Isoflurane and sevoflurane: Effects on mitochondrial function in the rat and human brain

By

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Oslo, November 2009

Ravi Bains

2. PAPERS INCLUDED IN THE THESIS

The thesis is based on the following papers referred to by their Roman numerals:

I. Moe MC, Bains R, Vinje ML, Larsen GA, Kampenhaug EB, Berg-Johnsen J.
Sevoflurane depolarizes pre-synaptic mitochondria in the central nervous system.
Acta Anaesthesiol Scand. 2004 May; 48(5): 562-8.

II. Bains R, Moe MC, Larsen GA, Berg-Johnsen J, Vinje ML.
Volatile anaesthetics depolarize neural mitochondria by inhibition of the electron transport chain.
Acta Anaesthesiol Scand. 2006 May; 50(5): 572-9.

III. Bains R, Moe MC, Vinje ML, Berg-Johnsen J.
Isoflurane induced depolarization of neural mitochondria increases with age.
Acta Anaesthesiol Scand. 2009 Jan; 53(1): 85-92.

IV. Bains R, Moe MC, Vinje ML, Berg-Johnsen J.
Sevoflurane and propofol depolarize mitochondria in rat and human cerebrocortical synaptosomes by different mechanisms.
Acta Anaesthesiol Scand. 2009 Jul 22. [Epub ahead of print].

3. ABBREVIATIONS

4-AP	4-aminopyridine
$\Delta\Psi_m$	Mitochondrial membrane potential
MPT	Mitochondrial permeability transition pore
VGCC	Voltage-gated Ca^{2+}-channels
FCCP	Carbonyl cyanide-p-(trifluoromethoxy)-phenylhydrazone
CNS	Central nervous system
K^+	Potassium
ER	Endoplasmic reticulum
$[Ca^{2+}]_i$	Free cytosolic Ca^{2+}
GABA	Gamma-amino-butyric acid
GA	General anaesthetics
IP_3	Inositol triphosphate
LGIC	Ligand-gated ion channels
5 HD	5-hydroxydecanoate
MAC	Minimal alveolar concentration
$MitoK_{ATP}$	Mitochondrial adenosine triphosphate - regulated potassium channels
NMDA	N-methyl-D-aspartate
ATP	Adenosine-5'-triphosphate
ADP	Adenosine diphosphate
PKC	Protein kinase C
ROS	Reactive oxygen species
OGD	Oxygen – and glucose deprivation
VA	Volatile anaesthetics

4. INTRODUCTION

4.1 General anaesthesia

General anaesthesia is a state defined by a reversible drug induced loss of consciousness, areflexia and analgesia (1). The discovery of general anaesthesia, in the mid 19th century, is considered a major milestone in medical history, making modern surgery possible by rendering the patient unaware of, and unresponsive to, painful stimulation. Although GA are administered to millions of patients each year (2), the exact mechanisms of unconsciousness still remain elusive.

Anaesthetics appear to act on the cell membrane by interacting with the main components, namely lipids, proteins and oligosaccharides (3). Meyer and Overton proposed the lipid theory by showing a close correlation between anaesthetic potency and lipid solubility at the beginning of the 20th century (4). However, interaction of anaesthetics with functional membrane proteins became evident as the activity of purified luciferase (a soluble lipid-free enzyme responsible for the luminescent reaction in firefly) was inhibited by GA (5).

The idea that anaesthetics act by disrupting lipid bilayers or some other nonspecific mechanisms is largely abandoned, and anaesthetics at clinical concentrations are now thought to exert their effects selectively by binding directly to specific proteins (5).

Much research have focused on identifying a particular brain region on which anaesthetics act to induce their effects (6). The most sensitive regions appear to be the thalamic sensory relay nuclei and the deep layer of the cortex to which these nuclei project. This constitutes the route taken by sensory impulses reaching the cortex, so inhibition can result in a lack of awareness of sensory input. As the anaesthetic

concentration is increased, all brain functions are affected, including motor control, reflex activity, respiration and autonomic regulation (7).

4.2 Cellular mechanisms of general anaesthesia

At the cellular level, the effect of anaesthetics is mainly to inhibit excitatory synaptic transmission. In 1906 Sherrington reported that chloroform blocked reflex activity in the spinal cord at lower concentrations than needed to inhibit impulse propagation along nerve fibres (8). Numerous studies have since implicated that inhibition of synaptic transmission could be due to reduced transmitter release, increased transmitter uptake, inhibition of the action of the transmitter, or reduced excitability of the postsynaptic cell (9, 10).

GA may effect the impulse conduction on several sites like: 1. Afferent nerve fibres, 2. Excitatory synapse, 3. Inhibitory synapse, 4. Postsynaptic neurone. Clinically relevant doses of isoflurane depress impulse conduction in thin, unmyelinated, afferent nerve fibres, whereas almost no effect is found on impulse propagation in thicker, myelinated fibres (11). Furthermore, isoflurane hyperpolarizes the postsynaptic neuron (12), making the cell more refractory to membrane depolarisation.

4.3 Molecular mechanisms of general anaesthesia

GA in supraclinical doses act non-specifically, leading to a variety of effects. In clinically relevant doses, however, GA are thought to exert its effects directly on proteins, possibly binding to a hydrophobic area of the protein and inhibiting its normal function (5). The most likely targets are VGCC, LGIC and second messenger systems.

The molecular target for intravenous anaesthetics, such as propofol, is identified as the $GABA_A$ receptor, with functional effects possibly depending on the receptor subunit composition (7). For VA, such as isoflurane and sevoflurane, there are currently three main candidates: 1. $GABA_A$ receptors, 2. Two-pore-domain K^+ channels, and 3. NMDA receptors. In addition, there are evidence for involvement of several minor targets, such as inhibitory glycine receptors and cyclic-nucleotide-gated (HCN) channels, which underlie the hyperpolarisation-activated cation current, and presynaptic Na^+ channel subtypes (7).

4.4 The presynaptic terminal and signal transduction

Depolarization of nerve terminals opens VGCC coupled to the exocytotic machinery and facilitates signal transduction by releasing neurotransmitters (13). Neurotransmitter release has been intensively studied since 1950, using the frog neuromuscular synapse as a model (14). The influx of Ca^{2+} further propagates Ca^{2+} release from internal stores, such as the ER and the mitochondria. The resulting increase in cytosolic Ca^{2+} initiates the fusion of neurotransmitter containing vesicles with the plasma membrane. The released neurotransmitter diffuses across the synaptic cleft and may interact with postsynaptic receptors, leading to either excitatory or inhibitory stimuli, depending on the neurotransmitter or postsynaptic receptor involved. The signal transduction process is terminated by enzymatic breakdown or reuptake into either the presynaptic terminal or adjacent glial cells (15).

4.5 Transmitter substances

The predominant excitatory neurotransmitters in the mammalian brain, glutamate and acetylcholine, allow the passage of Na^+ and Ca^{2+} and thereby depolarize the plasma membrane toward the threshold potential required for triggering an action potential (15). The major inhibitory neurotransmitters, GABA and glycine, usually affect Cl^- channel receptors and render the target cell more difficult to depolarize (16).

To summarize, the nerve terminal converts an electrical signal to a chemical signal, and the postsynaptic cell converts the chemical signal back to an electrical one. Anaesthetics may exert their effects by disrupting signal transduction along these pathways. Matthews and Quilliam in 1964, provided the first evidence that anaesthetics may reduce the nerve impulse induced amount of transmitter release (17). Considerable evidence now indicate that VA potentiate the effect of inhibitory postsynaptic receptors, predominantly the $GABA_A$-receptor, as well as depress the effect of excitatory postsynaptic receptors (18-25). Previous studies from our lab has also shown that isoflurane increase uptake velocity of glutamate into presynaptic terminals in the high affinity area (26).

4.6 Intracellular Ca^{2+} and voltage gated Ca^{2+} channels

Intracellular Ca^{2+} is an important second messenger that controls neurosecretion and neuronal excitability through at least five major classes of VGCC, which are known as the L, N, P/Q, R and T subtypes (27). Furthermore, cytosolic Ca^{2+}-homeostasis is regulated by the Ca^{2+}-ATPase and the Na^+/Ca^{2+} exchanger, which in neurons pump Ca^{2+} out of cytosol and intracellular organelles, including ER and mitochondria (13). In the ER, Ca^{2+} is controlled by IP_3 and ryanodine receptors, which after stimulation release

Ca^{2+} (Ca^{2+} dependent Ca^{2+} release), and by the Ca^{2+}-ATPase that actively pumps Ca^{2+} from the cytoplasm back into the ER (28).

Studies on the P/Q – and the N-types of the VGCC, which are the most influential in the Ca^{2+}-dependent presynaptic release of neurotransmitters have revealed only a modest sensitivity to VA (29-31). Furthermore, data indicate no significant effect of VA on basal $[Ca^{2+}]_i$ (30, 32). However, in studies on hippocampus slices and acutely dissociated neurons it has been reported an increased basal $[Ca^{2+}]_i$ in response to clinically relevant concentrations of isoflurane (33). In rat synaptosomes, isoflurane tended to increase the basal $[Ca^{2+}]_i$ (34).

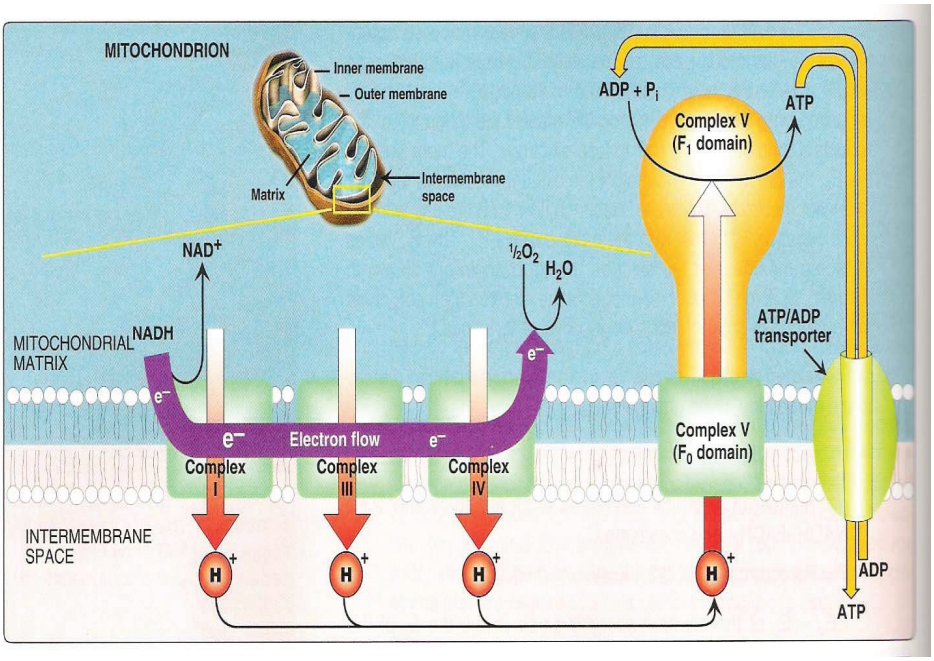
4.7.1 Mitochondrial function

The mitochondria play an essential role in cellular homeostasis. Structurally, they comprise of an outer membrane, that is freely permeable to most ions and small molecules, and an impermeable inner membrane with specialized carriers and transporting systems.

The electron transport chain is present in the inner mitochondrial membrane and is composed of five distinctive complexes responsible for shuttling electrons and ultimately ATP synthesis (27). Complexes I, II, and IV function as proton pumps, in series with respect to the electron flux and in parallel with respect to the proton circuit. Each complex in the respiratory chain receives or donates electrons to carrier enzymes, such as coenzyme Q and cytochrome C, and ultimately molecular oxygen and protons are reduced to form water at complex IV. The fall in redox potential during the electron flux generates an electrochemical proton gradient by pumping protons across the inner

mitochondrial membrane. The protons re-enter the mitochondrial matrix through the ATP synthesis at complex V and phosporylate ADP to ATP (Fig.1).

The electrochemical potential around –150 mV across the inner mitochondrial membrane therefore drives the three fundamental functions of mitochondria, namely ATP generation, Ca^{2+} uptake/storage, and generation/detoxification of ROS (27, 35-37). Furthermore, changes in mitochondrial Ca^{2+} regulate the enzymes in the tricarboxylic acid cycle.



Figur 1. Electron transport chain shown coupled to the transport of protons. Reprinted with approval of Lippincott Williams and Wilkins, WK Health, Philadelphia, USA.

4.7.2 Mitochondrial Ca^{2+} uptake and release

Mitochondrial uptake of Ca^{2+} during physiological Ca^{2+} signalling regulates mitochondrial metabolism, mainly by up regulating the dehydrogenases of the tricarboxylic acid cycle (pyruvate dehydrogenase, 2-oxoglutarate dehydrogenase and NAD^+ isocitrate dehydrogenase) (37, 38). The influx of Ca^{2+} into the matrix is dependent on the electrochemical gradient for Ca^{2+}, and low resting intramitochondrial Ca^{2+} concentration (39). The main route of entry to the matrix is the uniporter located in the inner membrane (40).

The Ca^{2+} extrusion from the mitochondrial matrix is an active process requiring either ATP hydrolysis or an extramitochondrial ion travelling down an electrochemical gradient (41, 42). In some tissues, Ca^{2+} is extruded from the mitochondria in exchange for Na^+. A Na^+/H^+ antiporter restores the mitochondrial Na^+ balance. Excessive mitochondrial Ca^{2+} accumulation in combination with oxidative stress, ATP depletion and high inorganic phosphate, may initiate opening of the MPT, resulting in dissipation of the $\Delta\Psi_m$ and ceasing of oxidative phosphorylation. The following ATP depletion may lead to both necrotic and apoptotic cell death (43).

Thus, the respiratory chain generates the $\Delta\Psi_m$ and that in combination with a mitochondrial/cytosolic Ca^{2+} gradient, provides the necessary driving force for mitochondrial Ca^{2+} uptake and release (37). There has also been suggested Na^+ independent Ca^{2+} efflux by either reversal of the uniporter (44) .

4.7.3 Mitochondria and ROS production

The mitochondria are the major intracellular source of ROS. Complex IV reduces molecular oxygen to water. However, about 5 % of this reaction may occur as a single

electron reduction at complex III, generating superoxide anion O_2^-. There is also evidence of a minor ROS production at complex I (45). The mitochondria are also vulnerable to ROS that may cause DNA strand breakage, peroxidation of polyunsaturated fatty acid chains and inhibition of the electron transport chain (45). Furthermore, ROS may increase Ca^{2+} release from intracellular stores, and thereby increase the mitochondrial uptake. Current evidence indicates that Ca^{2+}overload and ROS in combination may initiate MTP, although the interaction between them is not fully understood.

5. THE AIMS OF THE STUDY

The present study was conducted to investigate the effects of isoflurane and sevoflurane, the most commonly used VA in neuroanaesthesia (46, 47), on mitochondria from cerebrocortical synaptosomes. Mitochondrial function was studied with special emphasis on the complexes in the electron transport chain that are important to maintain the mitochondrial membrane potential. The effects of VA were then compared with the effects of the intravenous anaesthetic propofol. The specific aims were defined:

1. To assess whether sevoflurane affects mitochondrial function in the central nervous system, and to investigate whether an effect involves the activation of mitoK_{ATP}. (Paper I)

2. To compare the effects of isoflurane and sevoflurane on the $[Ca^{2+}]_i$ and the $\Delta\Psi_m$, and to explore whether clinically relevant concentrations affect the $\Delta\Psi_m$ by inhibition of the electron transport chain. (Paper II)

3. To investigate whether the mitochondrial sensitivity to anaesthetics is related to age and the relationship to intervention of the respiratory chain activity. (Paper III)

4. To investigate and compare the effects of sevoflurane and propofol on $[Ca^{2+}]_i$ and the $\Delta\Psi_m$ in rat and human cerebrocortical synaptosomes, and relate the changes to interventions in the electron transport chain. (Paper IV)

6. MATERIALS AND METHODS

6.1 The synaptosomal preparation

Isolated nerve terminals (synaptosomes) have been in use since the beginning of the 1960s (48) to investigate presynaptic function. Through a process of homogenization and centrifugation (49), the nerve terminals are pinched off and resealed (Fig.2). The synaptosomes contain mitochondria and are able to maintain the ATP levels for more than 6 hours when kept on ice (49, 50). Preserved in a medium with a low K^+ concentration, they maintain a membrane potensial of -60 mV to -80mV (51) and a cytoplasmic free Ca^{2+} concentration of 0.1-0.2 μM (52). The synaptosomal preparation is thus regarded energetically intact (13). There are two main experimental advantages using this preparation. First, it is the simplest preparation with an intact plasma membrane and cytoplasm including mitochondria, metabolic pathways, and machinery for uptake, storage, and release of neurotransmitter. Secondly, it is free from glial and neuronal cell body elements (53). The synaptosomal preparation can be made from the animal or human brain of any age, in contrast to the neonatal requirement of most neuronal cell cultures.

There are numerous mitochondria in the nerve terminals (54). Although not representative of an intact neuron, the synaptosomal preparation represents a simple model for investigating mitochondrial and cellular function (13, 54), and seems ideal when studying the effects of neuropharmacological agents on mitochondrial function in the brain. There are, however, differences in the function of mitochondria in the neuronal soma and mitochondria situated in the presynaptic terminal (55), which underline the

importance of studying mitochondrial function both in neurons and in presynaptic terminals in isolation.

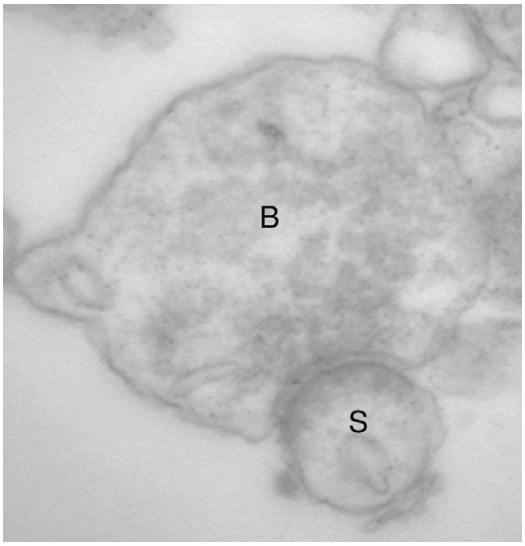


Fig. 2. Electron microscopic picture of the synaptosomal preparation from rat brain used in these thesis showing isolated presynaptic boutons (B). Some postsynaptic elements such as a postsynaptic spinae (S) connected to the presynaptic terminals in dense synaptic clefts. Magnification: X 64 000. With approval of Reidun Torp, Centre for Molecular Biology and Neuroscience, Oslo, Norway.

6.2 Measuring mitochondrial function using fluorescent probes

In the present work a time-course registration of functional mitochondrial parameters was studied using a fluorescence spectrophotometer (Hitachi Ltd., Tokyo, Japan). Synaptosomal $[Ca^{2+}]_i$ was measured as the fluorescence of fura-2 (Molecular Probes, the Netherlands), a ratiometric dye that upon binding to Ca^{2+} exhibits a shift in the excitation maximum (56). Fura-2 is excited at 340 nm and 380 nm of light, and the ratio of the emissions at those wavelengths is directly correlated to the amount of $[Ca^{2+}]_i$. An increase in the normalized fura-2 ratio thus indicates an increase in $[Ca^{2+}]_i$.

The $\Delta\Psi_m$, a central parameter of mitochondrial function, was measured as the fluorescence of JC-1, a lipophilic and cationic dye that exhibits potential-dependent accumulation in the negatively charged mitochondria. At low concentrations (low $\Delta\Psi_m$), JC-1 exists mainly in a monomeric form, emitting green fluorescence, whereas JC-1 in high concentrations (high $\Delta\Psi_m$) forms aggregates emitting red fluorescence. A reduction in the normalized JC-1 ratio indicates a depolarization of the mitochondrial membrane (57).

To assess whether the observed effect of VA on the $\Delta\Psi_m$ was disturbed by interference between the two fluorescence probes, single-dye experiments with JC-1 and sevoflurane 2 MAC were performed. Sevoflurane decreased the $\Delta\Psi_m$ in N-ACSF and in Ca^{2+} depleted ACSF to the same degree as in dual-probe experiments.

6.3 Inhibition of the respiratory chain

In order to investigate the mechanisms of isoflurane and sevoflurane action on the electron transport chain, complex I and V were inhibited by adding oligomycin 2 µg/ml and rotenone 2 µM

Oligomycin is a natural antibiotic isolated from *Streptomyces diastatochromogenes* which inhibits mitochondrial H^+-ATP synthase at complex V (58). Oligomycin at high concentrations may also inhibit the plasma membrane Na^+-K^+-ATPase.

Rotenone is a naturally occurring chemical from the roots of several tropical and subtropical plant species belonging to the genus *Lonchocarpus* or *Derris* with toxic properties towards humans and other mammals. Its toxic effect is due to a general

inhibition of cellular respiration. Specifically, rotenone inhibits the phosphoryl group transfer at complex I by inhibiting the transfer of electrons from iron-sulfur centers in complex I to ubiquinone, and thereby preventing the conversion of NADH into usable cellular energy (ATP)(59).

6.4 Membrane depolarization

The synaptosomal plasma membrane can be depolarized with K^+ producing a clamped depolarisation, which does not, however, mimic physiological conditions (60). A more physiological membrane depolarisation is evoked by the K^+ channel inhibitor 4-AP (13). Using 4-AP, repetitive activation of Ca^{2+} channels are produced, and most of the Ca^{2+} independent efflux of amino acids seen with K^+ evoked depolarization is avoided (61).

In the present investigations the mitochondrial uncoupler FCCP 1 μM was added after anaesthetic exposure in each experiment to attain maximum mitochondrial depolarization.

6.5 Anaesthetic agents

Sevoflurane is a methyl propyl ether, which was synthesized in 1968. The initial development was slow because of some apparent toxic effects and problems of biotransformation and stability with soda lime. The drug has been available for general clinical use in Japan since 1990 and has been more recently introduced in Western Europe. The blood/partion coefficient of sevoflurane is 0.69, which is about half of that of isoflurane (1.43) and closer to that of desflurane (0.42), making the rate of equilibration between alveolar and inspired concentration faster than for halothane, enflurane and isoflurane, but slower than for desflurane. The MAC value of sevoflurane

in adults is between 1.7 and 2% in O_2 and 0.6% in 60% N_2O (62). It is non-flammable and non-irritating.

Sevoflurane has established itself as an important neuroanaesthetic agent, as both cerebral autoregulation and perfusion pressure is better maintained with sevoflurane than with isoflurane (63, 64). Sevoflurane has similar effects on the CNS as halothane, isoflurane and desflurane. Intracranial pressure increases at high-inspired concentrations, but the effect is minimal over the 0.5-1.0 MAC range. Sevoflurane decreases arterial pressure, but cardiac output is well preserved over the normal anaesthetic maintenance dose. In summary, sevoflurane produces a smooth and fast induction and a rapid recovery with minimal effects on respiratory and circulatory parameters using a clinical anaesthetic concentrations.

Sevoflurane and all other anaesthetic agents, at supra-clinical doses, however, can cause death by loss of cardiovascular reflexes and respiratory paralysis. Increased epileptiform electroencephalographic activity and seizure-like motor activity are reported in association with sevoflurane administration. (65). There is also a theoretical risk of renal toxicity due to the inorganic fluoride ion

Isoflurane is a halogenated ether inhalation-agent that was initially synthesized in 1965. It is non-flammable, suitable for vaporization, and has a low biodegradation as only 0.17 % is metabolized, with rapid elimination and thus small toxicity (66). A low blood/gas partition coefficient of 1.4 provides rapid uptake, distribution and elimination (66). MAC is 1.3% in middle aged humans and 1.5% in rats (62). Cerebral blood flow is mainly unaltered at concentrations of isoflurane below 1 MAC, and cerebral oxygen

consumption is decreased under isoflurane anaesthesia (67). Thus, isoflurane has until recently been considered the inhalation anaesthetic of choice in neurosurgery (46, 47).

Propofol is a short acting intravenous agent, clinically available since 1977, mainly used for induction of anaesthesia and long-term sedation. Propofol is presented in a 1-2 % formulation consisting of an oil and water emulsion. The pharmacokinetic profile is characterized by a rapid distribution from blood to tissues, and an equally rapid clearance. Propofol is mainly and rapidly metabolized in the liver, but some minor metabolites are also detected in the urine. The total body clearance of propofol is greater than liver blood flow, indicating an extrahepatic metabolism (68).

7. SUMMARY OF RESULTS

7.1 Paper I

Recent studies have demonstrated that VA increase the ischemic tolerance of the heart by activating mitochondrial signalling pathways. In particular, stimulation of mitoK_{ATP} has been documented (8, 9). In this study we therefore wanted to test whether sevoflurane affects mitochondrial function in the brain by altering the $\Delta\Psi_m$, and to investigate whether activation of mitoK_{ATP} might be involved. The effects of sevoflurane on the $\Delta\Psi_m$ was related to alterations in $[Ca^{2+}]_i$.

In Ca^{2+}-containing medium, sevoflurane 1 and 2 MAC gradually decreased the normalized JC-1 ratio from 0.96 ± 0.04 in control to 0.92 ± 0.03 and 0.89 ± 0.04, representing a decrease in the $\Delta\Psi_m$ (n=9, $p<0.01$). Furthermore, sevoflurane 2 MAC increased $[Ca^{2+}]_i$. In Ca^{2+}-depleted medium, sevoflurane 1 and 2 MAC decreased the $\Delta\Psi_m$ while $[Ca^{2+}]_i$ remained unaltered. Sevoflurane 2 MAC attenuated the 4-AP induced decrease in the $\Delta\Psi_m$. When mitoK_{ATP} was blocked, adding the specific antagonist 5-HD 500 μM, the sevoflurane induced decrease in the $\Delta\Psi_m$ was attenuated, but not blocked. The depolarizing effect of sevoflurane compared to FCCP was calculated to 12.2 ± 3.1 % in Ca^{2+}-containing medium and 17.4 ± 1.7 % in Ca^{2+}-depleted medium (n=5).

This study suggests that sevoflurane in clinically relevant concentrations depolarizes the mitochondrial membrane in isolated CNS presynaptic terminals, and that this effect is not dependent on Ca^{2+}-influx to the cytosol, even though $[Ca^{2+}]_i$ in this situation was significantly altered. Opening of mitoK_{ATP} is only partly responsible for the depolarizing effect of sevoflurane, suggesting that additional mechanisms must be involved.

7.2 Paper II

Having observed that clinical concentrations of sevoflurane affects the $\Delta\Psi_m$ in neural mitochondria, we wanted to further elucidate the mechanisms. Earlier studies have demonstrated that both VA and barbiturates inhibit the mitochondrial respiration by interfering with complex I (69-71) and the oxidative phosphorylation at complex V (69, 72-74), and thereby proposing the mitochondria as one of the targets for anaesthetic agents. The $\Delta\Psi_m$ controls the generation of ATP and ROS, and sequestration of $[Ca^{2+}]_i$. The main purpose of the present study was to compare the effects of isoflurane and sevoflurane on the $\Delta\Psi_m$, and to explore whether clinical concentrations of these anaesthetics affect the $\Delta\Psi_m$ by inhibition of the electron transport chain.

Isoflurane 1 and 2 MAC decreased the normalized JC-1 ratio from 0.92 ± 0.03 in control to 0.86 ± 0.02 and 0.81 ± 0.01, respectively, reflecting a depolarization of the mitochondrial membrane (n=9). Isoflurane 2 MAC increased $[Ca^{2+}]_i$. In Ca^{2+}-depleted medium, isoflurane still decreased the $\Delta\Psi_m$ while $[Ca^{2+}]_i$ remained unaltered. The effect of isoflurane was more pronounced than for sevoflurane. Blocking complex V of the respiratory chain enhanced the isoflurane and sevoflurane induced mitochondrial depolarization, whereas blocking complex I and V decreased the $\Delta\Psi_m$ to the same extent in control, isoflurane and sevoflurane experiments.

This study demonstrates that isoflurane and sevoflurane may act as metabolic inhibitors by depolarizing presynaptic mitochondria through inhibition of the electron transport chain. Isoflurane seems to inhibit mitochondrial function more pronounced than sevoflurane. Both agents inhibit the respiratory chain sufficiently to cause ATP synthase reversal.

7.3 Paper III

We further wanted to investigate mitochondrial sensitivity to anaesthetics related to age. The sensitivity for GA increases with age, but the mechanism for this age related sensitivity is still unknown. The main purpose of the present study was thus to investigate the effects of isoflurane on $[Ca^{2+}]_i$ and the $\Delta\Psi_m$ in isolated presynaptic terminals from neonatal, adolescent, and adult rats. The effect was compared and related to intervention of the respiratory chain activity.

In neonatal rats isoflurane had no significant effect on the $\Delta\Psi_m$. In adolescent and adult synaptosomes, however, isoflurane 1 and 2 MAC decreased the $\Delta\Psi_m$. Isoflurane 2 MAC increased $[Ca^{2+}]_i$ in neonatal and adolescent rats, but not in adult synaptosomes. In Ca^{2+}-depleted medium, isoflurane still decreased the $\Delta\Psi_m$ while $[Ca^{2+}]_i$ remained unaltered. By blocking complex V of the respiratory chain, the isoflurane induced mitochondrial depolarization was enhanced in all age groups. Blocking complex I depolarized the mitochondria to the same extent as isoflurane 2 MAC, but without any additive effect.

This study demonstrates that isoflurane depolarizes the $\Delta\Psi_m$ of neural mitochondria in an age dependent manner by inhibition of the respiratory chain. The effect is more pronounced in the adolescent and adult than in neonatal rats. The increased mitochondrial sensitivity with age seems to be related to the reversed function of the ATP synthase.

7.4 Paper IV

Volatile and intravenous anaesthetics influence brain physiology differently. The mitochondrial membrane potential drives the main functions of mitochondria. Sevoflurane depolarizes neural mitochondria. There is still, however, limited information concerning the effects of anaesthetics on neural mitochondria in humans. The effects of

sevoflurane and propofol on $[Ca^{2+}]_i$ and the $\Delta\Psi_m$ was therefore compared in rat and human synaptosomes, and the changes related to interventions in the electron transport chain.

Sevoflurane and propofol decreased the $\Delta\Psi_m$ in rat synaptosomes in a dose dependent manner, and to the same extent by equipotent doses. Inhibition of complex V enhanced the depolarizing effect of sevoflurane 2 MAC, but not of propofol 100 μM. Neither sevoflurane nor propofol affected $[Ca^{2+}]_i$ significantly. Sevoflurane and propofol decreased the $\Delta\Psi_m$ in human synaptosomes to the same extent as in the rat experiments.

The depolarizing effect of propofol on the Ψ_m was more rapid in onset than that of sevoflurane. Whereas sevoflurane inhibits the respiratory chain sufficiently to cause ATP synthase reversal, the depolarizing effect of propofol seems to be related to inhibition of the respiratory chain from complex I-V.

8. DISCUSSION

The effects of isoflurane, sevoflurane and propofol on mitochondrial function was studied in cerebrocortical synaptosomes prepared from the female rat and human brain. The role of signalling pathways, such as activation of mitoK_{ATP}, and whether these anaesthetics affect the $\Delta\Psi_m$ by inhibition of the electron transport chain was investigated. The effects of VA was related to alterations in $[Ca^{2+}]_i$.The effects of VA were then compared with the effects of the intravenous anaesthetic propofol.

8.1 The effects of volatile anaesthetics on the mitochondrial membrane potential

Isoflurane and sevoflurane in concentrations corresponding to 1 and 2 MAC depolarized the $\Delta\Psi_m$ gradually in a dose dependent manner (paper I and II), while only 2 MAC, a clinically relevant concentration in induction of anaesthesia, increased $[Ca^{2+}]_i$ slowly. When extracellular Ca^{2+} was removed, isoflurane and sevoflurane 1 and 2 MAC still decreased the $\Delta\Psi_m$ without altering $[Ca^{2+}]_i$. The findings imply that most of the mitochondrial depolarization caused by the VA administration was independent of Ca^{2+}influx to the cytosol. In contrast, a much higher concentration of isoflurane (3.1 mM) had to be applied to cortical neuronal-glial cultures to produce a depolarization of the $\Delta\Psi_m$, and this was followed by irreversible cell damage (75).

The depolarizing effect of isoflurane is more pronounced than for sevoflurane. Still, when extracellular Ca^{2+} was removed, isoflurane decreased the $\Delta\Psi_m$ without affecting the $[Ca^{2+}]_i$, and the effect on the $\Delta\Psi_m$ was more pronounced than for sevoflurane. The degree of mitochondrial depolarization induced by VA thus seems to be related to altered

mitochondrial function rather than effects on Ca^{2+}-influx due to plasma membrane depolarization (13).

The K^+-channel inhibitor 4-AP or the mitochondrial uncoupler FCCP was added at the end of each experiment to simulate pre-synaptic membrane depolarization or to attain maximum mitochondrial depolarization, respectively. A rapid and substantial mitochondrial depolarization was achieved by FCCP, while membrane depolarization with 4-AP decreased the $\Delta\Psi_m$ moderately. Isoflurane and sevoflurane 2 attenuated the 4-AP evoked change in $[Ca^{2+}]_i$ and the $\Delta\Psi_m$. Compared with FCCP the depolarizing effect of isoflurane and sevoflurane was less and more protracted (approximately 27 % in Ca^{2+} depleted medium). The anaesthetics did not change the value of maximum mitohondria depolarization with FCCP, this emphasizes that the effects of isoflurane and sevoflurane is due to a reduction in the $\Delta\Psi_m$, and not unspecific additive effects on the JC-1 fluorescence signal. However, since isoflurane and sevoflurane had an intrinsic depolarizing effect on mitochondria, further studies using a shorter time of exposure could reveal whether VA attenuate 4-AP induced changes in the $\Delta\Psi_m$.

There is considerable evidence that opening of mitoK_{ATP}, either directly or by activation of PKC, is important in VA induced cardioprotection (76, 77). This effect has to some extent been attributed to a decrease in the $\Delta\Psi_m$ and mitochondrial uncoupling (76), as activation of mitoK_{ATP} allow K^+ to enter into the mitochondria (78). In the brain, the density of mitoK_{ATP} are higher than in both liver and heart tissue**,** and these channels play a central role in the regulation of mitochondrial matrix volume (79). In this study 5-HD (paper I), a specific mitoK_{ATP}-antagonist, tended to attenuate the anaesthetic induced decrease in the $\Delta\Psi_m$ in Ca^{2+}-containing medium, but without reaching statistical

significance. In Ca^{2+}-depleted medium, the sevoflurane induced decrease in the $\Delta\Psi_m$ was significantly attenuated by 5-HD. However, the $\Delta\Psi_m$ was still significantly decreased by the anaesthetic in the presence of 5-HD. Thus, even though sevoflurane is known to activate mitoK_{ATP}, the anaesthetic-induced mitochondrial depolarization found in this study is only partly due to activation of mitoK_{ATP}. These findings are in accordance with reports where opening of the cardiac mitoK_{ATP} depolarized the mitochondria only to a minor degree (78, 80).

Sevoflurane and propofol depolarized the neural mitochondria in rat and human synaptosomes to the same degree, but with a different pharmacokinetic profile. Whereas propofol depolarized the mitochondria rapidly, sevoflurane exerted its effect more gradually. The surgical specimens used in this study were collected from superficial cortical structures from patients subjected to a temporal lobe resection due to medically intractable epilepsy. The tissue is supposedly normal, but is exposed to ischemia during preparation that may produce a pool of nonviable synaptosomes with damaged mitochondria, and the results of the present study can therefore not be applied directly to the healthy human brain.

What mechanisms are responsible for the depolarization of the $\Delta\Psi_m$ in presynaptic mitochondria?

Complex I, III, and IV in the electron transport chain act as proton pumps that generate an electrochemical potential of about -150 mV across the inner mitochondrial membrane. The energy generated is used to produce ATP and to buffer intracellular Ca^{2+} whenever

the local $[Ca^{2+}]_i$ rises above a critical "set point" (27, 37, 81, 82). The $\Delta\Psi_m$ is therefore regarded a central parameter of mitochondrial function (37, 82). Mitochondrial depolarization can result from increased ATP demand or increased Ca^{2+}-influx (83). In both cases, mitochondrial ATP synthase at complex V would continue to function and maintain the cytosolic ATP/ADP ratio. Impaired electron transport or substrate supply, and enhanced inner membrane proton permeability would also depolarize the mitochondria (83). Furthermore, activation of other ionic channels in the mitochondrial membrane, such as mitoK_{ATP}, could to some degree reduce the $\Delta\Psi_m$ (84). Additionally, it must be kept in mind that synaptic and non-synaptic mitochondria differ somewhat in their metabolic properties. Notably, synaptic mitochondria have a lower respiration rate and complex IV activity, which may render them more susceptible to insults such as ischemia (85).

8.2 The effects of volatile anaesthetics on the electron transport chain

Mitochondrial depolarization can be caused by respiratory chain inhibition, impaired substrate supply, or enhanced inner membrane proton permeability. Under these conditions, cytosolic ATP is consumed as complex V operates in a reverse mode to extrude matrix protons in an attempt to maintain the $\Delta\Psi_m$ (27). In cells or subcellular fractions with active glycolysis, respiratory chain inhibition at complex I to IV will therefore cause only a partial depolarization, because ATP synthase reversal at complex V can maintain a suboptimal $\Delta\Psi_m$ (27). In the present thesis, the finding of a considerably more depolarized mitochondrial membrane during isoflurane and sevoflurane (paper II) experiments when complex V was blocked indicates that the

mitochondrial membrane was sufficiently depolarized by the anaesthetics to partly reverse the function of ATP synthase at complex V. These findings in presynaptic terminals are in coherence with the effect of barbiturates on neural mitochondria in rat cortical neural cultures (86).

In synaptosomes, rotenone inhibition of complex I causes a drop in ATP levels (27), rendering the cell function dependent on the glycolytic capacity for ATP supply (87). The present study demonstrates that isoflurane depolarizes the $\Delta\Psi_m$ to the same extent as rotenone (paper III). Isoflurane did not potentiate the effects of rotenone on the mitochondria, suggesting related uncoupling mechanism. FCCP was still able to decrease the $\Delta\Psi_m$, indicating residual mitochondrial function. These findings are in accordance with other studies indicating that complex I is the most sensitive complex of the electron transport chain to inhibition by VA in mammalian mitochondria as shown in cardiac mitochondria (87).

After blocking complex I and inhibiting the ATP synthase reversal at complex V, neither isoflurane nor sevoflurane at concentrations corresponding to 2 MAC did significantly decrease the $\Delta\Psi_m$ further. Still, FCCP was able to decrease the $\Delta\Psi_m$, indicating that there was not complete collapse of the $\Delta\Psi_m$. These data suggest that clinical concentrations of isoflurane and sevoflurane depolarize presynaptic mitochondria by inhibition of the electron transport chain. VA thus seem to act as weak metabolic inhibitors of mitochondrial respiration as suggested by researchers more than 30 years ago (70), and this effect induces a mild depolarization of presynaptic mitochondria even at clinical concentrations.

8.3 The effects of volatile anaesthetics on free cytosolic Ca^{2+}

Ca^{2+} regulation in synaptosomes ($[Ca^{2+}]_i$) was investigated by administration of saturated solutions of isoflurane and sevoflurane and testing immediate response on $[Ca^{2+}]_i$. In an environment with extracellular Ca^{2+}-concentration of 1.3 mM, isoflurane and sevoflurane 2 MAC increased the basal $[Ca^{2+}]_i$ (paper I and II). The findings are in accordance with studies using brain slices and dissociated neurons (33), while earlier experiments with synaptosomes have failed to demonstrate any significant increase in $[Ca^{2+}]_i$ after isoflurane exposure (33, 34, 88). In contrast to changes in the $\Delta\Psi_m$, the anaesthetics had the opposite effect on $[Ca^{2+}]_i$, as isoflurane 2 MAC caused a smaller increase in $[Ca^{2+}]_i$ than sevoflurane 2 MAC (84, 89). This is interesting as increased basal $[Ca^{2+}]_i$-levels augments the probability of inducing seizures (34). Epileptiform electroencephalographic activity and seizure-like motor activity have in fact been reported during sevoflurane anaesthesia (65).

Previous studies on the VGCC, which are the most influential in the Ca^{2+}-dependent presynaptic release of neurotransmitters, have revealed only a modest sensitivity to VA (5, 31). However, recent studies have shown that anaesthetics may attenuate the depolarization-induced Ca^{2+}-increase in the presynaptic independently of VGCC by inhibiting the Ca^{2+}-dependent Ca^{2+}-release from ER (90, 91).

In the present studies isoflurane and sevoflurane also increased basal $[Ca^{2+}]_i$ in synaptosomes with relatively high extracellular Ca^{2+}-concentration (Paper I & II), that increased the Ca^{2+}-load into the presynaptic terminals (Paper I). The anaesthetics could thus potentially inhibit Ca^{2+}-uptake into presynaptic Ca^{2+}-stores such as ER (28), as described in kidney cells (92), or attenuate mitochondrial Ca^{2+}-buffering (59), as

discussed later. However, the effects of isoflurane and sevoflurane on the $\Delta\Psi_m$ was independent of Ca^{2+}-influx to the cytosol. The effects of isoflurane and sevoflurane on cytosolic Ca^{2+}-homeostasis in the presynaptic terminal are interesting topics for further research.

8.4 The age related effects of volatile anaesthetics on mitochondria

Mitochondrial and synaptosomal Ca^{2+}-efflux rates decline with age, and the ability to bind Ca^{2+} within the mitochondria may also change (93). The consequences of increased cytosolic Ca^{2+} and altered mitochondrial Ca^{2+}-buffering may involve mitochondrial depolarization or an increased propensity toward activation of the MPT (94). Reduced $\Delta\Psi_m$ would furthermore lower the driving force for Ca^{2+} entry and clearance of cytosolic Ca^{2+} load.

The depolarizing effect of isoflurane on neural mitochondria is more pronounced in the adolescent and adult than in neonatal synaptosomes (paper III). Previously it has been shown that isoflurane enhances suppresion of excitatory synaptic transmission in the aged rat hippocampus (95), and that increased sensitivity of mature synapses to anaesthetic action is not due to altered nerve fibre conduction (96). Taken together these findings suggest the involvement of synaptic sites in the age dependent potentiation of anaesthetic action. A lower overall energy requirement in neonates and a different distribution of glutamate receptors might be involved in the observed difference in mitochondrial sensitivity (97, 98) .

In the present study the isoflurane induced depolarization was enhanced in all age groups when complex V was blocked. The neonatal rats thus preserved the $\Delta\Psi_m$ better during

isoflurane exposure than adolescent and adult rats, indicating a more pronounced reversal of ATP synthase at complex V in neonates. The increased mitochondrial sensitivity with age seems to be related to the reversed function of the ATP synthase of the electron transport chain.

The effect of isoflurane on $[Ca^{2+}]_i$ was not influenced by age. When extracellular Ca^{2+} was removed, isoflurane still decreased the $\Delta\Psi_m$, and the effect was more pronounced in the adolescent and adult than in neonatal synaptosomes. The age dependent isoflurane sensitivity thus seems to be linked to a mitochondrial site rather than changes in $[Ca^{2+}]_i$.

8.5 Volatile anaesthetics and clinical implications

In the present thesis, the neuroprotective possibilities of VA during OGD have not been studied. However, since VA, such as isoflurane and sevoflurane, are frequently used in patients at risk for ischemic tissue damage, a neuroprotective potential is of considerable clinical interest, and will be briefly discussed.

VA possess neuroprotective properties in both *in vivo* and *in vitro* animal models of ischemia (99, 100). One possible mechanism would be their ability to reduce neuronal excitability through enhanced inhibitory and depressed excitatory synaptic transmission (101-103). It has been reported that small bursts of ROS production may initiate preconditioning in the myocardium (104, 105), and that isoflurane induced such a ROS production by inhibiting the respiratory chain in myocardial mitochondria (106). VA may also precondition brain against ischemia by activating mito K_{ATP} (107, 108). In the present study, we demonstrate that isoflurane and sevoflurane inhibits the respiratory chain in neural mitochondria. Whether this effect could stimulate a burst of ROS

production, that in turn may act neuroprotective, is an interesting topic for further research.

The electrochemical potential across the mitochondrial inner membrane is important for cellular Ca^{2+}-regulation (80). During ischemia, when the cytosolic Ca^{2+}-concentration rapidly increases, mitochondria are vulnerable to a Ca^{2+}-overload known to trigger the lethal MPT (78, 109). The slow dissipation of the $\Delta\Psi_m$ produced by isoflurane and sevoflurane may attenuate ischemic mitochondrial Ca^{2+}-overload in the presynaptic terminal by lowering the driving force for Ca^{2+}-uptake (37, 110), and thus prevent the activation of necrotic and apoptotic pathways or attenuate the oxidative stress (111). In addition, a decrease in the $\Delta\Psi_m$ is known to reverse the ATP synthase leading to depletion of cytoplasmatic ATP, and is generally associated with neurotoxicity (37). Whether a slow mitochondrial depolarization may be a neuroprotective mechanism must therefore be interpreted with caution.

Recent studies have demonstrated that anaesthetic agents can protect the brain in experimental models both during ischemia and by preconditioning before an ischemic injury (112-114). Anaesthetic agents influence the pathophysiology of cerebral ischemia at multiple levels. VA reduce ischemia induced glutamate release, inhibit postsynaptic glutamate receptors (115), and enhance GABA mediated hyperpolarization (116). In addition, they increase the level of antiapoptotic proteins such as Bcl-2 (117) that reduce mitochondrial permeability transition, cytochrome C release and subsequent activation of apoptosis cascades.

Preconditioning has been demonstrated for isoflurane (108) and sevoflurane by activation of sarcolemmal and mitochondrial potassium–ATP channels, activation of adenosine, and

activation of signal transduction pathways such as ERK ½, Akt, PKC and P38 (118, 119). Furthermore, a recent investigation by Engelhard et al. observed increased neurogenesis by sevoflurane 4 weeks after cerebral ischemia (120), and suggesting the possibility that anaesthetics may effect post ischemic CNS regeneration.

Despite the vast number of studies exploring the cerebral effects of anaesthetics, it is yet not known whether some of the VA are neuroprotective in clinical settings (47, 114, 121). To obtain this kind of knowledge, it might be important to elucidate the molecular mechanisms of action of the different VA *in vitro*. This information could be utilized to design further *in vitro* and *in vivo* studies testing the neuroprotective potential of different anaesthetic agents during OGD and excitotoxic stimuli. VA induced neuroprotection is a crucial topic for future research, as revealing clinical relevant neuroprotective mechanisms could make an immense improvement in the outcome from brain injury and cerebrovascular diseases.

9. MAIN CONCLUSIONS

The main conclusions in the present thesis are:

1. Sevoflurane slowly depolarizes the mitochondrial membrane in isolated CNS presynaptic terminals, and this effect is not dependent on Ca^{2+}-influx to the cytosol. Opening of mitoK_{ATP} is only partly responsible for this depolarizing effect of sevoflurane.

2. Isoflurane and sevoflurane depolarize presynaptic mitochondria through inhibition of the electron transport chain. Isoflurane seems to inhibit mitochondrial function more pronounced than sevoflurane. Both agents inhibit the respiratory chain sufficiently to cause ATP synthase reversal.

3. Isoflurane depolarizes the $\Delta\Psi_m$ of neural mitochondria in an age dependent manner by inhibition of the respiratory chain. The effect is more pronounced in the adolescent and adult than in neonatal rats.

4. Sevoflurane and propofol at equipotent doses depolarize the mitochondria in rat and human nerve terminals to the same extent. The depolarizing effect of propofol on the Ψ_m was more rapid in onset than that of sevoflurane. Whereas sevoflurane inhibits the respiratory chain sufficiently to cause ATP synthase reversal, the depolarizing effect of propofol seems to be related to inhibition of the respiratory chain from complex I-V.

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