\psi_m$, mitochondrial membrane potential
Debio-025 = UNIL025 = MeAla^{3}EtVal^4-CsA, N-methyl-D-alanine-3-N-ethyl-valine-4-CsA
DMSO, dimethyl sulfoxide
EGTA, ethylene glycol-bis(β-aminoethylether)-N,N,N´,N´-tetraacetic acid
EM, electron microscopy
ER, endoplasmic reticulum
ETC, electron transport chain
$FADH_2$, 1,5-dihydro-flavin adenine dinucleotide
GSH, reduced glutathione
GSSG, oxidized glutathione
H_2O_2, hydrogen peroxide
IB, isolation buffer
JC-1, 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide
K_{ATP}, ATP-sensitive potassium channels
MCAO, middle cerebral artery occlusion
MeVal^4-CsA, N-methyl-valine-4-CsA
mitoK_{ATP}, mitochondrial ATP-sensitive potassium channels
MOPS, 3-(N-Morpholino)propanesulfonic acid
mPT, mitochondrial permeability transition
NAD^+, nicotinamide adenine dinucleotide, oxidized form
NADH, nicotinamide adenine dinucleotide, reduced form
$O_2^{\bullet-}$, superoxide
$ONOO^-$, peroxynitrite
$OH^\bullet$, hydroxyl radical
Pi, phosphate
Ppif$^{-/-}$, peptidylprolyl isomerase F (= CypD)
PKC, protein kinase C
ROS, reactive oxygen species
TBI, traumatic brain injury
NIM811 = MeIle^4-CsA, N-methyl-isoleucine-4-CsA
NMDA, *N*-Methyl-D aspartate
$NO^\bullet$, nitric oxide
ROS, reactive oxygen species
Smac, second mitochondrial activator of caspases
SOD, superoxide dismutase
TCA, tricarboxylic acid
TOM, protein translocase of the outer mitochondrial membrane
TPP^+, triphenylphosphonium cation

SUMMARY

There is currently no clinically available drug with neuroprotective properties to limit the evolving cell death following *e.g.* stroke or traumatic brain injury. The mitochondrial permeability transition (mPT) is a potential pathological mechanism causing cell death in the CNS. As the name implies, the mPT is defined by a sudden increase in permeability of the inner mitochondrial membrane, whose normal impermeable state is fundamental for the bioenergetic function of mitochondria. The objective of the present studies was to characterize the mPT phenomenon in isolated rodent brain mitochondria. Mitochondria serve an important role in cellular calcium homeostasis and buffer transient increases in calcium, but mitochondrial calcium overload is also the prime trigger for mPT. In the present studies, we found that brain mitochondria readily undergo changes attributable to mPT induction such as swelling, loss of membrane potential, uncoupling of oxidative phosphorylation and respiratory inhibition. The mitochondrial generation of reactive oxygen species (ROS) was also increased following mPT, and mitochondria became permeable to NAD(H).

Cyclosporin A (CsA) binds to the mitochondrial protein cyclophilin D (CypD) and can thereby inhibit mPT, an effect that is unrelated to its immunosuppressive action, and mPT in brain mitochondria was found to be highly sensitive to CsA inhibition. CsA has demonstrated prominent neuroprotective properties in several different animal models of neurological disease, and recent experiments subjecting mice lacking CypD to ischemia support the conclusion that the effect of CsA at least in this model is mediated by mPT inhibition. A library of CsA analogs was evaluated for mPT-inhibiting and ROS-reducing properties, and two newly developed non-immunosuppressive CsA analogs were found to be potent inhibitors of mPT already at nanomolar concentrations.

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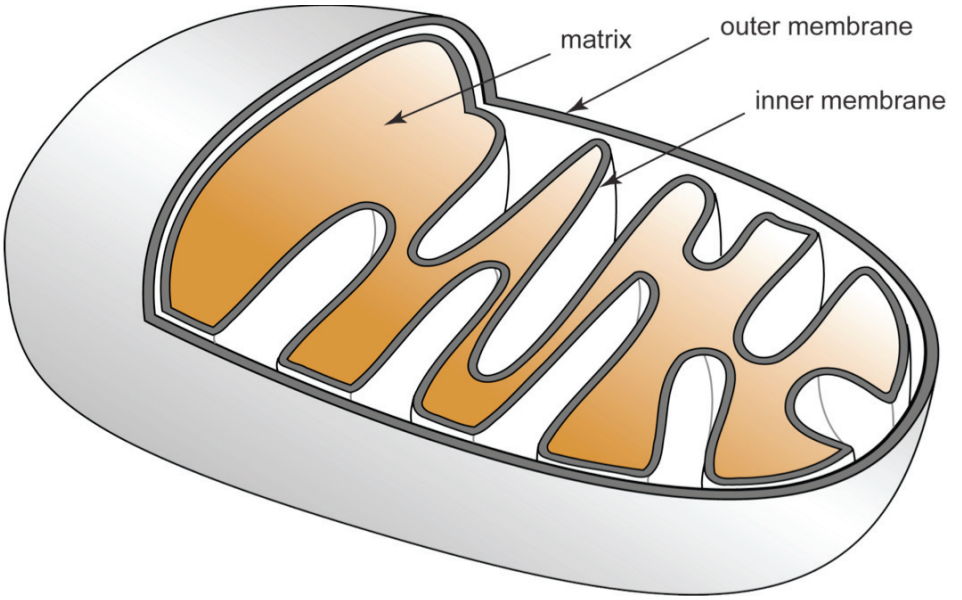

Figure 1. *Schematic drawing of a mitochondrion. Used under the terms of the GNU Free documentation license.*

SWEDISH SUMMARY – SVENSK SAMMANFATTNING

Hjärnan är ett energikrävande organ och förbrukar ca 20% av blodets syre trots att den bara utgör 1-2% av kroppsvikten. Mitokondrien är den del av cellen där syre och näringsämnen förbränns till koldioxid och vatten, och mitokondrien kallas ofta för cellens kraftstation. Denna process, den så kallade cellandningen, är kopplad till produktion av den energirika molekylen ATP som sedan används till energikrävande processer i cellen. Mitokondriens inre delar (dess matrix) omsluts av ett inre veckat lipidmembran. Detta omsluts i sin tur av ett yttre lipidmembran (Fig 1.). Det yttre membranet innehåller många porer som släpper igenom små molekyler, medan innermembranet är helt tätt, eller impermeabelt, för vattenlösliga molekyler.

Cellandningen, eller respirationen, i mitokondrien är en komplicerad process och den kräver just ett impermeabelt innermembran för att fungera. Näringsämnen bryts successivt ned i cellen till mindre beståndsdelar och i mitokondrien används den energi som finns lagrad i näringsämnena till att pumpa ut vätejoner (protoner) från mitokondriens matrix till utrymmet mellan inner- och yttermembranet. Eftersom innermembranet inte släpper igenom protoner leder detta till en skillnad i protonkoncentration (pH) över innermembranet, men också till en spänningsskillnad på grund av att protoner är laddade partiklar. Sammantaget utgör pH-skillnaden och spänningen en protondrivande kraft. Utpumpningen av protoner kan liknas vid en generator som alstrar kraft till en elektrisk krets. Den elektriska kraften i en krets kan användas för olika ändamål, t.ex. till att driva en motor. I mitokondrien används den protondrivande kraften främst för att omvandla molekylen ADP till ATP.

Hjärnans celler kräver mycket ATP för att kunna fungera. En störning av mitokondriernas normala funktioner kan därför leda till att cellerna de finns i skadas eller dör. En allvarlig störning som kan leda till celldöd är mitokondriens permeabilitets-transition (mPT). Liksom namnet antyder innebär mPT en övergång, eller transition, från mitokondriens normala impermeabilitet till en plötslig permeabilitet, d.v.s. flera molekyler som normalt inte kan passera över mitokondriens innermembran kan plötsligt flöda fritt, däribland protoner. Liksom en kortslutning av en elektrisk krets, innebär mPT att den protondrivande kraften och därmed ATP-bildningen helt upphör. Eftersom mitokondriens matrix innehåller väldigt mycket proteiner leder också mPT till att vatten flödar in osmotiskt och att mitokondrien svullnar.

Mitokondrien har också en viktig funktion i att buffra kalcium (Ca^{2+}). Kalciumkoncentrationen inne i cellen är normalt väldigt låg, ca 10000 gånger lägre än koncentrationen utanför cellen, och tillfälliga höjningar kan aktivera flera olika processer. Kalcium är därför en viktig signalmolekyl som kan styra olika funktioner i cellen. För höga eller långvariga kalciumökningar kan dock

vara mycket farligt för cellen, och då spelar mitokondriens förmåga att ta upp och lagra kalcium en viktig roll. I normala fall har mitokondrien stor kapacitet att lagra kalcium, men under situationer då t.ex. cellens energistatus är försämrat sjunker mitokondriens kapacitet att lagra kalcium och kalcium kan då istället orsaka att mitokondrien genomgår mPT.

Medicinen cyclosporin A (CsA) förhindrar mPT genom att binda till proteinet cyclophilin D (CypD) i mitokondrien. CypD katalyserar en omvandling i mitokondriens innermembran som leder till mPT, och genom att hämma CypD kan CsA motverka att mitokondrien genomgår mPT. CsA har flera effekter i människokroppen och används idag för ett helt annat ändamål, nämligen immunförsvarshämning så att det t.ex. är möjligt att transplantera in främmande organ utan att de stöts bort. Om CsA kommer in i hjärnan är det kraftigt skyddande mot flera typer av hjärnskador. I djurförsök minskar t.ex. CsA den skada som orsakas efter att blodtillförseln tillfälligt stoppas till hela eller delar av hjärnan (så som sker vid stroke) eller efter de tryck och krosskador som sker vid en traumatisk hjärnskada. CsA har också visats förlänga överlevnaden i möss som har en neurodegenerativ sjukdom liknande ALS (amyotrofisk lateral skleros).

Mycket av arbetet i denna avhandling bygger på hypotesen att den skyddande effekten av CsA vid olika hjärnskador delvis eller helt beror på förhindrandet av mPT. En del tidigare arbete har dock ifrågasatt att mPT verkligen finns eller har relevans i hjärnans mitokondrier eller att CsA har någon skyddande effekt.

I de två första delarbetena undersökte vi om framrenade mitokondrier från hjärnvävnad genomgår mPT när de blir exponerade för kalcium. Vi undersökte dels mitokondrier vars cellandning var avstängd och där kalcium har en relativt direkt effekt på de molekylära strukturer som ligger bakom mPT, och dels respirerande mitokondrier som gavs näringsämnen och aktivt tog upp och buffrade kalcium. Under båda situationerna svullnade mitokondrierna till följd av kalcium vilket tyder på att de genomgick mPT. Svullnaden var beroende av dosen av kalcium, men då mitokondrierna svullnade var responsen genomgående d.v.s. det tycktes inte finnas några olika undergrupper av mitokondrier som svarade fundamentalt olika på kalcium. I respirerande mitokondrier kunde svullnaden avbrytas genom att binda upp givet kalcium, och mitokondrierna återfick då till stor del sitt tidigare utseende och sin spänning över innermembranet. CsA var kraftfullt skyddande mot mPT i mitokondrier vars cellandning var avstängd, och hade en liknande effekt i respirerande mitokondrier om ADP var närvarande. Om ADP inte tillsattes hade CsA en mindre effekt mot kalciuminducerad svullnad, men hade desto större effekt på att förbättra återhämtningen hos mitokondrier där kalcium bands upp. Slutsatserna från de första arbetena var att hjärnmitokondrier generellt kan genomgå mPT och att CsA utövar en skyddande verkan mot mPT.

För att bättre kunna utvärdera om effekten av CsA i djurförsök beror på förhindrandet av mPT eller inte vore det önskvärt med mer specifika mPT-hämmare. För behandling av människor mot hjärnskador kan den immunförsvarshämmande effekten av CsA anses vara en biverkan. I det tredje delarbetet utvärderade vi två cyclosporinvarianter, NIM811 och UNIL025 (eller Debio-025), med hjälp av de metoder vi använde i de två första studierna. Både NIM811 och UNIL025 saknar immunförsvarshämmande verkan men är i övrigt strukturellt lika CsA. Vi fann att båda molekylerna förhindrade mPT på liknande sätt som CsA. Båda, men framför allt UNIL025, var också mer potenta än CsA, d.v.s. de förhindrade mPT vid lägre doser än CsA. Slutsatsen från det tredje delarbetet var att dessa molekyler är lovande farmaka både experimentellt för att bättre kunna undersöka mPT i celldödsprocesser men också förhoppningsvis som mer specifika läkemedel med mindre biverkningar hos människor.

Upphörd energiproduktion från mitokondrier kan uppenbarligen skada en cell om den blir omfattande. En ytterligare orsak till att mitokondrien kan orsaka cellskada och celldöd tros vara genom produktion av reaktiva syreprodukter (begreppet fria radikaler används ofta som synonym). Eftersom syrgasomvandlingen sker i mitokondrien kan också reaktiva syreprodukter bildas här. Mitokondrien har dock också kraftfulla avgiftningssystem, och om mitokondrien under normala förhållanden verkligen producerar en större mängd reaktiva syreprodukter som kan skada cellen är omtvistat. Det har dock föreslagits att kalcium kan orsaka ökad produktion av reaktiva syreprodukter. I det fjärde delarbetet visade vi att mitokondrien genererar mer reaktiva syreprodukter efter att den genomgått mPT. Kalcium i sig utan att mitokondrien genomgick mPT hade dock ingen ökande effekt. Eftersom den protondrivande kraften upphör när mitokondrien genomgår mPT försvinner också mycket av drivkraften för att generera reaktiva syreprodukter. Å andra sidan försvinner också en viktig del av avgiftningssystemet eftersom detta också är beroende av den protondrivande kraften. Slutresultatet blir att nettoproduktionen av reaktiva syreprodukter ökar och detta kan i sin tur leda till ytterligare skada i cellen. I arbetet testade vi också ett tjugotal cyclosporin-analoger och fann att deras förmåga att förhindra kalciuminducerad svullnad korrelerade med förmågan att förhindra kalciuminducerad produktion av reaktiva syreprodukter. Mitokondrier isolerade från mänsklig levervävnad uppvisade också liknade resultat som mitokondrier från råttlever och hjärna. Slutsatsen från fjärde delarbetet var att mPT är en situation då mitokondrier kan generera patologiska nivåer av reaktiva syreprodukter.

I de tidigare arbetena gav vi kalcium som en enstaka stor dos för att se om mitokondrierna genomgick mPT. I femte delarbetet gav vi istället kalcium som en långsam infusion. Därmed kunde vi noggrannare följa hur mitokondrierna

klarade av att buffra kalcium. Genom att vid infusion av kalcium dels titta på hur mitokondrierna behåller kalcium och dels se hur de förbrukar syrgas kunde vi se att det som begränsar mitokondriens förmåga att buffra kalcium verkligen är mPT. En viktig observation var dock att det som reglerar mPT inte nödvändigtvis behöver ha en motsvarande effekt i hur mycket kalcium mitokondrien klarar av att buffra. Det som aktiverar mPT är fritt kalcium inne i mitokondrien, men när mitokondrien aktivt tar upp kalcium binder den upp det mesta i komplex med fosfat och det blir därmed inaktivt. Det som ligger bakom att mitokondrien kan binda upp kalcium är dess basiska miljö (högt pH). Detta står i motsats till regleringen av mPT i sig. I studier av hur mPT aktiveras när kalciumupptag är avstängt har en sur miljö (lågt pH) visat sig vara skyddande. Det som troligtvis är viktigast i kroppen är kombinationen av dessa effekter och då finner vi att betydelsen av komplexbildningen av kalcium överväger. I det femte delarbetet finner vi nämligen att ett basiskt matrix är väsentligt för att hjärnmitokondrier ska kunna binda upp kalcium och undvika mPT.

Mitokondriens innermembran är också i normala fall impermeabelt för kalium (K^+) som finns i hög koncentration inne i cellen. Det har dock föreslagits att en typ av kaliumkanaler (mitoK_{ATP}) finns i mitokondrien och att de kan öppnas vid kortvariga blodflödesrubbningar. Dessa kanaler ger ett läckage av kalium in till mitokondriens matrix och det tros kunna skydda mitokondrierna vid nya episoder av blodflödesbrist, även om mekanismen för den skyddande effekten är okänd. Vi finner att om vi ökar läckaget av kalium med hjälp av en kemikalie som transporterar kalium ökar hjärnmitokondriers förmåga att buffra kalcium (Ca^{2+}). Detta tror vi beror på att matrix blir ytterligare basiskt p.g.a. en kompensationsmekanism för kaliumläckaget. Genom att göra matrix mer basiskt hålls nivån av fritt kalcium lågt och därmed minskas också risken att mPT aktiveras.

Mitokondrien har även en viktig roll i de celldödsprocesser som är nödvändiga under t.ex. utvecklingen av hjärnan eller i organ där celler byts ut. Denna typ av celldöd innebär en ordnad sorti som sker utan att orsaka störningar kring den döende cellen. Denna process brukar kallas för programmerad celldöd eller apoptos. Mellan mitokondriens membran finns flera celldödsaktiverande proteiner. När de finns i mitokondrien är de antingen inaktiva eller har en annan funktion. Om de däremot läcker ut i resten av cellen, aktiverar de celldödsprogram som successivt bryter ned cellen. Vid aktivering av mPT och den svullnad som följer kan mitokondriens yttermembran spricka och dessa celldödsproteiner läcka ut. Men det finns andra mekanismer som kan orsaka att dessa proteiner läcker ut, och det har varit mycket omdebatterat vilken roll mPT kan ha i programmerad celldöd. Eftersom denna typ av celldöd är aktiv och kräver en bevarad energiproduktion kan inte alla mitokondrier genomgå mPT. Då blir följden istället att cellen förlorar sin intakta struktur och miljö, med följd att dess innehåll läcker ut och orsakar inflammation. Denna celldöd kallas

för nekros. I celldödsprocesser som utvecklas efter akuta hjärnskador ser celldöden varken ut som nekros eller apoptos. Man kan tänka sig att en tillfällig mPT och svullnad av mitokondrien, så som den vi visar i andra delarbetet, eller mPT begränsat till en liten del av cellens mitokondrier kan starta långsammare celldödsprocesser som har inslag av energibrist, ökad produktion av reaktiva syreprodukter men även utsläpp av dödsproteiner. Dock tycks inte den rena formen av programmerad celldöd involvera mPT.

Studier med läkemedel såsom CsA tyder på att mPT kan spela en viktig roll för hur celldöden utvecklas efter flera typer av akuta hjärnskador men även en del kroniska. Skadan kan därmed begränsas om man ger läkemedel som hämmar aktiveringen av mPT. Med hjälp av genetiskt modifierade djur som saknar CypD har man också kunnat bekräfta att mPT är ansvarig för mycket av den hjärnskada som utvecklas i en djurmodell som efterliknar stroke hos människor. Användning av CsA eller dess mer specifika analoger begränsas dock för närvarande av att de inte på ett enkelt sätt passerar in i hjärnan. Vid traumatisk hjärnskada är dock den barriär som håller CsA utanför hjärnvävnaden skadad och kliniska prövningar i människor har påbörjats för att undersöka om CsA kan hjälpa dessa patienter. Även vid den neurodegenerativa sjukdomen ALS planeras CsA att ges direkt in till centrala nervsystemet i hopp om att minska sjukdomstakten.

BACKGROUND

Mitochondrial energy conversion and chemiosmotic coupling

Large amounts of energy are required to maintain normal neuronal activity in the central nervous system. The human brain accounts for 1-2% of the total body mass but is responsible for 20% of the body's total oxygen consumption (Erecinska & Silver 1989). Plasma glucose is the main substrate for the brain and deprivation of glucose or oxygen leads to functional failure and irreversible damage if prolonged (Gibbs *et al.* 1942, Lipton 1999, Siesjö 1992). ATP is the universal "energy currency" in the cell. ATP is a phosphate donor with a "high-energy" phosphate bond. The energy released when ATP is split into ADP and phosphate is utilized to drive various energy requiring processes in the cell. In the brain, a predominant part of energy metabolism is utilized to uphold the sodium and potassium gradients over the cell membrane, required for synaptic transmission (Whittam 1962). Glycolysis produces a minor part of the required ATP but the real mainspring of energy in eukaryotic cells is respiration, the enzymatic oxidation of fuel molecules by molecular oxygen, coupled to phosphorylation of ADP to ATP. The oxidative phosphorylation takes place in mitochondria and they can therefore be seen as the powerhouse of the cell. All three major foodstuffs, carbohydrate, fatty acids, and amino acids, are predominantly oxidized by the citric acid cycle, or the Krebs tricarboxylic acid (TCA) cycle. The main entry into the TCA cycle is through Acetyl-CoA. The acetyl group is broken down to CO_2 through a cyclic oxidation mechanism that was first deduced by Hans Krebs (Krebs & Johnson 1937) (which granted him the Nobel Prize in Physiology or Medicine in 1953). Redox energy is transferred from the TCA cycle in the form of NADH and $FADH_2$ to the electron transport chain (ETC) of the inner mitochondrial membrane. The ETC is an assembly of multiple carriers of electrons that are grouped into four multi-polypeptide complexes. NADH and $FADH_2$ are oxidized at the respiratory complexes I and II respectively, and electrons are ultimately carried to complex IV where oxygen is reduced to water.

The link between oxidation of metabolites and phosphorylation of ADP into ATP remained unsolved for many years until Peter Mitchell proposed the chemiosmotic theory (Mitchell 1961)(for which he was awarded the Nobel Prize in Chemistry in 1978). The chemiosmotic theory postulates an indirect coupling between the two processes of oxidation and phosphorylation that depends on the extrusion of protons over the inner mitochondrial membrane. The flow of electrons through the respiratory complexes of the inner mitochondrial membrane translocates positively charged protons from the matrix into the intermembrane or cristae space. This builds up a proton concentration difference (a ΔpH) and an electrical membrane potential ($\Delta\Psi_m$) which together results in an electrochemical proton gradient called

protonmotive force. The protonmotive force drives the synthesis of ATP as protons translocate back into the matrix through the F_1F_oATP synthase (or complex V), where the protonmotive force is converted into mechanochemical energy to drive ATP synthesis (Kagawa & Racker 1966). The protonmotive force thus couples oxidation and respiration with phosphorylation and this depends on the intactness of the inner mitochondrial membrane. In order for a protonmotive force to exist the inner mitochondrial membrane cannot be generally permeable to charged species and specific carriers and transport systems for ions and metabolites are required. As an intact inner mitochondrial membrane is the basis of efficient energy coupling, a change in permeability will have dramatic consequences.

Cell death in the brain

Cell death is commonly divided into apoptosis and necrosis, originally based on morphologic criteria (Kerr *et al.* 1972). Apoptosis is a regulated and energy-dependent cell death characterized by cell shrinking, chromatin condensation and fragmentation, membrane blebbing, and disintegration of the cell into membrane-enclosed apoptotic bodies. In contrast, necrosis is a passive process characterized by swelling of the cell and organelles due to the equilibration of solutes across the membranes ultimately leading to release of the cellular contents and inflammation. Apoptosis is usually the characteristic of programmed cell death, and this is a prominent feature in the immature brain as 50% of neurons are eliminated during normal brain development (Wieloch 2002).

The cell death following different pathological stresses in the central nervous system may not be confined into these strict divisions and the features of cell death may more depend on the underlying causes. Cell death following cerebral ischemia involves morphological changes that can neither be regarded as apoptotic or necrotic (Wieloch 2002). For instance, dying cells shrink but apoptotic bodies are not formed and inflammation is a prominent feature (van Lookeren Campagne & Gill 1996, Iadecola & Alexander 2001). Cell death following excitotoxicity (see below) has been defined as a separate entity in a recently reported classification of cell death (Kroemer *et al.* 2005).

That intact mitochondrial function is vital for the energy metabolism of the energy demanding brain is perhaps evident, but mitochondrial participation in cell death extends beyond energy metabolism. Mitochondria in excitable tissues serve an important role in cellular calcium homeostasis, and the redox centers in mitochondria are both potential generators and targets of harmful reactive oxygen species (ROS). Further, the mitochondrial intermembrane space harbors

several proteins that activate programmed cell death pathways once they are released to the cytosol.

Calcium homeostasis

Calcium was proposed to be the common denominator triggering brain damage in status epilepticus, ischemia and hypoglycemia (Siesjö 1981) following the findings that ischemia and status epilepticus trigger rapid translocation of calcium from extracellular to intracellular spaces in neuronal tissue (Nicholson *et al.* 1977) as well as accumulation of fatty acids (Rehncrona *et al.* 1982). Calcium ions regulate several cellular processes and are normally kept in the range 0.1 to 1 μM in the cytoplasm, *i.e.* 1000 to 10000 times lower than the extracellular concentration 1.2 mM. The sodium/calcium exchanger and a calcium ATPase at the plasma membrane uphold the large electrochemical calcium gradient. Calcium is also transported into the endoplasmic reticulum (ER) by a calcium ATPase and electrophoretically into mitochondria over a certain concentration threshold.

Glutamate is the primary excitatory neurotransmitter in the brain, and mediates calcium entry in the postsynaptic neuron primarily through activation of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and NMDA (*N*-Methyl-D aspartate) receptors. AMPA receptor activation increases the conductance of sodium which depolarizes the membrane. The depolarization relieves the magnesium inhibition of NMDA receptors, and glutamate binding causes opening of the channel to calcium and sodium, which enter the cell along their respective gradients. The finding that glutamate can induce neurotoxicity (Olney & Sharpe 1969) initiated the excitotoxicity hypothesis. Excitotoxicity with increased activation of glutamate receptors are implicated in many neurological diseases, spanning from acute disorders such as ischemia to chronic neurodegenerative diseases such as amyotrophic lateral sclerosis, and the neurotoxicity stem largely from the associated calcium toxicity (Meldrum & Garthwaite 1990).

Intracellular calcium can rise to severely elevated levels during excitotoxicity, in particular if energy metabolism is compromised. The calcium efflux pathways are energy-dependent and if sodium accumulates, the sodium/calcium exchanger may reverse, causing increased calcium uptake. During ischemia, the intracellular calcium levels can rise to 50-100 μM (Siesjö 1992). This will cause sustained activation of calcium-dependent enzymes including calpains which degrade the cytoskeleton and cleave several proteins involved in cell death (Yokota *et al.* 1995, Orrenius *et al.* 2003) and phospholipases that will cause an increase in fatty acids, particularly arachidonic acid (Bazan 1970). Several regulatory proteins are activated by calcium such as calmodulin (CaM)

and protein kinase C (PKC). The serine/threonine phosphatase calcineurin, which constitutes 1% of the total protein content in brain (Yakel 1997), have multiple substrates that can activate cell death pathways. Calcineurin dephosphorylates Bad (Wang *et al.* 1999), binds to Bcl-2 (Shibasaki *et al.* 1997) and activates neuronal nitric oxide synthase (nNOS), which is also activated by calcium itself (Snyder *et al.* 1998).

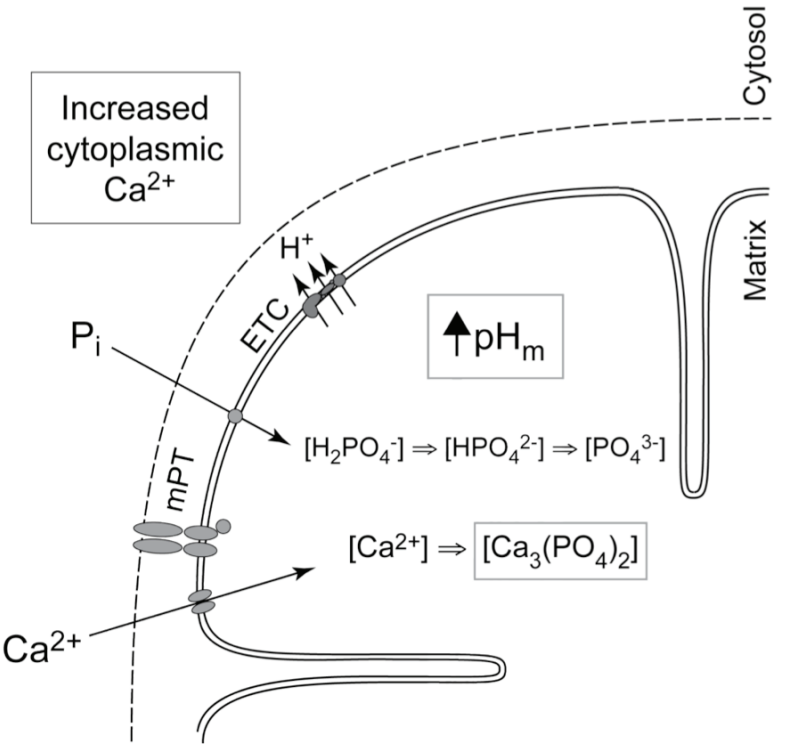

Figure 2. *Mitochondrial calcium buffering. Calcium is taken up into mitochondria above a certain cytoplasmic calcium concentration. Due to the alkaline matrix environment, the concentration of deprotonated phosphate (PO_4^{3-}) is high and this kind of phosphate forms tricalcium phosphate complexes with calcium. Free calcium is thus kept at a low concentration and mPT is normally not activated. (ETC = electron transport chain).*

Mitochondria can accumulate large amounts of calcium in presence of physiological levels of phosphate and adenine nucleotides. Mitochondrial calcium uptake serves both as a metabolic regulator of matrix dehydrogenases and as a buffer of cytoplasmic calcium transients. Calcium moves

electrophoretically into the mitochondrial matrix through the calcium uniporter above a certain cytoplasmic calcium concentration, in brain mitochondria approximately 0.5 μM. The rate of uptake is dependent on both $\Delta\Psi_m$ and the extramitochondrial calcium concentration. Phosphate enters mitochondria along with calcium and forms an osmotically inactive tricalcium phosphate complex, $Ca_3(PO_4)_2$ (Fig. 2). Both the transport of $H_2PO_4^{2-}$ and the two proton dissociations to PO_4^{3-}, the phosphate that complex Ca^{2+}, are dependent on pH. The concentration of PO_4^{3-} will therefore vary with the cube of the proton concentration. Due to the alkaline matrix environment, free Ca^{2+} will be kept at a low micromolar level despite accumulation of large concentrations of total calcium (Nicholls 2005).

Mitochondrial generation of reactive oxygen species (ROS)

Reactive oxygen species (ROS) are oxygen-derived molecules with a high reactivity toward other molecules. Free radicals are molecules with a free unpaired electron, denoted by (•), and these terms are usually used almost interchangeably although not all free radicals are ROS and vice versa. Reactive oxygen species play a physiological role in intracellular signaling, and can function as second messengers (Droge 2002) but are potentially harmful as they are capable of oxidative modifications of proteins, nucleic acids, polysaccharides and lipid membranes (Turrens 2003).

Several processes in the cell can produce ROS, *e.g.* xanthine and NAD(P)H oxidases. Mitochondria can generate ROS by incomplete reduction of molecular oxygen, in particular at complex I and III of the ETC. In contrast to the reduction of oxygen at complex IV, where all partially reduced intermediates are retained until full reduction is achieved, the other redox centers may leak electrons to oxygen, forming superoxide ($O_2^{\bullet-}$). Animals lacking mitochondrial superoxide dismutase (Mn SOD or SOD2), the first line of antioxidant defense against ROS, die within ten days of life due to oxidative damage (Li *et al.* 1995). This finding demonstrates that mitochondrial superoxide production is potentially very harmful but also that the endogenous detoxifying antioxidant mechanisms are very potent. SOD converts superoxide to hydrogen peroxide (H_2O_2), which in turn is converted to water by glutathione peroxidase. The latter process requires an uncompromised mitochondrial function as the substrate for glutathione peroxidase, reduced glutathione (GSH), is held reduced by NADPH which in turn is held highly reduced by the protonmotive force driven energy-linked nicotinamide nucleotide transhydrogenase (Hoek & Rydstrom 1988, Nicholls & Budd 2000).

Other potent ROS compounds may also be formed. Under conditions where nitric oxide ($NO^{\bullet}$) is present it may react with superoxide to form peroxynitrite

($ONOO^-$), and H_2O_2 may also form the hydroxyl radical ($OH^\bullet$) in presence of reduced transition metals, such as Fe^{2+} (Turrens 2003).

Mitochondrial generation of ROS is considered to participate in cell death cascades of both acute brain injuries and chronic neurodegenerative diseases, and the mitochondria are also themselves sensitive targets for ROS-induced pathological modifications (Beal 1996, Nicholls & Budd 2000). In models of excitotoxicity, increased ROS production in mitochondria has been demonstrated. It has been coupled to mitochondrial calcium loading but the mechanism linking calcium and ROS as well as the time course of ROS increase is not settled (Reynolds & Hastings 1995, Dugan *et al.* 1995, Vergun *et al.* 2001, Vesce *et al.* 2004, Nicholls 2004, Starkov *et al.* 2004).

Mitochondria and programmed cell death

Mitochondria host a number of proteins in the intermembrane space that are inactive or serve a normal physiological function in the mitochondria but participate in the death of the cell if they are released to the cytosol (Orrenius *et al.* 2007). The most well studied of these cell death proteins is cytochrome *c* which is part of the ETC in mitochondria. Once released it forms the apoptosome complex with apoptosis activation factor-1 (Apaf-1) and dATP which recruit and activate procaspase-9. Caspases are a family of proteases, which are proenzymes that require activation and subsequently orchestrate cell death. Activated caspase-9 in turn activates procaspase-3 and other effector procaspases that trigger a cascade of events leading to apoptotic cell death. Other intermembrane proteins include Smac (second mitochondrial activator of caspases), HtrA2/Omi and AIF (Apoptosis inducing factor). AIF can induce caspase-independent cell death and interestingly, AIF-linked chromatin condensation in the nucleus is facilitated by cyclophilin A (Cande *et al.* 2004)

The Bcl-2 family of proteins are important regulators of programmed cell death and include both survival and death factors, and the interplay between the opposing family members in response to various forms of intracellular stress will arbitrate whether a cell death cascade is initiated (Cory *et al.* 2003). The Bcl-2 proteins and caspases represent an evolutionary conserved pathway of programmed cell death and much of the understanding comes from studies of similar proteins in nematodes (Horvitz 1999). A majority of the Bcl-2 family proteins act on the level of mitochondria. Cleaving of Bid induces translocation, oligomerization and insertion of Bax and/or Bak into the outer mitochondrial membrane. The Bax/Bak insertion permeabilizes the outer membrane in a process that requires the protein translocase of the outer mitochondrial membrane (TOM) complex (Ott *et al.* 2007). This process renders the outer mitochondrial membrane permeable to cytochrome *c* and other proapoptotic

intermembrane proteins. The importance of Bax and Bak is illustrated by the resistance of cells lacking both of these proteins to most types of apoptotic stimuli (Wei *et al.* 2001). The antiapoptotic members of this protein family, *e.g.* Bcl-2 and Bcl-X_L, interact with Bax and Bak and prevent their oligomerization. ROS may also influence the Bax/Bak-induced cytochrome *c* release. Cardiolipin in the inner mitochondrial membrane anchors cytochrome *c* and peroxidation of cardiolipin increases the dissociation of the hemoprotein (Orrenius *et al.* 2007).

Mitochondria in ischemic preconditioning and tolerance

Ischemic preconditioning or ischemic tolerance are two terms for a phenomenon whereby a short non-injurious ischemic insult can greatly reduce the severity of a subsequent prolonged ischemia (Janoff 1964, Murry *et al.* 1986, Kitagawa *et al.* 1990). This adaptive response can be seen as a general biological phenomenon by which organisms respond with protective mechanisms to potentially recurring challenges (Dirnagl *et al.* 2003), or hormesis, a concept of biphasic dose-response relations that are seen in many different areas of research from toxicology to psychology (Calabrese 2007). A low dose of one type of stressful stimulus can also induce resistance to another, *e.g.* prior transient hyperthermia can protect against subsequent forebrain ischemia (Chopp *et al.* 1989).

Ischemic preconditioning mediates both a rapid adaptive response coming into effect within minutes to hours as well as an induced tolerance occurring over a longer time frame requiring gene activation and *de novo* protein synthesis. The former is extensively studied in cardiac ischemia and the latter in cerebral ischemia (Gidday 2006, Yellon & Downey 2003). Elucidating the mechanisms underlying these endogenous survival responses might enable a therapeutic opportunity to reduce tissue damage following ischemia by super-inducing or boosting the preconditioning pathways (Dirnagl *et al.* 2003, Gidday 2006, Yellon & Downey 2003). Mitochondria have been suggested to take center stage also in these pathways.

ATP-sensitive potassium channels (K_{ATP}) exist in plasma membranes. In pancreatic beta cells they serve an important function in the regulation of insulin secretion (Hattersley & Pearson 2006). The mitochondria have been proposed to contain similar ATP-sensitive potassium channels (mitoK_{ATP}), but pharmacologically distinct from the plasma membrane channels (Inoue *et al.* 1991, Garlid & Paucek 2003). The mitoK_{ATP} have been the focus of a large body of scientific work and are believed to be important mediators of the preconditioning effect. However, the evidence comes almost exclusively from studies with pharmacological compounds, mainly diazoxide and 5-

hydroxydecanoate (5-HD) (Yellon & Downey 2003, Ardehali & O'Rourke 2005). Ischemic preconditioning can be closely mimicked by the K_{ATP} opener diazoxide, which show a concentration-dependent selectivity for mitoK_{ATP} over plasma membrane K_{ATP} (Garlid *et al.* 1996, Liu *et al.* 1998). Both ischemic and diazoxide-mediated preconditioning can be blocked by 5-HD which is believed to inhibit the diazoxide-induced opening of mitoK_{ATP} (Jaburek *et al.* 1998). A role of mitoK_{ATP} is also implicated in different models of cerebral ischemia where pretreatment with diazoxide mediates neuronal protection in a 5-HD-sensitive manner (Liu *et al.* 2002, Domoki *et al.* 1999). Other mitochondrial potassium channels have also been suggested, such as a voltage-sensitive and a calcium-activated potassium channel, where ligands of the latter demonstrate a similar cardioprotective effect as ligands of mitoK_{ATP} (Siemen *et al.* 1999, Szabo *et al.* 2005, Xu *et al.* 2002, Hanley & Daut 2005).

Ischemic preconditioning and mitoK_{ATP} channel activation have been proposed to afford tissue protection by inhibiting mPT activation during reperfusion (Hausenloy *et al.* 2002, Hausenloy *et al.* 2004, Liu *et al.* 1998, Ardehali & O'Rourke 2005). Two suggested mechanisms linking mitoK_{ATP} to mPT are decreased calcium uptake due to depolarization of mitochondria and activation of PKCε, which in turn modulates the mPT pore components (Costa *et al.* 2006, Holmuhamedov *et al.* 1999). Decreased mPT activation has also been demonstrated in heart mitochondria from preconditioned animals following ischemia (Halestrap *et al.* 2007), but the mechanism is not known.

The mitochondrial permeability transition (mPT)

The mitochondrial permeability transition (mPT) is, as the name implies, a sudden transition in permeability of the inner mitochondrial membrane. As discussed above, an impermeable inner mitochondrial membrane is the basis for the chemiosmotic coupling of respiration and ATP production in mitochondria. The permeability increase following mPT mediates all solutes less than 1500 Da to equilibrate over the mitochondrial membranes. Therefore, extruded protons from the ETC will rapidly fall back into the matrix and respiration will be uncoupled with cessation of ATP production. The mitochondrial matrix is dense in proteins and the equilibration of electrolytes will cause an osmotic influx of water causing the matrix to swell. This may in turn cause a rupture of the outer mitochondrial membrane with release of intermembrane proapoptotic proteins.

Mitochondrial swelling has been noted since early studies of isolated mitochondria and in order to attain functioning mitochondria, a calcium chelator had to be present during the isolation process (Lehninger 1949, Hunter & Ford 1955). Swelling was often considered an artifact until seminal studies

by Douglas Hunter and Robert Haworth in the late seventies established the mPT as a tightly regulated and reversible phenomenon, and a pore formation was suggested (Hunter & Haworth 1979a, Hunter & Haworth 1979b, Hunter *et al.* 1976, Haworth & Hunter 1979). A decade later, it was discovered that the immunosuppressant and undecapeptide cyclosporin A (CsA) is a specific mPT inhibitor (Crompton *et al.* 1988, Broekemeier *et al.* 1989).

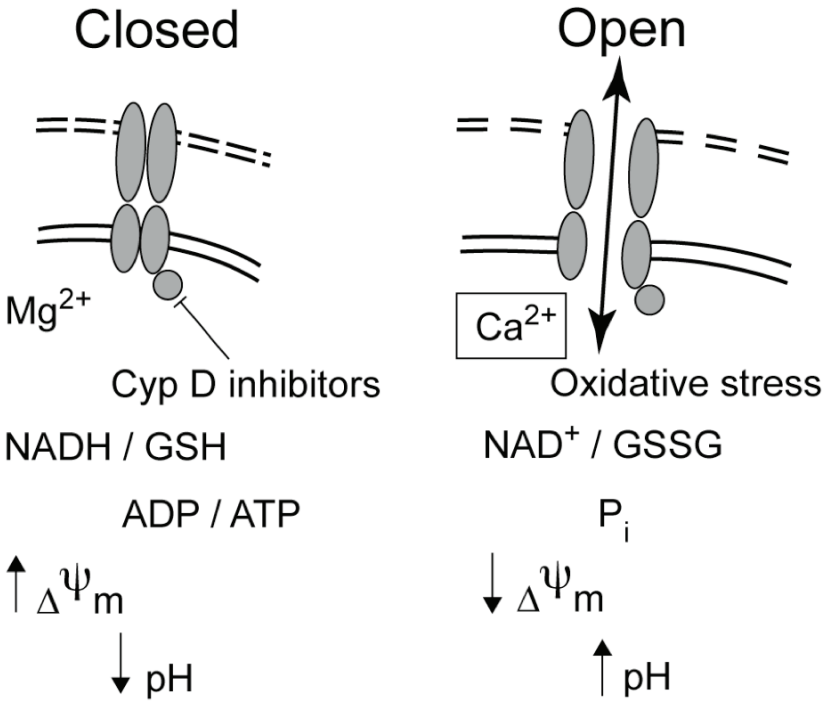

Figure 3. *Regulation of the mPT pore complex. Some important factors that stabilize the mPT pore components in a closed conformation are listed to the left whereas factors that increase the probability of an open conformation are listed to the right. Whether these factors will increase or decrease mPT activation in mitochondria actively buffering calcium will however also depend on their effects on calcium complex formation.*

Mitochondrial calcium overload is the prime trigger for mPT activation (Fig. 3). Calcium ions (Ca^{2+}) are believed to trigger a conformational change of the responsible protein(s). Other divalent cations, Mg^{2+}, Sr^{2+}, and Mn^{2+} competitively counteract the Ca^{2+} effect (Haworth & Hunter 1979, Bernardi *et al.* 1992). Protons (H^{+}) also tend to stabilize the mPT pore complex *per se* (but

simultaneously decrease the complexation of Ca^{2+}, see above). Several factors reflecting the bioenergetic status of mitochondria greatly influence the probability for mPT, most notably ADP and ATP levels. Both ADP and ATP, but most potently the former, dose-dependently reduce the sensitivity of mitochondria to undergo mPT. In contrast, phosphate increases the sensitivity. The redox status of pyridine nucleotides (NADH/ NAD^+) as well as glutathione (GSH/GSSG) influence the open/close state of the mPT complex. The oxidized status favor opening and this is considered to be caused by oxidation of critical dithiol groups on the mPT pore complex. Likewise, peroxides and sulfhydryl group reagents sensitize mitochondria towards mPT. An oxidative status or oxidative stress is thus considered to be the second important trigger for mPT besides calcium.

The target for CsA is the mitochondria-specific cyclophilin D (CypD). CypD has peptidylprolyl *cis-trans* isomerase activity and is thought to facilitate the calcium-triggered conformational change of the protein(s) forming the mPT pore (Halestrap & Davidson 1990). It therefore serves a regulatory role in mPT formation that can be overcome by increasing calcium or other inducing agents. CsA should therefore be seen as a desensitizer of mPT and not a blocker (Bernardi *et al.* 2006). The role of CypD is confirmed by experiments with mitochondria from *Ppif* $^{-/-}$ mice lacking CypD. These mitochondria have a higher threshold for mPT induction, similar to what is seen in mitochondria from control animals with CsA present, and display no additional response to CsA (Baines *et al.* 2005, Nakagawa *et al.* 2005, Basso *et al.* 2005, Schinzel *et al.* 2005).

Which molecular structure that may underlie mPT in the inner mitochondrial membrane has been a matter of controversy. The early observations that substrates transported by the adenine nucleotide translocase (ANT), ATP and ADP, inhibits mPT activation, whereas nucleotides not transported by ANT are without effect (Hunter & Haworth 1979a, Zoratti & Szabo 1995) lead to the proposal that ANT is the protein of the inner mitochondrial membrane that can be deformed into a non-selective high conductance channel by calcium ions (Halestrap & Davidson 1990). Also, the ANT inhibitor atractylate, which traps the ATP/ADP binding site facing the cytoplasmic side, promotes mPT, while the inhibitor bonkreate, which locks ANT facing the matrix side, inhibits mPT. Purified ANT reconstituted in artificial membranes can form a calcium-sensitive pore resembling the mPT pore (Brustovetsky & Klingenberg 1996, Brustovetsky *et al.* 2002). Calcium has been proposed to bind to a matrix site of ANT, inducing a structural change in the protein with pore formation. Alternatively, calcium could bind to negatively charged cardiolipin which may destabilize the ANT-cardiolipin interaction essential for normal ANT function (Hoffmann *et al.* 1994). ANT also contains cysteine residues that can be cross-

linked by oxidative stress or sulfhydryl-group reagents (McStay *et al.* 2002). Further supporting a role for ANT is that CypD has been shown to directly bind to ANT in a CsA-sensitive manner (Crompton *et al.* 1998, Woodfield *et al.* 1998)

Several lines of evidence thus support a fundamental role of ANT in mPT. However, mPT can occur in the absence of ANT. Liver mitochondria lacking ANT have been shown to exhibit CsA-sensitive mPT. The mPT was no longer sensitive to ANT ligands and the calcium dose required to induce mPT was considerably higher than in controls. This suggested that ANT merely have an important regulatory role and is not the essential component of mPT (Kokoszka *et al.* 2004). Another interpretation is that ANT is the normal mPT component but that other less abundant members of the mitochondrial carrier family can fulfill this role in the absence of ANT (Halestrap *et al.* 2007). Yet another view is that unfolded membrane proteins form the mPT and that ANT usually is the most abundant of these (He & Lemasters 2002).

The mPT is considered to be formed at contact sites between the inner and outer mitochondrial membranes (Crompton *et al.* 2002, Brdiczka *et al.* 2006). The outer membrane voltage-dependent anion channel (VDAC) has been found to have mPT like properties when incorporated into artificial membranes, and that it can bind to ANT (Szabo *et al.* 1993, Crompton *et al.* 1998). In spite of this, recent gene deletion studies show that it is not essential for mPT, and even does not seem to exert any important regulatory role (Krauskopf *et al.* 2006, Baines *et al.* 2007). Several other proteins, such as the mitochondrial creatine kinase, hexokinase and the peripheral benzodiazepine receptor, have been suggested to be part of the mPT pore and to regulate its activity (Beutner *et al.* 1998). The most widely accepted view is that mPT is accomplished through a dynamic multiprotein complex that involves regulatory proteins of the outer membrane but that it is primarily an inner membrane event. The main structure consists of ANT, CypD, and possibly VDAC, and this complex has the ability to attract several other proteins (Crompton 1999, Halestrap *et al.* 2007, Wieloch *et al.* 2007).

Permeability transition in isolated brain mitochondria

The large body of characterizing work on the mPT has been performed in mitochondria isolated from liver or heart tissue. Studies on mPT in isolated brain mitochondria are more recent and some of these suggest that the mPT of the brain has unique characteristics or that the phenomenon is absent or insignificant.

The cyclophilin D inhibitor CsA was found to be neuroprotective in models of cerebral ischemia and hypoglycemia in the mid and late nineties, and this suggested that the mPT may be an important pharmacological target in the brain (Uchino *et al.* 1998, Uchino *et al.* 1995, Li *et al.* 1997, Friberg *et al.* 1998). Although CsA has several molecular targets, most noteworthy the serine/threonine phosphatase calcineurin, the relevance of mPT was supported by a beneficial effect of the non-calcineurin inhibiting (non-immunosuppressive) CsA-analog $MeVal^4$-CsA (Matsumoto *et al.* 1999) and also by the lesser degree of protection afforded by another calcineurin inhibitor that lack mitochondrial targets (Drake *et al.* 1996, Uchino *et al.* 2002, Yoshimoto & Siesjö 1999, Friberg *et al.* 1998).

In a study directly comparing brain and liver mitochondria, Berman *et al.* found that brain mitochondria did not undergo mPT and swelling when exposed to the same levels of calcium, phosphate and oxidants as liver mitochondria. The absorbance decrease of brain mitochondria following mPT triggering was only 10-14% of that in liver mitochondria, and it was concluded that brain mitochondria are insensitive to mPT induction and swelling by calcium (Berman *et al.* 2000). In another series of studies, Andreyev *et. al.* noted that isolated respiring brain mitochondria suspended in a buffer containing levels of ADP and ATP matching the total levels of adenine nucleotides found in normal tissue homogenates did not undergo mPT in response to calcium, and the authors argued that brain mitochondria are relatively resistant to mPT induction (Andreyev & Fiskum 1999, Andreyev *et al.* 1998). Other studies have demonstrated an mPT phenomenon in brain mitochondria with swelling and loss of $\Delta\psi_m$ upon calcium administration (Friberg *et al.* 1999, Friberg *et al.* 1998), but some have failed to demonstrate more than a partial inhibition by CsA and concluded that CsA is a less potent inhibitor of mPT in brain mitochondria compared to other mitochondria (Brustovetsky & Dubinsky 2000b, Kristal & Dubinsky 1997, Kristal *et al.* 2000). A third suggested difference of brain mitochondria compared to other mitochondria is a more heterogeneous response to calcium and CsA. Kristián *et al.* found an incomplete swelling response to calcium and argued that brain mitochondria are heterogeneous in their response to calcium with resistant subpopulations (Kristián *et al.* 2000, Kristián *et al.* 2002). Similarly Lifshitz *et al.* proposed a population model of brain mitochondria in which they are subdivided into calcium-sensitive and insensitive as well as CsA-sensitive and insensitive populations, in order to explain the heterogeneous response of mitochondria from different brain regions to traumatic brain injury (Lifshitz *et al.* 2003).

OBJECTIVES

The overall objective of this thesis was to characterize the mitochondrial permeability transition phenomenon in brain mitochondria and to evaluate its role as a pharmacological target. The specific aims were:

1. To study if the mitochondrial permeability transition phenomenon can be detected in brain mitochondria similar to the phenomenon found in liver mitochondria (Paper I-II).

2. To determine if and under which conditions cyclosporin A inhibits the mitochondrial permeability transition in brain mitochondria (Paper I-II).

3. To evaluate the potencies of two non-immunosuppressive cyclosporin analogs and to screen a library of cyclosporin compounds for inhibition of permeability transition in brain mitochondria (Paper III-IV).

4. To investigate the effect of calcium overload and permeability transition on the generation of reactive oxygen species in rodent and human mitochondria (Paper IV).

5. To assess how permeability transition can be reliably detected, and determine the relationship between mitochondrial calcium retention and permeability transition (Paper I-V).

6. To explore how increased inner membrane potassium conductance influences brain mitochondrial calcium retention and how calcium retention can be related to matrix pH changes (Paper V).

METHODS

Animals
Animal procedures were approved by the Malmö/Lund Ethical Committee for Animal Research (M229-00, M221-03, M44-07). Adult male Wistar rats (300-500 g) or C57BL mice (20-30 g) were allowed *ad libitum* access to water and food prior to use. Tissues were removed following rapid decapitation of rats or cervical dislocation of mice (Paper I-IV) or alternatively, decapitation after a brief sedation with isoflurane (Paper V).

Human tissue
Human liver tissue was obtained from 4 male patients, 56-65 years old, undergoing liver resection due to colorectal cancer metastases. The human study was approved by the Ethical Committee of Hachioji Medical Center, Tokyo Medical University, permit number 12-01, and complies with the World Medical Association Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects and the EU Convention for the Protection of Human Rights and Dignity of the Human Being with Regard to the Application of Biology and Medicine: Convention on Human Rights and Biomedicine.

Materials
Amplex Red, Rhodamine 123, Calcium Green 5N and Fura 6F were purchased from Molecular Probes (Eugene, OR), Percoll solution, ^{3}H-acetate, ^{3}H-H_2O and ^{14}C-sucrose from Amersham Biosciences (Uppsala, Sweden) and Ready Safe scintillation cocktail from Beckman Coulter (Bromma, Sweden). NIM811 (MeIle^{4}-CsA) was kindly provided by Novartis (Basel Switzerland) and Debio-025 (UNIL025, MeAla^{3}EtVal^{4}-CsA) was from Debiopharm S.A. (Lausanne, Switzerland). Cyclosporin-A (CsA) and other cyclosporin analogs were provided by IVAX-Pharmaceuticals (Ceske Budejovice, Czech Republic). All other chemicals were from Sigma (St. Louis, MO).

Preparation of isolated brain mitochondria
Isolation of free, *i.e.* non-synaptosomal brain mitochondria was achieved using a Percoll gradient according to Sims, method B (Sims 1990), with slight modification. The brain was rapidly removed to ice-cold isolation buffer (320 mM sucrose, 2 mM EGTA, 10 mM trizma base, pH 7.4). Preparation was carried out under ice-cold conditions. Underlying structures and pia mater were removed from cortical tissue using curved forceps. The tissue (10% w/v) was homogenized in 1 ml isolation buffer (IB) containing 12% (v/v) Percoll, using a 2 ml Kontes teflon homogenizer, size 19, total clearance 0.05 mm (Vineland, NJ, USA). The homogenate was added on top of a Percoll gradient, 40% and 26% respectively, and centrifuged in a Beckman Optima MAX-E

Ultracentrifuge, 100.3 rotor (Palo Alto, CA, USA) at 30700 *g* for 7 min, yielding a dense fraction between the two lower Percoll layers (fraction 3). The latter was collected and diluted 1:4 with isolation buffer, followed by a washing step at 16700 *g* for 12 min. A last washing step containing isolation buffer and BSA (0.5 mg/ml) was performed in an Eppendorf microcentrifuge (Hamburg, Germany) at 7300 *g* for 7 min yielding a dense mitochondrial pellet that was resuspended in isolation buffer. All brain mitochondrial experiments were run within 5 h following decapitation.

Preparation of isolated liver mitochondria

Isolation of liver mitochondria was either performed using the Percoll gradient protocol described above, except smaller tissue pieces were homogenized (3-5% w/v) in order to get a demarcated fraction 3, or using a differential centrifugation protocol including a Percoll density step to remove contaminating membranes (Halestrap 1987). In the latter, liver tissue (10% w/v) was homogenized in ice-cold IB with 0.5% BSA using a 10 ml Kontes Teflon Homogenizer, size 22. The homogenate was centrifuged in a Beckman Avanti Centrifuge with F1010 rotor at 311 *g* for 10 min at 4°C. The supernatant was decanted and centrifuged at 7800 *g* for 5 min. The resulting pellet was resuspended in 8 ml isolation buffer with 19% (v/v) Percoll, and centrifuged at 11220 g for 10 min. The supernatant was removed and the pellet was resuspended in 8 ml isolation buffer and again centrifuged at 7800 *g* for 5 min.

Quantification of mitochondrial protein content

Mitochondrial protein content was determined using the Bradford method (Bradford 1976). Absorbance at 595 nm was determined in a Hitachi U-2800 Spectrophotometer (Tokyo, Japan) or a Bio-Rad Model 680 microplate reader (Bio-Rad Laboratories AB, Sundbyberg, Sweden) using BSA as standard.

Quantification of mitochondrial citrate synthase activity

Citrate synthase activity was followed spectrophotometrically at 412 nm by measuring the rate of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB, 0.1 mM) oxidation as acetyl-CoA (50 µM) and oxaloacetate (0.25 mM) is converted to citrate and CoA-SH in 50 mM Tris-HCl medium, pH 8.0, with 0.1% Triton-X100.

Mitochondrial swelling

Changes in mitochondrial volume and configuration were monitored by following changes in 90° light scattering at 520 nm in a Perkin-Elmer Luminescence Spectrometer LS-5B or LS-50B (Emeryville, CA, USA) with a temperature controlled cuvette holder and magnetic stirrer. The decrease in light scattering has been shown to closely parallel the percentage of the mitochondrial population undergoing swelling as a result of permeability

transition (Hunter & Haworth 1979a). Light scattering changes were followed in mitochondria under de-energized conditions at 28°C or under energized conditions in KCl- or sucrose-based buffers at 37°C. De-energized experiments were performed in a buffer containing 150 mM KCl, 20 mM MOPS, 10 mM trizma base, pH 7.3, the respiratory inhibitors rotenone (0.5 μM) and antimycin A (0.2 μg/ml), and the calcium ionophore A231872 (2 μM) to ensure equilibration of calcium across the inner mitochondrial membrane. For calcium concentrations up to 200 μM, 2 mM nitrilotriacetic acid (NTA) was added and free [Ca^{2+}] was calculated from the NTA buffering (Connern & Halestrap 1994). Experiments with energized mitochondria were performed in buffers containing either 125 mM KCl and 20 mM trizma base or 250 mM sucrose, 20 mM MOPS and 10 mM trizma base, pH 7.1. Since the buffering of trizma base is dependent on temperature, pH was set at 37°C. Both buffers included 2 mM P_i(K), 1 mM $MgCl_2$ and 1 μM EGTA with 5 mM malate and 5 mM glutamate as respiratory substrates. Transient swelling experiments were performed in KCl buffer and added calcium was chelated with EGTA after 2 min. The adenine nucleotide ADP (20 μM) and the ATP synthase inhibitor oligomycin (1 μg/ml) were present when evaluating cyclosporin A effects on the induction of mitochondrial swelling. The non-specific ionophore alamethicin was employed to induce a standardized swelling response independent of mPT. The degree of light scattering decrease following calcium exposure was divided by that induced by alamethicin (7.5 - 10 μg/ml) and is expressed as % of maximal (*i.e.* alamethicin-induced) swelling.

Mitochondrial membrane potential ($\Delta\Psi_m$)

All fluorescence experiments were performed under similar conditions and the buffers used for swelling experiments in the Perkin-Elmer Luminescence Spectrometer LS-50B. Three different fluorescent probes were used to monitor qualitative changes of $\Delta\Psi_m$. In Paper II, JC-1 at 200 nM and Safranin O at 1 μM were employed. JC-1 accumulation upon exposure to the negatively charged mitochondrial inside is accompanied by a shift in emission. Membrane potential was followed as the ratio of emission at 596 nm over 536 nm with excitation at 490 nm. Safranin O was excited at 485 nm and emission at 580 nm was followed. Rhodamine 123 at 100 nM was used in Paper IV and V with excitation and emission set to 490 nm and 528 nm, respectively. Accumulation of safranin O and rhodamine 123 in mitochondria is driven by $\Delta\Psi_m$, and results in quenching and decrease in their fluorescence. Depolarization results in dequenching of the signal, and consequently an increase in fluorescence. The protonophore carbonyl cyanide m-chlorophenylhydrazone (CCCP) was used at 0.5 - 1 μM to maximally depolarize the mitochondria.

NAD(P)H autofluorescence
The redox status of NAD(P)H was determined qualitatively by following its autofluorescence (excitation 340 nm, emission 460 nm). Antimycin A could not be used in this assay as it interfered with the NAD(P)H signal.

Mitochondrial production of reactive oxygen species (ROS)
Mitochondrial release of ROS was detected by following the H_2O_2-sensitive oxidation of 1 μM Amplex Red (N-acetyl-3,7-dihydroxyphenoxazine) to the fluorescent product resorufin in presence of horseradish peroxidase (HRP, 0.5 U/ml) and superoxide dismutase (SOD, 20 U/ml). Excitation and emission wavelengths were set to 560 nm and 590 nm, respectively. Known amounts of H_2O_2 were added to establish a calibration curve (in the presence of mitochondria without substrates). All the compounds used in the ROS experiments were tested under similar conditions without mitochondria to control for unspecific interactions with the probe. Several compounds had to be excluded and the respiratory inhibitors rotenone and antimycin A had to be prepared freshly or stored frozen in dimethyl sulfoxide (DMSO) due to such interactions. NADH could only be used at low concentrations (Votyakova & Reynolds 2004).

Mitochondrial calcium retention capacity
Mitochondrial calcium uptake and release were monitored using extramitochondrial calcium-sensitive fluorescent probes. Experiments were performed in energized KCl buffer as described above. In order to enable calcium uptake, the samples were supplemented with 200 μM ATP and 50 μM ADP in presence of 1 μg/ml oligomycin.
The excitation ratio of Fura 6F (340/380 nm with emission at 509 nm, 250 nM) or the fluorescence of Calcium Green 5N (excitation 506 nm and emission 532 nm, 100 nM) was followed as mitochondrial suspensions were continuously infused with 200 nmol $CaCl_2$/mg/min (10 μM/min). The start of calcium uptake was defined as the point where the experimental curve deviated from a control curve run with 1 μM ruthenium red present, blocking mitochondrial calcium uptake through the uniporter. Calcium retention capacity was calculated as the amount of infused calcium from the start of mitochondrial calcium uptake until start of maximal calcium release.

Mitochondrial respiration
Mitochondrial oxygen consumption was measured in airtight chambers using Clark-type oxygen electrodes. In Papers I-IV, experiments were performed in equipment from Hansatech (Norfolk, UK) with 0.1 mg mitochondria suspended in 0.4 ml KCl buffer at 30°C. Mitochondrial respiratory states were measured as defined by Chance and Williams (Chance & Williams 1955) except respiratory substrates (5 mM malate and glutamate) were present from start of

experiments. Respiratory control ratios were determined by dividing state 3 respiration following ADP addition with state 4 respiration after ADP was converted to ATP. In order to evaluate respiratory changes following mPT in more detail and to follow oxygen consumption of mitochondria during calcium infusion under similar conditions as calcium retention capacity experiments, a second set of experiments were performed in an Oroboros Oxygraph-2k. These experiments were run at 37°C in 2 ml KCl buffer using DatLab 4 software allowing on-line respiration rate output with high sensitivity, low noise and concentration-dependent background correction (Oroboros Instruments, Innsbruck, Austria) (Hütter *et al.* 2006).

Measurement of cytochrome *c* release and GSH content

An ELISA kit for detection of rodent cytochrome *c* (Quantikine® M, R&D Systems, Abingdon, UK) was employed to measure cytochrome *c* release. Mitochondrial samples were collected after experiments measuring light scattering or H_2O_2 production. Directly at the end of experiments, 1 mM EGTA was added to prevent further calcium insult. The samples were supplemented with a protease inhibitor cocktail (Sigma P-2714), rapidly chilled on ice and centrifuged at 7000 *g* for 10 min. After a second centrifugation of the supernatant at 436000 *g* for 60 min the supernatants and pooled pellets were tested for cytochrome *c* content. Total GSH content was determined using a Glutathione Colorimetric Detection Kit (ApoGSH^{TM}, BioVision, Mountain View, CA, USA), generating 2-Nitro-5-thiobenzoic acid from DTNB (5,5′-Dithiobis(2-nitrobenzoic acid) and GSH. GSH content was determined by measuring absorbance at 415 nm.

Matrix pH measurements

Changes in intramitochondrial pH were measured by determining the equilibrium distribution of the weak acid acetate (Nicholls 1974). The uncharged and permeant protonated species (HAc) will equilibrate over the mitochondrial membranes, while the impermeable anion (Ac^-) accumulates in an alkaline compartment such as the mitochondrial matrix due to increased dissociation. The pH difference can be determined by the relationship $\Delta pH = \log_{10} Ac^-_{ext}/Ac^-_m$ where ext and m refers to extra-matrix and matrix compartments respectively. A whole preparation of brain mitochondria (approx. 700 µg) was divided in half and incubated in ordinary KCl media supplemented with ^{3}H-labeled acetate (0.5 µCi or 18.5 kBq/ml) and ^{14}C-labeled sucrose (0.058 µCi or 2.1 kBq/ml). ^{14}C-sucrose does not permeate the inner mitochondrial membrane and was used to subtract non-matrix ^{3}H activity. Following incubation with either 3 pmol/mg valinomycin or vehicle (ethanol), the suspension was carefully layered on top of 800 µl silicone oil AR 110 and centrifuged at 20800 *g* for 2 min. Samples of supernatant (2 x 2 µl) were transferred to 500 µl H_2O before it was discarded together with the silicone oil.

The mitochondrial pellet was resuspended in 500 µl H_2O in scintillation vials (HDPE 24 ml, VWR) and 5 ml Ready Safe scintillation fluid was added. Samples were measured in a LS 6500 Scintillation counter (Beckman Coulter, Fullerton, CA). Similar experiments were performed with ^3H-H_2O instead of acetate for matrix volume estimates.

Electron microscopy

Mitochondrial samples were prepared following incubation in buffers or following exposure to calcium or alamethicin. The suspension was rapidly chilled and centrifuged in an Eppendorf microcentrifuge at 12000 *g* for 2 min. Samples were fixed in a solution containing 0.1 M Sörensen buffer, 1.5% Paraformaldehyde and 1.5% Glutaraldehyde over night, and then resuspended and washed in 0.1 M Sörensen buffer twice. The samples were further embedded in agarose, post-fixed in 1% osmium tetroxide, washed in 0.1 M phosphate buffer, dehydrated in graded series of acetone and embedded in Agar 100 which was polymerized for 48 h at 60°C. Sections, 50-70 nm, were cut in a LKB SuperNova Ultratome and stained with 4% uranylacetate followed by 0.5% lead citrate. Electron micrographs were obtained using a Philips CM 10 microscope (Eindhoven, The Netherlands).

Statistical analysis

Data is presented as mean ± SEM or SD for at least 3-4 separate experiments and analyzed with one-way ANOVA and the Bonferroni *post hoc* correction for multiple comparisons or unpaired students *t*-test using StatView 5.0 (SAS Institute Inc. Cary, NC). The level of statistical significance was set to 5%.

RESULTS AND CONCLUSIONS

Brain mitochondria exhibit a concentration-dependent sensitivity to calcium-induced permeability transition (Paper I-II)

In the first two studies, we examined if isolated brain mitochondria undergo permeability transition (mPT) in response to calcium challenges. In Paper I, brain mitochondria were studied under so called de-energized conditions where the respiratory complexes are inhibited and a calcium-ionophore is introduced so that added calcium equilibrates over the inner mitochondrial membrane. By measuring light scattering, mitochondria were found to swell upon calcium administration to an extent and rate dependent on the calcium concentration. The light scattering decrease at higher calcium concentrations was close to that induced by the non-specific pore forming drug alamethicin, and electron micrographs confirmed that the mitochondria underwent a configurational change following calcium. The response of brain and liver mitochondria was qualitatively similar, but much higher concentrations of calcium were needed in order to swell liver mitochondria under these conditions. In Paper II, mitochondria were studied under more physiologically relevant conditions with supplemented respiratory substrates. Under these conditions, mitochondria build up a protonmotive force and calcium uptake is elecrophoretic and hence dependent on the membrane potential ($\Delta\Psi_m$). Brain mitochondria were found to undergo extensive light scattering decrease in both KCl- and sucrose-based buffers with concomitant loss of membrane potential. In KCl medium the swelling response was very rapid and further, the light scattering decrease, the $\Delta\Psi_m$ loss and the morphological swollen appearance in electron micrographs were reversible to a large extent and dependent on the duration and concentration of the calcium insult. By using flow cytometry analysis of the mitochondria, it could be demonstrated that the swelling response and the recovery response were due to general shifts of the entire population of mitochondria rather than shifts in subpopulations of mitochondria.

Permeability transition in brain mitochondria is highly sensitive to cyclosporin A (CsA) inhibition under de-energized conditions, during transient swelling or in presence of ADP (Paper I-II)

The second objective of Paper I-II was to study if CsA inhibits mPT in brain mitochondria. Under de-energized conditions, there was a clear dose-dependent inhibition of calcium-induced swelling with significant effect already at 10 nM and an IC_{50} at 23 nM. The maximal inhibition at 1 μM was effective over a wide range of calcium concentrations. In respiring mitochondria in the absence of adenine nucleotides CsA only marginally prevented the onset of calcium-induced swelling but demonstrated a pronounced improvement of the recovery following removal of calcium. The resulting degree of swelling following a transient calcium insult was thus diminished by CsA. The dose-response curve

was very similar to that under de-energized settings and likewise, CsA had a significant effect over a wide range of calcium doses. In contrast to the effect in the absence of adenine nucleotides, CsA exhibited a prominent inhibition of the induction of swelling in presence of ADP and both light scattering and $\Delta\Psi_m$ decreases were prevented at a dose calcium that induced extensive swelling without CsA. In liver mitochondria, CsA inhibited induction of swelling in the absence of externally added ADP. The reversibility of swelling was however less prominent in liver mitochondria and the CsA effect following a transient calcium insult was less than that in brain mitochondria.

The non-immunosuppressive cyclosporin analogs NIM811 and Debio-025 (UNIL025) are potent inhibitors of permeability transition in brain mitochondria (Paper III)

In this study, we utilized the assays for permeability transition in brain mitochondria established in the first two studies and evaluated the potencies of two new non-immunosuppressive cyclosporin compounds, NIM811 (MeIle^4-CsA) and Debio-025 (UNIL025 or MeAla^{3}EtVal^4-CsA). By performing dose-response studies of the cyclosporin analogs and CsA under de-energized conditions, the non-immunosuppressive analogs were found to exhibit the same maximal inhibition but with higher potencies than CsA. They also afforded similar inhibition as CsA under respiring conditions. Flow cytometric analyses demonstrated that there were no cyclosporin-insensitive subpopulations of mitochondria.

Calcium-induced increase in mitochondrial generation of reactive oxygen species is caused by permeability transition (Paper IV)

Mitochondrial generation of reactive oxygen species (ROS) is implicated in several pathological states of the central nervous system and calcium has been linked to an increased generation even though several studies show a decreased ROS generation upon brain mitochondrial calcium loading. The objective of Paper IV was to investigate if mPT can be the cause of calcium-induced ROS generation. We demonstrate that calcium loading of rodent brain mitochondria or human liver mitochondria induces an increased H_2O_2 generation if the calcium loading causes mPT. If mPT is inhibited with CsA and ADP or CsA only (brain and liver, respectively), the same calcium loading either decrease or is without effect on ROS generation. A library of cyclosporin compounds was evaluated in a swelling assay and the relative potencies correlated well with inhibition of ROS increase upon calcium loading.

An increased ROS generation could also be seen in mitochondria following permeabilization with alamethicin. An obligatory role for calcium in the mechanism for ROS generation was thus not seen. Calcium did however induce a direct interference with respiration in permeabilized mitochondria and a high

dose calcium induced more ROS and inhibited respiration more than a low dose, even though the extent of light scattering decrease was similar. In permeabilized mitochondria, ROS generation correlated with availability of respiratory substrates, and inhibition of the respiratory complexes induced dramatic increases in ROS generation. An explanatory model is proposed where the ROS-inducing effect of calcium and permeability transition depends on the balance of ROS-reducing and ROS-inducing factors. The former include loss of $\Delta\Psi_m$ and NADH, and the latter loss of energy-dependent antioxidant scavenging and a disturbed electron flow through the respiratory complexes caused by cytochrome *c* loss and calcium-induced damage.

An increased K^+ conductance of brain mitochondria is beneficial for the ability to buffer large calcium challenges (Paper V)

In the final study of this thesis, we demonstrate that calcium retention capacity of isolated brain mitochondria can be enhanced by increasing the potassium conductance of the inner mitochondrial membrane. This was achieved by administering low doses of the potassium carrier valinomycin, selected to induce a $\Delta\Psi_m$ decrease similar to that by ADP. The calcium retention of brain mitochondria was determined by following the extramitochondrial calcium concentration during a continuous calcium infusion. Mitochondria maintained a steady-state calcium concentration until there was a rapid release of calcium. The CypD inhibitor $MeAla^3EtVal^4$-CsA improved the extent of calcium retention. By following mitochondrial oxygen consumption in a high-resolution respirometer we demonstrated that respiratory inhibition and increased permeability for pyridine nucleotides occurs at the time of rapid calcium release, indicative of mPT. Increasing mitochondrial potassium conductance assumedly influences several physiological parameters that classically are believed to increase the sensitivity towards mPT, such as decreased $\Delta\Psi_m$ and increased pH. Contrary to the protective direct effect of protons on the stability of the permeability transition complex, an alkaline matrix pH was found to be beneficial for mitochondrial calcium retention. Protonophores or the electroneutral proton/potassium exchanger nigericin which both decrease the pH of the matrix, reduced the calcium retention capacity of brain mitochondria. Decreasing the pH of the buffer from 7.1 to 6.6 similarly decreased the ability to store calcium. With nigericin present, increased calcium retention could be obtained by increasing the pH of the medium. Increasing the potassium conductance with valinomycin increased the accumulation of tritiated acetate, which equilibrate over the mitochondrial membranes according to ΔpH, but it will also depend on volume changes of the matrix. We propose that the mechanism underlying the beneficial effect of increasing potassium conductance includes an enhanced alkalinity of the matrix, which increases the uptake and deprotonation of phosphate and thus increases the ability of mitochondria to form inactive calcium-phosphate complexes. Keeping free

calcium ions low due to increased complexation may be more important than the effects of pH, $\Delta\Psi_m$ and phosphate *per se* in preventing activation of mPT.

UNPUBLISHED AND PRELIMINARY RESULTS

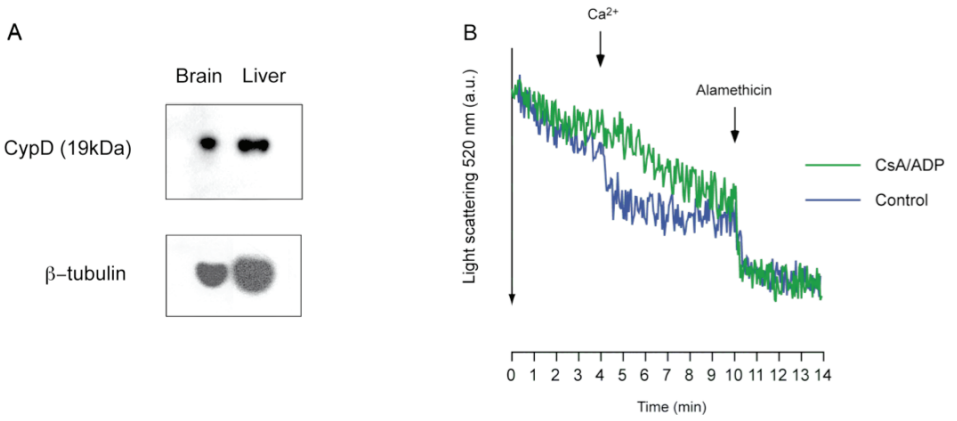

Figure 4. *Cyclophilin D (CypD) in human brain and liver homogenates, and calcium-induced mPT in isolated human brain mitochondria. A. Western blots of CypD and β-tubulin. Blots were probed with polyclonal anti-cyclophilin D and monoclonal anti-tubulin and detected using corresponding secondary horseradish peroxidase-labeled antibodies. 12 µg/lane was used for brain and 22 µg/lane for liver. (courtesy of L. Chen and F. Shibasaki) B. Changes in light scattering of isolated human brain mitochondria, 12.5 µg/ml in 2 ml KCl buffer with 5 mM malate and glutamate at 37°C. Calcium (200 µM) was administered where indicated to mitochondria with or without 1 µM CsA and 200 µM ADP. The non-specific ionophore alamethicin was thereafter added to induce a standardized swelling response.*

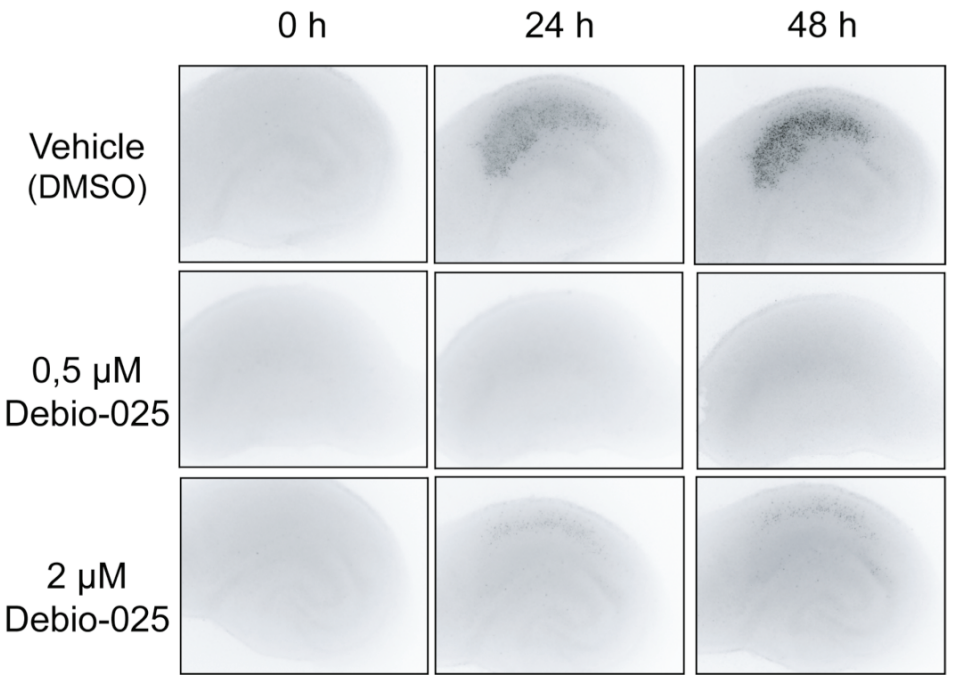

Figure 5. *Prevention of delayed cell death by the CsA analog Debio-025 in a model of in vitro ischemia of organotypic hippocampal slices (method described in Rytter et al. 2005). Representative photographs of hippocampal slices before ischemia and after 24 and 48 hours of recovery. Cell death was evaluated with the fluorescent cell death marker propidium iodide (PI). Debio-025 (also known as UNIL025 and MeAla*^{3}*EtVal*^{4}*-CsA) or vehicle (DMSO) was present in the medium from 20 h before experiment, during ischemia and in the recovery period.*

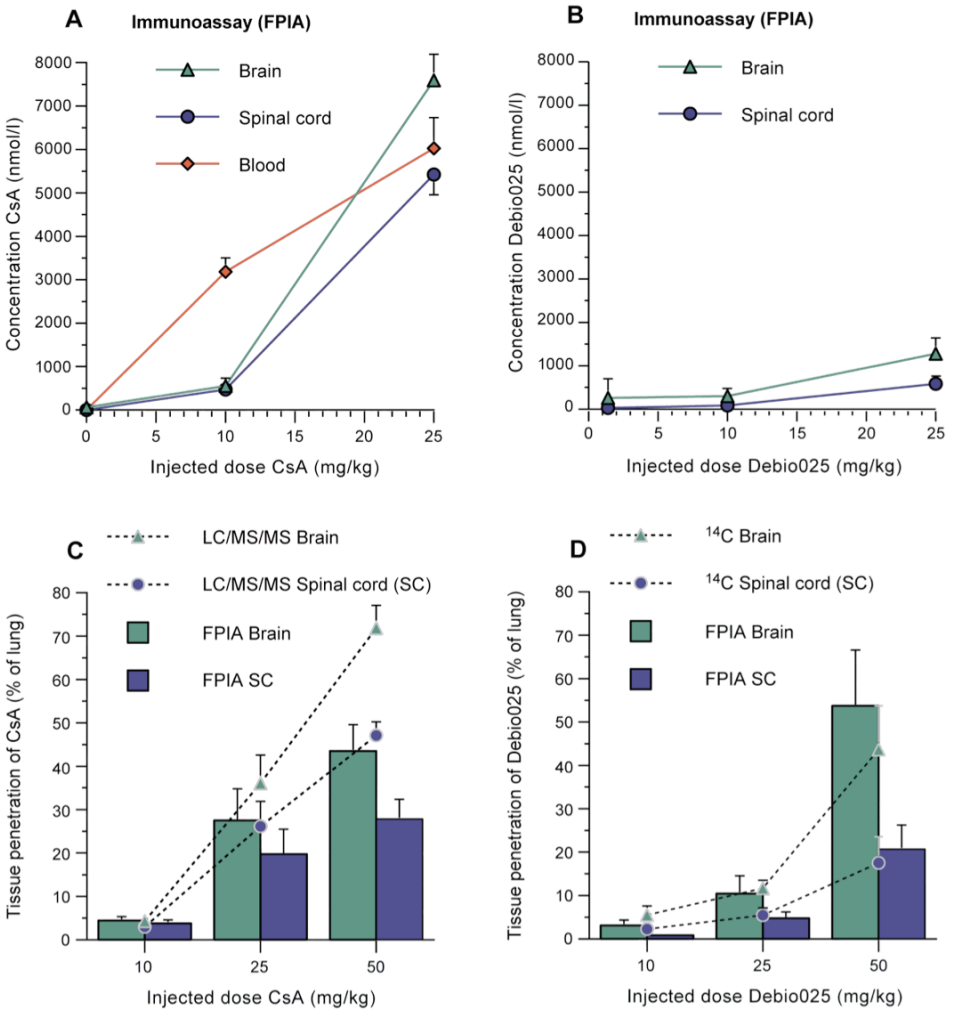

Figure 6. *Concentrations of cyclosporin A (CsA) and Debio-025 in the central nervous system following intravenous injections in rats. Samples were collected from animals 5 hours following injections. CsA was measured using a commercial fluorescence polarization immunoassay (FPIA) detecting CsA and metabolites (Abbot, Chicago, IL), or by using liquid chromatography with tandem mass spectrometry (LC/MS/MS). Debio-025 was measured in the same FPIA system by preparing a separate standard curve or by measuring activity of ^{14}C-labeled Debio-025 in a scintillation counter. A and B show tissue concentrations of CsA and Debio-025 measured by FPIA. C and D illustrate the relative tissue concentrations compared to lung concentrations. The injected doses of the compounds are given on the x-axis.*

DISCUSSION

Permeability transition in brain mitochondria

The results and conclusions from Paper I and II stand somewhat in contrast to some of the previous findings in isolated brain mitochondria mentioned in the background section. We find that mPT can be readily induced by calcium under various conditions and that different modes of mPT activation are CsA-sensitive, in particular if ADP is present.

The disparate conclusions are probably due to both methodological and conceptual differences. Assays of swelling commonly detect light scattering of mitochondrial suspensions at different angles, where 180° light scattering is similar to absorbance or transmission, as measured in standard laboratory spectrophotometers. It has been noted in early studies that 90° light scattering is much more sensitive to mitochondrial swelling and mPT induction than absorbance (Lehninger 1962, Hunter & Haworth 1979a). We have noted a clear difference between the relative extent of absorbance and 90° light scattering decrease following mPT in brain mitochondria, in particular when compared with the swelling induced by the non-specific ionophore alamethicin (unpublished results). The use of absorbance assays has probably contributed to the unexpected conclusion that brain mitochondria are resistant to calcium-induced mPT and swelling (Berman *et al.* 2000, Eliseev *et al.* 2007). The reported dissimilarities between mitochondria from different tissues in swelling assays may be due to physical properties such as size and density. Functional mPT assays measuring calcium retention, $\Delta\Psi_m$ and NAD(P)H redox status generally report similar qualitative responses following calcium-induced mPT, even though the relative effects of different inducers and inhibitors of mPT may be markedly different between tissues (Panov *et al.* 2007, Panov *et al.* 2004, Kristal *et al.* 2000, Chalmers & Nicholls 2003). The second line of argument against mPT in brain mitochondria derives from studies were mitochondria are suspended in medium containing a physiological level of ATP, around 3 mM. Brain mitochondria have been found to be resistant to calcium-induced mPT under these conditions, and it has been concluded that mPT is likely not activated by calcium in the brain under physiological conditions (Andreyev & Fiskum 1999, Andreyev *et al.* 1998). This is likely a probable scenario. Considering the bioenergetic consequences of mPT, activation under physiological conditions would likely be detrimental to the cell, even if mPT is limited to a few mitochondria or transiently induced. Transient mPT-induction has however been suggested to be a mechanism of fast calcium release from mitochondria and this has been postulated to represent a normal physiological function of the mPT pore (Gunter & Pfeiffer 1990, Bernardi & Petronilli 1996, Ichas & Mazat 1998). So-called pore-flickering has been demonstrated both in isolated mitochondria and in cells (Huser *et al.* 1998, Petronilli *et al.* 1999,

Gillessen *et al.* 2002), but if this actually represents a physiological condition or rather a transitional state prior to full induction, indicative of cell stress, has been debated (Crompton 1999). A role for mPT under pathological conditions in the cell is more likely, and relies on more solid experimental evidence. The pathological conditions where mPT is most convincingly indicated, such as ischemia, hypoglycemia and excitotoxicity, include a severe depletion of energy metabolites or an oxidative state in combination with increased calcium and possibly oxidative stress. The finding that mPT is not readily induced under physiological conditions *in vitro* does not preclude a prominent role for mPT *in vivo* under conditions where its activation would be favored.

The enhanced CsA effect by ADP in brain mitochondria has also been demonstrated by several other researchers. Panov *et al.* demonstrated that the combined effect of ADP and CsA in inhibiting mPT was synergistic and much greater than the sum of ADP and CsA separately (Panov *et al.* 2007, Panov *et al.* 2004). By following calcium loading of mitochondria in presence of adenine nucleotides, several other authors have found a significant effect of CsA (Chalmers & Nicholls 2003, Brown *et al.* 2006, Naga *et al.* 2007, Damiano *et al.* 2006, Shalbuyeva *et al.* 2007)

The conclusion that brain mitochondria have the ability to undergo mPT and that mPT in brain is CsA-sensitive, in combination with pharmacological studies indicating mPT in several models of neurological disease, support a role of mPT in neurological cell death. Recent studies using genetically modified mice that lack CypD (*Ppif* $^{-/-}$), the mitochondrial target for CsA, have provided strong support for this conclusion (Baines *et al.* 2005, Forte *et al.* 2007, Schinzel *et al.* 2005). The much-debated role for mPT in different cell death modes has also been clarified as will be discussed below. Despite these studies supporting a role of CypD in several pathologies of the brain, it has been suggested that CypD is downregulated in the mature rodent brain and that this explains the inability of brain mitochondria to swell (Eliseev *et al.* 2007). While much evidence in rodents argue against this hypothesis, it is possible that CypD downregulation occurs in the human adult brain, but this has not been studied. In a preliminary pilot experiment we have examined if CypD is present in the adult human brain and if isolated human brain mitochondria undergo mPT. Preliminary results indicate that respiring brain mitochondria contain CypD and undergo CsA-sensitive calcium-induced swelling (Figure 4).

In Paper II we also study whether the mPT phenomenon is similar within the whole brain mitochondrial population or if there are indications of subpopulations with different sensitivities to calcium and/or CsA, which has been proposed (Kristián *et al.* 2000, Kristián *et al.* 2002, Lifshitz *et al.* 2003). The conclusion of our studies was that activation of mPT as well as the

protection afforded by CsA and the recovery following a transient swelling were all rather homogenous responses distributed throughout the whole mitochondrial population and not selective responses of subpopulations. However, this conclusion cannot be extended to all brain mitochondria being similar in other senses, only that all mitochondria seem to possess the ability to undergo mPT and to be sensitive to CsA inhibition. Both in the de-energized assay in Paper I and in the energized assays in Paper II, the mitochondria behaved as though they individually have a certain probability to undergo mPT with a progressively larger part of the mitochondrial population undergoing swelling as the calcium challenge is increased. When comparing mitochondria from different subregions of the brain, different cell types, or even different cell compartments, several studies have noted differences in the activation of mPT. Thus even within the brain not all mitochondria are created equal.

Several studies have noted different susceptibilities of mitochondria from different brain regions to activation of mPT that have correlated with selective vulnerability in different disease models (Friberg *et al.* 1999, Mattiasson *et al.* 2003, Brustovetsky *et al.* 2003, Sullivan *et al.* 2004). Different mitochondrial contents of adenine nucleotides or CypD have been suggested to underlie these differences. Synaptic brain mitochondria have been found to be more susceptible to calcium overload and mPT than non-synaptosomal mitochondria (Brown *et al.* 2006, Naga *et al.* 2007). The authors attribute this difference to an increased content of CypD in synaptosomal mitochondria due to higher content of neuronal mitochondria, which they find have higher CypD content. The higher CypD level would accordingly sensitize mitochondria towards mPT. The latter conclusion stands somewhat in contrast to the findings of Bambrick *et al.* where CsA enhanced calcium loading in permeabilized cultured astrocytes but not in cultured cerebellar granule neurons (Bambrick *et al.* 2006). The discrepancy between the *in vivo* and the cell culture results may be explained by the finding that cultured neurons seem to downregulate CypD (Eliseev *et al.* 2007) and this could also perhaps explain some discrepancies in results with CsA in cultured neurons although this has not been evaluated. Even though the CypD content could partly explain the different abilities of synaptic and non-synaptic mitochondria to retain calcium, a preserved difference was found following maximal CsA inhibition of CypD and also in CypD knock-out mice (Naga *et al.* 2007). The remaining difference in calcium retention capacity was similar to that we have found between brain cortical and spinal cord mitochondria (Morota *et al.* 2007). In this study, spinal cord mitochondria were able to take up considerably less calcium than cortical brain mitochondria but the mPT-sensitivity to calcium in several other assays was similar. However, the set-point concentration for mitochondrial calcium uptake was different for the two populations of mitochondria. These findings indicate that several parameters of mitochondrial function that are not directly related to the

regulation of mPT may also influence the resistance of mitochondria towards calcium.

Detection of mPT in brain mitochondria

Throughout the studies of this thesis several different methods have been employed to evaluate the characteristics and consequences of mPT. A general view that has emerged is that the conclusions drawn from an experiment should follow the precision and specificity of the methods used. This is of particular importance when searching for and evaluating putative inhibitors of mPT or other pharmacological targets in mitochondria. However, several pitfalls of an assay may not be obvious.

The 90° light scattering assay has in our hands been a rapid and sensitive method for detecting morphological changes in brain mitochondria. Absorbance (or 180° light scattering) has been much less sensitive (unpublished observations). Methods evaluating functional parameters of mitochondrial integrity, such as NADH redox status and $\Delta\Psi_m$ are also sensitive to mPT changes and are in many cases a necessary complement to light scattering assays. An inhibition of mitochondrial swelling, readily detected in the light scattering assay, does not necessarily have to be caused by modulation of the mPT components or associated proteins. An inhibition can also be seen if mitochondrial calcium uptake is inhibited. If the inhibition of calcium uptake is due to specific interaction with the mitochondrial calcium uniporter this may be an important pharmacological target. If the inhibited calcium uptake is due to reduced electrophoretic uptake of the cation due to inhibition of respiration or extensive uncoupling of oxidative phosphorylation, the inhibition of mPT cannot be regarded to be a pharmacological target, rather a side effect of toxic dosage. The conclusion that the neuroprotective action of minocycline is caused by direct mPT inhibition is probably an example of the latter phenomenon (Teng *et al.* 2004, Wang *et al.* 2003, Zhu *et al.* 2002). Calcium-induced swelling of mitochondria, as detected in a light scattering assay, can be inhibited by high doses of minocycline. However, when monitoring functional parameters of mitochondria, an uncoupling and respiratory inhibition by minocycline was seen (Månsson *et al.* 2007). Under the same conditions in which minocycline prevented swelling, it also completely blocked calcium uptake. Further, the results of minocycline could be mimicked by using a high dose of a protonophore (CCCP), which completely uncoupled mitochondria and consequently prevented calcium uptake. Similar unspecific effects can probably be seen by several drugs if they are used at very high concentrations. We have therefore suggested that the minimal requirement for attributing mPT inhibition to a compound should include effects in several complementary assays

including prevention of depolarization as well as increased calcium retention capacity (Wieloch *et al.* 2007).

An integrative approach to evaluate mitochondrial calcium handling and mPT was introduced by Chalmers and Nicholls (Chalmers & Nicholls 2003). By following the extramitochondrial calcium concentration and continuously infuse the media with calcium, mitochondrial calcium retention capacity can be determined. Mitochondria start to take up calcium above a certain threshold and maintain an almost constant extramitochondrial calcium concentration until there is a rapid release of the accumulated calcium, which is considered to represent activation of mPT. This method has several advantages compared to traditional swelling methods. Firstly, the mitochondria take up calcium at a bioenergetic steady-state and rapid changes in mitochondrial redox status or membrane potential are avoided. Secondly, the assay provides a quantitative approach to evaluate mPT. Swelling or depolarization following administration of calcium bolus loads is often an all or nothing response of the whole mitochondrial population, and the quantification of mPT is somewhat arbitrary and often limited to either positive or negative effects. Using calcium retention capacity as an outcome allows for evaluation of both positive and negative effects over a wider modulatory range. Finally, mitochondrial calcium retention is dependent on an intact respiratory function as well as the ability to form inactive calcium-phosphate complexes. A parameter that may influence the mPT complex component towards one direction may influence the calcium buffering and thus the available free calcium in another, and the actual probability for mPT will depend on their relative importance. Important examples of this dual effect are demonstrated in Paper V for pH and $\Delta\Psi_m$. A low pH is considered to stabilize the mPT pore complex in a closed configuration, and this is apparent in mPT assays run under de-energized conditions (Halestrap 1991, Haworth & Hunter 1979) *i.e.* under situations where active calcium uptake and sequestration is inhibited. This is also true for brain mitochondria under similar conditions (Kristián *et al.* 2001, Costa *et al.* 2006, Friberg *et al.* 1999). In contrast, in functional and respiring mitochondria, a high matrix pH is required in order to form calcium-phosphate complexes and keep free calcium ions at a regulated low concentration (Nicholls 2005). Similarly, a high $\Delta\Psi_m$ is considered to stabilize mPT (Bernardi 1992), but if $\Delta\Psi_m$ is elevated in exchange for ΔpH by the electroneutral proton/potassium exchanger nigericin, calcium retention is dramatically reduced (Paper V).

The calcium retention assay, although sensitive for both adverse and beneficial influences on mitochondrial stability, lack somewhat in specificity. The improved calcium retention capacity of mitochondria in presence of CsA indicates that mPT is the limit of calcium sequestration (Chalmers & Nicholls 2003). By following light scattering or NAD(P)H autofluorescence during

calcium infusion, a decreased NAD(P)H fluorescence as well as decreased light scattering is seen at the time for calcium release (Paper V). These changes also indicate mPT, but this assumption is limited by the large artifactual NAD(P)H and light scattering signal that precedes the decrease.

The calcium sequestration of brain mitochondria with formation of calcium-phosphate complexes in the matrix causes an increase in the refractory index and the light scattering increases as more calcium is accumulated (Kristián *et al.* 2007). The increase in NAD(P)H fluorescence is likely also caused by the same artifactual phenomenon and not by an increased reduction state (Chalmers & Nicholls 2003). The decrease in NAD(P)H fluorescence and light scattering could therefore solely reflect calcium release and decreased amount of calcium-phosphate complexes. The release of calcium does not necessarily have to be caused by mPT, it could also reflect a reversal of the calcium uniporter (Zoccarato & Nicholls 1982).

In order to obtain a more specific correlation between the calcium release in the calcium retention capacity assays and activation of mPT, calcium infusion experiments were performed simultaneous to measurements of oxygen consumption in high-resolution respirometry equipment. At the time of calcium release, there was a concomitant inhibition of respiration and increased permeability to NAD^+ and NADH. Both the respiratory inhibition and the increased permeability to pyridine nucleotides are significant evidence for a permeability transition and thus confirmed that mPT can be regarded as the endpoint in the calcium retention assay.

In the pioneering characterizing studies of mPT by Hunter and Haworth, permeability changes were detected by following uncoupling of respiration (Hunter *et al.* 1976). A few years later, they introduced 90° light scattering as a more convenient tool to evaluate mPT (Hunter & Haworth 1979a). A few decades later, respiration remains the most powerful tool in evaluating mitochondrial integrity.

Reactive oxygen species and brain mitochondria

Mitochondria are often referred to as the main source of ROS in the cell, and the redox centers in the respiratory complexes are truly potential sites for tremendous ROS generation. At the same time, the antioxidant defenses in the mitochondria are very potent. Several other processes in the cell can produce ROS, *e.g.* oxidation of hypoxanthine or phospholipid degradation. Whether mitochondria actually are a net source of ROS or if they can function as a sink for ROS under normal conditions is not a trivial question to answer. Detection of ROS requires the use of probes that might have a higher scavenging affinity

than the endogenous antioxidants. Experiments with isolated mitochondria show that if the probes are added after mitochondria are removed from a suspension, no remaining ROS are detected (Nohl *et al.* 2003). Undisputedly though, there are numerous indications for ROS-mediated cell death in many diseases and it has also been suggested to be the cause of senescence (Nicholls & Budd 2000, Soane *et al.* 2007, Droge 2002). Certain conditions could favor the formation of very aggressive free radicals, such as formation of peroxynitrite ($ONOO^-$) in presence of superoxide and nitric oxide or formation of hydroxyl radical ($OH^\bullet$) if hydrogen peroxide reacts with transitions metals such as free iron.

Under which conditions brain mitochondria become a source for pathological ROS production was the focus of Paper IV. Calcium accumulation of mitochondria has been linked to increased ROS generation and several mechanisms have been suggested (Nicholls & Budd 2000, Soane *et al.* 2007). Our results in isolated brain and liver mitochondria show that calcium induces increased ROS generation if the mitochondria undergo mPT, but not if they remain intact. As several alterations following mPT tend to decrease ROS generation, such as abolished membrane potential, we argue that the simultaneous reduction in antioxidant capacity and a hampered electron transport through the respiratory complexes underlie the net increase in ROS. The mPT thus represents one condition in which mitochondria become a main source of ROS in the cell. Consequently, ROS as a result of mPT, in a larger or smaller proportion of the mitochondria in a cell, may be a significant contribution to the cell death pathways triggered upon its activation, adding to perturbations caused by diminished ATP production and release of proapoptotic proteins.

As oxidative stress is considered to trigger mPT, ROS release following mPT could cause more mitochondria to undergo mPT, a phenomenon known as ROS-induced ROS release (Zorov *et al.* 2000). While oxidants have a convincingly demonstrated role in triggering mPT in heart and liver mitochondria, the role in isolated brain mitochondria is less clear. The oxidants phenylarsine oxide and *tert*-butyl hydroperoxide have lacked effect on mPT in some studies but sensitized mitochondria in others (Kristal & Dubinsky 1997, Friberg *et al.* 1999, Kristián *et al.* 2001, Schönfeld & Reiser 2007). In our hands, high doses of H_2O_2 or *tert*-butyl hydroperoxide have not significantly sensitized brain mitochondria to mPT (unpublished observations).

Many of the uncertainties about the role of mitochondrial ROS generation in normal cellular function as well as in pathological states may be advanced by the recent development of mitochondrially targeted antioxidants. By conjugating an antioxidant such as ubiquinone to the triphenylphosphonium

cation (TPP^+), the antioxidant is specifically targeted to mitochondria. The lipophilic TPP^+ readily pass membranes and is accumulated in mitochondria due to the $\Delta\Psi_m$. A first generation compound, MitoQ, reduces ROS and protects against ROS-mediated damage in mitochondria *in vitro*, but it remains to be tested if it is an effective antioxidant *in vivo* (Cocheme *et al.* 2007).

Regulation of the permeability transition pore versus mitochondrial complexation of calcium

The primary objective of Paper V was to examine how potassium flux may influence mitochondrial calcium handling. Besides this, the results of Paper V emphasize the importance of evaluating mitochondrial responses to calcium overload from an integrated view and not solely from the regulation of the mPT pore. For example, it is firmly established that acidosis inhibits mPT activation, and mPT is thus not considered to open during ischemia when acidosis is prominent (Di Lisa & Bernardi 2006, Halestrap *et al.* 2007). The evidence for the acidosis mediated mPT inhibition comes almost exclusively from studies of mPT under de-energized conditions, *i.e.* under conditions when mitochondria do not actively sequester calcium (Bernardi *et al.* 1992, Halestrap 1991, Hunter & Haworth 1979a). While this setting may be a powerful experimental tool to evaluate isolated molecular interactions in mitochondria, it can not be considered to reflect the mitochondrial status in tissues. Even though mitochondria might be considered to be de-energized to some extent during ischemia, the calcium uptake leading to mPT will probably always be caused by active electrophoretic uptake. Our experiments demonstrate that less calcium is required in order to trigger mPT at low pH. Similar findings have also been obtained by Kristián *et al.* who founds that energized mitochondria became more sensitive to calcium-induced swelling at low pH (Kristián *et al.* 2001). This stands in sharp contrast to the effect of low pH on the mPT complex *per se*, and we have also confirmed this in our de-energized mitochondrial preparations (unpublished data). Given the importance of an alkaline matrix pH to keep free calcium ions low, the net effect of pH is probably more correlated to the effect on calcium complexation than to that on the mPT pore complex. The same line of argument can probably be used for several other factors, such as phosphate and $\Delta\Psi_m$. The effect of phosphate is similarly considered to be that of an increased tendency to activate mPT, but it is the availability of phosphate that determines the solubility of free calcium ions. Analogous to pH, phosphate is expected to have a similar dual effect. By increasing the external phosphate concentration from 2 mM to 5 mM, Chalmers and Nicholls found that mitochondria maintained a lower matrix concentration of free calcium, but despite this were sensitized to mPT (Chalmers & Nicholls 2003). In this case, the effect of phosphate on the mPT pore was probably more important than its effect on calcium complexation. In the case of $\Delta\Psi_m$, the mPT pore is often

defined as a voltage-dependent pore due to the sensitivity to the level of $\Delta\Psi_m$ (Bernardi *et al.* 2006, Zoratti & Szabo 1995). However, manipulation of $\Delta\Psi_m$ is usually achieved by using protonophores, and this will reduce the ΔpH as well, destabilizing any calcium-phosphate complexes formed, which may be equally if not more important for triggering mPT.

An integrated view on mitochondrial calcium handling is thus needed in order to predict what effect changing a parameter may have on mPT probability. Our work also demonstrates that mPT can be prevented by manipulating a physiological parameter, such as potassium conductance of mitochondria, and this strategy may be an alternative or additive strategy to prevent mPT *in vivo*.

How can increased potassium conductance be beneficial?
A partial uncoupling of mitochondria, so called mild uncoupling, has been suggested to mediate tissue protection under *e.g.* ischemia. A reduction of mitochondrial ROS generation as well as reduced electrophoretic uptake of calcium due to a decrease of $\Delta\Psi_m$ are two proposed mechanisms for how uncoupling could be beneficial. The first mechanism is based on the finding that mitochondrial ROS generation increases dramatically parallel to the reduction state of the redox centers in the ETC (Brand 2005, Andreyev *et al.* 2005). This is particularly evident when isolated mitochondria are feeded exclusively with the complex II substrate succinate, mediating so called reverse electron flow through complex I, but it has been questioned if this is true for mitochondria *in situ* (Tretter & Adam-Vizi 2007). Mitochondria within their physiological context in the cell are probably less hyperpolarized, due to constant ATP production than inactive mitochondria with saturated substrate supply, and a mild uncoupling in mitochondria with a lower $\Delta\Psi_m$ may reduce the energy-dependent antioxidative capacity as much as it reduces ROS.

The second mechanism for how a mild uncoupling may be beneficial to mitochondria, *i.e.* through reduced electrophoretic calcium uptake, was studied in detail in Paper V with an integrative view on mitochondrial calcium handling in focus. Mild uncoupling accomplished by using a protonophore was, in contrast to the above hypothesis, not beneficial for the calcium retention of brain mitochondria. Instead, it dose-dependently decreased the calcium sequestration. This effect was probably due to a decrease in ΔpH by the protonophore, decreasing the ability to form calcium complexes in the matrix. In contrast, uncoupling mitochondria with a potassium carrier, and thereby increasing the potassium cycling over the inner mitochondrial membrane, was beneficial and increased calcium retention.

We propose that a partial depolarization by increased potassium conductance results in a corresponding increase in ΔpH, and that this type of mild

uncoupling preserves or even enhances the alkaline environment in the matrix. Using an integrated view on mitochondrial calcium handling, we suggest that an enhanced alkalinization of the matrix improves mitochondrial calcium retention by increasing the availability of phosphate to complex-bind free calcium ions, thereby preventing mPT-activation. Consequently, opening of potassium channels in the inner mitochondrial membrane alkalinize the matrix and this in turn may increase the resistance of brain mitochondria to calcium overload in *e.g.* ischemia.

This suggestion is attractive but remains hypothetical as there is to date no substantial proof of specific mitochondrial potassium channels and mainly speculations of their conductance state when open. The most direct evidence for potassium channels comes from electrophysiological recordings. There are only a few studies of mitochondrial potassium channels published and they all suffer from a potential problem of contaminating membranes from other cellular sources than mitochondria. Isolated mitochondrial preparations always have contaminations, especially if they are not prepared using density gradients (Halestrap *et al.* 2007). None of the above mentioned studies report what means were taken to assure the purity of the preparations. The predominate proof comes from pharmacological studies, and the specificity of the pharmacological compounds used, such as diazoxide and 5-HD, have been questioned (Hanley & Daut 2005, Dröse *et al.* 2006, Halestrap *et al.* 2007). Another source of potassium flux in mitochondria have been suggested to be ANT, in particular in the absence of ADP or ATP or when it is trapped facing the cytoplasmic side (Halestrap *et al.* 2007). As ANT is considered to be the protein that can form the mPT pore, and mPT activation has been suggested to initially predominantly mediate electrophoretic potassium uptake via a lower conductance state than full-blown mPT (Brustovetsky & Dubinsky 2000a, Shalbuyeva *et al.* 2006, Halestrap *et al.* 2007), a short low-grade activation of mPT could possibly function as an ATP-sensitive potassium channel. In support of this is the hypothesis that transient mPT activation during preconditioning mediates the beneficial effect by reducing full-blown mPT during the actual ischemia (Hausenloy *et al.* 2004). An increased matrix alkalinization caused by the increased potassium flux during the short low-grade mPT could then underlie the increased resistance of the mitochondria to the calcium challenge in ischemia and reperfusion. However, experiments supporting such a course of events are needed in order to evaluate the merit of this hypothesis.

Permeability transition in cell death pathways

The role of mPT in cell death pathways has been a matter of debate. An outermost view on one side would be that it reflects a circumstantial phenomenon seen at the endpoint of cellular injury when no intervention is possible, and on the other side that it is a common denominator in cell death linking apoptosis to necrosis (Lemasters *et al.* 1998). The consequences of mPT with cessation of ATP production, loss of calcium homeostasis and generation of ROS implicate that it would lead to necrotic types of cell death, and a role for mPT in necrosis is fairly well established and accepted (Bernardi *et al.* 2006, Crompton 1999, Halestrap 2006). A prominent role for mPT in normal programmed apoptotic cell death as that seen during development seems unlikely. In particular after the findings that mice lacking CypD (*Ppif* $^{-/-}$) develop normally and cells from them respond normally to a range of apoptotic stimuli (Baines *et al.* 2005, Nakagawa *et al.* 2005, Schinzel *et al.* 2005). Further, cell lines overexpressing CypD are more prone to undergo mPT-mediated necrosis, but on the contrary seem resistant to some types of apoptotic cell death (Li *et al.* 2004). Even though mPT is not the mechanism by which apoptosis is mediated in the normal healthy animal, this does not preclude the possibility that mPT may be involved in triggering apoptotic cell death pathways under some pathophysiological conditions (Halestrap *et al.* 2007).

Since the programmed cell death pathways are energy-requiring processes, an involvement of mPT has to be either limited to a partial population of the mitochondria or it has to be only transiently induced. A sustained activation of mPT in all mitochondria would not only lead to a cessation of ATP production, it would also cause a reversal of complex V to utilize ATP in a futile attempt to build up protonmotive force, and as a consequence drain the cell of ATP produced during glycolysis.

In favor of a partial mitochondrial population undergoing mPT are observations of distinct microheterogeneity in cellular calcium transients. Upon stimulation of calcium release from endoplasmatic reticulum (ER), calcium reaches far higher concentrations at localized calcium "hot spots", which is believed to be where mitochondria associate with ER (Rizzuto *et al.* 1993). These microdomains of high calcium would then enable fast mitochondrial calcium uptake without inducing elevated calcium levels in the rest of the cell. Similar close apposition of mitochondria to plasma membrane calcium microdomains has also been suggested (Rizzuto *et al.* 2000). This implies that some mitochondria will be exposed to significant higher levels of calcium than others and that the probability of triggering mPT upon a pathological stimulus may vary considerably at different locations in the cell. A localized mPT may thus trigger cell death cascades that will not necessarily cause necrosis (Honda *et al.* 2005).

Likewise, a transient mPT, similar to that we and others have demonstrated *in vitro*, may trigger pathological cell death stimuli while preserving some ATP production (Paper II and (Shalbuyeva *et al.* 2006)). Evidence for such pathways *in vivo* remains mostly circumstantial, and it is not clear if such an mPT represents a low conductance state or the high conductance state permitting free equilibration of solutes <1500 Da. For example, Hausenloy and colleagues find evidence for a transient mPT mediating preconditioning in cardiac ischemia (Hausenloy *et al.* 2004). On the other hand, the classical high conductance permeability of mPT, which cause entrapment of deoxyglucose, has not been detected (Halestrap *et al.* 2007). In cultured hippocampal neurons, excitotoxic glutamate has been shown to induce reversible mitochondrial remodeling which was inhibited by cyclosporin compounds (Shalbuyeva *et al.* 2006). This may be related to mitochondrial morphological changes in global cerebral ischemia. In the model of transient global ischemia, cell death has the peculiar characteristic of being delayed, but the morphology of this ischemic cell death is neither apoptotic nor necrotic (Wieloch 2002). Both CsA and, at least partially, broad-spectrum inhibitors of caspases prevent cell death if administered shortly after reperfusion (Uchino *et al.* 1995, Chen *et al.* 1998, Himi *et al.* 1998). Mitochondria have been demonstrated to accumulate calcium shortly after ischemia (Simon *et al.* 1984, Zaidan & Sims 1994), and also to undergo a transient swelling following ischemia (McGee-Russell *et al.* 1970, Petito & Pulsinelli 1984), but if this represents transient activation of mPT is not known. Cytochrome *c* has been reported to be released within 30-60 min of reperfusion following global ischemia, and this as well as cell death were prevented by CsA (but not the calcineurin inhibitor FK506) (Domanska-Janik *et al.* 2004, Nakatsuka *et al.* 1999). Other studies have however noted prominent cytochrome *c* release and mitochondrial swelling only after 36 h following global ischemia, thus rather representing a secondary mitochondrial failure possibly correlating to cellular demise (Ouyang *et al.* 1999). Similar to localized mPT, a transient mPT can be speculated to be involved in more delayed cell death pathways which probably also include apoptotic mechanisms.

The prime triggers for mPT, calcium and ROS, as well as mPT itself may influence the cell death pathways governed by the Bcl-2 family proteins, and the Bcl-2 proteins may influence the triggering of mPT. The Bcl-2 family of proteins can modulate the cellular calcium homeostasis and thereby indirectly modulate the activation of mPT. For example, both Bcl-2 overexpression and Bax/Bak deletion alter cellular calcium handling, in particular by reducing the ER store of calcium, so that less calcium is released to mitochondria upon stimulation (Pinton *et al.* 2000, Scorrano *et al.* 2003). Caspase-3 can cleave proteins necessary for the calcium extrusion from the cell such as the plasma membrane calcium-ATPase and the sodium/calcium exchanger (Orrenius *et al.*

2003), which would increase the calcium challenge to mitochondria. Cytochrome *c* can also increase calcium-induced calcium release from ER by binding to inositol 1,4,5-trisphosphate (IP_3) receptors (Boehning *et al.* 2003). On the other hand, mitochondrial ROS generation can facilitate cytochrome *c* release by oxidation of cardiolipin and thereby destabilize their interaction (Ott *et al.* 2002). It has also been suggested that mPT may be responsible for structural reorganizations to ensure more rapid release of intermembrane proteins (Forte & Bernardi 2006). The cell death triggers following a pathological insult may therefore activate both pathways to different extents depending on the type of insult.

The mitochondrial permeability transition as a pharmacological target

The most common evidence for mPT as a mediator of cell death und thus its potential as a pharmacological target derives from studies using CsA. A neuroprotective effect by CsA can however not be taken as proof for an involvement of mPT. The inhibition of mPT by CsA is specific at the molecular level but the effect of CsA *in vivo* is not specific. The target for CsA in mitochondria is the mitochondria-specific cyclophilin, CypD. However, several other cyclophilins are found in human tissue, namely cyclophilin (Cyp) A, B, C and Cyp-40 (Waldmeier *et al.* 2003). The cyclophilins function as molecular chaperones and catalyze *cis-trans* isomerization of peptidyl-prolyl bonds. They are considered to be involved in folding of newly synthesized proteins and are suggested to mediate several other functions in different cellular compartments as well as in intercellular communication (Waldmeier *et al.* 2003). CypA is abundantly present in the cytoplasm and the complex of CypA and CsA in turn binds to the calcineurin enzyme and prevents its phosphatase activity. CsA is clinically used as an immunosuppressant to prevent rejection of transplanted organs and to treat autoimmune diseases. The immunosuppressive effect is mediated by calcineurin inhibition in T-helper cells, which prevents subsequent release of lymphokines and T-cell proliferation in cell-mediated immune responses (Borel *et al.* 1996). As described in the background section, calcineurin is also present in neurons and suppressing its activity has also been suggested to mediate neuroprotection. The neuroprotective effect of CsA may therefore be mediated by inhibition of calcineurin and not mPT, or a combination of the two. In order to dissect the relevant target for CsA, another calcineurin inhibitor without mitochondrial targets, FK506, has been used. An alternative approach has been to use a non-immunosuppressive (*i.e.* non-calcineurin inhibiting) cyclosporin analog. One such analog, $MeVal^4$-CsA, was previously available, and in Paper III, we evaluated two new non-immunosuppressive analogs, $MeIle^4$-CsA and $MeAla^3EtVal^4$-CsA. These analogs provide significant benefits over native CsA when evaluating the relevant pharmacological target, but they too inhibit the activity of other

cyclophilins. The difference from CsA lies in their inability to bind to calcineurin. The most specific evidence for an involvement of mPT in disease has come from recent studies using CypD knockout mice (*Ppif* $^{-/-}$), where the effect of the gene deletion in many cases has been similar to the protection afforded by CsA. Only a few animal disease models have however been tested in these genetic knock-outs so far. Another line of more direct evidence for mPT induction is the use of ^{3}H or ^{14}C labeled deoxyglucose, which is only taken up by mitochondria with increased permeability (Griffiths & Halestrap 1995).

A major obstacle in evaluating the neuroprotective effects of cyclosporin compounds is their limited penetration across the blood-brain barrier (BBB) (Tsuji *et al.* 1993, Lemaire *et al.* 1996, Tanaka *et al.* 2000). CsA and several other lipophilic compounds are actively extruded into the blood stream by the p-glycoprotein transporter present in the capillary endothelium. Different techniques can be used to overcome this problem. The BBB can be disrupted by physical damage to the tissue or osmotically through mannitol injection. The p-glycoprotein can also be saturated by using high systemic doses of CsA or high local doses by injecting it in the carotid artery. Alternatively, CsA can be administered directly into the cerebrospinal fluid. Only a few studies actually examine if adequate tissue doses have been reached and parallel to this is the lack of knowledge of the effective concentration range in the CNS. Consequently, a positive outcome of CsA treatment in an animal model can not be regarded as evidence of mPT involvement, and on the opposite side, a lack of effect can not be taken as evidence that mPT has not been involved, especially if CsA has not penetrated the BBB.

CsA and its analogs have however been one of the most convincing and broadly effective group of drugs displaying neuroprotective properties in several diverse models of acute and chronic neurological disease. Pharmacological studies have in particular implicated mPT in the pathogenesis of ischemia, hypoglycemia, traumatic brain injury (TBI) and amyotrophic lateral sclerosis (ALS).

Both in hypoglycemic coma and cerebral ischemia, several changes in the bioenergetic status arguably lead to conditions favoring mPT activation. In a model of global ischemia, cerebral tissue ATP levels have been demonstrated to decline to approximately 10% (from 3 mM to 300 μM) within 2 min (Folbergrová *et al.* 1990). The hydrolysis of adenine nucleotides is accompanied by a massive rise in tissue Pi (Crompton 1999) and in ischemia there is rapid cytoplasmic acidification due to the conversion from aerobic to anaerobic metabolism, whereas in hypoglycemia there is a rapid shift in intracellular redox status towards oxidation (Friberg & Wieloch 2002). Phospholipids are degraded and the free fatty acid levels increased, which

acidify the matrix, uncouple mitochondria and sensitize ANT towards mPT (Wieckowski *et al.* 2000, Wieckowski & Wojtczak 1998). Both hypoglycemia and ischemia involve excitotoxic-mediated damage, and in a model of excitotoxicity *in vivo*, NMDA injection into the striatum caused deoxyglucose entrapment in mitochondria (Zaidan *et al.* 2004). Cell death following hypoglycemic coma as well as mitochondrial swelling in granule cell dendrites are prevented by CsA, but not FK506 (Friberg *et al.* 1998, Ferrand-Drake *et al.* 1999, Ferrand-Drake *et al.* 2003).

In models of global ischemia, the CA1 neurons are selectively vulnerable, and CsA dramatically ameliorates their cell death both under normo- and hyperglycemic conditions if administered early after ischemia (Uchino *et al.* 1995, Uchino *et al.* 1998, Uchino *et al.* 2002, Li *et al.* 1997, Nakatsuka *et al.* 1999, Domanska-Janik *et al.* 2004). Some investigators have also found a neuroprotective effect of FK506 (Drake *et al.* 1996, Uchino *et al.* 2002), whereas others have not (Domanska-Janik *et al.* 2004). The non-immunosuppressive CsA analogs have not been thoroughly studied in global ischemia models. We have performed a pilot study with MeAla^{3}EtVal^4-CsA in rat global ischemia using two dose regimens, 10 mg/kg with disrupted BBB and 50 mg/kg with intact BBB. Neither of these treatments induced significant protection, but the study was inconclusive as there was no positive control and the brain tissue concentration was not measured (unpublished data). In a follow up study, the brain tissue concentration of CsA was compared to that of MeAla^{3}EtVal^4-CsA at 5 h following intravenous injections of the respective drugs (Figure 6). These results demonstrate that the BBB penetration of MeAla^{3}EtVal^4-CsA is significantly less than that of CsA, which in itself has a poor passage over the BBB. Thus, different routes of administration need to be tested in order to evaluate the *in vivo* neuroprotection of non-immunosuppressive analogs in the global ischemia model. In contrast, delayed CA1 cell death in hippocampal slices exposed to oxygen glucose deprivation is readily prevented by CsA and the non-immunosuppressive CsA analog MeIle^4-CsA, while FK506 is ineffective (Rytter *et al.* 2005). MeAla^{3}EtVal^4-CsA has also been evaluated in this model and displays a prominent effect against cell death (Figure 5). Additional evidence *in vivo* is needed in order to definitely conclude that mPT is essential in the pathogenesis and that it is a viable pharmacologic target in this type of delayed ischemic cell death.

In animal models of transient or permanent focal ischemia, CsA significantly reduces cerebral infarction when administered up to 3 h following reperfusion. The most robust effect has been observed with intracarotid CsA infusion following 5 min of reperfusion after 2h of middle cerebral artery occlusion (MCAO), where CsA virtually eliminated the infarct (Yoshimoto & Siesjö 1999, Yoshimoto *et al.* 2001). FK506 has been less effective in some models

(Yoshimoto & Siesjö 1999), but equally effective in others (Kuroda *et al.* 1999, Butcher *et al.* 1997). On the other hand, equal efficacy of MeVal^4-CsA and CsA in transient MCAO has also been demonstrated (Matsumoto *et al.* 1999). Strongly supporting an essential role for CypD in this model of ischemia is the finding that CypD knock-out mice display a dramatic reduction of brain infarct size following MCAO compared to their littermate controls (Schinzel *et al.* 2005), similar to the effect in cardiac ischemia (Baines *et al.* 2005, Nakagawa *et al.* 2005).

There is extensive documentation of a neuroprotective effect by CsA in different models of TBI. The shear forces will cause an immediate but also a delayed cell structure injury and a disruption of the BBB (Buki & Povlishock 2006). There is therefore probably an extended therapeutic time window and CsA can be administered by ordinary intravenous injection. In an impact acceleration model of TBI, CsA has been demonstrated to attenuate mitochondrial damage and reduce diffuse axonal injury when administered both intrathecally and intravenously following injury (Buki *et al.* 1999, Okonkwo & Povlishock 1999, Okonkwo *et al.* 2003). Different intravenous dose regimens of CsA in a cortical impact model have afforded improved mitochondrial integrity and energetic status as well as a significant reduction of lesion volume. An early bolus followed by continuous infusion of CsA has shown the most efficacy but one study also demonstrates a therapeutic window up to 24h (Scheff & Sullivan 1999, Sullivan *et al.* 1999, Sullivan *et al.* 2000a, Sullivan *et al.* 2000b). CsA has also exhibited neuroprotective effects in fluid percussion models (Sullivan *et al.* 2000a, Riess *et al.* 2001). FK506 has been less studied but has displayed neuroprotective effects in the impact acceleration model, but was ineffective in the cortical impact model (Singleton *et al.* 2001, Scheff & Sullivan 1999). There are thus indications that inhibition of mPT mediates a large part of the beneficial effect of CsA, but studies with non-immunosuppressive compounds or CypD knock-out animals in TBI models are lacking. The promising animal data have influenced two independent groups to initiate NIH sponsored human clinical trials of CsA administration to patients with severe traumatic brain injury in the United States, and the first safety studies show that CsA is well tolerated (Mazzeo *et al.* 2006, Empey *et al.* 2006).

A common feature of many neurodegenerative diseases is damage to mitochondria that contributes to the degenerative phenotype. In the common animal model for ALS, hSOD1-G93A mice, mPT inhibition seems to mediate extended survival. Mutation in the human SOD1 gene, which accounts for 20% of familiar ALS cases, does not seem to cause a loss or gain of its superoxide dismutase activity, but rather the cytosolic protein translocates into mitochondria. How it is toxic or why spinal cord mitochondria are selectively

vulnerable is not known, but the mutated SOD1 is speculated to interfere with several elements of normal mitochondrial function, ultimately leading to increased activation of the mitochondrial cell death pathways. In relation to mPT, calcium retention capacity of transgenic ALS mice has been found to be decreased prior to onset (Damiano *et al.* 2006) and spinal cord mitochondria also have a generally reduced calcium retention capacity compared to brain mitochondria (Morota *et al.* 2007). Intracerebroventricular injections of CsA every other day to late stage ALS mice doubled their remaining life-span after late stage diagnosis (Keep *et al.* 2001), and weekly injections before onset delayed the hindlimb weakness, prolonged physical performance and increased neuronal survival and life-span (Karlsson *et al.* 2004). Systemic administration of CsA to the same ALS model, but with an additional knock-out of the p-glycoprotein to facilitate CsA delivery to brain tissue, similarly increased life-span of the animals (Kirkinezos *et al.* 2004). Administration of FK506 to the hSOD1-G93A mice has however failed to improve outcome (Anneser *et al.* 2001). A human clinical trial in ALS patients has been performed with CsA, but the dose chosen was intended for immunosuppression and consequently is not expected to reach significant tissue levels in the spinal cord. A recent study with CypD knock-out mice demonstrates that the desensitization of mPT conveys protection of spinal cord axons in experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis (MS) (Forte *et al.* 2007). Intrathecal delivery of CsA or a non-immunosuppressive compound may thus prove to be beneficial in patients with ALS and MS.

CONCLUDING REMARKS

The studies of this thesis began with a project to evaluate newly synthesized versions of cyclosporin A (CsA), linked to a molecule that would facilitate its penetration into the brain. The rationale for this project was based on the hypothesis that inhibition of mPT mediates the neuroprotective properties of CsA seen in several animal models. The methods in Paper I were used for this purpose, screening the new derivatives for inhibition of mPT. While the initial project failed (the linker molecules interfered with the active sites of CsA, and a second generation of unstable constructs were toxic), an interest in mitochondria, and the role of mPT in particular, was born.

The conclusions in several publications that mPT in brain mitochondria does not seem to be relevant and that it is not sensitive to CsA did not appear to be in agreement with the results obtained in animal models. The results from our own succeeding studies have disputed these conclusions and hopefully our findings have helped to clarify the existence and the regulation of the mPT phenomenon in brain mitochondria. To grasp the role of mitochondria in the vast and often confusing field of free radical production and damage has been a challenge, but also a great source for learning. To look at mitochondrial calcium handling from an integrated view including both calcium sequestering and the direct regulation of mPT has been very rewarding, and it may lead to alternative approaches to increase mitochondrial calcium resistance. For the scientific field, the studies in animals lacking CypD, the mitochondrial target for CsA, have been very important and have clarified many issues concerning the importance of mPT in cell death both *in vitro* and in animal disease models.

Several natural and semi-synthetic CsA analogs have been tested throughout these studies with the hope to find a more specific compound with better penetration into the brain. It was a great pleasure to find that two non-immunosuppressive analogs were at least as potent as CsA in inhibiting mPT. However, as they do not seem to have an improved ability to cross the blood-brain barrier, the initial objective of improving delivery to the brain remains. For conditions where this barrier is breached, such as traumatic brain injury, or in patients where a continuous intrathecal administration may be feasible, such as patients with ALS, the present compounds may be fully adequate. It is therefore with great anticipation I look forward to future clinical trials with CsA or other drugs targeting mPT, to find out if inhibition of mPT can finally be a successful approach to limit neurological cell death.

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REFERENCES

Andreyev, A. and Fiskum, G. (1999) Calcium induced release of mitochondrial cytochrome c by different mechanisms selective for brain versus liver. *Cell Death Differ,* 6, 825-832.

Andreyev, A. Y., Fahy, B. and Fiskum, G. (1998) Cytochrome c release from brain mitochondria is independent of the mitochondrial permeability transition. *FEBS Lett,* 439, 373-376.

Andreyev, A. Y., Kushnareva, Y. E. and Starkov, A. A. (2005) Mitochondrial metabolism of reactive oxygen species. *Biochemistry (Mosc),* 70, 200-214.

Anneser, J. M., Gmerek, A., Gerkrath, J., Borasio, G. D. and Heumann, R. (2001) Immunosuppressant FK506 does not exert beneficial effects in symptomatic G93A superoxide dismutase-1 transgenic mice. *Neuroreport,* 12, 2663-2665.

Ardehali, H. and O'Rourke, B. (2005) Mitochondrial K(ATP) channels in cell survival and death. *J Mol Cell Cardiol,* 39, 7-16.

Baines, C. P., Kaiser, R. A., Purcell, N. H. *et al.* (2005) Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature,* 434, 658-662.

Baines, C. P., Kaiser, R. A., Sheiko, T., Craigen, W. J. and Molkentin, J. D. (2007) Voltage-dependent anion channels are dispensable for mitochondrial-dependent cell death. *Nat Cell Biol,* 9, 550-555.

Bambrick, L. L., Chandrasekaran, K., Mehrabian, Z., Wright, C., Krueger, B. K. and Fiskum, G. (2006) Cyclosporin A increases mitochondrial calcium uptake capacity in cortical astrocytes but not cerebellar granule neurons. *J Bioenerg Biomembr,* 38, 43-47.

Basso, E., Fante, L., Fowlkes, J., Petronilli, V., Forte, M. A. and Bernardi, P. (2005) Properties of the permeability transition pore in mitochondria devoid of Cyclophilin D. *J Biol Chem,* 280, 18558-18561.

Bazan, N. G., Jr. (1970) Effects of ischemia and electroconvulsive shock on free fatty acid pool in the brain. *Biochim Biophys Acta,* 218, 1-10.

Beal, M. F. (1996) Mitochondria, free radicals, and neurodegeneration. *Curr Opin Neurobiol,* 6, 661-666.

Berman, S. B., Watkins, S. C. and Hastings, T. G. (2000) Quantitative biochemical and ultrastructural comparison of mitochondrial permeability transition in isolated brain and liver mitochondria: evidence for reduced sensitivity of brain mitochondria. *Exp Neurol,* 164, 415-425.

Bernardi, P. (1992) Modulation of the mitochondrial cyclosporin A-sensitive permeability transition pore by the proton electrochemical gradient. Evidence that the pore can be opened by membrane depolarization. *J Biol Chem,* 267, 8834-8839.

Bernardi, P., Krauskopf, A., Basso, E., Petronilli, V., Blalchy-Dyson, E., Di Lisa, F. and Forte, M. A. (2006) The mitochondrial permeability transition from in vitro artifact to disease target. *Febs J,* 273, 2077-2099.

Bernardi, P. and Petronilli, V. (1996) The permeability transition pore as a mitochondrial calcium release channel: a critical appraisal. *J Bioenerg Biomembr,* 28, 131-138.

Bernardi, P., Vassanelli, S., Veronese, P., Colonna, R., Szabo, I. and Zoratti, M. (1992) Modulation of the mitochondrial permeability transition pore. Effect of protons and divalent cations. *J Biol Chem,* 267, 2934-2939.

Beutner, G., Ruck, A., Riede, B. and Brdiczka, D. (1998) Complexes between porin, hexokinase, mitochondrial creatine kinase and adenylate translocator display properties of the permeability transition pore. Implication for regulation of permeability transition by the kinases. *Biochim Biophys Acta,* 1368, 7-18.

Boehning, D., Patterson, R. L., Sedaghat, L., Glebova, N. O., Kurosaki, T. and Snyder, S. H. (2003) Cytochrome c binds to inositol (1,4,5) trisphosphate receptors, amplifying calcium-dependent apoptosis. *Nat Cell Biol,* 5, 1051-1061.

Borel, J. F., Baumann, G., Chapman, I., Donatsch, P., Fahr, A., Mueller, E. A. and Vigouret, J. M. (1996) In vivo pharmacological effects of ciclosporin and some analogues. *Adv Pharmacol,* 35, 115-246.

Bradford, M. M. (1976) A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. *Anal Biochem,* 72, 248-254.

Brand, M. D. (2005) The efficiency and plasticity of mitochondrial energy transduction. *Biochem Soc Trans,* 33, 897-904.

Brdiczka, D. G., Zorov, D. B. and Sheu, S. S. (2006) Mitochondrial contact sites: their role in energy metabolism and apoptosis. *Biochim Biophys Acta,* 1762, 148-163.

Broekemeier, K. M., Dempsey, M. E. and Pfeiffer, D. R. (1989) Cyclosporin A is a potent inhibitor of the inner membrane permeability transition in liver mitochondria. *J Biol Chem,* 264, 7826-7830.

Brown, M. R., Sullivan, P. G. and Geddes, J. W. (2006) Synaptic mitochondria are more susceptible to Ca2+ overload than nonsynaptic mitochondria. *J Biol Chem,* 281, 11658-11668.

Brustovetsky, N., Brustovetsky, T., Purl, K. J., Capano, M., Crompton, M. and Dubinsky, J. M. (2003) Increased susceptibility of striatal mitochondria to calcium-induced permeability transition. *J Neurosci,* 23, 4858-4867.

Brustovetsky, N. and Dubinsky, J. M. (2000a) Dual responses of CNS mitochondria to elevated calcium. *J Neurosci,* 20, 103-113.

Brustovetsky, N. and Dubinsky, J. M. (2000b) Limitations of cyclosporin A inhibition of the permeability transition in CNS mitochondria. *J Neurosci,* 20, 8229-8237.

Brustovetsky, N. and Klingenberg, M. (1996) Mitochondrial ADP/ATP carrier can be reversibly converted into a large channel by Ca2+. *Biochemistry,* 35, 8483-8488.

Brustovetsky, N., Tropschug, M., Heimpel, S., Heidkamper, D. and Klingenberg, M. (2002) A large Ca2+-dependent channel formed by recombinant ADP/ATP carrier from Neurospora crassa resembles the mitochondrial permeability transition pore. *Biochemistry,* 41, 11804-11811.

Buki, A., Okonkwo, D. O. and Povlishock, J. T. (1999) Postinjury cyclosporin A administration limits axonal damage and disconnection in traumatic brain injury. *J. Neurotrauma,* 16, 511-521.

Buki, A. and Povlishock, J. T. (2006) All roads lead to disconnection?--Traumatic axonal injury revisited. *Acta Neurochir (Wien),* 148, 181-193; discussion 193-184.

Butcher, S. P., Henshall, D. C., Teramura, Y., Iwasaki, K. and Sharkey, J. (1997) Neuroprotective actions of FK506 in experimental stroke: in vivo evidence against an antiexcitotoxic mechanism. *J Neurosci,* 17, 6939-6946.

Calabrese, E. J. (2007) Converging concepts: Adaptive response, preconditioning, and the Yerkes-Dodson Law are manifestations of hormesis. *Ageing Res Rev*.

Cande, C., Vahsen, N., Kouranti, I. *et al.* (2004) AIF and cyclophilin A cooperate in apoptosis-associated chromatinolysis. *Oncogene,* 23, 1514-1521.

Chalmers, S. and Nicholls, D. G. (2003) The relationship between free and total calcium concentrations in the matrix of liver and brain mitochondria. *J Biol Chem,* 278, 19062-19070.

Chance, B. and Williams, G. R. (1955) Respiratory enzymes in oxidative phosphorylation. III. The steady state. *J Biol Chem,* 217, 409-427.

Chen, J., Nagayama, T., Jin, K., Stetler, R. A., Zhu, R. L., Graham, S. H. and Simon, R. P. (1998) Induction of caspase-3-like protease may mediate delayed neuronal death in the hippocampus after transient cerebral ischemia. *J Neurosci,* 18, 4914-4928.

Chopp, M., Chen, H., Ho, K. L., Dereski, M. O., Brown, E., Hetzel, F. W. and Welch, K. M. (1989) Transient hyperthermia protects against subsequent forebrain ischemic cell damage in the rat. *Neurology,* 39, 1396-1398.

Cocheme, H. M., Kelso, G. F., James, A. M. *et al.* (2007) Mitochondrial targeting of quinones: therapeutic implications. *Mitochondrion,* 7 Suppl, S94-102.

Connern, C. P. and Halestrap, A. P. (1994) Recruitment of mitochondrial cyclophilin to the mitochondrial inner membrane under conditions of oxidative stress that enhance the opening of a calcium-sensitive non-specific channel. *Biochem J,* 302, 321-324.

Cory, S., Huang, D. C. and Adams, J. M. (2003) The Bcl-2 family: roles in cell survival and oncogenesis. *Oncogene,* 22, 8590-8607.

Costa, A. D., Jakob, R., Costa, C. L., Andrukhiv, K., West, I. C. and Garlid, K. D. (2006) The mechanism by which the mitochondrial ATP-sensitive K+ channel opening and H2O2 inhibit the mitochondrial permeability transition. *J Biol Chem,* 281, 20801-20808.

Crompton, M. (1999) The mitochondrial permeability transition pore and its role in cell death. *Biochem J,* 341, 233-249.

Crompton, M., Barksby, E., Johnson, N. and Capano, M. (2002) Mitochondrial intermembrane junctional complexes and their involvement in cell death. *Biochimie,* 84, 143-152.

Crompton, M., Ellinger, H. and Costi, A. (1988) Inhibition by cyclosporin A of a Ca2+-dependent pore in heart mitochondria activated by inorganic phosphate and oxidative stress. *Biochem J,* 255, 357-360.

Crompton, M., Virji, S. and Ward, J. M. (1998) Cyclophilin-D binds strongly to complexes of the voltage-dependent anion channel and the adenine nucleotide translocase to form the permeability transition pore. *Eur. J. Biochem.,* 258, 729-735.

Damiano, M., Starkov, A. A., Petri, S., Kipiani, K., Kiaei, M., Mattiazzi, M., Flint Beal, M. and Manfredi, G. (2006) Neural mitochondrial Ca2+ capacity impairment precedes the onset of motor symptoms in G93A Cu/Zn-superoxide dismutase mutant mice. *J Neurochem,* 96, 1349-1361.

Di Lisa, F. and Bernardi, P. (2006) Mitochondria and ischemia-reperfusion injury of the heart: Fixing a hole. *Cardiovasc Res*.

Dirnagl, U., Simon, R. P. and Hallenbeck, J. M. (2003) Ischemic tolerance and endogenous neuroprotection. *Trends Neurosci,* 26, 248-254.

Domanska-Janik, K., Buzanska, L., Dluzniewska, J., Kozlowska, H., Sarnowska, A. and Zablocka, B. (2004) Neuroprotection by cyclosporin A following transient brain ischemia correlates with the inhibition of the early efflux of cytochrome C to cytoplasm. *Brain Res Mol Brain Res,* 121, 50-59.

Domoki, F., Perciaccante, J. V., Veltkamp, R., Bari, F. and Busija, D. W. (1999) Mitochondrial potassium channel opener diazoxide preserves neuronal-vascular function after cerebral ischemia in newborn pigs. *Stroke,* 30, 2713-2718; discussion 2718-2719.

Drake, M., Friberg, H., Boris-Möller, F., Sakata, K. and Wieloch, T. (1996) The immunosuppressant FK506 ameliorates ischaemic damage in the rat brain. *Acta Physiol Scand,* 158, 155-159.

Droge, W. (2002) Free radicals in the physiological control of cell function. *Physiol Rev,* 82, 47-95.

Dröse, S., Brandt, U. and Hanley, P. J. (2006) K+-independent actions of diazoxide question the role of inner membrane KATP channels in mitochondrial cytoprotective signaling. *J Biol Chem,* 281, 23733-23739.

Dugan, L. L., Sensi, S. L., Canzoniero, L. M., Handran, S. D., Rothman, S. M., Lin, T. S., Goldberg, M. P. and Choi, D. W. (1995) Mitochondrial production of reactive oxygen species in cortical neurons following exposure to N-methyl-D-aspartate. *J Neurosci,* 15, 6377-6388.

Eliseev, R. A., Filippov, G., Velos, J., VanWinkle, B., Goldman, A., Rosier, R. N. and Gunter, T. E. (2007) Role of cyclophilin D in the resistance of brain mitochondria to the permeability transition. *Neurobiol Aging,* 28, 1532-1542.

Empey, P. E., McNamara, P. J., Young, B., Rosbolt, M. B. and Hatton, J. (2006) Cyclosporin A disposition following acute traumatic brain injury. *J Neurotrauma,* 23, 109-116.

Erecinska, M. and Silver, I. A. (1989) ATP and brain function. *J Cereb Blood Flow Metab,* 9, 2-19.

Ferrand-Drake, M., Friberg, H. and Wieloch, T. (1999) Mitochondrial permeability transition induced DNA-fragmentation in the rat hippocampus following hypoglycemia. *Neuroscience,* 90, 1325-1338.

Ferrand-Drake, M., Zhu, C., Gido, G. *et al.* (2003) Cyclosporin A prevents calpain activation despite increased intracellular calcium concentrations, as well as translocation of apoptosis-inducing factor, cytochrome c and caspase-3 activation in neurons exposed to transient hypoglycemia. *J Neurochem,* 85, 1431-1442.

Folbergrová, J., Minamisawa, H., Ekholm, A. and Siesjö, B. K. (1990) Phosphorylase alpha and labile metabolites during anoxia: correlation to membrane fluxes of K+ and Ca2+. *J Neurochem,* 55, 1690-1696.

Forte, M. and Bernardi, P. (2006) The permeability transition and BCL-2 family proteins in apoptosis: co-conspirators or independent agents? *Cell Death Differ,* 13, 1287-1290.

Forte, M., Gold, B. G., Marracci, G. *et al.* (2007) Cyclophilin D inactivation protects axons in experimental autoimmune encephalomyelitis, an animal model of multiple sclerosis. *Proc Natl Acad Sci U S A,* 104, 7558-7563.

Friberg, H., Connern, C., Halestrap, A. P. and Wieloch, T. (1999) Differences in the activation of the mitochondrial permeability transition among brain regions in the rat correlate with selective vulnerability. *J Neurochem,* 72, 2488-2497.

Friberg, H., Ferrand-Drake, M., Bengtsson, F., Halestrap, A. P. and Wieloch, T. (1998) Cyclosporin A, but not FK 506, protects mitochondria and neurons against hypoglycemic damage and implicates the mitochondrial permeability transition in cell death. *J Neurosci,* 18, 5151-5159.

Friberg, H. and Wieloch, T. (2002) Mitochondrial permeability transition in acute neurodegeneration. *Biochimie,* 84, 241-250.

Garlid, K. D. and Paucek, P. (2003) Mitochondrial potassium transport: the K(+) cycle. *Biochim Biophys Acta,* 1606, 23-41.

Garlid, K. D., Paucek, P., Yarov-Yarovoy, V., Sun, X. and Schindler, P. A. (1996) The mitochondrial KATP channel as a receptor for potassium channel openers. *J Biol Chem,* 271, 8796-8799.

Gibbs, E. L., Lennox, W. G., Nims, L. F. and Gibbs, F. A. (1942) ARTERIAL AND CEREBRAL VENOUS BLOOD. ARTERIAL-VENOUS DIFFERENCES IN MAN. *J Biol Chem,* 144, 325-332.

Gidday, J. M. (2006) Cerebral preconditioning and ischaemic tolerance. *Nature reviews,* 7, 437-448.

Gillessen, T., Grasshoff, C. and Szinicz, L. (2002) Mitochondrial permeability transition can be directly monitored in living neurons. *Biomed Pharmacother,* 56, 186-193.

Griffiths, E. J. and Halestrap, A. P. (1995) Mitochondrial non-specific pores remain closed during cardiac ischaemia, but open upon reperfusion. *Biochem J,* 307, 93-98.

Gunter, T. E. and Pfeiffer, D. R. (1990) Mechanisms by which mitochondria transport calcium. *Am. J. Physiol.,* 258, C755-C786.

Halestrap, A. P. (1987) The regulation of the oxidation of fatty acids and other substrates in rat heart mitochondria by changes in the matrix volume induced by osmotic strength, valinomycin and Ca2+. *Biochem J,* 244, 159-164.

Halestrap, A. P. (1991) Calcium-dependent opening of a non-specific pore in the mitochondrial inner membrane is inhibited at pH values below 7. Implications for the protective effect of low pH against chemical and hypoxic cell damage. *Biochem J,* 278, 715-719.

Halestrap, A. P. (2006) Calcium, mitochondria and reperfusion injury: a pore way to die. *Biochem Soc Trans,* 34, 232-237.

Halestrap, A. P., Clarke, S. J. and Khaliulin, I. (2007) The role of mitochondria in protection of the heart by preconditioning. *Biochim Biophys Acta,* 1767, 1007-1031.

Halestrap, A. P. and Davidson, A. M. (1990) Inhibition of Ca2(+)-induced large-amplitude swelling of liver and heart mitochondria by cyclosporin is probably caused by the inhibitor binding to mitochondrial-matrix peptidyl-prolyl cis-trans isomerase and preventing it interacting with the adenine nucleotide translocase. *Biochem J,* 268, 153-160.

Hanley, P. J. and Daut, J. (2005) K(ATP) channels and preconditioning: a re-examination of the role of mitochondrial K(ATP) channels and an overview of alternative mechanisms. *J Mol Cell Cardiol,* 39, 17-50.

Hattersley, A. T. and Pearson, E. R. (2006) Minireview: pharmacogenetics and beyond: the interaction of therapeutic response, beta-cell physiology, and genetics in diabetes. *Endocrinology,* 147, 2657-2663.

Hausenloy, D. J., Maddock, H. L., Baxter, G. F. and Yellon, D. M. (2002) Inhibiting mitochondrial permeability transition pore opening: a new paradigm for myocardial preconditioning? *Cardiovasc Res,* 55, 534-543.

Hausenloy, D. J., Yellon, D. M., Mani-Babu, S. and Duchen, M. R. (2004) Preconditioning protects by inhibiting the mitochondrial permeability transition. *Am J Physiol Heart Circ Physiol,* 287, H841-849.

Haworth, R. A. and Hunter, D. R. (1979) The Ca^{2+}-induced membrane transition in mitochondria. II. Nature of the Ca^{2+} trigger site. *Arch Biochem Biophys,* 195, 460-467.

He, L. and Lemasters, J. J. (2002) Regulated and unregulated mitochondrial permeability transition pores: a new paradigm of pore structure and function? *FEBS Lett,* 512, 1-7.

Himi, T., Ishizaki, Y. and Murota, S. (1998) A caspase inhibitor blocks ischaemia-induced delayed neuronal death in the gerbil. *Eur J Neurosci,* 10, 777-781.

Hoek, J. B. and Rydstrom, J. (1988) Physiological roles of nicotinamide nucleotide transhydrogenase. *Biochem J,* 254, 1-10.

Hoffmann, B., Stockl, A., Schlame, M., Beyer, K. and Klingenberg, M. (1994) The reconstituted ADP/ATP carrier activity has an absolute requirement for cardiolipin as shown in cysteine mutants. *J Biol Chem,* 269, 1940-1944.

Holmuhamedov, E. L., Wang, L. and Terzic, A. (1999) ATP-sensitive K+ channel openers prevent Ca2+ overload in rat cardiac mitochondria. *J Physiol,* 519 Pt 2, 347-360.

Honda, H. M., Korge, P. and Weiss, J. N. (2005) Mitochondria and ischemia/reperfusion injury. *Ann N Y Acad Sci,* 1047, 248-258.

Horvitz, H. R. (1999) Genetic control of programmed cell death in the nematode Caenorhabditis elegans. *Cancer Res,* 59, 1701s-1706s.

Hunter, D. R. and Haworth, R. A. (1979a) The Ca^{2+}-induced membrane transition in mitochondria. I. The protective mechanisms. *Arch Biochem Biophys,* 195, 453-459.

Hunter, D. R. and Haworth, R. A. (1979b) The Ca^{2+}-induced membrane transition in mitochondria. III. Transitional Ca^{2+} release. *Arch. Biochem. Biophys.,* 195, 468-477.

Hunter, D. R., Haworth, R. A. and Southard, J. H. (1976) Relationship between configuration, function, and permeability in calcium-treated mitochondria. *J Biol Chem,* 251, 5069-5077.

Hunter, F. E., Jr. and Ford, L. (1955) Inactivation of oxidative and phosphorylative systems in mitochondria by preincubation with phosphate and other ions. *J Biol Chem,* 216, 357-369.

Huser, J., Rechenmacher, C. E. and Blatter, L. A. (1998) Imaging the permeability pore transition in single mitochondria. *Biophys J,* 74, 2129-2137.

Hütter, E., Unterluggauer, H., Garedew, A., Jansen-Dürr, P. and Gnaiger, E. (2006) High-resolution respirometry--a modern tool in aging research. *Exp Gerontol,* 41, 103-109.

Iadecola, C. and Alexander, M. (2001) Cerebral ischemia and inflammation. *Curr Opin Neurol,* 14, 89-94.

Ichas, F. and Mazat, J. P. (1998) From calcium signaling to cell death: two conformations for the mitochondrial permeability transition pore. Switching from low- to high-conductance state. *Biochim Biophys Acta,* 1366, 33-50.

Inoue, I., Nagase, H., Kishi, K. and Higuti, T. (1991) ATP-sensitive K+ channel in the mitochondrial inner membrane. *Nature,* 352, 244-247.

Jaburek, M., Yarov-Yarovoy, V., Paucek, P. and Garlid, K. D. (1998) State-dependent inhibition of the mitochondrial KATP channel by glyburide and 5-hydroxydecanoate. *J Biol Chem,* 273, 13578-13582.

Janoff, A. (1964) Alterations in Lysosomes (Intracellular Enzymes) During Shock; Effects of Preconditioning (Tolerance) and Protective Drugs. *Int Anesthesiol Clin,* 2, 251-269.

Kagawa, Y. and Racker, E. (1966) Partial resolution of the enzymes catalyzing oxidative phosphorylation. IX. Reconstruction of oligomycin-sensitive adenosine triphosphatase. *J Biol Chem,* 241, 2467-2474.

Karlsson, J., Fong, K. S., Hansson, M. J., Elmer, E., Csiszar, K. and Keep, M. F. (2004) Life span extension and reduced neuronal death after weekly intraventricular cyclosporin injections in the G93A transgenic mouse model of amyotrophic lateral sclerosis. *J Neurosurg,* 101, 128-137.

Keep, M., Elmér, E., Fong, K. S. and Csiszar, K. (2001) Intrathecal cyclosporin prolongs survival of late-stage ALS mice. *Brain Res,* 894, 327-331.

Kerr, J. F., Wyllie, A. H. and Currie, A. R. (1972) Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British journal of cancer,* 26, 239-257.

Kirkinezos, I. G., Hernandez, D., Bradley, W. G. and Moraes, C. T. (2004) An ALS mouse model with a permeable blood-brain barrier benefits from systemic cyclosporine A treatment. *J Neurochem,* 88, 821-826.

Kitagawa, K., Matsumoto, M., Tagaya, M. *et al.* (1990) 'Ischemic tolerance' phenomenon found in the brain. *Brain Res,* 528, 21-24.

Kokoszka, J. E., Waymire, K. G., Levy, S. E., Sligh, J. E., Cai, J., Jones, D. P., MacGregor, G. R. and Wallace, D. C. (2004) The ADP/ATP translocator is not essential for the mitochondrial permeability transition pore. *Nature,* 427, 461-465.

Krauskopf, A., Eriksson, O., Craigen, W. J., Forte, M. A. and Bernardi, P. (2006) Properties of the permeability transition in VDAC1(-/-) mitochondria. *Biochim Biophys Acta*.

Krebs, H. A. and Johnson, W. A. (1937) Metabolism of ketonic acids in animal tissues. *Biochem J,* 31, 645-660.

Kristal, B. S. and Dubinsky, J. M. (1997) Mitochondrial permeability transition in the central nervous system: induction by calcium cycling-dependent and -independent pathways. *J Neurochem,* 69, 524-538.

Kristal, B. S., Staats, P. N. and Shestopalov, A. I. (2000) Biochemical characterization of the mitochondrial permeability transition in isolated forebrain mitochondria. *Dev Neurosci,* 22, 376-383.

Kristián, T., Bernardi, P. and Siesjö, B. K. (2001) Acidosis promotes the permeability transition in energized mitochondria: implications for reperfusion injury. *J Neurotrauma,* 18, 1059-1074.

Kristián, T., Gertsch, J., Bates, T. E. and Siesjö, B. K. (2000) Characteristics of the calcium-triggered mitochondrial permeability transition in nonsynaptic brain mitochondria: effect of cyclosporin A and ubiquinone O. *J Neurochem,* 74, 1999-2009.

Kristián, T., Pivovarova, N. B., Fiskum, G. and Andrews, S. B. (2007) Calcium-induced precipitate formation in brain mitochondria: composition, calcium capacity, and retention. *J Neurochem,* 102, 1346-1356.

Kristián, T., Weatherby, T. M., Bates, T. E. and Fiskum, G. (2002) Heterogeneity of the calcium-induced permeability transition in isolated non-synaptic brain mitochondria. *J Neurochem,* 83, 1297-1308.

Kroemer, G., El-Deiry, W. S., Golstein, P. *et al.* (2005) Classification of cell death: recommendations of the Nomenclature Committee on Cell Death. *Cell Death Differ,* 12 Suppl 2, 1463-1467.

Kuroda, S., Janelidze, S. and Siesjö, B. K. (1999) The immunosuppressants cyclosporin A and FK506 equally ameliorate brain damage due to 30-min middle cerebral artery occlusion in hyperglycemic rats. *Brain Res,* 835, 148-153.

Lehninger, A. L. (1949) Esterification of inorganic phosphate coupled to electron transport between dihydrodiphosphopyridine nucleotide and oxygen. *J Biol Chem,* 178, 625-644.

Lehninger, A. L. (1962) Water uptake and extrusion by mitochondria in relation to oxidative phosphorylation. *Physiol Rev,* 42, 467-517.

Lemaire, M., Bruelisauer, A., Guntz, P. and Sato, H. (1996) Dose-dependent brain penetration of SDZ PSC 833, a novel multidrug resistance-reversing cyclosporin, in rats. *Cancer Chemother. Pharmacol.,* 38, 481-486.

Lemasters, J. J., Nieminen, A. L., Qian, T. *et al.* (1998) The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. *Biochim Biophys Acta,* 1366, 177-196.

Li, P. A., Uchino, H., Elmér, E. and Siesjö, B. K. (1997) Amelioration by cyclosporin A of brain damage following 5 or 10 min of ischemia in rats subjected to preischemic hyperglycemia. *Brain Res.,* 753, 133-140.

Li, Y., Huang, T. T., Carlson, E. J. *et al.* (1995) Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat Genet,* 11, 376-381.

Li, Y., Johnson, N., Capano, M., Edwards, M. and Crompton, M. (2004) Cyclophilin-D promotes the mitochondrial permeability transition but has opposite effects on apoptosis and necrosis. *Biochem J,* 383, 101-109.

Lifshitz, J., Friberg, H., Neumar, R. W. *et al.* (2003) Structural and functional damage sustained by mitochondria after traumatic brain injury in the rat: evidence for differentially sensitive populations in the cortex and hippocampus. *J Cereb Blood Flow Metab,* 23, 219-231.

Lipton, P. (1999) Ischemic cell death in brain neurons. *Physiol Rev,* 79, 1431-1568.

Liu, D., Lu, C., Wan, R., Auyeung, W. W. and Mattson, M. P. (2002) Activation of mitochondrial ATP-dependent potassium channels protects neurons against ischemia-induced death by a mechanism involving suppression of Bax translocation and cytochrome c release. *J Cereb Blood Flow Metab,* 22, 431-443.

Liu, Y., Sato, T., O'Rourke, B. and Marban, E. (1998) Mitochondrial ATP-dependent potassium channels: novel effectors of cardioprotection? *Circulation,* 97, 2463-2469.

Mansson, R., Hansson, M. J., Morota, S., Uchino, H., Ekdahl, C. T. and Elmer, E. (2007) Re-evaluation of mitochondrial permeability transition as a primary neuroprotective target of minocycline. *Neurobiol Dis,* 25, 198-205.

Matsumoto, S., Friberg, H., Ferrand-Drake, M. and Wieloch, T. (1999) Blockade of the mitochondrial permeability transition pore diminishes infarct size in the rat after transient middle cerebral artery occlusion. *J. Cereb. Blood Flow Metab.,* 19, 736-741.

Mattiasson, G., Friberg, H., Hansson, M., Elmer, E. and Wieloch, T. (2003) Flow cytometric analysis of mitochondria from CA1 and CA3 regions of rat hippocampus reveals differences in permeability transition pore activation. *J Neurochem,* 87, 532-544.

Mazzeo, A. T., Kunene, N. K., Gilman, C. B., Hamm, R. J., Hafez, N. and Bullock, M. R. (2006) Severe human traumatic brain injury, but not cyclosporin a treatment, depresses activated T lymphocytes early after injury. *J Neurotrauma,* 23, 962-975.

McGee-Russell, S. M., Brown, A. W. and Brierley, J. B. (1970) A combined light and electron microscope study of early anoxic-ischaemic cell change in rat brain. *Brain Res,* 20, 193-200.

McStay, G. P., Clarke, S. J. and Halestrap, A. P. (2002) Role of critical thiol groups on the matrix surface of the adenine nucleotide translocase in the mechanism of the mitochondrial permeability transition pore. *Biochem J,* 367, 541-548.

Meldrum, B. and Garthwaite, J. (1990) Excitatory amino acid neurotoxicity and neurodegenerative disease. *TIPS,* 11, 379-387.

Mitchell, P. (1961) Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature,* 191, 144-148.

Morota, S., Hansson, M. J., Ishii, N., Kudo, Y., Elmér, E. and Uchino, H. (2007) Spinal cord mitochondria display lower calcium retention capacity compared with brain mitochondria without inherent differences in sensitivity to cyclophilin D inhibition. *J Neurochem*.

Murry, C. E., Jennings, R. B. and Reimer, K. A. (1986) Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation,* 74, 1124-1136.

Naga, K. K., Sullivan, P. G. and Geddes, J. W. (2007) High cyclophilin D content of synaptic mitochondria results in increased vulnerability to permeability transition. *J Neurosci,* 27, 7469-7475.

Nakagawa, T., Shimizu, S., Watanabe, T., Yamaguchi, O., Otsu, K., Yamagata, H., Inohara, H., Kubo, T. and Tsujimoto, Y. (2005) Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. *Nature,* 434, 652-658.

Nakatsuka, H., Ohta, S., Tanaka, J., Toku, K., Kumon, Y., Maeda, N., Sakanaka, M. and Sakaki, S. (1999) Release of cytochrome c from mitochondria to cytosol in gerbil hippocampal CA1 neurons after transient forebrain ischemia. *Brain Res,* 849, 216-219.

Nicholls, D. G. (1974) The influence of respiration and ATP hydrolysis on the proton-electrochemical gradient across the inner membrane of rat-liver mitochondria as determined by ion distribution. *Eur J Biochem,* 50, 305-315.

Nicholls, D. G. (2004) Mitochondrial dysfunction and glutamate excitotoxicity studied in primary neuronal cultures. *Curr Mol Med,* 4, 149-177.

Nicholls, D. G. (2005) Mitochondria and calcium signaling. *Cell Calcium,* 38, 311-317.

Nicholls, D. G. and Budd, S. L. (2000) Mitochondria and neuronal survival. *Physiol Rev,* 80, 315-360.

Nicholson, C., Bruggencate, G. T., Steinberg, R. and Stockle, H. (1977) Calcium modulation in brain extracellular microenvironment demonstrated with ion-selective micropipette. *Proc Natl Acad Sci U S A,* 74, 1287-1290.

Nohl, H., Kozlov, A. V., Gille, L. and Staniek, K. (2003) Cell respiration and formation of reactive oxygen species: facts and artefacts. *Biochem Soc Trans,* 31, 1308-1311.

Okonkwo, D. O., Melon, D. E., Pellicane, A. J., Mutlu, L. K., Rubin, D. G., Stone, J. R. and Helm, G. A. (2003) Dose-response of cyclosporin A in attenuating traumatic axonal injury in rat. *Neuroreport,* 14, 463-466.

Okonkwo, D. O. and Povlishock, J. T. (1999) An intrathecal bolus of cyclosporin A before injury preserves mitochondrial integrity and attenuates axonal disruption in traumatic brain injury. *J. Cereb. Blood Flow Metab.,* 19, 443-451.

Olney, J. W. and Sharpe, L. G. (1969) Brain lesions in an infant rhesus monkey treated with monsodium glutamate. *Science,* 166, 386-388.

Orrenius, S., Gogvadze, V. and Zhivotovsky, B. (2007) Mitochondrial oxidative stress: implications for cell death. *Annu Rev Pharmacol Toxicol,* 47, 143-183.

Orrenius, S., Zhivotovsky, B. and Nicotera, P. (2003) Regulation of cell death: the calcium-apoptosis link. *Nat Rev Mol Cell Biol,* 4, 552-565.

Ott, M., Norberg, E., Walter, K. M., Schreiner, P., Kemper, C., Rapaport, D., Zhivotovsky, B. and Orrenius, S. (2007) The Mitochondrial TOM Complex Is Required for tBid/Bax-induced Cytochrome c Release. *J Biol Chem,* 282, 27633-27639.

Ott, M., Robertson, J. D., Gogvadze, V., Zhivotovsky, B. and Orrenius, S. (2002) Cytochrome c release from mitochondria proceeds by a two-step process. *Proc Natl Acad Sci U S A,* 99, 1259-1263.

Ouyang, Y. B., Tan, Y., Comb, M., Liu, C. L., Martone, M. E., Siesjö, B. K. and Hu, B. R. (1999) Survival- and death-promoting events after transient cerebral ischemia: phosphorylation of Akt, release of cytochrome C and Activation of caspase-like proteases. *J Cereb Blood Flow Metab,* 19, 1126-1135.

Panov, A., Dikalov, S., Shalbuyeva, N., Hemendinger, R., Greenamyre, J. T. and Rosenfeld, J. (2007) Species- and tissue-specific relationships between mitochondrial permeability transition and generation of ROS in brain and liver mitochondria of rats and mice. *Am J Physiol Cell Physiol,* 292, C708-718.

Panov, A. V., Andreeva, L. and Greenamyre, J. T. (2004) Quantitative evaluation of the effects of mitochondrial permeability transition pore modifiers on accumulation of calcium phosphate: comparison of rat liver and brain mitochondria. *Arch Biochem Biophys,* 424, 44-52.

Petito, C. K. and Pulsinelli, W. A. (1984) Delayed neuronal recovery and neuronal death in rat hippocampus following severe cerebral ischemia: possible relationship to abnormalities in neuronal processes. *J Cereb Blood Flow Metab,* 4, 194-205.

Petronilli, V., Miotto, G., Canton, M., Brini, M., Colonna, R., Bernardi, P. and Di Lisa, F. (1999) Transient and long-lasting openings of the mitochondrial permeability transition pore can be monitored directly in intact cells by changes in mitochondrial calcein fluorescence. *Biophys J,* 76, 725-734.

Pinton, P., Ferrari, D., Magalhaes, P., Schulze-Osthoff, K., Di Virgilio, F., Pozzan, T. and Rizzuto, R. (2000) Reduced loading of intracellular Ca(2+) stores and

downregulation of capacitative Ca(2+) influx in Bcl-2-overexpressing cells. *J Cell Biol,* 148, 857-862.

Rehncrona, S., Westerberg, E., Akesson, B. and Siesjo, B. K. (1982) Brain cortical fatty acids and phospholipids during and following complete and severe incomplete ischemia. *J Neurochem,* 38, 84-93.

Reynolds, I. J. and Hastings, T. G. (1995) Glutamate induces the production of reactive oxygen species in cultured forebrain neurons following NMDA receptor activation. *J Neurosci,* 15, 3318-3327.

Riess, P., Bareyre, F. M., Saatman, K. E., Cheney, J. A., Lifshitz, J., Raghupathi, R., Grady, M. S., Neugebauer, E. and McIntosh, T. K. (2001) Effects of chronic, post-injury Cyclosporin A administration on motor and sensorimotor function following severe, experimental traumatic brain injury. *Restor Neurol Neurosci,* 18, 1-8.

Rizzuto, R., Bernardi, P. and Pozzan, T. (2000) Mitochondria as all-round players of the calcium game. *J Physiol,* 529 Pt 1, 37-47.

Rizzuto, R., Brini, M., Murgia, M. and Pozzan, T. (1993) Microdomains with high Ca2+ close to IP3-sensitive channels that are sensed by neighboring mitochondria. *Science,* 262, 744-747.

Rytter, A., Cardoso, C. M., Johansson, P., Cronberg, T., Hansson, M. J., Mattiasson, G., Elmer, E. and Wieloch, T. (2005) The temperature dependence and involvement of mitochondria permeability transition and caspase activation in damage to organotypic hippocampal slices following in vitro ischemia. *J Neurochem,* 95, 1108-1117.

Scheff, S. W. and Sullivan, P. G. (1999) Cyclosporin A significantly ameliorates cortical damage following experimental traumatic brain injury in rodents. *J Neurotrauma,* 16, 783-792.

Schinzel, A. C., Takeuchi, O., Huang, Z. *et al.* (2005) Cyclophilin D is a component of mitochondrial permeability transition and mediates neuronal cell death after focal cerebral ischemia. *Proc Natl Acad Sci U S A,* 102, 12005-12010.

Schönfeld, P. and Reiser, G. (2007) Ca2+ storage capacity of rat brain mitochondria declines during the postnatal development without change in ROS production capacity. *Antioxid Redox Signal,* 9, 191-199.

Scorrano, L., Oakes, S. A., Opferman, J. T., Cheng, E. H., Sorcinelli, M. D., Pozzan, T. and Korsmeyer, S. J. (2003) BAX and BAK regulation of endoplasmic reticulum Ca2+: a control point for apoptosis. *Science,* 300, 135-139.

Shalbuyeva, N., Brustovetsky, T., Bolshakov, A. and Brustovetsky, N. (2006) Calcium-dependent spontaneously reversible remodeling of brain mitochondria. *J Biol Chem,* 281, 37547-37558.

Shalbuyeva, N., Brustovetsky, T. and Brustovetsky, N. (2007) Lithium desensitizes brain mitochondria to calcium, antagonizes permeability transition, and diminishes cytochrome C release. *J Biol Chem,* 282, 18057-18068.

Shibasaki, F., Kondo, E., Akagi, T. and McKeon, F. (1997) Suppression of signalling through transcription factor NF-AT by interactions between calcineurin and Bcl-2. *Nature,* 386, 728-731.

Siemen, D., Loupatatzis, C., Borecky, J., Gulbins, E. and Lang, F. (1999) Ca2+-activated K channel of the BK-type in the inner mitochondrial membrane of a human glioma cell line. *Biochem Biophys Res Commun,* 257, 549-554.

Siesjö, B. K. (1981) Cell damage in the brain: a speculative synthesis. *J Cereb Blood Flow Metab,* 1, 155-185.

Siesjö, B. K. (1992) Pathophysiology and treatment of focal cerebral ischemia. Part I: Pathophysiology. *J Neurosurg,* 77, 169-184.

Simon, R. P., Griffiths, T., Evans, M. C., Swan, J. H. and Meldrum, B. S. (1984) Calcium overload in selectively vulnerable neurons of the hippocampus during and after ischemia: an electron microscopy study in the rat. *J Cereb Blood Flow Metab,* 4, 350-361.

Sims, N. (1990) Rapid isolation of metabolically active mitochondria from rat brain and subregions using percoll density gradient centrifugation. *J Neurochem,* 55, 698-707.

Singleton, R. H., Stone, J. R., Okonkwo, D. O., Pellicane, A. J. and Povlishock, J. T. (2001) The immunophilin ligand FK506 attenuates axonal injury in an impact-acceleration model of traumatic brain injury. *J Neurotrauma,* 18, 607-614.

Snyder, S. H., Lai, M. M. and Burnett, P. E. (1998) Immunophilins in the nervous system. *Neuron,* 21, 283-294.

Soane, L., Solenski, N. and Fiskum, G. (2007) Mitochondrial Mechanisms of Oxidative Stress and Apoptosis. In: *Handbook of Neurochemistry and Molecular Neurobiology - Brain Energetics. Integration of Molecular and Cellular Processes*, (A. Lajtha, G. E. Gibson and G. A. Dienel eds.), Vol. 5*,* pp. 703-734. Springer, New York, NY, USA.

Starkov, A. A., Chinopoulos, C. and Fiskum, G. (2004) Mitochondrial calcium and oxidative stress as mediators of ischemic brain injury. *Cell Calcium,* 36, 257-264.

Sullivan, P. G., Rabchevsky, A. G., Hicks, R. R., Gibson, T. R., Fletcher-Turner, A. and Scheff, S. W. (2000a) Dose-response curve and optimal dosing regimen of cyclosporin A after traumatic brain injury in rats. *Neuroscience,* 101, 289-295.

Sullivan, P. G., Rabchevsky, A. G., Keller, J. N., Lovell, M., Sodhi, A., Hart, R. P. and Scheff, S. W. (2004) Intrinsic differences in brain and spinal cord mitochondria: Implication for therapeutic interventions. *J Comp Neurol,* 474, 524-534.

Sullivan, P. G., Thompson, M. and Scheff, S. W. (2000b) Continuous infusion of cyclosporin A postinjury significantly ameliorates cortical damage following traumatic brain injury. *Exp Neurol,* 161, 631-637.

Sullivan, P. G., Thompson, M. B. and Scheff, S. W. (1999) Cyclosporin A attenuates acute mitochondrial dysfunction following traumatic brain injury. *Exp. Neurol.,* 160, 226-234.

Szabo, I., Bock, J., Jekle, A., Soddemann, M., Adams, C., Lang, F., Zoratti, M. and Gulbins, E. (2005) A novel potassium channel in lymphocyte mitochondria. *J Biol Chem,* 280, 12790-12798.

Szabo, I., De Pinto, V. and Zoratti, M. (1993) The mitochondrial permeability transition pore may comprise VDAC molecules. II. The electrophysiological properties of VDAC are compatible with those of the mitochondrial megachannel. *FEBS Lett,* 330, 206-210.

Tanaka, C., Kawai, R. and Rowland, M. (2000) Dose-dependent pharmacokinetics of cyclosporin A in rats: events in tissues. *Drug metabolism and disposition: the biological fate of chemicals,* 28, 582-589.

Teng, Y. D., Choi, H., Onario, R. C. *et al.* (2004) Minocycline inhibits contusion-triggered mitochondrial cytochrome c release and mitigates functional deficits after spinal cord injury. *Proc Natl Acad Sci U S A,* 101, 3071-3076.

Tretter, L. and Adam-Vizi, V. (2007) Uncoupling is without an effect on the production of reactive oxygen species by in situ synaptic mitochondria. *J Neurochem*.

Tsuji, A., Tamai, I., Sakata, A., Tenda, Y. and Terasaki, T. (1993) Restricted transport of cyclosporin A across the blood-brain barrier by a multidrug transporter, P-glycoprotein. *Biochem Pharmacol,* 46, 1096-1099.

Turrens, J. F. (2003) Mitochondrial formation of reactive oxygen species. *J Physiol,* 552, 335-344.

Uchino, H., Elmér, E., Uchino, K., Li, P. A., He, Q. P., Smith, M. L. and Siesjö, B. K. (1998) Amelioration by cyclosporin A of brain damage in transient forebrain ischemia in the rat. *Brain Res.,* 812, 216-226.

Uchino, H., Elmér, E., Uchino, K., Lindvall, O. and Siesjö, B. K. (1995) Cyclosporin A dramatically ameliorates CA1 hippocampal damage following transient forebrain ischaemia in the rat. *Acta Physiol Scand,* 155, 469-471.

Uchino, H., Minamikawa-Tachino, R., Kristian, T., Perkins, G., Narazaki, M., Siesjö, B. K. and Shibasaki, F. (2002) Differential neuroprotection by cyclosporin A and FK506 following ischemia corresponds with differing abilities to inhibit calcineurin and the mitochondrial permeability transition. *Neurobiol Dis,* 10, 219-233.

van Lookeren Campagne, M. and Gill, R. (1996) Ultrastructural morphological changes are not characteristic of apoptotic cell death following focal cerebral ischaemia in the rat. *Neurosci Lett,* 213, 111-114.

Vergun, O., Sobolevsky, A. I., Yelshansky, M. V., Keelan, J., Khodorov, B. I. and Duchen, M. R. (2001) Exploration of the role of reactive oxygen species in glutamate neurotoxicity in rat hippocampal neurones in culture. *J Physiol,* 531, 147-163.

Vesce, S., Kirk, L. and Nicholls, D. G. (2004) Relationships between superoxide levels and delayed calcium deregulation in cultured cerebellar granule cells exposed continuously to glutamate. *J Neurochem,* 90, 683-693.

Votyakova, T. V. and Reynolds, I. J. (2004) Detection of hydrogen peroxide with Amplex Red: interference by NADH and reduced glutathione auto-oxidation. *Arch Biochem Biophys,* 431, 138-144.

Waldmeier, P. C., Zimmermann, K., Qian, T., Tintelnot-Blomley, M. and Lemasters, J. J. (2003) Cyclophilin D as a drug target. *Curr Med Chem,* 10, 1485-1506.

Wang, H. G., Pathan, N., Ethell, I. M. *et al.* (1999) Ca2+-induced apoptosis through calcineurin dephosphorylation of BAD. *Science,* 284, 339-343.

Wang, X., Zhu, S., Drozda, M., Zhang, W., Stavrovskaya, I. G., Cattaneo, E., Ferrante, R. J., Kristal, B. S. and Friedlander, R. M. (2003) Minocycline inhibits caspase-independent and -dependent mitochondrial cell death pathways in models of Huntington's disease. *Proc Natl Acad Sci U S A,* 100, 10483-10487.

Wei, M. C., Zong, W. X., Cheng, E. H. *et al.* (2001) Proapoptotic BAX and BAK: a requisite gateway to mitochondrial dysfunction and death. *Science,* 292, 727-730.

Whittam, R. (1962) The dependence of the respiration of brain cortex on active cation transport. *Biochem J,* 82, 205-212.

Wieckowski, M. R., Brdiczka, D. and Wojtczak, L. (2000) Long-chain fatty acids promote opening of the reconstituted mitochondrial permeability transition pore. *FEBS Lett,* 484, 61-64.

Wieckowski, M. R. and Wojtczak, L. (1998) Fatty acid-induced uncoupling of oxidative phosphorylation is partly due to opening of the mitochondrial permeability transition pore. *FEBS Lett,* 423, 339-342.

Wieloch, T. (2002) Molecular mechanisms of ischemic brain damage. In: *Cerebral blood flow and metabolism*, (L. Edvinsson and D. N. Krause eds.), pp. 423-451. Lippincott Williams & Wilkins, Philadelphia.

Wieloch, T., Mattiasson, G., Hansson, M. J. and Elmér, E. (2007) Mitochondrial Permeability Transition in the CNS—Composition, Regulation, and Pathophysiological Relevance. In: *Handbook of Neurochemistry and Molecular Neurobiology - Brain Energetics. Integration of Molecular and Cellular Processes*, (A. Lajtha, G. E. Gibson and G. A. Dienel eds.), Vol. 5, pp. 667-702. Springer, New York, NY, USA.

Woodfield, K., Ruck, A., Brdiczka, D. and Halestrap, A. P. (1998) Direct demonstration of a specific interaction between cyclophilin-D and the adenine nucleotide translocase confirms their role in the mitochondrial permeability transition. *Biochem J,* 336, 287-290.

Xu, W., Liu, Y., Wang, S., McDonald, T., Van Eyk, J. E., Sidor, A. and O'Rourke, B. (2002) Cytoprotective role of Ca2+- activated K+ channels in the cardiac inner mitochondrial membrane. *Science,* 298, 1029-1033.

Yakel, J. L. (1997) Calcineurin regulation of synaptic function: from ion channels to transmitter release and gene transcription. *Trends Pharmacol Sci,* 18, 124-134.

Yellon, D. M. and Downey, J. M. (2003) Preconditioning the myocardium: from cellular physiology to clinical cardiology. *Physiol Rev,* 83, 1113-1151.

Yokota, M., Saido, T. C., Tani, E., Kawashima, S. and Suzuki, K. (1995) Three distinct phases of fodrin proteolysis induced in postischemic hippocampus. Involvement of calpain and unidentified protease. *Stroke,* 26, 1901-1907.

Yoshimoto, T. and Siesjö, B. K. (1999) Posttreatment with the immunosuppressant cyclosporin A in transient focal ischemia. *Brain Res,* 839, 283-291.

Yoshimoto, T., Uchino, H., He, Q. P., Li, P. A. and Siesjö, B. K. (2001) Cyclosporin A, but not FK506, prevents the downregulation of phosphorylated Akt after transient focal ischemia in the rat. *Brain Res,* 899, 148-158.

Zaidan, E., Nilsson, M. and Sims, N. R. (2004) Increased mitochondrial permeability in response to intrastriatal N-methyl-D-aspartate: detection based on accumulation of radiolabel from [3H]deoxyglucose. *Neurochem Res,* 29, 609-616.

Zaidan, E. and Sims, N. R. (1994) The calcium content of mitochondria from brain subregions following short-term forebrain ischemia and recirculation in the rat. *J Neurochem,* 63, 1812-1819.

Zhu, S., Stavrovskaya, I. G., Drozda, M. *et al.* (2002) Minocycline inhibits cytochrome c release and delays progression of amyotrophic lateral sclerosis in mice. *Nature,* 417, 74-78.

Zoccarato, F. and Nicholls, D. (1982) The role of phosphate in the regulation of the independent calcium-efflux pathway of liver mitochondria. *Eur J Biochem,* 127, 333-338.

Zoratti, M. and Szabo, I. (1995) The mitochondrial permeability transition. *Biochim. Biophys. Acta,* 1241, 139-176.

Zorov, D. B., Filburn, C. R., Klotz, L. O., Zweier, J. L. and Sollott, S. J. (2000) Reactive oxygen species (ROS)-induced ROS release: a new phenomenon

accompanying induction of the mitochondrial permeability transition in cardiac myocytes. *J Exp Med,* 192, 1001-1014.