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JAAKKO PAASONEN

OPTIMIZATION AND IMPLEMENTATION OF PRECLINICAL PHARMACOLOGIC FMRI FOR DRUG RESEARCH AND DEVELOPMENT

Optimization and Implementation of Preclinical Pharmacologic fMRI for Drug Research and Development

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ABSTRACT

Functional magnetic resonance imaging (fMRI) is a modern biomedical imaging method, which allows a non-invasive assessment of brain function. The detection of brain activity is based on the coupling between neuronal activity, energy consumption, and blood flow; fMRI techniques sensitive to local changes in blood flow, volume, and oxygenation represent surrogate markers for neuronal activity. While fMRI studies can be conducted safely with human subjects, preclinical experiments in animals are invaluable in many situations, e.g. in early phase drug development. The aim of this thesis was to improve the preclinical fMRI methodology and exploit these improvements in preclinical drug research.

Anesthesia is a typical requirement in preclinical fMRI studies, as imaging does not tolerate movement of the subject. Anesthesia, however, suppresses brain functions and can directly interfere with the object being investigated. Therefore, the present work aimed to devise an optimal anesthesia protocol. When the experimental results were assessed in the light of a critical literature review, it was concluded that urethane seems to be the most suitable anesthetic for non-recovery pharmacologic fMRI studies, while isoflurane and medetomidine may represent the best options for follow-up and recovery protocols.

Another issue complicating the assessment of anesthetized subjects is the varying depth of anesthesia. In the present thesis, it was shown that spontaneous fluctuations in baseline fMRI signal can provide valuable information related to the responsiveness of the subject to external stimuli, most likely reflecting the depth of anesthesia. Such readily implementable tool for estimating the level of anesthesia is more convenient than the invasive measurements of electrophysiological neuronal activity.

By exploiting the optimized anesthesia protocols, pharmacologic fMRI was applied in a multimodal study investigating the effects of phencyclidine on rat brain. The present work revealed that phencyclidine alters brain networking and dopamine levels dose-dependently, associated with different schizophrenia-like symptom classes. These findings can provide a sound foundation for devising more specific models for schizophrenia in drug development.

Taken together, the present thesis provides a basis for improved fMRI methodology, and encourage the exploitation of pharmacologic fMRI in brain and drug research.

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TIIVISTELMÄ

Toiminnallinen magneettikuvaus (TMK) on moderni biolääketieteen kuvantamismenetelmä, joka mahdollistaa kehoon kajoamattoman aivojen toiminnan tutkimuksen. Aivoaktivaation havaitseminen perustuu hermosolujen toiminnan ja verenvirtauksen kytkeytymiseen toisiinsa; TMK:ssa mittaustekniikka, joka on herkkä havaitsemaan paikallisia muutoksia verisuonistossa, kuvaa siis epäsuorasti hermoston aktivoitumista. Vaikka TMK voidaan suorittaa turvallisesti ihmisillä, ovat eläinkokeet usein korvaamattomia, varsinkin lääkekehityksen aikaisissa vaiheissa. Tämän työn tarkoituksena oli kehittää prekliinisen TMK:n menetelmiä, ja hyödyntää niitä aivosairauksiin liittyvässä lääketutkimuksessa.

Prekliinisissä TMK:ssa eläin usein nukutetaan. Nukutusaineet kuitenkin lamaavat aivotoimintoja, ja voivat vaikuttaa TMK:n tuloksiin. Tässä tutkimuksessa vertailtiin seitsemää nukutusainemenetelmää, ja osoitettiin uretaanin olevan hyvä nukutusaine niissä lääkeaineisiin liittyvissä TMK:n tutkimuksissa, joissa eläimen herättämistä ei vaadita. Mikäli toipuminen on toivottavaa, isofluraani ja medetomidiini ovat hyviä vaihtoehtoja.

Toinen nukutusaineiden aiheuttama ongelma on nukutuksen syvyyden vaihtelu, joka voi vaikuttaa aivoaktivaation luonteeseen häiritsevästi. Tässä työssä osoitettiin, että TMK:lla mitattava aivotoiminnan perustason signaali voi tarjota tietoa aivojen kyvystä reagoida ulkoiseen ärsykkeeseen, heijastaen todennäköisesti nukutuksen syvyyttä. Kyseinen menetelmä on huomattavasti helpompi toteuttaa nukutuksen syvyyden arvioimiseksi kuin esim. kehoon kajoava aivojen sähköisen toiminnan mittaus.

Hyödyntämällä saavutettuja tuloksia, TMK sisällytettiin laajaan tutkimukseen, jossa tutkittiin fensyklidiinin vaikutuksia aivojen toimintaan. Työssä osoitettiin, että fensyklidiini muuttaa aivojen toimintaa, verkostoitumista, ja dopamiini-tasoja annosriippuvaisesti, ja muutokset ovat yhteydessä fensyklidiinin aiheuttamiin erilaisiin skitsofrenian kaltaisiin oireisiin. Näihin tuloksiin perustuen pystytään kehittämään tarkempia skitsofrenian oiremalleja uusien skitsofrenialääkkeiden kehitystä varten.

Tämän väitöskirjan tulokset tarjoavat lukuisia parannuksia prekliinisen TMK:n menetelmiin, ja korostavat TMK:n soveltuvuutta ja tärkeyttä aivotutkimuksessa ja lääkekehityksessä.

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Contents

Abbreviations

2PK	Two-pore-domain K^+ ion channel
AC	α-Chloralose
ACh	Acetylcholine
AMPA	α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ASL	Arterial spin labeling
ATP	Adenosine triphosphate
BBB	Blood-brain-barrier
BOLD	Blood oxygenation level dependent
CBF	Cerebral blood flow
CBV	Cerebral blood volume
CNS	Central nervous system
CT	X-ray computed tomography
DA	Dopamine
dHb	Deoxygenated hemoglobin
DMN	Default mode network
EEG	Electroencephalography
FC	Functional connectivity
fMRI	Functional magnetic resonance imaging
GABA	Gamma-amino butyric acid
GLM	General linear model
Hb	Oxygenated hemoglobin
ICA	Independent component analysis
ISO	Isoflurane
LC	Locus coeruleus
LFP	Local field potential
MABP	Mean arterial blood pressure
mPFC	Medial prefrontal cortex
MRI	Magnetic resonance imaging

MT	Magnetization transfer
nAChR	Nicotinic acetylcholine receptor
NE	Norepinephrine
NIfTI	Neuroimaging Informatics Technology Initiative
NMDA	N-methyl-D-aspartate
NMR	Nuclear magnetic resonance
PCP	Phencyclidine
PET	Positron emission tomography
phMRI	Pharmacologic functional magnetic resonance imaging
RF	Radiofrequency
ROI	Region of interest
rsfMRI	Resting-state functional magnetic resonance imaging
SCZ	Schizophrenia
SPM8	Statistical Parametric Mapping 8, software
VASO	Vascular-Space-Occupancy

1 Introduction

The brain has been one of the most fascinating and most widely investigated topics due to its complex and enigmatic nature (Bear et al. 2001). Over the centuries, or even millennia, the curiosity of humankind has driven science forward as investigators seek answer to questions such as how do we see, hear, feel, move, remember, or forget; researchers from different disciplines have dedicated their lives to understanding the nervous system. Despite the long history underpinning brain research and the major contributions of different specialists, the interdisciplinary approach, neuroscience, is a relatively young, yet revolutionary branch of science (e.g., The Society for Neuroscience was only founded in 1969). It was eventually understood that unraveling the function of brain would require cross-disciplinary cooperation, bringing together scientists from different backgrounds to form one of the fastest growing scientific disciplines.

In addition to the general biological interest, an understanding of brain function is particularly crucial in the health sciences. As advances in healthcare have lengthened human lifespan all around the world, the burden of neurological disorders has simultaneously increased, and these have even been claimed to form "one of the greatest threats to public health" (World Health Organization 2006). A longer lifetime allows time for the appearance of many slowly developing central nervous system (CNS) diseases, several of which are severely debilitating and ultimately lethal. Therefore, an understanding of how the brain functions is crucial if one wishes to unravel the pathophysiology behind neurodegenerative diseases. This knowledge provides a foundation for 1) the prevention of disease development and progression, 2) efficient diagnostics, and 3) development of effective treatments.

Our knowledge related to brain function has expanded remarkably during the last decades. The mechanisms of several basic neural functions, such as cellular communication via either electrical activity or neurochemical transmission, are relatively well understood. However, despite the huge contribution and input of the neuroscience community, our current understanding of brain function is far from being complete. One of the factors definitely hindering clarification is the lack of efficient tools for studying the living brain. Although providing relatively detailed information, the value of many classical *in vitro* and *ex vivo* research techniques is limited since complex brain functions are sub-sectioned into smaller pieces, without gaining a true overall view. As several brain diseases have been recognized to include complicated and widespread changes, the importance of macroscale investigations cannot be underestimated. On the other hand, it is a formidable challenge to conduct an *in vivo* study of the brain as a whole, including deep brain regions without disturbing its normal structure and function; macrolevel studies of electrical activity, neuropharmacology, or brain networking *in vivo* are far from straightforward or simple tasks. Therefore, improved methods are constantly being sought in biomedical research to accelerate progress in understanding brain function.

One of the most important technical milestones in medical diagnostics and brain research was the introduction of X-ray computed tomography (CT) in the 1970s; this represented a revolution in biomedical imaging (Raichle and Mintun 2006). Importantly, the introduction of CT encouraged the developmental work of other techniques, and CT was quickly followed by parallel modalities such as magnetic resonance imaging (MRI) and positron emission tomography (PET). For instance, the basic principles of nuclear magnetic resonance (NMR) physics were already established in 1940s, but the ability to obtain a cross-sectional MRI image from the NMR signal was not devised until 30 years later in the 1970s (Raichle 2009). MRI attracted particularly high interest due to its outstanding soft tissue contrast and lack of ionizing radiation. Not surprisingly, the developers of NMR physics and researchers behind MRI image formation concept were later recognized by awards of Nobel prizes.

In addition to superb image quality, contrast, and safety in structural imaging, MRI has proved to be much more than an improved type of microscope for studying brain anatomy. It was speculated already in the late 1970s that MRI would be capable of providing insights into many diverse topics i.e. tissue chemistry, perfusion, and metabolism (Raichle 2009). The complex nature of signal formation in MRI is distinct from other imaging modalities, and different signal preparation steps have enabled further developmental work to acquire even more advanced imaging sequences. Indeed, the MRI repertoire has advanced so that today it displays a wide range of valuable applications, e.g., blood flow and volume measurements, functional imaging of brain activity, mapping of neural tracts, and spatially localized spectroscopy for metabolite investigations. In fact, versatility can be considered one of the greatest advantages of MRI, and is likely one of the main reasons why MRI has swiftly become a standard and widely-used method in clinical and preclinical diagnostics and brain research.

Functional MRI (fMRI) is an MRI approach, which has had a remarkable impact on unraveling brain functions. Interest in functional brain imaging, or the study of certain aspects of brain function or activity, was already emerging in the 1980s with the novel possibilities offered by PET (Raichle 2009). PET, however, required the use of radioligands and exhibited poor spatiotemporal resolution, which stimulated the development of alternative techniques. MRI enthusiasts were working intensively to replicate a similar experimental setup as described for PET with their more versatile technique. Finally, at the beginning of 1990s, several groups independently published successful functional imaging experiments, which had been conducted with MRI.

The best known approach is the blood oxygenation level dependent (BOLD) contrast (Ogawa et al. 1990, Ogawa et al. 1992, Ogawa et al. 1993), which exploits the different magnetic properties of oxyhemoglobin and deoxyhemoglobin as a way of detecting regional neural activity to some external sensory stimulus (e.g., auditory or visual) with good spatiotemporal resolution. Only a few years later, the spontaneous fluctuations in the baseline BOLD signal were postulated to originate from intrinsic brain activity (Biswal et al. 1995), which stimulated even more the scientific interest in fMRI. Since then, the fully translational BOLD fMRI approach has been utilized in tens, perhaps even hundreds, of thousands preclinical and clinical *in vivo* MRI investigations.

Despite the exponentially increasing number of fMRI implementations, several methodological issues still remain unexplored. As fMRI is an indirect technique aiming to measure the activity of nervous system, it is particularly important to characterize the cascade and coupling between neural activity and fMRI signal. For example, pharmacologic fMRI (phMRI) is a promising fMRI subtype, where the brain activation profile of a neuroactive drug is investigated during drug development and for research purposes. However, the contribution of pharmacologically modulated electrophysiological activity to the fMRI signal is poorly characterized. This link requires the use of simultaneous and invasive electrophysiological recordings in combination with phMRI, and although such technically challenging studies are feasible they demand highly controlled preclinical approaches.

Despite the numerous advantages offered by the fMRI animal experiments, they also include some disadvantages. Preclinical fMRI investigations usually require anesthesia so that the subjects, in this case animals, keep still during imaging, and this must be achieved without inducing unnecessary stress. This introduces a major confounding factor in animal fMRI experiments. What are the effects of different anesthetics on baseline brain activity or on the way how brain responds to external stimuli? Is the use of anesthesia acceptable or does it invalidate the experimental results? Is one particular anesthetic more suitable than others for preclinical fMRI? How should the results be interpreted if anesthesia has been used? If the use of anesthesia is unavoidable, are there any tools or parameters to be used for controlling its effects? Some of these are the key questions which present work intended to answer, in order to improve the preclinical fMRI methodology.

Although the underlying mechanisms behind the fMRI signal formation are yet not fully understood and some unresolved issues remain, the significance of fMRI for brain research is undeniable. The strength of fMRI lies in its possibility of being combined with classical research methods. The opportunity to link certain behavior or modulated neurotransmission in healthy or diseased brain to macro-level brain function or networking is an extremely fascinating, yet mostly unexploited avenue in neuroscience research. This kind of approach would be extremely beneficial in unraveling the pathological mechanisms in complex CNS diseases, such as schizophrenia (SCZ), or in validating preclinical disease models for drug development purposes. Therefore, a part of the present work explored the possibility of incorporating a carefully optimized preclinical fMRI component into an interdisciplinary and multimodal study investigating the characteristics of a commonly used preclinical model of SCZ; the intended goal was to facilitate development of novel drugs for this devastating disease.

2 Review of the Literature

The first part of this chapter (2.1) reviews succinctly the composition, communication, energy consumption, metabolism, and neurovascular coupling of the brain at the level necessary for the context of fMRI and phMRI. Additionally, mechanisms of anesthesia, commonly used anesthetics in preclinical fMRI, their effects on brain function, and pathophysiological changes in schizophrenic brain will be discussed. More thorough reviews of brain structure and function (e.g., Bear et al. 2001), energy consumption and metabolism (e.g., Magistretti and Allaman 2015), neurovascular coupling (e.g., Attwell et al. 2010), the mechanisms of anesthetics (e.g., Franks 2008), preclinical anesthetics (e.g., Lukasik and Gillies 2003), the effects of anesthetics on fMRI (e.g., Masamoto and Kanno 2012), and schizophrenic brain (e.g., Kahn and Sommer 2015) are readily available in literature.

In the second part of this chapter (2.2), the basics of NMR, common fMRI contrasts, and common fMRI applications, phMRI and resting-state fMRI (rsfMRI), are briefly reviewed. Here too, more exhaustive books (e.g., Huettel et al. 2004) and reviews (e.g., Salmeron and Stein 2002, Fox and Raichle 2007, Jenkins 2012, Lu and Stein 2014) related to these topics can be found in the literature.

2.1 BRAIN STRUCTURE AND FUNCTION

2.1.1 Composition and neural communication

Neural tissue is chiefly composed of two broad categories of cells: neurons and glia (Bear et al. 2001). The numbers of these cells (~85 billion of each) has been suggested to be roughly equal in the adult human brain (Azevedo et al. 2009). Neurons are responsible for detecting changes in the body and its environment, reacting to these changes by communicating with other neurons, and transmitting responses to the detected changes. Glial cells are mainly supporting cells providing insulation and energy substrates for neurons (Bear et al. 2001), but, according to emerging evidence, they also participate in neuronal signaling (Kettenmann and Verkhratsky 2008).

A typical neuron (Figure 1) can be divided into three parts: soma, axon, and dendrite(s) (Bear et al. 2001). The conduction of information in neurons, which typically occurs in axons, is based on electrical excitability. At rest, a voltage gradient is maintained across the cell membrane. If a stimulus occurs, the ions on the both sides of the membrane become redistributed, leading to a decrease in the voltage gradient. If the decrease, or depolarization, exceeds a predefined voltage threshold, an action potential will be triggered. After full depolarization, the original electric potential is gradually restored in a process called repolarization. Once initiated, the action potential passes down the nerve´s cell membrane until it reaches the end of the axon. The nature of action potential propagation is always similar e.g. in its size and duration. Therefore, the information is coded in the frequency and pattern of the action potentials, and in the distribution of excited (or firing, spiking, discharging, impulse firing) neurons.

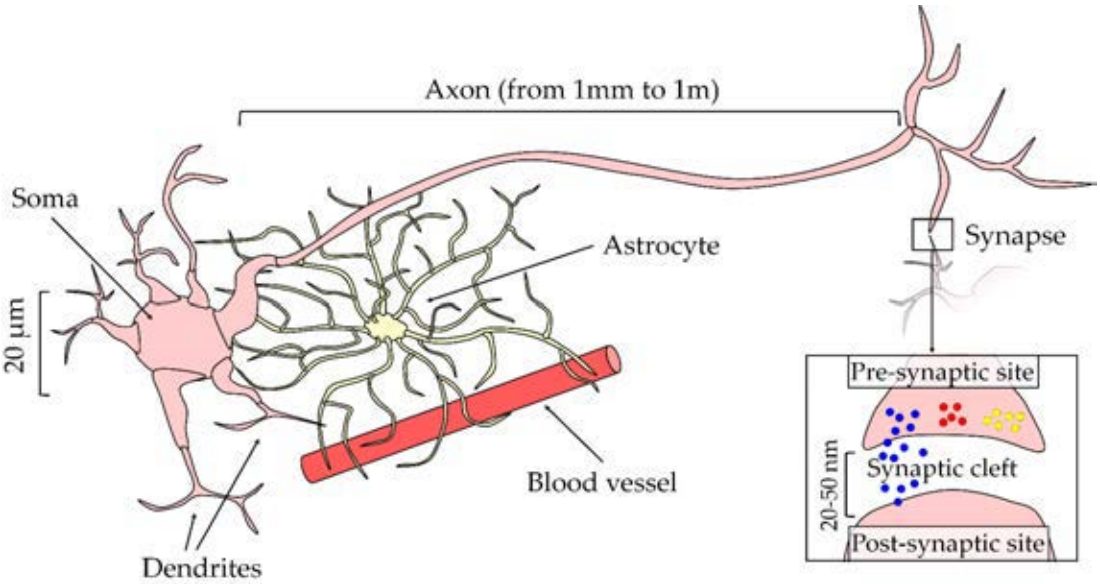

Figure 1. Simplified illustration of different parts (soma, dendrites, and axon) of a single neuron, an astrocyte (glial cell), and a synapse showing the release of neurotransmitter.

As the function of the CNS is based on the complex interplay between neurons, it is essential to transfer the information further in the large-scale network. The signaling between neurons is called synaptic transmission (Bear et al. 2001). The synapse is a specialized junction, which typically occurs between the pre-synaptic axonal terminal and the post-synaptic dendrite (Pakkenberg et al. 2003). It has been estimated that a single cortical neuron in human brain has roughly 7000 individual synapses, which means that there may be a quadrillion (10^{15}) synapses in a human brain. Despite the enormous amount of neurons in the brain (85 billion), the gigantic number of synapses has led to estimations that any single neuron is able to make a contact with any other neuron through six interconnections at the most (Drachman 2005).

Two subtypes of synapses exist: electrical and chemical (Bear et al. 2001). The greatest advantage of electrical synapse is its speed, since it allows the instantaneous transmission of action potentials from one cell to the next. In addition, the signal can be transmitted bi-directionally. Nonetheless, the vast majority of the synapses in adult mammalian brain involve the release of chemicals. In the chemical synapse, the electric information arriving at the presynaptic site is converted into a chemical form and this is delivered to the postsynaptic site across a synaptic cleft, i.e. across an intercellular gap (Figure 1). The presynaptic cellular site contains synaptic vesicles, which store the chemical compounds, i.e. neurotransmitters, required for the signal transmission across the synaptic cleft. The release of neurotransmitters into synaptic cleft is triggered by the incoming action potential. Subsequently, the released neurotransmitters bind to specific receptor proteins located in the postsynaptic membrane, inducing receptor-specific intracellular changes in postsynaptic activity.

More than 100 chemical substances have been recognized to be involved in synaptic transmission (Bear et al. 2001, Purves et al. 2001). The compounds can be classified in different ways, perhaps the division based on size into peptides and small-molecule neurotransmitters being the simplest. It is common to further divide the small-molecule

neurotransmitters into amino acids (e.g., gamma-amino butyric acid (GABA), glycine, and glutamate) and amines (e.g., acetylcholine (ACh), dopamine (DA), norepinephrine (NE), and serotonin). Subsequently, the individual neurotransmitters define the numerous neurotransmitter systems, such as GABA system (GABAergic), acetylcholine system (cholinergic), glutamate system (glutamatergic), and DA system (DAergic). In addition to the signaling function, the concept of the neurotransmitter system includes all the cellular processes related to the signaling, such as the synthesis, packaging, release, reuptake, and degradation of the neurotransmitter.

The nature of neurotransmitters targeting directly the electrical excitability of the postsynaptic neuron can be categorized as either excitatory or inhibitory (Bear et al. 2001). For instance, glutamate mediates the majority of the excitatory neurotransmission in the brain, while GABA is involved in most of the inhibitory actions. Generally, if a neurotransmitter induces depolarization and subsequently triggers an action potential in the postsynaptic neuron, it has an excitatory effect. In contrast, if the postsynaptic cellular site becomes hyperpolarized and is less likely to be depolarized, the neurotransmitter has an inhibitory effect. In addition to direct binding, several other neurotransmitter signaling mechanisms are known. For example, a neurotransmitter may bind to receptors that modulate the function of other receptors through intracellular mechanisms (e.g., enhancement or inhibition).

Because of the great amount of different neurotransmitters and signaling mechanisms, chemical neurotransmission offers enormous amount of different possibilities to code the presynaptic information to the post-synaptic site (Bear et al. 2001, Purves et al. 2001). This feature makes the chemical neurotransmission far more versatile compared than its electrical counterpart. Signal transmission with small-molecule neurotransmitters is also fast; compounds are released from vesicles in <1 ms after the action potential invades the axonal terminal. The release of peptides, however, requires typically a serie of action potentials, and is therefore considerably slower (>50 ms).

The exploration of chemical neurotransmission has dramatically increased our understanding of CNS function during the past 30-40 years (Bear et al. 2001, Purves et al. 2001). Importantly, the new information has revealed effective chemical pathways through which to influence brain activity. This has proved to be particularly important for health sciences since several CNS diseases are associated with imbalances in neurotransmitter levels. Neuropharmacology, i.e. the study of the effects of drugs on receptor systems, has identified options where dysfunctions in neurotransmission may be compensated. Drugs altering brain function and inducing changes in behavior, mood, or perception are named specifically as psychotropic or psychoactive drugs.

Traditionally, the chemical compounds, or drugs, affecting synaptic transmission have been extracted from plants (e.g., nicotine), although synthetic compounds (e.g., phencyclidine, PCP) have become common during the modern era. In most cases, drug molecules are small lipid-soluble compounds that can reach neuronal tissue by transmembrane diffusion across the blood-brain-barrier (BBB) (Banks 2009). Drugs can target several parts of the neurotransmitter system, such as the synthesis, receptor binding, and breakdown of

neurotransmitter, and subsequently influence and modulate the synaptic signal transmission (Bear et al. 2001, Purves et al. 2001). Many well-known drugs have their mechanism of action via direct binding to a post-synaptic receptor. Such drug molecules can induce either similar post-synaptic activity as the corresponding neurotransmitter (in which case the drug is called a receptor agonist), or block the receptor to prevent (or inhibit) the normal receptor function (in which case it is a receptor antagonist).

The discovery of action potentials over one hundred years ago, which eventually led to the concepts of synapses and neurotransmitters, has been one of the major reasons why neurobiological research has so heavily focused on the neurons (Kettenmann and Verkhratsky 2008, Lauritzen et al. 2012). As the very first electrophysiological recording techniques revealed inactivity of glial cells, neurons were considered to be the cells solely responsible for communication in nervous tissue.

However, work during the most recent decades has started to emphasize the important role of glial cells in neuronal communication and synaptic signaling, as advances in research techniques have made it possible to examine glial function in more detail. For instance, it was found out that glial cells actually are excitable and can relay information; however, their diffusion-based conduction mechanism is fundamentally very different from that encountered in neurons. The information in glial cells is coded within the intracellular Ca^{2+} levels, and the information exchange can occur either spontaneously within glia or in conjunction with adjacent neurons.

The magnitude of conduction speed is very different between the cell types: in neurons it can be as fast as milliseconds whereas in glia it tends to take seconds or even minutes. Nevertheless, it is now widely acknowledged that brain function arises from the interplay and signaling of both neuronal and astrocytic networks. The most abundant glial cell type in the brain is the astrocyte, which belongs to the class of macroglial cells (Bear et al. 2001, Kettenmann and Verkhratsky 2008). Astrocytes have numerous important tasks, such as the regulation of the volume and ion concentrations of the extracellular space in neural tissue, the regulation of the neuroprotective barrier between CNS and the rest of the body, the protection of neurons from metabolic damage, the supply of energy substrates, as well as functioning as supporting matrices for neurons and their junctions (Kettenmann and Verkhratsky 2008, Magistretti and Allaman 2015).

The macrostructure and function of CNS is very similar across mammals (Bear et al. 2001). For example, similar neuronal circuitries have been observed to contribute to baseline activity (Lu et al. 2012) and behavior (Balleine and O'Doherty 2010) in rodents and humans. Nevertheless, significant differences are found, e.g., in the numbers of cells (Herculano-Houzel 2009), cytoarchitecture (Vogt and Paxinos 2014), and information conduction or processing pathways (Craig 2009).

2.1.2 Neuroenergetics

The human brain is small compared to the total body mass (~2 %), but nonetheless, as much as 20 % of the energy produced in the body at rest is consumed by the brain (Attwell et al. 2010, Rich and Brown 2016). The proportional energy demand of the brain is considerably

smaller in other vertebrates: only ~2 % in rodents and 9-12 % in nonhuman primates (Mink et al. 1981, Magistretti and Allaman 2015).

Glucose is the main source of energy for brain under normal physiologic conditions (Magistretti and Allaman 2015, Rich and Brown 2016); glucose is readily available in blood, or in astrocytes as a stored form, glycogen (Raichle and Mintun 2006). Glucose can be metabolized to carbon dioxide (CO_2) and water along a metabolic pathway, which begins with glycolysis and ends with oxidative phosphorylation (Falkowska et al. 2015). The intermediate product between glycolysis and oxidative phosphorylation is pyruvate, which is converted into lactate when pyruvate production is faster than its consumption in oxidative phosphorylation.

Adenosine triphosphate (ATP) molecules are produced as a consequence of glycolysis and oxidative phosphorylation; these provide the chemical energy for brain functions. One glucose molecule can produce 2 ATP molecules in glycolysis, whereas ~30 ATP molecules are produced in oxidative phosphorylation. Therefore, oxidative phosphorylation is a far more efficient process, and the majority of the energy produced in brain is provided by oxidative phosphorylation. However, glycolysis does not require oxygen and is a much faster process than oxidative phosphorylation. These are important advantages when neural activity and energy demands are rapidly increasing.

The majority of the energy consumption in brain (up to 80 %) is devoted to processes involved in neural signaling (Raichle and Mintun 2006, Engl and Attwell 2015). At rest, neural signaling consumes roughly an equivalent amount of energy as a human leg muscle while running a marathon (Attwell and Laughlin 2001). Synaptic activity is believed to utilize 80 % of the energy, with glial cell processes consuming the rest (Engl and Attwell 2015, Magistretti and Allaman 2015). For the most part, ATP molecules are required to drive the ionic pump activity, which maintains (or re-establishes) the electrochemical gradients across cell membranes, and enables the development and progression of action potentials in neuronal tissue (Attwell and Laughlin 2001, Magistretti and Allaman 2015, Rich and Brown 2016).

The energy consumption is not uniform across neurotransmitter systems (Attwell and Laughlin 2001). For instance, postsynaptic effects of inhibitory neurons are likely to induce significantly smaller energy demands than postsynaptic effects of excitatory neurons. Furthermore, the numbers of excitatory synapses are ~9 times higher than the number of inhibitory synapses. In addition to differences between neurotransmitter systems, there are major regional differences in energy consumption. For example, grey matter, dominated by excitatory glutamatergic synapses, has a considerably higher energy consumption than the brain on average.

Even though the synaptic transmission in neurons consumes the vast majority of the energy in the brain, the non-neuronal cells have a central role in neuroenergetics (Engl and Attwell 2015, Falkowska et al. 2015, Magistretti and Allaman 2015). The great energy requirement at synapses poses also a huge energetic burden, and therefore effective supportive cellular mechanisms are required. It appears that astrocytes are the key cells that maintain the

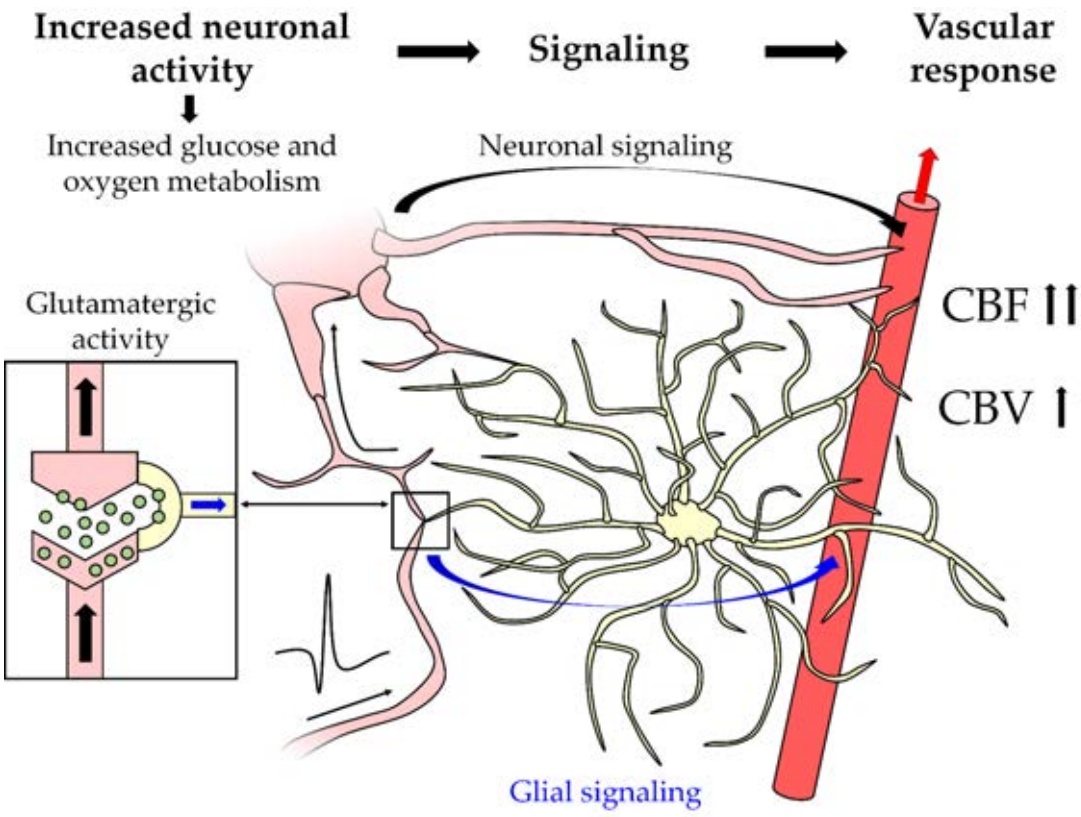

Figure 2. Hypothetical neurovascular coupling mechanisms. CBF, cerebral blood flow; CBV, cerebral blood volume.

coupling between neuronal activity and delivery of the energy substrates. For instance, astrocytes store glycogen; the energy stored in that molecule can be transferred to neurons during rapidly increased energy requirements (Brown 2004). Moreover, some of the cellular energy metabolism steps, such as glycolysis and lactate production, appear to have been outsourced to astrocytes as these functions are only minimally expressed in neurons (Falkowska et al. 2015, Magistretti and Allaman 2015).

2.1.3 Neurovascular coupling

As neural activity is highly energy demanding, the functions of neurons and glia are sensitive to glucose and oxygen deficiencies (Attwell et al. 2010). Attwell et al. (2010) further postulated that the tight coupling between neural activity and blood circulation, named neurovascular coupling, is thus a key factor in maintaining brain function and preventing neural cell damage and death. Due to the neurovascular coupling, the oxygen- and glucose-rich blood flow is enhanced in those regions expressing increased activity and energy consumption. This localized hemodynamic response is also known as a functional hyperaemia.

The underlying cellular mechanisms of neurovascular coupling are not fully understood, and several hypotheses have been postulated (Attwell et al. 2010). The first theories suggested that the increase in local blood flow would be driven by a negative-feedback system; a metabolic signal generated by neurons, such as ATP consumption and subsequent change in

glucose, O_2, or CO_2 levels, would directly regulate blood flow. Intuitively, local CO_2 changes appeared to be a reasonable basis to explain blood flow regulation, as CO_2 affects the H^+ concentration and thus the pH in blood, and this would subsequently dilate vessels and adjust blood flow. However, recent evidence has indicated that the pH in the extracellular space increases during increased neural activity (e.g., Makani and Chesler 2010). This is because the metabolically produced CO_2 is washed quickly away, and increased ionic pump activity elevates the extracellular pH. Additionally, the manipulations of O_2 (Mintun et al. 2001) and glucose (Powers et al. 1996) levels in blood have produced results conflicting with the negative-feedback theory (Attwell et al. 2010).

As metabolic signaling does not appear to be a valid mechanism to account for functional hyperaemia, alternative hypotheses have been presented (Attwell et al. 2010). The current general opinion proposes that synaptic signaling, particularly glutamatergic signaling, is closely involved in the cellular mechanisms of neurovascular coupling; blood flow is regulated by a feedforward system in which neurons send regulatory signals to blood vessels, either directly or indirectly. One of the key factors supporting the concept of a feedforward system (instead of a negative-feedback system) is the observation that blood flow increases roughly four times more than the energy consumption during neural activation (Lin et al. 2010). This mismatch between the amount of required and provided energy substrates indicates that the absolute energy demand or metabolism are not regulating the blood flow, as would be expected in a negative-feedback system, and it is more likely that the blood flow is controlled unidirectionally by the neuronal activity (Attwell et al. 2010). The fundamental reason for such an intense increase of cerebral blood flow compared to energy consumption, however, remains unclear. It has been suggested to function as a proactive function, which can be advantageous during certain pathological conditions.

While the discussion related to the mechanisms of neurovascular coupling is tilting towards neural and synaptic signaling, the role of astrocytes in functional hyperaemia is also increasingly being emphasized (Attwell et al. 2010, Magistretti and Allaman 2015). Firstly, astrocytes have morphological features that indicate that these cells have central role in neuroenergetics and neurovascular coupling; they envelop synapses and have long processes that reach the capillary. The outer surfaces of cerebral capillaries are practically filled with the astrocytic endfeet. Secondly, astrocytes express cellular activities that are thought to be reflections of communication between neurons and vascular cells. For instance, astrocytes express proteins that can detect glutamatergic synaptic activity (Figure 2). After gaining the information of increased glutamatergic activity in synapses, astrocytes signal the smooth muscle cells to dilate the capillaries to increase local blood flow, and subsequently couple the neuronal activity with energy requirements by taking up, metabolizing, and delivering energy substrates from blood.

In addition to glutamate, several other signaling agents have been proposed to be involved in these cellular mechanisms (Attwell et al. 2010, Magistretti and Allaman 2015). For example, astrocytic signaling and release of vasoactive agents after glutamatergic input are mostly based on arachidonic acid derivatives. In addition to lactate (metabolite of pyruvate) (Ido et

al. 2001) and prostaglandin (metabolite of arachidonic acid derivatives), adenosine (metabolite of ATP) (Ko et al. 1990) is known to affect local blood flow regulation.

Although glutamatergic and astrocyte-mediated signaling are thought to be essential in neurovascular coupling (Figure 2), complementary signaling mechanisms have been recognized (Attwell et al. 2010, Magistretti and Allaman 2015). For instance, neurons are able to influence the vasculature directly by releasing nitric oxide or vasoactive peptides. However, the exact contributions of the direct and indirect (e.g., astrocyte-mediated) pathways remain unclear as both paths are activated by the same source, synaptic activity, and therefore they are very difficult to investigate separately.

Nevertheless, the direct and indirect pathways are thought to have varying importance in different brain regions, or even within different neural networks in the same region. In addition to the predominant glutamatergic neurovascular modulation, other neurotransmitters, such as GABA (Kocharyan et al. 2008), have been suggested to be involved in neurovascular coupling. The list of agents contributing functional hyperaemia is, however, incomplete, and further investigations are required (Attwell et al. 2010, Magistretti and Allaman 2015). Nevertheless, the diversity in neurovascular signaling approaches and agents is most likely necessary to establish unique coding strategies, for different neurovascular demands under different tasks and conditions.

The neuronal activity-induced changes in blood flow are typically very local, and do not display a spatial spread of vasodilation (Masamoto and Kanno 2012). It has been shown that regions around activated areas exhibit inhibitory vasoconstriction, and therefore improve the blood flow focally at activation sites (Devor et al. 2007). However, the cellular mechanisms behind this phenomenon remain unknown (Masamoto and Kanno 2012).

2.1.4 Anesthesia – mechanisms and impact on brain function

Anesthesia is a temporary physiological state, in which a reversible loss of consciousness is induced after the administration of a CNS-inhibitive drug (also known as an anesthetic). Typically, anesthesia has several states where loss of consciousness is defined as the first state where the subject is unable to respond to verbal communication; at higher concentrations anesthetics are able to induce a state where the anesthetized subject does not respond to noxious stimuli (Franks 2008). The induction of unconsciousness is a characteristic of all anesthetics, while other features of anesthesia, such as analgesia, amnesia, and muscle relaxation, are dependent on the anesthetic being administered (Franks 2008, Chau 2010).

Several millions of patients are anesthetized annually, as modern surgical procedures would not be feasible without the pain relief and muscle relaxation provided by anesthetics (Franks 2008, Chau 2010). In addition to their wide use in human subjects, anesthetics have been exploited in veterinary medicine and preclinical animal experiments for similar reasons (Lukasik and Gillies 2003). Additionally, the prevention of motion of animals is essential in several imaging modalities, such as in MRI, as motion severely deteriorates the image quality (Masamoto and Kanno 2012). Awake and restrained animals are likely to experience significant stress during such measurements in the noisy environment of the device (Lahti et

al. 1998), and thus stress and the effect of stress on results can be minimized by using anesthesia (Lukasik and Gillies 2003).

The first anesthetics (e.g., nitrous oxide and the first barbiturates) were discovered around 150 years ago, and since then, dozens of anesthetic agents have been introduced (Chau 2010). Although all anesthetics are capable of inducing similar levels of unconsciousness, their chemical structure vary greatly. The question of how such different molecules, and such diverse compounds from simple inert gases to complex steroids, can induce similar anesthetic endpoints, has puzzled pharmacologists for several decades, and is still a mystery awaiting ultimate resolution (Franks 2008). The constantly increasing information has led to one conclusion – the mechanisms behind anesthesia-induced unconsciousness are remarkably more complex than previously thought (Chau 2010).

Hypotheses related to the mechanisms of anesthetics are introduced and debated regularly. The first ideas, emerging roughly a century ago, suggested that all anesthetics have a common non-specific mechanism of action targeting the lipid layers in neural cell membranes, subsequently disrupting neural functions (Chau 2010). This hypothesis, however, has been mainly rejected and replaced by the concept that anesthetics bind to certain receptor proteins directly (Franks 2008). Although numerous ion channels, enzymes, and receptor systems have been systematically investigated, only a few of them appear to be directly involved in the mechanisms of anesthesia (Franks 2008). Additionally, the amount of affected protein types and the magnitude of these effects appear to be very diverse among anesthetics, which complicates the subject even more (Franks 2008, Chau 2010). Some of the identified (or hypothesized) cellular level key targets will be briefly discussed in the following paragraphs.

First, many years ago it was postulated that inhibitory GABA receptors (particularly the subtype A ($GABA_A$) receptors) could be considered as potential binding sites for anesthetics, and subsequently mediate the anesthetic effects (e.g., Nicoll 1978). Indeed, there is substantial evidence supporting the fact that the GABAergic system is involved in anesthetic mechanisms, as almost all general anesthetics potentiate GABAergic neurotransmission, and directly bind and activate GABA receptors at higher concentrations (Franks 2008). Additionally, increasing numbers of modern genetic studies have shown that point mutations in GABA receptor subtypes can affect the anesthetic outcome of several anesthetics (for review, see e.g., Franks 2008). The importance of the GABAergic system in anesthesia mechanisms, however, varies among anesthetics.

The second plausible molecular level target for anesthetics is a group of two-pore-domain K^+ ion channels (2PK) (Nicoll and Madison 1982). It has been found that 2PKs are modulated by the inhalation anesthetics (Patel et al. 1999) and the genetic modification of 2PK channels can furthermore modulate the anesthetic effects of these agents (Heurteaux et al. 2004). The exact role of 2PKs in brain function is not fully understood, but they are thought to modulate neuronal excitability (Franks 2008). Therefore, any change in 2PK function could hypothetically hyperpolarize the cell membrane, and subsequently disturb the propagation of neuronal signal. 2PKs, however, are not a common target for all anesthetics as several intravenous anesthetics have no effect on 2PK functions.

The glutamatergic system is the third possible molecular site for accounting for the actions of anesthetics (Franks 2008). In particular, N-methyl-D-aspartate (NMDA) receptors are thought to be involved (Flohr et al. 1998). Several inhalation anesthetics induce inhibitory effects on NMDA receptors (Franks 2008), and NMDA antagonists, such as ketamine or PCP, can induce loss of consciousness at high concentrations (Carter 1995). Additionally, studies with transgenic mice have indicated that certain NMDA receptor subtypes can indeed modulate the outcome of anesthesia (e.g., Sato et al. 2005). It is likely, however, that NMDA inhibition or antagonism does not solely mediate the effects of anesthetics, and additional mechanisms are most likely involved (Franks 2008).

In addition to the three above-mentioned molecular level targets, several others have been introduced. For instance, the enhancement of inhibitory glycine receptors (Harrison et al. 1993) in brain stem and spinal cord has been postulated to be involved in the mechanisms of volatile anesthetics (Franks 2008). The inhibition of cyclic-nucleotide-gated channels, e.g., in motor (Sirois et al. 2002) and thalamocortical (Ying et al. 2006) neurons, might mediate the effects of some anesthetics, such as some volatile agents and propofol. The anesthetic-induced presynaptic inhibition of Na^+ may also account for decreased glutamatergic signaling occurring after propofol or isoflurane (ISO) anesthesia (Ouyang et al. 2003).

The above discussion comprises only a glimpse of the hypothesized and to some extent identified cellular targets of anesthetics; it is apparent that the molecular targets vary greatly and it is difficult to draw any general conclusions about anesthesia mechanisms, or how these diverse changes ultimately lead to a loss of consciousness. Therefore, the more recent anesthesia research has included an evaluation of macroscale changes observed in brain regions, neuronal pathways, and functional networks in subjects gradually fading into unconsciousness. When the molecular level information is combined with the macrolevel changes, valuable clues can be obtained about the neural mechanisms of unconsciousness and anesthesia (Franks 2008).

One of the key elements in understanding anesthesia mechanisms is the concept that neurophysiology and brain activity during anesthesia display many similarities to natural non-rapid-eye-movement sleep (Tung and Mendelson 2004, Franks 2008). For instance, neurophysiologic factors, such as circadian rhythm (Munson et al. 1970) and sleep deprivation (Tung et al. 2002), can modulate both sleep and the outcome of anesthesia (Tung and Mendelson 2004). Additionally, accumulated sleep debt can dissipate under anesthesia (Tung and Mendelson 2004). Therefore, understanding of sleep mechanisms can help to solve the underlying macroscale mechanisms of anesthesia, as the same neuronal pathways might control the sleep and wakefulness and be targeted by anesthetics (Franks 2008, Chau 2010). Next, the most common region- and pathway-specific differences between awake and unconscious brain will be briefly discussed.

Recent imaging studies have consistently indicated that the activity of thalamus is suppressed during anesthesia (Franks 2008). The thalamus is essential in controlling the information exchange between the periphery and cortex (Huguenard and McCormick 2007). The information flow from the periphery to cortex when the subject is awake is maintained by the constant depolarization of thalamocortical neurons by several arousal nuclei (Franks

2008). During the transition from wakefulness to deep non-rapid-eye-movement sleep, thalamocortical neurons gradually shift to low-frequency bursting mode. The low-frequency bursting activity spreads to most of the thalamic and cortical regions, resulting in decreased peripheral information processing within the cortex. Therefore, it is reasonable to postulate that alterations in thalamic network functions, such as in thalamocortical pathway (Alkire et al. 2000), are highly relevant factors in the mechanisms of unconsciousness (Franks 2008). The anesthetic-induced modulation of thalamic 2PKs and $GABA_A$ receptors are thought to be involved in the hyperpolarization of thalamic neurons, which subsequently leads to disturbed thalamocortical activity and loss of consciousness.

Although thalamic functions appear to be essential in controlling the level of consciousness, it is the cerebral cortex (especially frontal and parietal regions) that is shut down during sleep and anesthesia (Franks 2008). The role of cortical neurons in the loss of consciousness and anesthesia is, however, still largely unclear. Most of the receptor-level targets of anesthetics are abundantly expressed in cortex, and are affected directly by anesthetics, at least *in vitro* (Lukatch and MacIver 1996, Hentschke et al. 2005). Hypothetically, anesthetics could decrease the activity of cortical neurons and inhibit corticothalamic projections, leading to a more suitable cortical state to allow the low-frequency bursting activity (Franks 2008). Additionally, it might be that the loss of consciousness arises from similar mechanisms during both anesthesia and sleep, but with different contributions from thalamic and cortical suppressions.

In addition to the direct region-specific inhibition of neuronal cellular activity, the anesthesia-induced modulation of arousal and sleep pathways is an alternative hypothesis to explain anesthesia (Franks 2008). Several excitatory networks, originating from arousal nuclei either in thalamus or hypothalamus, promote wakefulness, while distinct inhibitory pathways, mostly involving GABAergic neurons, are able to inhibit the arousal pathways to stimulate and maintain sleep. Therefore, the control of wakefulness is a constant interaction between these two network categories, where a switch to another dominant network can induce a relatively rapid change in the state of arousal (Saper et al. 2001). As the decrease of the level of arousal is similar in anesthesia and sleep, they might also induce similar effects on arousal networks (Franks 2008). This hypothesis is supported by the fact that anesthesia-induced loss of consciousness can be reversed pharmacologically, e.g., by cholinergic treatments that strongly activate the arousal pathway.

Taken together, several cellular and macroscale level mechanisms have been proposed to contribute to anesthesia-induced unconsciousness, and this progress has slowly started to shed light on the mystery of anesthetic actions (Franks 2008). Even although the exact mechanisms remain unclear, there is strong evidence that the activation or inhibition of specific receptor proteins is essential in mediating the effects of anesthetics, and furthermore, that thalamus has a key role in controlling the level of arousal. The anesthesia-induced thalamic modulation may originate from direct effects on thalamic neurons, or from the modulation of arousal-sleep pathways. Additionally, the direct effects of anesthetics on cortical neurons may make some contribution to the loss of consciousness.

2.1.5 Schizophrenia

SCZ is a chronic neuropsychiatric disease affecting approximately 1 % of the world´s population (Pratt et al. 2012). It was described in detail for the first time already in 1893 (see (Kahn and Keefe 2013). The wide range of symptoms in SCZ are typically divided into three major categories: positive, negative, and cognitive. Positive symptoms include hallucinations and delusions; negative symptoms consist of deficits in social functioning, inability to experience pleasure, and lack of motivation; cognitive deficits include deficits in memory, attention, perception, and social cognition (Pratt et al. 2012). Even though the symptoms of SCZ have been relatively well characterized, there has been only minor progress in the treatments or prognosis during the last 50 years for this severe mental disorder (Kahn and Keefe 2013). The currently available drugs are able to alleviate the positive symptoms (Pratt et al. 2012, Kahn and Sommer 2015), but have little or no effect on negative and cognitive symptoms (Pratt et al. 2012).

SCZ is often classified as a psychotic disorder (Kahn and Keefe 2013). The classification, however, may be misleading as abnormalities in brain development and function may be detected already a decade before the onset of any psychotic episodes. The key symptoms during the time preceding positive symptoms include deficits in learning and memory functions, implying that the fundamental nature of the disease is cognitive. Additionally, there is emerging evidence that the disease progression starts already in childhood before adolescence, or possibly even during pregnancy (Kahn and Sommer 2015), and the susceptibility to disease may be enhanced by early life stress (Jawahar et al. 2015).

The early onset of disease development in SCZ is supported by the significantly smaller intracranial volume observed in patients with SCZ (Haijma et al. 2013). The increase in the intracranial volume, or the size of the skull, is dependent on brain growth, which plateaus at approximately the age of 14 (Courchesne et al. 2000), and therefore it has been postulated that in SCZ there are already disturbances in brain development before that age (Haijma et al. 2013). In addition to the decreased brain growth until adolescence, the abnormal brain development continues further as both grey and white matter volumes are significantly smaller in SCZ patients compared to healthy controls, even when the smaller intracranial volume is taken into account (Haijma et al. 2013). The decreases in grey and white matter volumes, compared to healthy subjects, appear to progress similarly until the onset of psychotic symptoms, when the loss of white matter seems to terminate (Hulshoff Pol and Kahn 2008). The loss of grey matter, however, continues (Kahn and Sommer 2015).

The loss of grey and white matter during SCZ is not uniform throughout the brain (Cahn et al. 2006). The decline in the amount of white matter is thought to originate from axonal or glial damage in specific association fibers (Mandl et al. 2013, Kahn and Sommer 2015), as well as from a reduced number of oligodendrocytes in frontal cortex (Hof et al. 2003) and hippocampus (Schmitt et al. 2009). The grey matter loss has been speculated to include mainly cortical thinning, e.g., in frontal and temporal brain regions, resulting in decreased integrity of cortical areas (Cahn et al. 2006, Kahn and Sommer 2015).

The pathophysiological mechanisms of SCZ are being intensively investigated, and currently at least three major interacting processes are thought to be involved: dysfunction of the

DAergic system, dysfunction of the glutamatergic system, and proinflammatory status of the brain (Kahn and Sommer 2015). The initial interest in the DAergic system rose already in the 1950s, when the chlorpromazine (potent DA receptor D2 antagonist) was found to have antipsychotic effects (Kahn and Sommer 2015).

Imbalances in the DAergic system (e.g., in mesolimbic and mesocortical pathways) are thought to originate mostly from disturbed presynaptic DA processes, such as DA uptake, synthesis, and release capacities (Howes et al. 2009, Howes et al. 2011, Kahn and Sommer 2015). Additionally, increased DA binding capacity has been observed (Kahn and Sommer 2015). Most of the above observations related to disturbed DAergic functions were conducted in striatal regions, and are thought to be associated with the positive symptoms in SCZ (Howes and Kapur 2009). Even though the genetically-induced increase of striatal DA receptors led to cognitive deficits in a mouse model (Bach et al. 2008), the contribution of DAergic dysfunction to the negative and cognitive symptoms in humans remains unclear (Kahn and Sommer 2015).

In contrast to the DAergic system, a disruption of the glutamatergic system (NMDA receptor hypofunction) has been hypothesized to be a key element in the cognitive impairment occurring in SCZ (Anticevic et al. 2012, Kahn and Sommer 2015). The plasticity of NMDA receptors has a vital role in brain maturation; during brain development, NMDA receptors undergo structural changes that improve their functionality to respond to environmental stimuli (Kahn and Sommer 2015). Incorrect changes in NMDA receptor subtype compositions, e.g., induced by inflammation (Samuelsson et al. 2006), stress (El-Khodor et al. 2004), or hypoxia (Kaur et al. 2006), may well be associated with the onset of cognitive impairment and SCZ development (Kahn and Sommer 2015).

Another supporting factor for the relevance of a disrupted NMDA system in SCZ pathophysiology is the observation that NMDA antagonists are able to induce the full spectrum of SCZ-like symptoms (Kahn and Sommer 2015). The disturbed NMDA receptor system can disrupt inhibitory GABAergic feedback interneurons, which subsequently leads to decreased control and loss of synchrony of glutamatergic neurons (Lewis et al. 2005). The levels of some enzymes related to GABAergic interneurons and GABA synthesis are indeed decreased in patients diagnosed with SCZ (Curley et al. 2011), and SCZ patients have also expressed deficits in GABA receptor physiology (Hyde et al. 2011). Deficient function of GABA-mediated inhibitory mechanism can subsequently lead to abundant DAergic activity in subcortical networks, such as in the mesolimbic pathway (Kahn and Sommer 2015), and to global hyperactivity of non-NMDA glutamatergic neurons (Large 2007). For instance, glutamatergic neurons originating from hippocampus can induce strong excitatory activity in striatal DA neurons (Floresco et al. 2001). Therefore, decreased NMDA-mediated neurotransmission can increase subcortical DA levels and contribute to the positive symptoms (Howes and Kapur 2009), but also to the cognitive symptoms as neural synchrony is significantly decreased (Kahn and Keefe 2013).

Although both GABAergic interneurons and NMDA hypofunction are recognized to be associated with SCZ pathophysiology and increased subcortical DA levels, it is not known which neurotransmitter system is the primary reason for disease development; are

GABAergic neurons affected by NMDA hypofunction as described above, or is it a functional disorder in GABA interneurons inducing aberrant NMDA receptor functions (Kahn and Sommer 2015)? Nevertheless, these changes appear to ultimately lead to increased subcortical DA levels (and positive symptoms), and provide an explanation why psychotic symptoms are not expressed until several years after the appearance of cognitive deficits (Kahn and Keefe 2013).

In addition to specific changes in neurotransmission, a proinflammatory brain state has been linked with SCZ (Stevens 1982), and may be associated with some of the symptoms (Kahn and Sommer 2015). Acute inflammation can turn into a chronic form, which can be very harmful for the immature brain (Kahn and Sommer 2015). In developing brain, it is mainly the microglial cells that are responsible for immune responses; while microglia are reacting to inflammation, their neuroprotective and supporting actions are reduced to secondary tasks (Feigenson et al. 2014, Kahn and Sommer 2015). The lack of normal glial functions may thus affect the brain development and contribute to the pathophysiology of SCZ.

2.2 PRECLINICAL FUNCTIONAL MAGNETIC RESONANCE IMAGING

2.2.1 Nuclear magnetic resonance

The magnetic properties of the nuclei of atoms in a sample or subject placed in external magnetic field represent the basis for the physical phenomenon of NMR (Huettel et al. 2004). The abundance of hydrogen in the human body (mainly as a part of water) is one of the main reasons why the vast majority of MRI studies have been based on the imaging of hydrogen. Certain other atoms, such as carbon, fluorine, and phosphorus can also be exploited in MRI investigations.

As hydrogen has only a single proton in its atomic nucleus, it has an inherent quantized angular momentum, spin, and can be considered as a small magnet (Huettel et al. 2004). The quantum number for hydrogen spin is ½, which means that the spin has two possible energy states in an external magnetic field (B_0). In the absence of the B_0 field, the spins are typically randomly oriented. When the B_0 field is applied, spins take on a gyroscopic motion, precession, around an axis determined by the B_0 field, and they are distributed into either low-energy (parallel to B_0) or high-energy (antiparallel to B_0) states. Because of the lower energy of the parallel state, there are always more spins in the parallel than in the antiparallel state. The proportion of spins in different states is dependent on temperature and magnetic field strength, as shown in Equation 1 (Boltzmann distribution):

$$\frac{P_P}{P_A} = \mathrm{e}^{\frac{\Delta E}{k_B T}}, \tag{1}$$

where P_P is the probability for a spin to be parallel with B_0, P_A the probability for a spin to be antiparallel with B_0, ΔE the energy difference between energy states, k_B Boltzmann´s constant, and T temperature. A higher temperature provides more thermal energy for spins and a higher probability to belong to a high-energy state, while a higher magnetic field increases the energy difference between energy states, and therefore favors the low-energy state.

As there are different amounts of spins aligned in opposite directions, a net magnetization is generated along the B_0 field. The net magnetization can be considered as a vector, which has both longitudinal (parallel or antiparallel with B_0) and transverse (perpendicular to B_0) components. Therefore, the ratio of spins in different orientations (Equation 1) determines the strength of longitudinal magnetization vector. When an equilibrium state is present in the B_0 field, the net magnetization vector consists mainly of longitudinal components, as the variability in transverse components cancels out any significant sum effects in transverse plane.

The magnetization in the equilibrium state, however, is not measurable, and to produce a measurable signal the equilibrium needs to be disturbed (Huettel et al. 2004). This is usually done by applying another external magnetic field (typically named as B_1 in MRI). The oscillating B_1 field provides electromagnetic energy that is absorbed by some of the low-energy spins, leading to their transition from a low-energy to a high-energy state, and subsequently disturbing the thermal equilibrium. This process is also known as the excitation of spins. Excitation typically induces phase coherence among the spins along the B_1 field in the transverse magnetization component, leading to a detectable sum vector in the transverse plane. When the presence of B_1 is removed, the excited spins start to return to the original state and release the absorbed energy, simultaneously causing measurable changes in the transverse magnetization component. These time-varying changes during NMR signal reception period form the basis for MRI image formation.

The maximal on-resonance excitation of spins requires a radiofrequency (RF) pulse (B_1 field) that oscillates with a frequency characteristic for each nucleus, known as the Larmor frequency (ν). The Larmor frequency is dependent on the gyromagnetic ratio (γ), which is a constant for given nucleus, and magnetic field B_0, as shown in Equation 2:

$$\nu = \frac{-\gamma B_0}{2\pi} . \tag{2}$$

The excitation (or generation of oscillating B_1 field) is conducted with an RF coil, in which the electromagnetic field is generated by a current driven in a loop of wire. After excitation, the changes in magnetic field are measured with a receiver coil, tuned to Larmor frequency. During the signal receiving period, the time-varying transverse magnetization component induces measurable currents into the receiver coil, consisting also of loop of wire(s). The longitudinal magnetization component (M_z) recovers to its original state (M_0) according to the T_1 time constant during time (t), as shown in Equation 3:

$$M_z = M_0 \left(1 - \mathrm{e}^{\frac{-t}{T_1}}\right) . \tag{3}$$

The detectable NMR signal originates from the transverse magnetization component (M_{xy}), which decays after excitation as the spins gradually lose their phase coherence. The decay is dependent on a time constant T_2, as shown in Equation 4:

$$M_{xy} = M_0 \mathrm{e}^{\frac{-t}{T_2}} . \tag{4}$$

The T_2 signal decay is, however, dependent on multiple factors that are commonly categorized into either dynamic or static interactions. The time constant including both of these factors is termed as T_2^*, while in T_2 the dephasing effect of static field inhomogeneities (T_2') is removed with a 180° refocusing pulse. T_2^* is the most commonly used weighting in functional imaging, as it has the highest sensitivity to the BOLD signal. The relationship between T_2, T_2^*, and T_2' is shown in Equation 5:

$$\frac{1}{T_2^*} = \frac{1}{T_2} + \frac{1}{T_2'} \quad . \tag{5}$$

The relaxation times (T_1 or T_2) are characteristic for spins in different environments, and therefore provide the fundamental information related to the spin surrounding and dynamics in a certain spatial location (Huettel et al. 2004). A simple NMR signal acquisition, however, provides a signal from the whole sample, and is not enough to separate signal sources originating from different sources to produce a three-dimensional MRI image. Therefore, the signal requires spatial coding steps before or during its acquisition. In MRI, the signal localization is achieved with the use of gradient coils, which generate additional, controllable temporary magnetic fields in all three dimensions during imaging. Firstly, the resonance frequency of spins at different locations can be modulated by influencing the spatial distribution of field strength in one dimension. Subsequently, a certain subpopulation of spins can be excited by selecting a narrow-frequency excitation pulse. Secondly, gradient coils allow the encoding of two remaining dimensions by modulating the frequency and phase of spins in different locations. These preparations confer the required individuality on signals originating from different spatial locations, and contain the necessary information with which to construct a three-dimensional MRI image.

2.2.2 Functional MRI contrasts

In fMRI, stimuli-evoked or intrinsic brain activity is investigated by observing surrogate markers, namely changes in blood oxygenation level, flow, and volume. Therefore, all fMRI contrasts rely on the mechanisms of neurovascular coupling (Attwell et al. 2010, Masamoto and Kanno 2012); increases in neural activity and energy demand lead to the enhanced delivery of oxygen and glucose to the activation site (as discussed in detail in chapter 2.1.3). If brain energy consumption is the major source for fMRI signals, then fMRI baseline would reflect mainly glutamatergic post-synaptic activity and the action-potential related ion currents (see chapter 2.1.3) (Attwell and Laughlin 2001). Indeed, the fMRI responses have been shown to correlate well with local field potentials (Logothetis et al. 2001, Huttunen et al. 2008).

The stimuli-induced changes in local neuronal spiking rate and energy consumption are, however, relatively small (up to 10 % but typically less than 5 %) compared to task- or stimuli-free state (Scholvinck et al. 2008, Attwell et al. 2010, Raichle 2015). Such small changes in neuronal activity would be difficult to detect but, fortunately, the increase in blood flow is roughly 4-fold greater than required to meet the needs of the neurons (Lin et al. 2010), facilitating the indirect detection of neural activity by fMRI. A typical fMRI investigation exploits one of the three major approaches: BOLD, cerebral blood volume (CBV), or cerebral blood flow (CBF) fMRI, which will be discussed briefly below.

BOLD is the most commonly utilized contrast in fMRI. For example, roughly 52 % of phMRI studies have measured the BOLD signal (Haensel et al. 2015). The contrast exploits the different magnetic properties of oxygenated hemoglobin (Hb) and deoxygenated hemoglobin (dHb) (Huettel et al. 2004). When hemoglobin binds oxygen (oxyhemoglobin, Hb), the complex becomes more diamagnetic. When oxygen is released from hemoglobin (deoxyhemoglobin, dHb), the complex becomes more paramagnetic. Fully deoxygenated blood has roughly a 20 % greater intensity of magnetization (or magnetic susceptibility) in the magnetic field compared to fully oxygenated blood, providing the fundamental mechanism for BOLD contrast.

The paramagnetic properties of dHb shorten the transverse relaxation times of spins around dHb, leading to a faster decay in the NMR signal. Therefore, an MRI sequence sensitive to differences in transverse relaxation times displays a higher signal in regions around Hb and a lower signal in regions around dHb. Based on these observations, one could expect that increased cerebral metabolism and oxygen consumption would increase the dHb levels in blood during neural activation, and subsequently decrease the fMRI signal. The fMRI signal, however, typically increases at activation sites. As mentioned before, the neuronal activity increases local CBF excessively (delivery of oxygen > consumption of oxygen), which increases the proportional amount of local Hb. As local CBF is dependent on local CBV (Grubb et al. 1974), neuronal activity also increases local blood volume as well as the absolute amount of diamagnetic Hb, emphasizing even more the local changes in the magnetic field and the BOLD signal (Huettel et al. 2004).

The second common method is the CBV-weighted imaging, where the stimuli-induced changes in blood volume are followed with fMRI (Belliveau et al. 1991, Rosen et al. 1991, van Bruggen et al. 1998). Approximately 37 % of phMRI studies report the use of some CBV-weighted fMRI protocol (Haensel et al. 2015). The contrast in CBV fMRI is typically based on the administration of an intravascular contrast agent, e.g., a small superparamagnetic iron oxide, which similarly to paramagnetic dHb increases the magnetic susceptibility, reduces transverse relaxation time, and decreases the fMRI signal (van Bruggen et al. 1998, Huettel et al. 2004).

First, the contrast agent, which typically has a half-life of several hours, distributes into the blood circulation, and decreases the fMRI signal in the vasculature to a baseline level. During neural activity, the local increase in blood volume can be detected as a further decrease in the fMRI signal, because of the local increase of CBV and paramagnetic contrast agent. Even though the variation in CBV is relatively small compared to changes in CBF (Grubb et al. 1974), the use of a contrast agent enhances the blood volume changes to a detectable level, mainly because of the large surface of capillary bed expressing the changes in magnetic susceptibility (van Bruggen et al. 1998).

In addition to the use of contrast agents, CBV can be measured with Vascular-Space-Occupancy (VASO) fMRI, which exploits the different T_1 relaxation times between blood and parenchyma (Lu et al. 2003, Lu and van Zijl 2012); the signal originating from blood can be eliminated with an inversion pulse, and during fMRI stimuli decreased parenchymal signal suggests increased CBV, with an assumption that the total brain volume remains same.

The third fMRI approach, the functional measurement of CBF, has been used only rarely; less than 5 % of phMRI investigations mapped the neuronal responses by exploiting blood perfusion fMRI (Haensel et al. 2015). A typical functional CBF measurement exploits arterial spin labeling (ASL) technique (Huettel et al. 2004). In ASL, an MRI image pair is acquired. Before the acquisition of the first image, a labeling pulse is applied to the neck of the subject to suppress (or saturate) the MRI signal originating from spins in arterial blood. Next, the suppressed spins in blood travel upstream to the brain, from which an image is acquired. This image reveals the signal loss in those regions to which the saturated arterial blood travelled. Additionally, there is a signal loss in the imaging slice because of the magnetization transfer (MT) effect of the labeling pulse; the labeling pulse at the neck is experienced as an off-resonance (water) pulse in imaging slice. This kind of pulse saturates spins in the macromolecule pool in the imaging slice, and because of the exchange of magnetization between a macromolecule and water pools, the water signal is suppressed in the imaging slice. The second MRI image in ASL acquisition is obtained in similar way to the first image, except that the labeling pulse is placed above the head with an equal distance to imaging slice; with this approach, the labeling pulse does not induce any signal loss because of the saturated spins in arterial blood, but induces a similar MT effect in the imaging slice. Therefore, a CBF map indicating the regions and amount of blood flow can be produced by subtracting these two images. In functional measurements, several image pairs are acquired continuously to detect changes in regional CBF, which could reflect neuronal activity.

Although the exact relations between the different fMRI contrasts remain unclear, CBV and CBF are known to be tightly coupled (Grubb et al. 1974, Shen et al. 2008) and changes in CBF and CBV reflect the changes in BOLD (Ogawa et al. 1993, Mandeville et al. 1998, Silva et al. 1999). It is also known that the different contrasts or sequences emphasize different vascular components (Duong et al. 2001, Kim et al. 2007, Shen et al. 2008), which may be necessary to be taken into account in experimental design and data interpretation. For example, the gradient-echo echo-planar imaging BOLD signal includes a significant extravascular contribution of the large draining veins (Ogawa et al. 1993, Lee et al. 1999); the location of these veins may be distant from the activated neuronal region, thus reducing the spatial specificity of the method. In contrast, spin-echo echo-planar imaging BOLD signal is less sensitive to the large vessels, as the extravascular dephasing effects induced by large draining veins are refocused by a 180° pulse. Therefore, the BOLD signal acquired with a spin-echo sequence represents better the changes in the capillary bed, especially at high magnetic fields.

BOLD has been the most popular choice of the three main fMRI approaches, mostly because of its straightforward implementation; contrast is based on an endogenous contrast agent, hemoglobin, and it does not require any invasive procedures (Steward et al. 2005, Kim and Bandettini 2010). However, changes in the BOLD signal, induced by neuronal activation, are typically only few percentages, and similar changes may originate from hardware noise and changes in physiology (e.g., changes in heart rate and respiration) (van Bruggen et al. 1998). Additionally, the qualitative nature of the BOLD approach is a notable limitation (Silva et al. 1999). In contrast to BOLD, CBV and CBF are physiological parameters that are at least semi-quantitative (Steward et al. 2005), and as absolute values are obtained, comparison of results between different studies is more straightforward. Nevertheless, both CBV and CBF have their own limitations. In CBV-weighted imaging, the injection of exogenous contrast agent is

unavoidable, and the elimination of contrast agent complicates data analysis. With CBF, the temporal resolution is often poor (~10 s) in comparison to BOLD and CBV (~1-2 s), and the mapping of CBF from whole-brain is technically challenging, resulting in limited spatial coverage and resolution.

2.2.3 Anesthesia in preclinical fMRI

As mentioned above, the majority of the preclinical MRI studies have been conducted under general anesthesia, mainly to prevent the motion and stress of subjects during scanning (Lukasik and Gillies 2003). Subjects might also undergo surgical procedures immediately before imaging, and the maintenance of anesthesia-induced analgesia is therefore necessary. The use of anesthesia in fMRI experiments, however, induces a fundamental level conflict; the ultimate intention is to investigate and localize brain activity, but brain activity has been modulated by the anesthesia, leading to a non-responding behavioral state. General anesthetics are known to suppress both spontaneous and evoked electrical brain activity (Steward et al. 2005). Therefore, the effects of anesthetics on fMRI signal formation (Figure 3), and the extent to which anesthesia interferes with fMRI studies have been extensively studied.

As there are several physiological steps between neuronal activity and fMRI signal changes, there are also several different levels at which anesthetics can cause interference in the fMRI measurements. In the context of fMRI, anesthetics can modulate the 1) baseline neural processing, including spontaneous activity, metabolism, and blood flow, 2) neural responses to various stimuli, 3) neurovascular coupling mechanisms, and 4) vascular reactivity (Masamoto and Kanno 2012). Therefore, it is reasonable to expect that the hemodynamic responses will differ in the conscious and anesthetized conditions, as was recently shown with marmosets (Liu et al. 2013a). The variability in fMRI results, however, is not limited to these two main conditions; neural activity, physiological state, and subsequent hemodynamic responses vary greatly even among different anesthesia protocols (Haensel et al. 2015). The neuronal spiking during somatosensory stimulus has been shown to be modulated by anesthesia in a protocol-dependent manner (e.g., Huttunen et al. 2008), as does

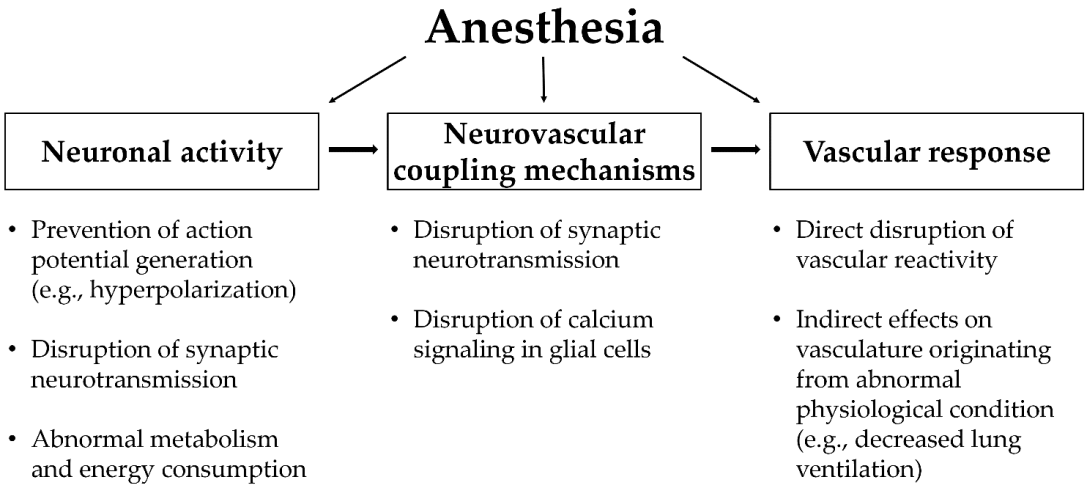

Figure 3. Possible confounding effects of anesthetics on neural processing and neurovascular coupling mechanisms relevant to functional magnetic resonance imaging experiments. CBF, cerebral blood flow; CBV, cerebral blood volume.

the hemodynamic response (e.g., Lindauer et al. 1993, Huttunen et al. 2008, Sommers et al. 2009, Liu et al. 2013a, Schroeter et al. 2014). Similar observations have been made in phMRI experiments (Abo et al. 2004, Bruns et al. 2009, Hodkinson et al. 2012, Liu et al. 2012).

When the use of anesthesia is unavoidable, as it is in numerous preclinical fMRI settings, caution is required in the selection of anesthetic; the varying fMRI responses under the different anesthesia protocols can be traced most likely from the characteristic mechanisms of each anesthetic. Therefore, it is reasonable to assume that some anesthetics would be more suitable for certain study designs than others, or for fMRI in general. Some compounds may affect mainly microvascular responses, while others directly affect vascular physiology (Masamoto and Kanno 2012); some anesthetics may induce extensive bursting activity in cortex, while others have more modest cortical effects; some drugs may suppress breathing, while some others tend to induce hyperventilation, etc. These characteristics are extremely valuable information, as they provide a means to improve the study design; with brief preliminary screening, several alternative compounds can be rapidly excluded, and importantly, the most suitable candidates can be selected. Many different anesthetics have been used in imaging studies (Lukasik and Gillies 2003), and therefore some of the commonly used anesthetics in preclinical fMRI will be briefly reviewed.

α-Chloralose (AC) is a long-acting anesthetic, which has traditionally been the most commonly used anesthetic agent in fMRI experiments. Despite its long history, most of its anesthetic mechanisms still remain unclear (Balis and Monroe 1964, Lees 1972, Garrett and Gan 1998). At low concentrations AC potentiates $GABA_A$ activity, while at high concentrations $GABA_A$ receptors are directly activated by AC (*in vitro* experiments, (Garrett and Gan 1998, Wang et al. 2008). The binding site of AC in the $GABA_A$ receptor complex is, however, unclear and appears to be different from many other anesthetics (Garrett and Gan 1998). AC did not affect glutamate-, glycine-, or ACh-mediated currents in the hippocampal neuron at low concentrations (Wang et al. 2008). In contrast, the activity of DAergic neurons appeared to be suppressed (Nieoullon and Dusticier 1980), and this may make a contribution to its wide range of inhibitory effects.

The use of AC in phMRI studies is not common; less than 8 % of studies report AC as a primary or secondary anesthetic agent (Haensel et al. 2015). In contrast to its occasional use in pharmacological studies, AC has been exploited in somatosensory stimulation studies because it is believed to preserve the metabolic coupling and to evoke only minimal cardiovascular effects (Ueki et al. 1992). However, convulsions, acidosis, and hypothermia are recognized potential side effects of AC (Balis and Monroe 1964). Additionally, decreased cerebrovascular reactivity to some endogenous substances, such as carbon dioxide (Sandor et al. 1977), may occur under AC anesthesia, and the level of analgesia under AC anesthesia may not be sufficient for surgical procedures (Silverman and Muir 1993). AC is considered a non-recoverable anesthetic, but a few recent functional studies with rats have indicated that it may be possible to devise a recovery protocol (Luckl et al. 2008, Alonso Bde et al. 2011).

Isoflurane (ISO) belongs to the group of inhalation anesthetics. It is one of the golden standard anesthetics in structural imaging, but it is also being increasingly exploited in functional studies (Lukasik and Gillies 2003). For instance, almost 44 % of the preclinical

phMRI studies were conducted under ISO anesthesia until 2013 (Haensel et al. 2015). When studies using halothane are added, the total amount of studies using an inhalation anesthetic is as high as 77 % (Haensel et al. 2015). The popularity of ISO in MRI experiments originates from its several advantageous properties: administration is easy, both induction and recovery are fast, the level of anesthesia is easily controllable and stable throughout measurements, and there do not appear to be any clear contraindications preventing follow-up studies (Lukasik and Gillies 2003). ISO induces also sufficient muscle relaxation without evoking convulsions (Eger 1984, Lukasik and Gillies 2003). Nevertheless, inhalation anesthetics cause vasodilation and decrease heart rate, mean arterial blood pressure (MABP), and breathing rate (Van Aken and Van Hemelrijck 1991, Lukasik and Gillies 2003, Masamoto and Kanno 2012). Additionally, there may be inhibition of neurotransmission-modulated vasodilation (Toda et al. 1992). These drawbacks are significant confounding factors in fMRI, as the anesthesia-induced vasodilation decreases the relative vascular response to neural activation or other vasoactive compounds (Sicard et al. 2003, Sicard and Duong 2005), subsequently hindering the detection of fMRI signal changes (Masamoto and Kanno 2012).

The exact mechanisms of action of all inhalation anesthetics are unclear (Lukasik and Gillies 2003). The inhibition of CNS activity has been speculated to originate from the enhancement of GABAergic and inhibition of cholinergic, serotonergic, and glutamatergic neurotransmitter systems (Campagna et al. 2003, Masamoto et al. 2009). For example, several neuronal cholinergic receptors are inhibited by as much as 70-90 % at clinically relevant ISO concentrations (Violet et al. 1997, Dong et al. 2006), while some other subtypes remain unaffected (Flood et al. 1997, Flood and Role 1998). The dose-dependent vasodilation appears to be partly modulated through direct actions of ISO on smooth muscle cells in cerebral vessels (Iida et al. 1998). Additionally, high doses of inhalation anesthetics may open BBB (Tetrault et al. 2008), and thus increase CBV and drug concentrations in neural tissue, and complicate data interpretation. The dose of inhalation anesthetics may also affect the stimuli-induced fMRI signal changes (Marcar et al. 2006).

Medetomidine (MED) is a relatively new sedative agent used in veterinary and preclinical animal experiments (Lukasik and Gillies 2003, Sinclair 2003). Several groups have incorporated MED into their fMRI protocols (Weber et al. 2006, Pawela et al. 2009, Williams et al. 2010, Liu et al. 2012, Kalthoff et al. 2013); based on brain connectivity analyses and suitability for longitudinal studies, MED may be more suitable than AC or ISO for fMRI experiments. Despite the increasing amount of supporting findings, the use of MED in phMRI experiments is still relatively rare (less than 2 %) (Haensel et al. 2015). MED induces safe and non-terminal sedation providing muscle relaxation, analgesia, and anxiolysis. Additionally, its acceptable range of anesthesia depth may be wider than that of other anesthetics (Williams et al. 2010), and anesthesia can be reversed rapidly by administration of its antagonist, atipamezole (Sinclair 2003). Therefore, repeated anesthesia induction and follow-up studies are feasible (Weber et al. 2006, Pawela et al. 2009). However, unwanted physiological effects, such as bradycardia, reduced cardiac output, changes in MABP, vasoconstriction, hypothermia, reduced breathing rate, and reduced CBF are observed in MED-sedated animals (Sinclair 2003). Moreover, due to its poor analgesic properties additional anesthetics need to be used during surgical procedures (Sinclair 2003) and sedation for periods longer than 3 h requires an optimized dosing protocol (Pawela et al.

2009). In addition, the anesthesia level under MED is dependent on the stress level at the beginning of anesthesia (Sinclair 2003).

As MED, or its active enantiomer dexmedetomidine, is a highly specific α_2-agonist and thus, pharmacologically an exceptionally selective anesthetic agent (Sinclair 2003), the mechanisms of actions may be more straightforward to understand, at least in comparison to anesthetics with diffuse effects. MED is known to bind α_2-adrenoceptors in the specific parts of brainstem, such as in pons and locus coeruleus (LC) (Correa-Sales et al. 1992, Lakhlani et al. 1997, Nelson et al. 2003, Pawela et al. 2009). The LC has a high density of α_2-receptors and is an important modulator of vigilance (Delagrange et al. 1993, Aston-Jones et al. 1994). Hyperpolarization induced by α_2-agonist leads to a decreased firing rate of noradrenergic neurons in the LC and further inhibition of norepinephrine (NE) release, which is necessary for arousal (Nelson et al. 2003, Sinclair 2003). These effects are likely to contribute to the disturbed thalamocortical activity (or the activation of endogenous sleep pathway), and subsequent loss of consciousness.

Thiobutabarbital (TBB) is a long-acting anesthetic, which is a member of the diverse group of barbiturates (Booth 1988, Koskela and Wahlstrom 1989). Barbiturates have been used as anesthetic agents for several decades (Dundee and Riding 1960), but their use in clinical settings has declined due to safety concerns, and their popularity in preclinical experiments is also decreasing; less than 4 % of phMRI studies have been conducted under barbiturate anesthesia (Haensel et al. 2015). Nevertheless, as a single dose of TBB can provide long-lasting anesthesia (Frey 1961), it is an interesting option for fMRI.

Barbiturates have effects on multiple receptor systems; the release of several neurotransmitters, such as ACh, NE, and glutamate, is inhibited, while $GABA_A$ receptors are directly activated and GABA-mediated currents enhanced by barbiturates (Ho and Harris 1981). Additionally, high barbiturate concentrations may significantly disturb calcium uptake at nerve endings (Lukasik and Gillies 2003). These cellular effects most likely contribute to the suppression of cortical and thalamic brain activity (Booth 1988).

The depth of barbiturate-induced unconsciousness may vary from mild sedation to surgical level anesthesia among different substances and doses (Booth 1988, Lukasik and Gillies 2003). The dose of barbiturates correlates with the BOLD response amplitude, and can even alter the sign of the stimuli-induced signal change (negative/positive) if the dose is high enough (Martin et al. 2000). Additionally, the therapeutical window of barbiturates is typically narrow, and these drugs induce several unwanted physiological effects, such as decreases in cardiac output, respiration, MABP, body temperature, CBF, and oxygen uptake in neural tissue (Booth 1988, Lindauer et al. 1993, Lukasik and Gillies 2003, Masamoto and Kanno 2012). The direct effects of barbiturates on cerebral vasculature are still controversial (vasoconstriction vs. vasodilation) (Masamoto and Kanno 2012).

Urethane (URE) is a traditional anesthetic in electrophysiological and pharmacological investigations (Boyland and Rhoden 1949, Maggi and Meli 1986) but still used for several reasons; it provides steady and long-lasting (even up to 24h) surgical level anesthesia with a single dose, it affects only minimally breathing and heart rate, MABP is only moderately

decreased, it provides good muscle relaxation, and several brain regions and autonomic functions remain relatively unaffected (Boyland and Rhoden 1949, Maggi and Meli 1986, Field et al. 1993). Additionally, while electroencephalographic patterns recorded under general anesthesia typically lack the alternation between different states, as it occurs during natural sleep, the electroencephalographic behavior under URE uniquely exhibits these cyclic changes (Tung and Mendelson 2004, Clement et al. 2008).

Despite the numerous recognized advantages, only ~8% of phMRI studies have reported the use of URE during measurements (Haensel et al. 2015). The unpopularity may originate from certain disadvantages: URE is a potential carcinogen and mutagen, which has to be taken into account during its handling (Field and Lang 1988, Maggi and Meli 1986). Additionally, administration may cause necrosis in intra-abdominal organs, and the anesthetic protocol is therefore considered as terminal (Field and Lang 1988). The impact of different sleep-like brain states to the response to external stimulus is also unclear. There do seem to be some differences, at least in the propagation of excitation waves (Wanger et al. 2013).

At the cellular level, URE has only modest effects on multiple receptor systems; typically anesthetic-enhanced $GABA_A$ activity is only slightly (20-30 %) potentiated, and similar effects on glycine receptors have been observed (Maggi and Meli 1986, Hara and Harris 2002). Similarly, the activities of glutamate, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and nicotinic ACh receptors (nAChR) receptors are only minimally modulated (10-20 %) (Maggi and Meli 1986, Hara and Harris 2002). Therefore, it has been proposed that the anesthetic effect of URE originates from the combined effects on multiple targets: minimal enhancement of inhibitory neurotransmission and minimal inhibition of excitatory neurotransmission (Hara and Harris 2002). Based on these effects and expression of natural sleep-like electrical brain activity, some investigators have claimed that URE may be better choice for several electrophysiologic and pharmacologic studies than many other alternative anesthetic substances (Maggi and Meli 1986, Hara and Harris 2002, Masamoto and Kanno 2012).

As discussed above, several anesthetics alter physiology leading to changes in several parameters, such as body temperature, respiration, MABP, and heart rate (Steward et al. 2005). These physiological variables, or their indirect effects, have been shown to affect the fMRI signal (Steward et al. 2005), but in contrast to the effects of anesthetics on CNS, this "physiological noise" can be measured and corrected relatively easily (Jenkins 2012). Such corrections are therefore highly recommended, as the quality of fMRI data can be significantly improved.

Almost all anesthetics decrease body temperature, which is perhaps the most commonly controlled physiological parameter during fMRI measurements. The insertion of a rectal temperature probe is straightforward and fast, and most of the animal holders of modern MRI scanners have a built-in warm water circulation system for heating purposes. Over 91 % of phMRI studies have reported the monitoring and controlling of body temperature (Haensel et al. 2015). Although body temperature has direct effects on CBF and cerebral energy consumption (Carlsson et al. 1976, McCulloch et al. 1982), these effects are only modest compared to the direct effects of anesthesia (Steward et al. 2005).

The respiration rate is typically decreased by anesthetics. Insufficient ventilation may have serious consequences, e.g. leading to hypercapnia, hypoxia, vasodilation, increased CBF, and decreased fMRI responses (Sicard et al. 2003, Sicard and Duong 2005, Steward et al. 2005). Therefore, the evaluation of lung ventilation is extremely important. The analysis of arterial blood samples for blood gas values provides a standard and reliable approach to determine the sufficiency of respiration. Approximately 63 % of phMRI studies have implemented blood gas analysis in their experimental protocol (Haensel et al. 2015). In several cases, the blood gases have either been fluctuating or not optimal during the fMRI session, and artificial ventilation is required to increase or stabilize the lung ventilation and gas exchange. In addition to enabling the adjustments of blood gas values, a mechanical ventilation protocol including the administration of a muscle relaxant prevents the spontaneous or stimuli-induced changes in breathing rate that could well mask the fMRI responses (Xu et al. 2000). Approximately 52 % of phMRI studies stated that they adopted artificial ventilation, and furthermore in 54 % of studies allowing spontaneous breathing, the investigators were at least monitoring the breathing rate (Haensel et al. 2015). However, blood sampling and ventilation protocols usually require invasive procedures and complicate the experimental design (Steward et al. 2005). Subsequently, follow-up studies including recovery are likely to be more difficult, even impossible.

The anesthetic-induced changes in cardiovascular parameters, such as in heart rate and MABP, may also contribute to the fMRI signal (Steward et al. 2005). A pharmacologically induced increase in MABP was shown to correlate well with the fMRI signal (Tuor et al. 2002). Based on the findings of Tuor et al. (2002), the NE-induced increase in MABP, however, needs to be relatively high (>55 mmHg) to evoke significant fMRI signal changes that have a good correlation with MABP. Additionally, the NE-induced fMRI signal changes in CNS may originate from neurons whose purpose is to detect changes in MABP (Verberne and Owens 1998, Steward et al. 2005). In contrast to the study of Tuor et al. (2002), the administration of cocaine methiodide, which similarly to NE does not cross the BBB but increases MABB, induced only weak and scattered fMRI signal changes in brain (Luo et al. 2003). Despite the inconsistent findings, anesthesia protocols inducing varying level of MABP may require the utilization of MABP measurements to exclude the possibility that a change in MABP has contributed to the fMRI signal. Unfortunately, the reliable measurement of MABP, often requires arterial cannulation, which complicates the experiment. A more common and easier approach to measure cardiovascular parameters is the recording of heart rate, which can be obtained either by electrodes or pulse oximetry. Heart rate, and its possible changes, can give a crude estimation of the physiological status of subject, for example related to the depth of anesthesia (Steward et al. 2005). Roughly two out of three phMRI studies have monitored MABP and/or heart rate (Haensel et al. 2015).

As briefly demonstrated above, several characteristics related to the pharmacological effects and physiological characteristics have been recognized for different anesthetics; these details can be exploited in fMRI study design to select the most appropriate anesthetic and to avoid severe interactions. The selection of the anesthetic and its dose, however, is always a compromise between pros and cons. An interesting approach to minimize some of the unwanted effects is to use a combination of several anesthetic agents (Lukasik and Gillies 2003). Two substances targeting different receptor systems may potentiate each other´s

anesthetic effects, and individual drugs can be administered at significantly lower doses resulting in fewer side effects (Lukasik and Gillies 2003). For instance, the combination of MED and ISO was introduced recently for stimuli fMRI, and the authors reported that unwanted effects of both anesthetics were reduced (Fukuda et al. 2013). The diverse use of different anesthetics, doses, and their combinations, however, has a significant disadvantage: the lack of comparability of results. One has to be careful when drawing conclusions based on results obtained with different experimental protocols (Masamoto and Kanno 2012). For example, a considerable body of fMRI data has been obtained under AC anesthesia, but the majority of these observations may be reproducible only under exactly the same experimental conditions.

Even though some anesthesia protocols may appear suitable and yield good responses, they may be temporally unstable (Masamoto and Kanno 2012) or not reproducible in different animal strains or species (Lukasik and Gillies 2003). For instance, fMRI responses under AC anesthesia varied time-dependently during a 6 h period, and these changes were paralleled by similar changes in electrophysiological activity (Austin et al. 2005). Due to their stable tissue concentration, constantly administered inhalation anesthetics may be temporally more stable than injectable anesthetics (Lukasik and Gillies 2003).

In summary, the anesthesia-induced confounding factors in fMRI emerge mainly from the suppression of region-specific neural activity and receptor systems, from the disturbance of molecular level neurovascular coupling mechanisms, and from the alterations in physiological parameters (Steward et al. 2005, Masamoto and Kanno 2012). In contrast, it seems that anesthetics exert only moderate direct effects on vascular reactivity and on the temporal dynamics of neurovascular coupling (Masamoto and Kanno 2012). Even though anesthesia does not diminish the hemodynamic responses completely, as shown by a great number of preclinical fMRI studies (Steward et al. 2005), it is still one of major issues in fMRI experiments and requires considerable attention.

2.2.4 Pharmacologic fMRI

In phMRI, the acute effects of CNS-active drugs are investigated by exploiting the fMRI technique. Typically, phMRI studies attempt to localize activation sites and temporal dynamics of drug responses, e.g., in the characterization of a drug´s pharmacodynamics, BBB-permeability, or in finding appropriate dose ranges of a novel drug candidate (Leslie and James 2000, Jenkins 2012, Jonckers et al. 2013). The first phMRI investigations were conducted in the mid-1990s (e.g., Silva et al. 1995, Chen et al. 1997), and already these early investigations showed that phMRI demonstrated a good correlation with cerebral glucose utilization, release of neurotransmitters, and behavior (Leslie and James 2000). Importantly, the phMRI signal was found to correlate better with drug actions in CNS and their consequences (secondary neurotransmission, behavior, etc.) than with absolute drug concentration in tissue, a clear advantage of this approach compared to other forms of functional imaging (Stein et al. 1998, Jenkins 2012). Approximately a decade later, it was confirmed that electrophysiological activity correlated well with the phMRI signal during local lidocaine administration (Rauch et al. 2008). The correlation between fMRI signal and electrophysiological activity during intravenous drug administration, however, has not been investigated yet.

Table 1. Different substances (*n*=60) studied in 129 rat phMRI investigations. The compounds investigated ≥4 times (*n*), or in >3 % of studies, are highlighted. Reviewed from data represented by Haensel et al. (2015).

Compound	*n*	%	Compound	*n*	%	Compound	*n*	%
2-Deoxy-D-glucose	1	0.8	**Fluoxetine**	**4**	**3.1**	GR-73632	1	0.8
7-OH-DPAT	2	1.6	Formalin	1	0.8	Nicotinamide	1	0.8
Adenosine (or close analog)	2	1.6	Gabaculine	1	0.8	**Nicotine**	**5**	**3.9**
Amphetamine	**25**	**19.4**	Gabapentin	2	1.6	PG-01037	1	0.8
Apomorphine	3	2.3	Ghrelin	2	1.6	**Phencyclidine**	**6**	**4.7**
Aripiprazole	1	0.8	Guanfacine	1	0.8	Pilocarpine	2	1.6
Atomoxetine	1	0.8	**Heroin**	**4**	**3.1**	Quinelorane	1	0.8
Bicuculline	2	1.6	HU210	1	0.8	Quinpirole	1	0.8
Bromocriptine	1	0.8	Ketamine	2	1.6	Reboxetine	1	0.8
Bupropion	1	0.8	Arginine	1	0.8	Remifentanil	1	0.8
Capsaicin	1	0.8	Tetrahydropalmatine	1	0.8	Rivastigmine	1	0.8
Celecoxib	1	0.8	LY-404187	1	0.8	SB-269970	1	0.8
Citalopram	1	0.8	**m-Chlorophenylpiperazine**	**4**	**3.1**	SB-277011	1	0.8
Cocaine (or close analog)	**14**	**10.9**	Mecamylamine	1	0.8	Scopolamine	1	0.8
CP55940	1	0.8	Memantine	1	0.8	Serine	1	0.8
Diazepam	1	0.8	**Methylphenidate**	**4**	**3.1**	Sulpiride	1	0.8
Dihydrexidine	1	0.8	MK-801	2	1.6	Tolperisone	1	0.8
Ethanol	1	0.8	Modafinil	1	0.8	Vigabatrin	1	0.8
Fenfluramine	2	1.6	Morphine	1	0.8	Yohimbin	1	0.8
FG-7142	1	0.8	Naloxone	1	0.8	Zymosan	1	0.8

Traditionally, phMRI studies have focused on drugs (agonists or antagonists) that affect specific neurotransmitter systems, e.g., serotonergic, DAergic, glutamatergic, or opioid systems (Steward et al. 2005). In addition to the exploration of the direct effects of drugs, phMRI can be combined with other fMRI experiments, where the drug-induced modulation of a neuronal response to some other stimulus is investigated (Jenkins 2012). In some cases, the drug-induced fMRI responses may be significantly more interesting after an acute or a chronic drug pretreatment period, such as in preclinical models of addiction (Jenkins 2012), anxiety (Kalisch et al. 2004), or SCZ (Pratt et al. 2012).

The review of data presented by Haensel et al. (2015) reveals the use of 60 different compounds in 129 phMRI measurement series (Table 1). The following criteria [in addition to the criteria reported by Haensel et al (2015)] were applied while compiling the table: Measurements were conducted with anesthetized rats, study included acute administration of a drug (pretreatments not included), and acute response was followed with MRI (pre-/post-treatment changes in resting-state not included). The most common substances appear to be two stimulant neuroactive drugs amphetamine and cocaine, which account for over 30 % of the studies, and as many as 42 of 60 (70 %) compounds have been investigated only once (each contributing 0.8 % to all measurements). The extensive use of the same compounds (cocaine and amphetamine) in phMRI experiments may reflect methodological optimization rather than research interest in the effects of one of these drugs.

Either nicotine or PCP has been investigated in every almost tenth phMRI study (9 %, Table 1). Nicotine, the most addictive substance present in tobacco, is an nAChR agonist that acutely enhances cognition and attention (Gozzi et al. 2006), while PCP is an NMDA receptor antagonist that induces SCZ-like symptoms (psychosis, and cognitive and social deficits) in both animals and humans (Gozzi et al. 2008b, Pratt et al. 2012). Both of these drugs have been extensively studied and exploited in neuroscience. Nicotine is an important substance in studies related to the neurobiological and pharmacological mechanisms of addiction and tolerance (Gozzi et al. 2006, Zuo et al. 2011) In contrast, PCP is the most common pharmacological tool in animal models of SCZ, providing a basis for preclinical pathophysiology investigations and drug development (Pratt et al. 2012).

The rat phMRI studies involving acute administration of either nicotine (Choi et al. 2006, Gozzi et al. 2006, Schwarz et al. 2007, Li et al. 2008, Zuo et al. 2011) or PCP (Risterucci et al. 2005, Gozzi et al. 2008b, Gozzi et al. 2008c, Gozzi et al. 2008a, Bruns et al. 2009, Gozzi et al. 2010, Hackler et al. 2010, Broberg et al. 2013) are listed in Table 2. Interestingly, all nicotine and PCP studies (where anesthesia has been used) were conducted under inhalation anesthesia, even though inhalation anesthetics are known to significantly affect cholinergic (primary target of nicotine) and glutamatergic neurotransmission (primary target of PCP) (see chapter 2.2.3). As discussed in the previous chapter, anesthesia can have considerable effects on fMRI studies by disturbing neurovascular coupling and neural activity, but the impact can be even more crucial in phMRI measurements; drugs under investigation may undergo direct interactions with anesthetics, or drugs may share the same cellular level targets as the anesthetics, and these mechanisms can significantly modulate the observed phMRI response. For example, cocaine administration under AC resulted in an increase in CBF, but under ISO anesthesia there was a decrease in CBF (Du et al. 2009). Therefore, a knowledge of the pharmacodynamics of both the drug being studied and the anesthetic agent used to immobilize the animal is essential in phMRI study design. Despite the importance of choosing a suitable anesthesia protocol in phMRI, only a few groups have compared their

Table 2. Rat phMRI studies including acute administration of nicotine or phencyclidine.

Drug	Dose (mg/kg)	Route	fMRI contrast	B_0	TR (s)	Follow-up (min)	Anesthetic	Dose	Rat strain	1st Author	Year
Nicotine	0.07	i.v.	CBV	4.7/9.4T	64	25	Halothane	1.0-1.5%	SD	Choi	2006
Nicotine	0.35	i.v.	CBV	4.7T	40/80	25-45	Halothane	0.8%	SD	Gozzi	2006
Nicotine	0.35	i.v.	CBV	4.7T	40	15-20	Halothane	0.8%	SD	Schwarz	2007
Nicotine	0.14	i.v.	BOLD	4.7T	32	25	Awake	-	SD	Li	2008
Nicotine	0.03/0.10/0.30	i.v.	CBV	9.4T	30	25	Isoflurane	1.7-1.8%	SD	Zuo	2011
PCP	0.1/0.3/1.0	i.v.	ASL	4.7T	150	60	Isoflurane	2.0-2.5%	W	Risterucci	2005
PCP	0.5	i.v.	CBV	4.7T	40	20	Halothane	0.8%	SD	Gozzi	2008
PCP	0.5/1.0	i.v.	CBV	4.7T	80	20	Halothane	0.8-1.0%	SD	Gozzi	2008
PCP	0.5	i.v.	CBV	4.7T	40	30	Halothane	0.8%	SD	Gozzi	2008
PCP	0.1/0.3/1.0	i.v.	ASL	4.7T	60	30-60	Isoflurane	2.0-2.3%	W	Bruns	2009
PCP	0.5	i.v.	CBV	4.7T	40	30	Halothane	0.8%	SD	Gozzi	2010
PCP	5.6	i.p.	BOLD	7T	0.4	30	Isoflurane	1.0%	SD	Hackler	2010
PCP	0.5	i.v.	CBV	4.7T	23	30	Isoflurane	0.8%	LH	Broberg	2013

ASL, arterial spin labeling; BOLD, blood oxygenation level dependent; CBV, cerebral blood volume; LH, Lister hooded; PCP, phencyclidine; SD, Sprague-Dawley; TR, repetition time for different time points; W, Wistar.

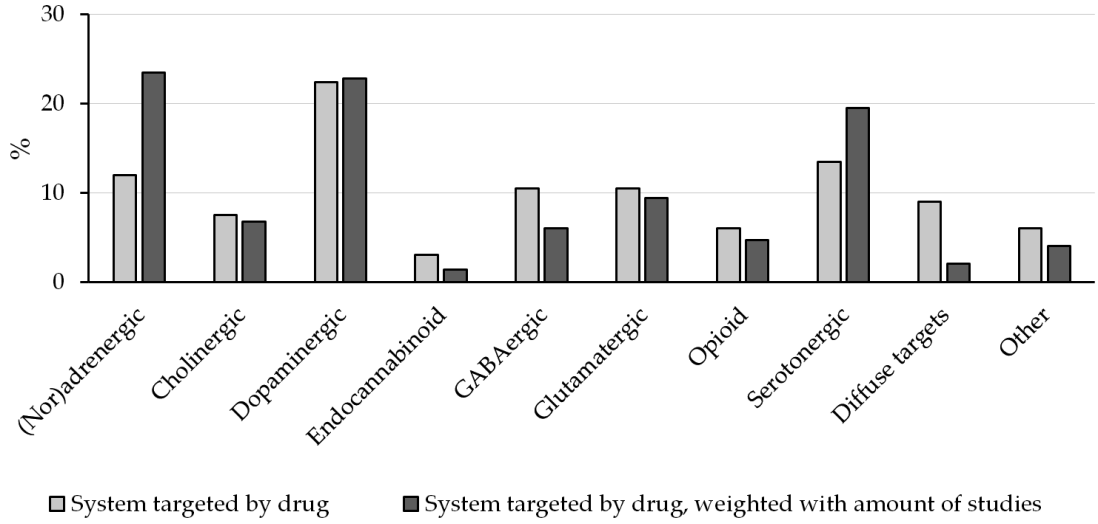

Figure 4. Primary neurotransmitter systems targeted by drugs in rat phMRI studies. The light grey bars indicate the distribution of 60 compounds (Table 1), while dark grey bars indicate the weighted distribution, where the amount of studies (n=129) has been taken into account. Reviewed from data represented by Haensel et al. (2015).

phMRI results under different anesthetics (e.g., Abo et al. 2004, Bruns et al. 2009, Du et al. 2009, Hodkinson et al. 2012, Liu et al. 2012). It is also important to remember that drugs may affect neurovascular coupling mechanisms similarly to anesthetics, and subsequently mask the hemodynamic response to neuronal activity (Hyder et al. 2002, Bourke and Wall 2015).

The drugs used in rat phMRI studies (Table 1) can be roughly categorized according to which neurotransmitter system they target (Figure 4). In most cases, only the primary transmitter system was used in the categorization, but in a few exceptions where the drug strongly interferes with two transmitter circuitries (for example cocaine: DA and serotonin systems) the secondary system has also been taken into account. The most common circuitries targeted by drugs during phMRI appear to be the (nor)adrenergic (~25 %), DAergic (~20 %), and serotonergic (~20 %) systems. Compounds having diffuse targets (such as 2-deoxy-D-glucose, arginine, formalin, and zymosan) have been studied only rarely (~2 %). Other systems, which include adenosine, growth hormone secretagogue receptor, and tachykinin receptor 1 systems, have been only rarely stimulated or suppressed during phMRI measurements (together ~4 %). These observations indicate that the majority of published phMRI experiments have focused on major neurotransmitter systems, which is most likely due to the fact that these systems evoke detectable fMRI signal changes more easily; the activation of minor systems, or peptide- or hormone-based neurotransmission, may lead to either too small or too slow changes in brain energy consumption to be detected with fMRI.

Even though the drugs studied in phMRI may be highly specific and target only a single neurotransmitter system, it is important to remember that neuropharmacological studies are typically not studies of a single circuitry, in fact the opposite is the case since the complex organization of neural networks is based on an intimate interplay between different systems. For instance, highly selective drugs modulating DAergic system may subsequently affect serotonergic, GABAergic, glutamatergic, and cholinergic circuitries (Jenkins 2012). The simultaneous manipulation of multiple receptor systems may considerably complicate the

interpretation of phMRI data (Liu et al. 2007), because only a net signal from many sources is obtained. For example, a phMRI investigation related to the acute effects of caffeine may be problematic (Bourke and Wall 2015). Caffeine is a CNS stimulant that modulates adenosine receptor activity, leading to changes in alertness and performance (Wesensten et al. 2002). These effects are mediated through antagonism of adenosine A_1 and A_{2A} receptors (Diukova et al. 2012). However, caffeine can also modulate the DAergic, noradrenergic, cholinergic, serotonergic, and endocannabinoid systems (Ferre 2008, Diukova et al. 2012). In addition to its effects on multiple neurotransmitter circuitries, the binding of caffeine to A_{2A} receptor induces vasoconstriction (Diukova et al. 2012), and thus the net effect observed in the fMRI signal may be dependent on the regional distribution of different receptor subtypes (vasoconstriction through A_{2A} vs. CNS stimulation through A_1 and A_{2A}) (Bourke and Wall 2015). Therefore, the interpretation of phMRI results obtained with even a well-known and extensively studied substance may not be straightforward, not to mention even the possible challenges encountered with experimental compounds having unknown effects on receptor systems and vasculature.

It is also noteworthy that if, as is suspected, fMRI signal changes follow closely brain energy consumption, then the main contributor to the changes in signal intensity may be the excitatory post-synaptic activity of glutamatergic signaling since it has a dominant role in excitatory neurotransmission, and also in the energy consumption of synaptic transmission (Attwell and Laughlin 2001). Therefore, the fMRI signal changes in phMRI studies may contain a pronounced glutamatergic contribution despite the fact that the administered drug has some other neurotransmitter systems as its primary target, which is the case in roughly 90 % of the studies (Figure 4).

Despite the disadvantages, it is generally agreed that non-invasive phMRI is an important method that offers a safe, unique, informative, and translational approach since it is possible to conduct repeatable drug investigations across species, with good temporal and spatial resolution (Leslie and James 2000, Steward et al. 2005, Jenkins 2012, Bourke and Wall 2015); most of the shortfalls in phMRI methodology can be compensated by appropriate study design (Bourke and Wall 2015) and complementary methods (e.g., microdialysis, electrophysiological measurements, or behavioral tests). Some of the disadvantages can even be considered as strengths of the method: phMRI can measure the total outcome of a pharmacologic stimulus (including all effects on neurotransmitter systems), and is not restricted to only localizing the receptor binding sites. When this property is combined with the sensitive endogenous hemodynamic contrast, phMRI may even allow the detection of small molecular level changes that are not achievable with other *in vivo* methods (Jenkins 2012).

2.2.5 Resting-state fMRI

As discussed in chapters 2.1.2 and 2.2.2, the brain accounts for a substantial proportion of the body´s total energy consumption at rest, and the consumption is typically not increased by more than 5 % while performing some task. Therefore, the vast majority of brain activity occurs spontaneously, making this spontaneous activity highly relevant topic for investigations aiming to further our understanding of brain function (Fox and Raichle 2007).

The investigation of spontaneous fluctuations in physiological parameters in brain is not a new concept, as the spontaneous temporal changes in oxygen availability and CBF have been studied since the 1950s (e.g., Clark et al. 1958, Moskalenko et al. 1964). These early investigations revealed region-specific irregular rhythmic fluctuations, which occurred at very low frequencies (<0.1Hz) and were distinct from breathing or heart rate. Several decades later in the 1990s, similar spontaneous activity was detected in fMRI BOLD signal. The factor that aroused scientific interest in spontaneous fMRI signal fluctuations was the whole-brain coverage achieved with MRI; the measurement was not spatially restricted compared to implanted electrodes (Smucny et al. 2014). More importantly, the analysis of whole-brain spontaneous BOLD activity revealed that several brain regions had very similar resting-state activities (Biswal et al. 1995). These findings led to revolutionary proposals that these region-specific spontaneous low frequency hemodynamic fluctuations may indirectly represent intrinsic neuronal activity and functional connectivity (FC) (Biswal et al. 1995, Lu and Stein 2014). Therefore, it was postulated that resting-state fMRI (rsfMRI) could be the long-awaited tool allowing researchers to non-invasively investigate brain as a dynamically active system consisting of connections between regions and/or networks (Smucny et al. 2014).

The early task-free fMRI investigations, or rsfMRI studies, investigated functional networks in the motor, auditory, and visual cortices in human brain (Biswal et al. 1995). These observations were confirmed later by many others, as well as the identification of several new networks (Fox et al. 2005, Smith et al. 2009), such as the most extensively studied network to date, the default mode network (DMN) (Greicius et al. 2003). Importantly, networks with similar spatial distributions have been observed across species (Smucny et al. 2014) and several core networks are preserved across arousal states (Nallasamy and Tsao 2011), which together suggest that resting-state activity is a fundamental and intrinsic feature of brain function.

There are extensive advantages associated with rsfMRI and the technique also has many applications. The acquisition of rsfMRI data is technically relatively fast and easy to perform and the measurement is conducted during rest; no external task or stimulus is required from the subject (Fox and Raichle 2007). Therefore, rsfMRI data can be obtained from patients that are unwilling or incapable to perform tasks, and from animals that cannot perform similar tasks as humans during fMRI (Fox and Raichle 2007, Smucny et al. 2014). Additionally, the rsfMRI provides information of whole-brain activity, whereas task-based fMRI experiments only focus on certain brain region(s).

Even though rsfMRI is a relatively recently introduced development, altered resting-state activity has already been recognized in several CNS diseases, such as in Alzheimer´s disease, multiple sclerosis, bipolar disorder, depression, SCZ, attention deficit hyperactivity disorder, autism, and epilepsy (see (Fox and Raichle 2007, Lu and Stein 2014, Smucny et al. 2014), and many of the changes in FC have been shown to correlate well with behavioral changes (Lu and Stein 2014). Additionally, several other states, such as addiction (Sutherland et al. 2012), sleep, and anesthesia (Nallasamy and Tsao 2011), have been found to affect the parameters of FC. These findings strongly indicate that the rsfMRI has a true potential in biomedical imaging, and have encouraged the adoption of this method in diagnostics, pathophysiology investigations, treatment evaluation, and drug development (Smucny et al. 2014).

Importantly, the translational feature of rsfMRI can be widely exploited in preclinical work. For example, studies done in humans have revealed that a dysfunction of several networks, such as salience, auditory, and cortico-subcortical networks, is associated with the symptom severity in SCZ. Therefore, rsfMRI offers a unique approach to exploit biomarkers, characterize and improve disease models to match the connectivity deficits in humans, and to evaluate the effects of novel treatments on brain connectivity in preclinical drug development.

In addition to the changes observed during different disease states, connectivity parameters are known to be affected by pharmacological treatments. A few early studies studied changes in FC preceding an acute or subchronic serotonergic, dopaminergic, glutamatergic, or cholinergic modulation (see (Smucny et al. 2014). Such studies can open new frontiers in phMRI experiments, as in addition to the acute drug response the long-term changes in FC can be evaluated. Subsequently, the drug-induced changes in brain connectivity can be associated with certain behavioral changes or deficits. Nevertheless, only a few exploratory studies have exploited such possibilities (Lu and Stein 2014, Smucny et al. 2014).

As mentioned above, anesthesia and arousal state modulate the resting-state activity. Several anesthetic or sedative agents, such as AC (Lu et al. 2007), ISO (Liu et al. 2013c), midazolam (Kiviniemi et al. 2005), thiopental (Kiviniemi et al. 2000), sevoflurane (Peltier et al. 2005), and propofol (Liu et al. 2013b, Barttfeld et al. 2015) have been found to affect FC parameters in a dose-dependent manner. Even though some of the studies have reported conflicting results, it is generally thought that only part of the networks (or connections) are suppressed by anesthetics, while the function of certain others are preserved even under deep anesthesia (Nallasamy and Tsao 2011, Bettinardi et al. 2015). For example, the DMN remains mainly functional during deep propofol (Bettinardi et al. 2015) and midazolam (Greicius et al. 2008) anesthesia, while several other networks covering cortical and subcortical regions are suppressed (Liu et al. 2013b). Somewhat similar observations have been made during MED sedation (Akeju et al. 2014). However, the anesthesia-induced changes appear to vary greatly between different anesthesia protocols (Nallasamy and Tsao 2011, Grandjean et al. 2014, Jonckers et al. 2014), which most likely originates from the different mechanisms of action of anesthetic agents. This includes also significant changes in MABP, which can strengthen or weaken the vasomotor fluctuations (Hudetz et al. 1992, Kiviniemi et al. 2005).

Based on these observations, two conclusions can be made: 1) a certain degree of resting-state activity is preserved through arousal states, suggesting that rsfMRI studies of subjects in a coma, vegetative state, sleep, under sedation, and under anesthesia are meaningful and informative, and 2) rsfMRI studies can shed light on the complex network-level mechanisms of anesthetics and loss of consciousness. Theoretically, one could argue that the rsfMRI parameters should provide information related to the responsiveness and status of the brain; in fact a few studies have already explored this topic by using somatosensory stimulus or a task-based activation (e.g., Lu et al. 2007, Kannurpatti et al. 2012).

Since the introduction of rsfMRI, several groups have been exploring the coupling between spontaneous BOLD fluctuations and electrophysiological neural activity. It is well known that the majority of electrical activity in brain occurs at remarkably higher frequencies than

0.1Hz, and thus investigators have examined whether such low frequencies reveal anything about brain state or connectivity. The best explanation to date is perhaps that the changes and fluctuations in high frequency ranges (e.g., 60-100Hz) have good correlations with the spectral power of the low frequencies (see (Fox and Raichle 2007). Although the exact biophysical mechanisms underlying the rsfMRI signal remain still unclear (Zhou et al. 2014), evidence is emerging to suggest that the spontaneous fluctuations are indeed surrogate markers for neural activity, and not just random noise (Fox and Raichle 2007). For example, the rsfMRI signal displays a good correlation with the lower band powers of the electroencephalography (EEG) signal (Lu et al. 2007, Hiltunen et al. 2014), spontaneous BOLD activity correlates well with infraslow local field potentials (LFPs) (Pan et al. 2013), and the calculated connectivity strength is similar between BOLD and spiking activity-related intracellular calcium levels (Vazquez et al. 2014). Additionally, axonal disconnection leads to region-specific diminished resting-state activity (Zhou et al. 2014).

Despite wide support, intense debate, even complete skepticism related to the reliability of the whole rsfMRI concept, still exists (Morcom and Fletcher 2007), as the method suffers from methodological issues and technical shortfalls. For example, one could ask how to control resting-state across subjects, or even within a single subject? The mental state, stream of consciousness, and vigilance would be expected to induce huge variability in the "resting-state" baseline. One could say that any rsfMRI measurement is just a poorly controlled task-based fMRI study (Fox and Raichle 2007). One argument has been postulated i.e. that the brain has two layers of cognitive processing, where the first layer consists of conscious self-referential mental activities, and second layer is concerned with core features of intrinsic activity that are not sensitive to changes in task processing (Fransson 2006). Therefore regardless of mental activities, the second layer should always represent "resting-state" and contribute to rsfMRI signal. Based on the concept introduced by Fransson (2006) and definitions of anesthesia (Nallasamy and Tsao 2011), the temporary loss of perception and self-referential mental processes could potentially bring the subject closer to the "resting-state".

From a more technical point of view, several authors have been concerned about the confounding effect of noise in rsfMRI studies (Lu and Stein 2014). Basically there are three main sources for the noise in fMRI measurements: scanner, motion of subject, and non-neuronal physiological activity. The noise induced by scanner hardware can be characterized with phantom measurements and small motions can be typically corrected with post-processing tools, but physiological noise originating from cardiovascular variables and breathing can be more problematic. The sampling frequency typically utilized in fMRI measurements (from 0.5Hz to 1Hz) is insufficient for the reliable detection of non-neural physiological fluctuations, at least in preclinical environment. Therefore, the physiological parameters have to be assessed separately with devices capable of measuring at higher sampling frequencies in order that they can be removed from the rsfMRI signal with linear regression approaches (Iannetti and Wise 2007). If physiological data are not available or cannot be measured, data-driven methods, such as independent component analysis (ICA), can be exploited to remove non-neuronal signal contributions (Steward et al. 2005). Despite these concerns, the impact of non-neuronal physiological noise appears to be minor in preclinical investigations, as shown in several studies (e.g., Majeed et al. 2009, Kalthoff et al. 2011). Additionally, recently developed ultra-fast imaging techniques can help to disentangle

non-neuronal physiological processes from neuronal fluctuations (Zahneisen et al. 2011, Asslander et al. 2013, Jacobs et al. 2014, Kiviniemi et al. 2016).

In summary, rsfMRI has demonstrated its potential in brain research by detecting functional network structures, and by providing system-level biomarkers for brain function during healthy and disease conditions. However, the biophysiological mechanisms underpinning the low frequency fluctuations still remain unclear, and the characterization of exact neuronal source(s) of the underlying resting-state activity will demand a considerable effort from the biomedical imaging community (Lu and Stein 2014). Fortunately, the noise removal, processing, and analysis (e.g., seed-based correlation and ICA) of rsfMRI data are rapidly evolving fields, and more sophisticated approaches are introduced regularly.

3 *Aims of the Study*

The general aim of this PhD thesis was to improve preclinical fMRI methodology, especially by implementing preclinical fMRI into drug research and development. There were the following specific aims:

1. To compare seven different anesthesia protocols in an acute nicotine challenge phMRI studies in rats.

As discussed earlier, the common requirement of anesthesia is one of the most problematic factors in preclinical fMRI experiments. Therefore, the main aim of study **I** was to compare phMRI results obtained under different anesthesia protocols, and explain the results in the light of the present neuropharmacological knowledge. Additionally, different fMRI contrasts were compared. These results can significantly help and guide researchers planning phMRI studies in future.

2. To utilize functional connectivity (FC) as a way to evaluate brain hemodynamic responsiveness in anesthetized rats.

The aim of study **II** was to investigate whether rsfMRI could be used to estimate the hemodynamic responsiveness of anesthetized rats. It is known that the depth of anesthesia affects rsfMRI parameters. Therefore, brain connectivity could also display a direct relationship with the brain responsiveness. If there were such a readily implementable measure, it could significantly improve the quality of preclinical fMRI studies, as both the outcome of anesthesia and brain responsiveness could be estimated during data interpretation.

3. To characterize phencyclidine-induced schizophrenia (SCZ)-like symptoms in rats by exploiting phMRI, rsfMRI, dopamine measurements, and behavioral tests.

The results of the studies **I** and **II** were utilized to establish an optimized fMRI protocol adopted in study **III**, where the aim was to characterize the dose-dependency of PCP-induced SCZ-like symptoms by exploiting a novel multimodal approach. As the preclinical models used to assess new drug candidates for SCZ have limited translational validity, a thorough characterization of an existing model could provide more specific applications for the development of novel drugs for the treatment of SCZ.

4 Materials and methods

This chapter describes the animal preparations, MRI experiments, and data processing and analyses of the original publications **I**, **II**, and **III**. Additionally, the materials and methods of microdialysis measurements and behavioral tests used in study **III** are explained concisely.

4.1 ANIMALS

All animal experiments were done with rats in the facilities of University of Eastern Finland. All procedures were approved by the National Animal Experiment Board (license numbers ESAVI-2011-002037, ESAVI-2012-932, ESAVI-2013-6446, and ESAVI-2014-7259), and conducted in accordance with the European Commission Directive guidelines (2010/63/EEC).

Adult male Wistar rats (**I**: *n*=79, 265-440g, **II**: *n*=47, 265-408g, and **III**: *n*=293, 240-385g) were used in all studies. Studies **I** and **II** exploited partly the same animals. If not stated otherwise, the rats were group-housed in cages in an animal room having an ambient temperature of 22±2°C, humidity of 50-60 %, and regular light-dark cycle of 12/12 h. Food and water were available *ad libitum*, if not stated otherwise.

4.1.1 Surgical procedures

Surgical procedures were performed with standard aseptic techniques, according to the recommended general and local anesthesia guidelines. A sufficient depth of anesthesia was confirmed by observing hind limb withdrawal reflexes, palpebral reflexes, and breathing rate before making the initial incisions. Normal body temperature (~37°C) was maintained by using a feedback-controlled heating pad during the surgery. Post-operative care was provided for procedures including recovery (*in vivo* microdialysis experiments in study **III**).

All rats undergoing the MRI experiments (**I**, **II**, and **III**) were first anesthetized with isoflurane (5 % for induction and 2 % for maintenance), delivered in a carrier gas mixture consisting of N_2/O_2 with proportions of 70/30. Small polyethylene cannulas were inserted into the femoral artery for blood sampling purposes, and into the femoral vein for the administration of pharmaceuticals. Both femoral veins were cannulated in animals that required constant delivery of intravenous anesthetic during the MRI measurement (**I** and **II**). A small tracheal tube was inserted when mechanical ventilation was used (**I**, **II**, and **III**). Additionally, a small craniotomy was performed, and an LFP electrode was implanted into the somatosensory cortex of six animals (**I)** to record neuronal electric activity simultaneously with fMRI acquisition. After completion of the surgical procedures, anesthetized animals were transferred to a warm rat holder in the MRI scanner and the head was fixed firmly with a bite bar and earplugs.

In addition to the MRI experiments, surgical procedures were conducted in study **III** prior to *in vivo* microdialysis experiments. In these experiments, the anesthesia was induced and maintained with a mixture of ketamine (60 mg/kg, i.p.) and MED (0.4 mg/kg, i.p.). When an

appropriate depth of anesthesia was reached, a guide cannula for a microdialysis probe was inserted into either medial prefrontal cortex (mPFC) or striatum. After surgery, atipamezole (0.5 mg/kg, s.c.) was used as an anti-sedative agent, and buprenorphine (0.02 mg/kg, s.c.) and carprofen (5.0 mg/kg, s.c.) were used to relieve post-operative pain. The animals were allowed to recover alone in cages for 3-5 days before the microdialysis experiments.

4.1.2 Anesthesia protocols

Several different anesthesia protocols were investigated in studies **I** and **II**, and the obtained results guided the selection of anesthesia protocol for the fMRI experiments in study **III**. Microdialysis experiments and behavioral tests in study **III** were conducted in awake animals.

Studies **I** and **II** included the following protocols after surgical procedures: AC (60 mg/kg initial bolus + additional 30 mg/kg bolus every 1 h, i.v.), ISO (1.3 %, inhaled), MED (0.01 mg/kg initial bolus + 0.1 mg/kg/h infusion, i.v.), TBB (140 mg/kg, i.p.), and URE (1250 mg/kg, i.p.). With all anesthetics, mechanical ventilation was applied to provide sufficient lung ventilation and normal blood gas values (**I** and **II**). Ventilation volumes were adjusted for each anesthesia protocol in separate bench testing experiments. A muscle relaxant, pancuronium bromide (~1 mg/kg initial bolus + ~0.5 mg/kg additional bolus every 1 h, i.v.), was administered to inhibit spontaneous breathing activity occurring against the ventilator. Additionally, the adequacy of protocols including spontaneously breathing subjects were evaluated with two anesthetics, TBB and URE, in study **I**. Inhaled air was the same N2/O2 70/30 mixture for all subjects, including a mechanically ventilated and some spontaneously breathing animals.

The rats undergoing fMRI measurements in study **III** were anesthetized with URE (1000 mg/kg, i.v.), as the results of study **I** together with the literature review indicated that URE may well be the most suitable anesthetic for phMRI investigations. Additionally, the results from study **II** indicated that intraperitoneal administration of anesthetic may induce higher variability to brain connectivity compared to the intravenous route; between-subject variability in intraperitoneal administrations can lead to substantially different extents of absorption of anesthetic (Svendsen 2005), and subsequently to significantly different depths of anesthesia. This was later confirmed by comparing FC of rats anesthetized with intraperitoneal and intravenous URE (unpublished data); the rats anesthetized with intravenous urethane had significantly smaller variance in FC data. Therefore, the intravenous route was used in study **III**. No clear correlation was observed between anesthetic depth and BOLD responses under URE anesthesia (**II**), which may suggest either robust responsiveness under various anesthesia levels, or disturbance of different sleep-like states in FC (Zhurakovskaya et al. 2016). All rats in study **III** were mechanically ventilated, and the muscle relaxant (pancuronium bromide, 0.5 mg/kg/h, i.v.) was administered as a constant infusion throughout the fMRI measurements.

4.1.3 Physiology monitoring

Arterial blood was sampled (sample size of 0.15 ml) twice during each fMRI experiment, and analyzed immediately for pCO_2, pO_2, sO_2, and pH values. Based on the results of the first blood sample, mechanical ventilation or inhaled O_2 were adjusted if the blood gas values

were not within the normal physiologic range. A second sample was used to estimate the physiological condition of each animal after the phMRI experiment. An MRI-compatible monitoring system was used for real-time physiology monitoring, typically covering rectal temperature, heart rate, breathing rate, exhaled CO_2, and oxygen saturation. The rats were kept warm throughout the measurements by circulating warm water in the rat holder of the MRI scanner. If the warm water circulation was insufficient, a blanket was placed over the animal. In addition to these standard procedures during the fMRI experiments, MABP was measured from three rats in study **III** to gain a crude estimate of any possible changes in MABP after an acute high-dose phencyclidine challenge.

4.2 MRI EXPERIMENTS

All MRI experiments were conducted in the A.I.Virtanen Institute for Molecular Sciences, University of Eastern Finland, in the preclinical MRI facilities maintained by the Biomedical NMR research group.

4.2.1 Hardware

Two different MRI systems were used. The majority of the measurements in study **I**, and all experiments in studies **II** and **III** were conducted with a horizontal 7T Bruker Pharmascan. In the simultaneous electrophysiological and fMRI measurements in study **I**, a horizontal 9.4T Varian system was used. This was because the Faraday cage around the 9.4T system provided a better basis for electrophysiological measurements. For excitation, quadrature resonator volume coil (Bruker) and actively decoupled volume radiofrequency coil (Varian) were used. For signal reception, rat brain quadrature surface coils were used with both magnets. The MRI systems were operated and data recorded either with Paravision 5.1 (7T) or Varian DirectDrive™ console (9.4T).

4.2.2 Anatomical imaging

Prior to each MRI session, a pilot scan was performed to adjust the center of the cerebrum of the animal close to the isocenter of the MRI scanner. Subsequently, an automated global shimming procedure was done, following a fieldmap-based local shimming implementation for a voxel (8 x 12 x 15 mm^3) covering the cerebrum. Anatomical images were acquired during each MRI experiment (**I**, **II**, and **III**). Although the anatomical data sets were not shown or exploited in the original publications **I** and **II**, it is important to visually inspect the brain for any spontaneous abnormalities that could influence neuronal functions.

The high-resolution anatomical images were acquired with a 7T Bruker system by using the TurboRARE-T2 sequence with whole-brain coverage. The following parameters were used: repetition time 4.7 s, echo time 16.1 ms, effective echo time 48.4 ms, echo train length 8, field-of-view 5.0x5.0 cm^2, matrix size 512x512, 98x98 μm^2 in-plane resolution, and 30 slices with a thickness of 0.75 mm. A similar, T_2-weighted fast spin-echo sequence was used to obtain anatomical images with the 9.4T Varian system: repetition time 3.0 s, echo time 16.0 ms, effective echo time 48.0 ms, echo train length 8, field-of-view 5.0x5.0 cm^2, matrix size 512x512, 98x98 μm^2 in-plane resolution, and ~30 slices with a thickness of 0.75 mm.

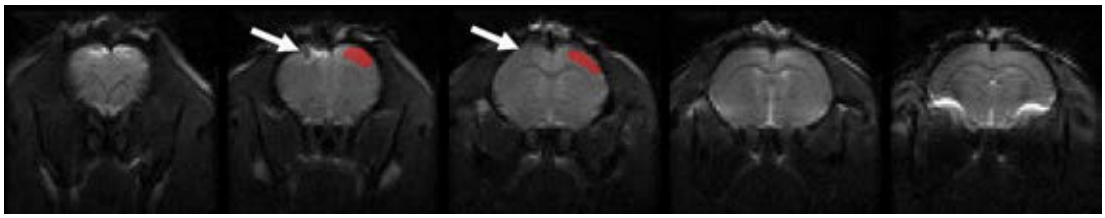

Figure 5. Original spin-echo echo planar imaging images from simultaneous local field potential and fMRI measurements at 9.4T in study **I**. Five representative 1.5 mm thick slices (anterior-posterior, left-right) are shown. The location of electrode (or minor susceptibility-induced distortion) is shown with white arrows. Red region indicates the contralateral region-of-interest for pharmacologic MRI analysis.

4.2.3 Functional imaging

The majority of the fMRI measurements in study **I**, and all fMRI measurements in studies **II** and **III** utilized BOLD contrast as an indirect measure of neuronal activity. A subset of animals in study **I** were imaged by using CBV-weighted protocol or functional CBF measurements.

Before functional imaging, 1st and 2nd order global shimming was typically performed. Subsequently, a field map –based shimming implementation was exploited to optimize the local shim in the brain. In all measurements done with 7T, the local shim voxel was 8 x 12 x 15 mm^3, which covered roughly the whole cerebrum. A smaller voxel (4 x 8 x 11mm^3) covering the interhemispheric cortical regions around the electrode was used in simultaneous LFP and BOLD measurements at 9.4T, as the interest was in the somatosensory cortex where the electrode was implanted.

All functional imaging in this thesis were based on single-shot spin-echo echo planar imaging sequences. As discussed earlier, spin-echo has higher specificity because of the decreased contribution of draining vessels (Lee et al. 1999). Additionally, the image deformation induced by electrodes and subsequent magnetic field inhomogeneities can be minimized by using spin-echo sequences in simultaneous LFP and fMRI measurements (see Figure 5). The fMRI parameters used in each original publication are listed in Table 3. The CBF

Table 3. The parameters of single-shot spin-echo echo planar imaging sequences used in functional imaging in this thesis.

Study	Contrast	B_0	Bandwidth (kHz)	TR (s)	TE (ms)	FOV (cm)	Matrix	In-plane resolution (μm)	Slices (number x mm)
I	BOLD	7T	250	2.0	45	2.5 x 2.5	64 x 64	391 x 391	9 x 1.5
	CBF	7T	250	6.0	24	3.5 x 3.5	64 x 64	547 x 547	5 x 2.0
	CBV	7T	250	2.0	24	2.5 x 2.5	64 x 64	391 x 391	9 x 1.5
	BOLD / LFP	9.4T	250	4.0	35	2.5 x 2.5	64 x 64	391 x 391	15 x 1.5
II	BOLD	7T	250	2.0	45	2.5 x 2.5	64 x 64	391 x 391	9 x 1.5
III	BOLD	7T	200	2.0	45	2.5 x 2.5	64 x 64	391 x 391	11 x 1.5

BOLD, blood oxygenation level dependent; CBF, cerebral blood flow; CBV, cerebral blood volume; FOV, field-of-view; LFP, local field potential; TE, echo time; TR, repetition time.

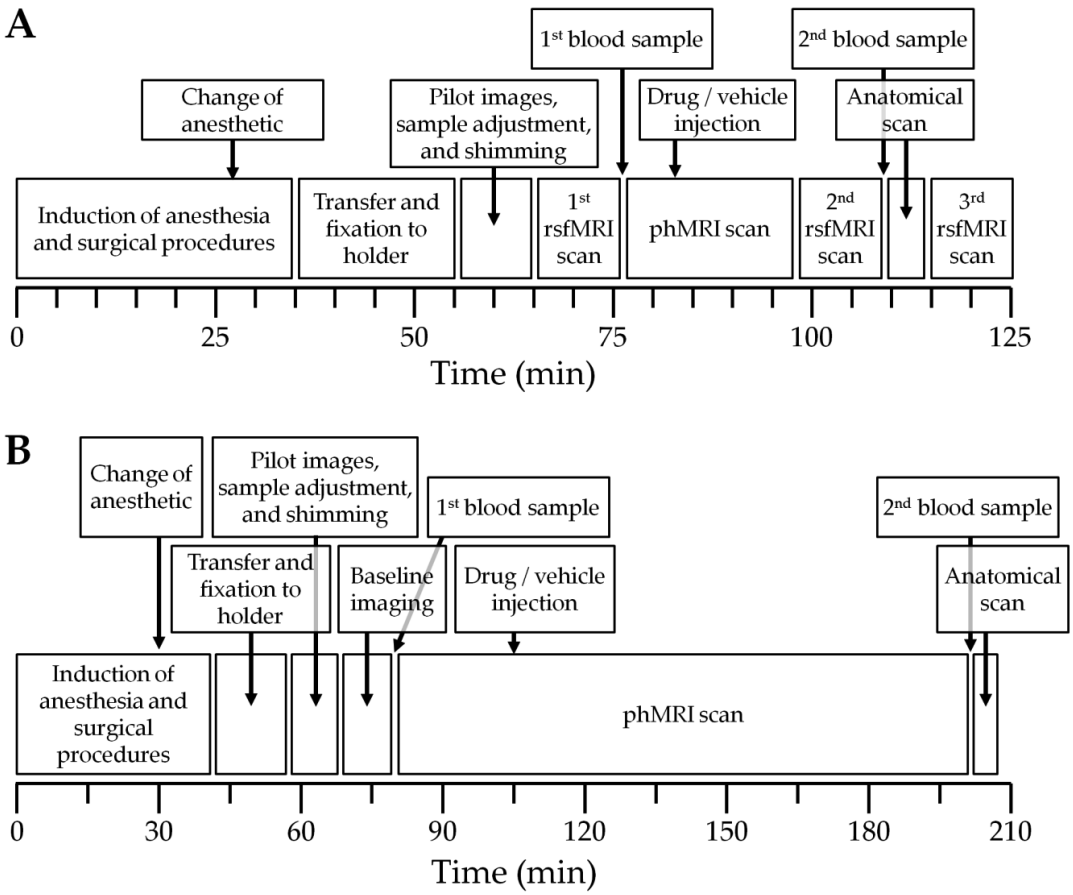

Figure 6. Schematic illustrations of typical timeline of fMRI experiments in studies **I** and **II** (A), and in study **III** (B).

measurements were based on double adiabatic inversion arterial spin labeling technique (Alsop and Detre 1998).

In studies **I** and **II**, nicotine (0.25 mg/kg tartrate salt, 88 μg/kg free base, i.v.) was administered to rats during the phMRI scan. In study **III**, PCP (1, 2, 3, or 5 mg/kg, hydrochloride salt, s.c.) was injected instead of nicotine. Physiological saline was administered to control animals. To obtain CBV-weighted contrast in study **I**, superparamagnetic ferumoxytol (25 mg/kg) was given to rats ~10 min before starting fMRI acquisition. Additionally, for a subset of animals (n=8) in study **II**, forepaw stimulations (1-4 per paw per subject) were performed with the following parameters: 0.3 ms pulse length, 10 Hz frequency, 1.2 mA current, and 3x30 s pulse trains with a 1 min baseline between trains. Each forepaw stimulation fMRI scan included 180 volumes (6 min), and forepaws were stimulated one at a time.

The timelines of typical phMRI/rsfMRI experiments in studies **I**-**III** are shown in Figure 6. Most of the experiments in study **I** and **II** closely followed the scheme in Figure 6A. In study **I**, a few experiments included pharmacological pretreatment (mecamylamine, a nAChR antagonist, 2 mg/kg, i.v.), which increased the length of the experiments by about 30 min. In study **II**, a subset of animals underwent complementary forepaw stimulation experiments, which also increased the length of measurements by about 30 min. The design of MRIb

experiments in study **III** is shown in Figure 6B. After completion of the MRI measurements, rats were immediately sacrificed by receiving 5 % ISO, an intravenous bolus of concentrated potassium chloride, and cervical dislocation.

4.3 *IN VIVO* MICRODIALYSIS EXPERIMENTS

In addition to fMRI experiments, study **III** included *in vivo* microdialysis experiments to determine extracellular DA and PCP concentrations during the acute PCP challenge from two brain regions associated with disturbed neurotransmission in SCZ. The results were subsequently compared with fMRI findings.

Prior to microdialysis experiments, a guide cannula was surgically implanted (see chapter 4.1.1). Before the start of the experiments, rats were transferred to a microdialysis bowl, and a microdialysis probe, perfused with artificial cerebrospinal fluid (0.5 μl/min), was inserted into the brain through the implanted guide cannula. Collection of microdialysate was started after 15 h of washout time, and a 1 h stabilization period (2.0 μl/min). The baseline samples were collected for the first 60 min, after which PCP (1, 2, or 3 mg/kg, s.c.) or saline was injected. The sample collection, as 20 min fractions, was continued for 5 h. For PCP quantification, the microdialysis probe was calibrated with the retrodialysis by drug approach (Bouw and Hammarlund-Udenaes 1998). DA levels were quantified by using an ultra-high performance liquid chromatography system with electrochemical detection. PCP concentrations were quantified by liquid chromatography coupled with an electrospray ionization triple quadrupole mass spectrometer.

4.4 BEHAVIORAL TESTS

Study **III** included also behavioral tests, which aimed to complement the fMRI and microdialysis experiments.

4.4.1 Locomotor activity

The rats were given PCP (1, 3, or 5 mg/kg, s.c.) or saline. Subsequently, the rats were transferred to open field arenas, and locomotor activity was recorded for 2 h. The distance traveled was calculated offline by using EthoVision XT 8.5 video tracking software.

4.4.2 Social interaction

The rats were housed alone in cages for 4-7 days prior to the social interaction test. A pair of rats, unfamiliar to each other, received the same treatment (PCP 1.5 mg/kg or saline, s.c.) 45 min before the test. Subsequently, rats were placed in open field arena, and social interaction behavior was recorded for 10 min. Data were analyzed with EthoVision XT 8.5 software by a researcher blind to the treatment. The total time for social interaction, including sniffing, following, walking around a partner, or mutual grooming, was calculated. Passive or aggressive interactions were not considered as social interaction.

4.4.3 Novel object recognition

Prior to the novel object recognition tests, rats were habituated to open field arenas for two days (10 min/day). Two similar objects (A1 and A2, 10±2 cm), made of metal and glass, were placed in the arena before the first experiment. Rats were treated with either PCP 2.0 mg/kg or saline 45 min before the test, and allowed to investigate the identical objects for 3 min. After the first experiment, the objects A1 and A2 were changed to objects A3 and B1, where A3 was similar to A1 and A2, and B1 novel. The test was repeated with new objects 24 h after the first day. Licking, sniffing or touching the object were considered as object exploration, while leaning against, turning around, or sitting on the object were not. Data were recorded and analyzed with EthoVision XT 8.5 software.

4.5 DATA PREPROCESSING, ANALYSES, AND STATISTICAL TESTS

All fMRI data were preprocessed by using the same pipeline shown in Figure 7. First, the fMRI data provided by the scanner software were converted to the more standard NIfTI (Neuroimaging Informatics Technology Initiative) format by using Aedes (aedes.uef.fi). All subsequent preprocessing was done with Statistical Parametric Mapping 8 (SPM8, www.fil.ion.ucl.uk/spm). As the acquisition of multiple slices in fMRI measurements can lead to considerable time differences between slices (up to ~1.8s), the timing of slices was corrected with a slice-timing correction. However, the correction was not made, if the maximum difference between first and last acquired slice was less than 750ms. Next, data were motion-corrected. Although the majority of the experiments were done with paralyzed subjects, and non-paralyzed animals did not show clear movement, motion correction was important for removing hardware-based spatial drifts. Low-frequency drifts (up to 0.015Hz) in fMRI data originate typically from scanner instabilities, and exist in all samples (Smith et al. 1999, Steward et al. 2005). The subsequent smoothing (2x2 voxel full-width at half-maximum Gaussian kernel) averaged out noise, and enhanced the detection of non-random events in the signal. In the last step, the fMRI data were co-registered to a reference brain, which was selected from the acquired data. The same reference brain was used for co-registrations in all BOLD studies.

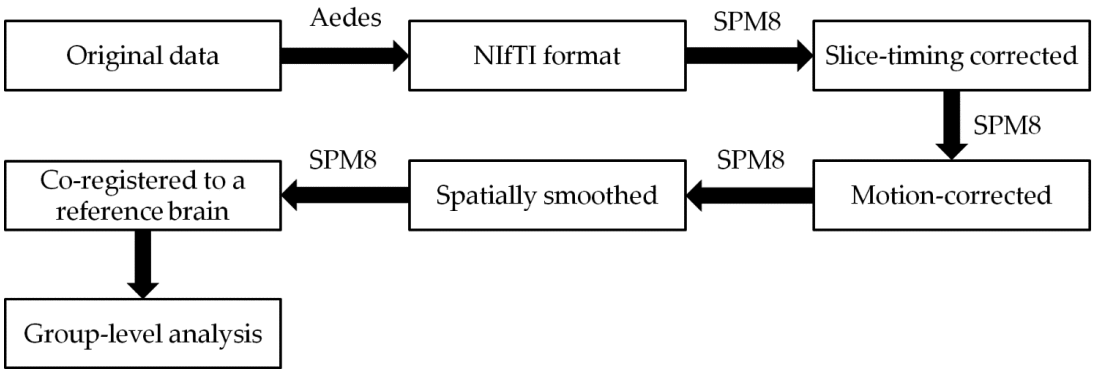

Figure 7. Schematic illustration of the standard fMRI data preprocessing pipeline exploited in all original publications. NIfTI, Neuroimaging Informatics Technology Initiative; SPM8, Statistical Parametric Mapping version 8.

The majority of the fMRI analyses and group-level average or median calculations were done with custom-built Matlab (2011a) codes, and visual inspections and preparations of MRI images for publications were done with Aedes. Regions of interest (ROIs) were drawn on the reference brain according to an anatomic atlas (Paxinos and Watson 1998). The phMRI responses in study **I** were localized with the ICA toolbox (GIFT, version 2.0a). The more traditional approach, general linear model (GLM), was not used, because the response profiles varied extensively between groups; the aim of study **I** was to compare anesthesia protocols, and any chosen block model would have favored some groups more than others. Therefore, a model-free and data-driven alternative (ICA) was implemented for phMRI analyses. In study **III**, the drug-induced responses were localized with voxel-wise group-level t-tests between drug treatment and control for each time-point. The BOLD responses in study **III** were slowly evolving, making the GLM approach with block design again unsuitable. Additionally, the long (2 h) fMRI scan led to confounding baseline drifts, which complicated even more the use of GLM. The average non-linear baseline drift was estimated from saline group ROI time series, and subsequently removed from all subjects for visualization of ROI time courses (**III**).

FC was analyzed by exploiting coherence (**II**) or the more common correlation (**III**) calculations between predefined ROIs. Prior to FC calculations, the low-frequency linear drift was removed from the fMRI time series, and data were low-pass filtered at 0.15Hz. The comparison between correlation values and different coherence frequency ranges (Figure 8) calculated from the same data indicated that the correlation and coherence analyses produce similar information at lower coherence frequencies (0.01-0.05Hz). Therefore, this range was used as a measure for FC in study **II**. However, Figure 8 also shows that at higher coherence frequencies, the information with respect to the correlation may differ. Therefore, coherence analysis is more informative approach in rsfMRI analysis than traditional correlation analysis.

Statistical tests were performed either with GraphPad Prism (Version 5.03) or Matlab (Version 2011a or 2014a), and statistical significance was determined using p-values <0.05, <0.01, <0.005, and <0.001. As the group sizes in studies **I** and **II** were relatively small (~5-10 subjects), mostly nonparametric measures (median ± quartiles) and statistical testing (e.g.,

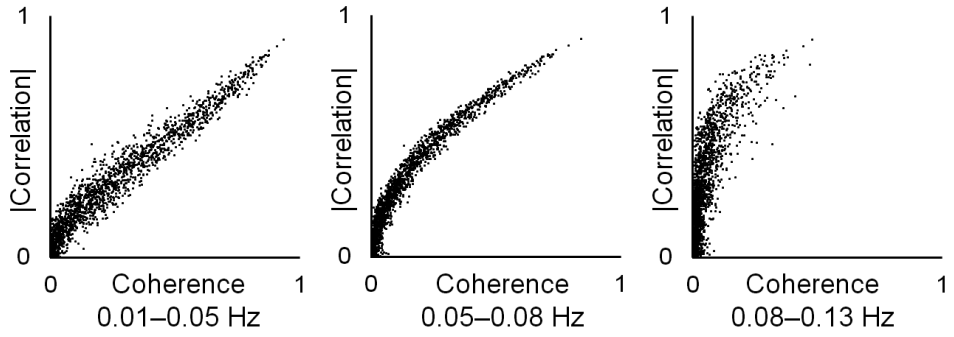

Figure 8. Relationship between correlation and coherence values at different coherence frequencies. Correlation and coherence values were obtained from 91 ROI pairs at three different frequency ranges from 10 thiobutabarbital-anesthetized rats. The values in lower coherence frequency range (0.01-0.05 Hz) highly resemble the correlation values, while the similarity decreases along the higher coherence frequencies.

Mann-Whitney U test, Spearman rank correlation, and Kruskal-Wallis ANOVA) were applied. However, paired t-tests were used when correlation matrices were compared (**II**). In study **III**, the group sizes were generally larger compared to studies **I** and **II**, especially in locomotor activity measurements and novel object recognition tests (~20-30 subjects in each group). Therefore, parametric measures (average ± standard error of mean) and testing (e.g. t-test, Pearson correlation, and ANOVA) were applied when performing statistical tests for the behavioral data (**III**). For consistency, parametric measures and methods were used also for the fMRI and *in vivo* microdialysis data in study **III**. In all individual analyses, multiple comparison corrections (e.g., false discovery rate, Tukey´s, or Dunnett's post-hoc tests) were made.

5 Results

Different fMRI approaches were exploited to compare anesthesia protocols for phMRI (**I**), to investigate the possibility of using rsfMRI as a tool to measure brain responsiveness (**II**), and to characterize phencyclidine-induced dose-dependent functional changes in brain (**III**). Additionally, *in vivo* microdialysis experiments and behavioral tests complemented the fMRI findings in study **III**.

The analyses of arterial blood samples indicated that the blood gas values of subjects undergoing fMRI experiments were within the normal physiological range throughout the measurements (**I**, **II**, and **III**), except in the spontaneously breathing TBB group in study **I** (Kruskal-Wallis, $p<0.005$), where hypercapnia was observed.

5.1 COMPARISON OF ANESTHESIA PROTOCOLS FOR NICOTINE PHMRI (I)

All rats receiving acute administration of nicotine (88 µg/kg, i.v.) showed a robust BOLD signal changes, while rats receiving saline did not display any responses. Apart from the TBB group, the responses were consistent within each anesthesia protocol. In the TBB group, two clearly different response profiles were observed (Figure 9), and animals were subsequently divided into two groups – TBB subgroup 1 (n=4, clear positive responses) and TBB subgroup 2 (n=6, mainly negative responses). Maximum BOLD signal changes among ROIs were significantly different between TBB subgroups 1 and 2 (Mann-Whitney, $p<0.001$).

The spatial localization of nicotine-induced signal changes in brain was rather similar among the various anesthesia protocols, although some differences were observed in cortical and subcortical regions. However, the inspection of BOLD time series showed obvious

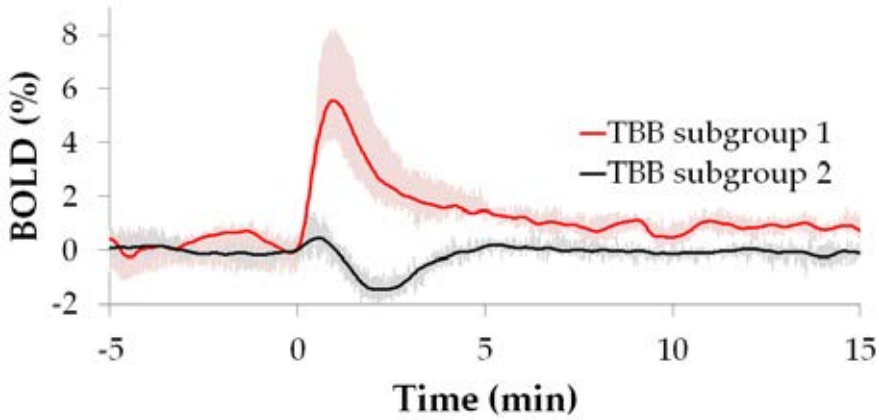

Figure 9. Median blood oxygenation level dependent (BOLD) signal measured from the somatosensory cortex of mechanically ventilated thiobutabarbital-anesthetized (TBB) rats (total *n*=10). Shaded regions indicates the interquartile ranges. Nicotine (88 µg/kg, i.v.) was given at time zero.

Table 4. Summary of the results obtained from anesthesia protocol comparison (**I)**. The blood oxygenation level dependent (BOLD) signal changes were studied after an acute nicotine (88 µg/kg, i.v.) challenge. The following measures, or means, were used for categorization purposes: group-level average area under curve (AUC) values for BOLD responses; visual comparison of group-level activation maps to autoradiography results (Mugnaini et al. 2002); and group-level average time (seconds) for BOLD responses to reach peak, and decay to half maximum. For cortical AUC, values <300 = poor, 300-500 = moderate, 501-800 = good, and >800 = excellent. For subcortical AUC, values (a.u.) <250 = poor, 250-325 = moderate, 326-400 = good, and >400 = excellent. The rise of the BOLD signal to peak in subcortical regions was categorized as follows: values (seconds) <230 = fast, 230-240 = intermediate, and >240 = slow. The thresholds for subcortical response decay (seconds) were <275 = fast, 275-290 = intermediate, and >290 slow

		AC	ISO	MED	TBB SB	TBB SG1	URE SB	URE
Cortical regions	BOLD responses	Poor	Moderate	Good	Moderate	Good	Excellent	Excellent
	Localization compared to binding sites	Poor	Good	Good	Good	Good	Good	Good
	Response rise	Intermediate	Slow	Slow	Fast	Fast	Fast	Fast
	Response decay	Fast	Slow	Slow	Fast	Intermediate	Slow	Slow
Subcortical regions	BOLD responses	Moderate	Poor	Good	Good	Good	Good	Excellent
	Localization compared to binding sites	Good	Poor	Moderate	Good	Good	Good	Good
	Response rise	Intermediate	Slow	Slow	Fast	Fast	Fast	Fast
	Response decay	Slow	Intermediate	Intermediate	Fast	Fast	Fast	Fast

AC, α-chloralose; BOLD, blood oxygenation level dependent; ISO, isoflurane; MED, medetomidine; SB, spontaneously breathing; SG1, subgroup 1; TBB, thiobutabarbital; URE, urethane.

differences between the groups. For instance, the mean (± standard deviation) positive BOLD signal changes obtained from somatosensory cortex were as follows: AC, 1.0±0.9 %; ISO, 2.6±0.5 %; MED, 3.4±1.2 %; TBB (spontaneously breathing), 4.9±1.0 %; TBB subgroup 1, 6.2±3.7 %; URE (spontaneously breathing), 10.0±4.3 %; and URE 7.8±2.7 %. At the group-level, significant differences in response amplitudes were observed in several cortical regions, interpreted as anesthesia protocol-dependent BOLD responses to nicotine. Although the mean responses were indicative of different BOLD responses to nicotine also in subcortical ROIs, no significant differences were observed in any subcortical region (ANOVA and Tukey´s multiple comparison post-hoc test). This may be, at least partly, because of the lower amount of nAChRs and lower signal-to-noise ratio of deeper brain structures, which may hinder the detection of differences in BOLD responses.

In addition to the response amplitudes, differences between anesthesia protocols were observed in several other parameters, such as in the temporal development of the BOLD response. For example, the mean (± standard deviation) times for BOLD responses to reach its peak in hippocampus after the acute nicotine challenge were as follows: AC, 242±19s; ISO, 253±27s; MED, 255±23s; TBB (spontaneously breathing), 221±4s; TBB subgroup 1, 228±6s; URE (spontaneously breathing), 228±23s; and URE 221±2s; the fastest BOLD peak in hippocampus was observed in the TBB group, while the slowest was acquired in the MED group (~30s difference, $p<0.01$, Mann-Whitney test).

Table 4 summarizes the characteristics of nicotine-induced fMRI signal changes obtained under different anesthesia protocols. Clear and significant differences were observed

between anesthesia protocols, and the highest and most robust BOLD responses were unquestionably acquired under URE anesthesia (with both mechanically ventilated and spontaneously breathing animals). As URE is used only rarely in phMRI experiments (see chapter 2.2.3), we characterized further the drug-induced hemodynamic changes by conducting CBV-weighted and CBF measurements to estimate the relationship between BOLD, CBV, and CBF under URE anesthesia. Additionally, the coupling between electrophysiological activity and drug-induced hemodynamic responses under URE anesthesia was determined with simultaneous LFP and BOLD measurements.

The nicotine-induced responses were consistent between BOLD, CBV, and CBF contrasts under URE anesthesia. The maximum responses among ROIs showed high linear correlation between measures (BOLD vs. CBV r^2=0.73, BOLD vs. CBF r^2=0.70, and CBV vs. CBF r^2=0.88; $p<0.001$ in all cases). Calculations of cerebral metabolic rate of oxygen, based on the separate BOLD, CBV, and CBF measurements, indicated a transient increase of ~40 % after nicotine injection, which is in good agreement with previous findings (Hyder et al. 2000). In simultaneous BOLD and LFP measurements, nicotine decreased the spectral power at lower frequencies (0.3-1.4 Hz), but increased spectral power at higher frequencies (13-70 Hz); a good correlation (r^2=0.43, $p<0.001$) was observed between cortical LFP (spectral power of 13-70 Hz) and BOLD time series, similar to what has been reported earlier (Logothetis et al. 2001, Kayser et al. 2004, Mukamel et al. 2005, Niessing et al. 2005, Viswanathan and Freeman 2007,

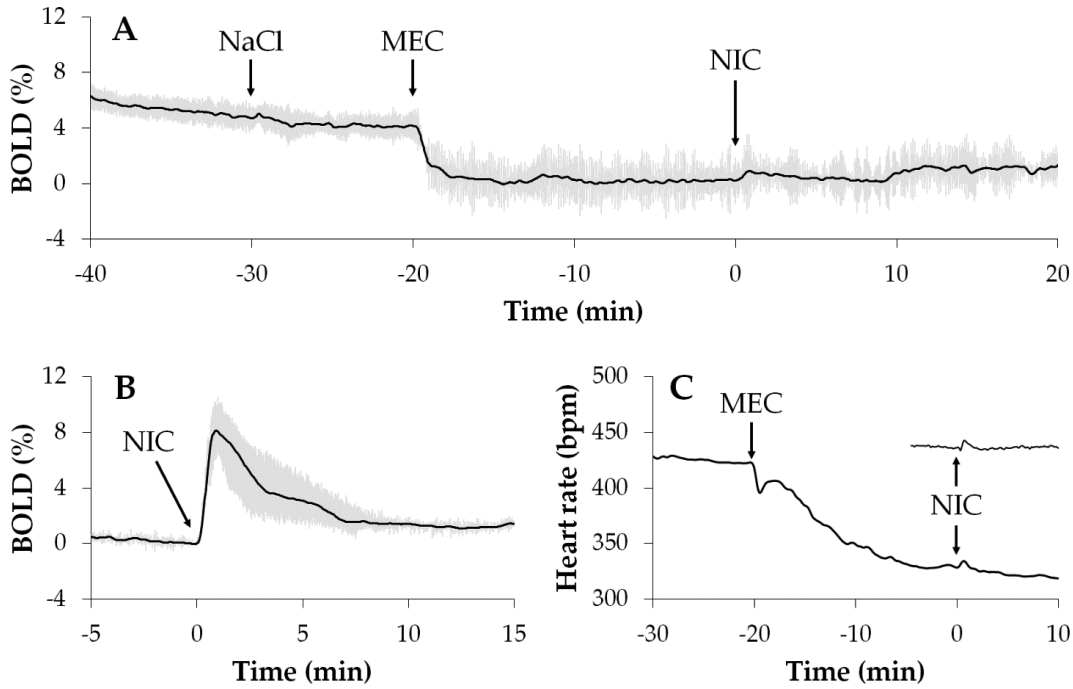

Figure 10. Blood oxygenation level dependent (BOLD) time series (A, n=2; B, n=6), and heart rate time series (C, n=2) during mecamylamine (2 mg/kg, i.v.) and nicotine (88 µg/kg, i.v.) treatments in urethane-anesthetized rats. Mecamylamine, given 20 min prior to nicotine, blocked the nicotine-induced hemodynamic response (A and B). Mecamylamine decreased heart rate (C). However, similar nicotine-induced increase of 1.0-1.5 % in heart rate was detected with and without mecamylamine pre-treatment (C). BOLD, blood oxygenation level dependent; MEC, mecamylamine; NaCl, sodium chloride; NIC, nicotine.

Goense and Logothetis 2008, Lippert et al. 2010). These experiments confirmed that a neuronal source underlies the fMRI signal changes, and that normal hemodynamic coupling is preserved under URE anesthesia.

As expected, the nicotine-response was successfully blocked by pre-treatment with a nAChR antagonist mecamylamine (Figure 10, compare A and B) at the receptor level. Even with the pre-treatment, similar increases (1-2 %) in heart rates were detected (Figure 10C). This observation suggests that the nicotine-induced increase in cardiac output in the present work is not contributing to BOLD responses in brain, further supporting the neural origin of nicotine-induced BOLD responses during URE anesthesia.

5.2 FUNCTIONAL CONNECTIVITY AND HEMODYNAMIC RESPONSIVENESS (II)

As rsfMRI data was obtained during the nicotine phMRI experiments (**I**), it was possible to evaluate the coupling between phMRI responses and FC (**II**). The initial rsfMRI analysis of TBB-anesthetized rats indicated a similar grouping pattern to that obtained in study **I** with acute nicotine challenge; the rats with a clear and positive nicotine response (TBB subgroup 1) had significantly (t-test, $p<0.001$) stronger coherence (0.01-0.05 Hz) values between ROIs (0.43±0.24) than the rats with negative responses (TBB subgroup 2; 0.11±0.11) (Figure 11). Differences were observed also in higher coherence frequency ranges (>0.05 Hz), as shown in Figures 11 and 12. These findings 1) provided a possible explanation for the different nicotine response types obtained with TBB-anesthetized rats (**I**), and 2) pointed to a robust relationship between parameters of FC and brain hemodynamic responsiveness to external stimuli.

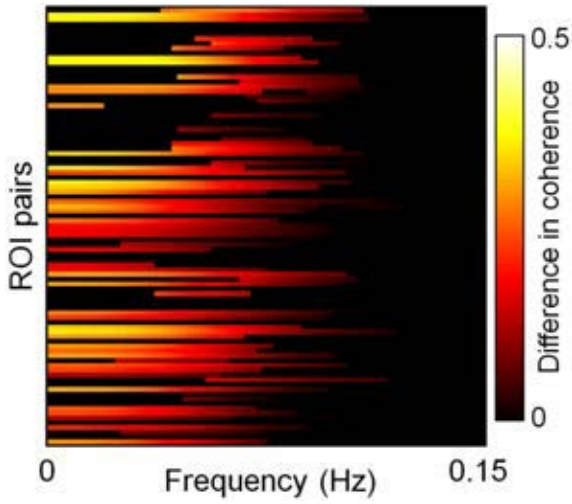

Figure 11. The group-level differences in coherence spectra ($p<0.05$, false discovery rate corrected) between thiobutabarbital subgroups 1 and 2. Total of 91 region of interest pairs (formed from 14 regions) are distributed on the y-axis, while frequency range (0.00-0.15Hz) is on x-axis, and the absolute difference in mean coherence values is color coded (black<red<yellow). Only statistically significant differences are shown. ROI, region of interest.

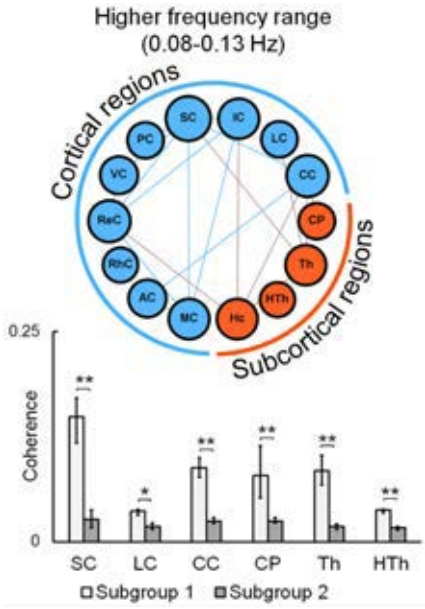

Figure 12. The group-level differences in functional connectivity between thiobutabarbital subgroups 1 and 2 at higher coherence frequency range (0.08-0.13 Hz). Edges in graph represent the differences ($p<0.005$, false discovery rate corrected) in region-specific coherence values between subgroups. Averaged coherence of six representative brain regions are shown below the graph (Mann-Whitney, $*p<0.05$, $**p<0.01$). AC, auditory cortex; CC, cingulate cortex; CP, caudate putamen; Hc, hippocampus; HTh, hypothalamus; IC, insular cortex; LC, limbic cortex; MC, motor cortex; PC, parietal association cortex; ReC, retrosplenial cortex; RhC, rhinal cortex; SC, somatosensory cortex; Th, thalamus; VC, visual cortex.

The analysis of Spearman´s rank correlation coefficients showed evident coupling between the coherence values and subsequent nicotine-induced AUC values in TBB animals ($\varrho = 0.45$-0.85 including all ROIs). Additionally, good correlation values were observed under ISO (e.g., $\varrho = 0.43$-0.97 in cortical ROIs) and MED (e.g., $\varrho = 0.49$-1.00 in subcortical ROIs) anesthesia. In contrast, the correlation coefficients were only poor or negligible in AC and URE groups, indicating no clear association between FC and BOLD responses under these anesthetics. The poor correlation between AUC and FC values in AC group may originate from the weak nicotine responses (**I**), while in URE group the different sleep-like states induced by URE may induce variability to connectivity values (Zhurakovskaya et al. 2016)

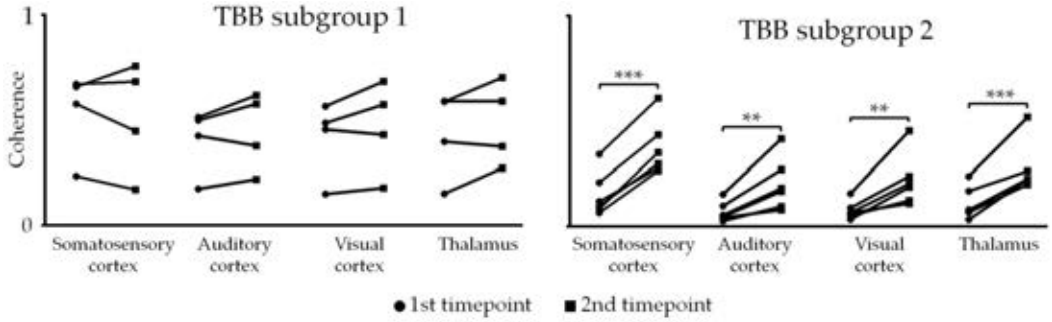

Figure 13. Functional connectivities (0.01-0.05 Hz) of representative regions plotted from thiobutabarbital (TBB) subgroups 1 and 2. After 1 h, the connectivity increases in subgroup 2 (Mann-Whitney, $**p<0.01$, $***p<0.001$) to a level where significant differences between subgroups in these regions were no longer detected.

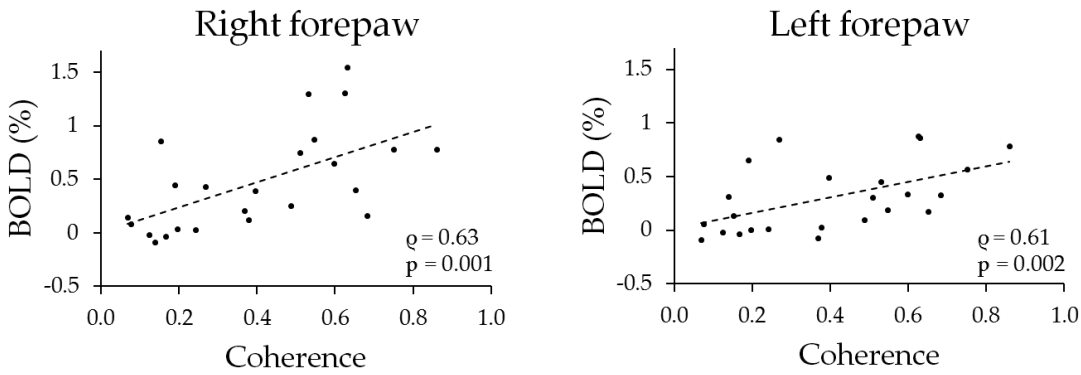

Figure 14. Spearman´s rank correlation coefficients (ρ) between mean increases in blood oxygenation level dependent (BOLD) signal intensity during forepaw stimulation period and preceding functional connectivity, both measured from primary somatosensory cortex. Data were obtained from both paws of six rats (four stimulation sessions per paw at 22-min intervals).

that hinders the detection of the coupling. Therefore, the coupling could still exist if higher nicotine dose would have been used (in case of AC) or larger amount of subjects would have been included for experiments (in case of URE).

The repeated acquisition of rsfMRI data (Figure 6A) enabled the evaluation of temporal changes in FC under different anesthetics. After a 1-h interval, changes were observed in three groups: AC, TBB subgroup 2, and URE (paired t-test, $p<0.001$). The progression of FC in individual rats in TBB subgroups is shown in Figure 13. With ISO, MED, and TBB subgroup 1, no significant temporal changes in FC were detected. As the temporal changes were observed mainly in groups receiving only a single dose of anesthetic and not in groups having constant administration of anesthetic, these results imply that rsfMRI is capable of detecting changes in anesthesia depth, attributable to metabolism of anesthetic.

As FC was found to change during prolonged measurements with certain anesthetics, we evaluated whether the coupling between FC and stimuli-induced BOLD responses would remain at different time points. A significant correlation was found between mean forepaw responses and FC (Figure 14) during repeated measurements, indicating that the coupling was prolonged in its nature. Additionally, rats that underwent both nicotine challenge and forepaw stimulations exhibited consistent findings. For example, a rat with low initial coherence tended to display a negative BOLD response to nicotine and a weak forepaw response. Approximately 1 h later, the coherence values were significantly higher (paired t-test, $p<0.001$), and the forepaw response had also become clear.

5.3 EFFECTS OF PHENCYCLIDINE ON NEURAL ACTIVITY AND BEHAVIOR (III)

The amplitude and extent of PCP-induced brain activation increased dose-dependently. The inspection of statistical activation maps suggested that at PCP 1.0 mg/kg, there were no significant differences between PCP and control groups. At PCP 2.0 mg/kg, activation was localized in mPFC, nucleus accumbens, minor striatal regions, and discrete cortical regions. At PCP 3.0 mg/kg, activation covered the whole striatum and large parts of thalamus.

Additionally, the cortical activation was remarkably enhanced compared to PCP 2.0 mg/kg. At PCP 5.0 mg/kg, as compared to PCP 3.0 mg/kg, enhanced activation was observed only in posterior cortical regions, indicating that a maximum spatial response had been reached already at PCP 3.0 mg/kg. The findings were similar in the group-level time series and AUC calculations; time courses and AUC values at mPFC were similar at PCP doses 2.0, 3.0, and 5.0 mg/kg, while a clear dose-dependent increase was seen in the visual cortex, suggesting that certain frontal brain regions are fully activated already by a low PCP dose but that the activation of posterior regions requires higher PCP doses.

The PCP-induced acute fMRI signal changes were fully supported by PCP and DA levels in the brain extracellular fluid (*in vivo* microdialysis experiments). Unbound PCP levels increased dose-dependently in both mPFC and striatum. However, PCP was found to increase extracellular dopamine levels in mPFC already at the 2.0 mg/kg dose, while PCP 3.0 mg/kg was required to increase dopamine levels in striatum. A good temporal correlation was observed between extracellular dopamine levels and BOLD signal changes (R^2~0.6, $p<0.001$).

The FC analysis indicated that brain networks were disturbed by PCP; the global connectivity decreased as a function of PCP dose (Figure 15). Several pathways originating from key regions associated with SCZ pathophysiology were affected in dose-dependent manner, such as connections originating from thalamus, striatum, mPFC, nucleus accumbens, and hippocampus (logistic regression analysis, $p<0.05$). Additionally, the integration and connectivity of cortex was severely affected (logistic regression analysis, $p<0.001$). Mesocortical and mesolimbic connections, which are dysfunctional in SCZ, were disturbed in a dose-dependent manner (logistic regression analysis, $p<0.05$), similarly to hippocampal connections (logistic regression analysis, $p<0.05$). The analysis of interhemispheric connectivity indicated that the integration of hippocampus was significantly decreased already by PCP 2.0 mg/kg (one-way ANOVA, Dunnett´s multiple comparison, $p<0.05$).

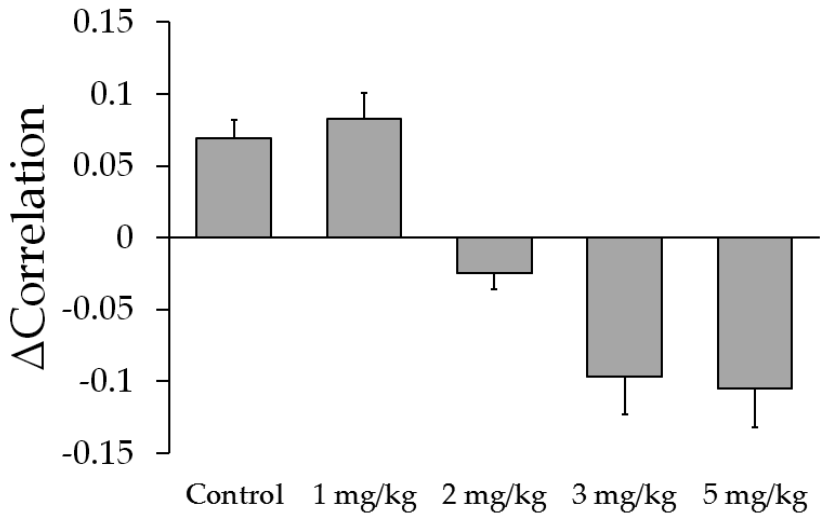

Figure 15. Changes in global functional connectivity (ΔCorrelation) 1h after treatment. Correlation values were averaged from 12 regions of interest from each subject (n=7/group).

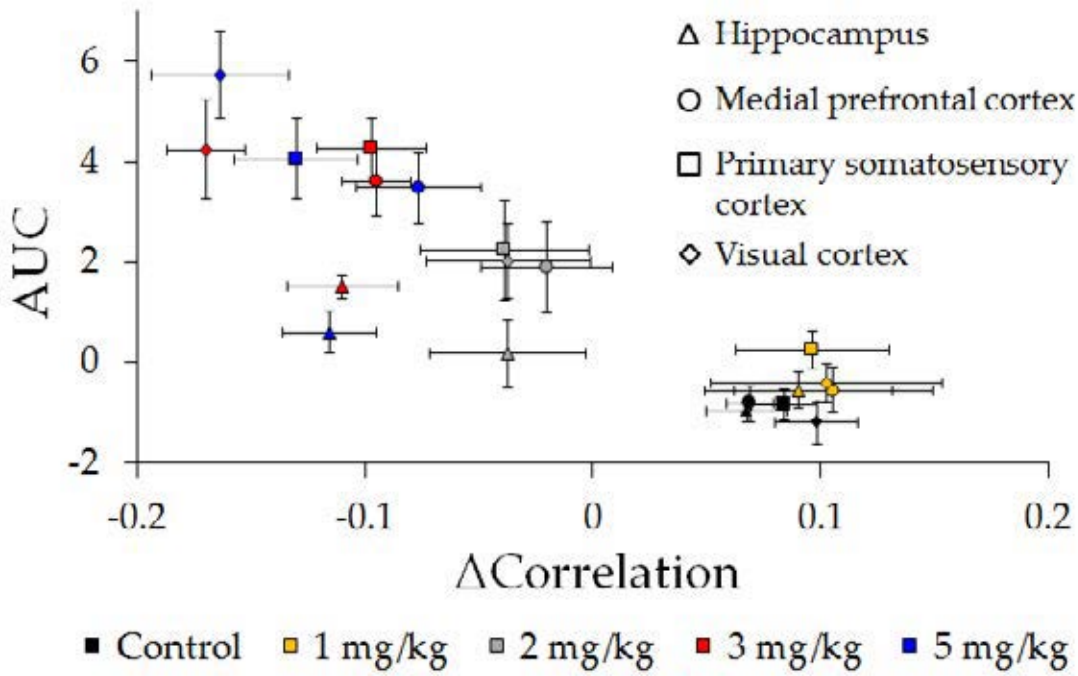

Figure 16. The effects of phencyclidine on blood oxygenation level dependent signal (measured as area under curve, AUC) and averaged functional connectivity in four regions of interest. Color of marker indicates grouping, and shape of marker region of interest.

The effects of different PCP doses on both AUC and FC in four representative regions are shown in Figure 16, which implies that a region-specific increase in AUC is not a necessity for disturbed connectivity. For example, the AUC changes in hippocampus were only minor throughout the dose range, but there was a considerable effect of PCP on FC (Δcorrelation) in hippocampus. In contrast, AUC appeared to increase in primary somatosensory cortex after the administration of PCP 1.0 mg/kg, but there was no clear decrease in connectivity.

In behavioral tests, high PCP doses increased locomotor activity (3.0 and 5.0 mg/kg against saline, t-test $p<0.001$ 2 h after treatment). Additionally, the locomotor activity exhibited excellent temporal cross-correlation with the decreased FC in the motor cortices ($R^2=0.8$, $p<0.001$). In the novel object recognition test, rats treated with 2.0 mg/kg PCP had lower (t-test, $p<0.001$) discrimination between familiar and novel objects than saline-treated rats. In the social interaction test, rats treated with 1.5 mg/kg PCP investigated each other significantly less (t-test, $p<0.05$) than control rats.

Acute subcutaneous administration of PCP 5.0 mg/kg increased peripheral MABP only mildly (~15 mmHg). The increase in MABP was observed at ~5 min after the injection, while BOLD signal only reached its maximum amplitude around 30-45 min. Therefore, the low correlation between MABP and BOLD signal (R^2~0.2) suggests that the PCP-induced increase in MABP does not make any major contribution to the PCP-induced fMRI signal changes in brain.

6 Discussion and Conclusions

The present work compared the effects of different anesthesia protocols on acute nicotine-induced BOLD signal changes (**I**). Additionally, the relationship between baseline FC and stimuli-induced BOLD signal changes under different anesthetics was investigated (**II**). Studies **I** and **II** were intended to improve the preclinical phMRI methodology by providing a means to minimize some of the confounding effects induced by anesthetics. The results, together with the literature review, provide a basis for the selection of anesthetic for phMRI experiments (**I**), and a simple tool for measuring the brain hemodynamic responsiveness to external stimuli (**II**). Therefore, the findings of studies **I** and **II** can significantly improve the quality of preclinical phMRI studies, when the use of anesthesia is unavoidable.

In the third part of this thesis, an optimized preclinical fMRI protocol was implemented into the multimodal characterization of PCP-induced SCZ-like symptoms in rats (**III**). PCP-induced dose-dependent changes in biomarkers in neural activity were detected for different SCZ-like symptom classes, and the alterations detected in fMRI and *in vivo* microdialysis experiments paralleled the changes in selected behavioral tests. By exploiting the results of study **III**, the existing PCP-model can be applied better for inducing different SCZ-like symptom classes, and be incorporated into drug development programs seeking improved drugs to treat SCZ.

6.1 THE IMPACT OF ANESTHESIA PROTOCOL ON PHMRI RESPONSES

Even although the nicotine-induced spatial BOLD signal changes in rat brain varied with the different anesthesia protocols (**I**), most of the activated regions exhibited a good correspondence with the known nicotine binding and activation sites (Clarke et al. 1985, London et al. 1988, McNamara et al. 1990, Mugnaini et al. 2002, Chin et al. 2008). As previous studies have indicated, in contrast to higher doses 300-350 μg/kg, low doses of nicotine (≤100 μg/kg) do not produce significant changes in MABP (Linville et al. 1993, Gozzi et al. 2006, Uchida and Hotta 2009), and as vehicle injections did not induce detectable signal changes, it can be argued that the majority of the nicotine-induced changes in BOLD signal originate from the CNS actions of nicotine (88 μg/kg, **I**). Therefore, the differences between groups, in both spatial and temporal activation profile, most likely arise from the different pharmacological actions of anesthetics; each anesthetic exerts its own unique effect on neuronal circuits and physiology, and/or distinctive interactions with nicotine, and these effects and/or interactions were reflected in the different amplitude, location, or nature of the nicotine BOLD responses. A practical example of the remarkably different effects of anesthetics on physiology is the difference in the required ventilation volume between groups to maintain steady-state parameters. Rats anesthetized with URE required >50 % greater ventilation volume per minute than rats treated with MED to maintain normal blood gas values, which may indicate indirectly a large difference in baseline functionality, metabolism, and/or energy consumption between these anesthetics; a lower level of baseline

neural activity leads to a lower amount of energy consumption and CO_2 production, and a lower requirement of lung ventilation.

The nicotine-induced BOLD signal changes under AC anesthesia were low, which is in good agreement with previous nAChR stimulation studies (Skoubis et al. 2006, Chin et al. 2008). As the anesthetic mechanisms of AC are mostly unknown (see chapter 2.2.3), the pharmacological basis for negligible nicotine responses remains unclear. Nevertheless, these findings, together with the unknown anesthesia mechanisms, suggest that AC may not be the optimal choice for phMRI investigations. Indeed, AC has been rarely administered in phMRI studies (<8 %) (Haensel et al. 2015).

The nicotine-induced BOLD signal changes under ISO, which has been the anesthetic selected in almost half of the phMRI studies (Haensel et al. 2015), were moderate in the cortical brain regions but poor in the subcortical regions. The moderate cortical response is somewhat surprising, as ISO is known to heavily suppress several nAChR subtypes and induce vasodilation (see chapter 2.2.3), which should lead to remarkably decreased activation and impaired detection of activation, respectively. This raises a question – is the BOLD response to nicotine mediated by certain nAChR subunits that are not affected by ISO? The spatial distribution of BOLD activation interestingly resembles the distribution of the α7-subtype nAChRs (Mugnaini et al. 2002), which is only minimally modulated by ISO *in vitro* (Flood et al. 1997). The cortical blood flow is, however, thought to be modulated by other subtypes (Uchida and Hotta 2009), such as $\alpha 4\beta 2$. As far as we are aware, acute nicotine challenge has not been investigated before with phMRI under ISO anesthesia. There are few studies (Choi et al. 2006, Gozzi et al. 2006) which utilized halothane, an anesthetic which has a similar mechanism of action to ISO (Violet et al. 1997, Campagna et al. 2003); these reported findings similar to those observed in the present study.

We do not believe that there are any previous acute nicotine phMRI experiments done under MED anesthesia. Although MED is not a common choice in phMRI studies (<2 %) (Haensel et al. 2015), the BOLD responses obtained in the MED group were generally good; the spatial correspondence with nicotine binding and activation sites was at least moderate, and BOLD response amplitudes were good throughout the brain. The responses were, however, not the highest or clearest in the present comparison study. One of the possible reasons for this may be the central role of LC in MED-induced loss of consciousness. LC expresses most of the nAChR subtypes; *in vitro* experiments have shown that nicotine increases the firing rate in the LC, and nicotine-induced membrane depolarization peaks at approximately 1 min and returns to the original level in 10–15 min (Egan and North 1986), which fits well into the present phMRI data. As the nicotine-induced increase in CBF is also partly mediated by the NE release from LC (Uchida et al. 2002), the inhibition of neurons in LC by MED may impact on several pathways that eventually lead to impaired nicotine-induced neuronal activation and reduced changes in the fMRI signal. Additionally, the strong MED-induced vasoconstriction, as discussed in chapter 2.2.3, may also compromise the hemodynamic responsiveness.

TBB has not been used in fMRI or phMRI experiments before, but it was selected for this comparison study due to its ability to induce long-lasting anesthesia after a single

intraperitoneal administration (Frey 1961, Koskela and Wahlstrom 1989). Even though the results obtained with optical imaging have not encouraged the use of TBB in functional studies (Lindauer et al. 1993), the rats anesthetized with TBB displayed mostly good and robust BOLD signal changes to the nicotine challenge. Interestingly, despite the hypercapnic pCO_2 values (~70 mmHg) the spontaneously breathing TBB group responded strongly to nicotine leading to similar maximum median response values compared to the ventilated TBB subgroup 1 although they had significantly lower pCO_2 values (~40 mmHg). One possible reason for this observation is that the pH remained in a reasonable range in both groups (~7.30 in spontaneously breathing TBB and ~7.45 in TBB SG1). The mechanism for hypercapnic vasodilatation is probably related to the direct effect of extracellular hydrogen ions, and neither molecular CO_2 nor bicarbonate ions have independent vasoactivity (Kontos et al. 1977a, Kontos et al. 1977b). Supporting data was obtained in forepaw stimulation experiments, where BOLD and CBF responses with mild acidosis (pCO_2 49 mmHg, pH 7.30) were comparable to those under normal conditions (Sicard and Duong 2005). In contrast, the response has been almost entirely eliminated in animals exhibiting severe acidosis (pCO_2 70 mmHg, pH 7.15) (Sicard and Duong 2005). Nevertheless, the findings in study **I** indicate that TBB at selected dose suppresses the respiratory function excessively so that it is difficult to maintain a normal physiological condition.

Another interesting observation in TBB animals was the division of ventilated animals into two subgroups due to their different nicotine-induced BOLD responses. There are possible explanations for this variability: 1) narrow therapeutical window of barbiturates (Flecknell 1996) and 2) intraperitoneal administration route (Svendsen 2005). According to Svendsen (2005), intraperitoneal injections can lead to varying absorption of drug between subjects. Additionally, intraperitoneal administration of barbiturates causes irritation that can further affect the absorption of anesthetic agent (Svendsen 2005). When a drug´s absorption is dependent on two varying factors, and combined with a narrow therapeutic window, it is not surprising that there were remarkable variances in CNS effects. High barbiturate concentrations are known to suppress calcium uptake in synapses and subsequently the release of neurotransmitters, such as ACh and NE, in contrast low barbiturate concentrations can enhance the release of ACh in brain (Ho and Harris 1981). These cellular mechanisms may provide, at least partly, an explanation for the different BOLD responses to nicotine in TBB group.

The most robust and highest nicotine-induced BOLD signal changes were measured in URE-anesthetized rats, as expected based on the literature review. Additionally, both URE protocols resulted in similar blood gas values and BOLD responses, which suggests that the more straightforward protocol exploiting spontaneously breathing animals is comparable to the protocol involving mechanical ventilation. If a follow-up protocol is, however, required, the results indicate that both MED and ISO provided good BOLD responses to nicotine, while responses under AC anesthesia were negligible and high variability in results under TBB anesthesia was observed.

As URE has been only rarely used in phMRI experiments (~8 %) (Haensel et al. 2015), the phMRI responses during URE anesthesia were characterized more carefully by using BOLD, CBV, and CBF contrasts, and in simultaneous LFP recordings with BOLD measurements. As

the phMRI experiments were conducted with different fMRI approaches, the applicability of fMRI contrasts for nicotine phMRI experiments will be also discussed briefly.

6.2 FUNCTIONAL MRI CONTRASTS IN THE NICOTINE CHALLENGE PHMRI

The ROI analysis (**I**) revealed good correlations between fMRI contrasts, suggesting normal relationship between BOLD, CBV, and CBF during URE anesthesia (Grubb et al. 1974, Ogawa et al. 1993). The spatial distribution of activation maps were similar between BOLD and CBF. This is consistent with previous studies, which have detected a good correlation between these methods with forepaw stimulation (Silva et al. 1999, Lee et al. 2002). In CBV data, the activation was slightly more widespread in deeper brain structures. This is most probably due to the higher sensitivity and better contrast-to-noise ratio, which make it possible to detect changes in brain regions with a poorer signal-to-noise ratio. A similar finding was reported in a cocaine phMRI study, in which activation was more easily detected in subcortical regions with CBV contrast than with BOLD (Mandeville et al. 2001).

The dynamics of nicotine responses were highly consistent with the different contrasts. However, the temporal resolutions (2 s for BOLD and CBV, and 12 s for CBF) did not allow for comparison of sub-second dynamics between these three methods. It has been suggested that temporal dynamics with slowly evolving pharmacologic activations are approximately the same between different fMRI contrasts (Mandeville et al. 2001), which is supported by these present findings. The CBV response to nicotine was comparable to findings reported by Gozzi et al. (2006), while much longer CBV responses were recorded by Choi et al. (2006); the differences most likely originate from the different anesthesia protocols in these two studies. It does seem that acute nicotine CBF measurements have not been performed with fMRI before, but studies with a laser Doppler flowmetry method have been published (Linville et al. 1993, Uchida et al. 1997). The responses in those studies were similar in magnitude but different in duration from our CBF results. However, optical imaging collects the signal only from the observable surface of the cortex while the hemodynamic response measured with fMRI varies in the different cortical layers (Shen et al. 2008). Therefore, the results are not directly comparable.

To summarize the results of BOLD, CBV, and CBF phMRI experiments under URE anesthesia, a normal relationship with the different contrasts was observed, and each approach provided rather equivalent spatial and temporal information. BOLD proved to be an excellent non-invasive method with good spatial and temporal resolution, specificity, and sufficient sensitivity. In the CBF measurements, the dynamics of pharmacologic activation were slow enough to be reliably followed by its low temporal resolution and the spatial distribution of the activation was large enough to be detected with a more coarse resolution. CBV exhibited the highest contrast-to-noise ratio and sensitivity, but its imaging requires the injection of a contrast agent. With smaller nicotine doses (or another compound), the CBV method may provide the necessary enhancement for reliable detection of drug actions. Therefore, the CBV-weighted contrast can be beneficial in experiments where the expected

fMRI response is low, e.g., while investigating low doses of drugs or stimulating minor neurotransmitter systems.

6.3 SIMULTANEOUS ELECTROPHYSIOLOGIC MEASUREMENTS WITH PHMRI

As all fMRI contrasts are indirect measures of neuronal activity, it is extremely advantageous to confirm the fMRI findings with complementary methods, such as electrophysiologic measurements. Measuring LFP is an invasive microelectrode recording method, in which the signal reflects postsynaptic signal processing within a few millimeters of the electrode tip (Mitzdorf 1987, Juergens et al. 1999, Logothetis et al. 2001). In the simultaneous BOLD and microelectrode measurements, the correlation with the hemodynamic response is better with LFPs than with single- or multi-unit activity (Logothetis et al. 2001), and therefore LFPs were selected for simultaneous examination with BOLD during the nicotine challenge.

As described earlier, a good correlation was observed between BOLD and LFP power (13-70 Hz), which is also widely acknowledged. Additionally, nicotine administration was found to decrease spectral power in the lower frequencies (0.3-1.4 Hz), which is supported by earlier observations (Ebenezer 1986). The negative relationship between low frequencies and BOLD signal changes is also a previously reported phenomenon (Mukamel et al. 2005, Niessing et al. 2005). Administration of the nAChR antagonist mecamylamine did not alter the frequency power at 0.3–1.4 Hz, whereas the power at 13–70 Hz was drastically suppressed, with about a 3 % simultaneous decrease in the fMRI signal intensity. Mecamylamine decreases MABP (Biesold et al. 1989), but the simultaneous electrophysiologic data suggested that the decrease in BOLD had a neuronal source. Changes in MABP may well be in the range of cerebral autoregulation, and the nAChR antagonism by mecamylamine is not associated with the changes in MABP (Biesold et al. 1989). The mecamylamine-induced suppression of cholinergic activity, possibly seen in both LFP power (13–70 Hz) and BOLD signals, is consistent with the concept of BOLD reflecting synaptic input and signal processing (Logothetis et al. 2001, Logothetis 2002). The association between a negative BOLD response and decreased neuronal activity has been previously demonstrated in monkeys and with visual stimuli (Shmuel et al. 2006); our findings indicate that the corresponding relationship exists also in anesthetized rats with pharmacologically induced neuronal suppression.

Taken together, simultaneous recordings of BOLD and LFP demonstrated an association between hemodynamic changes and electrical activity evoked by pharmacologic challenges. The BOLD signal resembled the observed LFP spectral power (13–70 Hz) in both pharmacologically produced neuronal stimulation and suppression at the somatosensory cortex of URE-anesthetized rats. Furthermore, these observations combined with earlier findings (Gsell et al. 2006, Rauch et al. 2008) indicate that the use of fMRI with simultaneous electrophysiologic recordings and pharmacologic modulation of brain may provide a valuable technique for clarifying the neurophysiologic source of BOLD, and for research into the role of neurotransmitters, such as ACh, glutamate, or DA, in the functional response of the brain to different stimuli.

Based on the results of the present work (**I**) and the literature review, the use of URE in terminal phMRI experiments can be recommended. First, URE appears to affect neurotransmitter systems only mildly, preserving several neural and physiologic functions (Maggi and Meli 1986, Hara and Harris 2002). Second, anesthesia with URE displays many similarities to natural sleep (Tung and Mendelson 2004, Clement et al. 2008), which has not been reported for the other anesthetics. Third, mechanical ventilation, which is commonly a necessity in fMRI experiments, is not required to maintain normal blood gas values (**I**), most likely because several autonomic functions are preserved under URE anesthesia. Fourth, a single administration is sufficient to evoke a prolonged anesthesia (Maggi and Meli 1986), which is often desirable in fMRI. Fifth, consistent findings were observed between different fMRI contrasts, and between electrophysiological neuronal activity and the BOLD signal during acute pharmacologic challenges (**I**). Additionally, findings in study **III** indicated good correspondence between the BOLD signal, obtained in URE-anesthetized rats, and extracellular drug levels in tissue obtained with in vivo microdialysis measurements from conscious animals. However, if the study design requires recovery or follow-up studies, the use of either MED, ISO, or their combination (Lukasik and Gillies 2003, Fukuda et al. 2013) may be a better from the anesthetics investigated in study **I**.

6.4 ANESTHESIA PROTOCOL, CONNECTIVITY, AND FMRI RESPONSES

The coupling between FC and hemodynamic responsiveness to stimuli under anesthesia has not been thoroughly investigated, even though evidence is emerging that the level of anesthesia is coupled with parameters in brain connectivity (Nallasamy and Tsao 2011). Therefore, FC could theoretically provide a measure for the depth of anesthesia in fMRI experiments, which further would help to optimize the anesthesia protocol, find the proper temporal window for stimulation studies, provide relevant information to rule out outliers, and subsequently reduce the variance in fMRI data.

In study **II**, a clear coupling between FC and BOLD responses was observed with several anesthetics. Perhaps the most interesting observation was done in the ventilated TBB group, in which two distinct response types to acute nicotine challenge were observed (**I**); similar division of animals in FC analysis (**II**) supported the discussion that the observed differences in nicotine responses originated from the variance in the induction of TBB anesthesia (see Chapter 6.1 for discussion). Additionally, the FC of TBB-anesthetized rats converged during the 1-h follow-up period. This observation supports further the different absorption and distribution rates of anesthetic between subjects; rats having rapid absorption and distribution of the anesthetic are initially in deeper anesthesia due to the narrow therapeutic window (Figure 17). Because of the metabolism, the level of anesthesia decreases and FC increases with time. The exact cellular mechanisms inducing the remarkable differences in fMRI signal changes under TBB anesthesia are not clear, but they may be related to barbiturate-induced disruption of calcium signaling (Ho and Harris 1981). Spontaneous BOLD fluctuations (Du et al. 2014), neurovascular coupling mechanism, and excitatory neurotransmission (Ho and Harris 1981) are all at least partly calcium-mediated. Therefore, strong calcium inhibition can considerably suppress the changes in the rsfMRI and fMRI signals by disturbing both neurovascular coupling mechanisms and neurotransmission.

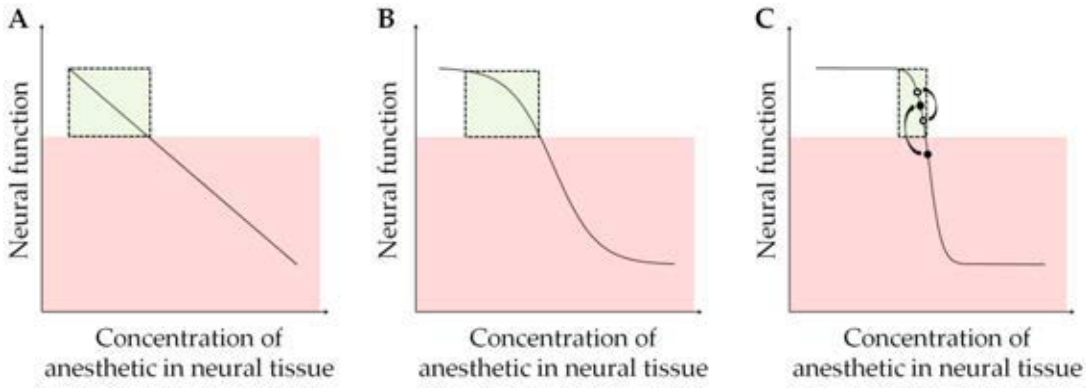

Figure 17. Three simplified relationships (A, B, and C) between the level of neural functions relevant for fMRI signal detection (e.g., neurotransmission or neurovascular coupling mechanisms) and the concentration of anesthetic in neural tissue. The green area indicates the window, where the anesthetic starts to inhibit neural processes and induce anesthesia, while fMRI responses are still detected. The red area represents deep anesthesia and inhibited responses. The widest window (A) allows more variability in the concentration of anesthetic in tissue, while with a narrow window (C) the dosing must be very precise. Several barbiturates are known to have narrow therapeutic windows, and even a small variation in tissue concentration may have resulted in distinct responsiveness. However, the correct window for activation studies is eventually reached due to metabolism of the anesthetic (see black circle in C).

TBB was not the only anesthetic expressing evident coupling between nicotine-induced BOLD responses and FC; good correlation values were also observed during ISO and MED anesthesia (**II**). Similar findings, however, were not found with AC and URE anesthesia, but the concept of coupling between FC and BOLD responses may still hold for all anesthetics if stimuli are optimized for the anesthesia protocol, larger group sizes are used, and a longer period for rsfMRI analysis is acquired (e.g., to separate different sleep-states under URE). Nevertheless, significant temporal changes in baseline connectivity were observed under both AC and URE anesthesia, which is intuitively expected as anesthetics administered with injections are being continually metabolized towards lower concentrations. Therefore, the follow-up of FC in study **II** supports the existing data (Pawela et al. 2009) that rsfMRI is able to detect temporal changes in brain connectivity, most likely arising from the changes in the brain tissue concentration of anesthetic and depth of anesthesia.

We believe that this is the first time when the correlation between FC and BOLD responses has been investigated by using pharmacologic stimuli (**II**). A few groups have touched on this topic by using forepaw stimulation in rats (Lu et al. 2007, Pawela et al. 2009). In the present thesis, the correlation between connectivity and phMRI responses was corroborated with repeated forepaw stimulation experiments under TBB anesthesia (**II**), where similar correlation values to phMRI experiments were obtained for FC and BOLD coupling. Therefore, the findings in the current thesis, together with earlier studies, suggest that the coupling is valid with different types of stimuli, which further emphasizes the importance of coupling between FC and BOLD responses.

The contributions of physiological noise (e.g., spontaneous movement, breathing, and heart rate) to the rsfMRI results in study **II** were expected to be minimal; it has been shown that cardiac noise has only a minor impact on FC, whereas actual movement is the main confounding contributor to rs-fMRI analysis in preclinical experiments (Majeed et al. 2009,

Kalthoff et al. 2011). There were no significant temporal changes in heart rates caused by any of the anesthetics, and by using a paralyzing agent and mechanical ventilation, spontaneous movement was prevented. The effect of low dose of nicotine at the second rsfMRI time point was also most likely minimal; because of the rapid metabolism, very low activity can be measured from rodent brain after 1 h, and acutely nicotine-increased DA levels have been reported to have typically decayed within 45 min (Hansson and Schmiterlow 1962, Marshall et al. 1997).

Taken together, the results of study **II** strongly indicate that the analysis of FC can provide a readily implementable biomarker for brain hemodynamic responsiveness to external stimuli and it can monitor the progression of anesthesia. First, a clear coupling between nicotine-induced BOLD signal changes and the preceding FC was observed with several anesthetics, and reasonable explanations were found for those cases where coupling was not observed. Second, the coupling was corroborated with supplementary forepaw stimulation studies, which produced similar findings to the pharmacologic challenge. Third, FC changed significantly between 1-h interval time points in groups that received anesthetic as initial boluses, while groups receiving constant administration did not display these changes. Fourth, the forepaw stimulation experiments suggested that the coupling is preserved temporally: when FC increased, BOLD responses increased. Therefore, connectivity analysis of baseline periods, or the addition of short rsfMRI scans into the fMRI study design can be highly recommended; it can provide invaluable information related to the state of the animal and explain different responses between subjects, as shown in studies **I** and **II**.

6.5 PHENCYCLIDINE-INDUCED SCHIZOPHRENIA-LIKE SYMPTOMS IN RAT

The results of study **III** strongly indicate that in rats, PCP induces the many SCZ-like symptom classes in a dose-dependent manner; at lower PCP doses (1.0-2.0 mg/kg) the drug-induced moderate changes were localized in the frontal and cortical brain regions thought to be involved in the cognitive and negative symptoms, while at higher PCP doses (3.0-5.0 mg/kg) the PCP-induced neuronal modulation was strong and widespread, hinting of dominant positive SCZ-like symptoms. The discussion related to study **III** is summarized in Figure 18.

At lower doses (1.0-2.0 mg/kg), PCP activated fronto-cortical brain regions in both fMRI and *in vivo* microdialysis experiments, while deeper subcortical areas remained unaffected (**III**). Subsequently, the connectivities of frontal cortex and hippocampus were disturbed; abnormal functions of these regions have been associated with cognitive symptoms in patients with SCZ (Friston and Frith 1995, Peled et al. 2001, Meyer-Lindenberg et al. 2005, Large 2007, Gozzi et al. 2008b, Millan and Bales 2013, Dawson et al. 2015). The novel object recognition test (**III**) indicated that PCP at the 2.0 mg/kg dose significantly disturbed recognition memory functions, further supporting the fMRI and microdialysis findings and the manifestation of SCZ-like cognitive deficits at lower PCP doses (1.0-2.0 mg/kg).

In addition to cognitive deficits, the changes induced by the lower PCP doses (1.0-2.0 mg/kg) occurred in brain regions, such as hippocampus, frontal cortex, and prefrontal cortex (**III**), which have been associated with negative symptoms in patients with SCZ (Russell et al. 2000, Crespo-Facorro et al. 2001, Potkin et al. 2002, Winograd-Gurvich et al. 2006). The social interaction test (**III**) confirmed that a low PCP dose (1.5 mg/kg) decreased social interaction behavior, corroborating the fMRI and microdialysis conclusions that low PCP doses (1.0-2.0 mg/kg) may induce SCZ-like negative symptoms.

At higher PCP doses (3.0-5.0 mg/kg), the neuronal activation (or modulation) was more global and reached to deeper brain structures (**III**); subcortical changes in DA neurotransmission are thought to contribute to the positive symptoms in SCZ (Gozzi et al. 2008b, Pratt et al. 2012). In addition to strong acute changes, high PCP doses (3.0-5.0 mg/kg) heavily disrupted FC, which may had led to a similar neurobiological condition to that suggested to prevail in SCZ patients (Lynall et al. 2010, Dawson et al. 2015, Kim et al. 2016). Based on clinical observations in hallucinating SCZ patients (Oertel-Knochel et al. 2014, Rolland et al. 2015), disturbed connectivity in mesolimbic and auditory networks, which were both detected in study **III**, could well be reflections of the positive SCZ-like symptoms. The presence of PCP-induced positive SCZ-like symptoms was also tested by recording locomotor activity (**III**), and a significant increase in distance travelled was induced by PCP 3.0 and 5.0 mg/kg, but not by 1.0 mg/kg.

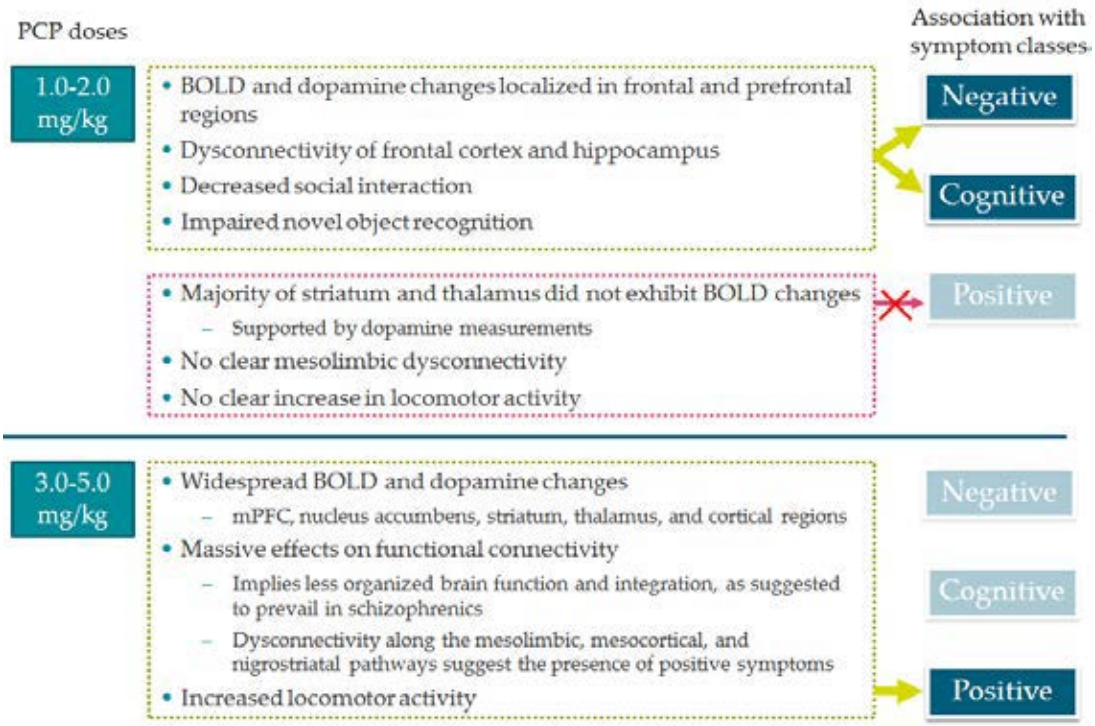

Figure 18. Summary of the findings of study **III**, and how they reflect different schizophrenia-like symptom classes in rat. BOLD, blood oxygenation level dependent; mPFC, medial prefrontal cortex; PCP, phencyclidine.

In study **III**, all of the experiments were performed in conscious animals except for the fMRI measurements, which may confound the data interpretation as results from different modalities were combined. Based on the results of study **I**, the URE anesthesia protocol was exploited in the fMRI experiments in study **III**. As the aim was to investigate the effects of acute PCP (an NMDA antagonist) on rat brain, the fact that URE exerts only mild effects on several glutamatergic receptors (Hara and Harris 2002) was a factor further favoring the choice of this anesthetic agent. The results of study **III** indicated that the URE protocol provided a robust basis for PCP phMRI studies: a high correspondence was observed between fMRI measurements and *in vivo* microdialysis experiments (conducted with awake animals). However, exact interactions between URE and PCP, and how URE affects PCP-induced changes in FC remain unknown, and the anesthesia-induced confounding factors and their contribution cannot be completely ruled out.

Even though study **I** showed that spontaneously breathing URE animals produced similar phMRI results to ventilated animals, mechanical ventilation was still applied in study **III**; the major benefit here was that the muscle relaxant completely prevented motion and ensured that high quality rsfMRI analyses covering long periods could be acquired.

The results of study **II** suggested that the different sleep-like states may induce variance to FC obtained under URE anesthesia. To deal with this issue, long periods in phMRI scan (900 volumes, 30 min) were reserved for connectivity analysis. With this approach, the contribution of different states was assumed to be averaged out. The mean FC was indeed within a narrow range among the groups, and there were no pre-treatment differences. The long temporal window would also have allowed the separation of sleep-like states, should this be desired during the analysis. Even though FC under URE anesthesia was found to change temporally (**II** and **III**), the anesthesia-related increase in connectivity strength was assumed to be similar across the subjects, and was therefore considered as an insignificant factor in study **III**.

Taken together, the observations in study **III** suggest that the higher PCP doses (3.0-5.0 mg/kg) induce positive SCZ-like symptoms in rat, while these markers were absent at the lower PCP doses (1.0-2.0 mg/kg). Therefore, the PCP dose range of 1.0-2.0 mg/kg can be used to rather specifically induce negative and cognitive SCZ-like symptoms in rat, without the confounding effects of positive symptoms (Figure 18). These findings can significantly improve the use of PCP in modeling of negative and cognitive SCZ-like symptoms and thus facilitate the development of novel drugs for SCZ.

6.6 CONCLUSIONS AND FUTURE VIEWS

Different fMRI modalities, including phMRI and rsfMRI, have unarguably made a significant impact on brain research during the last two decades. This was further supported by the present work, where it was shown that with simple optimization steps (**I**, **II**) preclinical fMRI is indeed a powerful brain research tool that can have a significant impact on drug development (**III**) and subsequently on clinical environment. In summary, the present work suggests that

I) URE may be the best anesthetic for preclinical phMRI experiments, if the recovery of the animal from experiment is not required. BOLD responses to nicotine under URE anesthesia were similar to those reported in conscious animals, and were well associated with electrophysiological activity and nAChR distribution. If recovery or follow-up studies are required, then ISO and MED (or their combination) may represent the best alternatives.

II) the preclinical rsfMRI acquisition can provide invaluable information related to the brain responsiveness during anesthesia. This information can be exploited in both fMRI study design and data interpretation, as resting-state acquisition can follow the progression of anesthesia, and subsequently the progression and level of responsiveness to external stimuli. Negligible or unexpected responses can possibly be explained with the fluctuating level of functional connectivity during the experiments.

III) the preclinical phMRI and rsfMRI methods can detect distinct changes in brain activity and connectivity after acute PCP administration, which resemble the chronic pathophysiological changes observed in patients with SCZ. Additionally, the preclinical neuroimaging measures display a good correspondence with the *in vivo* microdialysis data and behavioral tests. It was shown, that by optimizing the dose of phencyclidine, different SCZ-like symptom classes can be highlighted in animal model of SCZ. This information can be directly exploited in the preclinical testing of the effects of novel drug candidates for SCZ.

Despite recent progress, the full potential of preclinical (or clinical) fMRI has surely still not been reached, and there are still many methodological improvements to be expected. For instance, one possibility is to try combinations of different anesthetics and doses to minimize the effect of anesthesia, as well as finding novel ways to optimize fMRI contrast or parameters by developing improved imaging sequences, and development of more sophisticated MRI hardware for better temporal/spatial resolution. Other options would be to devise robust awake animal imaging setups, or improve rsfMRI/phMRI data preprocessing and analysis methods. Additionally, the exact neurobiological sources and mechanisms underpinning the fMRI signal, especially rsfMRI signal, are still far from being clear, which hinders the fMRI data interpretation. These are only some of the methodological possibilities, and their achievement will require extensive cooperation across disciplines and between many research groups. Additionally, experts in neurobiology, neurology, and neuropharmacology should be challenged to pose the most interesting questions to focus the evolution of the fMRI technique; it could be argued that advances in fMRI may provide revolutionary insights how healthy brain functions and reveal causes of many devastating neurological diseases.

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JAAKKO PAASONEN

Functional magnetic resonance imaging (fMRI) is a modern research tool that has furthered our understanding of brain function. In this thesis, commonly-used anesthesia protocols in preclinical fMRI were compared, and an optimized protocol was devised for drug research that could alleviate the burden of brain diseases. With careful study design, fMRI can provide novel approaches for narrowing the gap between human diseases and animal models, and for testing the effects of novel drug candidates.

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