Thesis for doctoral degree (Ph.D.) 2011

EFFECTS OF SEVOFLURANE ANESTHESIA ON COGNITIVE FUNCTION AND CHOLINERGIC SIGNALING

Andreas Wiklund

Karolinska Institutet

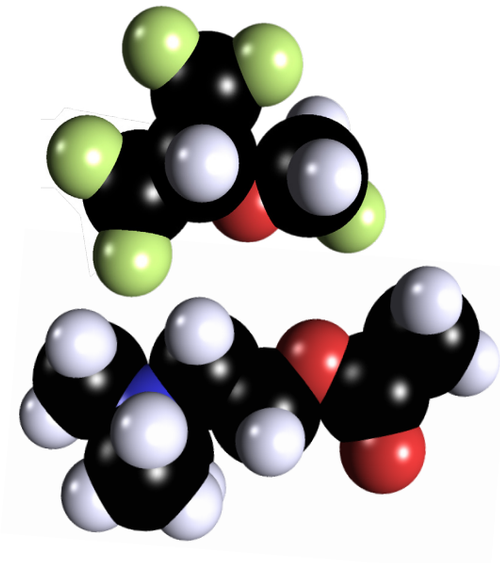
Thesis for doctoral degree (Ph.D.) 2011

EFFECTS OF SEVOFLURANE ANESTHESIA ON COGNITIVE FUNCTION AND CHOLINERGIC SIGNALING

Andreas Wiklund

Thesis for doctoral degree (Ph.D.)
2011

EFFECTS OF SEVOFLURANE ANESTHESIA ON COGNITIVE FUNCTION AND CHOLINERGIC SIGNALING



Andreas Wiklund

Karolinska Institutet

From the Department of Physiology and Pharmacology,
Section of Anesthesiology and Intensive Care Medicine
Karolinska Institutet, Stockholm, Sweden

EFFECTS OF SEVOFLURANE ANESTHESIA ON COGNITIVE FUNCTION AND CHOLINERGIC SIGNALING

Andreas Wiklund
M.D.

Karolinska Institutet

Stockholm 2011

Cover image: One molecule of sevoflurane and one of acetylcholine.

Published by Karolinska Institutet.
Printed by Larserics Digital Print AB.

ISBN 978-91-7457-449-4

To my family

Ever tried, ever failed. No matter. Try again. Fail again. Fail better.
(Samuel Beckett)

ABSTRACT

In recent years, concerns have been raised regarding the potential negative impact of anesthetic drugs in postoperative cognitive dysfunction. This thesis is an attempt to contribute to the understanding of the underlying mechanisms. Previous animal studies have suggested that volatile anesthetics can cause long-lasting cognitive alterations in various settings. Based on the strong association between cholinergic function and cognitive performance, we have focused our studies on the long-term effects of volatile anesthetics on cholinergic neurotransmission.

By using a combination of laboratory animal experiments and molecular biology techniques in cultured cells, we have described effects of sevoflurane anesthesia on cognitive function and cholinergic signaling. Our studies on spontaneous exploratory behavior, anxiety, object memory and analyses of behavioral organization in mice show that for some aspects of cognitive performance sevoflurane can cause long-lasting effects, while other aspects are unaltered by anesthesia. We have shown that age and preexisting cholinergic dysfunction are factors that influence the results. A normal cholinergic system, as represented by young wild type mice, seems to protect from behavioral alterations after anesthesia.

Our results suggest that sevoflurane causes a sustained attenuation in acetylcholine-induced phosphorylation of important intracellular kinases. We have linked our findings to signaling via muscarinic acetylcholine receptors, and we have also demonstrated that the effect of sevoflurane is not likely exerted by reducing the receptor population on the cell surface, nor by altering the total amount of receptor protein available for ligand binding.

The overall conclusion from the results of this thesis is that under certain experimental conditions, sevoflurane induces long-lasting alterations both in animal behavior and in cellular signaling.

Key words: Postoperative cognitive dysfunction, sevoflurane, acetylcholine, nicotinic acetylcholine receptors, muscarinic acetylcholine receptors, anesthesia, episodic-like memory, spatial memory

LIST OF PUBLICATIONS

I. Wiklund A, Granon S, Cloëz-Tayarani I, Faure P, le Sourd A-M, Sundman E, Changeux J-P, Eriksson L.I
Sevoflurane Anesthesia Alters Exploratory and Anxiety-like Behavios in Mice Lacking the B2 Nicotinic Acetylcholine Receptor Subunit
Anesthesiology 2008; 109: 790-8

II. Wiklund A, Granon S, Faure P, Sundman E, Changeux J-P, Eriksson L.I
Object memory in young and aged mice after sevoflurane anaesthesia
NeuroReport 2009, 20: 1419-1423

III. Wiklund A, Gustavsson D, Ebberyd A, Sundman E, Schulte G, Jonsson Fagerlund M, Eriksson L.I
Prolonged attenuation of acetylcholine-induced phosphorylation of ERK 1/2 in PC12 cells following sevoflurane exposure
Submitted to Acta Anaesthesiologica Scandinavica: AAS-11-0459

IV. Wiklund A, Gustavsson D, Ebberyd A, Mkrtchian S, Jonsson Fagerlund M, Eriksson L.I, Schulte G
Muscarinic acetylcholine receptor gene expression profile in PC12 cells following sevoflurane exposure
Manuscript

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ACh	Acetylcholine
AChR	Acetylcholine receptor
AKT	AKT serine/threonine-protein kinase
CABG	Coronary artery bypass graft
DAG	Diacylglycerol
DSM-IV	Diagnostic and statistical manual of psychiatric disorders IV
ERK	Extracellular signal regulated kinase
GABA	Gamma-aminobutyric acid
GPCR	G protein-coupled receptor
ISPOCD	International study on postoperative cognitive dysfunction
mAChR	Muscarinic acetylcholine receptor
MAPK	Mitogen activated protein kinase
MWC	Monod-Wyman-Changeux
nAChR	Nicotinic acetylcholine receptor
NGF	Nerve Growth Factor
NMDA	N-methyl D-aspartate
PC12	Pheochromocytoma cell line 12
PCR	Polymerase chain reaction
PI3K	Phopsphoinositide-3 kinase
POCD	Postoperative cognitive dysfunction
qPCR	Quantitative real time polymerase chain reaction
β_2KO	Beta-2-knock out

INTRODUCTION

BACKGROUND:

In recent years, a gradually increasing attention among anesthesiologists has been directed to cognitive deficits, such as learning or memory impairment, which might occur in patients after surgery. Recent studies have demonstrated that as many as 26% of patients show measurable degrees of cognitive deterioration at one week after surgery, and that 10% of patients still show cognitive deficits three months later (Moller, Cluitmans et al. 1998). These particular cognitive deficits in which there is a temporal relation between surgical intervention and cognitive problems are denominated postoperative cognitive dysfunction (POCD).

According to official statistics from the National Board of Health and Welfare (Socialstyrelsen), a total of 1108627 patients underwent surgery in Sweden in 2009 alone. With a growing number of surgical procedures performed each year, together with an increasingly elderly population, the number of people presenting with POCD will also likely increase.

This thesis project emanates from the questions surrounding this phenomenon, and was conceived in an attempt to shed some light on one of the most crucial questions, namely the role of anesthesia drugs for the development of POCD. Thus, in an attempt to contribute with some of the highly sought for pieces of the mechanistical puzzle of POCD, we have addressed the question of measurable and reproducible long-term effects on cholinergic signaling and cognitive function.

POSTOPERATIVE COGNITIVE DYSFUNCTION (POCD)

Early on in the history of anesthesia, concerns were raised on the potential drawbacks from anesthetic agents, and that there would be negative aspects of exposure to anesthesia as well. In a seminal paper by Bedford in the Lancet in 1955 (Bedford 1955), several case descriptions of cognitive dysfunction accompanied his hypothesis that anesthetic drugs might be one of the factors causing the problem.

With the development of cardiac surgery and the use of extracorporeal circulation techniques, there was also an increased concern for the cognitive problems that seemed to be associated with this specific type of surgery (Willner, Rabiner et al. 1976; Shaw, Bates et al. 1987; Mills 1993). For a long time, cognitive problems after cardiac surgery were attributed to the

specific situation of by-pass circulation, with microemboli as the most common and likely culprit. However, since cognitive deficits were also observed in patients undergoing non-cardiac surgery, the question of underlying mechanisms was soon open again.

In 1998, a large multicenter study by Moller and coworkers, demonstrated that as many as 26% of patients undergoing non-cardiac surgery would present measurable cognitive deficits at one week postoperatively, and that 10% of patients would still show cognitive deficits at three months after surgery (Moller, Cluitmans et al. 1998). Several studies followed, and in 2008, Monk and co-workers presented even higher numbers of POCD at hospital discharge. In their study, POCD was present in as many as 41.4% of elderly (60 years and older), in 30.4% in middle-aged (40 to 59 years) and in 36.6% in young (18 to 39 years) patients, when measured at 1 week after surgery. At three months after surgery, POCD was still present in 5.7% of young patients, in 5.6% of middle-aged patients, and in 12.7% of elderly patients (Monk, Weldon et al. 2008). A list of selected studies on the incidence of POCD in non-cardiac surgery is presented in table 1.

It has even been stated that POCD would be associated with increased mortality, risk of leaving the labor market prematurely and that it would lead to increased dependency on social transfer payments (Johnson, Monk et al. 2002; Rudolph, Jones et al. 2007; Monk, Weldon et al. 2008; Steinmetz, Christensen et al. 2009). Taken together, this translates to a substantial cost for postoperative delirium and cognitive dysfunction (Deiner and Silverstein 2009; Steinmetz, Christensen et al. 2009).

In summary, there is overwhelming documentation that POCD does indeed exist (Perouansky and Hemmings 2009). Nevertheless, since it often presents itself only as a subtle persistent deterioration in cognitive performance after a surgical intervention, in clinical reality, it can be difficult to diagnose and is therefore quite often foreseen (Maze and Todd 2007).

Table 1: Incidence of postoperative cognitive dysfunction in patients (modified from Newman et al 2007):

Study	POCD at 1 week (%)	POCD at 3 months (%)
Moller, Cluitmans et al. 1998	25.8% vs. 3.4%	9.9% vs. 2.8%
Dijkstra, Houx et al. 1999	27% vs. 6%	8% vs. 2% (NS)
Rasmussen and Moller 2000	32.7% vs. 3.4	9.4% vs. 2.8%
Ancelin, de Roquefeuil et al. 2001	71% (no control)	56% (no control)
Jonsson et al, 2002	19.2% vs. 4.0%	6.2% vs. 4.2% (NS)
Monk et al, 2008	30-41% vs. 2.8-5.1%	16-39% vs. 6.3%

Risk factors for POCD

Insofar, the major risk factors associated with the development of POCD seem to be high age, low educational level, history of previous cerebral vascular accident and the presence of cognitive dysfunction at hospital discharge (Ancelin, de Roquefeuil et al. 2001; Monk, Weldon et al. 2008). Also, preexisting cognitive dysfunction prior to surgery seems to be of importance (Silverstein, Steinmetz et al. 2007; Bekker, Lee et al. 2010).

In addition, medical conditions such as alcohol abuse (Hudetz, Iqbal et al. 2007; Hudetz, Patterson et al. 2009) thyroid disease have also shown to affect the risk panorama (Newfield 2009) as has metabolic syndrome. Cortisol levels have been suggested to be associated with POCD (Rasmussen, O'Brien et al. 2005; Pearson, de Vries et al. 2010). Recently, preoperative depression and poor executive function were presented as two independent risk factors (Smith, Attix et al. 2009).

Furthermore, surgical trauma, inflammation, postoperative pain, analgesic regimen and many other associated factors seem to play a non-negligible part in the development of POCD. (Newfield 2009) A recent study has shown that differences in cognitive performance is much more strongly related to the surgical intervention than to the type of anesthetic regimen used (Ancelin, de Roquefeuil et al. 2010).

Table 2: Risk factors associated with POCD at 3 months after surgery.

Probable	**Possible**	**Not likely**
Advanced age	Opioid treatment	ICU-stay
Low educational level	Proinflammatory mediators	Hypercapnia
POCD at discharge	Cortisol secretion	Hypoxia
Previous stroke	Pain	Hypotension
Preop cognitive impairment	Psychological factors	
Preoperative depression	Anesthesia	
	Surgical category	

Proper analgesic treatment seems to be protective of POCD (Morrison, Magaziner et al. 2003) after hip replacement surgery. Also analgesic adjuvant therapy with the NMDA-receptor antagonist ketamine seems to be protective following coronary artery bypass surgery (CABG) surgery (Hudetz, Iqbal et al. 2009). The use of regional anesthetic techniques is advocated in patients with cognitive decline prior to surgery (Halaszynski 2009). In CABG patients, a recent study has shown an increase in amyloid proteins and an association with cognitive decline similar to that of Alzheimer's disease (Evered, Silbert et al. 2009). A recent study in non-cardiac surgery patients has demonstrated a robust postoperative neuroinflammatory response, with striking similarities to the inflammatory

pattern seen in Alzheimer patients. Even more interesting, their results suggest that anesthetic management could be of relevance for modulating this response (Tang, Baranov et al. 2011).

Cognitive function:

”Cogito, ergo sum*” Descartes famous words on how thinking proves our existence are a good introduction to cognition. Descartes gave his view on thinking in his work ”Les principes de la philosophie”, which was published in 1644:

*“Par le mot de penser, j’entends tout ce qui se fait en nous de telle sorte que nous l’apercevons immédiatement par nous-mêmes ; c’est pourquoi non seulement entendre, vouloir, imaginer, mais aussi sentir, est la même chose ici que penser. “***

The word “cognition” is derived from the latin word cognoscere, which means ”to know” or “to think”. In general terms, cognition is the collective awareness and the integration of sensory inputs from all organs into our conscious awareness of our surroundings. It also incorporates learning, memory, logic thinking, verbal fluency, communication skills and many more aspects of brain function. This makes the term difficult to easily define, and thus makes it elusive when it comes to how it should be measured and quantified. The results of testing cognitive function will be highly dependent on which aspect of cognitive function is being studied (Newman, Stygall et al. 2007) and is also highly dependent on the context in which it is tested.

Therefore, most studies on POCD have used various neuropsyhological test batteries covering several aspects of cognitive function, in order to assess the global level of cognitive function. The term POCD infers a temporal relation to surgery in which the cognitive decline is preceded by a surgical procedure. Furthermore, the diagnosis requires a comparison between the cognitive performance prior to and following the intervention in order to discern the contribution to the cognitive decline by the surgical procedure itself.

For the purpose of evaluating and comparing data, most studies rely on comparing the Z score, as described in the International Study of Postoperative Cognitive Dysfunction (ISPOCD) study by Moller et al (Moller, Cluitmans et al. 1998).

**”I think, therefore I am”*
***“By the word thinking, I intend everything that is perceived by ourselves; that is why not only hearing, wanting, imagining, but also feeling is the same thing as thinking”*

Several separate cognitive function tests are summarized to a composite Z score, which is used to assess global cognitive change (Monk, Weldon et al. 2008). A negative change in cognition, when significant, is now commonly referred to as postoperative cognitive dysfunction (POCD) (Deiner and Silverstein 2009).

The most commonly used tests can be grossly divided into two groups, of which the first includes tests aiming at evaluating executive function. Tests aiming at assessing concentration, processing speed and attention would fall into this category. Examples of such tests are the Stroop color-word test, Concept shifting task and the Letter-digit coding test. Into the second category would fall tests aiming at assessing learning and memory such as Visual verbal learning test with immediate and delayed recall of words.

One of the domains of cognitive function most susceptible to neurodegenerative diseases, such as Alzheimer's disease, is the episodic memory (Belleville, Sylvain-Roy et al. 2008). This can be characterized as explicit memory of information on what, when and where, and also as a contextual memory of the relationships between these variables. In animal studies it is most commonly referred to as episodic-like memory.

Although at a first glance somewhat similar, postoperative delirium and postoperative cognitive dysfunction are regarded as two separate entities (Rudolph, Jones et al. 2007; Rudolph, Marcantonio et al. 2008; Deiner and Silverstein 2009; Robinson, Raeburn et al. 2009). The definition of delirium as described in the Diagnostic and Statistical Manual of Mental Disorders fourth edition (DSM-IV) points out the following main characteristics for the diagnosis: First, a change in mental status involving reduction of the awareness of the environment and disturbances in attention. Second, hallucinations or cognitive symptoms such as disorientation and memory deficits often occur.

Links between general anesthesia and POCD

Still today, more than one and a half century after the introduction of general anesthesia, the underlying mechanisms are not fully understood. To the layman, anesthesia is often explained as a state of unconsciousness or sleep. But, it is also a state of amnesia, analgesia and immobility. Most commonly general anesthesia is achieved using a combination of drugs providing each of the desired qualities; anesthetics for unconsciousness and amnesia, opioids or local anesthetics for analgesia, and muscle relaxants for immobility. It is often noted in the clinical setting that although the patient has regained full consciousness after anesthesia, amnesia can still be most

present. This postoperative amnesia has been attributed residual effects of anesthetics, and might last several hours following anesthesia.

General anesthetics fall into one of two categories. First, there are the volatile anesthetic agents, such as sevoflurane, isoflurane and desflurane, which are generally used to maintain anesthesia and more rarely used for induction. Second, there are the intravenous agents, such as propofol or barbiturates, which are most commonly used for induction of anesthesia, rather than maintenance. Although some compounds share similar features with respect to receptor affinity in the central nervous system, it is intriguing to realize how such a wide range of chemically very diverse compounds can cause a virtually identical effect when it comes to altering the level of consciousness.

Anesthetics are believed to act via inhibiting transmission via excitatory receptors and potentiating transmission via inhibitory receptors. Although some anesthetic agents display narrow receptor affinities, e.g. propofol, which acts mainly on inhibitory $GABA_A$ receptors, others, e.g. volatile anesthetics such as sevoflurane, display promiscuous receptor affinities, binding to a large population of structurally diverse receptors. (Campagna, Miller et al. 2003) Anesthetics thus interact with neurotransmitter release, excitability of neuronal membranes, and by affecting signaling in key brain structures, the state of unconsciousness is reached. The exact neurobiochemical processes leading to this state are still to date not fully elucidated ((Campagna, Miller et al. 2003; Grasshoff, Rudolph et al. 2005; Franks 2008).

Sevoflurane

Sevoflurane (1,1,1,3,3,3-hexafluoro-2-(fluoromethoxy)propane) is one of the most commonly used volatile anesthetics worldwide. It is a highly fluorinated methyl isopropyl ether, with a boiling point of 58.6°C and a molecular weight of 200g/M. Like most volatile anesthetics, the mechanism of action is unclear, but likely mediated by effects on a wide array of receptors, among which can be mentioned potentiation of $GABA_AR$, glycineR, and potassium channels, and simultaneous inhibition of nAChR, mAChR, NMDAR, AMPAR, serotoninR and voltage gated sodium channels (Alkire, Hudetz et al. 2008)

Does anesthesia cause POCD?

There is still controversy regarding whether or not general anesthesia is to be suspected as a cause of POCD (Hudson and Hemmings 2011). Although several studies have failed to show differences between anesthetic regimens (Wu, Hsu et al. 2004), some evidence point in the direction that general anesthesia might be worse for POCD than regional anesthesia (Rasmussen, Johnson et al. 2003; Weber, Friedl et al. 2009; Mason, Noel-Storr et al. 2010). In a study comparing propofol and sevoflurane for the maintenance of anesthesia no differences in cognitive performance were found between the two anesthesia groups, but there was a difference compared to non-anesthetised controls (Rohan, Buggy et al. 2005).
In conclusion, no adequately powered study has so far investigated the relation in POCD between intravenous and volatile anesthetics in humans. Instead, the strongest evidence for long-lasting effects of anesthetics on cognitive function is found in animal studies. These show important differences between several types of anesthetics, which will be discussed in the following chapter.

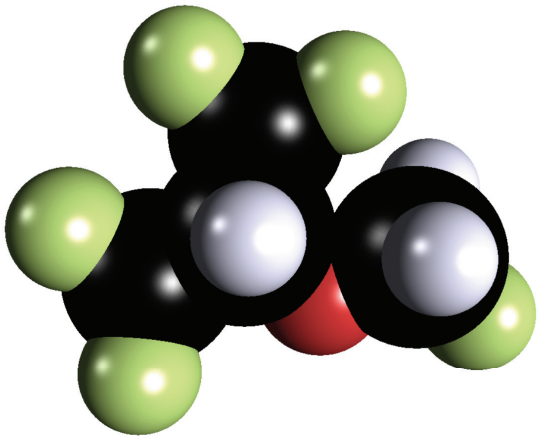


Figure 1: A schematic model of the molecular structure of sevoflurane. Black represents carbon atoms, fluoride atoms are green, hydrogen ions are white and the oxygen atom is red. Image was made in Pc3DViewer freely available from PubChem.

ANIMAL AND CELLULAR MODELS OF POCD

It is possible to grossly divide animal models for cognitive function into two categories: conditioned and unconditioned tests. The first category of tests all use some form of reinforcer, either as reward or punishment, as a means of increasing the sensitivity of the test. Unconditioned tests do not require reinforcers. Instead, they rely on the natural and spontaneous behaviors of the animal, and are considered more sensitive to subtle changes in behavior than conditioned tests (Ennaceur and Delacour 1988).

Several studies in rats have shown persistent memory and learning difficulties following general anesthesia. The effects outlast by far what would be predicted from the pharmacokinetic profile of the investigated compounds (Culley, Baxter et al. 2003; Culley, Baxter et al. 2004; Crosby, Culley et al. 2005). It seems that type of compound also matters, for example did not propofol alter spatial cognitive performance in aged rats (Lee, Culley et al. 2008). Although most studies point at long-lasting effects, lasting days to weeks, of exposure to anesthetics, not all indicate that learning and memory is impaired. Some even demonstrate improved spatial learning after exposure to volatile anesthetics (Komatsu, Nogaya et al. 1993; Komatsu, Nogaya et al. 1998; Butterfield, Graf et al. 2004; Rammes, Starker et al. 2009)

There are some conflicting findings on anesthetic exposure and neurotoxicity in vivo and in vitro. In rats, in which behavioral alterations might last for several weeks, no increase in cell death in brain regions has been observed after general anesthesia with isoflurane (Stratmann, Sall et al. 2010). In contrast, others have demonstrated that prolonged (6h) exposure to isoflurane induces caspace pathways and increases apoptosis in brain cells (Xie, Dong et al. 2006). Another cause for concern with respect to anesthetic involvement in POCD is the finding that volatile anesthetics, again in contrast to propofol, seem to be linked to enhanced aggregation and toxicity of the Alzheimer disease-associated amyloid beta protein (Eckenhoff, Johansson et al. 2004; Xie, Dong et al. 2006). In relation to this, an increased interest has been directed against the connection between neuroinflammation and cognitive deterioration (Caza, Taha et al. 2008; Xie, Zhang et al. 2009; Hu, Ou et al. 2010; Hudetz, Patterson et al. 2011).

The effects of anesthetics seem to be more pronounced in aged animals. A recent study has shown astrocytosis, increased beta-amyloid protein and increased phosphorylation of tau in aged mice subjected to general anesthesia and surgery. (Wan, Xu et al. 2010).

Adding surgery to the scene makes the image even more complex since pro-inflammatory cytokines seem to have a strong influence on cognitive function (Terrando, Rei Fidalgo et al. 2010). Proinflammatory cytokine dependent activation of glial cells increase neuroinflammation (Wan, Xu et al. 2007). In fact, in operated rats, anesthesia technique has showed to be of less importance than the type of surgical trauma (Cao, Ma et al. 2010).

In conclusion, animal studies support the assumption that anesthetics induce long-lasting physiological changes, which can be observed as alterations in cognitive performance. These deficits are often noted in functions such as memory, attention and speed of information processing, areas, which are closely related to the actions of cholinergic neurotransmission. This will be further explored in the next chapter.

CHOLINERGIC NEUROTRANSMISSION

In 1926 Otto Loewi performed an experiment where he demonstrated that the vagus nerve signaled though a water-soluble compound first described as *"vagusstoff"*, but soon characterized as acetylcholine. Ten years later, Otto Loewi and Henri Dale, were rewarded the Nobel Prize in Physiology or Medicine *"for their discoveries relating to the chemical transmission of nerve impulses"*. Acetylcholine was thus the first neurotransmitter to be described (Tansey 2006).

Distribution of cholinergic neurons

As a neurotransmitter, acetylcholine acts at multiple targets in the central and peripheral nervous system, in the neuromuscular junction, in the visceral motor system and also functions as a modulator of the immune system (Wessler and Kirkpatrick 2008; Rosas-Ballina and Tracey 2009). Although cholinergic neurons comprise less than one percent of neurons in the central nervous system, they are involved in a wide range of neural functions due to their widespread distribution in the brain. As illustrated in figure 2, virtually every part of the brain receives projections from cholinergic neurons, which extend over long distances. An interesting feature about the distribution of cholinergic neurons is how it interacts in a relatively consistent manner of motor versus sensory, which topographically translates to a ventral to dorsal orientation of projections in both the brain and the spinal cord (Woolf and Butcher 2010). From the basal forebrain population of cholinergic neurons widespread projections reach almost the entire cortex. These neurons are believed to play a central role in selective attention, learning, memory, perception and consciousness (Woolf and Butcher 2010). Ascending cholinergic neurons originating in the mesopontine region and projecting to prefrontal and other cortical areas also play an important role in higher

cognitive functions. Their main mode of action is to gate sensory input, attention and levels of consciousness, and via interaction with primarily serotonergic signaling cholinergic signaling plays an important role in learning (Klinkenberg, Sambeth et al. 2010). There are also cholinergic projections with more local distribution in basal brain areas where they are believed to play a role in the important circuitry mediating rhythmicity to brain signaling. (Woolf and Butcher 2010) Cholinergic neurotransmission interacts with most other receptor systems and has important modulatory effects on dopaminergic, glutamatergic, serotonergic as well as GABA-ergic signaling (Perry, Walker et al. 1999).

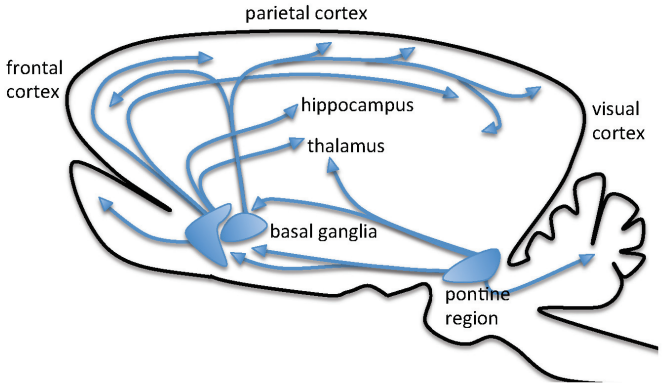


Figure 2: **A schematic drawing of cholinergic projections in rat brain**. Adapted from Woolf and Butcher, 1986). Cholinergic neurons exhibit two major types of organization, local circuits and projections. One major constellation of projecting cholinergic neurons is found in basal forebrain. Another important constellation is located in the pontine region. Despite the relative scarcity of cholinergic neurons, almost every part of the brain receives cholinergic input.

Acetylcholine and cognition

Cholinergic neurotransmission is clearly implicated in cognitive deterioration both in normal aging as well as in neurodegenerative disorders such as Alzheimer's disease (Bohnen, Kaufer et al. 2003; Nordberg 2006). In this disease there is a substantial loss of cholinergic neurons (Jurgensen and Ferreira 2010), and a reduction in brain nAChR content, which has been demonstrated to correlate well to the degree of cognitive dysfunction (Sabri, Kendziorra et al. 2008). For many years, the mainstay treatment of cognitive decline in Alzheimer's has been cholinesterase inhibitors, and more recently drugs acting directly at cholinergic receptors (Lleo, Greenberg et al. 2006). Procholinergic treatment with physiostigmine has proven beneficial for

cognitive performance in rodents (Wuppen, Oesterle et al. 2010). Currently, cholinergic receptor agonists or allosteric modulators are being tested as therapeutics (Hurst, Hajos et al. 2005). Moreover, pharmacological modulation of both nicotinic and muscarinic cholinergic functions in various psychiatric disorders can modify cognitive function and reduce symptoms (Araki, Suemaru et al. 2002; Poirier, Canceil et al. 2002; Newhouse, Singh et al. 2004).

As previously mentioned, cholinergic neurotransmission is involved in attention, but also in particular aspects of learning and memory processes, and especially in the encoding of new memories. During learning of a new task, acetylcholine levels are highest in the beginning of the learning period, and gradually become lower with habituation. A biphasic role for acetylcholine in learning and memory has also been demonstrated, in which high levels of acetylcholine are necessary for encoding, but where low levels of acetylcholine facilitate retrieval of memories. Acetylcholine has thus been proposed to set the brain in a recording mode, rather than a playback mode, when it comes to learning and memory (Hasselmo and Sarter 2011). Cholinergic signaling via mAChRs has been indeed been shown to be important to the consolidation of new memories, but of less importance in the retrieval of already stored memories (Warburton, Koder et al. 2003). In addition, acetylcholine signaling is necessary for cellular plasticity and the formation of new synapses necessary for learning (Woolf and Butcher 2010).

Anesthesia and cholinergic signaling

There are several interesting links between the cholinergic system and the action of anesthetics. Several different volatile anesthetics have shown to affect cholinergic signaling *in vitro* (Franks and Lieb 1994; Violet, Downie et al. 1997; Flood and Coates 2002; Tassonyi, Charpantier et al. 2002; Chiara, Dangott et al. 2003). *In vivo* experiments have demonstrated that isoflurane reduces acetylcholine release in the brain (Dong, Fukuda et al. 2006), and that this effect is even more pronounced in aged animals (Jansson, Olin et al. 2004). Interference with prefrontal mAChRs can significantly alter arousal from anesthesia (Dennison, Anthony et al. 1987; Demarco, Baghdoyan et al. 2004). Since, cholinergic neurons are believed to contribute to the complex regulation of the oscillations in the brain that regulate sleep-wake cycles, contribute to perception and are involved in the global synchronization of brain functions (Woolf and Butcher 2010) it has been suggested that cholinergic neurotransmission is involved in the regulation of consciousness.

Acetylcholine receptors

The subtypes of neuronal acetylcholine receptors are divided into nicotinic acetylcholine receptors (nAChRs) and muscarinic acetylcholine receptors (mAChRs), as illustrated in figure 3. Nicotinic AChRs are ligand-gated ion channels composed of five transmembranous subunits of which there are nine different α-subunits (α_2- α_{10}) and three β-subunits (β_2-β_4) (Wevers 2010) The most common types of nAChR in the central nervous system are the α_7 and the $\alpha_4\beta_2$ subtypes. While $\alpha_4\beta_2$ are widely distributed over the entire brain, α_7 are mainly located in hippocampus, amygdala, hypothalamus and restricted layers of the cerebral cortex (Graef, Schonknecht et al. 2011).

Table 3: Major acetylcholine receptor subtypes

Receptor	Type	Localisation	Effector
M_1	Muscarinic	Cortex, Hippocampus	Gq-coupled, IP3
M_2	Muscarinic	Brainstem, thalamus, cerebellum	Gi-coupled, cAMP
M_3	Muscarinic	Enteric NS, CNS	Gq-coupled, IP3
M_4	Muscarinic	Striatum	Gi-coupled, cAMP
M_5	Muscarinic	Substantia nigra	Gq-coupled, IP3
α_7	Nicotinic	Hippocampus, amygdala	Ca^{2+} influx
$\alpha_4\beta_2$	Nicotinic	Widespread in CNS	Na^{+}-influx

Nicotinic AChRs are ion channels with different selectivity for cations. The homomeric α_7-nAChR is highly selective for Ca^{2+} ions, whereas the heteromeric $\alpha4\beta_2$-receptor is a Na^{+} selective channel. Thus activation of α_7-nAChR act excitatory, and activation of $\alpha4\beta_2$-nAChRs modulate membrane potential and increases excitability. While ionotropic nAChRs mediate fast synaptic transmission, the metabotropic mAChRs are G protein-coupled and mediate slower responses, lasting seconds to minutes.

Classification of G proteins is made based upon the subunit composition, of which there are four types: G_s, G_i, G_q and $G_{12/13}$. It is thus the receptor-associated G protein that determines the nature of the response upon agonist binding to the receptor. Receptor-induced activation of G_s proteins stimulates adenylyl cyclase activity, opens Ca^{2+}-channels and closes Na^{+}-channels. mAChRs belong to either G_q (M_1, M_3, M_5), or G_i (M_2, M_4). The G_i family inhibits adenylyl cyclase, closes Ca^{2+}-channels and opens K^{+}-channels. Activation of G_q leads to increased activity in phospholipase C and IP3/DAG, which in turn regulates intracellular Ca^{2+}-signaling. $G_{12/13}$ proteins are involved in cytoskeletal remodeling, via Rho family GTPase signaling (Minami and Uezono 2006)

Of the different mAChR subtypes, M_1 receptors are the most abundant subtype in cortex and hippocampus and are believed to be involved in cognition and neuroinflammation (Fisher, Pittel et al. 2003). M_2 receptors

are found presynaptically mainly in the brainstem, thalamus and cerebellum, and they also modulate acetylcholine release in the prefrontal cortex. Presynaptically located M_3 receptors modulate cortical dopamine release M_4 receptors are mainly located in the striatum, and are involved in regulation of dopaminergic signaling. The still rather poorly characterized M_5 receptors seem to play a role in substance abuse (Graef, Schonknecht et al. 2011).

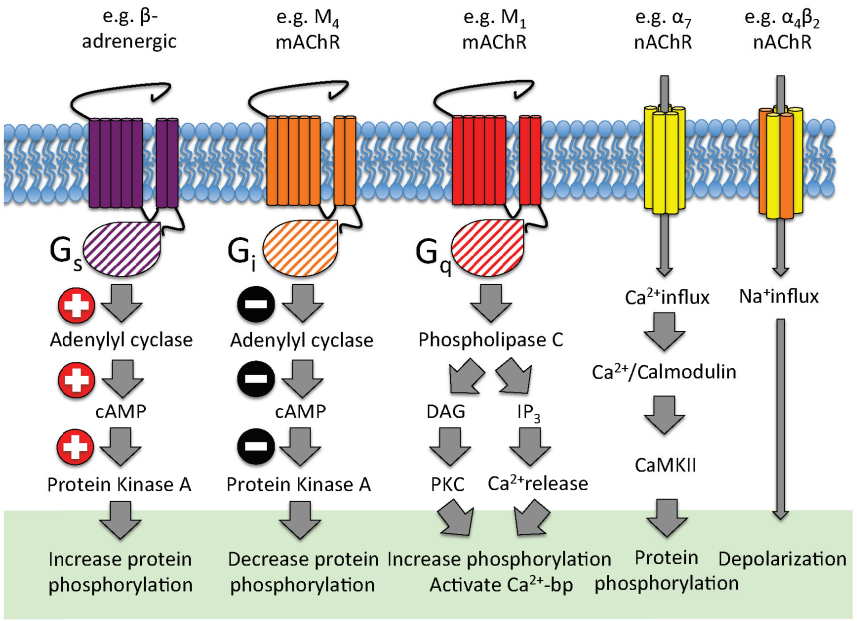


Figure 3: **Effector pathways downstream of G protein-coupled receptors and ligand gated ion channels.** The leftmost receptor is coupled to a Gs protein, which increases cAMP as its second messenger, and exemplified by a β-adrenergic receptor. The Gi protein coupled to the next receptor reduces cAMP levels by inhibiting adenylyl cyclase activity, illustrated by a muscarinic acetylcholine receptor (mAChR) of the M_4-type. The Gq protein activates a different second messenger pathway, involving IP3 and DAG (diacylglycerol), exemplified by an M_1-mAChR. To the right, the two most abundant nicotinic acetylcholine receptors (nAChRs) in the central nervous system are illustrated. The α7 nAChR is a highly selective Ca^{2+}-channel protein, and the $\alpha_4\beta_2$ nAChR is a selective Na^{+}-channel.

Mechanisms of acetylcholine receptor modulation

Physiological synaptic modulation can be achieved in a number of ways. Modulation of neurotransmitter release from the presynaptic neuron can be the first step in altering synaptic transmission. This may be done by changing the firing frequency of the neuron, changing the dynamics of neurotransmitter reuptake, synthesis and degradation or modifying ion conductance of channel proteins. Indeed, by using microdialysis *in vivo* in rats it has been demonstrated that volatile anesthetics significantly attenuate acetylcholine release from presynaptic terminals in the brain (Jansson, Olin et al. 2004).

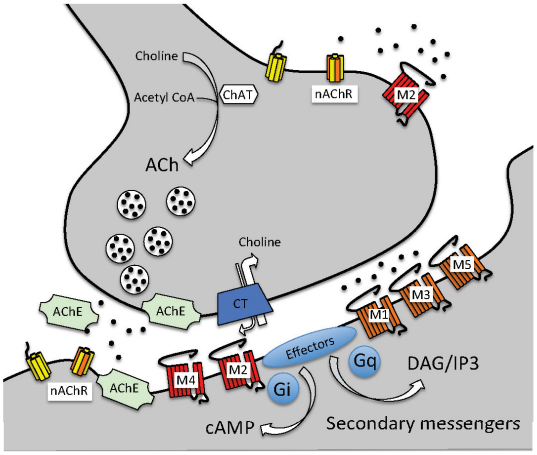


Figure 4: **Model of a cholinergic synapse** (modified from Cooper, Bloom and Roth: Biochemical Basis of Neuropharmacology, 2003) with an example of presynaptic and postsynaptic distribution of the various cholinergic receptor subtypes. Acetylcholine is relseased in quantas from the presynaptic nerve ending, and diffuses into the synaptic cleft, where interaction with receptors takes place. The released acetylcholine is metabolized by acetylcholinesterases present in the synaptic cleft, which in turn terminates the action of ACh. Choline is recycled into the presynaptic terminal.

To regulate the signal strength independently of neurotransmitter release, cells can also adjust the amount of receptors available for agonist binding. By reducing receptor protein synthesis, increasing receptor degradation or by internalizing the receptor, thus making it unavailable for agonist interaction, the postsynaptic neuron can reduce the synaptic transmission. Pharmacological studies have demonstrated that volatile anesthetics alter gene expression in rat hippocampus (Culley, Yukhananov et al. 2006; Sekine, Matsumoto et al. 2006; Kalenka, Gross et al. 2010) and also might alter cellular protein synthesis directly (Kvolik, Glavas-Obrovac et al. 2005).

Allosteric modulation

Allosteric modulation is achieved by binding of a substance to another site, i.e. a regulatory site, than the actual agonist-binding site on the receptor, i.e. the active site. As a consequence, the geometry of the protein is altered, which in turn affects the affinity for the ligand. Such alterations in receptor configuration can produce changes in receptor function in both a positive and a negative way with regards to the effects following agonist binding. Two major models of allosteric regulation exist, the concerted Monod-Wyman-Changeux (MWC) model (Monod, Changeux et al. 1963), or the sequential model (Koshland, Nemethy et al. 1966). Common for both models is that protein subunits exist in two distinct conformational states, either tensed or relaxed, of which the relaxed state binds agonists more readily. Binding of an allosteric modulator to a regulatory site increases or decreases the binding affinity of the ligand to the active site, as illustrated in figure 5. Thus, allosteric regulation is a way to modulate the intracellular response to endogenous ligands.

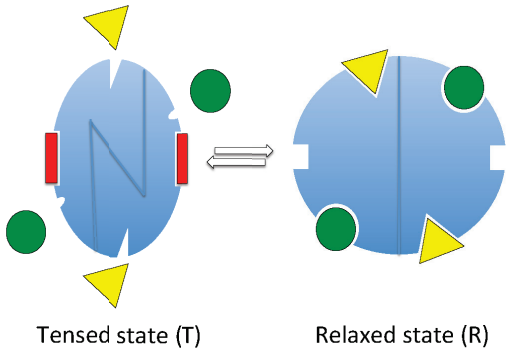


Figure 5: **Allosteric modulation illustrated by a simplified MWC-model**. Adapted from Changeux (Changeux and Edelstein 2005). Binding by an allosteric inhibitor (red bars) to an inhibitory regulatory site stabilizes the receptor in the tense state, thus preventing agonist (yellow triangles) binding. Agonist binding to the active site in the relaxed state will stabilize the structure in a configuration that favors binding of substrate (green circles) to the effector site of the receptor protein

Insofar, most studies point in the direction of volatile anesthetics act as allosteric modulators of receptor function. Anesthetic binding to allosteric sites on receptor proteins interfere with the modes of motion essential to protein functions (Szarecka, Xu et al. 2007) and thereby alters receptor signaling. Volatile anesthetics have distinct allosteric effects on ligand-gated ion channels, such as nAChRs and GABA receptors (Young, Oshiki et al. 1981; Nury, Van Renterghem et al. 2011). In some cases the allosteric site is within the ion channel, and binding of anesthetic to the allosteric site will

also obstruct the lumen, as is the case for nAChRs and volatile anesthetics (Flood et al. 2008). This has been repeatedly demonstrated for both ligand gated ion channels (Raines, Rankin et al. 1995; Raines 1998; Raines and Zachariah 1999) as well as for metabotropic receptors, such as mAChRs (Forman, Miller et al. 1995; Durieux 1996; Nietgen, Honemann et al. 1998; Weigl and Schreibmayer 2001). Anesthetics can also competitively inhibit the function of a protein, as for the protein firefly luciferase, in which anesthetics competitively block substrate binding to the enzyme (Franks and Lieb 1984; Franks, Jenkins et al. 1998; Eckenhoff, Tanner et al. 2001). Overall, a common feature of anesthetics seems to be that they enhance signaling via inhibitory receptors and impair signaling mediated via excitatory receptor systems.

Intracellular signaling pathways related to acetylcholine receptors

On of the most extensively studied intracellular pathways is the Mitogen Activated Protein Kinase (MAPK)-pathway. Here, an extracellular signal is relayed down from the receptor via a series of kinases down to a Extracellular Signal Regulated Kinase (ERK), of which there are several isoforms. The most extensively studied isoforms are ERK 1/2. Phosphorylation of ERK 1/2 activates the enzyme, which in turn leads to alteration of transcription factors such as CREB, cFos, and others.

Another pathway of interest is the one involving PI3K/AKT and mTOR. Activation of AKT or PKB, as it also called, can come from multiple sources, of which G protein-coupled receptors is one alternative. The AKT pathway is believed to be involved in cell survival and apoptosis. In oncology, this signaling pathway has received major interest since many tumor cells have constantly active AKT, and might depend on this for survival. More of relevance to this thesis is that exposure to volatile anesthetics increase phosphorylation of AKT, which in this context has been implicated in the long-lasting effects of pharmacological cardiac preconditioning (Wang, Traystman et al. 2008).

There are also other signaling pathways originating from G proteins, such as the PI3K/AKT pathway. Having listed these different pathways, it is important also to note that considerable crosstalk between different signaling pathways occurs when converging pathways share the same effector proteins or transcription factors.

AIMS OF THE THESIS:

The overall aim of this thesis was to evaluate the effects of sevoflurane anesthesia on cognitive function and cholinergic signaling.

The specific aims were:

I: To investigate sevoflurane-induced alterations of exploratory and anxiety-like behavior in young and aged mice with a pre-existing nicotinic cholinergic dysfunction.

II: To investigate the effect of sevoflurane anesthesia, age and pre-existing nicotinic cholinergic dysfunction on object memory.

III: To describe the effects of sevoflurane exposure on long-term alterations in cholinergic signaling and intracellular pathways.

IV: To investigate if sevoflurane exposure alters cellular acetylcholine receptor surface expression, gene transcription or amount of receptor proteins.

MATERIALS AND METHODS:

Animals

For the behavioral studies in paper I and II we used C57BL/6 mice, both wild type and genetically modified mice. The genetically modified mice were deficient of the gene for the β_2 nAChR subunit. They displayed a distinct behavioral phenotype with hyperactivity, abnormal social behavior, but with preserved spatial learning (Picciotto, Caldarone et al. 2001) (Granon, Faure et al. 2003). Young mice were 3-4 months of age. Aged mice were 15-18 months of age at the time of experiments. In total 30 young wild type, 20 aged wild type, 28 young β_2KO and 22 aged β_2KO-mice were used for the experiments in paper I and II.

Anesthesia

For anesthesia, animals were placed in a small plexiglass cylinder, into which a mixture of oxygen and sevoflurane was fed via nylon tubing. Temperature control was achieved via external heating, and animal body temperature was monitored continuously via a rectal thermometer. Respiratory rate was monitored every five minutes during anesthesia.
In a series of pilot animals prior to the study in paper I, it was determined that sevoflurane at 2.6% was a suitable concentration, at which the respiratory rate was sufficient to prevent hypoxia. Blood oxygen saturation was continuously monitored with a neonatal pulse oximetry probe, but only in the pilot animals. This was due to technical issues on attaching the probe to the thigh of the mouse without affecting spontaneous locomotion afterwards.

Open field

A common way of analyzing rodent behavior is to study movements in an open field (Ammassari-Teule, Fagioli et al. 1994). In our studies we used a circular open field to investigate both spontaneous exploratory activity and anxiety in the animal. The trajectories of the mice in the open field were recorded using an automated video tracking system. Based upon movement velocity, the information was broken down into navigatory or exploratory behavior. An index, called exploratory index, was calculated by dividing the time spent in slow exploration with the time spent in fast navigation.
Further analysis of the way the mice organized their trajectories and their behavior was made by dividing the behavior into active (A) or inactive (I) periods, and also dividing the location of the animal into central (C) or peripheral (P) relating to the position within the circular open field. Thus, a two-letter code (CA, CI, PA or PI) could be attributed to the animal behavior

at every time-point during the experimental session. By further analyzing the sequence of codes we were able to compare patterns of behavioral organization. Since our aim was to detect subtle changes in animal behavior, we believed that unconditioned tests of normal exploratory behavior would be the most sensitive and most suitable for our hypotheses. The details of the experimental procedure are available in the materials and methods section in paper I.

Elevated plus maze

Although the open field test gives some information on anxiety levels, as expressed in time avoiding the central part of the open field, the elevated plus maze test aims more specifically at evaluating anxiety-like behavior (File 2001). This test consists of a cross-shaped platform, with two arms with walls (closed arms) and two without (open arms), and elevated some 50 centimeters above the ground. The test provides information on anxiety levels, as expressed in time that the animals venture out on the open arms. It also gives information on locomotor activity, expressed in number of transitions between the closed arms. The higher the anxiety level, the less time will the animal venture out on the open arms. Since this test is conceived to be stressful to the animals, we only performed it once, at the end of the experimental session. How we performed the tests is described in detail in paper I.

Novel object memory

A common and very strong feature of rodent behavior is a high degree of novelty exploration. The object memory test is based on this innate motivation to explore novelty (Ennaceur and Delacour 1988) and is a test for episodic-like memory in animals. It requires a learning phase, during which the animal is presented with two identical objects placed some distance apart, so that in order to explore both objects, the animal has to move actively across the cage to familiarize with the objects. The learning phase in our study lasted two times five minutes, and was separated by a one-minute rest in the home cage. The memory phase of the test is done the following day by replacing one of the familiar objects with a new one, similar in shape and size, but still clearly distinguishable from the familiar object. The theory behind this test states that if the animal has a memory of the familiar object, it will spend more time exploring the novel object instead of the familiar remembered object. Equal time exploring both objects would in the test situation indicate a lack of memory of the object from the day before. An independent observer analyzed video recordings of the sessions and documented the exploration time at each object. The experimental procedure is extensively described in the material and methods section in paper II.

Radioligand binding

In paper I we performed radioligand studies to investigate whether sevoflurane anesthesia would alter the agonist affinity to nicotinic acetylcholine receptor subtypes in the brains of exposed animals. Coronal brain sections were incubated with either epibatidine, for labeling of high affinity nAChRs; alfa-bungarotoxine, for labeling of low affinity nAChRs; or hemicholinium, for labeling of choline uptake sites. Exposure lasted eight days, and was followed by image analysis of each ligand to obtain an optical density, corresponding to binding to the receptor.
The work was laborsome but yielded high-resolution images, and demonstrated that sevoflurane anesthesia did not alter agonist affinity to nicotinic receptors, when investigated 24 hours after exposure. For a complete description of the method, please refer to paper I.

Cell culture

PC12 cells, derived from a rat pheochromocytoma cell line have been extensively studied as a model system for cholinergic neurons and also for effects of anesthetics (Kress, Muller et al. 1991). In our studies we used undifferentiated PC12 cells of subclone I. Prior to experiments we performed a characterization of the cells, which revealed that although the undifferentiated PC12 cells contained mRNA for both nAChRs and mAChRs, only stimulation of muscarinic receptors elicited a measurable degree of phosphorylation of the kinases we wanted to study. Due to this finding, we concluded that the cells displayed a functional muscarinic profile, and that the nAChRs were not fully operational. It is known that differentiation of PC12 cells with nerve growth factor (NGF) causes the receptor population to switch to a more neuronal type.

Immunoblotting

The key experimental method in my experiments has been the immunoblotting of cellular protein structures. We have used standard techniques in which cells were lysed using SDS-buffer containing ß-mercaptoethanol and then subsequently sonificated. Samples were loaded onto 10% SDS-PAGE gels and separated by gel electrophoresis (60 minutes at 200V) before transfer (60 minutes at 105V) onto polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Germany). After blocking (5% fat-free milk in TBST) for 60 minutes, immunoblots were incubated with primary antibodies in room temperature for 60 minutes, or at 4°C overnight, depending on the type of primary antibody used. After washing in TBST, immunoblots were subsequently incubated with secondary HRP-conjugated antibodies for 60 minutes at room temperature and developed.

Images were captured digitally, and optical density of bands was quantified using special software. Optical density of bands representing the protein of interest was determined, and values were normalized against the level of a housekeeping protein for comparisons, internal loading and quality control. For comparisons between exposed and control samples, ratios were calculated pair-wise on each membrane. For details, please see the materials and methods section in paper III and IV.

PCR

In our experiments we have bee using two different PCR techniques. In paper III we used regular PCR to demonstrate the presence of DNA for the various acetylcholine receptors and receptor subtypes. In paper IV we used quantitative real time PCR, which allows quantification of the number of gene copies present in the cells from the beginning. This allowed for comparison and quantification of the different receptors and receptor subtypes and their relation to sevoflurane exposure. For details of the procedure, see paper III.

Biotinylation assay

In paper IV, we used a biotinylation assay to determine the population of mAChRs localized to the cell membrane versus the population in the cell cytoplasm. With this technique, surface proteins are first covalently attached to biotin, which is a water-soluble B-complex vitamin (vitamin B7). After biotinylation of surface proteins, cells are lysed and the lysate is passed through a column coated with Neutravidin, which binds the biotinylated proteins. After washing of the column, the biotinylated proteins are eluded by adding a strong acid solution, and in the following step, the proteins are denatured with SDS and finally analysed using standard immunoblotting techniques. This method is described in detail in paper IV.

Table 4: General techniques, methods and materials used in the different papers constituting this thesis:

Method:	Paper:
Open field	I, II
Elevated plus maze	I, II
Novel object memory	II
Radioligand binding (Autoradiography)	I
Cell culture	III, IV
Immunoblotting	III, IV
RT-PCR	III
Quantitative PCR	IV
Biotinylation assay	IV

Essentially, all models are wrong, but some are useful
(George E. P. Box)

RESULTS AND COMMENTS:

PAPER I:

In paper I we studied how exposure to sevoflurane anesthesia affected spontaneous exploratory behavior in young and aged mice, with or without cholinergic dysfunction. We demonstrated that both young and aged mice with a pre-existing cholinergic dysfunction altered their behavior after sevoflurane anesthesia. In mice lacking β_2-containing nAChRs, a reduced locomotor behavior and an increased exploratory behavior was seen after anesthesia. These changes were not observed in mice with a normal cholinergic receptor population. Investigations of nAChR density could not explain the differences between the groups.

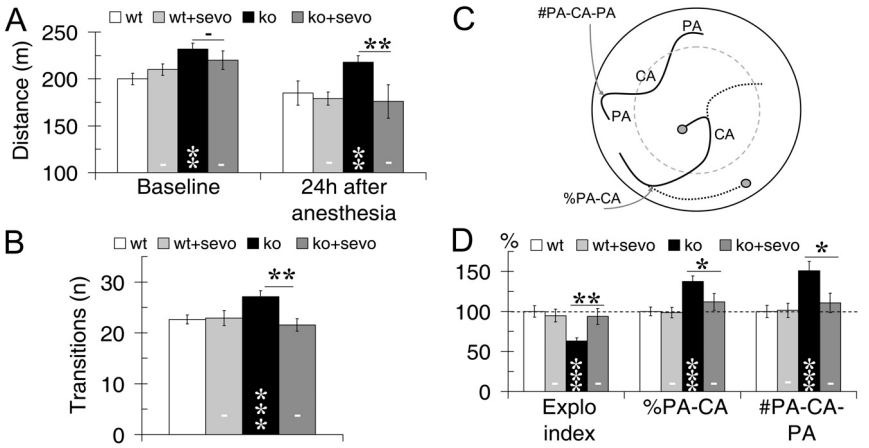


Figure 1 from paper I: Locomotor activity and temporo-spatial organization of trajectories in young mice.

(A) Distance moved in the open field before (baseline) and 24 h after anesthesia. Anesthesia reduced the distance moved in β_2KO mice ($p<0.05$) but not in WT mice (n.s.).

(B) Activity in the plus maze expressed as number of transitions between the closed arms of the maze. Anesthesia reduced locomotor activity in β_2KO mice ($p<0.05$), but not in WT mice (n.s.).

(C) Spatio-temporal organization of trajectories in the open-field. The number of large movements from the peripheral area (PA) across the central area (CA) of the open field is indicated by #PA-CA-PA. The conditional probability of a transition from a PA to a CA state, i.e. the probability to move from the periphery to the centre, is indicated by %PA-CA.

(D) Spatio-temporal organization of trajectories in young mice. All values are compared relative to the WT control group, which is set to 100%. Exploration index (explo index) provides information on the repartition of fast versus slow movements in the open field and is obtained by dividing time spent at slow movement speed with time spent in fast

movement speed. Young control β_2KO mice differed significantly from young control WT regarding exploration index ($p<0.01$) (D, left), %PA-CA ($p<0.01$) (D, middle) and #PA-CA-PA ($p<0.01$) (D, right). After sevoflurane anesthesia, exploration index (D, left), %PA-CA (D, middle) and #PA-CA-PA (D, right) in young β2KO mice were no longer significantly different from young WT mice. No effect of anesthesia on these behavioral parameters was demonstrated in young WT mice. Statistical significance for planned comparisons between control and anesthesia groups are indicated above the corresponding horizontal lines. Symbols within vertical bars indicate statistical significance for planned comparison to WT control. (wt=wild type control group (n=20); wt+sevo=wild type anesthesia group (n=11); ko=β2 knock out control group (n=18); ko+sevo=β2 knock out anesthesia group (n=8) * P<0.05; ** P<0.01; *** P<0.001; - P>0.05)

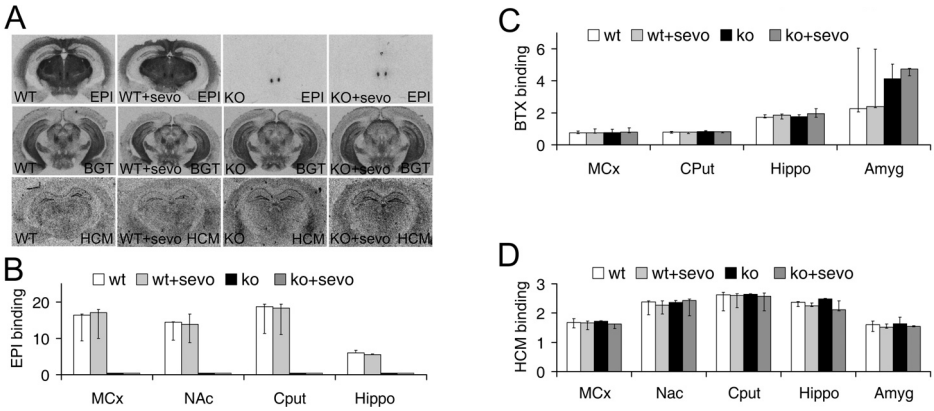


Figure 5 from paper I: Autoradiography

(A) Representative film autoradiograms of [^{125}I]-epibatidine binding at bregma level -2.7mm (top), [^{125}I]-α-bungarotoxin binding at bregma level -3.4mm (middle) and [^{3}H]-hemicholinium binding at bregma level -2.3mm (bottom) of adult wild type mice in control group (WT), wild type mice in anesthesia group (WT+sevo), mutant mice in control group (KO) and mutant mice in anesthesia group (KO+sevo). Residual binding of [^{125}I]-epibatidine to non-β_2-containing nAChRs in certain nuclei, such as the fasciculus retroflexus (fr), is observed in β_2KO mice.

(B) Quantification of [^{125}I]-α-bungarotoxin binding to low affinity nAChRs.

(C) Quantification of [^{125}I]-epibatidine binding to high affinity nAChRs.

(D) [^{3}H]-hemicholinium binding. Values are expressed in arbitrary units as median±interquartile range. Exposure to sevoflurane did not alter the density of either high or low affinity binding sites in any region, nor did it alter the regional cholinergic transmission indexed by the choline transporter. (Amyg=Amygdala; CPut=Caudate Putamen; Hippo=Hippocampus; MCx=Motor Cortex; NAc=Nucleus Accumbens; wt=wild type control group (n=5); wt+sevo=wild type anesthesia group (n=5); ko=β2 knock out control group (n=5); ko+sevo=β2 knock out anesthesia group (n=5))

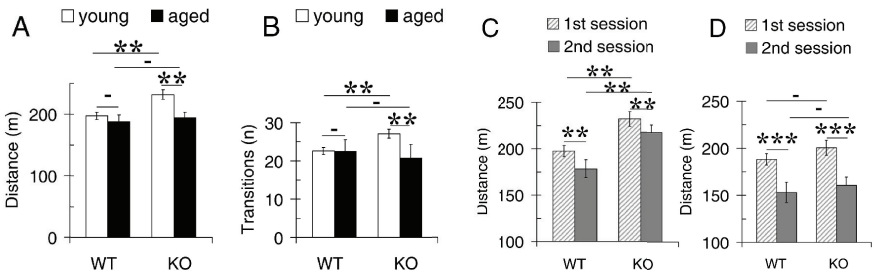


Figure 4 from paper I: Effects of age, repeated measurements and genotype.
(A) Comparison of locomotor activity in the open field between young and aged mice.
(B) Effects of age on transitions in elevated plus maze.
(C) Habituation effect among young mice in open field.
(D) Habituation effect in aged mice. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

PAPER II:

In paper II we demonstrated that the anesthesia procedure reduces episodic-like memory in mice in a similar manner in both normal wild type mice and in mutant mice lacking β_2-containing nAChRs. Here, the effect of interfering with memory consolidation either by handling or by anesthesia could be of relevance to the results. We demonstrated that long-term object memory in mice is not dependent on β_2-containing nAChRs. Furthermore, for the same reasons as in paper I, both young and aged mice were studied, and the results showed that object memory was not affected negatively by age. The animals tested for object memory in paper II were the same as tested for open field and anxiety like behavior in paper I.

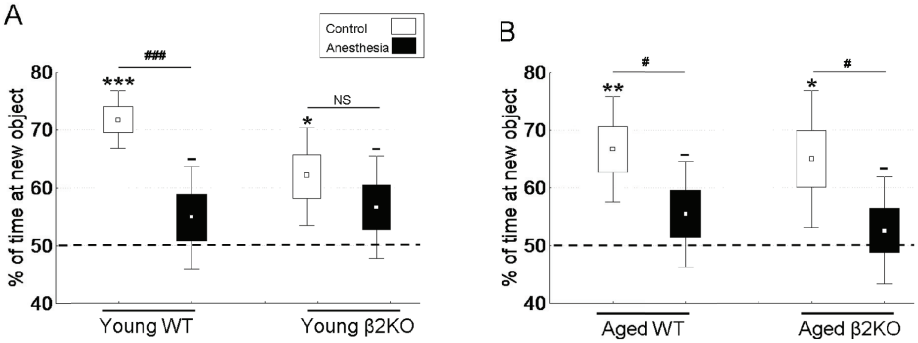


Figure 1 from paper II: Object memory testing in young and aged mice
Object memory index in young mice (A) and aged mice (B) is reduced in both WT and β_2KO after anaesthesia. * indicates $p<0.05$ compared to chance level of exploration. # indicates $p<0.05$ for difference between control and anaesthesia.

PAPER III:

In paper III, PC12 cells expressing functional mAChRs were exposed to sevoflurane for two hours, and then tested with respect to acetylcholine-induced phosphorylation of ERK1/2. The results showed that cholinergic signaling is affected for a prolonged time after sevoflurane exposure, even when sevoflurane concentration in the culture medium is very low.
Phosphorylation of AKT was increased during sevoflurane exposure but rapidly returned to control levels after exposure. The dynamics for return to baseline levels of phosphorylation was different between ERK 1/2 and AKT.

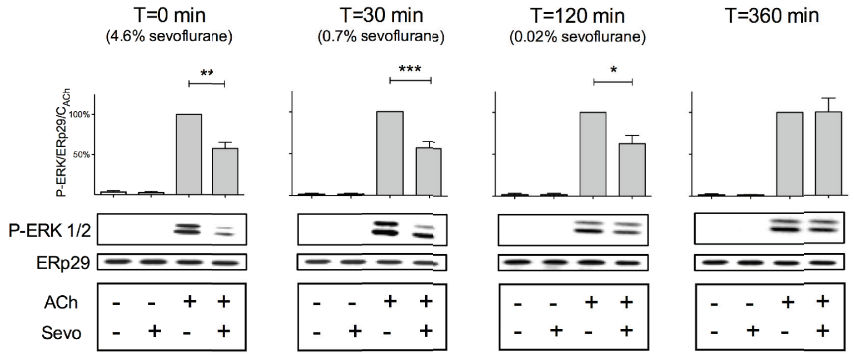


Figure 4 from paper III: Acetylcholine-induced phosphorylation of ERK 1/2.
Comparison between sevoflurane-exposed cells and control cells following after exposure to 5% sevoflurane for 2 hours. Below the graphs are representative western blots of phosphorylated ERK 1/2 and ERP29. ERP29 was used as an internal loading control. N=4-6 for all measurements. * $p<0.05$, ** $p<0.01$, *** $p<0.001$. Data are presented as mean±SEM.

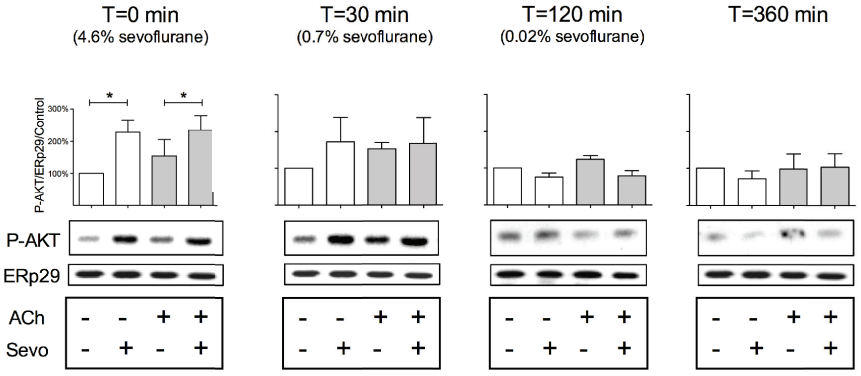


Figure 5 from paper III: Phosphorylation of AKT
AKT phosphorylation after stimulation with 10uM acetylcholine in sevoflurane-exposed and non-exposed cell cultures. Below the graphs are representative western blots of phosphorylated AKT and ERp29. Data are presented as mean±SEM, n=4-6 for all measurements. *$p<0.05$

PAPER IV:

In paper IV we investigated if sevoflurane exposure would alter receptor localization, quantity or synthesis. Our results demonstrated a sustained reduction in acetylcholine-induced ERK 1/2 phosphorylation, as in paper III. We could demonstrate that internalization of mAChRs does not occur due to sevoflurane exposure alone, and therefore could not explain the attenuated response. mRNA for M_1 mAChRs was increased after exposure. However, this mRNA increase did not affect the receptor protein content, and can thus not explain the attenuation of response. On might speculate that an increase in mRNA is a compensatory mechanism for a reduction in functional receptor proteins, but we have yet no support for this hypothesis. We thus concluded that the attenuation of acetylcholine-induced ERK 1/2 phosphorylation was not likely due to changes in receptor localization or receptor quantity.

Fig 3

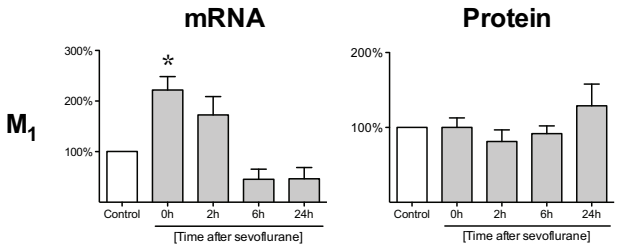
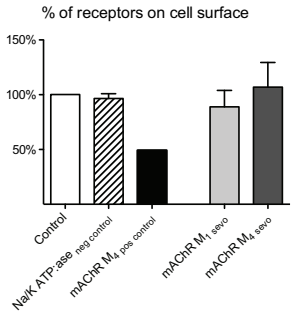


Figure 3 from paper IV: mRNA and protein content in PC12 cells following sevoflurane exposure for M_1 mAChRs. Shaded bars indicate change in sevoflurane-exposed cells compared to the non-exposed controls indicated by the white bar. The increase in mRNA for M_1 mAChRs does not lead to a significant increase in receptor protein. Error bars are ±SEM, * indicates $p<0.05$ from control. N=3-4 in all graphs.

Figure 4 from paper IV: Effects of sevoflurane exposure on surface localisation of mAChRs. Shaded bars indicate M_1 and M_4 mAChRs in sevoflurane-exposed cells compared to non-exposed controls. Striped bar indicate the negative control Na^+/K^+ ATPase α-1, which is a membrane bound protein. The black bar indicates the positive control of M_4 mAChRs stimulated with the mAChR selective agonist charbachol, which causes internalization of half the muscarinic receptor population. Error bars are ±SEM, n=3., except for positive control in which n=1.

Fig 4



DISCUSSION:

In this thesis we have shown that under certain experimental conditions, sevoflurane induces long-lasting alterations both in animal behavior and cellular signaling. Our principal aim has been to experimentally address the clinical problem of postoperative cognitive dysfunction (POCD) with special reference to cholinergic signaling.

The cholinergic hypothesis for cognitive deficits after anesthesia

After carefully studying the available literature on the matter, we concluded that cholinergic neurotransmission would be a highly interesting field to further explore, in terms POCD. We founded this hypothesis upon some striking similarities between the cognitive deficits seen in Alzheimer's dementia and the descriptions of cognitive dysfunction in the postoperative period. As previously mentioned, episodic memory deficits are a hallmark of early cognitive impairment, of which some of the features relate to altered cholinergic transmission. (Sabri, Kendziorra et al. 2008) As mentioned in the introduction, anticholinergic properties of anesthetics are well described, as is the potential for anesthetics to act as allosteric modulators on cholinergic receptors, both nAChRs and mAChRs.

In this thesis, we have showed that cholinergic status of animals is of relevance for the effect of exposure to anesthesia. In paper I, we demonstrate that mice lacking the β_2-subunit of the nAChR display behavioral alterations after anesthesia, while mice with normal cholinergic signaling do not. In paper I we speculate on the underlying mechanisms to this observation, and conclude that it can either be that a normal cholinergic system protects against behavioral alterations, or that a pre-existing cholinergic dysfunction could facilitate sevoflurane-induced disturbances in neurotransmission. A drawback of using genetically modified animals, which carry the gene alteration since conception, is of course the possibility of compensatory mechanisms changing the function in other systems, which might affect the results.

Re-expression experiments in mice lacking the β_2-subunit have shown that restoring the receptor population in very localized areas of the brain can reverse the behavioral effects (Maskos, Molles et al. 2005). This suggests that the effects of sevoflurane on behavior as observed in paper I, might in fact relate to changes in very specific neuronal circuits, and do not necessarily mirror a global effect on the whole brain. In this context it is interesting to note that although previous studies have demonstrated that β_2KO do not differ in anesthetic requirements and MAC value, we demonstrated in paper I that they did take longer time to recover from

anesthesia. Here, we noted that the time from discontinuation of sevoflurane administration to return of the righting reflex was longer in mice lacking the β_2-subunit of the nAChR. One possible hypothesis for this effect is that β_2-containing nAChRs play a role in neuronal circuits mediating recovery from anesthesia, reflecting the role of the cholinergic system in mediating arousal.

Chronic administration of nicotine reduces hyperactivity in β_2KO mice in similar, although not necessarily identical ways, as the effect of sevoflurane anesthesia in paper I. Hence, we speculate on the possible involvement on other nAChRs, notably the α_7-nAChR, in the pseudo-normalization of the β_2KO hyperactive phenotype. With a location on presynaptic nerve terminals in modulatory pathways, alterations of α_7-nAChR performance could have far-reaching consequences by changing the function on several other signaling pathways.

Two important conclusions on the cholinergic system in episodic-like memory could be drawn from the results in paper II. First, both young and aged wild type and β_2KO mice that had not been anesthetized displayed excellent object memory, suggesting that signaling via receptors containing the β_2-subunit is not crucial for long-term retention of object memories. Secondly, anesthetized wild-type mice and anesthetized β_2KO showed equally poor object memory retention, indicating that a preexisting nicotinic cholinergic deficit did not worsen object memory performance. This in turn suggested that other pathways than the nicotinic acetylcholine pathway should also be probed in search for the underlying mechanisms of episodic-like object memory.

In paper III and IV, we demonstrated that even very low concentrations of sevoflurane might perturb acetylcholine signaling in cultured cells. This is of relevance since it has been shown that residual concentrations of anesthetics can remain in the body of patients for extended periods of time, and that the rate of elimination drops as the concentration falls. In fact, elimination is asymptotic (Lockwood 2010). In our cell culture experiments, we shift focus towards the effects on the mAChRs, rather than the nAChRs. One might even argue that the effects of sevoflurane on mAChRs are as interesting as the effects on α_7-nAChR, since these receptors are potential targets for the effects observed in β_2KO mice after anesthesia.

The contribution of anesthetics versus the effects of systemic inflammation and surgery

For obvious reasons, large-scale studies in humans with the objective of finding the role of anesthetics in POCD are cumbersome. Such studies will always have to deal with the effects of surgery and systemic inflammation as confounders. Indeed, even studies comparing differences between general

and regional anesthesia are obscured by the admixture of sedative doses of anesthetics to the group receiving regional anesthesia (Rasmussen, Johnson et al. 2003). Still, the contribution of anesthetic drugs to POCD is a question of utmost importance. We therefore decided at an early stage that we would investigate the role of anesthesia and separate this from the contribution of surgical stress or inflammation. The possibility to isolate the role of anesthesia from that of the confounders is a very good reason for doing an experimental study. Hence, we decided that neither surgery nor blood sampling would be performed, to avoid triggering a systemic inflammatory reaction in the animals. We also decided upon a combination of unconditioned tests: spontaneous exploration of an open field, object memory and elevated plus maze. We share the belief that these tests are more suitable and possess a higher sensitivity for detecting subtle changes in cognitive function.

To reduce stress levels in the animals during the experimental sessions, we provided enriched environment in the cages and let the animals get used to our presence and handling, by taking care of all the steps in animal handling, from changing the bedding in the cages, to feeding and watering the animals. In addition, the contribution of stress to the results was controlled for by including a sham group of animals, which was subjected to identical handling as the anesthesia group with the only exception that they were not anesthetized in the anesthesia chamber. During anesthesia, we carefully and continuously monitored body temperature and respiratory rate of the animals. All anesthetized animals were handled equally and since there are no differences in anesthetic requirement between b2KO and wild type, to our judgment, the mice did receive an equal anesthetic dose.

With this setup, we were able to detect behavior alterations following sevoflurane anesthesia in young and aged mice with a genetically modified cholinergic system. These changes were present even though no surgery had been done to the animals, nor had we initiated any systemic inflammatory response. Mice with a normal cholinergic system did not display any behavioral changes in the open field or plus maze tests, suggesting a protective role of cholinergic signaling on cognitive changes after anesthesia.

In contrast to others (Terrando, Rei Fidalgo et al. 2010) who have showed that anesthesia does not cause memory deficits in wild type mice, our results from the episodic-like memory test demonstrated that wild type and mutant mice were both affected by sevoflurane anesthesia. This reflects the complexity of behavioral testing in animals. First of all, different memory tests do not necessarily reflect the same type of memory processing. For example, tests involving strong associative conditioning learning are likely

to involve brain areas, such as the amygdala, which are not as strongly associated with unconditioned exploratory behavior tests. Furthermore, as illustrated by our own models, differences in the time allowed for memory consolidation may influence the results. Hence, contradictory results regarding the effects of anesthetics on memory function are merely an indication of the tremendous complexity involved in interpreting and designing tests of cognitive function in animals.

In our studies, mice were anesthetized for two hours, spontaneously breathing sevoflurane at 2.6% mixed with oxygen. Anesthesia drastically lowered the breathing frequency, and although we had controlled for hypoxia, other respiratory related physiological changes, such as hypercapnia, might have occurred. Nevertheless, since both wild type and β_2KO were anesthetized according to the same protocol, and since the MAC-value is not different between wild type and β_2KO (Flood, Sonner et al. 2002), the observed behavioral differences are not likely derived from differences in physiological parameters during anesthesia. Sevoflurane at 2.6% is a concentration that is routine in human anesthesia practice, and was chosen to be able to keep the mice spontaneously breathing for two hours. It corresponds to approximately 1 minimal alveolar concentration in C57BL6 mice, and given that no painful stimulus was given to the mice during anesthesia, it would probably be considered as a relatively profound level of anesthesia. Pilot studies evaluating the sevoflurane concentration revealed that higher concentrations lowered the respiratory rate down to levels that would risk causing hypoxia in the animals. Temperature regulation in very small animals such as mice can be difficult in the laboratory. Anesthesia caused a rapid drop in animal body temperature and active warming was absolutely necessary. We accomplished this via continuous rectal temperature monitoring and a heating lamp. To conclude, animals were anesthetized according to an identical protocol. Respiratory rate and body temperature was monitored in all animals. No significant difference were found between wild type mice and β_2KO mice, which suggests that they did reach the same depth of anesthesia and thus the same exposure to the anesthetic.

Of course, it could be argued that the behavioral effect in β_2KO was due to incomplete washout of sevoflurane from the brain compartment at 24 hours after anesthesia. If this would apply, it still would not explain the differences between wild type and β_2KO mice. However, such an assumption would support our reasoning in paper III, in which we speculate that the effects on acetylcholine induced phosphorylation on ERK 1/2 is actually due to residual sevoflurane in the medium.

The effect of age.
Since age is one of the risk factors most clearly associated to POCD, we wanted to investigate the effect of age in our animal models. Hence, we included not only young mice between 3-4 months of age, but also elderly mice between 15-18 months of age, which for C57BL/6 mice is a considerable age. Previous studies in animals have demonstrated that high age increases the risk for cognitive alterations after anesthesia (Culley, Baxter et al. 2003; Culley, Baxter et al. 2004; Crosby, Culley et al. 2005).

Prior to the experiments, we thus assumed that aged mice would be more susceptible to the effects of anesthesia, and that this would be even more obvious in the β_2KO group. Instead, the characteristic phenotype of β_2KO-mice became attenuated with increasing age, maybe associated with the accelerated aging of these animals. In paper I we therefore we proposed that aging itself had an effect on hyperactivity in β_2KO-mice, and that this would be a consequence of the accelerated aging observed in these animals.

For a memory to become a long-term memory, it requires encoding, which is done during the consolidation phase. Interfering with memory consolidation will affect the long-term memory storage. In paper I, the first open field session was performed 24 hours before anesthesia and the second was done 24 hours after anesthesia. For this test, animals thus had 24 hours to consolidate the memory of the open field, and the results showed that all groups displayed habituation from session I to session II. Habituation in this context, is reduced exploratory activity, and can be interpreted as long-term memory of the test apparatus.

On the other hand, this time frame was radically different for the object memory test, evaluating the episodic-like memory. Here, the learning sessions were performed immediately before induction of anesthesia, allowing only one minute between the end of the learning session and the induction of anesthesia. Testing of object memory was performed 24 hours after anesthesia, as for the open field test. While all animals, irrespective of age or genotype, showed habituation in the open field, only the control groups showed long-term memory for the objects. As shown in paper II, age was not was attenuated in all anesthesia groups, and unaffected in the control groups. Here, even the sham groups were affected, indicating that the stress of being transferred into the anesthesia chamber might have an influence on memory consolidation, in addition to the potential effect of anesthesia.

To conclude the discussion of paper I, we state that sevoflurane anesthesia altered exploratory and anxiety-like behavior in both young and aged β_2KO but not in wild-type mice of any age. It is further possible to argue that an

intact cholinergic system seems to protect against anesthesia-induced behavioral changes, as well as age-related changes.

An important conclusion on the influence of age in episodic-like memory can be drawn from the results in paper II. Here, both young and aged wild type and β_2KO mice that had not been anesthetized displayed excellent object memory, suggesting that signaling via receptors containing the β_2-subunit is not crucial for long-term retention of object memories. It also shows that both wild type and β_2KO mice have preserved object memory even in senescence.

Long-lasting effects of anesthetics on cellular signaling

It has previously been described that volatile anesthetics have profound and long-lasting effects on various intracellular signaling pathways (Culley, Yukhananov et al. 2006; Sekine, Matsumoto et al. 2006; Kalenka, Gross et al. 2010). In this thesis, we show that under certain conditions, anesthesia can induce effects both on behavior and in cellular function. There might be several explanations to this, and an obvious reason is that there is residual anesthetic drug affecting signaling pathways. It has been debated that the accumulation of anesthetics in the body of patients can be quite considerable during a normal anesthesia, and that elimination takes longer time than expected.

Measuring acetylcholine-induced phosphorylation of ERK 1/2 provided information on gross intracellular effects, and allowed for characterization of the exposure-effect relation. The gain in sensitivity for sevoflurane-induced changes in signaling pathways simultaneously reduced the possibility to identify the exact location of the effect. A great number of intracellular signaling pathways converge to ERK 1/2. There is also a considerable crosstalk between signaling pathways, making it virtually impossible to control for all the possible intermediary factors leading to a particular cellular response. Since phosphorylation of ERK 1/2 can be achieved in many ways, one must be very cautious in assigning a specific pathway to an observed effect.

This problem was addressed in the following way: To ascertain that the signal obtained from phosphorylation of ERK 1/2 was a specific muscarinic signal, we stimulated cells both with selective muscarinic receptor agonists, oxotremorine, and nicotinic receptor agonists, nicotine. These experiments showed a clear muscarinic receptor mediated phosphorylation of ERK 1/2, and an absent nicotinic response. In addition, we performed quantitative PCR analysis to determine the relative distribution of mAChRs, which showed predominance for the M_1 and the M_4 subtypes.

Due to the complicated relationship between anesthetic potency and temperature, we performed all experiments at 37°C. A comparison between acetylcholine-induced phosphorylation of ERK 1/2 in exposed and non-exposed cell cultures revealed a distinct and sustained attenuation of ERK 1/2 phosphorylation in sevoflurane-exposed cultures. The attenuation of acetylcholine-induced phosphorylation of ERK 1/2 persisted more than two hours after exposure, and was not restored to control levels until six hours after exposure.

In contrast to the sustained effects on ERK 1/2, sevoflurane exposure induced a transient phosphorylation of AKT. Such effects on AKT from anesthetic exposure are previously described, and are considered to be of relevance to the pharmacological preconditioning effects seen in cardiomyocytes. Albeit transient, it is possible that phosphorylation of AKT might still have long-lasting effects on gene transcription, not measured in this study. Instead, the conclusions of paper III were that sevoflurane exposure affects cellular intracellular signaling differentially for ERK1/2 and for AKT. While sevoflurane exposure induced a sustained attenuation in acetylcholine-induced ERK 1/2 phosphorylation, it caused a transient increase in phosphorylation of AKT.

Paper IV was designed as an attempt to pinpoint the location for the effect observed in paper III. Here, we chose to start from the cellular surface, at the level of the muscarinic receptors. The aim of was to investigate if sevoflurane exposure would alter the mAChR population on the cell surface. An acute internalization of mAChRs during sevoflurane exposure followed by a gradual restoration of surface receptors could possibly explain the dynamics in paper III. Therefore, a biotinylation assay was chosen to quantify surface bound receptors. We also quantitatively measured messenger RNA for the two most abundant muscarinic receptors, the M_4 and the M_1, to investigate possible alterations in gene transcription. Indeed, sevoflurane exposure increased levels of M_1 mRNA, but this was not reflected in increased receptor protein content. Protein levels were assessed using standard immunoblotting techniques. The results showed that while stimulation with a muscarinic agonist caused internalization of mAChRs, there was no difference in surface bound muscarinic receptor protein between sevoflurane exposed and control cell cultures. Hence, we concluded that neither mAChR internalization, nor altered mAChR receptor content, would likely explain the attenuation of acetylcholine induced ERK 1/2 phosphorylation following sevoflurane exposure.

CONCLUSIONS:

The overall conclusion from the results of this thesis is that under certain experimental conditions, sevoflurane induces long-lasting alterations both in animal behavior and cellular signaling. This is based on the following:

We conclude that sevoflurane anesthesia alters exploratory and anxiety-like behavior in mice lacking β_2-containing nAChRs. We suggest that brain circuits involved in organization of locomotor behavior or anxiety should be further investigated, as should the possible pharmacologic modulation of the effect and the cellular origin.

Furthermore, we have shown that post-training exposure to anesthesia causes similar long-term object memory impairment in both wild type and mutant mice lacking β_2-containing nAChRs. This effect is seen when considering the combination of anesthesia and handling of the animals. We have shown that signaling through β_2-containing nAChRs is not critically involved in long-term object memory in mice. In our results, we did not find any effect of age on temporospatial or object memory in wild type mice.

In our cellular model, we have shown that sevoflurane exposure perturbs signaling in a dual manner. On one hand, sevoflurane causes a sustained attenuation of acetylcholine-induced ERK 1/2 phosphorylation that persists even at sevoflurane concentrations close to detection level. On the other hand, AKT phosphorylation is increased two-fold during sevoflurane exposure, with a rapid return to baseline values after exposure.

We have also shown that the mechanism behind the attenuation of acetylcholine-induced ERK 1/2 phosphorylation is not likely related to alterations in mAChR gene expression, receptor protein content or amount of receptors on the cell surface.

ACKNOWLEDGEMENTS:

I would like to express my deepest gratitude to all who supported and inspired me during the work with this thesis, and especially to:

My supervisor, **Lars I Eriksson**, for making it all possible. For creating a scientific environment with tremendous opportunities for creative and novel ideas. For unfailing optimism and positive energy. For unrivalled networking skills and for seemingly being able to open any door.

My co-supervisor **Gunnar Schulte**, for holding me by the hand while treading carefully amidst kinases and receptors in the jungle of intracellular signaling pathways. For being open-minded and pragmatic, almost like an anesthesiologist...

My co-supervisor **Eva Sundman**, for constructive criticism and for always taking time to read through my many early drafts and helping me find directions in my writing.

Malin Jonsson Fagerlund, my co-author and room mate in the lab. For most valuable intellectual contributions to the last part of my thesis project, and for sharing your impressive scientific knowledge. For clear-eyed views on problem-solving.

Professor **Jean-Pierre Changeux**, my co-author for the first papers. Former head of the Unité de Recepteurs et Cognition at the Pasteur Institute. For so generously sharing your absolutely stunning scientific knowledge and for making it possible for me to work for some time in your amazing lab in Paris. For this I am ever grateful!

Professor **Sylvie Granon**, my co-author and for being the one teaching me everything I know on behavioral studies in animals. For your social skills and for making me feel as a part of the great Pasteur team!

Mes plus profondes gratitudes à tout le monde à l'Institut Pasteur: **Isabelle Cloëz-Tayarani, Uwe Maskos, Philippe Faure, Nicolas, Pierre, Sandra** et tout l'ensemble des gens qui contribuait a créer une athmosphère tout à fait exceptionelle. Mes gratitudes sincères à **Monika Mameli-Engvall** pour ses efforts à m'enseigner des bonnes habitudes du café à l'italienne! Je vous remercie tous, infiniment!

Anette Ebberyd, co-author, for creating such a warm and welcoming atmosphere in the lab. For always overlooking my lab skills (or rather lack of lab skills…). For your good mood and good company! For arranging the most outstanding fredagsfika, every week!

Souren Mkrtchian, co-author, for critically reviewing and constructively commenting our projects!

Daniel Gustavsson, co-author, for all the help and excellent collaboration in the lab. For great discussions on life and other matters and for getting me in physical shape again, after years of deterioration…

All my friends in the lab corridor and in the research group, especially: **Jessica Kåhlin**, for always sharing your marvelous positive energy to people in need! **Karin Eriksson**,

for great discussions on various matters... **Karsten Ahlbeck**, for all the good laughs. Actually, there are 11 types of people… **Åsa Konradsson Geuken** for providing tremendous energy to everyone in our corridor! You rock! **Pernilla Nyström**, for contributing to a super work environment in the lab and for reviving my track and field memories! **Carina** and **Mikaela** for helping me out when I got lost in the downstairs corridor … Special thanks to **Eva Lindgren**, for teaching me how to make PC12 cells thrive! **Mike**, for that he took himself the time to read through my swenglish…

Jonas Blixt, for listening to my frustrated monologues when everything felt hopeless...

Per Lindestam, for lending me a cool device for paper I and II and for making me start listening to live music again!

Rolf Sandin for interesting scientific discussions in San Diego and on the plane back to Sweden. I really appreciate your wonderful scientific curiosity!

Lars Irestedt, for being who you are. A role model for every anesthesiologist!

Bertil Fredholm, for sharing your vast knowledge and views on research.

Eddie Weitzberg och **Jon Lundberg** for your contagious way of dealing with scientific challenges!

Sten Walther, for making me choose anesthesiology as my discipline and for simply being a true and genuinely inspiring scientist!

Alliance Française, **Olof Norlanders minnesstiftelse** and **KI fonder** for granting me financial support during my time in the lab in Paris.

Claes Frostell, **Eva Selldén**, **Kristina Hambraeus-Jonzon** at **ANOPIVA**, for creating an environment in which science is not forgotten. **Kirsi Dolk**, for your magnificent ability to accommodate all my wishes in combining clinical and laboratory work. Soon, I will be reporting in for full time clinical duty! **Ingeborg Gottlieb-Inacio, Birgitta Lundqvist-Huzell, Kerstin Larsson, Kerstin Staffansson, Kristina Nordh-Claesson** and **Ann Norberg** for excellent administrative support!

All my **colleagues, nurses** and **assistant nurses** at the Department of Anesthesiology and Intensive Care Medicine (**ANOPIVA**) I am really fortunate to be working at what is probably the best anesthesia department in the entire world, and it is you guys who make all the difference!

My wonderful friends in **Attackinvest**, for the good times!

My **mother**, my **father** and my **sister**. For being there.

My beloved family: **Gabriella**, **Agda** and **Tage**. You are everything.

REFERENCES:

Alkire, M. T., A. G. Hudetz, et al. (2008). "Consciousness and anesthesia." Science 322(5903): 876-880.

Ammassari-Teule, M., S. Fagioli, et al. (1994). "Radial maze performance and open-field behaviours in aged C57BL/6 mice: further evidence for preserved cognitive abilities during senescence." Physiol Behav 55(2): 341-345.

Ancelin, M. L., G. de Roquefeuil, et al. (2001). "Exposure to anaesthetic agents, cognitive functioning and depressive symptomatology in the elderly." Br J Psychiatry 178: 360-366.

Ancelin, M. L., G. de Roquefeuil, et al. (2010). "Long-Term Post-Operative Cognitive Decline in the Elderly: The Effects of Anesthesia Type, Apolipoprotein E Genotype, and Clinical Antecedents." J Alzheimers Dis.

Araki, H., K. Suemaru, et al. (2002). "Neuronal nicotinic receptor and psychiatric disorders: functional and behavioral effects of nicotine." Jpn J Pharmacol 88(2): 133-138.

Bedford, P. D. (1955). "Adverse cerebral effects of anaesthesia on old people." Lancet 269(6884): 259-263.

Bekker, A., C. Lee, et al. (2010). "Does mild cognitive impairment increase the risk of developing postoperative cognitive dysfunction?" Am J Surg 199(6): 782-788.

Belleville, S., S. Sylvain-Roy, et al. (2008). "Characterizing the memory changes in persons with mild cognitive impairment." Prog Brain Res 169: 365-375.

Bohnen, N. I., D. I. Kaufer, et al. (2003). "Cortical cholinergic function is more severely affected in parkinsonian dementia than in Alzheimer disease: an in vivo positron emission tomographic study." Arch Neurol 60(12): 1745-1748.

Butterfield, N. N., P. Graf, et al. (2004). "The effect of repeated isoflurane anesthesia on spatial and psychomotor performance in young and aged mice." Anesth Analg 98(5): 1305-1311, table of contents.

Campagna, J. A., K. W. Miller, et al. (2003). "Mechanisms of actions of inhaled anesthetics." The New England journal of medicine 348(21): 2110-2124.

Cao, X. Z., H. Ma, et al. (2010). "Postoperative cognitive deficits and neuroinflammation in the hippocampus triggered by surgical trauma are exacerbated in aged rats." Prog Neuropsychopharmacol Biol Psychiatry.

Caza, N., R. Taha, et al. (2008). "The effects of surgery and anesthesia on memory and cognition." Prog Brain Res 169: 409-422.

Changeux, J. P. and S. J. Edelstein (2005). "Allosteric mechanisms of signal transduction." Science 308(5727): 1424-1428.

Chiara, D. C., L. J. Dangott, et al. (2003). "Identification of nicotinic acetylcholine receptor amino acids photolabeled by the volatile anesthetic halothane." Biochemistry 42(46): 13457-13467.

Crosby, C., D. J. Culley, et al. (2005). "Spatial memory performance 2 weeks after general anesthesia in adult rats." Anesth Analg 101(5): 1389-1392.

Culley, D. J., M. Baxter, et al. (2003). "The memory effects of general anesthesia persist for weeks in young and aged rats." Anesth Analg 96(4): 1004-1009, table of contents.

Culley, D. J., M. G. Baxter, et al. (2004). "Impaired acquisition of spatial memory 2 weeks after isoflurane and isoflurane-nitrous oxide anesthesia in aged rats." Anesth Analg 99(5): 1393-1397; table of contents.

Culley, D. J., R. Y. Yukhananov, et al. (2006). "Altered hippocampal gene expression 2 days after general anesthesia in rats." Eur J Pharmacol 549(1-3): 71-78.

Deiner, S. and J. H. Silverstein (2009). "Postoperative delirium and cognitive dysfunction." Br J Anaesth 103 Suppl 1: i41-46.

Demarco, G. J., H. A. Baghdoyan, et al. (2004). "Carbachol in the pontine reticular formation of C57BL/6J mouse decreases acetylcholine release in prefrontal cortex." Neuroscience 123(1): 17-29.

Dennison, R. L., Jr., B. L. Anthony, et al. (1987). "Effects of halothane on high affinity agonist binding and guanine nucleotide sensitivity of muscarinic acetylcholine receptors from brainstem of rat." Neuropharmacology 26(8): 1201-1205.

Dijkstra, J. B., P. J. Houx, et al. (1999). "Cognition after major surgery in the elderly: test performance and complaints." Br J Anaesth 82(6): 867-874.

Dong, H. L., S. Fukuda, et al. (2006). "Excitatory and inhibitory actions of isoflurane on the cholinergic ascending arousal system of the rat." Anesthesiology 104(1): 122-133.

Durieux, M. E. (1996). "Muscarinic signaling in the central nervous system. Recent developments and anesthetic implications." Anesthesiology 84(1): 173-189.

Eckenhoff, R. G., J. S. Johansson, et al. (2004). "Inhaled anesthetic enhancement of amyloid-beta oligomerization and cytotoxicity." Anesthesiology 101(3): 703-709.

Eckenhoff, R. G., J. W. Tanner, et al. (2001). "Cooperative binding of inhaled anesthetics and ATP to firefly luciferase." Proteins 42(4): 436-441.

Ennaceur, A. and J. Delacour (1988). "A new one-trial test for neurobiological studies of memory in rats. 1: Behavioral data." Behav Brain Res 31(1): 47-59.

Evered, L. A., B. S. Silbert, et al. (2009). "Plasma amyloid beta42 and amyloid beta40 levels are associated with early cognitive dysfunction after cardiac surgery." Ann Thorac Surg 88(5): 1426-1432.

File, S. E. (2001). "Factors controlling measures of anxiety and responses to novelty in the mouse." Behav Brain Res 125(1-2): 151-157.

Fisher, A., Z. Pittel, et al. (2003). "M1 muscarinic agonists can modulate some of the hallmarks in Alzheimer's disease: implications in future therapy." J Mol Neurosci 20(3): 349-356.

Flood, P. and K. M. Coates (2002). "Sensitivity of the alpha7 nicotinic acetylcholine receptor to isoflurane may depend on receptor inactivation." Anesth Analg 95(1): 83-87, table of contents.

Flood, P., J. M. Sonner, et al. (2002). "Heteromeric nicotinic inhibition by isoflurane does not mediate MAC or loss of righting reflex." Anesthesiology 97(4): 902-905.

Forman, S. A., K. W. Miller, et al. (1995). "A discrete site for general anesthetics on a postsynaptic receptor." Mol Pharmacol 48(4): 574-581.

Franks, N. P. (2008). "General anaesthesia: from molecular targets to neuronal pathways of sleep and arousal." Nature reviews. Neuroscience 9(5): 370-386.

Franks, N. P., A. Jenkins, et al. (1998). "Structural basis for the inhibition of firefly luciferase by a general anesthetic." Biophysical journal 75(5): 2205-2211.

Franks, N. P. and W. R. Lieb (1984). "Do general anaesthetics act by competitive binding to specific receptors?" Nature 310(5978): 599-601.

Franks, N. P. and W. R. Lieb (1994). "Molecular and cellular mechanisms of general anaesthesia." Nature 367(6464): 607-614.

Graef, S., P. Schonknecht, et al. (2011). "Cholinergic receptor subtypes and their role in cognition, emotion, and vigilance control: an overview of preclinical and clinical findings." Psychopharmacology 215(2): 205-229.

Granon, S., P. Faure, et al. (2003). "Executive and social behaviors under nicotinic receptor regulation." Proc Natl Acad Sci U S A 100(16): 9596-9601.

Grasshoff, C., U. Rudolph, et al. (2005). "Molecular and systemic mechanisms of general anaesthesia: the 'multi-site and multiple mechanisms' concept." Curr Opin Anaesthesiol 18(4): 386-391.

Halaszynski, T. M. (2009). "Pain management in the elderly and cognitively impaired patient: the role of regional anesthesia and analgesia." Curr Opin Anaesthesiol 22(5): 594-599.

Hasselmo, M. E. and M. Sarter (2011). "Modes and models of forebrain cholinergic neuromodulation of cognition." Neuropsychopharmacology : official publication of the American College of Neuropsychopharmacology 36(1): 52-73.

Hu, Z., Y. Ou, et al. (2010). "Inflammation: a bridge between postoperative cognitive dysfunction and Alzheimer's disease." Med Hypotheses 74(4): 722-724.

Hudetz, J. A., Z. Iqbal, et al. (2009). "Ketamine attenuates post-operative cognitive dysfunction after cardiac surgery." Acta Anaesthesiol Scand 53(7): 864-872.

Hudetz, J. A., Z. Iqbal, et al. (2007). "Postoperative cognitive dysfunction in older patients with a history of alcohol abuse." Anesthesiology 106(3): 423-430.

Hudetz, J. A., K. M. Patterson, et al. (2011). "Postoperative cognitive dysfunction after noncardiac surgery: effects of metabolic syndrome." Journal of anesthesia 25(3): 337-344.

Hudetz, J. A., K. M. Patterson, et al. (2009). "A history of alcohol dependence increases the incidence and severity of postoperative cognitive dysfunction in cardiac surgical patients." Int J Environ Res Public Health 6(11): 2725-2739.

Hudson, A. E. and H. C. Hemmings, Jr. (2011). "Are anaesthetics toxic to the brain?" British journal of anaesthesia 107(1): 30-37.

Hurst, R. S., M. Hajos, et al. (2005). "A novel positive allosteric modulator of the alpha7 neuronal nicotinic acetylcholine receptor: in vitro and in vivo characterization." The Journal of neuroscience : the official journal of the Society for Neuroscience 25(17): 4396-4405.

Jansson, A., K. Olin, et al. (2004). "Effects of isoflurane on prefrontal acetylcholine release and hypothalamic Fos response in young adult and aged rats." Exp Neurol 190(2): 535-543.

Johnson, T., T. Monk, et al. (2002). "Postoperative cognitive dysfunction in middle-aged patients." Anesthesiology 96(6): 1351-1357.

Jurgensen, S. and S. T. Ferreira (2010). "Nicotinic receptors, amyloid-beta, and synaptic failure in Alzheimer's disease." J Mol Neurosci 40(1-2): 221-229.

Kalenka, A., B. Gross, et al. (2010). "Isoflurane anesthesia elicits protein pattern changes in rat hippocampus." J Neurosurg Anesthesiol 22(2): 144-154.

Klinkenberg, I., A. Sambeth, et al. (2010). "Acetylcholine and attention." Behav Brain Res.

Komatsu, H., J. Nogaya, et al. (1993). "Memory facilitation by posttraining exposure to halothane, enflurane, and isoflurane in ddN mice." Anesth Analg 76(3): 609-612.

Komatsu, H., J. Nogaya, et al. (1998). "Repetitive post-training exposure to enflurane modifies spatial memory in mice." Anesthesiology 89(5): 1184-1190.

Koshland, D. E., Jr., G. Nemethy, et al. (1966). "Comparison of experimental binding data and theoretical models in proteins containing subunits." Biochemistry 5(1): 365-385.

Kress, H. G., J. Muller, et al. (1991). "Effects of volatile anesthetics on cytoplasmic Ca2+ signaling and transmitter release in a neural cell line." Anesthesiology 74(2): 309-319.

Kvolik, S., L. Glavas-Obrovac, et al. (2005). "Effects of inhalation anesthetics halothane, sevoflurane, and isoflurane on human cell lines." Life Sci 77(19): 2369-2383.

Lee, I. H., D. J. Culley, et al. (2008). "Spatial memory is intact in aged rats after propofol anesthesia." Anesthesia and analgesia 107(4): 1211-1215.

Lleo, A., S. M. Greenberg, et al. (2006). "Current pharmacotherapy for Alzheimer's disease." Annu Rev Med 57: 513-533.

Lockwood, G. (2010). "Theoretical context-sensitive elimination times for inhalation anaesthetics." Br J Anaesth 104(5): 648-655.

Maskos, U., B. E. Molles, et al. (2005). "Nicotine reinforcement and cognition restored by targeted expression of nicotinic receptors." Nature 436(7047): 103-107.

Mason, S. E., A. Noel-Storr, et al. (2010). "The impact of general and regional anesthesia on the incidence of post-operative cognitive dysfunction and post-operative delirium: a systematic review with meta-analysis." J Alzheimers Dis 22 Suppl 3: 67-79.

Maze, M. and M. M. Todd (2007). "Special issue on postoperative cognitive dysfunction: selected reports from the journal-sponsored symposium." Anesthesiology 106(3): 418-420.

Mills, S. A. (1993). "Cerebral injury and cardiac operations." Ann Thorac Surg 56(5 Suppl): S86-91.

Minami, K. and Y. Uezono (2006). "Gq protein-coupled receptors as targets for anesthetics." Current pharmaceutical design 12(15): 1931-1937.

Moller, J. T., P. Cluitmans, et al. (1998). "Long-term postoperative cognitive dysfunction in the elderly ISPOCD1 study. ISPOCD investigators. International Study of Post-Operative Cognitive Dysfunction." Lancet 351(9106): 857-861.

Monk, T. G., B. C. Weldon, et al. (2008). "Predictors of cognitive dysfunction after major noncardiac surgery." Anesthesiology 108(1): 18-30.

Monod, J., J. P. Changeux, et al. (1963). "Allosteric proteins and cellular control systems." Journal of molecular biology 6: 306-329.

Morrison, R. S., J. Magaziner, et al. (2003). "Relationship between pain and opioid analgesics on the development of delirium following hip fracture." J Gerontol A Biol Sci Med Sci 58(1): 76-81.

Newfield, P. (2009). "Postoperative cognitive dysfunction." F1000 medicine reports 1.

Newhouse, P., A. Singh, et al. (2004). "Nicotine and nicotinic receptor involvement in neuropsychiatric disorders." Curr Top Med Chem 4(3): 267-282.

Newman, S., J. Stygall, et al. (2007). "Postoperative cognitive dysfunction after noncardiac surgery: a systematic review." Anesthesiology 106(3): 572-590.

Nietgen, G. W., C. W. Honemann, et al. (1998). "Influence of anesthetics on endogenous and recombinantly expressed G protein-coupled receptors in the Xenopus oocyte." Toxicology letters 100-101: 319-327.

Nordberg, A. (2006). "Emerging biology of the cholinergic system across the spectrum of Alzheimer's disease." Int Psychogeriatr 18 Suppl 1: S3-S16.

Nury, H., C. Van Renterghem, et al. (2011). "X-ray structures of general anaesthetics bound to a pentameric ligand-gated ion channel." Nature 469(7330): 428-431.

Pearson, A., A. de Vries, et al. (2010). "Cerebrospinal fluid cortisol levels are higher in patients with delirium versus controls." BMC Res Notes 3: 33.

Perouansky, M. and H. C. Hemmings, Jr. (2009). "Between Clotho and Lachesis: how isoflurane seals neuronal fate." Anesthesiology 110(4): 709-711.

Perry, E., M. Walker, et al. (1999). "Acetylcholine in mind: a neurotransmitter correlate of consciousness?" Trends Neurosci 22(6): 273-280.

Picciotto, M. R., B. J. Caldarone, et al. (2001). "Neuronal nicotinic acetylcholine receptor subunit knockout mice: physiological and behavioral phenotypes and possible clinical implications." Pharmacol Ther 92(2-3): 89-108.

Poirier, M. F., O. Canceil, et al. (2002). "Prevalence of smoking in psychiatric patients." Prog Neuropsychopharmacol Biol Psychiatry 26(3): 529-537.

Raines, D. E. (1998). "Conformational transitions of the nicotinic acetylcholine receptor as a model for anesthetic actions on ligand-gated ion channels: single and sequential mixing stopped-flow fluorescence studies." Toxicol Lett 100-101: 163-168.

Raines, D. E., S. E. Rankin, et al. (1995). "General anesthetics modify the kinetics of nicotinic acetylcholine receptor desensitization at clinically relevant concentrations." Anesthesiology 82(1): 276-287; discussion 231A-232A.

Raines, D. E. and V. T. Zachariah (1999). "Isoflurane increases the apparent agonist affinity of the nicotinic acetylcholine receptor." Anesthesiology 90(1): 135-146.

Rammes, G., L. K. Starker, et al. (2009). "Isoflurane anaesthesia reversibly improves cognitive function and long-term potentiation (LTP) via an up-regulation in NMDA receptor 2B subunit expression." Neuropharmacology 56(3): 626-636.

Rasmussen, L. S., T. Johnson, et al. (2003). "Does anaesthesia cause postoperative cognitive dysfunction? A randomised study of regional versus general anaesthesia in 438 elderly patients." Acta Anaesthesiol Scand 47(3): 260-266.

Rasmussen, L. S. and J. T. Moller (2000). "Central nervous system dysfunction after anesthesia in the geriatric patient." Anesthesiol Clin North America 18(1): 59-70, vi.

Rasmussen, L. S., J. T. O'Brien, et al. (2005). "Is peri-operative cortisol secretion related to post-operative cognitive dysfunction?" Acta Anaesthesiol Scand 49(9): 1225-1231.

Robinson, T. N., C. D. Raeburn, et al. (2009). "Postoperative delirium in the elderly: risk factors and outcomes." Ann Surg 249(1): 173-178.

Rohan, D., D. J. Buggy, et al. (2005). "Increased incidence of postoperative cognitive dysfunction 24 hr after minor surgery in the elderly." Can J Anaesth 52(2): 137-142.

Rosas-Ballina, M. and K. J. Tracey (2009). "The neurology of the immune system: neural reflexes regulate immunity." Neuron 64(1): 28-32.

Rudolph, J. L., R. N. Jones, et al. (2007). "Independent vascular and cognitive risk factors for postoperative delirium." Am J Med 120(9): 807-813.

Rudolph, J. L., E. R. Marcantonio, et al. (2008). "Delirium is associated with early postoperative cognitive dysfunction." Anaesthesia 63(9): 941-947.

Sabri, O., K. Kendziorra, et al. (2008). "Acetylcholine receptors in dementia and mild cognitive impairment." Eur J Nucl Med Mol Imaging 35 Suppl 1: S30-45.

Sekine, S., S. Matsumoto, et al. (2006). "Changes in expression of GABAA alpha4 subunit mRNA in the brain under anesthesia induced by volatile and intravenous anesthetics." Neurochem Res 31(3): 439-448.

Shaw, P. J., D. Bates, et al. (1987). "Long-term intellectual dysfunction following coronary artery bypass graft surgery: a six month follow-up study." The Quarterly journal of medicine 62(239): 259-268.

Silverstein, J. H., J. Steinmetz, et al. (2007). "Postoperative cognitive dysfunction in patients with preoperative cognitive impairment: which domains are most vulnerable?" Anesthesiology 106(3): 431-435.

Smith, P. J., D. K. Attix, et al. (2009). "Executive function and depression as independent risk factors for postoperative delirium." Anesthesiology 110(4): 781-787.

Steinmetz, J., K. B. Christensen, et al. (2009). "Long-term consequences of postoperative cognitive dysfunction." Anesthesiology 110(3): 548-555.

Stratmann, G., J. W. Sall, et al. (2010). "Isoflurane does not affect brain cell death, hippocampal neurogenesis, or long-term neurocognitive outcome in aged rats." Anesthesiology 112(2): 305-315.

Szarecka, A., Y. Xu, et al. (2007). "Dynamics of firefly luciferase inhibition by general anesthetics: Gaussian and anisotropic network analyses." Biophysical journal 93(6): 1895-1905.

Tang, J. X., D. Baranov, et al. (2011). "Human Alzheimer and Inflammation Biomarkers after Anesthesia and Surgery." Anesthesiology.

Tansey, E. M. (2006). "Henry Dale and the discovery of acetylcholine." Comptes rendus biologies 329(5-6): 419-425.

Tassonyi, E., E. Charpantier, et al. (2002). "The role of nicotinic acetylcholine receptors in the mechanisms of anesthesia." Brain Res Bull 57(2): 133-150.

Terrando, N., A. Rei Fidalgo, et al. (2010). "The impact of IL-1 modulation on the development of lipopolysaccharide-induced cognitive dysfunction." Critical care 14(3): R88.

Violet, J. M., D. L. Downie, et al. (1997). "Differential sensitivities of mammalian neuronal and muscle nicotinic acetylcholine receptors to general anesthetics." Anesthesiology 86(4): 866-874.

Wan, Y., J. Xu, et al. (2007). "Postoperative impairment of cognitive function in rats: a possible role for cytokine-mediated inflammation in the hippocampus." Anesthesiology 106(3): 436-443.

Wan, Y., J. Xu, et al. (2010). "Cognitive decline following major surgery is associated with gliosis, beta-amyloid accumulation, and tau phosphorylation in old mice." Crit Care Med.

Wang, L., R. J. Traystman, et al. (2008). "Inhalational anesthetics as preconditioning agents in ischemic brain." Curr Opin Pharmacol 8(1): 104-110.

Warburton, E. C., T. Koder, et al. (2003). "Cholinergic neurotransmission is essential for perirhinal cortical plasticity and recognition memory." Neuron 38(6): 987-996.

Weber, C. F., H. Friedl, et al. (2009). "Impact of general versus local anesthesia on early postoperative cognitive dysfunction following carotid endarterectomy: GALA Study Subgroup Analysis." World J Surg 33(7): 1526-1532.

Weigl, L. G. and W. Schreibmayer (2001). "G protein-gated inwardly rectifying potassium channels are targets for volatile anesthetics." Molecular pharmacology 60(2): 282-289.

Wessler, I. and C. J. Kirkpatrick (2008). "Acetylcholine beyond neurons: the non-neuronal cholinergic system in humans." British journal of pharmacology 154(8): 1558-1571.

Wevers, A. (2010). "Localisation of pre- and postsynaptic cholinergic markers in the human brain." Behav Brain Res.

Willner, A. E., C. J. Rabiner, et al. (1976). "Analogical reasoning and postoperative outcome. Predictions for patients scheduled for open heart surgery." Arch Gen Psychiatry 33(2): 255-259.

Woolf, N. J. and L. L. Butcher (2010). "Cholinergic systems mediate action from movement to higher consciousness." Behav Brain Res.

Wu, C. L., W. Hsu, et al. (2004). "Postoperative cognitive function as an outcome of regional anesthesia and analgesia." Reg Anesth Pain Med 29(3): 257-268.

Wuppen, K., D. Oesterle, et al. (2010). "A subchronic application period of glucocorticoids leads to rat cognitive dysfunction whereas physostigmine induces a mild neuroprotection." J Neural Transm 117(9): 1055-1065.

Xie, G., W. Zhang, et al. (2009). "Relationship between perioperative inflammatory response and postoperative cognitive dysfunction in the elderly." Med Hypotheses 73(3): 402-403.

Xie, Z., Y. Dong, et al. (2006). "The common inhalation anesthetic isoflurane induces apoptosis and increases amyloid beta protein levels." Anesthesiology 104(5): 988-994.

Xie, Z., Y. Dong, et al. (2006). "Isoflurane-induced apoptosis: a potential pathogenic link between delirium and dementia." J Gerontol A Biol Sci Med Sci 61(12): 1300-1306.

Young, A. P., J. R. Oshiki, et al. (1981). "Allosteric effects of volatile anesthetics on the membrane-bound acetylcholine receptor protein." Molecular pharmacology 20(3): 506-510.

C’est qui est fait, n’est plus a faire!