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# **Propofol changes the cytoskeletal function in neurons**

**An experimental study in cortical cultures**

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Cover picture: Cortical rat neurons stained med anti-tubulin  $\beta$  III antibodies and visualised with fluorescence microscopy (Marcus Renberg). Adapted to cover and text by Per Lagman, Mediacenter, Linköping.

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*To Natalija, Nicole and Noah*

*The way to know life is to love many things.*

*Vincent van Gogh*

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# ABSTRACT

Every day, general anaesthetics are given to a large number of patients around the world but the cellular mechanisms of how anaesthetics act is still not clear. General anaesthetics cause the intended unconsciousness, amnesia and immobility in patients, but also side effects such as a decrease in mean arterial pressure and arrhythmia, both of which contribute to complications such as heart damage and stroke. With more knowledge of the mechanism of anaesthetic drugs, these complications could be reduced.

It has been shown that anaesthetics cause a disruption of the thalamocortical connectivity and brain network connectivity. How the network communication is disrupted however is not known. Propofol and thiopental are both intravenous anaesthetic drugs used widely in clinical anaesthesia. They bind to the GABA<sub>A</sub> receptor and enhance its function.

The cytoskeleton helps the cell to maintain its shape and participate in cellular movement and transport. Cellular transport to and from a neuron's cell body and periphery is performed by motor proteins that move vesicles, organelles and proteins along cytoskeletal tracks. We have previously shown that propofol causes a reorganisation of the cytoskeleton protein actin in neurons, but we were further interested to study the effects of propofol and thiopental on the cytoskeletal function of cultured cortical rat neurons.

Our results show that propofol and thiopental cause neurite (axon and dendrite) retraction. Propofol's effects were time- and dose-dependent, and can be reversed when propofol is removed. We were able to inhibit propofol-induced neurite retraction if we stabilised actin by blocking either the motor protein myosin II or the GABA<sub>A</sub> receptor. We have previously shown that a small GTP-binding protein, RhoA, inhibits propofol-caused actin reorganisation. Propofol-induced neurite retraction was mediated via a downstream effector of RhoA, ROK, which induces phosphorylation of the myosin light chain and increases contractility. Furthermore, we have shown that propofol causes a switch from anterograde to retrograde transport and increases the average velocity of the moving vesicles in neurites. The propofol-induced retrograde vesicle transport was GABA<sub>A</sub> receptor-mediated.

Orexin A is a neuropeptide which regulates the sleep/awake cycle and has also been shown to reduce anaesthesia in animals when given intracerebroventricularly. We found that orexin A reverses propofol and thiopental-induced neurite retraction and actin reorganisation. Moreover, we

have shown that the orexin A inhibition of propofol-induced neurite retraction is mediated via the PLD/PKC intracellular signalling pathway. Propofol and thiopental decreased the tyrosine phosphorylation of the intermediate cytoskeletal protein vimentin which is reversed by orexin A.

Taken together, these results suggest that propofol causes a time- and dose-dependent, reversible and GABA<sub>A</sub> receptor-mediated neurite retraction in cultured cortical rat neurons. Propofol also causes a switch from anterograde to retrograde vesicle transport in neurites. Orexin A reverses propofol and thiopental-induced neurite retraction and cytoskeletal reorganisation. Orexin A inhibits propofol-induced neurite retraction via the PLD/PKC intracellular signalling pathway.

# POPULÄRVETENSKAPLIG SAMMANFATTNING

Den cellulära mekanismen för hur narkosmedel verkar är fortfarande oklar. Detta trots att narkosmedel används dagligen för att söva patienter vilka ska genomgå kirurgi. Narkosmedel ger förutom sömn också smärtfrihet och immobilisering. Vanliga biverkningar är blodtrycksfall och hjärtpåverkan vilket kan leda till allvarliga komplikationer som hjärtinfarkt och hjärtsvikt. Med ökad kunskap om verkningsmekanismen av narkosmedel kan vi också minimera biverkningar och komplikationer.

Propofol är ett av våra vanligaste narkosmedel, både för att inleda narkos och för att underhålla narkos.

Alla studier i denna avhandling har utförts på nervceller från råttor i cellkultur. Nervcellerna har exponerats för narkosmedlet propofol och därefter studerats i ljusmikroskop och med immuno-histofluorescens (specifika proteiner som görs synliga genom att man sätter på en antikropp som är specifik för proteinet, en så kallad primär antikropp och därefter en sekundär som sätter sig på den primära och avger ett fluorescent ljus). Detta gör att det blir synligt i mikroskop.

Hela hjärnan består av otaliga nätverk där nervcellerna är förbundna med varandra med sina utskott och kommunicerar på det sättet, det vill säga skickar budskap mycket snabbt till varandra. Studier med fMR (magnetresonans) på människa har visat att detta nätverk/kommunikation är försämrade då försökspersonen har fått narkos. Framförallt är kontakten mellan thalamus och cortex (en central del av hjärnan och hjärnbarken) försämrade eller brutna. Vad som ligger bakom den brutna förbindelsen som ses vid narkos är oklart.

Vi har i våra studier visat att narkosmedlet propofol orsakar ett tillbaka dragande av nervcellens utskott, det vill säga förbindelsen från cell till cell har blivit försämrade. Kommunikationen mellan nervceller fungerar inte fullt ut. Detta har vi visat genom att använda ljusmikroskop och immunohistofluorescenta tekniker där vi har märkt in vissa proteiner och sett hur det har skett ett tillbaka dragande av utskotten, vilka förbinder nervcellerna med varandra. Vi har vidare visat att denna effekt är både tids- och dosberoende och att effekten försvinner då narkosmedlet propofol sköljs bort från cellkulturen. Vi har också visat att transporten inne i cellen, från

cellkroppen ut perifert, av transmittorsubstanser och andra proteiner hämmas av propofol. Vanligtvis går denna transport till största delen från cellkroppen ut perifert. I periferin används de här ämnena för att upprätthålla kommunikationen till nästa cell, de har alltså en viktig funktion. När nervcellen exponeras för narkosmedlet propofol vänder denna transport och istället går den till största delen ut från periferin in till cellkroppen och det med en större hastighet än vanligtvis. Effekten av propofol är alltså av två slag, dels tillbaka dragande av nervutskotten och dels förändrad riktning av den viktiga transporten av proteiner och transmittorsubstanser.

I hjärnan finns signalsubstansen Orexin A vilken är viktig för reglering av vakenhet och sömn. Tidigare studier har visat att Orexin A kan reversera narkoseffekten.

Vi har visat att Orexin A motverkar narkosmedlet propofols effekter på nervcellen. Då nervcellen exponeras för propofol och Orexin A så sker inte tillbaka dragande av nervutskotten

Sammanfattningsvis visar de här resultaten att narkosmedlet propofol orsakar en receptor medierad tillbaka dragande av nervcellens utskott och att transporten ändrar riktning från cellkropp ut perifert till det omvända. Signalsubstansen Orexin A kan förhindra tillbakadragande av nervutskott via en intracellulär signal. Dessa resultat innebär att vi troligen har kommit ett steg närmare att finna fram hur narkosmedel verkar och möjligen också ett nytt medel för att reversera narkoseffekten.

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# ABBREVIATIONS

AE	Acetic acid
AMPA	$\alpha$ -amino-3-hydroxy-5-methylisoxazol-4-propanoic acid receptor
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BA	Barbiturate acid
BSA	Bovine serum albumin
[Ca <sup>2+</sup> ] <sub>i</sub>	Intracellular Ca <sup>2+</sup> concentration
CCD	Charge-coupled device camera
CCM	Ca <sup>2+</sup> -containing medium
CNS	Central nervous system
CSF	Cerebrospinal fluid
DAG	Diacylglycerol
DMR	Dorsal membrane ruffles
EC <sub>50</sub>	Half maximal effective concentration
ECL	Enhanced chemiluminescence
EEG	Electroencephalography
FCS	Foetal calf serum
FIPI	5-fluoro-2-indolyl des-chlorohalopamide
fMRI	Functional magnetic resonance imaging
GABA <sub>A</sub> R	$\gamma$ -aminobutyric acid type A receptor
GTP	Guanosine triphosphate
IC <sub>50</sub>	Half maximal inhibitory concentration
IP	Immuno-precipitation
LHA	Lateral hypothalamus
LPA	Lysophosphatidic acid
MAC	Minimally alveolar concentration
MLC	Myosin light chain
MLCK	Myosin light chain kinase
NA	Numeric aperture
Na <sub>2</sub> CO <sub>3</sub>	Sodium bicarbonate
NMDAR	N-methyl-D-aspartate receptor
NREM	Non-rapid eye movement
NSE	Neuron-specific enolase
OA	Orexin A
PA	Phosphatidic acid

PBS	Phosphate buffered saline
pEC <sub>50</sub>	Negative logarithm of the EC <sub>50</sub>
PET	Positron-emission tomography
PFA	Paraformaldehyde
PI <sub>3</sub> -kinase	Phosphoinositide 3-kinase
PKC	Protein kinase C
PLD	Phospholipase D
PMA	Phorbol 12-myristate 13-acetate
Ptyr	Anti-phosphotyrosine antibody
PVDF	Polyvinylidene fluoride
RhoA	Ras homolog gene family, member A
ROK	Rho-associated coiled coil-forming protein kinase
SDS	Sodiumdodecylsulphate
TREK-1	Stretch-dependent potassium channels

# LIST OF PAPERS

This thesis is based on the following papers which will be referred to by their Roman numerals:

**I Propofol causes neurite retraction in neurons**

Dean Turina, Vesa M Loitto, Karin Björnström, Tommy Sundqvist, Christina Eintrei. *British Journal of Anaesthesia* 2008; 3:374-379

**II Propofol alters vesicular transport in rat cortical neuronal cultures**

Dean Turina, Karin Björnström, Tommy Sundqvist, Christina Eintrei. Propofol alters vesicular transport in rat cortical neuronal cultures. *Journal of physiology and pharmacology*. 2011; 62:119-124

**III Orexin A reverses propofol and thiopental induced cytoskeletal rearrangement in primary cortical neuronal culture**

Dean Turina, Alenka Glavas, Karin Björnström (submitted manuscript)

**IV Orexin A inhibits propofol-induced neurite retraction by a PLD-dependent mechanism in neurons**

Dean Turina, Karin Björnström, Tommy Sundqvist, Christina Eintrei (submitted manuscript)



# INTRODUCTION

## General aspects of the anaesthesia mechanism

The discovery of general anaesthetics has had a significant impact on the development of surgery and medicine in general during the last century. General anaesthetics are chemically diverse compounds yet their net effect upon administration is always the same: unconsciousness, amnesia and immobility (1). Different intravenous (propofol, thiopental, ketalar, etomidate) and volatile agents (sevoflurane, isoflurane, halothane, desflurane, xenon), but also benzodiazepines in high concentrations, are capable of causing anaesthesia. Despite the widespread use of general anaesthetics, the mechanisms by which they produce their effects in the central nervous system are still poorly understood.

On a cellular level, we do know that anaesthetics reduce synaptic transmission and profoundly decrease neuronal activity in isolated cortical networks (2). At the nervous system level, general anaesthetics cause a decrease in brain glucose metabolism (3, 4). They act at multiple anatomic sites and reduce the number of network connections (5). But the mechanism of how the network communication is reduced by general anaesthetics is still unknown. Despite our limited knowledge of how anaesthetic agents produce their molecular, cellular and functional effects, they are used on a daily basis in surgeries worldwide. In Sweden, approximately 7% of its nine million population annually undergo some form of surgical intervention which usually requires anaesthesia (approximately 600 000 anaesthesias per year).

Anaesthesia is today considered relatively safe but, still, some patients do suffer complications. These complications are dominated by effects on the cardiovascular system (6). An antidote would be a valuable tool that could counteract the side effects of anaesthetics and shorten awakening as well enable the physician to make more frequent neurological assessments of intensive care patients. A possible antidote is orexin A (OA), a hypothalamic neuropeptide regulating wake-sleep cycles which is known to be involved in the emergence from anaesthesia (7). Thus a clearer understanding of the sites and mechanisms of the anaesthetic drug action is required in order to reduce

complication rates and open new ways for developing anaesthetic agents with improved pharmacological properties.

## Intravenous anaesthetics

### *Propofol*

Propofol (2,6-diisopropylphenol) is an intravenous anaesthetic agent which has been used widely in daily clinical practice for sedation and general anaesthesia since its introduction into clinical practice in 1986. Propofol is known to affect the  $\gamma$ -aminobutyric acid type A receptor (GABA<sub>A</sub>R) by stepping up the release of GABA (8) and also directly activate the receptor (9). It has been shown that propofol and other anaesthetics modulate synaptic transmission (10) and depress neurosecretion (11). Molecular studies have provided evidence that propofol enhances the GABA<sub>A</sub>R activity at concentrations relevant for anaesthesia (2). In addition to propofol's postsynaptic effects (10), it has been shown that propofol enhances GABA<sub>A</sub>R-mediated presynaptic inhibition (12).

Laboratory studies have shown that propofol induces tyrosine phosphorylation in the GABA<sub>A</sub>R  $\beta_2$  and  $\beta_3$ -subtypes in the cell membrane and the cytoskeletal fraction, and differs in distribution of the  $\beta_2$  subtypes compared to GABA (13). Propofol mediates a reversible and concentration-dependent actin reorganisation and causes actin cytoskeleton to form ring structures in neurons (14). The actin ring formation is transcellular and dependent on the influx of extracellular calcium. No ring structures are produced by GABA however, when co-applied with propofol, a synergetic effect is seen (15). Propofol, but not GABA, also phosphorylates tyrosine residues in actin in the cell membrane and cytoskeletal fraction, and this effect is calcium dependent (15). Maximal phosphorylation was observed after two minutes which is consistent with the time of maximum chloride ion flow caused by propofol (16). Propofol also increases the content of F-actin in the neurons (17). This shows that the interaction with the cell cytoskeleton is important for propofol's effects since phosphorylation of actin occurs within two minutes, while the restructuring of actin to the visible ring structures reaches its maximum much later, and therefore is probably not relevant to propofol's anaesthetic effect (14). Propofol also causes an increase in the

neurons intracellular calcium concentration, and this is caused by both the influx from the extracellular environment and the release from the intracellular stores (14). The release from the intracellular stores is regulated by a tyrosine kinase that can be inhibited by herbimycin A (18). The intracellular signal used by propofol to cause the rings involves Rho, Rho-kinase, phosphoinositide 3-kinase (PI<sub>3</sub>-kinase) and tyrosine kinase (18).

## *Thiopental*

Thiopental is a very fast acting barbiturate which enhances the binding of GABA to the GABA<sub>A</sub>R and thereby increases the chloride currents. At low concentrations, thiopental potentiates GABA currents and, at higher concentrations, thiopental directly activates the receptor (19). It has long been thought that the molecular site of action of barbiturates at GABA<sub>A</sub>R resides within the  $\beta$  subunit but new data suggests a possible role for the  $\delta$  subunit as a target for thiopental action on GABA<sub>A</sub>R (20). S-thiopental causes a greater potentiation of GABA<sub>A</sub>R expressed in *Xenopus* oocytes, followed by the R-isomer (21). Thiopental, at clinically relevant concentrations, also inhibits recombinant nicotinic acetylcholine receptors which may depress neurotransmission (22). However, barbiturates facilitate the release of GABA from presynaptic nerve terminals and enhance inhibition (23). Thiopental causes a decrease in calcium entry into neurons after a glutamate-stimulated increase in  $[Ca^{2+}]_i$  (24). Thiopental attenuated N-methyl-D-aspartate (NMDA) and  $\alpha$ -amino-3-hydroxy-5-methylisoxazol-4-propanoic acid (AMPA)-mediated glutamate toxicity in hippocampal slices *in vitro* (25) and in rat cerebral cortices *in vivo* (26). In addition, thiopental attenuates ischaemia-induced intracellular calcium increases in the hippocampus and cortex (27).

## *Ketamine*

Ketamine is an intravenous anaesthetic with potent sedative, hypnotic and analgesic properties (28). Ketamine causes a dissociation between the talamocortical and limbic areas of the brain (29). Ketamine has no effect on GABA<sub>A</sub>R and exhibits its effect by an interaction with the N-methyl-D-aspartate receptor (NMDAR) (30). Ketamine is known for its ability to

preserve respiration and haemodynamic stability, but also has psychomimetic effects (28) which are, in practice, often mimicked by the simultaneous application of a benzodiazepine drug. The effect of ketamine is stereoselective and the dextro-S-isomer of ketamine has approximately three- to fourfold the potency of the levo-R-isomer (30). It has been shown that ketamine has a cytotoxic effect on developing neurons (31) and that even low concentrations of ketamine could impair the dendritic arbor development of immature GABAergic neurons (32).

# **MECHANISMS OF ACTION OF GENERAL ANAESTHETICS**

## **Molecular action**

The molecular mechanism of general anaesthetics is still, more than 150 years after the first anaesthetic was administered, to a large extent, unknown. At the beginning of the 1900s, Meyer and Overton (33, 34) showed that the potency of anaesthetics was dependent on their lipid solubility. This later gave rise to the so-called "lipid theory" which suggested that the cell membrane was a primary target for anaesthetics (35, 36). Observed lipid solubility of anaesthetics has initiated a hypothesis on the unitary mechanism of anaesthetic action. However the lipid theory does not explain the "cut-off" phenomenon of anaesthetics (37) in which larger molecules no longer increase in anaesthetic potency even though their solubility in lipids increases (38). The cut-off phenomenon has also been noted with the use of inhalation anaesthetics (39). In 1984, Franks and Lieb showed that the function of the purified lipid-free enzyme luciferase was impaired according to the potency of the anaesthetic, as predicted by the Meyer-Overton rule, and they suggested that the anaesthetic agents interact with proteins where the binding site has a limited volume and an anaesthetic could fit (40). The interior part of such a protein pocket will still need to have a lipophilic milieu, either through the amino acid residues of the protein itself or interference with a lipid bilayer of the cell (41). This is called the "protein theory" and suggests that proteins are the main target of anaesthetics. The protein theory does not exclude the old knowledge that fat solubility is important for the anaesthetic because mutation studies have shown that, if a hydrophobic amino acid is replaced by a hydrophilic, they can no longer interact anaesthetically with GABA<sub>A</sub>R (39).

A common binding site for anaesthetics has been found on the upper transmembrane part of the bacterial pentameric ligand-gated ion channel, with a cavity opening into the lipid bilayer (42). Ligand-gated ion channels, in particular GABA<sub>A</sub>Rs, NMDARs and glycine receptors, are thought to be among the most relevant molecular targets for general anaesthetics (43).

## ***The GABA<sub>A</sub> receptor***

GABA is the main inhibitory neurotransmitter in the mammalian CNS. It is a neurotransmitter in the brain which helps to stimulate relaxation and sleep (44). The pentameric GABA<sub>A</sub>R contains different subunits ( $\alpha, \beta, \gamma, \delta$ ) which combine to form a chloride channel that is normally closed but can be opened by GABA (45). A common subunit composition is  $2\alpha:2\beta:1\gamma$  (46). When activated, the receptor opens to allow chloride ions to flow into the cell. This causes hyperpolarisation which reduces the probability of propagating a new action potential throughout the neuron and reduces excitability. Tyrosine phosphorylation of the receptor is required for its function (47). Receptors are located at inhibitory synapses but are also present extrasynaptically on the soma, the dendrites and the axon (48). Whereas synaptic GABA<sub>A</sub>Rs underlie classical "phasic" GABA<sub>A</sub>Rs-mediated inhibition (inhibitory postsynaptic currents), tonic GABA<sub>A</sub>Rs-mediated inhibition results from the activation of extrasynaptic receptors through low concentrations of ambient GABA (49). Neurotransmitter receptors clustering at postsynaptic membranes are thought to represent a critical parameter for neuronal transmission (50). The clustering of synaptic GABA<sub>A</sub>Rs significantly affects synaptic transmission (51), and the clustering of extrasynaptic GABA<sub>A</sub>Rs modulates tonic inhibition with a concomitant reduction in the amplitude of tonic currents and diminished inhibition (52). Most general anaesthetics bind to the GABA<sub>A</sub>Rs (53) and glycine receptors (54) (mainly in the spinal cord). GABA<sub>A</sub>Rs are highly sensitive to positive allosteric regulation by propofol, barbiturates, sedative benzodiazepines, volatile anaesthetics, neuroactive steroids and a variety of other neurodepressive drugs (9, 55-57). Upon GABA<sub>A</sub>Rs, an increased inhibitory action is caused by the augmentation of the GABA binding (isoflurane) (55), (propofol) (58) and, in higher doses, a direct activation (propofol) (9), and benzodiazepines (19). Agonist-induced currents by propofol are affected by a point mutation of the  $\beta 2$  (59) and  $\beta 3$  (60) subunit of the GABA<sub>A</sub>R. It has been identified that a mutation in the GABA<sub>A</sub>  $\alpha$  subunit abolishes the action of barbiturates but not etomidate (61).

## ***The NMDA receptors***

The volatile anaesthetics xenon (62), nitrous oxide (laughing gas) (63) as well as the intravenous anaesthetic ketamine interact with NMDARs on the postsynaptic membrane (63). They suppress excitatory, glutamate-mediated

synaptic transmission by blocking NMDARs inhibition of its excitatory effect. The drugs that interfere with the NMDA receptor all show a pain-reducing effect as exemplified by nitrous oxide that is used for pain relief during labour. The reduced pain sensation is due to the non-competitive inhibition of the NR2B subtype of the receptor (64). The NMDA receptor system is also influenced by GABA and, in mice, when GABAergic inhibition of the NMDAR is removed, pain is increased (65). It has been shown that even volatile anaesthetics known to interact with GABA<sub>A</sub>R – isoflurane, sevoflurane and desflurane – reversibly inhibit recombinant NMDARs (66). Propofol depressed glutamate release, glutamate uptake from rat synaptosomes and volatile anaesthetics suppressed the excitatory glutamatergic transmission (67). This suggests that general anaesthetics, in addition to a postsynaptic action at receptor level, also act via the presynaptic inhibition of glutamate release.

## **Cellular action**

An extracellular signal propagates in the cell via the interaction of the drug with the receptor and amplification through several subcellular steps which include second messenger events. Cellular events elicited by anaesthetics include changes in second messengers such as [Ca<sup>2+</sup>]<sub>i</sub> (13, 15, 68), G-proteins (18), kinases (13, 18) and phosphatases (69).

Anaesthetics have been described to cause both an increase (13, 70), and also inhibit an increase, in [Ca<sup>2+</sup>]<sub>i</sub> (71-73). This is thought to be because of differences in experimental models, the type of cells used and temperature (74). Isoflurane can inhibit glutamate stimulated [Ca<sup>2+</sup>]<sub>i</sub> increases (75) thereby reducing the excitatory effect of glutamate. Propofol, thiopental and ketamine cause relaxation of the porcine airway smooth muscle by reducing [Ca<sup>2+</sup>]<sub>i</sub> (76). The combined use of Rho-associated coiled coil-forming protein kinases (ROK) inhibitors and propofol causes greater relaxation in rat bronchial smooth muscle (77). Results from our laboratory have shown that, in cultured cortical rat neurons, propofol causes an increase in [Ca<sup>2+</sup>]<sub>i</sub> by influx from both the extracellular and intracellular stores (14). Halothane causes calcium release from intracellular stores via the ryanodine receptor channel (78). An increase in [Ca<sup>2+</sup>]<sub>i</sub> is important for the activation of other cellular messengers as well as the regulation of actin.

Phosphorylation is an important mechanism which turns many protein enzymes in the cell on and off. Enzymes kinases and phosphatases are involved in the process of phosphorylation and dephosphorylation, and work

together as modulators of different proteins. Their targets are serine, threonine or tyrosine residues. Reversible phosphorylation of the GABA<sub>A</sub> receptor is important for its function (47, 79). Propofol causes an increase in tyrosine phosphorylation of the  $\beta 2$  and  $\beta 3$  subunits of the GABA<sub>A</sub> receptor, and differs from GABA in the subunits phosphorylated at the cell membrane (15). It has been shown that propofol's inhibitory effect on the NMDAR current in the primary cortical neurons is due to the inhibition of NR1 subunit phosphorylation (80). Propofol, isoflurane and ketamine reduced the *in vivo* phosphorylation of glutaminergic and dopaminergic receptors (69).

### ***The cytoskeleton and motor proteins***

Neurons contain an elaborate array of protein fibres that establish cell shape, provide mechanical strength, and facilitate locomotion and intracellular transport of organelles. The cytoskeleton is made up of three kinds of protein filaments: actin filaments (also called microfilaments), intermediate filaments and microtubules. Microtubules are cytoskeletal elements which molecular motors use like trails for the long-distance delivery of cellular cargos in neurites. Actin filaments function as tracks for the local movement and positioning of organelles and vesicles as well as the establishment of cell shape (81). Vimentin is an intermediate filament that links cytoskeletal proteins, often in close relation to the cell membrane. Vimentin is phosphorylated upon tyrosine (82), and is regulated by the kinases Src (83) and Rho-kinase (84). The cytoskeletal proteins participate by shaping the neuron, dividing the cell into specialised compartments and participating in cell movement. The axonal microfilament system is subject to strong contractile forces generated by the motor protein myosin, and those forces are counterbalanced by forces between the microfilament and microtubule arrays generated by cytoplasmic dynein (85). Propofol, not GABA, causes neuronal actin rearrangement to form actin rings or circular dorsal membrane ruffles (DMR) (14, 15, 18), and involves activation of the small G-protein RhoA and its downstream effector molecules to propagate signal transduction (18). After activation of the tyrosine kinase receptor, the cell membrane folds and moves like a wave to the cell interior where it forms DMR (86). Propofol-induced actin rearrangement is dependent on calcium and involves tyrosine kinase (18). The actin ring structure process has been described after initiation with different stimulus such as growth factors (87). Isoflurane interacts with actin and RhoA, and causes impaired organisation of actin stress fibres (88). Isoflurane also inhibits actin

reorganisation in dendritic spines (89) however, in this study, intravenous anaesthetics did not inhibit dendritic actin, suggesting different mechanisms for volatiles and intravenous anaesthetics. Taken together, these studies point to the cytoskeleton as an important part of the anaesthetic cellular mechanism.

Intracellular transport is driven by molecular motors that move along cytoskeletal tracks, and is responsible for delivering organelles, vesicles and macromolecular complexes to various destinations inside the cytoplasm (90). Axonal proteins and Golgi-derived vesicles are formed in the neuronal cell body and are shipped along the axon by a process called axonal transport. This movement is essential for the growth and survival of axons, and continues throughout the life of the nerve cell (91). For microtubule-based transport, kinesin motors generally drive transport towards the plus ends of microtubules in the cell periphery whereas cytoplasmic dynein ferries cargo to the microtubule minus ends in the cell centre (92, 93).

## ***Neurite retraction***

The normal function of the nervous system depends on precise connections that connect individual neurons together in a neuronal network. The selective retraction of axons, without the loss of the parent neurons, occurs during normal development of the nervous system as well as in response to injury and disease (94). In hibernating mammals, neurons exhibit a dramatic form of plasticity during torpor, with dendritic arbors retracting as body temperature cools and then re-growing rapidly as it rises. In the awake state, the neurites return to their pre-hibernation locations (95). Neurite retraction is also a hallmark of neurotrauma (96). Recent investigations indicate that retraction is an active process mediated by alterations in actin filament structure and motor protein-based force generation (85, 97). Several anaesthetic agents, including propofol, profoundly decrease ongoing neuronal activity in isolated cortical networks (2). It has been shown that propofol induces growth cone collapse and neurite retraction in chick explant culture (98). In order to maintain the proper function of the nervous system, precise connections that wire individual neurons together into an orderly network, have to be established. During the development of the nervous system, selective retraction of newly formed axons occurs extensively to eliminate inappropriate axonal projections (94). Hibernating mammals experience a temperature-dependent retraction of the neural microstructure throughout the brain, and recovery of the

microstructure to fixed parameters is rapid and complete on return to eutheria. Retraction is also an early axonal response to injury (96, 99).

## **The brain – the global effects of anaesthetics**

General anaesthetics act on different neuroanatomical targets in the nervous system. They cause amnesia and unconsciousness by acting at brain level, and immobility through action at spinal level (100, 101).

General anaesthetics strongly reduce neuronal activity in an isolated cortical network (2) and modulate synaptic transmission, and decrease neurosecretion (10). They strengthen synaptic inhibition, or reduce synaptic excitation, in several regions of the brain (102, 103). Positron-emission tomography (PET) and electroencephalography (EEG) studies have shown that most anaesthetics significantly decrease global cerebral blood flow (104, 105) and glucose metabolism (106). This study shows that general anaesthetic effects in the brain are mediated by different neuroanatomical structures. Both cortical (107) as well as subcortical (thalamus) (108, 109) structures are target sites for anaesthesia-induced loss of consciousness (Figure 1). For example, propofol decreases regional activity in the medial thalamus, cuneus, and posterior cingulate and orbitofrontal cortices in humans (104). The amygdala, the part of the brain which plays a role in memory, and emotional and autonomic functions, is also important for an anaesthetic-induced amnesia (110). However ketamine increases global metabolism in the brain (111) and produces unconsciousness without depressing the cortex or thalamus (112, 113). This shows that depression of neuronal activity in the thalamocortical system is not necessarily the only mechanism for general anaesthetic evoked unconsciousness. General anaesthetics preferentially affect synaptic transmission rather than axonal conduction (114) by agent-specific postsynaptic and/or presynaptic mechanisms (115).

Studies show that general anaesthetics may disrupt cortical integration and cause a disruption of corticothalamic connectivity (116, 117). Functional magnetic resonance imaging (fMRI) suggests that propofol-induced unconsciousness could be linked to a breakdown of the brain's temporal architecture and a disruption of the connectivity of the brain network which is

important for conscious perception (5). EEG and fMRI analysis suggest a significant reduction in the number of network connections during propofol anaesthesia (118) and sedation (119), as well as during sevoflurane anaesthesia (120). Those effects could be linked to the observed anaesthetic effect on neural plasticity.

## ***Sleep and anaesthesia***

Natural sleep and general anaesthesia share several common features including deactivation of the subcortical structures, a similar electroencephalographic pattern, and a reduction of sensory input and motor output. Similar patterns of neuronal activity are observed during non-rapid eye movement (NREM) sleep (121) and general anaesthesia e.g. deactivation of the thalamus (105), while their neuronal endpoints differ. Brainstem sleep-promoting neurons are also associated with the mechanism of anaesthesia-induced unconsciousness (122). Furthermore, a recovery process similar to that which occurs during sleep takes place during propofol anaesthesia (123). Kelz and colleagues demonstrated that hysteresis or neural inertia is attributed to the tendency of the brain to resist state (or phase) transitions between conscious and anaesthetic-induced unconscious states (124).

It appears that anaesthesia induction and emergence utilise different neural paths. Recent data demonstrates that the induction and emergence paths through which anaesthetic-induced unconsciousness arise and dissipate are not identical (124). Hypothalamic neuropeptide orexin-A, involved in the control of sleep and wakefulness, is also linked to emergence from general anaesthesia (7, 125). Hence the deactivation of thalamocortical pathways is important for the induction of anaesthesia, and hypothalamic pathways which control sleep-wakefulness are involved in emergence from anaesthesia. As stated in the paragraph above, although not identical, sleep and general anaesthesia share common features. Furthermore, a recovery process similar to that which occurs during sleep takes place during propofol anaesthesia (122). Despite the similarity, sleep and anaesthesia are distinct in their mechanisms and neural targets. Animal findings suggest that commonly used general anaesthetics are damaging to developing neurons and cause significant neuronal deletion in vulnerable brain regions (126). Several studies suggest an association between early exposure to anaesthesia and long-term cognitive impairment (127-129). Anaesthesia causes a breakdown of the brain's temporal architecture and a disruption of the connectivity of the brain

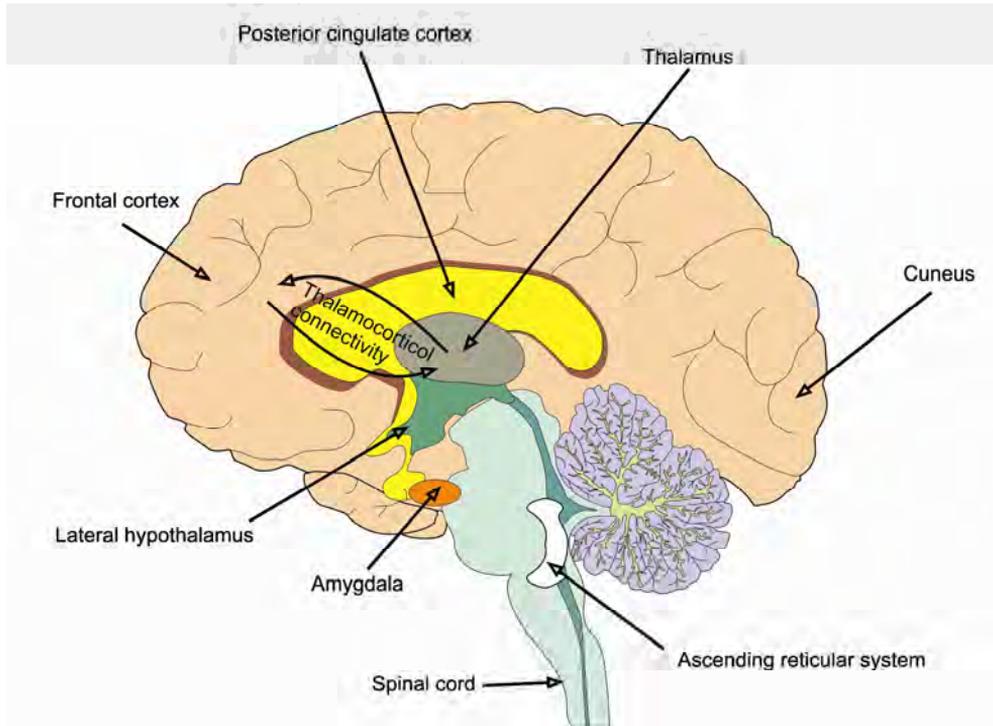
network which is important for conscious perception. This effect could be linked to observed anaesthetic effects on neural plasticity.

## *The orexinergic system*

The orexins/hypocretins are novel peptide neurotransmitters which were first characterised in 1998 (130, 131). The cell bodies of the neurons releasing them are localised exclusively in the posterior and lateral hypothalamus although they send out projections which innervate the whole central nervous system. A reduction of orexin/hypocretins levels occurs in most human cases of the sleep disorder narcolepsy (132). The hypothalamus is now recognised as a key centre for sleep regulation, with hypothalamic neurotransmitter systems providing the framework for therapeutic advances (4).

Hypocretin-orexin neurons are in an intrinsic state of membrane depolarisation which promotes their spontaneous activity. It is proposed that wakefulness and associated energy expenditure depend on this property, and that it allows the hypocretin-orexin neurons to maintain a tonic excitatory influence on the central arousal and peripheral sympathetic systems (5). Hypothalamic orexin/hypocretins express a robust innervation of the spinal cord (6). The orexinergic system has a potential role in the modulation of nociceptive transmission (7).

Orexin A, also known as hypocretin-1, is a novel neuropeptide which is highly important in the regulation of sleep/wakefulness regulation, feeding behaviour and energy homeostasis (130). Orexin neurons which produce orexin A and orexin B are exclusively localised in the lateral hypothalamus (LHA) although they send out projections which innervate the entire central nervous system (CNS) (133). These peptides bind to two G(q)-coupled receptors, termed  $OX_1R$  and  $OX_2R$  (134). A reduction of orexin levels occurs in most human cases of the sleep disorder narcolepsy (132). It has been shown that intra-techal OA induced EEG arousal without sympathetic cardiovascular activation in the isoflurane-anaesthetised rat, and that orexin A might influence the depth of anaesthesia (135).



**Figure 1. Brain structures and neuronal pathways involved in anaesthetic-induced amnesia and loss of consciousness.** Disruption of corticothalamic connectivity (116, 117) is important for the induction of anaesthesia and lateral hypothalamic pathways which control the sleep-wakefulness involved in emergence from anaesthesia (7, 125). (Illustration courtesy of Madu Batuwangala)

## **The spinal cord – anaesthetic effects**

Several studies in experimental animals have shown that general anaesthetics produce immobility by decreasing the transmission of noxious information ascending from the spinal cord to the brain (136-138). Eger and colleagues introduced the MAC (minimally alveolar concentration) concept in 1965 as quantification of the potency of a volatile anaesthetic. They defined MAC as the partial pressure of an inhalational anaesthetic in the lungs, at which 50% of the population of nonrelaxed patients remained immobile during surgically-induced stimulus (139, 140). Propofol and volatile anaesthetics induce immobility by enhancing GABA<sub>A</sub>R-mediated synaptic inhibition (141, 142). NMDARs and AMPARs (143), glycine receptors (54) and TREK-1 potassium channels(144) mediate the volatile anaesthetic reduction of motor output. The  $\alpha$ -2 adrenoreceptor agonists inhibit synaptic transmission in the rat spinal cord dorsal horn (145) and reduce the MAC of volatile anaesthetics (146). Descending signals from the brain modify the immobilising actions of general anaesthetics in the spinal cord (147) whereas ascending signals from the spinal cord contribute to the hypnotic actions of general anaesthetics in the brain (148). It suggests an interaction between general anaesthetic pathways in the spinal cord and the brain. Propofol and isoflurane act on the ventral horn neurons (149) while sevoflurane acts on dorsal horn neurons (150) to produce immobility. This shows that few targets within the spinal cord are susceptible to immobilising action by different anaesthetic agents. These results show that immobility is not a consequence of limited input into the spinal cord nor is it dependent on control impulses from the brain. Therefore a major issue is to understand is how the nociceptive signalling persists through the dorsal horn during propofol, halothane and isoflurane anaesthesia (151), and allows cerebral control over motor movement (152) while motor response is completely attenuated. There are only a few molecular targets that could explain MAC and this gives rise to the hypothesis that different general anaesthetics produce a common effect (immobility) in the spinal cord by non-specific actions (153).

# AIMS

The overall aim of this thesis was to investigate the effects of the intravenous anaesthetics propofol, thiopental and ketamine on cytoskeletal function in cultured cortical rat neurons.

The specific aims of the enclosed studies were to:

- Study the effect of intravenous anaesthetics on neuronal morphology and the cytoskeleton structure (Papers I and III).
- Investigate the effect of propofol on vesicle transport in neuronal neurites (Paper II).
- Study parts of the intracellular signalling events provoked by propofol (Papers I and IV).
- Study the role of orexin A on the intravenous anaesthetics effects of cell morphology, cytoskeleton structure, function and intracellular signalling pathways (Papers III and IV).



# MATERIAL AND METHODS

## *Cell culture (Papers I-IV)*

These studies were approved by the Ethics Committee for Animal Research at Linköping University. In the present study, primary cultures of rat neurons were obtained as described by Hansson and Rönnbäck (154). In short, newborn Sprague-Dawley rats were decapitated and the cortices were dissected free. The cortex tissue was sieved through a nylon mesh (80  $\mu\text{m}$ ) into Dulbecco's modified Eagle's medium and supplemented with 20% foetal calf serum (FCS), glucose (30 mM), insulin (5  $\mu\text{g ml}^{-1}$ ), glutamine (2 mM) and penicillin/streptomycin (10 000 U/ml). The cells were cultured on poly-L-lysine-coated 25 mm  $\phi$  sterile coverslips or on 25  $\text{cm}^2$  culture flasks at 37°C in a humidified atmosphere of 95% air/5%  $\text{CO}_2$ . After 24 h, the FCS concentration of the medium was changed to 10% and maintained at that level for seven days. 10  $\mu\text{M}$  cytosine-1- $\beta$ -d arabinofuranoside was added to new 10% media on the sixth day for 24 h to suppress growth of the glial cells. Thereafter the cultures received a new medium containing 5% FCS every second day. The cells were used between days 10 and 35.

## *Microscopy*

In Paper I, the coverslips with cell cultures were mounted in a RC-21B closed bath imaging chamber and the cells were observed by light microscopy using a Zeiss Axiovert 200 inverted microscope equipped with an alpha Plan-Fluar 100x/1.45 numerical aperture (NA) oil immersion objective (Carl Zeiss, Microimaging GmbH, Göttingen, Germany). In Papers II-IV, we used a Zeiss Axiovert 135M differential interference contrast microscope equipped with a 40x 1.3 NA objective and a ProgRes C10plus CCD camera (Jenoptik, Jena, Germany). The temperature in the cell culture was maintained at 37°C using an objective heater (Pecon GmbH, Erbach, Germany). A timelapse series was initiated wherein cell images were obtained at 1 min intervals between 0 and 15 min.

Each cell culture was rinsed twice in a  $\text{Ca}^{2+}$ -containing medium (CCM) (155) before use. In Paper I, cells were exposed to 0.02, 0.2, 2 and 20  $\mu\text{M}$  propofol or intralipid (20 mg  $\text{ml}^{-1}$ ). The GABA<sub>A</sub>R inhibitor bicuculline (30  $\mu\text{M}$ ) and F-actin stabilising agent, phalloidin (5  $\mu\text{M}$ ), were added 10 min prior to the propofol. A myosin II inhibitor, blebbistatin, was added 3 min prior to exposure with propofol. To observe reversibility, the propofol was washed out from the culture media after a 2 min exposure and the cells were observed for 20 min. In Paper III, the experiments started with CCM (5 min) to establish a steady state. Thereafter either propofol (20  $\mu\text{M}$ ) or thiopental (0.1-1 mM) was added 10 s before time zero for 5 or 10 min respectively. This was followed by the addition of the solvent for OA (acetic acid [AE, 0.001%]) or OA (10 nM), and the neurite was followed for a further 10 min. In inhibition experiments, cells were CCM-treated with OA (10 nM) 1 min before propofol (20  $\mu\text{M}$ ) for 10 min. OA (10 nM) only,  $\text{Na}_2\text{CO}_3$  (150  $\mu\text{M}$ ) or barbiturate acid (BA) (1 mM) were used as controls.

In Paper IV, the neurite length was measured after CCM for 5 min to establish the steady state, followed by propofol (2  $\mu\text{M}$ ) administration. To inhibit ROK, the cells were incubated with 80  $\mu\text{M}$  1-5 (-isoquinolinesulfonyl) homopiperazine (HA-1077) (156) in cell culturing media for 40 min prior to incubation with CCM/HA-1077 (5 min) to establish the steady state. Thereafter, propofol (2  $\mu\text{M}$ ) was added and the neurite was followed for a further 10 min. To study the effects of propofol, OA (10 nM) was added 1 min before propofol (2  $\mu\text{M}$ ) and used as the control. A phospholipase D (PLD) inhibitor, 5-fluoro-2-indolyl des-chlorohalopmide (FIPI, 30 nM)(157) was added to the cell culturing media for 60 min prior to steady state measurement in CCM/FIPI (5 min). Thereafter, OA (10 nM) was added 1 min before propofol (2  $\mu\text{M}$ ) and the neurite was followed for a further 15 min. To block kinase C (PKC), cells were incubated with CCM (5 min) followed by staurosporine (158) (3 nM) for 5 min. This was followed by the addition of OA (10 nM) or AE for 1 min before propofol (2  $\mu\text{M}$ , 10 min). The PKC activator, 100 nM phorbol 12-myristate 13-acetate (PMA) was added to the cells 3 min prior to propofol addition (2  $\mu\text{M}$ ) for a further 15 min. For control experiments, treatment with FIPI (100 nM) and staurosporine (3 nM) continued for a total of 15 min.

## *Video microscopy*

In Papers II-III, differential interference contrast images of cells were captured and transformed into video images using a ProgRes C10plus charge-coupled device (CCD) camera (Jenoptik, Jena, Germany) and a video image processor

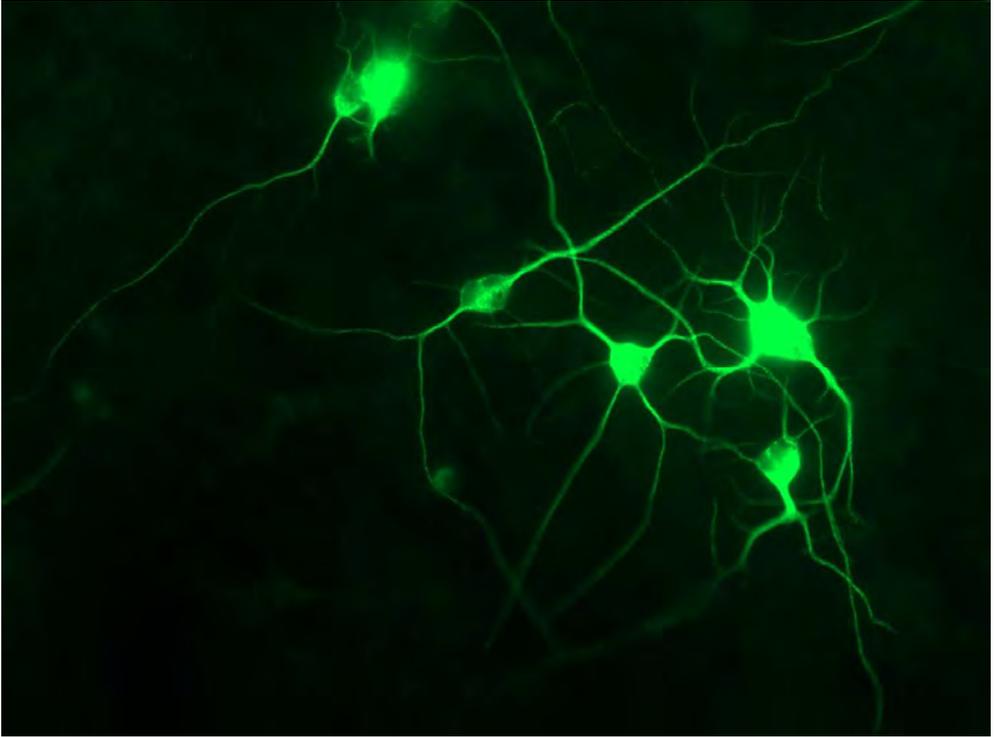
(Hamamatsu Photonics, Tokyo, Japan). Video images were displayed on a video monitor and stored on tape using a video recorder. We analysed only superficial cells which were laid on a glial cell layer. In Paper IV, we analysed cells directly from the timelapse series without using a video recording. The final processing of all images was done using Adobe PhotoShop 6.0 (Adobe Systems, San Jose, CA) and ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, MD, USA, <http://rsb.info.nih.gov/ij/>, 1997-2005).

## *Vesicle tracking (Paper II)*

In Paper II, the timelapse series was captured from video images and the interval between measurements were 1 min. The velocity of the individual vesicles was determined using the Manual Tracking plug-in for ImageJ software. Vesicle movement was analysed by the tracking of clearly visible vesicles (not clustered) in cultured cortical neurone neurites before and during pharmacological manipulation. The distance between the two consecutive frames, or between the initial and the final tracking points, was used to determine speed and average speed. Anterograde vesicle movement in neurites was defined as vesicles moving towards the cell periphery, and retrograde movement as vesicles moving towards the cell body. Bidirectional vesicle movement was defined as the equal number of movements in both directions. Cells were exposed to propofol, CCM or the lipid vehicle. The dynamics of the vesicle movement were observed each minute, starting with 5 min in CCM to determine normal vesicle movement. Thereafter either propofol (2  $\mu$ M) or CCM was added, and the vesicles were followed for 10 min. Propofol was added 10 s before time zero when images were captured. The vesicles were studied after the first 5 min in CCM, followed by GABA<sub>A</sub>R inhibitor, gabazine (7  $\mu$ M) for 5 min prior to a 10 min exposure of propofol (2  $\mu$ M). The propofol group was treated as described above and the control cells were exposed to gabazine (7  $\mu$ M) for a total of 15 min. To observe reversibility, the medium containing propofol was removed after a 2 min exposure and replenished with CCM, and the cells were observed for 18 min.

## ***Cytoskeletal structure identification (Paper III)***

Cells growing on coverslips were rinsed twice in CCM, pH 7.4 at 37°C, followed by propofol (16.8 µM), the lipid vehicle, OA, or the AE, 0.001% as described below at 37°C. In dose response reversal experiments, OA ( $10^{-12}$ - $10^{-7}$  M) was added after 1 min of propofol exposure for another 20 min in the presence of propofol or, for inhibition experiments, pre-incubated 1 min before propofol exposure for 20 min. To test if propofol causes any rebound effect, OA was given after 1 min of propofol exposure, and experiments terminated after 30 s - 35 min. To evaluate the effects of a brief OA exposure, OA ( $10^{-12}$ - $10^{-7}$  M) was added after 15 min of propofol treatment for the last 5 min. Effects of OA only were tested at  $10^{-12}$ - $10^{-7}$  M. Solvents were tested for the longest appropriate time at the highest concentration. In barbiturate experiments, thiopental (1 mM, corresponding to an induction dose (159, 160)), the non-anaesthetic barbituric acid (BA, 1 mM), or the solvent for thiopental  $\text{Na}_2\text{CO}_3$  (150 µM) were added for 2-30 min. Ketamine (30 µM) was applied for 20 min. For reversal experiments, OA (10 nM) was applied after 1 min of thiopental exposure, as described above. Stimulation was stopped by adding 4% paraformaldehyde (PFA)/phosphate buffered saline (PBS) for 20 min at 37°C. Thereafter cells were permeabilised (saponin 1%/PBS, 5 min at room temperature (rt)). Actin was labelled with Alexa-546-conjugated phalloidine (1:300 in 1% saponin/PBS) for 30 min in a dark humidified chamber (rt), rinsed 2x5 min in PBS and 1x5 min in distilled water. Vimentin was labelled using goat anti-vimentin anti-body (1:500) and Alexa-488-conjugated secondary antibody (1:400). Neurons were identified with mouse anti-tubulin-β3 antibody (1:500) followed by Alexa-546-conjugated antibody (1:400) (Figure 2). All antibodies were diluted in 1% saponin/PBS (with the addition of 1% bovine serum albumin (BSA) for primary antibodies) and incubated for 45 min as described above. Coverslips were mounted using a fluorescent mounting medium, mixed with DAPI ( $1.5 \mu\text{L}^{-1}$ ) onto glass slides. To ensure consistency in the results, coverslips were viewed (63x oil-fluorescence objective, NA 1.4 (Carl Zeiss, Germany)) in random order by the same person who was blinded for the various treatments. At least 100 cells were counted per coverslip and the percentage of cells containing actin rings was calculated. Vimentin and tubulin-β3 marked cells were manually evaluated.



**Figure 2.** Cultured rat cortical neurons stained with anti-tubulin  $\beta$  III antibodies and visualised with fluorescence microscopy.

### ***Analysis of cellular proteins and mass spectrometry (Paper III)***

Cells in 25 cm<sup>2</sup> culture flasks were washed twice in CCM then incubated with propofol (20  $\mu$ M) or thiopental (1 mM) for 5 min before the addition of OA (10 nM) for 10 min in the presence of the anaesthetics, with their respective solvent used as controls, at 37°C in a waterbath. After removal of the stimulation medium, an ice-cold lysis buffer with phosphatase inhibitors (250  $\mu$ l) was added. Subsequent procedures were carried out at 4°C or on ice (13). Cells were scraped off into the lysis buffer, homogenised and the cell lysate centrifuged (2x10 min, 200 g) to remove remaining intact cells and nuclei. Protein concentration was measured by spectrophotometry and samples were diluted with lysis buffer to equalise relative protein concentration in each experiment. 70  $\mu$ l of lysate was mixed with the sample buffer (161), followed

by heating for 15 min at 65°C and thereafter frozen until analysed. The remaining lysate was used for immuno-precipitation (IP) by anti-phosphotyrosine (PTyr) antibody (8 µl, 1 h) followed by protein A-Sepharose (200 µl, 20 mg·ml<sup>-1</sup>, 1h) at continuous rotation. IPs were recovered by centrifugation (9 000 g, 10 min), washed three times with lysis buffer, the pellet diluted with a 200 µl sample buffer, heated and frozen. 70 µl of the first IP-supernatant was mixed with the sample buffer, heated and frozen. Frozen samples were heated at 65°C for 10 min and then separated on homogenous 7.5% polyacrylamid gels in the presence of sodiumdodecylsphate (SDS) (13). Proteins in the gel were blotted with a polyvinylidifluoride (PVDF) membrane, blocked in 5% BSA/PBS, incubated with PTyr antibodies (1:500 in 1% PBS-BSA) or vimentin antibodies (1:2000) for (2h, rt or overnight, 4°C). The membrane was washed six times (PBS-Tween 0.05 %) and incubated with peroxidase-linked goat anti-mouse antibodies (1:5000-10000 in 0.05% PBS-Tween, 1 h, rt). After extensive washing in PBS-Tween, the membrane was incubated with enhanced chemiluminiscence (ECL) Western blotting detection reagents and exposed to ECL hyperfilm. In order to remove bound antibodies, some membranes were washed with 100 mM 2-mercaptoethanol, 2% SDS and 62.5 mM Tris-HCl (pH 6.7) for 30 min at 50°C, blocked and reprobred with a new antibody.

Whole cell lysates, IP or the IP-supernatants were separated as described above, stained with Coomassie blue G-250 for 20 min at room temperature, and destained with 5% AE/5% ethanol in water until the protein bands were clearly visible. Bands with changed phosphorylation were excised from the gel, and in-gel (162) digestion was performed with the sequencing grade modified trypsin. The peptides obtained were analysed on MALDI-MS and data cross-matched using the Mascot database.

## ***Data quantification and statistical analysis***

In Papers I-IV, measurement obtained from a single neurite is defined as (n=1) and neurons were obtained from at least three different rat litters in each experimental group. In Papers I and II-IV, the neurite retraction was calculated by subtracting the final length from the initial length. In Paper I, a neurite length at time zero was used as a reference. However propofol was added to the cultures, 10-15 s before time zero. Neurite length was measured manually using Adobe PhotoShop 6.0 (Adobe Systems, San Jose, CA) by an observer who was blinded.

Because even a short exposure to propofol has an effect on neurites, in Papers III-IV, a neurite length at time -1 min was used as a reference (100%). The neurite length was measured manually by an observer who had not done the experiments. In Paper I, the dose-response curves for neurite retraction were analysed by non-linear curve fit. The negative logarithm of the propofol concentration that produced half (pEC<sub>50</sub>) of the maximal neurite retraction was approximated by a non-linear regression analysis of the experimental data. EC<sub>50</sub> values were determined by fitting the data to a sigmoidal logistic equation using the programme Prism 4.0 software (GraphPad Software, San Diego, CA). The dose-response curves were fitted using the following equation:

$$Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\log \text{EC}_{50} - X) * \text{Hillslope}))}$$

Overall significant differences between conditions were determined through the analysis of variance for repeated measurements (ANOVA). Our decision to use ANOVA was due to multiple comparisons of time and treatment, and allowed us to avoid false significance. Post hoc comparisons were performed by the Bonferroni test for multiple comparisons. In Paper I, the values were expressed as the mean  $\pm$  standard deviation (SD) and, in Papers II-IV, as the mean  $\pm$  standard error of the mean (SEM). In Paper II, the values were expressed also as the mean  $\pm$  SEM since the measures were mean of means, so that the calculated SD is actually the same as SEM. In Paper III, linear regression analysis was performed to evaluate if a linear slope was seen between retraction and time. A  $p < 0.05$  was considered statistically significant in this thesis. All statistical analyses and graphing were done using Prism 4.0 software (GraphPad Software, San Diego).



## RESULTS AND DISCUSSION

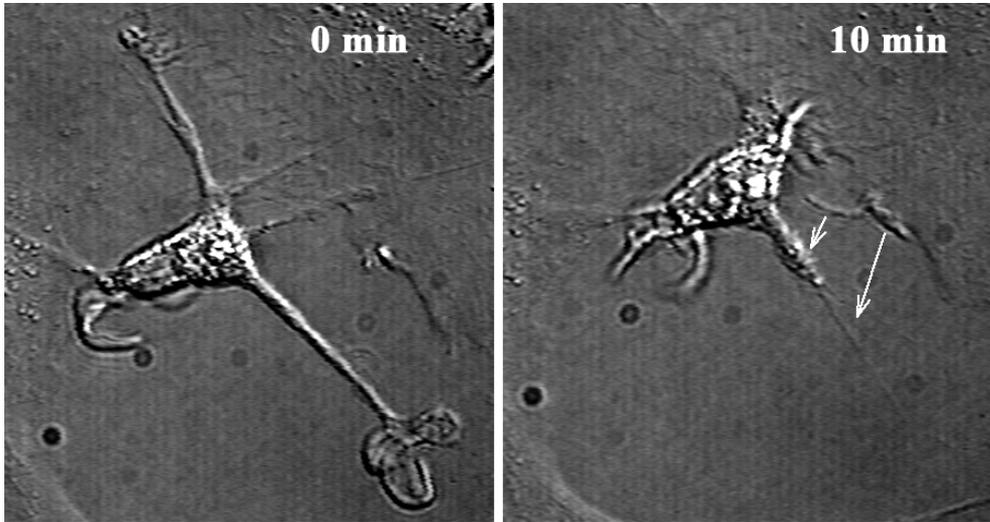
Neurite retraction is an important function of individual neurons and neural plexuses during normal and pathological conditions in developing and mature neurons. The neurite retraction is a highly regulated and active process mediated by cytoskeleton and motor proteins (85, 97).

Propofol causes a reorganisation of actin in cultured rat neurons and glial cells (14). However, the effect of propofol on neurites in cortical cell cultures is not known. OA is an endogenous peptide regulating wakefulness and it is known to interact with anaesthetics. Thus the studies included in the present thesis aim to elucidate the mechanisms and intracellular signalling of propofol-induced neurite retraction and investigate whether or not OA interferes with this retraction.

### *Propofol and thiopental but not ketamine induce neurite retraction and retrograde vesicle transport in cortical neuronal cultures*

Previous results from our laboratory showed propofol induces reorganisation of the neuronal cytoskeleton in cortical neuronal cultures (14). Furthermore, propofol induces growth cone collapse and neurite retraction in chick explant culture (98) and alterations in in vitro dendritic development of isolated developing GABA-positive interneurons (163). We chose to investigate whether or not propofol, thiopental or ketamine change neurite response dynamics in cultured cortical rat neurons. We found, in Papers I and III, that propofol and thiopental cause a neurite retraction in a cortical neuronal rat cultures (Figure 3). Our results in Paper II suggest that a propofol-induced neurite retraction initiates movement of the neurite cytoplasm towards the cell body and causes retrograde vesicle movement. Propofol-induced neurite retraction was dose- and time-dependent, reversible and GABA<sub>A</sub>R-dependent. No retrograde vesicle transport was seen after adding the GABA<sub>A</sub>R blocker, gabazine. If actin remodelling was inhibited or myosin ATPase was blocked, no retraction was seen after propofol treatment. The neurite left a thin trailing remnant and this was used to guide the neurite re-extension once propofol was removed from the cells. Neurite retraction has also been observed in

hibernating animals, indicating that such a reversible process is not only seen during pharmacological manipulation of the brain (95).



**Figure 3. The effects of propofol on neurite morphology.** *Timelapse differential interference contrast image of a rat cortical neurite. Dynamics of neurite retraction and morphological changes 10 min after addition of propofol (20  $\mu$ M). The long arrow shows a trailing remnant and the short arrow shows a retraction bulb.*

No effects on actin or neurite retraction were observed by ketamine, the non-anaesthetic barbiturate, barbituric acid, or solvents for the drugs, showing that this is an anaesthetic effect dependent on the GABA<sub>A</sub>R.

Intracellular transport is essential for neuronal function and communication between neurons. In addition to the postsynaptic effect on GABA<sub>A</sub>R(164), propofol also enhances pre-synaptic inhibition (165). In Paper II, we observed that 84% of all moving vesicles moved retrogradely when exposed to propofol versus control medium (10%), where most of vesicles moved anterogradely. The vesicles exposed to propofol continued to move retrogradely over 10 min with accelerated speed over the observed time course. Propofol treatment caused a significant increase in the average retrograde velocity of moving vesicles ( $0.025 \pm 0.004 \mu\text{m/s}$  in propofol-treated neurons versus control neurons  $0.011 \pm 0.002 \mu\text{m/s}$ ) (mean  $\pm$  SEM;  $p < 0.05$ ). Five percent of vesicles in the control medium showed bi-directional

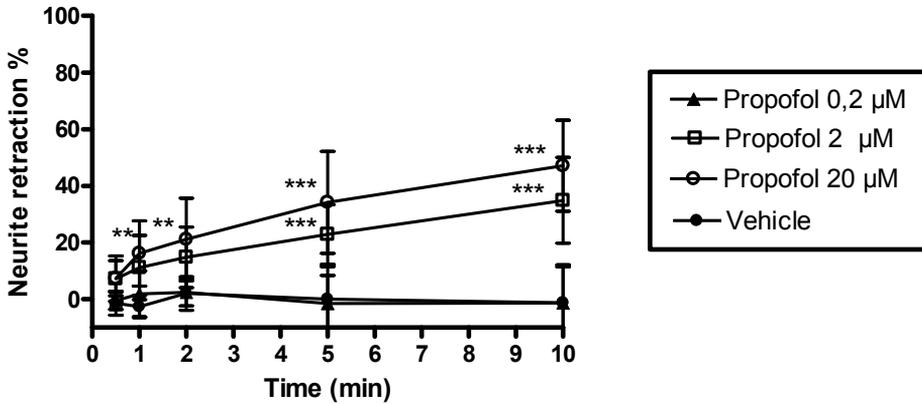
movement versus 11% of vesicles in propofol treated neurons. Our observation showed that 9 min after wash-out of propofol, vesicles decelerated, and 12 min after wash-out, began to move anterogradely. In an experiment, mitochondria were labelled with Mitotracker® and tracked over the time after exposure to propofol (Figure 6). Surprisingly, there was no statistical difference in mitochondrial movement after the addition of propofol compared to the control group (student's scientific project – N. Westerberg). These results were unexpected and may indicate that the retrograde vesicle transport induced by propofol is a selective and active process mediated by motor proteins.

An fMRI study has shown that propofol causes a breakdown of the brain's temporal architecture and a disruption of the connectivity of the brain network which is important for conscious perception (5). EEG and fMRI analyses suggest a significant reduction in the number of network connections during propofol anaesthesia (118) and sedation (119). Taken together, the disruption of the connectivity between neurons could be linked to an observed anaesthetic effect on neural plasticity. Propofol-induced neurite retraction may cause a reduction of the connectivity in the brain networks and inhibit neurotransmitter release.

Propofol's ability to induce neurite retraction was studied using two different light microscopy techniques. In Paper I, we measured neurite length by using timelapse light microscopy. In addition, propofol-induced neurite retraction and retrograde vesicle transport were observed in Papers II-IV by using differential interference contrast light microscopy.

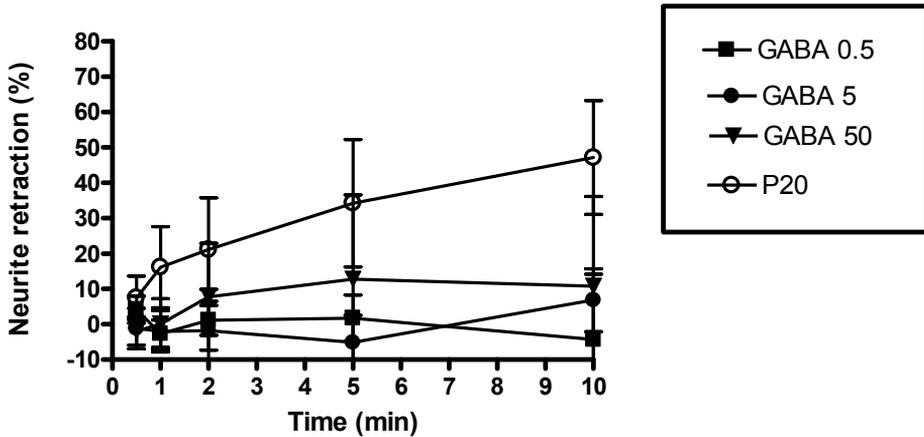
There are some differences regarding the propofol concentrations used in Papers I-IV. Firstly, in Paper I, we used a dose response study to determine which propofol concentration correlates best with the effect on neurite response dynamics. Our results showed that propofol concentrations of 2 and 20  $\mu\text{M}$  cause neurite retraction, and subclinical concentrations 0.2 and 0.02  $\mu\text{M}$  had no effect on neurite length (Figure 4). In inhibiting experiments in Papers I and III, we used propofol concentration 20  $\mu\text{M}$  and, in Papers II and IV, we used 2  $\mu\text{M}$ . The plasma concentration of propofol during the induction of anaesthesia in humans has been reported up to 30  $\mu\text{M}$  (166). In vivo, the majority of propofol is bound to serum proteins (97-98%) (167). The concentration of propofol in cerebrospinal fluid (CSF) in humans is 1.6% of the plasma concentration (168). Comparing clinically measured plasma concentrations of propofol with concentrations achieved at cellular level in vitro remains challenging. Our decision to use higher propofol concentrations

was because we wanted to show that OA is capable of reversing even a high concentration of propofol's effect.



**Figure 4. Effect of propofol on neurite length.** Cell cultures were exposed to 0.2, 2 and 20  $\mu$ M propofol or vehicle intralipid and assessed over 10 min. Values are expressed as the percentage neurite retraction over 14 experiments for each propofol concentration and six experiments for vehicle.

The endogenous substance GABA is a neurotransmitter which helps stimulate relaxation and sleep. It has been shown that propofol induces tyrosine phosphorylation of subtypes of the GABA<sub>A</sub>R  $\beta$  subunit different to GABA (15). In lines with those results, we have shown that GABA itself does not cause neurite retraction (Figure 5). The time-response results from Paper I show that there is already a significant retraction of neurites after one minute and the reverse process begins 10 min after the propofol wash-out. We believe that the timescale is not far from that of clinical anaesthesia. It takes between 45 seconds and two minutes for patients to fall asleep when given propofol. The time for awakening for patients is also within the timeframe we have seen. It must be taken into account that clearance via blood flow is more rapid than in vitro and an extensive lung extraction of propofol has been demonstrated in vivo (169).



**Figure 5. Effect of GABA on neurite length.** Cell cultures were exposed to 0.5, 5 and 50  $\mu\text{M}$  GABA or propofol 20  $\mu\text{M}$  and assessed over 10 min. Values are expressed as the percentage neurite retraction over six experiments for each GABA concentration and 14 experiments for propofol.

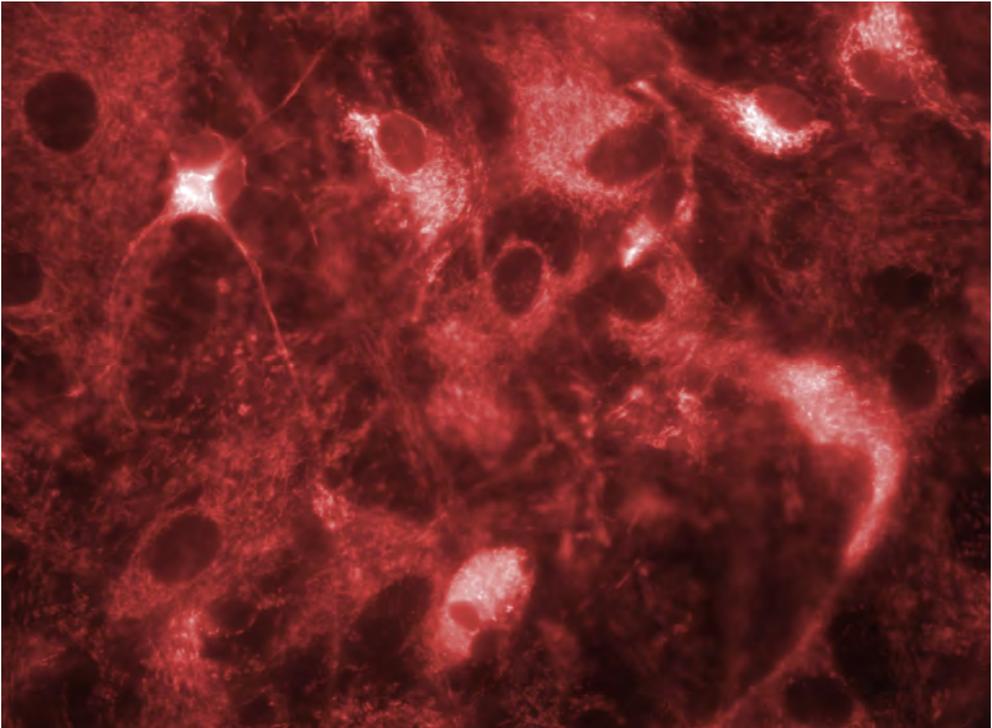
### *Propofol-induced neurite retraction and retrograde vesicle transport is GABA<sub>A</sub>R-mediated and dependent on actomyosin contractility*

Our group has previously shown that propofol induces tyrosine phosphorylation in the GABA<sub>A</sub>R  $\beta 2$  and  $\beta 3$ -subtypes (15). In Paper I, propofol-induced retraction of neurites was blocked by the GABA<sub>A</sub>R antagonist bicuculline and the importance of GABA<sub>A</sub>R is reinforced by the findings in Paper II that propofol-induced retrograde vesicle transport was blocked by GABA<sub>A</sub>R antagonist gabazine. Gabazine concentration of 7  $\mu\text{M}$  (170) could block propofol's effect on vesicle transport suggesting that this effect is mediated by synaptic GABA<sub>A</sub>R without affecting extrasynaptic GABA<sub>A</sub>R. This demonstrates the GABA<sub>A</sub>R role in propofol cell signalling.

Axon retraction is dependent on an interaction between myosin and actin. In axon, there is a balance between contractile forces generated by actomyosin, and contraregulatory forces generated by microtubule and dynein motor proteins (85). Previous results from our laboratory show that propofol mediates calcium-dependent, reversible actin reorganisation (14). In Paper I, we could inhibit propofol-induced neurite retraction by using phalloidin, the

F-actin stabilising agent, and the myosin II ATPase activity inhibitor, blebbistatin (171). This shows that propofol-induced neurite retraction is an active process dependent on myosin II and actin.

Taken together, our results reinforce the hypothesis that propofol-induced neurite retraction may cause a disconnection between neuronal cells and consequently an interruption of the vesicle transport to the synapses. This laboratory data might explain the observed reduction in the brain neuronal connectivity.

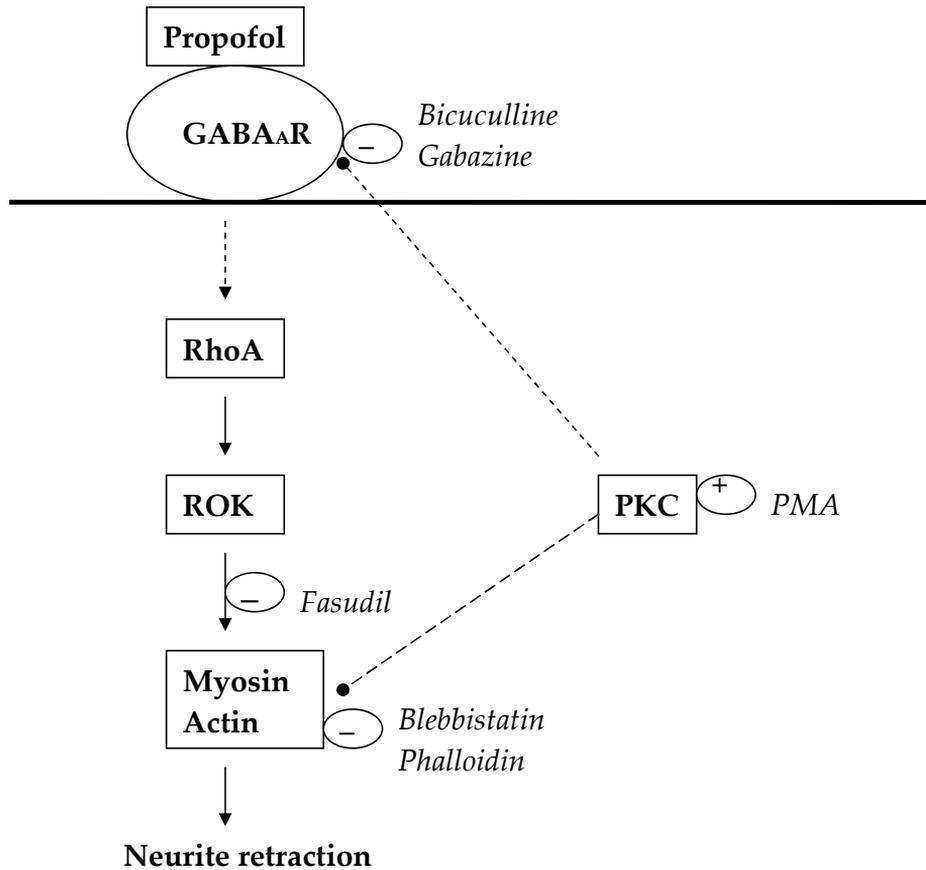


**Figure 6. Mitochondrial distribution in cortical rat cultures.** *Mitochondria are stained by using the MitoTracker® and shown as white spots in neurons on the surface, and glial cells and neurons on the bottom of the cortical cell.*

## ***Propofol-induced neurite retraction is mediated by the ROK-myosin intracellular signalling pathway***

In neuronal cells, neurite retraction is regulated by a GTPase RhoA-dependent mechanism, and suppression of RhoA and its downstream kinase ROK prevents the retraction of established axons *in vivo* (172) and *in vitro* (173). ROK activated by the RhoA increases phosphorylation of myosin regulatory light chains (MLC) by phosphorylating and inhibiting the myosin light chain (MLC) phosphatase. The motor protein myosin II is regulated by myosin light chain kinase (MLCK) that directly phosphorylates myosin regulatory light chains (94). An actin-myosin-dependent retraction has been observed for the lysophosphatidic acid (LPA) and thrombin (174) in actin rearrangements (175). In Papers I and II, we have shown that propofol-induced neurite retraction is dependent on signalling from GABA<sub>A</sub>R to actin and myosin II. In Paper IV, we have further investigated the intracellular signalling pathway involved in propofol-induced neurite retraction. A ROK inhibitor HA-1077, PKC activator PMA, blocked the neurite retraction induced by propofol. We have previously shown that Rho and ROK are important steps in propofol's signalling cascade as observed in the actin cytoskeleton (18).

PKC is a family of protein kinase enzymes that are involved in controlling the vital function of other proteins. In neurons, PKC is involved in the modulation of ion channels (176), the desensitisation of receptors (177) and the enhancement of neurotransmitter release (178). It has been shown that PKC $\epsilon$  reduces the sensitivity of GABA<sub>A</sub>R to intravenous anaesthetics and ethanol (179, 180). PKC $\epsilon$  decreases the amount of GABA<sub>A</sub>R at the cell surface and attenuates GABA<sub>A</sub>R currents (181). In cortical neurons, PKC inhibitors abolish phosphorylation of the GABA<sub>A</sub>R  $\beta$ 3 subunit and increase receptor activity, whereas activators of PKC enhance  $\beta$ 3 phosphorylation, leading to a decrease in channel activity (182). In accordance with this, our results show that PKC activation is important for propofol-induced neurite retraction in cortical neuronal cells. Taken together, we propose that it is the intracellular signalling pathway for propofol-induced neurite retraction: propofol binds to GABA<sub>A</sub>R and causes neurite retraction through the RhoA-ROK-myosin pathway. Furthermore, activation of PKC plays an inhibitive role during the neurite retraction caused by propofol (Figure 7).



**Figure7. Proposed pathway of propofol-induced neurite retraction.** Propofol binds to GABA<sub>A</sub>R and causes neurite retraction through the RhoA-ROK-myosin pathway. The PKC plays an inhibitive role in neurite retraction. The sites of action of the GABA<sub>A</sub>R inhibitors, bicuculline and gabazine, the RhoA inhibitor, HA-1077, the PKC activator PMA, the myosin II inhibitor, blebbistatin and the F-actin stabilising agent are shown. Dashed lines represent the pathways that remain to be verified.

## ***Orexin A reverses propofol and thiopental-induced neurite retraction and actin reorganisation***

Propofol and thiopental causes morphological changes of actin by forming a ring structure or DMR (14, 18). Membrane folding is initiated by activating the actin polymerisation (183) and actin rearranges underneath the membrane ruffle in a circular way, in which actin folds the membrane into tubes. The tubes are used for pulling down and sequestering receptors into the cell (184). The tubes are closed, forming vesicles that are either degraded or recirculated to the membrane. We believe that actin rings could be used by the cells for fast recruitment of the receptors upon stimulation by anaesthetics. In Papers III and IV, we investigate whether or not OA has a role in propofol and thiopental induced-actin reorganisation and neurite retraction. OA is a neuropeptide produced by neurons in the lateral hypothalamus. They send projections throughout the CNS to regulate the sleep-wake cycle as well as autonomic and neuroendocrine functions (130, 131). It has been shown that intracerebroventricular administration of OA reduces the anaesthetic effect of isoflurane, thiopental and ketamine in rats (135, 185, 186). Inhibition of orexinergic signalling does not affect induction in anaesthesia but delays emergence from volatile anaesthesia in rats (7). In Paper III, we have shown that OA reverses actin ring formation and neurite retraction induced by propofol or thiopental when applied after the anaesthetics, and inhibits these effects if given in advance of the anaesthetics. When added 5 min after propofol, OA dose-dependently reverses and, when added 1 min before propofol, OA respectively blocks the actin ring formation ( $IC_{50}$  of 0.96 nM and 0.19 nM). There is five-fold higher OA concentration needed to block, as opposed to reverse, the actin effects of propofol. This suggests the initiation of propofol cytoskeletal changes in vitro to be OA sensitive. Contrary to this data, it has been shown that the inhibition of orexinergic signalling does not affect induction but delays emergence from volatile anaesthesia in rats (7). No rebound effect of propofol is seen when OA is added after 1 min of propofol exposure, and the effect is sustained up to 35 min. These results suggest that OA reverses cellular morphology and cytoskeleton changes caused by propofol and thiopental.

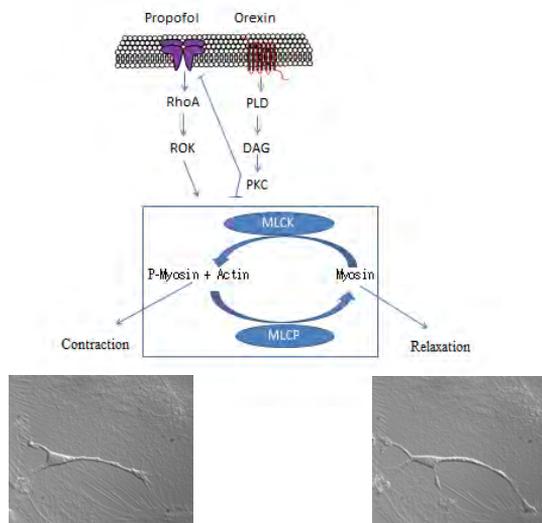
## ***Orexin A increases tyrosine phosphorylation of vimentin and reverses vimentine structure changes caused by propofol and thiopental***

Vimentin is an intermediate filament that links actin and tubulin, often in close relation to the cell membrane (187). In Paper III, OA increases the phosphorylation of vimentin (50 kDa) in both propofol and thiopental-treated cells. The vimentin band is doubled, with the major tyrosine phosphorylation taking place on the lower band. After OA treatment, the amount of vimentin is increased in the upper band and decreased in the lower band compared to anaesthetically stimulated cells. Such a shift in the ratio between the higher and lower molecular weight vimentin could be explained by the anaesthetics' effect on the remodelling of the cytoskeleton, and the OA effect could be to restabilise vimentin. Supporting this data, the morphology of vimentin is changed from granular to smooth distribution when anaesthetically dosed cells are further treated with OA, as seen with a fluorescent microscope. It has been shown that vimentin has the ability to modulate signal transduction in the cell (187). The activation of RhoA resulted in the ROK-dependent collapse of the filamentous vimentin network and translocation of ROK to the cell periphery in fibroblasts (188). This shows that vimentin can act as scaffolds by regulating spatial distribution of signalling molecules and influencing their ability to phosphorylate their targets. Thus OA could, via restabilising of vimentin, also change propofol and thiopental's intracellular signalling and contribute to a reversal of their cellular effects.

## ***Orexin A inhibits propofol-induced neurite retraction by a PLD/PKC-dependent mechanism in neurons***

In Paper IV, we continued to study the effects of OA on propofol-induced neurite retraction and we wanted to elucidate the intracellular signalling involved. We found that the PLD inhibitor FIFI and PKC inhibitor staurosporine prevent the inhibitory effect of OA on neurite retraction induced by propofol. OA interacts with two G(q)-coupled receptors (130, 131) which induce a rise of intracellular calcium (130) and activation of phospholipase (PLC) and PLD (189), leading to the production of phosphatidic acid (PA) which is further metabolised to lysophosphatidic acid (LPA) and diacylglycerol (DAG) (190). DAG activates PKC and facilitates translocation of

PKC from the cytosol to the plasma membrane (191). Based on these results, we propose an intracellular signalling pathway for OA inhibition of propofol-induced neurite retraction involving the PLD/PKC pathway. Suppression of the RhoA pathway occurs by the regulatory domain of PKC (192). The staurosporine's effect on OA inhibition of propofol-induced neurite retraction cannot be explained by the inhibitory effect of PKC on RhoA because staurosporine inhibits the PKC catalytic domain independently of the regulatory domain of the enzyme (158). It has been shown that activators of PKC enhance GABA<sub>A</sub>R  $\beta$ 3 subunit phosphorylation leading to a decrease in channel activity (182). Thus the possible explanation of PKC suppression of the propofol-induced neurite retraction is via GABA<sub>A</sub>R modulation of sensitivity to propofol. The other possibility is that PLD/PKC activation directly suppresses actomyosin-mediated contractility (Figure 8).



**Figure 8. Proposed pathway for OA inhibition of propofol-induced neurite retraction.** Propofol causes neurite retraction via binding to GABA<sub>A</sub>R and through the RhoA/ROK pathway. OA inhibits propofol-induced neurite retraction via binding to OXR and activation of PLD/PKC, resulting in a diminishment of GABA<sub>A</sub>R sensitivity to propofol and inactivating the RhoA pathway. PKC activation could also interfere directly with actomyosin-mediated contractility.



## LIMITATIONS

There are some limitations in the studies that should be mentioned. The findings in Papers I-IV were obtained from *in vitro* experiments which may not consequently replicate *in vivo* conditions. One has to be aware that it is difficult to extrapolate data obtained from laboratory-based studies to clinical settings. Our cultures consisted of mixed neuron-glia cells and we could analyse only superficial cells lying on a cell layer. The cells were chosen based on our judgment that their morphology resembled that of neurons and was therefore a subjective choice. Only neurites which lay within an optical microscopy field could be analysed. In Paper I, we used neuron-specific enolase (NSE) and estimated that our cultures comprised 75-80% neurons (154). However, by using more recently developed and specific neuronal markers, postnatal cell cultures have been shown to be dominated by glial cells and comprise 20-30% neurons (193). Thus it is possible that in our studies not all analysed cells were neurons. Our cultures comprise approximately 70% excitatory neurons and 30% inhibitory neurons (personal communication, M. Renberg). Therefore it is possible that, in our project, the majority of analysed cells were excitatory neurons. Furthermore, the cells have been analysed between days 10 and 35, and it has been shown that the postnatal neurons in cultures have a maximum number of synapses between days 22-25.



## CONCLUSIONS

The general conclusion from the present thesis is that propofol and thiopental causes neurite retraction and change the cytoskeletal organisation in cultured rat cortical neurons.

More specifically, the results show that:

- Propofol causes a dose- and time-dependent, reversible retraction of cultured cortical neuron neurites.
- The GABA<sub>A</sub>R blocker, bicuculline, the myosin II blocker, blebbistatin, and the F-actin stabilising agent, phalloidin, all inhibit the propofol-mediated neurite retraction.
- Images of retracted neurite are characterised by a retraction bulb and a thin trailing membrane remnant.
- Propofol causes a GABA<sub>A</sub> receptor-mediated switch from anterograde to retrograde transport and increases the average velocity of the moving vesicles in neurites.
- Propofol causes neurite retraction via binding to GABA<sub>A</sub>R and through the ROK-myosin intracellular signalling pathway.
- Orexin A reverses the cellular effects known to be mediated via the GABA<sub>A</sub>R of both propofol and thiopental, and this process involves the cytoskeletal proteins actin and vimentin.
- PLD-PKC activation is important for the orexin A inhibition of propofol-induced neurite retraction.



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