PETTERI STENROOS

FUNCTIONAL MAGNETIC RESONANCE IMAGING OF THE BRAIN IN ANESTHETIZED AND AWAKE RATS
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ABSTRACT

Functional magnetic resonance imaging (fMRI) is a versatile non-invasive imaging tool for measuring whole-brain function both in response to a stimulus or at rest. Via neurovascular coupling, resting-state functional magnetic imaging makes it possible to detect synchronized neuronal activity between brain regions which can be thought to collectively form resting-state networks. Altered network activity, in disease or disorders can be effectively studied in preclinical fMRI settings by using animal models.

Anesthesia extensively modulates resting-state connectivity, preventing the effective transfer of information between several brain regions. In this study, the direct effects of six anesthetics on rat brain resting-state connectivity were evaluated and compared to connectivity measured in the awake state. The results suggest that connectivity measured during propofol and urethane anesthesia most resembled the connectivity measured in the awake state; in contrast, the connectivity measured in the presence of isoflurane and medetomidine least resembled the connectivity measured in the awake state. The long-term effects of isoflurane anesthesia on functional connectivity were also evaluated. After one month from the initial single 3h exposure, strengthened functional connectivity between thalamo-cortical and hippocampal-cortical connections was found, suggesting isoflurane evoked brain plasticity.

Awake resting-state fMRI provides inferences about the intrinsic and spontaneous brain activities, e.g. cognition and memory, which are generally suppressed by anesthetics. The translational value of preclinical fMRI can be increased by utilizing awake animals. In this study, novel awake rat fMRI protocols and imaging techniques were developed and validated. A novel and freely available 3D-printable restraint kit was developed which allowed relatively stress-free imaging of fully awake rats after a short habituation period. Based on the measured corticosterone level, heart rate and movement, rats were able to adapt to the imaging environment within four days. Functional imaging with the standard echo planar imaging technique was also compared with a recently developed
Multi-band SWeep Imaging with a Fourier Transformation (MB-SWIFT) imaging technique. MB-SWIFT was demonstrated to be more suitable for awake rat imaging, causing less animal motion and being less sensitive to body motion related image artefacts.

In summary, the results emerging from this thesis improve preclinical fMRI methodologies by introducing optimized resting-state fMRI protocols for use in either awake or anesthetized rats. These results can increase the translational value of preclinical fMRI in the future.

_Medical Subject Headings: Functional Neuroimaging; Magnetic Resonance Imaging; Brain; Thalamus; Hippocampus; Cerebral Cortex; Neuronal Plasticity; Animals, Laboratory; Rats; Anesthesia; Anesthetics; Wakefulness; Restraint, Physical; Adaptation, Physiological_
TIIVISTELMÄ


verrattiin tavanomaisen kaikkuvaustekniikan ja äskettäin kehitetyn Multi-band SWEEP Imaging with Fourier Transformation (MB-SWIFT) -kuvantamistekniikan välillä. MB-SWIFT:n osoitettiin soveltuvan paremmin hereillä olevien rottien kuvantamiseen, koska se aiheutti vähemmän eläinten liikettä ja oli vähemmän herkkä kehon liikkeestä aiheuttaville kuvantamishäiriöille.


Luokitus: WL 141.5.M2, WL 141.5.N47, WN 185, WO 275, QV 81, QY 58, QY 60.R6
Yleinen suomalainen ontologia: toiminnallinen magneettikuvaus; aivot; koe-eläimet; rott (laji); nukutus; nukutusaineet; valvominen; sopeutuminen
ACKNOWLEDGEMENTS

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Kuopio, September 2020

Petteri Stenroos
LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following original publications:


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## ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>AMPA</td>
<td>$\alpha$-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid</td>
</tr>
<tr>
<td>ASL</td>
<td>Arterial spin labeling</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
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<tr>
<td>BOLD</td>
<td>Blood oxygenation level dependent</td>
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<tr>
<td>BS</td>
<td>Burst suppression</td>
</tr>
<tr>
<td>$B_0$</td>
<td>Static magnetic field</td>
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<td>CBV</td>
<td>Cerebral blood volume</td>
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<td>DMN</td>
<td>Default mode network</td>
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<td>EEG</td>
<td>Electroencephalography</td>
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<td>FC</td>
<td>Functional connectivity</td>
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<tr>
<td>FDR</td>
<td>False discovery rate</td>
</tr>
<tr>
<td>fMRI</td>
<td>Functional magnetic resonance imaging</td>
</tr>
<tr>
<td>GABA</td>
<td>Gamma-amino butyric acid</td>
</tr>
<tr>
<td>GE</td>
<td>Gradient echo</td>
</tr>
<tr>
<td>GluA1</td>
<td>Glutamate receptor 1</td>
</tr>
<tr>
<td>ICA</td>
<td>Independent component analysis</td>
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<tr>
<td>LFP</td>
<td>Local field potential</td>
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<tr>
<td>MB-SWIFT</td>
<td>MultiBand SWEEP Imaging with Fourier Transformation</td>
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<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>NIfTI</td>
<td>Neuroimaging Informatics Technology Initiative</td>
</tr>
<tr>
<td>NMDA-R</td>
<td>N-methyl-D-aspartate receptor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
<td>-------------------------------------------</td>
</tr>
<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
</tr>
<tr>
<td>NREM</td>
<td>Non-rapid eye movement</td>
</tr>
<tr>
<td>POCD</td>
<td>Postoperative cognitive dysfunction</td>
</tr>
<tr>
<td>REM</td>
<td>Rapid eye movement</td>
</tr>
<tr>
<td>RF</td>
<td>Radiofrequency</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>rsfMRI</td>
<td>Resting-state functional magnetic resonance imaging</td>
</tr>
<tr>
<td>SE-EPI</td>
<td>Spin-echo echo-planar imaging</td>
</tr>
<tr>
<td>SPM8</td>
<td>Statistical Parametric Mapping 8, software</td>
</tr>
<tr>
<td>TE</td>
<td>Echo time</td>
</tr>
<tr>
<td>TR</td>
<td>Repetition time</td>
</tr>
<tr>
<td>$T_1$</td>
<td>Longitudinal relaxation time</td>
</tr>
<tr>
<td>$T_2$</td>
<td>Transversal relaxation time</td>
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Wakefulness can be described as a brain state where an individual is expressing cognitive or behavioral responses to the external world. When awake, an individual is conscious of the environment or of him/herself. Wakefulness differs from sleep in many aspects, ranging from different expression of neuromodulators and genes, and activity of brain regions and circuits maintaining awareness and responsiveness. Even at rest, certain areas of the brain are busy, actively processing daydreams or diffusively monitoring the environment. In a more active state, the awake brain can process an enormous amount of information, but in a selective way. In the active state, the brain selectively focuses attention on external stimuli that are consecutively processed, giving rise to brain functions such as sensory perception, cognition, emotion, learning and behavior (Bear et al., 2007).

Although measurement of brain rhythms by electroencephalography (EEG) was discovered already in 1924 (see Haas, 2003), the assessment of brain wide networks, including subcortical nuclei, was only made possible by the invention of functional imaging techniques of positron emission tomography (PET) in the 1980s (see Raichle, 2009), and functional magnetic resonance imaging (fMRI) in 1990 (Ogawa et al., 1990). Of these techniques, functional magnetic resonance imaging proved to be far superior, based on its better temporal and spatial resolution and non-invasive nature, without the need to inject any contrast agents.

Functional magnetic resonance imaging is especially suitable for translational research as the same techniques can be utilized in animals and humans. Preclinical studies allow one to study altered brain activity in a versatile manner, for example by utilizing various animal disease models. Moreover, more invasive study designs can be combined with fMRI where the brain activity can be studied in response to the stimulation of specific brain region. Recently, research interest has shifted more towards studying large-scale brain networks, and the possibility to either manipulate them or to discover new translational biomarkers for the altered networks in response to disease or disorders.

New discoveries in preclinical network studies have been long hindered by the necessity for anesthetizing the animals during the functional imaging. Anesthetics have been thought to be necessary for reducing stress and motion in the animal. However, the use of anesthesia inevitably suppresses cognitive functions that are typically expressed in the awake state. Thus, the anesthetic state is more reminiscent of the state of reversible coma than the awake state (Brown et al., 2010). Thus, interesting brain functions, such as cognitive processing, responsiveness to external stimulus or learning cannot be effectively studied in anesthetized animals. Furthermore, these brain functions are in many cases the most extensively influenced by neurological diseases and disorders (Baudic et al., 2006; Blumenfeld, 2012; Mazzoni et al., 2012). Today, there is increasing interest towards awake
animal functional imaging and many novel imaging techniques have been developed to study fully awake animals.

In this study, we aimed to study the differences between resting-state functional connectivity of rats measured either with several commonly used preclinical anesthetics or in the awake state. We developed and utilized state-of-the-art awake imaging protocols and imaging techniques to improve awake fMRI methodologies. The results from this thesis can provide more opportunities in the future for neuroscientists to conduct experiments to examine various brain functions of the awake and conscious brain.
2 REVIEW OF THE LITERATURE

2.1 FUNCTIONAL MAGNETIC RESONANCE IMAGING

2.1.1 Nuclear magnetic resonance

Nuclear magnetic resonance (NMR) is based on the concept of nuclear spins and the nuclear resonance effect in the magnetic field. In a magnetic field, the equilibrium state of the spins can be disturbed by applying radio frequency (RF) energy at the resonance frequency of the spins, and this disruption can be utilized to measure NMR signal (de Graaf 2007).

Spin is an intrinsic quantum mechanical property of elementary particles. A proton is made of elementary particles called quarks. Each proton has two up quarks and one down quark, which collectively give the proton the net spin quantum number of +1/2. For different nuclei, the spin number can be a half-integer, an integer or zero, depending on the mass number and charge. Particles with half-integer spins and integer spins have an intrinsic spin angular momentum and a magnetic moment. In an external magnetic field, spin angular momentum will cause the spins to precess along the field (longitudinal plane by definition) at a specific frequency called the Larmor frequency, according to the Larmor equation (Eq. 1).

\[ w = \gamma B_0 \]  

(Eq. 1)

where \( w \) is angular frequency, \( \gamma \) is a nuclei specific gyromagnetic ratio and \( B_0 \) is the main magnetic field strength. The magnetic moment of spins causes them to more likely orient along the main magnetic field \( (B_0) \), towards their lower energy state. The distribution of spins in the magnetic field is given by Boltzmann distribution (Eq. 2):

\[ N^+ / N^- = \exp\left[ \frac{\Delta E}{kT} \right] = \exp\left[ \frac{hv}{kT} \right] \]  

(Eq. 2)

where \( N^+ \) and \( N^- \) are the number of spins in the lower and high energy states, \( \Delta E \) is the energy difference between the two states, \( k \) is the Boltzmann constant (1.381 x 10-23 joules/K), and \( T \) is the absolute temperature in Kelvin, \( v \) is the precession frequency and \( h \) is Planck’s constant. Accordingly, the stronger the magnetic field and spin frequency, the larger the portion of the spins that become aligned along the main magnetic field, thus increasing the MR signal.

However, spin precession along the main field cannot be directly detected. By applying a near field radio frequency (RF) pulse with amplitude \( B_1 \), perpendicular to main magnetic field at the Larmor frequency, the equilibrium state of spins can
be distorted. The length, shape and magnitude of the RF pulse dictate the degree of distortion of the spin system. For example, by applying a 90-degree RF pulse, the net orientation of the spin system is tipped perpendicular to the main field to the transverse plane. At the same time, spins start to precess coherently about the transverse plane. After the RF-pulse, coherent precession of the tipped spin system can be detected by a coil as an induced electromotive force. The detected signal is called free induction decay (FID), as immediately after the RF pulse stops, the spin system starts to relax back to an equilibrium state along the main field ($T_1$ relaxation, longitudinal relaxation), and simultaneously along the transverse plane ($T_2$ relaxation, transverse relaxation).

The rate of relaxation is dependent on the local tissue environments, for example dictated by local magnetic fields, proton exchange or spin-spin interactions. $T_1$ relaxation is, by definition, the time it takes for 63% of the net magnetization to relax back along the main field. $T_1$ relaxation is mainly caused by spin-spin interactions dictated by the tumbling rate approximately at the speed of the Larmor frequency of molecules, which can be described by the spectral density function. $T_2$ relaxation is, by definition, the time it takes for 37% of the transverse magnetization to disappear. $T_2$ relaxation is mainly caused by spin-spin interactions, proton exchange at low tumbling rates and diffusion in local field gradients. In reality, transverse relaxation is also attributable to static field inhomogeneities such as instrumental imperfections or local magnetic field gradients (e.g. iron, air cavities, deoxyhemoglobin). The total transverse relaxation including also static dephasing is called $T_2^*$ ($T_2$-star).

The image cannot be formed by the FID itself. For this reason, additional magnetic fields are required to cause controlled distortions in the x-, y- and z-directions in the main field. These distortions are enabled by applying current through additional loops of wire or conductive sheets, creating magnetic field gradients along the bore. With gradients, resonance frequency and/or the phase of the spins can be made to be proportional to spatial positions. With a Fourier transform, frequency components from the signal can be separated, and temporarily stored in a spatial frequency domain called k-space. In 2-dimensional imaging, multiple FIDs are acquired at a particular phase encoding level to fill the k-space. In echo planar imaging (EPI), following the RF-excitation, the k-space is rapidly filled with consecutively acquired echoes with varying phase accumulation to construct the image. Finally, by applying an inverse Fourier transformation, the image can be formed (Buxton 2002).

### 2.1.2 Functional MRI contrast

**Neurovascular coupling**

Functional contrast in fMRI is based on local signal intensity changes in consecutive MR images, caused by hemodynamic changes in blood volume, flow and
oxygenation following neuronal activity. This coupling between neuronal activity and subsequent vascular responses is called neurovascular coupling.

Even in the resting state, the brain consumes about 20% of total oxygen (Magistretti and Pellerin, 1999) and 60% of total glucose (Garrett and Grisham, 1997). Oxygen and glucose are needed for constant ATP production, of which most (60% to 70%) is used to maintain the Na+/K+ membrane potentials required for the generation of the action potential. In addition to neurons, glia cells have a constant metabolic drive, which is dependent on the neuronal activity (Jha and Morrison, 2018). Energy consumption causes variations in the concentration of diffusive ionic and molecular vasoactive metabolic by-products such as potassium, nitric oxide, adenosine, carbon dioxide, and arachidonic acid. Subsequently, these ions or metabolic by-products repolarize or depolarize vascular smooth muscle cells, causing either vasodilation or vasoconstriction, respectively. Glial cells are hypothesized to be an important link in the release of vasoactive agents (Raichle and Mintun, 2006). Furthermore, neurons can directly modulate neurovascular coupling through the release of vasoactive products, or by direct neuronal innervation. Current evidence suggests that synaptic activity and neuronal spiking both correlate to vascular responses. However, should synaptic activity and neuronal spiking become dissociated, then synaptic activity correlates more with vascular responses than spiking activity correlates with (Gandhi et al., 1999; Logothetis et al., 2001; Mathiesen et al., 1998; Rauch et al., 2008; Viswanathan and Freeman, 2007).

Vasodilation has been found to almost linearly change blood velocity and flux through decreases in vascular resistance. Following neuronal activity, more oxygen and glucose are transported to the capillaries around the activated areas, by an increase in arterial and (to a lesser extent) vein volume and flow (Drew et al., 2011). Changes in volume, flow and oxygenation of blood can be detected as MRI intensity changes; these form the basis of fMRI functional contrast. Changes in flow and oxygenation are commonly followed by a delay of a couple of seconds from neuronal activity in awake rodents (Drew et al., 2011; Gao et al., 2015; Kim et al., 2013; Martin et al., 2006) probably due to the slow diffusion and uptake of neurovascular mediators, but this is heavily dependent on which brain regions are being assessed (Devonshire et al., 2012) and the consciousness state (Aksenov et al., 2015; Martin et al., 2006).

**Blood oxygen level dependent contrast**

The most common fMRI technique utilizes intrinsic blood oxygenation level dependent (BOLD) contrast. Following the increase in neuronal activity and blood flow, the relative concentration of deoxyhemoglobin is decreased, and that of oxyhemoglobin increased in capillaries, venules and veins. Relative deoxyhemoglobin will decrease because the amount of delivered oxygen exceeds the oxygen demand of neural tissue (Fox and Raichle, 1986). As oxyhemoglobin is
diamagnetic and deoxyhemoglobin is paramagnetic, the relative decrease in deoxyhemoglobin increases the tissue transverse relaxation time. BOLD effects can be exploited by standard gradient echo (GE) or spin echo (SE) techniques, such as GE and SE echo planar imaging (EPI) techniques. In SE-EPI, a 180-degree RF-pulse is used to rephase the dephased spins caused by static field inhomogeneities. This refocusing works best around the large veins. However, the spins that are moving through the small-scale local magnetic field gradients around the small capillaries cannot be rephased, thus SE-EPI based BOLD T2-contrast is better localized around the small capillaries. In GE-EPI, because of the lack of a 180-degree RF-pulse, static field inhomogeneities around the veins are not reversed, and the BOLD T2*-contrast appears also around larger draining veins that can be far from the activated region (Buxton 2002). Both techniques have their inherent advantages, as SE-EPI is spatially more selective to the location of neuronal activation whereas GE-EPI has a better contrast-to-noise ratio. In addition to measuring only a BOLD effect, SE- and GE-techniques are always somewhat sensitive to blood flow (Gao and Liu, 2012) and volume (Mandeville et al., 1998), although both are dependent on the field strength, RF-coil design and measurement parameters.

The vascular changes following neural activation have a lag time (Hirano et al., 2011; Silva et al., 2007). Several BOLD hemodynamic response models have been illustrated (Buxton et al., 1998; Miller et al., 2001; Uludag et al., 2004), although the lag time is affected by anesthesia (Aksenov et al., 2015) and heavily dependent on the species (Andrea Pisauro et al., 2013; Tsagaris et al., 1969) and the stimuli used (Lewis et al., 2018). In humans, the response to external stimuli typically starts at 1-2 s and peaks after 4-5 s (Lewis et al., 2018). In rats, BOLD responses are faster, typically peaking at 2 s in awake, and at 4 s in anesthetized rats, having their typical full width at half-maximum of around 1 s in awake and 4 s in anesthetized states (Martin et al., 2006). However, even faster responses have been found in response to ultrashort stimulation (Hirano et al., 2011). The BOLD signal amplitude changes are typically 1-10% percent of the signal in response to stimulation (Ogawa et al., 1992) while resting-state signal changes are even smaller (1-2%) (Biswal et al., 1995). The coupling between neuronal activity and BOLD response have been found to be either linear, where BOLD magnitude increases monotonically with the summed neural activity (Li and Freeman, 2007; Logothetis et al., 2001, Zhang 2009) or in a nonlinear manner (Birn 2005, Liu 2010, Zhang 2008, Lewis 2018). Nonlinear BOLD responses have been typically found in fast stimulation paradigms (inter-stimulation-interval < 4-6 s) and these are mainly attributable to the large vessels (Birn 2005, Liu 2010, Zhang 2008), whereas microvasculature contributes mainly to the linear responses (Zhang 2009).

Excitatory and inhibitory neuronal activity can both contribute to positive BOLD responses, although excitatory activity accounts for the vast majority of the response (~80%) (Aksenov et al., 2015). The negative BOLD response is a more debated subject, but it could be receiving a contribution from neuronal suppression (Lauritzen et al., 2012; Stefanovic et al., 2004) or from large neuronal activation,
depending on the brain region or the brain state (Schridde et al., 2008) or by a vascular stealing effect (Poublanc et al., 2013).

**Cerebral blood flow and volume**

The most commonly used fMRI technique applied to measure non-invasively cerebral blood flow throughout the brain, is called arterial spin labeling (ASL) (Koretsky, 2012). It is based on acquiring two parallel images of the brain: a labelled image taken after a short period when blood water spins moving towards the brain are inverted or saturated, and a control image without the magnetic labeling but with a similar magnetization transfer effect and then after subtracting the images, a perfusion map is acquired. ASL has an advantage over traditional BOLD contrast by providing a more direct estimate of the neuronal activity but it suffers from poorer contrast-to-noise ratio and temporal resolution (Donahue and Jezzard, 2010).

The measurements of cerebral blood volume are traditionally based on injection of an intravascular contrast agent to enhance blood T₂ or T₂* relaxation in the vasculature localized around the sites of neuronal activity. Intravascular contrast agents such as paramagnetic monoamine iron oxide nanoparticles (Weissleder et al., 1991) can be used to infer CBV changes if the concentration of the agent in the blood remains constant (Smirnakis et al., 2007). In addition, noninvasive techniques such as vascular space occupancy have been developed (Lu et al., 2003). CBV techniques have the advantage over BOLD contrast by having either a higher contrast-to-noise ratio or better gray matter localization.

A recently developed technique, Multi-band SWEEP Imaging with Fourier Transformation (MB-SWIFT) (Idiyatullin et al., 2015) has been demonstrated to be well suited for cerebral blood flow contrast fMRI (Lehto et al., 2017). MB-SWIFT is a modification of the original SWIFT (Corum et al., 2007). SWIFT is a 3D radial MRI pulse sequence with large excitation and readout bandwidths, close to zero echo time and minimal gradient switching steps during data acquisition. In MB-SWIFT, multiple side bands are exploited to create a large bandwidth excitation profiles. Due to close to zero echo time, the functional contrast of MB-SWIFT likely originates from in-flow effects of blood (Lehto et al., 2017), in contrast to traditional T₂* BOLD-effects with EPI-techniques. Additionally, close to zero echo time makes possible the visualization of hard tissues with very short transverse relaxation times. Lately, MB-SWIFT has been used in the context of deep brain stimulation of the rat, where minimal susceptibility artefacts were produced from a tungsten wire deep electrode (Lehto et al., 2017). Furthermore, the acquired fMRI responses were comparable and functional signal-to-noise ratios even higher than those obtained with standard spin-echo EPI techniques (Lehto et al., 2017).

### 2.1.3 Resting-state functional magnetic resonance imaging

Resting-state functional magnetic imaging (rs-fMRI) makes it possible to detect synchronized intrinsic hemodynamic changes, which are thought to collectively
form resting-state networks (Biswal et al., 1995). Resting-state fMRI can have a remarkable clinical value, as diseased patients may have a poor capability to perform tasks inside a magnet. Importantly, a compromised network activity is associated with several disorders such as Alzheimer’s (Wang et al., 2007) or Parkinson’s disease (Ghahremani et al., 2018), epilepsy (Rajpoot et al., 2015) or schizophrenia (Lynall et al., 2010). rs-fMRI differs from task or stimulus fMRI techniques in the sense that the patient is lying still, i.e. not performing any task or being stimulated with any external stimulus. Therefore, rs-fMRI allows the detection of intrinsic brain activities where the mind is spontaneously producing self-referential events e.g. related to memory, imagination, inner speech or planning (Fransson, 2006). Most notable and well-known networks are the default mode (Greicius et al., 2003), attention (Fox et al., 2006), salience and executive networks (Seeley et al., 2007). The default mode network is thought to represent intrinsic self-referential activity during the resting condition. However, while performing a task, the default mode network is typically suppressed, while task-related networks are activated. In addition to these networks, many other networks have been detected such as frontoparietal (Zanto and Gazzaley, 2013), thalamocortical (Yuan et al., 2016), and somatomotor (Thomas Yeo et al., 2011) networks.

Resting-state networks are typically evaluated from fMRI data obtained at minimum in 5-10 min or longer scanning periods, where stationary, relatively strong connectivity between brain regions can be observed. Recently, dynamic rs-fMRI has been examined as a way of detecting much faster temporal patterns in a time scale of seconds (Gu et al., 2019). The dynamic evaluation of resting-state networks is thought to extract richer information in functional networks and enable discovery of transient rapidly changing brain states not detected by the standard static analysis.

Generally, there are two standard types of analysis of resting-state fMRI data; region of interest (ROI) based and data driven techniques. In the ROI-based analysis, specific seed regions in the brain are defined, and the correlations between the seeds or voxels in the brain are calculated. Data driven techniques, such as independent component (ICA) or principal component analyses (PCA), try to separate statistically independent time-courses into subcomponents which can represent spatial resting-state networks. There are pros and cons associated with each of these analytical methods. ROI-based techniques rely on some existing hypothesis about network activity (e.g. how it responds to altered conditions or to disease,) and compared to data driven techniques, they can provide better statistical strength by avoiding the problem of multiple comparisons. However, by concentrating on specific connections, they can fail to detect relevant brain network changes. Data driven techniques, on the other hand, do not rely on preconceptions, and can be used to reveal novel networks in different conditions (Cole et al., 2010), and additionally, are well suited to separate time-courses originating from experimental imperfections. Nevertheless, the selection of different algorithm parameters can largely impact the acquired results.
2.1.4 Complementary techniques for functional magnetic resonance imaging

While fMRI is currently one of the best techniques to measure whole-brain activity with high spatial resolution, other techniques like electroencephalography (EEG), local field potential (LFP) or optical imaging techniques can provide complementary information.

EEG is a technique to directly measure the electrical activity of neurons. It is usually performed in humans by placing electrodes on top of the scalp; in animals, it usually refers to a technique where electrodes are placed on the surface of the skull/dura. Most of the detected signal originates from the post-synaptical ion flow generated from the synchronous activity of millions of cortical, perpendicularly oriented, pyramidal neurons. Because of the excellent temporal resolution, in the scale of milliseconds (up to ~130Hz), EEG can be used to detect rapid changes in brain dynamics of spontaneous activity or responses to stimuli. However, as the measuring electrodes have a low impedance and collect the voltage generated by a large volume and are relatively far away from the area of activity, the detected signal suffers from a poor signal-to-noise ratio and can be a mixture from multiple sources. In practice, human EEG detects only signals from the cerebral cortex. Preclinical studies allow more invasive measurements with which to measure local extracellular field potentials (LFP) since electrodes are actually placed inside the brain tissue. Compared to EEG, LFP provides more detailed inferences about the activity of the precise brain regions of interest. Moreover, measurements with high impedance electrodes allow to measure action potentials from single neurons but these highly local measurements are poorly suited for neuronal network studies. However, optical imaging techniques, like calcium imaging, can achieve even single-cell spatial resolution over a large area. In calcium imaging, either chemical or genetically encoded calcium indicators change their fluorescence properties after the binding of a calcium ion. Therefore, the change in fluorescence can be used to measure brain activity in a living animal with a very high spatial and temporal resolution (Wang et al., 2003).

Importantly, these techniques can be supplemented with the fMRI (EEG/fMRI, opto/fMRI), combining the temporal and spatial specificity of the electrophysiological or optical techniques with the spatial coverage of the fMRI. When recording simultaneously at high temporal and spatial resolution, transient brain events (e.g. epileptic seizures), neural oscillations (e.g. alpha waves) or brain state (e.g. sleep states) can be reliably detected and combined with neural network level changes. In addition, simultaneous measurements make it possible to determine the neural origin of hemodynamic signals, this is a topic still under intensive research (Liu et al., 2011; Ma et al., 2016; Magri et al., 2012; Schölvinck et al., 2010; Shmuel and Leopold, 2008) However, combining these modalities is far from straightforward. For example, in simultaneous EEG/fMRI, electrode distortions or MRI gradient switching artefacts are among the greatest confounding factors affecting the interpretations of both the MRI image and the EEG signal. These fundamental problems can be eased by advanced artefact correction.
methods, selection of the most suitable recording electrodes or improvements in MRI acquisition methods.

2.2 FUNCTIONAL BRAIN IMAGING IN ANESTHETIZED ANIMALS

2.2.1 General mechanisms of action of anesthetics

The maintenance of awake brain function and circuits involves a subtle balance between excitatory and inhibitory neuronal activities (E/I balance) (Havlicek et al., 2017; Taub et al., 2013; Zhou and Yu, 2018) and effective connectivity (Moon et al., 2015; Rosanova et al., 2012; Tononi, 2004) which collectively form consciousness and the individual’s responsiveness to external stimuli (Franks, 2008). General anesthetics work by acting in the central nervous system to induce unconsciousness and a lack of awareness to painful stimuli. The common mechanisms for all general anesthetics are the modulatory effects on 1) neurotransmitter gated ion channels at postsynaptic terminals or 2) directly on nerve fibers. In general, they act by either enhancing inhibitory or suppressing excitatory receptors. General anesthetics can be subdivided according to their mechanism of action. The mode of action of GABA agonist or GABA allosteric modulators is by binding to GABA receptor sites, subsequently inducing negatively charged Cl⁻ transportation inside the cell, causing hyperpolarization of the cell, which inhibits the generation of action potentials. Common GABAergic anesthetics include inhalational anesthetics such as isoflurane, sevoflurane and desflurane, and other anesthetics such as propofol, barbiturates and benzodiazepines. In contrast, excitatory receptors such as NMDA and AMPA receptors trigger a depolarization of the cell through positively charged ions, such as Ca²⁺ and Na⁺, which enhances the generation of action potentials. Common NMDA antagonists, which suppress the activity of these receptors, include ketamine, phencyclidine and nitrous oxide which typically cause a condition called dissociative anesthesia. In addition, other mechanisms of actions of anesthetics include alpha-2 adrenergic receptor agonists (e.g. medetomidine) and potassium channel activators (e.g. halothane).

The binding of anesthetic agents at the neurotransmitter receptors or at ion channels alters resting postsynaptic potential (-70mV) to either more positive (depolarization) or negative (hyperpolarization). Postsynaptic potential is a graded potential, meaning that potentials from multiple synapses are summated in the neuronal body, and if the threshold at the axonal hillock is exceeded, then an action potential along the axon is generated to signal to other neurons. In the case of inhibition, the activation threshold is not exceeded, and the signal is not transmitted forwards. During deep anesthesia, there is a reduction in the firing rate of both inhibitory and excitatory cells in the cortex (Taub et al., 2013), possibly regulated by thalamo-cortical inputs (Beierlein et al., 2003; Hirata and Castro-Alamancos, 2010). During deep anesthesia, the E/I balance has been demonstrated to shift more
towards inhibition, detected as a relative increase in both amplitude and width of inhibitory synaptic events (Taub et al., 2013). These rather complicated changes in neuronal firing rate patterns and E/I balance have been found to be important for normal information integration in the brain maintaining consciousness. During anesthesia, this integration of information is disrupted, leading to an anesthesia specific type of unconsciousness (Lee et al., 2009).

### 2.2.2 Effects of anesthetics on functional magnetic resonance imaging

#### Neurovascular coupling

Functional magnetic resonance imaging has been typically performed in anesthetized animals to decrease stress and motion related artefacts. However, in addition to the fact that anesthetics affect neuronal activation, they also change the basal metabolic rate (Buchsbaum et al., 1989), and both of these processes can impact on the neurovascular coupling mechanisms observed as altered hemodynamic responsiveness (Paasonen et al., 2017). However, even in the awake state, there is no consensus about which neural oscillations are the most important in driving the hemodynamic changes detected in fMRI. It has been suggested that slowly varying EEG oscillations in delta band frequency (1-4 Hz) (Hanbing Lu et al., 2007) or the overall power over a wide frequency range (Leopold et al., 2003) make the main contributions to hemodynamic responses but also the contribution of fast oscillations in the gamma range (30-90 Hz) has been demonstrated to have an impact (Magri et al., 2012). As most anesthetic agents typically shift neuronal oscillations towards lower frequencies (e.g. delta band), this subsequently change hemodynamic response dynamics by typically delaying and suppressing the responses (Martin et al., 2006; Wu et al., 2016a). Moreover, optical imaging studies have shown that both arterial and veins dilation in anesthetized subjects is altered in response to a stimulus (Martin et al., 2006). Moreover, different anesthetics or the dosage of anesthesia can change these responses, which makes the interpretation of the results even more complicated.

#### Physiology

Anesthetics can suppress both breathing and heart rates of animals, therefore changing partial pressure of carbon dioxide (pCO$_2$) and oxygen (pO$_2$) and decreasing blood pH. Increased pCO$_2$, or decreased pO$_2$, can be caused by different factors, for instance, by hypoventilation or a compromised breathing rhythm, which are controlled by the breathing center in the brainstem. Several anesthetics e.g. opiates, volatile anesthetics, and hypnotics (Bigatello and Pesenti, 2019) are known to affect breathing patterns. The subsequent changes in pCO$_2$, pO$_2$ or pH are detected in the body by either peripheral chemoreceptors, which are sensitive to pCO$_2$ and pO$_2$ changes, or by central chemoreceptors, which are sensitive to changes in pCO$_2$ and pH. As an example, centrally detected hypercapnia leads to
increased blood flow and an elevated respiratory rate, while peripherally detected hypercapnia can trigger increases in blood pressure and cardiac output. Therefore, changes in blood pCO₂ or pO₂ can alter the hemodynamic responses and change responses to stimuli (Cohen et al., 2002) or resting-state connectivity (Chang and Glover, 2009; Nasrallah et al., 2015). In order to stabilize the physiological state of the animal, mechanical ventilation is needed with most anesthetics to maintain normal and constant blood gas values. However, anesthetics can also either directly, or indirectly via vasoactive products, affect the ion channels in the endothelium or smooth muscle in the blood vessel walls (Akata, 2007), which can further compromise hemodynamic responses.

2.2.3 Effects of anesthetics on functional connectivity

Anesthetics can disturb the interpretations of functional connectivity (FC) or detection of brain networks. For example, alterations in E/I balance, brain region specific hemodynamics or in receptive field size (Armstrong-James and George, 1988) can substantially change spatiotemporal hemodynamic patterns, which can become evident as an altered functional connectivity between brain regions (Grandjean et al., 2014; Jonckers et al., 2014; Kiviniemi et al., 2005; Xiao Liu et al., 2013a; H. Lu et al., 2007; Ma et al., 2018; Pawela et al., 2009; Peltier et al., 2005; Williams et al., 2010).

The performance of functional connectivity analysis, studying the intrinsic brain function, has been long complicated in preclinical experiments due to need for anesthesia. Lately, breakthroughs in awake animal imaging have made it possible to study the influence of anesthetics on intrinsic brain networks and cognition.

Anesthesia induced unconsciousness

Although most general anesthetics alter neurotransmission at the whole brain level, they can influence certain brain regions more than others. This can lead to abnormal global coordination of information transfer between the brain regions and an altered state of consciousness (Brown et al., 2011, 2010; Franks, 2008; Purdon et al., 2015). The causal reason for anesthesia induced unconsciousness (AIU) has been speculated to originate from affected large scale brain networks (Moon et al., 2015; Tononi, 2004). AIU can resemble unconsciousness from other origins such as slow-wave sleep or a vegetative state. For example, in the transition from the awake state to slow-wave sleep, decreased activity in thalamo-cortical (Hale et al., 2016), fronto-parietal (Spoormaker et al., 2012) and cortico-cortical (Spoormaker et al., 2010) networks has been detected. Typically, higher-order brain regions, e.g. those involved in cognitive functions and expressing strong FC, are most extensively influenced by anesthetics than lower-order sensory regions (Greenberg et al., 2008; Liang et al., 2015; Schmidt and Konishi, 1998; Sellers et al., 2013; Wu et al., 2016a). Therefore, cortical regions are typically more affected than subcortical regions, and
furthermore, in the cortex, frontal areas are more affected than sensory areas. AIU can therefore be a result in a loss of the typical awake brain topological FC organization (Hutchison et al., 2014; Wang et al., 2010; Wu et al., 2016b, 2016a) or specificity (Xiao Liu et al., 2013b).

**Changed functional networks**

In seed-based correlation or ICA based studies, the anesthesia induced loss of connectivity is typically seen as decreased FC inside the cortex (Schroeder et al., 2016), between anterior and posterior cortex (Hamilton et al., 2017; Schrouff et al., 2011), as well as between cortex and subcortical regions (Velly 2007), whereas bilateral cortical FC is typically preserved (Jonckers et al., 2014; Majeed et al., 2009; Pawela et al., 2008; Wang et al., 2011).

Anesthesia typically reduces small-range (Xiao Liu et al., 2013b; Wu et al., 2016a) connectivity and thus FC becomes spatially less localized (Hamilton et al., 2017). This can be seen as a breakdown of the typical FC nodes, or a disturbance of the typical FC patterns (Boly et al., 2012; Xiao Liu et al., 2013b; Xiping Liu et al., 2013). Moreover, several anesthetics such as isoflurane or propofol, can cause brain activity to shift to a burst suppression mode with two states: either no activity (suppression) or high-amplitude peaks (burst), evoking an apparent high cortical synchronization (Kenny et al., 2014; Zhang et al., 2019). This kind of anesthesia was reported to cause an increased “randomness” and decreased modularity (Liang et al., 2014) and to decrease effective information transfer between brain regions (Hamilton et al., 2017). The detection of biologically relevant brain subnetworks seems to be compromised by most anesthetics. In independent component analysis, this can be seen as a weakened integration within networks (Schrouff et al., 2011) or changed effective connectivity between typical brain networks (Bukhari et al., 2017). Dynamic FC analysis has revealed that anesthesia can dose-dependently modulate the number of unique dynamic brain states and decrease the number of state transitions (Hutchison et al., 2014).

**Preserved functional networks**

Even though anesthetics are capable of modulating most of the resting-state networks, certain intrinsic networks, or topological FC organization, driven by preserved and constant metabolic activity of neurons, are present even during an unconscious state (Liang et al., 2015). The connectivity pattern of networks such as associative cortical network, default mode network, subcortical networks, or lateral cortical networks have been detected in the presence of various anesthetics (Bukhari et al., 2017; Hutchison et al., 2010; Lu et al., 2012; Vincent et al., 2007) and some anesthetics have been found to mimic the FC pattern detected in the awake state (Williams et al., 2010).
However, FC during AIU is thought to be reduced down to a state that resembles the anatomical connectivity (Barttfelda et al., 2015, Ma et al., 2017) and to lose the typical awake brain temporal variability (Hutchison et al., 2014). Due to the loss of temporal variability, certain brain networks can be even more preserved under anesthesia, which can subsequently increase the detection power of certain, otherwise transient networks than can be detected in awake animals. Moreover, study designs using anesthetized animals can be of clinical value e.g. by using disease model animals or by implementing invasive stimulation schemes, where the use of awake animals is not possible. However, anesthesia can significantly complicate the detection of biologically relevant networks, thus compromising clinical translatability.

2.2.4 Isoflurane – the most common preclinical anesthetic

Isoflurane is one of the most commonly utilized general anesthetics in preclinical work. It has high potency and stability and is easy to use for anaesthesia maintenance. However, isoflurane can evoke side-effects such as respiration depression, reductions in blood pressure, vasodilation, and elevated airway irritation (Wren-Dail et al., 2017). In the clinic, isoflurane has nowadays been largely replaced by sevoflurane or desflurane, mainly because of the good safety record and less irritative nature of these agents.

Pharmacokinetics

Isoflurane, as an inhalation anesthetic, is absorbed into the bloodstream by diffusion. The minimum alveolar concentration which is enough to cause a loss of reactivity to a painful stimulus in adult rats is 1.22-1.35% (Orliaguet et al., 2001). The effect time of isoflurane is dependent on the ratio of the alveolar concentration to the inspired concentration over time (Stock et al., 2013). In comparison to other volatile anesthetics, isoflurane has a relatively high solubility in blood, thus increasing blood equilibrium time. Nonetheless, the relative alveolar uptake of isoflurane is rather rapid as 50% of the relative alveolar concentration is reached within ~2 mins. Inhalation drugs in general are delivered rapidly to the vessel-rich compartments, including the brain. Since isoflurane has a high brain-blood partition coefficient, it is quickly distributed to the brain tissue where its anesthetic effects take place.

Mechanism of action

The current evidence indicates that the main mechanism of action of isoflurane is enhancement of inhibitory GABA-A receptor activity (de Sousa et al., 2000; Garcia et al., 2010; Jones et al., 1992). Other postulated mechanisms of action include suppression of excitatory glutamatergic NMDA, AMPA and kainate receptors (de Sousa et al., 2000; Dong et al., 2006), suppression of cortical interneurons (Ferron et
al., 2009), Ca\textsuperscript{2+} and K\textsuperscript{+} channel currents (Buljubasic et al., 1992) and thalamocortical neurons in the ventrobasal thalamus (Ying et al., 2009).

**Burst suppression**

In the presence of isoflurane concentrations of 1.25-2.0%, brain activity shifts to a burst suppression state with quasi-periodical peaks and silent states (Derbyshire et al., 1936; Hudetz and Imas, 2007; Xiao Liu et al., 2013b). The BS phenomenon has been thought to be initiated by a depletion of extracellular calcium stores (Amzica, 2009), diminished cortical inhibition (Ferron et al., 2009) and an overall decrease in metabolic and neuronal activity (Ching et al., 2012). Even though BS activity is detected in the unconscious brain, the brain is thought to attempt to recover normal neuronal dynamics and exchange of information during the burst phase (Ching et al., 2012; Japaridze et al., 2015). Accordingly, it has been reported that cortical bursts are initiated by rhythmic thalamocortical oscillations (Steriade et al., 1994; Zhang et al., 2019) or can be triggered by subthreshold sensory stimuli (Kroeger et al., 2013).

**2.2.4.1 Isoflurane – long-term effects**

In addition to the initial isoflurane evoked changes in brain dynamics or FC, isoflurane has been found to exert long-term effects in brain activity, behavior, memory and gene-expression (Colon et al., 2017). Recently, several preclinical experiments have been conducted to reveal the effects of isoflurane on gene expression or behavior (Colon et al., 2017). Despite the extensive research on this subject, there are still contradictory findings in the literature about the possible long-term consequences; these are possibly attributable to the large variability in anesthesia concentrations, repetitiveness of anesthesia, combination of multiple anesthetics, or the age of the subjects being anesthetized. However, it has been found that neurodegeneration and behavioral deficits might be initiated by anesthetic agents and the effects can be pronounced with agents that act through a combination of both NMDA and GABA-A receptors (Fredriksson et al., 2007), which is also considered as a mechanism of action of isoflurane.

**Gene and protein expression**

Protein expression is a highly dynamic and complex process. In response to anesthesia, the initial changes in protein expression can further influence several downstream mechanisms leading to short- or long-term changes in the expression of other genes and proteins. In most studies, exploring the relatively short-term influence of isoflurane, gene and protein expression have been found to change within a period of hours to several weeks (Culley et al., 2006, 2004; Lin and Zuo, 2011; Lowes et al., 2017; Zhang et al., 2014). Signs of elevated neuroinflammation or
apoptosis have been some of the most common findings in preclinical gene expression studies (Cao et al., 2012; Ge et al., 2015; Kong et al., 2013; Zhang et al., 2015). The developing brain may be more susceptible to long-term changes, but changes have been also detected in the adult or aged brain (Colon et al., 2017).

**Behavior, memory and brain function**

Usually the brain is able to recover fully from anesthesia, and normal brain function is stabilized within weeks to months (Ii et al., 2016; Rammes et al., 2009; Uchimoto et al., 2014). However, if the stimulus is repeated or sufficiently strong, behavioral changes and altered brain function may be evident in the long-term or, potentially, even for the lifespan (Figure 1). As an analog example, multiple bursts of protein synthesis in response to environmental cues are needed for memory formation, to support neuronal growth and synaptic plasticity (Alberini, 2009; Costa-Mattioli et al., 2009). Thus, with regard to isoflurane anesthesia, repetitive or even a single anesthetic treatment can lead to prolonged functional, behavioral or memory impairments, especially in hippocampal dependent memory tasks (Kodama et al., 2011; Zhong et al., 2015) (However see Walters et al., 2016). Notably, as the isoflurane evoked BS pattern is highly distinct from typical neuronal oscillations, it is not surprising that this type of neural activity can be involved in brain plasticity (Broad et al., 2016). In addition to apoptotic or inflammatory activation, neural plasticity may contribute to the detected changes in behavior, learning and memory. Indeed, a correlation has been found between changes in learning and the upregulation of NMDA-Rs (Rammes et al., 2009) or altered regulation of GluA1-containing AMPA-Rs- trafficking (Uchimoto et al., 2014), supporting the link between neural plasticity and brain function. Furthermore, the possibility has been raised that isoflurane may be involved in the development of a condition called post-operative cognitive dysfunction (POCD). Even though the main reason for generation of POCD has been hypothesized to be the inflammatory responses to surgery (Safavynia and Goldstein, 2019), data also point at a potential role of anesthesia evoked POCD related symptoms (Geng et al., 2017). In addition to direct modulation of neuronal plasticity, isoflurane can also affect the brain through indirect mechanisms. Interestingly, at high doses, isoflurane is known to open the blood brain barrier (Tétrault et al., 2008), thus leaving the brain tissue exposed to peripheral inflammatory responses (Safavynia and Goldstein, 2019). Thus, even if not having a direct causal role, isoflurane can potentially contribute to POCD together with the surgical operation.

In contrast, also positive or no responses between isoflurane anesthesia and behavioral outcome have been reported in several animal studies (Alkire et al., 2005; Callaway et al., 2012; Rammes et al., 2009; Stratmann et al., 2010). Especially, deep anesthesia and BS have been found to have even protective effects on POCD in humans (Chen et al., 2017; Deiner et al., 2015). There has been a debate about the
potential cerebral protection effects of BS during surgery through decreased cerebral metabolism (Ching et al., 2012) (However see. Roach et al., 1999).

![Diagram](image)

Figure 1. Anesthesia can have both acute and chronic effects on brain circuits. Figure adapted from (Colon et al., 2017).

### 2.3 FUNCTIONAL BRAIN IMAGING IN AWAKE ANIMALS

#### 2.3.1 Challenges

Since the invention of BOLD fMRI technique (Ogawa et al., 1990) preclinical fMRI studies have used anesthetized animals to examine the brain function. Utilization of awake animals was nonexistent partly because of concerns of head motion related artefacts in low contrast-to-noise fMRI signal, and the influence of stress on resting-state networks or responses to stimuli. The willingness to undertake awake animal imaging was also low because of the lack of standardized fMRI methods for examining awake animals. Recently, technical improvements in hardware, such as improvements in gradient performance or RF coil designs, new inventions in pulse sequences, and more advanced preprocessing and analyzing techniques have made awake animal imaging a feasible approach (Bammer et al., 2005; Oakes et al., 2005; Power et al., 2014). Importantly, new hardware allows the use of large bandwidth imaging, making it less susceptible to animal movement (Bradley et al., 2018). Furthermore, new innovations have emerged for animal restraint and protocols reducing animal stress. Reports of awake fMRI in several animal species have been published i.e. rats (Hagino et al., 1998), mice (Desai et al., 2011), rabbits (AM et al., 2000) or non-human primates (Dubowitz et al., 1998). Rats have been the preferred
species for conducting awake animal fMRI studies due to their relatively larger brain volume as compared to mice. However, mice do have some advantages over rats and have been used successfully in different awake restraint setups with minor motion observed (Dasai et al., 2011, Madularu et al., 2017, Harris et al., 2015, Han et al., 2019). For example, because there is a large repertoire of genetically modified mouse strains, they can be especially suitable for specific disease model studies.

2.3.2 Approaches

Head and body restraint

Several early awake fMRI studies have used either unhabituated or curarized rats which were restrained in the MRI bed (Khubchandani et al., 2003; Lahti et al., 1999; Peeters et al., 2001). Restraint methods can be generally divided into methods using a head implant or dedicated head restraint holders to fix the head and/or the body. Each method has its own benefits and limitations. Head fixation which entails the insertion of a head implant, can provide a firmer head fixation and more a reliable setup for simultaneous intracranial methodologies such as simultaneous electrophysiological or optical measurements. However, a surgical operation is needed before the experiment, which can induce additional stress or evoke inflammatory responses and predispose the animals to prolonged anesthesia with potential long-term effects. Furthermore, the head implant must be secured tightly to the skull with screws and acrylic cement to tolerate head motion generated forces on the implant which can possibly cause susceptibility artefacts. Therefore, more commonly, head restraint holders have been used to fix the animal’s head and/or the body. The most common restraint setups include a cylindrical head holder together with plastic body tube (Lahti et al., 1999). Head holders typically include a nose cone, lateral head supports, a neck support or a bite bar to restrain the head in all linear and rotational directions. The body can be placed into a body tube (Lahti et al., 1999) or restrained with body clothing or rubber bands (Chang et al., 2016; Tsurugizawa et al., 2009) with limbs either taped together or left free.

Habituation and stress

Unhabituated awake animals tend to move and experience stress due to restraint and loud MRI noises (King et al., 2005). Motion related artefacts on MRI images or stress induced changes in brain function or animal behavior can induce artificial correlations or modulate the commonly observed resting-state or stimulus induced networks (Blanchard et al., 2001; Dopfel and Zhang, 2018; Guedri et al., 2017; Power et al., 2012). Therefore, animals need a habituation period in order to become used to the MRI environment and noise before the actual MR imaging. The time of each habituation session and the total days can vary greatly depending on animal species, protocol and individual animals. Typically, a habituation time for awake rats lasts from 1 to 2 weeks. The actual sufficient amount of animal training can be
dictated by the measured stress indicators, such as motion, breathing and heart rate or by analyzing stress hormone levels. The most widely used estimator of stress has been a general observation of animal motion (Lahti et al., 1999). However, animal motion alone does not necessary reflect the intrinsic state that the animal is experiencing. The first study to examine the effect of a lengthy habituation protocol (1-8 days) on corticosterone levels, the main rodent glucocorticoid, breathing and heart rate and motion in awake restrained rats (King et al., 2005) revealed that rats can become habituated to restraint and to MRI noise as early as three days with 90 min habituation sessions on each day. Other supplementary stress indicators can be used, such as ultrasonic vocalization (Popik et al., 2012; Reed et al., 2013) or long-term behavioral stress indicators (Chu et al., 2016). Furthermore, the fact that animals are typically capable and willing to perform complicated cognitive tasks during the restraint (Han et al., 2019; Schwarz et al., 2010) can be used as an indicator that there is not the presence of excessive stress.

2.3.3 Applications

Lately, multiple fMRI protocols have emerged for examining resting-state brain function or responsiveness to various external stimulations in naïve or disease-model rats (Table 1).
<table>
<thead>
<tr>
<th>Sequence</th>
<th>MRI</th>
<th>Application</th>
<th>Protocol</th>
<th>Habituation</th>
<th>Body of restraint</th>
<th>Head of restraint</th>
<th>Reference</th>
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</thead>
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<tr>
<td>GE</td>
<td>GE</td>
<td>Hypothalamic response to BDRO and BROMO</td>
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<td>days 10 min to 7-10 days</td>
<td>No</td>
<td>No</td>
<td>Sicard 2003</td>
</tr>
<tr>
<td>CO2</td>
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<td>Electrical forepaw stimulation</td>
<td>Curt squads</td>
<td>No, rats were curared</td>
<td>No</td>
<td>No</td>
<td>Lahti 1999</td>
</tr>
<tr>
<td>ASL</td>
<td>single</td>
<td>Novel fMRI method for awake rats</td>
<td>Plastic tube</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>Lahti 1999</td>
</tr>
<tr>
<td></td>
<td>ASL, single</td>
<td>No</td>
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<td>No</td>
<td>No</td>
<td>No</td>
<td>Lahti 1999</td>
</tr>
</tbody>
</table>

Table 1. Summary of previous awake rat fMRI protocols and applications. The search was conducted in PubMed by key words “awake rat fMRI” OR “conscious rat fMRI”.

- The ear chamber was conducted in PubMed by key words “awake rat fMRI” OR “conscious rat fMRI”.
- Habituation protocol
- Effects of bromocriptine (BROMO) and haloperidol (HPD)
<table>
<thead>
<tr>
<th>Study</th>
<th>Region</th>
<th>Contrast</th>
<th>Task</th>
<th>Duration</th>
<th>Controls</th>
<th>Implant</th>
<th>Methodology</th>
</tr>
</thead>
</table>
| Positive BOLD changes in thalamus. | 2 | GE-EPI | Sleep-induced increase in 
frontal-parietal cortex and 
thalamus and other areas. | From few mins to few days, 7 days | No | A Teflon plate | Reduced stress indicated by changes in 
respiration rate, heart rate, and 
corticosterone levels. |
| Negative BOLD changes in temporal and motor cortices. | 2 | GE | Sleep-regulating simultaneous EEG/MRI | From few mins to few days, 7 days | A Teflon plate | Restrained with four lateral bars. |
| Positive BOLD responses in the prefrontal cortex, nucleus accumbens, dorsal striatum, sensory cortex, hippocampus, thalamus, and midbrain areas. | 2 | SE-EPI | Cocaine administration | 90 min/day, 3 days | Similar to Lahti 1999 | Similar to Lahti 1999 |
| Changes to relative CBV in several regions including the cingulate, somatosensory, motor, auditory, and prefrontal cortices and in the thalamus and the periaqueductal gray/dorsal raphe. | 2 | ASL/GE-EPI | Novel habituation protocol for awake rats. Resting state fMRI. | 90 min/day, 4-8 days | Similar to Lahti 1999 | Similar to Lahti 1999 |
| Discrete dose-dependent relationship was evident. Activation in the area postrema and nucleus tractus solitarius consistent with concentration that induced emesis in ferrets. | 2 | Iron oxide contrast agent, GEMS | Nicotinic acetylcholine receptor agonist (ABT-594) | 7, 30, 60 min/day, 4 days | Similar to Lahti 1999 | Similar to Lahti 1999 |
| Dose-dependent relationship was evident. Activation in the area postrema and nucleus tractus solitarius consistent with concentration that induced emesis in ferrets. | 2 | Iron oxide contrast agent, GE | Apomorphine and ABT-594 (causing emesis in ferrets) | 7, 30, 60 min/day, 4 days | Similar to Lahti 1999 | Similar to Lahti 1999 |

**Tenney 2003**

**Khubchandani 2005**

**Febo 2005**

**King 2005**

**Skoubis 2006**

**Chin 2006**
| Hypoxia-induced CBF and BOLD reductions are smaller in awake relative to anesthetized rats at low pO2, but similar at high pO2. |
| ASL, single-shot GE-EPI |

**Hypoxia responses**

- Similar to Lahti 1999
- Similar to Duong 2007
- Increase in CBV to ethanol challenge.
- Presence of anesthesia promoted a significant preferential flow to subcortical areas not seen in the awake condition.

- Monocrystalline iron oxide nanocolloid, GE-EPI

**Ethanol response**

- 90 min/day, 4 days
- Similar to Lahti 1999

**Cannabinoid receptor 1/2 agonist produced a dose-related, region-specific activation in the brain agreeing with autoradiographic cannabinoid receptor 1 density binding maps.**

- FSE
- Cannabinoid receptor 1/2 agonists 7, 30, 60, 60 min/day, 4 days
  - Similar to Lahti 1999
  - Similar to Luo 2007
  - Chin 2008

**Brain areas of lateral hypothalamus, medial basal amygdala, forebrain cortex and anterior thalamic nuclei were activated.**

- MS-FSE
- Aggressive motivation to male intruder 60 min/day, 2 days
  - Similar to Lahti 1999
  - Similar to Ferris 2008

**Corn oil emulsion-induced BOLD signal increase in brain regions, including the bilateral amygdala, hippocampus and the ventral tegmental area.**

- FLASH
- Response to gut corn oil emulsion 30 to 90 min/day, 5 days
  - Similar to Tsurugizawa 2009

<p>| Elastic bands | Head implant (acrylic resin, plastic anchoring screws) Restrained with four lateral bars. | Similar to Lahti 1999 |
| Fers 2008 | Similar to Lahti 1999 | Similar to Lahti 1999 |
| Tsumagawa 2009 | Similar to Lahti 1999 | Similar to Lahti 1999 |</p>
<table>
<thead>
<tr>
<th>System</th>
<th>Response Induced Pain</th>
<th>Capsaicin-Activated Putative Pain Neuronal Circuit</th>
<th>Papez Circuit and Habenular System</th>
<th>Reward Anticipation</th>
<th>Residing Resting State (MRI)</th>
<th>Similar to Tsurugizawa 2009</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multi-Slice (FSE)</td>
<td>40 min/day, 1 week</td>
<td>30 to 90 min/day, 5 days</td>
<td>15 to 90 min/day, 8 days</td>
<td>Days 6, 15 to 90 min/day, 8 days</td>
<td>Days 6, 15 to 90 min/day, 8 days</td>
<td>Days 6, 15 to 90 min/day, 8 days</td>
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<tr>
<td>Similar to Tsurugizawa 2009</td>
<td>Similar to Tsurugizawa 2009</td>
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<tr>
<td>Similar to Lahti 1999</td>
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<td>Similar to Lahti 1999</td>
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<tr>
<td>Similar to Kulkarni 2012</td>
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<td>Similar to Kulkarni 2012</td>
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<tr>
<td>Similar to Tsurugizawa 2012</td>
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<tr>
<td>Similar to Zhang 2010</td>
<td>Similar to Zhang 2010</td>
<td>Similar to Zhang 2010</td>
<td>Similar to Zhang 2010</td>
<td>Similar to Zhang 2010</td>
<td>Similar to Zhang 2010</td>
<td>Similar to Zhang 2010</td>
</tr>
</tbody>
</table>

Increase in functional activity in the hippocampus, forebrain cortex and lateral hypothalamus within minutes of administration.

Response to stress hormone corticosterone 90 min/day, 2 days

Similar to Lahti 1999

Ferris 2010

Multiple cortical and subcortical regions including the prefrontal cortex, thalamus and retrosplenial cortex showed synchronous variation. Regions crucial to cognitive and emotional information processing.

Processing emotional information crucial to cognitive and emotional topographic fundamental consensus networks in functional regions including the frontal multicortical and subcortical regions increase in functional activity in the human brain.

Similar to Lahti 1999

Zhang 2010

Functional networks in rats conserve fundamental topological properties that are also seen in the human brain.

Similar to Lahti 1999

Liang 2011

Caudate-putamen, anterior insular cortex, hippocampus, ventral pallidum, nucleus accumbens and medial preoptic area were activated during light presentation (preconditioned to ethanol).

Similar to Tsurugizawa 2009

Similar to Tsurugizawa 2009

Tsurugizawa 2012

Different brain responses in animals exposed to banana, rosy and citrus odors compared to almond odor.

Similar to Lahti 1999

Similar to Lahti 1999

Kulkarni 2012

Capsaicin activated the putative pain neuronal circuit, Papez circuit and habenular system.

Similar to Lahti 1999

Similar to Lahti 1999

Yee 2015
Suitable for awake fMRI protocols. Adaptation to restraint in 8 days.

**Novel fMRI method for awake rats, air puff stimulation, resting state fMRI**

- Patterns of brain activity change in the limbic system and reward systems, the salience and introspective socioaffective networks, and several additional stress and social behavior-associated nuclei.
- Patterns of brain activity differ between conscious and unconscious states.
- Specific dRSFC patterns working as a switch between conscious and unconscious states.
- Changes in the dRSFC patterns differ between the hypertensive rat and the other strains.
- Specific dRSFC patterns are less similar from the structural map when comparing conscious and unconscious states.

<table>
<thead>
<tr>
<th>Method</th>
<th>Resting State</th>
<th>Hypertensive Rat</th>
<th>Early Life Social Stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Snuggle Sack</td>
<td>Similar to Lahti 1999</td>
<td>Similar to Lahti 1999</td>
<td>Similar to Lahti 1999</td>
</tr>
<tr>
<td>Head Implant</td>
<td>Similar to Lahti 1999</td>
<td>Similar to Lahti 1999</td>
<td>Similar to Lahti 1999</td>
</tr>
<tr>
<td>GE-EPI</td>
<td>15 to 60 min/day, 7 days</td>
<td>15 to 90 min/day, 8 days</td>
<td>15 to 60 min/day, 8 days</td>
</tr>
<tr>
<td>GE-EPI</td>
<td>30 min/day, 8-10 days</td>
<td>60 min/day, 8 days</td>
<td>60 min/day, 8 days</td>
</tr>
</tbody>
</table>

Changes in the limbic system and reward systems, the salience and introspective socioaffective networks, and several additional stress and social behavior-associated nuclei.
Enhanced thalamic, hypothalamic, hippocampal and somatosensory cortex responses to mechanical stimulation of the face. Altered functional connectivity in networks, previously identified in clinical chronic pain.

GE-EPI

Responses to inflammatory cocktail, migraine-like rat model

Similar to Lahti 1999

Air-puff stimuli in neuropathic pain model

Similar to Chang 2016

Delta FC was proportionally dependent on the FC strength across all connections. Relative ΔFC at each anesthetized condition was exclusively negative across all connections.

GE-EPI

Responses to oxytocin

Similar to Lahti 1999

Intracerebroventricular (1μg/5μl) oxytocin induced sex differences in BOLD activation in multiple brain regions. Nucleus accumbens and prefrontal brain areas displayed abnormal activity to normally innocuous stimuli, regardless of sex differences in oxytocin-induced differences in neuronal activity and connectivity.

GE-EPI

Resting state fMRI, wakefulness and graded levels of consciousness

Similar to Lahti 1999

Rat brain has a topological organization similar to humans. Functional similarities exist in brain regions.
<table>
<thead>
<tr>
<th>Study</th>
<th>Method</th>
<th>Treatment</th>
<th>Duration</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lahti 1999</td>
<td>GE-EPI</td>
<td>Sumatriptan - naproxen treatment, migraine-like rat model</td>
<td>60min, 3 days</td>
<td>Similar to Lahti 1999</td>
</tr>
<tr>
<td>Bishop 2019</td>
<td>GE-EPI</td>
<td>Positive fMRI response to (S)-ketamine in the cortex, nucleus accumbens and striatum. Negative fMRI responses to (R)-ketamine in various brain regions.</td>
<td>15 to 45 min/day, 4 days</td>
<td>Similar to Lahti 1999</td>
</tr>
<tr>
<td>Masaki 2019</td>
<td>3D-printable head holder</td>
<td>Curarization of rats</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stenroos 2018</td>
<td>GE-EPI</td>
<td>Validation of MB-SWIFT sequence for awake rat fMRI</td>
<td>Similar to Stenroos 2018</td>
<td></td>
</tr>
<tr>
<td>Liu 2019</td>
<td>GE-EPI</td>
<td>Positive fMRI response to (S)-ketamine and (R)-ketamine</td>
<td>15 to 90 min/day, 7 days</td>
<td>Similar to Lahti 1999</td>
</tr>
<tr>
<td>Paasonen 2020</td>
<td>MB-SWIFT</td>
<td>MB-SWIFT enabled near whole-brain resting state functional parcellation.</td>
<td>15 to 60 min/day, 7 days</td>
<td>Similar to Stenroos 2018</td>
</tr>
</tbody>
</table>

**Notes:**
- MB-SWIFT: Multi-band Steady-State Free Precession Imaging Technique
- GE-EPI: Gradient Echo-Planar Imaging
- SE-EPI: Spin-Echo Planar Imaging
As illustrated, multiple awake applications or new restraint methods have been utilized to study resting-state activity, diseases or symptoms (e.g. seizures or epilepsy), various drug responses (e.g. nicotine, alcohol, cocaine, apomorphine, cannabinoids), or different brain states (e.g. spontaneous sleep, hypoxia). Furthermore, many of these studies have compared the brain activity between anesthetic and awake states. Different stimulation applications have been successfully used with awake rats to obtain significant responses at multiple brain sites at the whole-brain level, which cannot always be achieved with anesthetized rats (Paasonen et al., 2016; Martin et al., 2006). Furthermore, recent awake resting state studies have managed to construct whole brain resting-state functional parcellation of the brain (Becerra et al., 2011; Liang et al., 2015; Liu et al., 2019, Ma 2018), again something not commonly achieved with anesthetized animals.

### 2.3.4 Awake brain networks

The brain networks of awake rats share the typical network organization of other animal species (Gao et al., 2017). With data driven analysis, several awake rat brain networks have been visualized; these resemble those which are commonly detected in humans (Ma et al., 2018). In addition, seed-based analysis has suggested that the cingulate cortex works as a central hub in the awake rat brain (Ma et al., 2018). Graph-theory models have indicated that the rat brain constitutes small-world and has strong community structures that resemble the fundamental topological organization of the human brain. As discussed in chapter 1.2.3., FC strength or organization can be vastly different in the anesthetized state, from the awake state complicating the detection of the correlated or anticorrelated brain regions important for awake brain function. Furthermore, awake animal imaging makes it possible to track dynamic brain states (Chang et al., n.d.; Hutchison et al., 2014) related to behavior or arousal, opening the door for more complicated fMRI study designs.

### 2.3.5 Prospects and limitations

**Stress**

Two of the greatest concerns in awake animal imaging are the experienced stress and motion of animals. Even though animals have been habituated to restraint and MRI noise, it can be difficult, if not impossible, to achieve a complete stress-free environment in the magnet. However, based on carefully planned experimental conditions the animal stress can be minimized. The ability to elicit stress related functional responses in awake rats (Dopfel and Zhang, 2018b; Ferris and Stolberg, 2010) can be used as an indicator that the imaging-related stress does not mask the effects of the novel stressor being studied. Brain activation can be elicited in brain
regions such as amygdala, hippocampus and prefrontal cortex, which are known to be susceptible to activation due to stress (McEwen et al., 2016).

**Motion**

Motion can disturb the fMRI signal in unpredictable ways in different brain regions, and transient motion can induce evident disturbance in the fMRI signal (Power et al., 2012). Even minor motion, less than the voxel size, can introduce an artificial correlation between brain regions, with proximal and laterally orientated connections being affected the most in humans (Power et al., 2012). Therefore, motion related artefacts can induce inconsistencies in the obtained intrinsic FC patterns (van Dijk et al., 2012) and artificial correlations can complicate interpretations emerging from connectivity measures. Although various realignment paradigms (e.g. linear rigid transformation or non-linear methods) are commonly used in standard fMRI analyzing pipelines, several additional corrections are typically needed. First, it is recommended to perform motion scrubbing where all the data points with detectable motion are removed. Then, after the standard realignment steps, a nuisance regression of the non-neural related signal is generally performed. Regressors are typically selected from 6 motion parameters (x, y, z, roll, pitch, yaw) estimated from the realignment steps. Additionally, a non-neural related signal from white matter and ventricles can be used as a regressor. Global signal regression has been shown to effectively reduce residual motion from the data, however, introducing a risk of removing neural related signals. Lastly, before the evaluation of FC, band-pass filtering between 0.01 to 0.15-0.20 Hz is typically done to reduce residual heart and breathing rate or scanner related artefacts. Many additional techniques, such as the use of multi-echo acquisition (Kundu et al., 2012), ICA denoising (McKeown et al., 2005) or wavelet despiking (Patel et al., 2014) methods, are being reviewed.
3 AIMS OF THE STUDY

The main aims of this thesis were to develop and implement novel resting-state fMRI methods with which to study brain connectivity in awake rats, and to evaluate the acute and chronic effects of anesthetics on resting-state connectivity. The specific aims of this thesis are as follows:

1. To study the long-term effect of isoflurane anesthesia on brain function

Cognitive or behavioral dysfunctions are common findings after invasive surgical operations involving general anesthetics. Furthermore, in preclinical studies, general anesthetics are routinely used during the surgical or measurement protocols. Therefore, the aim of Study I was to evaluate the long-term effects of the most commonly used preclinical anesthetic isoflurane by functional magnetic resonance imaging, local field potential measurements and gene expression analysis in rats. It was hypothesized that the results from this study would reveal the potential long-term modulatory consequences of isoflurane on brain function.

2. To develop and alternative awake rat fMRI method and compare resting-state connectivity of rats under awake state to anesthetic state

Under general anesthesia, the level of arousal and reactivity to external stimuli is heavily modulated. It is important to know how each anesthetic agent modulates the brain function in comparison with the awake state. In preclinical fMRI, awake animals need to be restrained inside the magnet, and habituated to awake imaging protocols. Therefore, the first aim of Study II was to develop a novel, low-stress, fMRI method with which to examine the brain resting-state connectivity of awake and lightly sedated rats. The aim of Study III was to compare the resting-state connectivity of rats imaged under the most commonly used preclinical anesthetics to connectivity measured under awake state. It was hoped that the results from these studies would provide valuable information of the influence of preclinical anesthetics on brain connectivity which can help researchers choose the most suitable imaging protocol for their fMRI study designs.
3. To improve awake rat fMRI methodology by implementing a novel MB-SWIFT sequence

Multiband-SWIFT is a unique and novel MRI sequence. With its silent nature and high insensitivity to motion, the working hypothesis was that this sequence would be especially well suited for fMRI studies with awake animals. Therefore, the aim of Study IV was to improve the awake rat fMRI imaging method by determining the use of MB-SWIFT sequence in awake rat resting-state connectivity studies, and to compare the sequence with the standard SE-EPI sequence.
4 SUBJECTS AND METHODS

4.1 ANIMALS

Animal work was approved by the Animal Experiment Board in Finland and implemented according to the guidelines set by the European Commission Directive 2010/63/EU. A total of 125 Wistar rats were used in Studies I-IV according to Table 2. The rats were either single or group-housed in individual ventilated cages (IVC), maintained on a 12/12 h light-dark cycle at room temperature of 22±2 °C and humidity of 50-60%. Food and tap water were available ad libitum.

Table 2. Overview of study protocols used in Studies I-V.

<table>
<thead>
<tr>
<th>Study</th>
<th>Subjects</th>
<th>Awake/anesthetics</th>
<th>Surgical procedures</th>
<th>Measurements</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Male Wistar rats, n=42, 419 ± 15 g</td>
<td>Isoflurane (1.8% before measurements, 1.3%, 2.0%, 3.0% during measurements)</td>
<td>Electrode implantation, tracheostomy, femoral vein/artery catheterization, perfusion + brain dissection</td>
<td>Resting-state fMRI, LFP, mRNA-sequencing</td>
</tr>
<tr>
<td>II</td>
<td>Male Wistar rats, n=23, 320 ± 25 g</td>
<td>Awake, isoflurane (0.5 and 1.3%)</td>
<td>Tracheostomy, femoral vein/artery catheterization</td>
<td>Resting-state fMRI, open field and sucrose preference test</td>
</tr>
<tr>
<td>III</td>
<td>Male Wistar rats, n=70, 235-440 g</td>
<td>Awake, propofol (7.5 mg/kg), α-chloralose (60 mg/kg, i.v.), isoflurane (1.3%), medetomidine (0.1 mg/kg/h, i.v.), ISO/MED (0.06 mg/kg/h i.v. MED and 0.5-0.6% ISO), urethane (1.25 g/kg, i.p.)</td>
<td>Tracheostomy, femoral vein/artery catheterization for anesthetized rats</td>
<td>Resting-state fMRI</td>
</tr>
<tr>
<td>IV</td>
<td>Male Wistar rats, n=13, 418 ± 23 g</td>
<td>Awake, isoflurane (1.3-2.0%)</td>
<td>Electrode implantation</td>
<td>Resting-state fMRI, EEG</td>
</tr>
</tbody>
</table>
4.2 SURGICAL PROCEDURES

4.2.1 Tracheostomy and femoral vein/artery catheterization (I-III)

Rats were mechanically ventilated and catheterized under anesthesia for blood sample withdrawal for the fMRI and/or LFP measurements. First, a small incision was made in the hind leg and small cannulas were inserted into femoral vein and artery and secured in place with sutures. Then, an incision was made in the neck and a small cannula was inserted into the trachea. The rat was then placed into an animal holder where the tracheal cannula was attached to the ventilator (MA1-7061, Harvard Apparatus Inc.). A bolus injection (i.v.) of muscle relaxant, pancuronium bromide (Pavulon™, Organon, Oss, Netherlands), was given until the animal stopped spontaneous breathing, and dosing was subsequently continued (~1 mg/kg/h, i.v.) throughout the study.

4.2.2 Electrode implantation (I, IV)

Epidural or/and deep electrodes were inserted acutely immediately before the measurements. First, the skull was exposed and cleaned with saline and hydrogen peroxide (3%) and left to dry. Small holes (~1 mm in diameter) were drilled into the skull according to electrode positions. In Study I, screw electrodes (BN 650, Bossard Holding AG, Switzerland) were inserted into the dura bilaterally over the left (S1L) and right (S1R) (AP:-1, ML: +/- 3 mm) somatosensory cortex and over the cerebellum (AP:-12, ML: - 2 mm) as a reference. A stainless steel double wire electrode (50 µm diameter, 600 µm tip separation, Nr.15168, California Fine Wire company, CA, USA) was implanted into the right hippocampus for DG (AP: -3.8, ML: +1.6, DV: -4.3 mm) and for CA1 (AP: -3.8, ML: +1.6, DV: -3.7 mm). In Study IV, polytetrafluoroethylene coated silver wire electrodes (0.2 mm bare wire diameter, AG549511, Advent Research Materials, Oxford, UK) were bent into an L-shape and placed on the dura bilaterally over the somatosensory (S1) cortex (AP −2.12; ML 2.5) and over the cerebellum (AP:-12, ML: -2 mm) as a reference. A silver wire (0.2 mm bare wire diameter) was placed under the neck skin to act as a ground in Studies I and IV.

4.2.3 Perfusion and brain dissection (I)

The rat was anesthetized with isoflurane (5% for induction and maintenance) for the perfusion. A needle was inserted into the left ventricle and an incision was made in the right vena cava. The rat was perfused with ice-cold NaCl for 4 min with a flow speed of 30 ml/min. After the perfusion, the head was decapitated, and the brain extracted, and the specimens of somatosensory cortices and hippocampus were dissected out. Brain samples were snap frozen in liquid nitrogen stored at -80°C.
4.3 HABITUATION PROTOCOL

An awake rat habituation protocol was developed in Study II and the same protocol was utilized in Study IV. A detailed description of the habituation protocol can be found in original Publication II (Stenroos et al. 2018). First, 3D-printable restraint parts were designed with a CAD-software (Autodesk 123D Design) to fit to the Bruker Biospin MRI rat bed. The 3D-printed restraint parts were padded with foam to reduce irritation to the parts touching the animal’s skin.

Briefly, the rat was first anesthetized with isoflurane and placed on the heating mat where the preparation steps were performed. Fore- and hind- paws were taped together, and the animal was wrapped in foamed plastic. Foamed plastic was taped around the rat’s shoulders and hind paws. Silicone ear plugs were inserted to protect the animal from loud gradient switching noises coming from the magnet. Next, the animal was moved to a rat bed where restraint parts were inserted. Vertical head motion was reduced by a nose cone, lateral motion by cheek supports, and neck or breathing related motion by a neck support. A final tape was placed around the rear body to reduce excessive body motion. A mock surface RF-coil was placed on top of the rat and then it was placed inside a mock scanner tube. The isoflurane inhalation was terminated, and habituation to restraint and MRI noises started. MRI noise were played back through a loudspeaker (JBL LSR308, Harman International Industries, Stamford, CT, USA) at a sound pressure level (SPL) equivalent to the MRI sequence as described in original Publications II and IV. In Study II, movement, breathing and heart rate and blood corticosterone levels were monitored throughout the habituation and MRI periods. The habituation schedule for Studies II and IV is illustrated in Figure 3. After the habituation session, the rat was anesthetized with isoflurane and removed from the restraint holder. During MRI sessions, a similar restraint protocol was used.
Figure 2. The awake habituation scheme (A) used in Study II, and the 3D-printable restraint holder (B). The rat restraint kit includes a Bruker rat bed (1), a padded shoulder/neck support (2), a sliding sledge with a bite bar (3), padded cheek supports (4), and a padded nose cone (5).

### 4.4 MRI MEASUREMENTS

#### 4.4.1 Hardware

MRI measurements were conducted with horizontal Bruker Biospin 7 T Bruker Pharmascan system operated with ParaVision 5.1 software (Bruker Biospin, Ettlingen, Germany) (I, II, III), and with horizontal 9.4 T bore magnet, with either 12-cm or 21-cm ID gradient coil set, interfaced with an Agilent DirectDRIVE console (Palo Alto, CA, USA) and operated by VnmrJ 3.1 (IV). For signal transmission and acquisition, rat brain quadrature surface coil and a quadrature resonator volume coil were used (I, II, III), or a custom-made (in-house made or by Neos Biotec, Pamplona, Spain) surface transmit-receive RF coil (IV).
4.4.2 Anatomical and functional imaging

Before the measurements, the animals were anesthetized according to protocols described in Figure 3. All fMRI measurements were done under resting state conditions with either anesthetized, lightly sedated or awake rats. In Study IV, measurements were additionally done with anesthetized rats where intentional body motion was induced by pulling strings, while the head was tightly fixed to the holder. Prior to anatomical and functional imaging, shimming was conducted with either Bruker or Agilent automatic 3-D field map method (I, II, III, IV) or by manual shimming (IV).

In Studies I, II and III fast spin-echo based TurboRare T2-weighted anatomical images were acquired before or after the functional imaging with the following parameters: repetition time 4.7 s, echo-train length 8, echo spacing 16 ms, effective echo time 48 ms, field-of-view 5.0 × 5.0 cm, bandwidth of 46.875 kHz, matrix size 512 × 512, and 30 slices with a thickness of 0.75 mm. In Study IV, fast spin echo based multi-slice (FSEMs) T2-weighted anatomical images were acquired with the following parameters: repetition time 3 (3.2) s, number of echoes 8, echo spacing 12 ms, effective echo time 48 ms, averages 4, bandwidth 62.5 kHz, matrix size 256 × 256, field-of-view 3.5 × 3.5 (4.0 × 4.0) cm², and 20–30 slices with a thickness of 0.5–1.0 mm. Values in parentheses above were used with the 21-cm gradient coil set.

In Studies I, II and III, functional imaging was performed with a single-shot spin-echo echo planar imaging sequence with the following parameters: repetition time 2 s, echo time 45 ms, bandwidth of 250 kHz, matrix size 64 × 64, field-of-view 2.5 × 2.5 cm, 9–11 slices with a thickness of 1.5 mm with outer volume suppression. In Study IV, functional imaging was acquired with MB-SWIFT and SE-EPI sequences. The parameters applied for the MB-SWIFT were as follows: repetition time 0.97 ms, 2000 spokes per volume, temporal resolution of 1940 ms, excitation/acquisition bandwidths 192/384 kHz, matrix size 64³, field-of-view 3.5 × 3.5 × 6.4 (4.0 × 4.0 × 6.4) cm³, and flip angle 6°. The parameters for the SE-EPI were as follows: repetition time 2–4 s, echo time 35 ms, bandwidth 208 kHz, matrix size 64 × 64 (64 × 32), field-of-view 3.5 × 3.5 (4.0 × 4.0) cm², and 8–15 slices with a thickness of 1.5 mm. Values in parentheses above were used with a larger 21-cm gradient coil.

After the measurements, the rats used in awake or lightly sedated imaging were returned to their cages (II, IV) and rats used for imaging under anesthesia were sacrificed with 5% isoflurane, administration of KCl (i.v.), and/or cervical dislocation (I, II, III, IV).
4.5 ELECTROPHYSIOLOGY

4.5.1 Hardware

Electrophysiological measurements were recorded with SciWorks data acquisition system (Datawave Technologies, Loveland, CO, USA) with a 2050 Hz sampling rate (I), and with BrainAmp MR system (Brain Products GmbH, Gilching, Germany)
with a preamplifier (10 x amplification; Multi Channel Systems, Reutlingen, Germany) with a 5000 Hz sampling rate (IV).

4.5.2 Recording

In Study I, a similar anesthetia protocol was used as in the fMRI measurements (Figure 3). In Study IV, the rats were first anesthetized with 5% isoflurane, and recording was conducted under 1.3–2.0% isoflurane. After the measurements, the rats were sacrificed with 5% isoflurane (I, IV), administration of KCl (i.v.) (I), and cervical dislocation (I, IV).

4.5.3 Simultaneous EEG-fMRI (I, IV)

Simultaneous EEG/fMRI measurements were conducted under 1.3% and 2.0% isoflurane anesthesia with a subgroup of rats (n=7) (unpublished data for Study I) and in Study IV (n=3).

Rats were implanted with a chronic EEG implant (unpublished data for Study I). First, the skull was exposed and cleaned and holes for the electrodes were drilled according to chapter 3.2.2. Additionally, small holes were drilled throughout the skull to facilitate bone cement adhesion (Palacos R + G, Heraeus Medical, Hanau, Germany). Polytetrafluoroethylene coated silver wire electrodes (0.2 mm bare wire diameter, AG549511, Advent Research Materials, Oxford, UK) were bent into a spiral shape and placed on the dura bilaterally over the somatosensory (S1) cortex (AP −2; ML 2.5) and cerebellum (AP:−12, ML: ± 2 mm) as a reference and ground. The electrodes were attached to the skull with Vetbond glue. The golden pins at the other ends of the electrodes were fitted to a plastic pedestal. Next, a layer of bone cement was applied and after a drying period, additional layers of dental cement (Selectaplus, DeguDent GmbH, Hanau, Germany) was applied. After a waiting period of one week, simultaneous EEG-fMRI experiments were conducted. Before the measurements, the animals were mechanically ventilated and catheterized in order to maintain normal blood gas values, similarly as done in Study I. EEG was recorded with a BrainAmp MR system (Brain Products GmbH, Garching, Germany) with a 5000 Hz sampling rate. Functional magnetic resonance imaging was performed with the following parameters: repetition time 2000 ms (acquisition within 650 ms and 1450 ms break for artefact free EEG), echo time 45 ms, bandwidth of 250 kHz, matrix size 64 × 64, field-of-view 2.5 × 2.5 cm, 9 slices with a thickness of 1.5 mm.

In Study IV, the electrodes were implanted acutely before the fMRI measurements using similar methods, except that bone/dental cement was not used. EEG was recorded according to 4.5.1. and MRI was conducted according to 4.4.2.
4.6 RNA-SEQUENCING (I)

Total RNA was extracted from the brain samples from somatosensory cortex and hippocampus with the Direct-zol™ RNA Miniprep (Zymo Research, Irvine, CA, USA) according to the manufacturer’s guidelines. Briefly, samples were homogenized in cold phosphate buffered saline (PBS) and TRIzol Reagent, and RNA was extracted after several filtering and washing steps with Zymo-Spin IIC columns. Lastly, RNA was eluted with DNase/RNase-free water.

Messenger-RNA (mRNA) was sequenced with a stranded mRNA-seq protocol (Finnish Functional Genomics Centre, University of Turku, Turku, Finland). First, the quality of the RNA samples was ensured by Advanced Analytical Fragment Analyzer and its concentration was measured with Qubit® Fluorometric Quantitation, Life Technologies. An RNA library was made by TruSeq® Stranded mRNA Sample Preparation, Illumina. Sequencing was performed using Illumina HiSeq 3000 with a read length of 50 bp and 260-320 M reads per sample.

4.7 STRESS MEASUREMENTS

4.7.1 Acute stress (II)

Plasma corticosterone level, heart and respiratory rate, and movement were measured each day during the habituation and fMRI sessions to estimate the acute stress in awake and lightly sedated animals caused by the habituation protocol (II). Respiratory rate (breaths/min) was measured with a pressure sensor placed under the rat and the heart rate (beats/min) with ECG electrodes (during habituation) or pulse oximetry sensor (during fMRI). Movement (counts/min) was analyzed from either the digital videos recorded during the habituation periods or from MRI images. After the habituation or imaging sessions, 150 µl of blood were drawn from the lateral tail vein, and corticosterone levels were later analyzed by corticosterone immunoassay (Corticosterone rat/mouse ELISA Cat. No.: RTC002R, Demeditec Diagnostics, Kiel, Germany) and a microtiter plate reader (Labsystems Multiskan MS, Vantaa, Finland) at absorbance of 405 nm.

4.7.2 Long-term stress (II)

To study potential depression and anxiety of rats caused by awake habituation, open field, and sucrose preference tests were run, and the body weight was measured 10 days after awake fMRI with a subgroup of rats (n = 5 habituated + 5 control rats). In the open-field test, the rat was placed in the circular plastic arena (diameter 100 cm), and the movement of the rat were video-recorded for 10 min. Distance travelled, rearing/grooming time and the time spent in different zones of the arena were analyzed with Ethovision XT 7.1 (Noldus, Wageningen, The Netherlands). In the sucrose preference test, the consumption of sucrose (1%) and
regular tap water were measured for 2 days, to analyze the percentage of sucrose water consumption. Each day, the water bottles were weighted, and positioning of the bottles was switched.

4.8 DATA ANALYSIS

When comparing groups, the results are shown as mean ± SEM. If not stated otherwise, Student’s two-sample t-test assuming equal variances and false discover rate (FDR) were used to account for multiple comparisons in all group level comparisons. In the group level functional connectivity analysis, the obtained correlation values (r-values) were normalized to z-values prior to calculating averages and performing statistical tests. Subsequently, the z-values were returned to r-values.

4.8.1 fMRI

Preprocessing

Functional MRI data (I, II, III) were first converted to NIfTI (http://aedes.uef.fi/), then slice-timing corrected, motion-corrected, spatially smoothed (2 × 2 voxel full-width at half-maximum Gaussian kernel), and finally co-registered to a reference brain (previously acquired SE-EPI image) with SPM8 and Matlab (Version 2011a, The Mathworks Inc., Natick, MA, USA). The motion of the awake rats was evaluated by visual inspection of volumes from raw data and by mass-center displacement values obtained from SPM8. Data series with motion larger than the voxel size (0.391 mm) were excluded from the analysis. Motion scrubbed 10-min data were then preprocessed again to evaluate FC. In order to demonstrate the success of motion correction steps, translational (x, y, z) and rotational parameters from individual animals and average translational values were illustrated.

MB-SWIFT data (III) were first reconstructed with SWIFT package 2018 (https://www.cmrr.umn.edu/swift/index.php) using correlation, gridding, and three iterations of the FISTA algorithm (Beck and Teboulle, 2009). The intensity bias caused by intensity gradient in the MB-SWIFT images was removed from the images using an N4ITK bias correction (Tustison et al., 2010). Volumes were motion corrected using Advanced Normalization Tools (ANTS, http://stnava.github.io/ANTs/) (Avants et al., 2011), and three nuisance regressors for both translation and rotation were computed. For co-registration, anatomical FSEMS images of each subject were first co-registered to a reference FSEMS image with ANTs, and the acquired transformations were applied to each realigned MB-SWIFT image. Finally, volumes with motion were excluded from the data. Motion was evaluated by visual inspection of volumes from the raw data, and by mass-center displacement values obtained by ANTs. Data series having motion more than 0.1% of voxel size and less than continuous 45 motion-free volumes were
excluded from the analysis. In order to demonstrate the success of motion correction steps, translational (x, y, z) and rotational parameters from individual animals and average translational values were illustrated.

Additionally, body motion induced $B_0$ changes were evaluated in isoflurane anesthetized rats from the raw or preprocessed images during MB-SWIFT or SE-EPI acquisition. Cortical signal variation between MB-SWIFT and SE-EPI were subsequently compared during the motion periods.

**Functional connectivity**

Whole brain functional connectivity was evaluated by a seed-based correlation (I, II, III, IV), voxel-wise correlation (I, II, III), and by independent component analysis (II, IV).

Before the analysis, the data were band-pass filtered at 0.01–0.15 Hz. Pearson correlation coefficients were calculated between time-series obtained from individual voxels or the selected regions of interests. For the seed-based correlation analysis, the 12 region of interests (ROIs) were as follows: medial frontal cortex (mFC), motor cortex (MC), somatosensory cortex (SC), visual cortex (VC), auditory cortex (AC), retrosplenial cortex (RC), nucleus accumbens (NAc), striatum (Str), hippocampus (HC), medial thalamus (ThM), ventrolateral thalamus (ThVL), and hypothalamus (HTH) (I-IV). The correlation was calculated from the time-series of 300 motion-free volumes (10 min) in each anesthesia or awake period (I-IV). Additionally, 12 ROIs were divided into 92 smaller ROIs, and the correlations of ROI time-series were used in graph theory-based complex-network analyses (Brain Connectivity Toolbox, https://sites.google.com/site/bctnet/) (III). Average correlations were obtained from the calculated correlation coefficients by taking an average from all cortico-cortical, cortico-striatal, hippocampal-cortical and thalamo-cortical connections (I). Additionally, partial correlation coefficients were calculated by using motion correction parameters as nuisance regressors (II, IV).

Functional connectivity in awake rats was evaluated by group-ICA (ICA; FSL MELODIC, https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/MELODIC) with manually drawn brain masks (II and IV). The number of components which had to be computed ranged from 30-50, according to previous awake rat studies (Becerra et al., 2011; Liang et al., 2011). Computed components that were located only at brain surfaces, inside large vessels, or only unilaterally, or were anatomically poorly localized, were excluded from the analysis to minimize the inclusion of artificial non-neural components.

**4.8.2 Electrophysiology**

Data were analyzed with Spike, version 8 (CED Ltd, Cambridge, England)) and Matlab (R2011a and R2017b, Mathworks Inc., Natick, MA, USA). In Study I, cortico-cortical and cortico-hippocampal correlation, and burst suppression activity were
evaluated to study brain connectivity. Additionally, the EEG-fMRI cross-correlation was determined in order to study the correlation between isoflurane caused BS activity and the fMRI signal.

**Correlation and burst suppression**

In Study I, the LFP signal was first normalized, band-stop filtered at 49-51 Hz by using a notch filter, and band-pass filtered at 1-90 Hz by using a 2nd order Butterworth filter (Spike2). The inter channel correlation was measured from the LFP signal by determining LFP envelope amplitude of the full band signal between the electrodes (see Liu et al. 2013). First, the normalized full-band LFP signal was converted to the LFP envelope by taking the absolute value of a Hilbert transform (Hilbert function) in Matlab. The envelope was then convolved with a double-gamma hemodynamic response function (HRF) with a 4-s lag and a negative undershoot which was constructed accordingly to findings by Martin et al. (Martin et al. 2006, Neuroimage). The convolved signal was then low-pass filtered below 0.15 Hz and down-sampled to 0.5 Hz to match the sampling rate of MRI. Finally, the Pearson correlation (r) was calculated to compare the detrended signals during the 10 min isoflurane periods. Correlation matrices of control and treated groups were created separately, and groups were compared by subtracting the correlation values of the control group from the treated group (Figure 6B).

Isoflurane induced burst suppression (BS) occurrence rate (bursts/s), duration of suppression periods (s) and burst amplitude (µV) were also evaluated for each isoflurane concentration. First, BS activity was analyzed with an in-house modified Matlab script FindRipples (http://fmatoolbox.sourceforge.net/Contents/FMAToolbox/Analyses/FindRipples.html). Burst occurrence rate and suppression durations from cortex and hippocampus were calculated by taking an average from the detected epochs from the left and right somatosensory cortices and from the DG and CA1 regions in hippocampus, respectively. When studying the amplitude of the burst activity, the suppression periods were first removed from the signal. Then, the root mean square (rms) function was used to obtain the amplitude for the remaining signal, for each isoflurane period.

**EEG-fMRI**

In unpublished data as a part of Study I, the correlation between EEG and fMRI signals (see Liu et al. 2013) was analyzed in the presence of isoflurane concentrations of 1.3% and 2.0%. First, the EEG signal was denoised from gradient switching artefacts using a wavelet-ICA method (Sheoran et al. 2014). A discrete stationary wavelet decomposition (swt-function in MATLAB) and a fast ICA algorithm (https://research.ics.aalto.fi/ica/fastica/) were used to obtain independent components. The components containing artefacts were removed and an inverse discrete stationary wavelet transform was used to recover the artefact-free signal.
Denoised EEG data were filtered, Hilbert transformed, and convolved with a HRF similar that described above. The convolved signal was then low-pass filtered below 0.15 Hz and down sampled to 0.5 Hz to match the sampling rate of MRI. To extract the burst suppression activity from the fMRI signal, ICA was used, and the extracted BS signal was compared with the HRF-convolved EEG envelopes. Finally, the BOLD and EEG signals were detrended and normalized, and the cross-correlation (r) was calculated between the signals in 300-volume data. The correlations in the primary somatosensory cortex in the presence of 1.3% and 2.0% isoflurane concentrations were calculated by taking an average of maximum cross-correlation values from the left and right hemispheres.

In Study IV, the cortical EEG signal was analyzed using similar methods as described above. MB-SWIFT and EEG signals were detrended, normalized, and the signals were aligned by a maximizing cross-correlation. A cross-correlation was calculated by using 50 volume windows with 10 volume steps. In addition, a gradient switching artefact on raw and denoised EEG, and electrode caused a susceptibility artefact on MRI image were evaluated for MB-SWIFT data and compared to SE-EPI data according to Publication IV.

4.8.3 Stress

The plasma corticosterone level, respiratory and heart rate, movement, and weight were analyzed with ANOVA for repeated measures and Dunnett’s post-hoc tests using GraphPad Prism. Differences in corticosterone levels between consecutive days were evaluated by comparing each habituation/MRI day to the baseline level. Differences in the heart rate, movement and weight between days were evaluated by comparing each habituation/MRI day to habituation day 1.

4.8.4 Gene expression

A detailed description of the gene expression analysis is provided in Publication I. In brief, after RNA-sequencing, sequencing reads were aligned with the rat reference transcriptome, transcript abundance quantification was performed (Kallisto v. 0.44.0) and transformed to gene-level counts (R package tximport version 1.10.1, R3.5.1) from the somatosensory cortex and hippocampus of each animal. Finally, the counts were pre-filtered (at least 3 samples with a count of 5 or higher) and normalized (R package DESeq2 version 1.22.2). The differentially expressed genes, in fold changes, between the groups were analyzed with the likelihood ratio test in DESeq2. A gene ontology classification was performed in the Panther classification system (PANTHER14.1).
5 RESULTS

The main finding in this thesis was that anesthetic agents modulate the resting-state connectivity of the rat brain both acutely and over the long-term comparing ed to the awake state. Another main finding was that resting-state connectivity can be measured relatively stress-free by using the novel awake rat fMRI method, and that the MB-SWIFT sequence can be more optimal for awake rat imaging than the standard EPI-sequences.

5.1 LONG-TERM CHANGES IN CONNECTIVITY, LFP AND GENE EXPRESSION AFTER ISOFLURANE TREATMENT

5.1.1 Isoflurane induced burst suppression activity

Functional MRI and local field potential measurements were measured in the presence of three different isoflurane concentrations of 1.3%, 2.0% and 3.0%. Each concentration of isoflurane induced burst suppression pattern which could be observed in LFP and fMRI (Figure 4). Similar to earlier findings (Liu et al.), the inter-burst interval of BS was shorter at 1.3% isoflurane and increased when there were high isoflurane concentrations of 2.0% and 3.0%. The intra-burst frequency was most prominent in the 1-40 Hz frequency range and the duration of each burst varied between 1-2 s. The average correlation between EEG envelope and BOLD signal acquired from somatosensory cortex was $0.24 \pm 0.03$ and $0.38 \pm 0.04$ with isoflurane concentrations of 1.3% and 2.0%, respectively.

When comparing isoflurane treated and control groups in Study I, no differences were found in BS patterns of occurrence rate, duration of suppression periods, or burst amplitude in somatosensory cortex (1.3%: $p>0.47$; 2.0%: $p>0.9354$; 3.0%: $p>0.22$) or in hippocampus (1.3%: $p>0.3778$; 2.0%: $p>0.8596$; 3.0%: $p>0.13$) in any isoflurane period (Figure 4).
Figure 4. Example of isoflurane induced burst suppression pattern detected from LFP and fMRI measurements from three representative rats (A, B and C). A typical burst suppression pattern was detected during three isoflurane concentrations of 1.3%, 2.0% and 3.0% from LFP (A), and from fMRI BOLD signal (B). Aligned EEG envelope and BOLD signals obtained from the primary somatosensory cortex in a representative animal (C).

5.1.2 Functional connectivity changes in response to isoflurane treatment

Average cortico-cortical, cortico-striatal, hippocampal-cortical and thalamo-cortical connectivities in the control and treated groups are illustrated in Figure 5. Significant increases were observed in average hippocampal-cortical correlation in the 2.0% period, and thalamo-cortical correlation in the 2.0% and the second 1.3% periods.
Based on 12-ROI fMRI correlation matrices, connectivity was significantly increased in 2.0% isoflurane period and slightly increased in the 2nd 1.3% isoflurane period (Figure 6A). Most of the changes were detected in hippocampal-cortical and thalamo-cortical connectivity. Accordingly, based on the LFP inter-channel correlation, hippocampal-cortical connectivity was significantly increased (Figure 6B).
Figure 6. 12-ROI fMRI correlation differences (A) and LFP correlation (B) from control and isoflurane treated rats during 1.3% 1st, 2.0%, 1.3% 2nd and 3.0% isoflurane anesthesia. * = p-value < 0.05.

Based on 12-ROI FC matrix, the thalamo-cortical connectivity was significantly changed in 10 CCs (10/12) in the 12-ROI FC matrix (p<0.05). There was also a significant increase in two thalamo-cortical CCs (2/12) in the second 1.3% isoflurane period. The hippocampal-cortical connectivity was significantly increased in five CCs (5/6) in the 2.0% isoflurane period (p<0.05). There was also a significant increase in one hippocampal-cortical CC (1/6) in the second 1.3% isoflurane period. To validate the observed changes in the fMRI connectivity, LFP connectivity was evaluated from hippocampal and cortical electrodes. Based on the LFP connectivity matrix, connectivity was increased in the second 1.3% period and appeared to be increased in the first 1.3% period and in the 2.0% period.

5.1.3 Gene expression

To explain connectivity findings, a whole genome RNA-sequencing was conducted. The expressions of 20 cortical genes were found to differ in response to isoflurane treatment (see Publication I). The expression was downregulated in 7 genes (7/20) (p<0.031) and upregulated in 13 genes (13/20) (p<0.049). Based on gene ontology classification, three genes belonged to the signal transmission class (Slc32a1, Best1, Scn9a), one to protein kinase class (Hipk2), and one to bitopic protein class (Snn).
5.2 UTILIZATION OF THE NOVEL AWAKE RAT FMRI METHOD

5.2.1 Stress and movement

The stress experienced by the rats decreased during the habituation period based on plasma corticosterone level, movement and heart rate (Figure 7A). Furthermore, there was no evidence of elevated long-term stress visible after 10 days from the awake imaging, as based on open field and sucrose preference tests or by weight (Figure 7B).

![Graphs of stress indicators](image)

Figure 7. Initial stress indicators of corticosterone, movement and heart rate (A) and long-term stress indicators obtained from open field and sucrose preference tests and from weight (B). * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001.

In awake animals, corticosterone concentrations (ng/ml) increased significantly from the baseline level on habituation day 1 (p = 0.014) indicating an elevated stress level due to awake habituation. However, the corticosterone level returned to baseline in the awake group on habituation day 4 (p = 0.75) and remained at the baseline level on MRI day 1 in both awake and lightly sedated animals (p = 0.25 and p = 0.35, paired t-test). The corticosterone concentration did differ statistically on MRI day 2 from MRI day 1 (p = 0.064, paired t-test). In the lightly sedated group, there was no clear difference between the baseline (117 ± 20 ng/ml) or the 1st habituation day (168 ± 27 ng/ml) (p = 0.066, paired t-test). Movement occurrences (counts/min) decreased significantly already during habituation day 2 (p = 0.002) as compared to habituation day 1. Lightly sedated rats moved significantly less during MRI day 1 as compared to awake rats (p < 0.001).
The heart rate decreased significantly on habituation day 4 and also on MRI day 1 as compared to habituation day 1 (p < 0.018). With respect to the long-term stress indicators, there were no differences in locomotor activity in the open field test, sucrose preference, or in the animals’ body weight (p > 0.05).

The amount of head motion of awake rats during the fMRI was evaluated from mass center displacement values provided by SPM (Figure 8) after the motion scrubbing procedure. The average motion occurrences of awake rats were 1.00 ± 0.58 events/min, the average motion 2.8 ± 0.2% relative to voxel size, and the maximum single head motion was 31.5 ± 0.06% relative to voxel size. No notable head motion was observed in the anesthetized rats. When comparing the average head motion of awake rats to anesthetized rats, motion differed significantly in X- (p < 0.001, two-sample t-test) and Y- (p < 0.001, two-sample t-test) directions, and in the rotational directions of roll (p < 0.01, two-sample t-test) and yaw (p < 0.001, two-sample t-test).

![Figure 8. Mass center displacement values of awake rats (n=6) provided by SPM after motion scrubbing. Each animal is represented by different color by translational (relative to voxel size) x, y, z and rotational (degrees) roll, pitch, yaw parameters.](image)

### 5.2.2 Physiology under different anesthetics and awake state

All physiologic parameters were within the normal range and no differences were detected in body weight or blood gas values (pCO₂, pO₂, and sO₂, one-way ANOVA and Tukey’s multiple comparison test) between the groups (see Publication III). However, the heart rates were characteristically significantly
decreased in the protocols using medetomidine (see. Lukasik and Gillies, 2003) when compared to other protocols.

5.2.3 Resting-state functional connectivity under different anesthetics and awake state

Based on group-ICA, the awake rat brain image was parcellated to multiple cortical FC modules. Mostly cortical components were found; out of these, 34/50 were estimated to be anatomically well-localized, i.e. not motion related or localized in veins. Near whole cortical coverage was achieved, with the strongest components being found in prefrontal (#1: medial frontal, #2: lateral frontal) insular (#3), somatomotor (#4), visual (#5), cingulate (#6) cortices. Based on a ROI-correlation analysis, the FC pattern obtained from awake rats consisted mainly of moderate to strong CCs. Moderate to strong average CCs were observed in cortico-cortical (0.44 ± 0.03), cortico-striatal (0.29 ± 0.04), hippocampal-cortical (0.22 ± 0.05), and thalamo-cortical (0.31 ± 0.03) connections. Low CCs were observed only in nucleus accumbens and hypothalamus, possibly because of a lower SNR acquired from deep brain structures.

![Figure 9. Independent components obtained from awake rats during SE-EPI. The analysis was set to run with 50 components, of which the 6 anatomically best localized components are shown. The statistical z-score maps are overlaid on anatomical T2-weighted images.](image)

In the awake group, there were no significant differences between the FC after the first and second week of training (p > 0.99) (Figure 10A). In the lightly sedated group, FC was significantly weaker between medial thalamus-motor, medial
thalamus-somatosensory, and medial thalamus-visual cortex as compared with the awake group (Figure 10A).

The connectivity changes between the anesthesia groups compared to the awake group are visualized at the group level in the 12-ROI correlation difference matrices (Figure 10B) and in the average correlation box plots (Figure 11). In the isoflurane group, several cortico-cortical CCs (15/15) were strengthened while cortico-subcortical (23/36) and subcortico-subcortical CCs (5/15) were suppressed. In the medetomidine group, the FC was globally suppressed (40/45) as compared with the awake group. In the isoflurane-medetomidine group, many subcortico-subcortical CCs (8/15), cortico-subcortical CCs (18/36), but only a few (5/15) cortico-cortical CCs were suppressed. In the α-chloralose group, relatively few CCs were suppressed in cortico-subcortical (8/36), subcortico-subcortical (5/15), and cortico-cortical (5/15) connections. In the propofol group, there were no statistical differences in any CCs when compared to the awake group. In the urethane group, overall, only 4 connections were suppressed. In the post-mortem group, CCs were nonexistent (0.02 ± 0.01) and almost all CCs differed from the awake group as expected.

The complex network analysis is illustrated in Figure 11B. Modularity was reduced in the isoflurane group (58%) when compared to the awake group. The mean degree was decreased in the isoflurane + medetomidine (15%) and medetomidine (23%) groups. Mean distance was slightly increased in the α-chloralose (30%), urethane (24%), and isoflurane + medetomidine (36%) groups, and considerably increased in the medetomidine (58%) group. The mean clustering coefficient was considerably increased in the isoflurane (93%) group and decreased in the medetomidine (61%) group.
Figure 10. 12-ROI correlation matrices in awake and lightly sedated rats (A) and correlation difference between awake and anesthetized rats (B). Awake rats were imaged after one or two weeks of habituation. Lightly sedated rats were imaged in the presence of 0.5% isoflurane anesthesia. Note the different color scale in B. * = p-value < 0.05.
5.3 RESTING-STATE FUNCTIONAL CONNECTIVITY OF AWAKE RATS ACQUIRED WITH MULTI-BAND SWIFT MRI SEQUENCE

5.3.1 Movement

First, the body motion-induced artefacts were evaluated with the subgroup of anesthetized head fixed rats. Body motion induced signal changes during SE-EPI and MB-SWIFT fMRI are illustrated from raw data in Figure 12A. Motion induced apparent brain shifts and squeezing were apparent in the SE-EPI images and manifested by cortical signal dropout and a higher signal variation during the induced periods of motion when compared to MB-SWIFT data. Apparent motion (∼2–3 mm ventral) was confirmed by visual inspection of images during the motion period. In contrast, cortical MB-SWIFT signal was minimally affected during the motion periods. Additionally, raw SE-EPI data was compared with the motion-corrected SE-EPI data to investigate the success of motion correction on data quality.
While a standard rigid ANTs motion correction failed to correct all the motion induced artefacts, an affine ANTs combined with regression of the 12 motion correction parameters improved the data quality significantly (Figure 12B). However, 2 of the 6 datasets were still affected by motion related artefacts.

Figure 12. Signal changes during controlled body motion experiments during SE-EPI and MB-SWIFT. The raw signal was compared between SE-EPI and MB-SWIFT sequences (A) and the effect of motion correction alone and together with motion regression was evaluated from SE-EPI data (B).

Awake rats moved on average 0.48 ± 0.23 events/min which was significantly less than in Study II (1.00 ± 0.58 events/min) (p=0.03, t-test). The head motion of individual awake rats in MB-SWIFT experiments was evaluated (Figure 13A) and the average translational values were compared with SE-EPI data (Figure 13B) of Study II. Based on the head motion parameters in the MB-SWIFT data, the observed motion levels were considerably lower (~15 times) than in awake SE-EPI experiments. In MB-SWIFT measurements, out of 750 volumes, only 8% had to be discarded due to motion.
Figure 13. Mass center displacement values of individual awake rats (n=9) during resting-state fMRI MB-SWIFT acquisition (A) and average translational value comparisons between resting-state fMRI MB-SWIFT (n=6) and EPI-acquisitions (B) after motion scrubbing. Each animal is represented by different color by translational (relative to voxel size) x, y, z and rotational (degrees) roll, pitch, yaw parameters.

5.3.2 Sound pressure

Sound pressure levels and sound spectrograms of MB-SWIFT sequences differed significantly from the 8- and 15-slice EPI sequences (Publication IV, Figure 1) and. Peak SPL (84.9 ± 1.7 dB) and average SPL (63.6 ± 1.7 dB) were clearly lower during the MB-SWIFT sequence when comparing to peak and average SPLs in either 8-
slice (116.9 ± 0.6 dB and 72.0 ± 5.4 dB) or 15-slice (116.8 ± 0.7 dB and 83.3 ± 4.3 dB) EPI sequences (p < 0.005). The measured peak sound pressure was 32.0-dB higher in SE-EPI, which represents 39.8 times higher sound pressure and 9.2 time higher loudness (see Schomer 1978). The measured average sound pressure was 8.4 dB in 8-slice EPI and 19.7 dB higher in 15-slice EPI, which corresponds to 2.6 and 9.7 times higher sound pressure, and 1.8 and 3.9 times higher loudness. Sound spectrograms demonstrated that the MB-SWIFT produced noise in a narrower and lower frequency range, up to 15kHz, while SE-EPI induced higher frequencies, up to 22kHz. Importantly, rats have their highest auditory sensitivity around 12–24 kHz (Borg, 1982).

5.3.3 Resting-state functional connectivity

Based on the group-level ICA, resting-state networks could be parcellated at the near whole-brain level (Figure 14). Anatomically well-localized components were detected including the cortical components of anterior frontal (#1), medial frontal (#2), orbital frontal (#3), motor (#4), insular (#5), somatosensory (#6), auditory (#7), visual (#8), retrosplenial (#9) and subcortical components of striatum (#10) and thalamus (#11). However, a hippocampal component was detectable only partly and limited to ventral part of the hippocampus. Based on the 12-ROI correlation matrix (Figure 15), the FC pattern observed from awake rats consisted of moderate-to-strong cortico-cortical, cortico-subcortical and subcortical-subcortical CCs. Additionally, a moderate-to-strong correlation (0.3-0.7) was detected inside and between these components in prefrontal, lateral frontal, parietal cortices and subcortex (Publication IV) which point to the formation of subnetwork and effective information transfer between these subnetworks.
Figure 14. Independent components obtained from awake rats during MB-SWIFT. The analysis was set to run with 30 components, of which the 11 anatomically best localized bilateral components are shown. On the top left, all 11 components are shown together. The statistical z-score maps are overlaid on anatomical T2-weighted images.
5.3.4 Simultaneous EEG/fMRI

SE-EPI-induced artefacts on raw EEG (16 ± 6.5 mV) were estimated to be an order of magnitude higher in amplitude than those induced by MB-SWIFT (original publication IV, one representative artefact per rat). However, artefacts observed during MB-SWIFT (original Publication IV, Figure 6) and SE-EPI (data not shown) could be effectively removed with standard artefact-removal approaches.

Compared with SE-EPI, MB-SWIFT was more insensitive to the image distortions caused by the surgical operations or the presence of EEG electrodes. Compared with the anatomical FSEMS image, the SE-EPI image shows vertical stretching and signal pile-up artefacts in the cortex close to the measuring electrodes and disturbed brain shape due to air cavities in the lower brain regions. The MB-SWIFT image does not display similar artefacts.

During both the fast and slow burst suppression patterns occurring in isoflurane anesthesia, a good correlation between the EEG and MB-SWIFT fMRI signals was observed i.e. the average group-level correlation was 0.57 ± 0.04.
DISCUSSION AND CONCLUSIONS

6.1 ISOFLURANE ANESTHESIA ALTERS LONG-TERM RESTING-STATE CONNECTIVITY OF RATS

Thirty days after a single isoflurane treatment, we could detect evidence of anesthesia-induced connectivity changes in fMRI and LFP, which suggests that long-term changes had occurred in brain function. According to the fMRI, FC increased between thalamo-cortical and hippocampal-cortical circuits in the animals which had undergone isoflurane treatment. The LFP measurement further confirmed these results i.e. there was strengthened connectivity between hippocampus and cortex.

6.1.1 Isoflurane associated brain plasticity

Functional connectivity

Both the suppression and burst phases of BS could have consequences for long-term brain plasticity. The induction of suppression has been linked to reduced metabolic activity of neurons due to reduced neuronal ATP metabolism, which can lead to suppression of neuronal firing (Ching et al., 2012). Furthermore, in an attempt to restore normal brain circuits or via reductions in cortical inhibitory control, neuronal activity momentarily shifts into a cortical bursting pattern. In Study I, exposure to an isoflurane concentration of 1.8% led to modulated brain function which was still apparent one month later in both fMRI and LFP. We detected changes with the 2.0% isoflurane, where BS is the dominant feature of neuronal activity and also with the 1.3% isoflurane, where the characteristic pattern evoked by isoflurane i.e. a low frequency electrical oscillation frequency, was more evident. This indicates that changes had occurred in both BS connectivity and connectivity from other origins, such as delta connectivity. Indeed, when BS activity was subjected to regression in the FC analysis, FC changes were still prominent in most brain connections. Furthermore, FC connectivity was found to be changed in the LFP alpha and beta bands and appeared to be altered also in the delta band (Publication I). It remains to be determined whether these functional changes have a role in disturbed memory function or behavior typical for POCD.

Gene expression

In our study, we found changes in relatively few genes (20) compared to previous studies which have examined more transient effects of isoflurane (Bunting et al., 2015; Culley et al., 2006; Ding et al., 2017; Edmands et al., 2013; Lowes et al., 2017;
Zurek et al., 2014). After a long waiting period of one month, it would be expected that only the most strongly altered gene expression levels would still be evident. Isoflurane causes initial hippocampal and cortical cell apoptosis and triggers also inflammatory responses but these are typically suppressed within weeks after treatment (Cao et al., 2012; Ge et al., 2015; Kong et al., 2013; Zhang et al., 2015). In addition, behavioral studies in experimental animals have demonstrated that the hippocampal dependent memory has typically stabilized back to baseline levels within weeks to one month from the initial isoflurane treatment (Culley et al., 2004; Lin and Zuo, 2011; Uchimoto et al., 2014; Zhang et al., 2014; Zhong et al., 2015). However, the results from our studies support the concept that the changes in cortical gene expression, which are involved in signal transmission, are still evident one month after exposure to the anesthetic. These cortical gene changes could be potentially involved in the brain functional changes that were detected.

All the genes found in signal transmission classes in which there were significant differences between the groups, are involved in neuronal signaling mechanisms. The Slc32a1 gene is needed for efficient GABA transmission in neurons, Best1 codes for a calcium activated anion channel expressed in astrocytes which can function as a GABA transporter (Oh and Lee, 2017), and Scn9a codes for a voltage dependent sodium channel needed for the generation of action potentials. It is known that isoflurane can suppress the activity of GABAergic interneurons, possibly increase astrocytic glutamate transportation (Ferron et al., 2009) in cortical interneurons, or directly modulate the generation of action potentials (Ou et al., 2020). Therefore, isoflurane can trigger changes in these neuronal signaling mechanisms that are potentially manifesting long-term changes in gene expression.

6.2 ANESTHETICS ALTERS RESTING-STATE CONNECTIVITY OF RATS

6.2.1 Resting-state network changes under anesthesia measured with standard echo planar imaging technique

Awake rs-fMRI

The measured FC in the awake rats with EPI sequence showed clear functional connectivity within and between the cortical and subcortical brain regions. Typical awake resting-state connectivity patterns were observed with both ROI-based and ICA-based methods. Based on the ROI-analysis, medium to high thalamo-cortical, fronto-parietal, cortico-cortical and bilateral connectivity was observed reminiscent of typical awake brain FC, similar to earlier findings (Ma et al., 2018). The complex network analysis revealed a high mean degree and short mean distance in awake rats, which is suggestive of a network structure with multiple connections and effective paths. The network analysis indicated also high modularity in the awake brain, especially when compared to isoflurane anesthesia. In addition, anatomically
and functionally relevant components were found in group-ICA, including frontal, somatomotor, insular, cingulate and visual cortices. These brain regions play roles in conscious brain functions, such as planning, decision making, working memory, motor initiation, consciousness, vision, reward and emotional processing. As the BOLD signal was apparent over a wide frequency range in the cortex and thalamus in awake rats (Publication III), this is believed to reflect the high temporal variability typical for conscious subjects (Baria et al., 2018).

**Awake vs anesthetics**

All anesthetics were found to modulate several aspects of FC when compared to the awake state. First, thalamo-cortical FC was suppressed by most of the anesthetics according to the ROI-based analysis. The thalamus is generally considered as a central hub for relaying peripheral information flow to cerebral cortex, and therefore critical for the regulation of consciousness (Mashour and Alkire, 2013). Similar to earlier studies, the higher order cortical areas were affected, i.e. there were changes in FC in fronto-parietal and cortical connections. In addition, connections involved in frontal parts of the default mode network indicated a generally weakened FC strength. Regions in the frontal DMN pathways are associated with several important central processes such as motivational drive, social behavior, mood control, and sensory processing (Raichle, 2015), which all can be related to consciousness. However, pathways in the posterior parts of DMN indicated somewhat even strengthened FC when the anesthetized rats were compared to the awake group. Interestingly, these regions are associated with recollection functions in the memory network; these are activated in the evening and possibly during the early stages of sleep in humans (Raichle, 2015; Shannon et al., 2013), i.e. highlighting the similar characteristics between anesthesia and sleep. Based on the graph-analysis, network-level structures in modularity, mean degree, mean distance and mean clustering coefficient were altered by most anesthetics, reflecting a disturbed information transfer between brain modules. Spontaneous BOLD signal spectral power was significantly weakened and showed anesthetic-specific narrow-ranged peaks in cortex and thalamus, which can be a reflection of less dynamic FC.

Isoflurane (1.3%) anesthesia heavily masked the FC observed in the awake state by inducing synchronous cortico-cortical, cortico-striatal burst suppression fluctuations and silencing cortico-subcortical and within subcortical FC. In the network level analysis, low modularity and a high clustering coefficient were observed, possibly indicating burst-suppression behavior. These isoflurane specific FC alterations are highly consistent with previous studies (Kalthoff et al., 2013; Liu et al., 2011; Williams et al., 2010) but notably, can be minimized by using low doses of isoflurane (Xiao Liu et al., 2013b). Accordingly, when animals were lightly sedated with 0.5% isoflurane, most of the FC was preserved and appeared topologically very similar to the FC measured under awake state. However, some
thalamo-cortical connections were still affected, which can explain the reduced awareness suggested by animal’s physiological parameters (Figure 7) behavior.

The FC pattern when animals received the combination of isoflurane (0.5-0.6%) and medetomidine (0.06 mg/kg/h) better resembled the data measured from awake rats when they were compared the results obtained with isoflurane (1.3%) or medetomidine (0.1 mg/kg/h) alone with a higher concentration. Intracortical FC was only mildly affected and resembled the FC pattern in the awake state, i.e. similar to earlier findings (Brynildsen et al., 2017; Lu et al., 2012). However, thalamo-cortical and subcortical FC was only partly preserved. In network level analyses, a lower mean degree and a higher mean distance were observed, indicative of compromised information transfer. Nevertheless, these results favor the use of isoflurane/medetomidine anesthesia in preclinical fMRI experiments, especially when studying the cortex.

The FC in the presence of medetomidine (0.1 mg/kg/h) was globally clearly weakened when compared to the awake state. Intracortical, cortico-striatal and thalamo-cortical connectivity was suppressed most, while the influence on bilateral FC was moderate (see Kalthoff et al., 2013; Pawela et al., 2008; Williams et al., 2010; Zhao et al., 2008) but still distinct from the FC detected in the awake state. In network level analyses, a lower mean degree and clustering coefficient and a higher mean distance were observed, possibly evidence of scattered brain modules and compromised efficiency of cortical and subcortico-cortical information transfer.

The FC is the presence of α-chloralose (60 mg/kg) resembled moderately the FC measured in the awake state. Intracortical and thalamo-cortical connectivity appeared to be well-to-moderately preserved although the FC was globally weakened (see Baek et al., 2016; H. Lu et al., 2007; Williams et al., 2010). In network level analyses, however, a higher mean distance was detected, possibly representing weakened information transfer. These findings suggest that α-chloralose is a potential anesthetic when conducting whole-brain rs-fMRI studies.

The FC pattern in the presence of propofol (7.5 mg/kg + 45 mg/kg/h) resembled in many ways the FC pattern in the awake state. Intracortical connectivity was strong but less specific, and thalamo-cortical FC was largely maintained (see Xiping Liu et al., 2013; Tu et al., 2011) and there were no differences compared to the awake group in network level analyses. However, in the propofol group, CCs had significantly higher standard deviations; this was likely caused by changes in the depth of anesthesia (Liu et al., 2013b), which can hinder the detection power. Overall, propofol is a promising anesthetic for whole-brain preclinical rs-fMRI studies.

The FC pattern in the presence of urethane (1250 mg/kg) also highly resembled the FC pattern detected under the awake state. Intracortical and thalamo-cortical connectivity was mainly stable and specific, although a couple of striatal, thalamic and cortical bilateral connections were affected. Similar findings of preserved cortico-hippocampal (Wilson et al., 2011) and thalamo-cortical (Zhurakovskaya et al., 2016) connectivity have been reported earlier. In addition, the correlation was
reduced slightly throughout the brain. In network level analyses, a higher mean distance was observed, possibly reflecting compromised bilateral information transfer. Urethane is known to induce sleep-like brain-wave transitions, with REM-like and NREM-like states, which can introduce additional variation in the FC data. Overall, FC was only mildly modulated by urethane anesthesia, and thus urethane appears a promising anesthetic for whole-brain preclinical rs-fMRI studies, if alternating brain states are taken into account.

Concentration and dosing methods were selected based on previous literature; these have been widely used methods in preclinical fMRI. However, only one concentration and dosing method per anesthetic agent was used in this study, and there could well be differences in the outcomes when different methods are used.

6.2.2 Implementation of awake rat fMRI protocol

Many of the existing awake imaging methods have several shortcomings with regard to both technical feasibility and animal welfare. Most of the approaches require dedicated RF coils, thus complicating the replication of the protocols by other investigators. In addition, the use of ear bars, the lack of ear protection and soft restraint materials, and the use of long and non-progressive habituation periods may induce acute pain or long-term stress (see Publication II).

Our 3D printed restraint kit combined with a standard Bruker rat bed and surface coil provides an easy and affordable option for awake rat imaging. In addition, the 3D printable template can be further modified for different beds and animal sizes or to different coil configurations. The habituation protocol was relatively fast (4 days) and simple for adoption by researchers accustomed to handling animals. All preparation steps, after the initial anesthesia induction, took less than 10 min per animal. The restraint kit is suitable for animals weighing from 200 to ~500 g and appropriate restraint part sizes for larger animals are also freely available (see Publication II).

Even though resting-state connectivity was acquired from fully awake rats, there can be potentially some left-over effects of the isoflurane used in the preparation phases in both the habituation and measurement sessions. As demonstrated in study I, isoflurane can induce long-term changes in functional connectivity, meaning that some caution must be exerted in the interpretation of the results. As isoflurane is known to dilate blood vessels and suppress respiratory function, some potentially prolonged vascular effects cannot be ruled out. Therefore, we kept the concentration of isoflurane and preparation times as low a level as possible and only started the imaging with a ~10-min waiting period after the cessation of anesthesia, and until there was a clear change in breathing and heart rate indicating that the animals were in an awake condition. Additionally, the recordings from the first 5-min were discarded.
6.2.3 Handling movement and stress of awake animals

Motion

In awake rat imaging, habituation of the animal to the MRI setting is necessary, as untrained rats move considerably more during the first days of restraint (Figure 7). In our habituation protocol, the motion started to decrease rapidly from the first habituation day and decreased to a level of ~1 body motion/min on the MRI day. Occasional small body movements did not typically influence the head position, which indicated that we had achieved a robust fixation with the restraint kit. To minimize the body motion influencing the head position, elastic body foam seemed to work sufficiently well to reduce excessive motion. The neck block also seemingly further reduced breathing related head motion. The typical head motion was usually less than half of the voxel size, and the standard motion correction steps (motion scrubbing, SPM realignment) were sufficient to provide relatively motion-free data (Figure 8).

Awake rats, naturally, moved significantly more than anesthetized rats (see Publication II). Nevertheless, the amount of movement in our experiments (2.8 ± 0.2% relative to voxel size) when related to voxel size was similar to the movement of patients in a clinical fMRI setting (4-10% relative to voxel size) (Power et al., 2012, 2014) and equal or less in comparison with several other awake rat experiments (King 2005, Chang 2016). If it is necessary to avoid motion completely, one can consider using lightly sedated animals with a shortened habituation period. As shown in Figure 7, motion levels were considerably smaller, starting already from the first habituation day with rats sedated with 0.5% isoflurane when compared to fully awake rats.

Stress

We measured several stress indicators i.e. plasma corticosterone level, movement, and heart rate. Since these all decreased during the habituation, we estimate that 3D-printed restraint kit is well-suited for the acclimatization of awake rats. The plasma corticosterone concentration was relatively low even on habituation day 1 (see Chang et al., 2016; King et al., 2005) and had returned to the baseline level by habituation day 4. Furthermore, the reduced corticosterone levels cannot be explained by reduced corticosterone production in adrenal cortex over these four days, as the corticosterone production has been previously demonstrated to be considerably higher in consecutive stressful days (Chang et al., 2016; Stockham, 1964). Motion also decreased to low levels (0–350 μm) as compared to previous reports from King (King et al., 2005) (0–550 μm) and Chang (Chang et al., 2016) (0–1,300 μm) on the MRI day. Moreover, after 4 days, the heart rate decreased to ~400 beats/min, which is within the normal range of adult Wistar rats during the active day phase (Zhang and Sannajust, 2000). Additionally, no signs of long-term anxiety or depression were observed based on open field and sucrose preference tests.
conducted 10 days after the awake imaging session. Habituated and control rats performed similarly in terms of locomotor activity, rearing, and time spent in the center of open-field, and in the sucrose preference tests. The non-elevated long-term stress markers indicate habituation had not been caused by learned helplessness (Seligman and Beagley, 1975) a state which has been linked with depression-like symptoms (Maier and Watkins, 2005). As prolonged restraint has been linked to enhanced chronic cognitive dysfunction or anxiety-like behaviors (Guedri et al., 2017), initially brief and gradually increased habituation periods can be beneficial for preventing chronic stress. Nonetheless, the rats cannot be stated to be fully stress-free in the awake imaging setting. If it is essential to avoid any excess stress, then the use of lightly sedated rats is a feasible option. As shown in Figure 7, corticosterone levels decreased to baseline levels already on day 2 and the heart rate was significantly lower on the MRI day in rats at 0.5% isoflurane than in awake rats.

Rats in the awake group were single housed to habituate them to the environment where rats were restrained one individual at a time. This was thought to decrease the stress experience during the restraint as rats would not be exposed to other conspecifics. However, prolonged isolation can induce long-term changes in stress and behavior (Hatch et al., 1963) which could introduce additional variation in the results comparing to the animals in the anesthetized group that were group housed.

6.3 MULTIBAND SWIFT CAN BE MORE SUITABLE FOR AWAKE RAT FMRI COMPARED TO ECHO PLANAR IMAGING

In Study IV, the MB-SWIFT sequence was compared with the SE-EPI sequence in awake rat fMRI with respect to MRI noise, motion level, motion artefacts, and resting-state FC.

6.3.1 Noise

The measured peak and average sound pressures in MB-SWIFT were considerably lower than those observed with SE-EPI. Furthermore, MB-SWIFT allows for continuous data acquisition with a more stable sound pressure level than typical EPI sequences. Interestingly, it has been shown that non-continuous fMRI acquisition can have significant disturbing effects on FC (Langers and Van Dijk, 2011). Furthermore, the higher frequencies produced by SE-EPI can potentially be more disturbing to awake rats compared to the frequencies produced by MB-SWIFT, as rats have their highest auditory sensitivity (12–24 kHz) around the frequencies produced by SE-EPI.
6.3.2 Motion

Controlled body movement experiments were performed in rats with head fixed to demonstrate how body motion related B0-field shifts would affect the fMRI time series. It is known that with EPI fMRI acquisition, body motion, such as breathing, can induce field offsets (Kalthoff et al., 2011) which can induce spatial shifts, signals reductions, or image distortions, especially in the phase encoding direction (Jezzard, 2012). A post-hoc motion related artefact removal can be difficult even with modern motion correction approaches (Zaitsev et al., 2017), especially complicating ultra-high field studies (Jezzard, 2012). The MB-SWIFT signal was only minimally affected by controlled body motions in comparison to the SE-EPI signal (Figure 12). Special features MB-SWIFT sequence such as its high bandwidth, close to zero echo-time and radial sampling are particularly suitable for imaging in awake animals. The high acquisition bandwidth of MB-SWIFT in all spatial encoding directions together with a close to zero echo time decreases field distortion induced image deformations or voxel displacements. Furthermore, the radial sampling used in the MB-SWIFT sequence has been shown to allow better resilience to motion than Cartesian sampling (Glover and Pauly, 1992).

The average motion occurrence rate (0.48±0.23 events/min) of awake rats measured with MB-SWIFT was approximately half that of the occurrence rate observed in the awake rats in Study II (1.00±0.58 events/min). Overall, the translational movement of rats was typically even less than 1/10 of the voxel size, and only 8% of the volumes needed to be removed due to excessive motion. Notably, after the motion correction procedures, apparent motions less than 1/100 of the voxel size were typically detected (Figure 13A). When comparing average motion parameters of MB-SWIFT data to SE-EPI data (study II), a considerable difference (~15 times) in the motion level was detected (Figure 13B). It is noteworthy that even though the motion corrections in Studies II and IV were done with a different software, the major differences in motion cannot be fully explained by different performance of the software.

Taken together, the observed reduced physical and apparent motion level in MB-SWIFT is most likely caused by reduced sound pressure level, continuous data acquisition and non-sensitivity of MB-SWIFT to body motion related B0 changes.

6.3.3 Functional connectivity

Overall, the functional connectivity results obtained with MB-SWIFT and data driven ICA-method and ROI-correlation method provided near whole-brain functional parcellation and moderate-to-good correlation values between and within cortical and subcortical regions.

The spatial organization of several ICs was very similar to previous work done in awake animals implementing standard EPI-techniques (Liang 2011, Becerra 2011, Ma 2018). In addition, several ICs overlapped with the brain regions believed to be included in DMN (Lu 2012), including medial frontal, retrosplenial, anterior...
cingulate, and parietal cortices. However, the network was not detected as a whole, which could be caused by preserved cognitive or sensory processing in the awake rats. However, MB-SWIFT enabled near whole-brain functional parcellation with a relatively small group size (n=9). With standard EPI techniques, this has not been commonly achieved with even larger group sizes (typically n=15-42, see original Publication IV). EPI, being more susceptible to animal motion, can hinder the reliable detection of biologically relevant ICs due to the high number of motion-related artificial ICs on the brain surface. This was also observed in our studies, as many artificial motion-related or non-specific unilateral components together with components localized in vessels (16/50 of components) were detected in the EPI data, whereas only a few clearly non-neural or non-specific ICs were detected in the MB-SWIFT data (7/30).

Furthermore, based on the ROI-based analysis, correlation values were also in good agreement with earlier awake studies as we detected moderate-to-good fronto-parietal (Liang et al., 2015; Smith et al., 2017; Zhang et al., 2010), cortico-striatal (Smith et al., 2017) and cortico-cortical (Zhang et al., 2010) correlations.

6.3.4 Simultaneous EEG/fMRI

Thanks to only minor artefacts in the MB-SWIFT fMRI images during simultaneous EEG measurements, the whole brain, including regions close to the measuring electrodes, could be analyzed. To summarize, it appeared that MB-SWIFT can be effectively used to study neurovascular coupling mechanisms in the resting state, as there was a very clear correlation between the HRF-convolved EEG envelope and MB-SWIFT fMRI signal during isoflurane-induced burst-suppression activity.

6.4 CONCLUSIONS AND FUTURE PROSPECTS

As demonstrated in this thesis, there are clear differences in resting-state brain activity between awake and anesthetized rats, and anesthesia can modulate brain activity even over the long-term. Although some anesthetics can be said to preserve brain activity such that it resembles the conscious state, each anesthetic agent has its unique modulatory effect on brain function and connectivity. In addition, as these effects are further modulated by anesthesia, specific long-term functional changes, the interpretations drawn from preclinical fMRI studies using anesthetized animals can be complicated and compromised. If anesthesia is used, one needs to be careful with the study design, i.e. taking into account the selection of an appropriate anesthetic agent most suitable for the study aims, knowledge of the suitable dose and effect duration, knowledge of modulatory effect on brain region-dependent neurovascular coupling mechanisms as well as taking care of animal physiology. As anesthesia can be unavoidable in certain MRI study protocols utilizing more
invasive and potentially unpleasant procedures, it is important to consider the above-mentioned factors.

However, when studying the brain functional networks at the whole-brain level, with the aim of exploring cognition, awareness, memory and behavior etc., there are justifiable reasons for utilizing fully awake animals. Indeed, there has been a growing interest in conducting awake animal fMRI imaging. Even though there have been and still are certain limitations in awake animal imaging, which are mainly related to animal motion and stress, improved MRI sequences, post-processing techniques, and imaging and habituation applications have been developed and there are several active research projects aiming to reduce these obstacles. The usage of fully conscious animals opens many exciting possibilities for incorporation into a variety of different fMRI study schemes, such as in the assessment of animal behavior. By broadening fMRI study design possibilities, preclinical fMRI can hopefully even further increase its translational value in the future. It can also be predicted that multimodal neuroimaging techniques where fMRI imaging techniques are combined with various forms of stimulation (e.g. deep brain electrical, optogenetic or transcranial magnetic stimulation) or other brain measuring techniques (e.g. calcium and ultrasound imaging, microelectrode arrays) will become increasingly popular in the near future.
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APPENDICES

ORIGINAL PUBLICATIONS (II-IV)
ORIGINAL PUBLICATIONS (II – IV)

Stenroos P, Paasonen J, Salo RA, Jokivarsi K, Shatillo A, Tanila H, Gröhn O.

Awake Rat Brain Functional Magnetic Resonance Imaging Using Standard Radio Frequency Coils and a 3D Printed Restraint Kit

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Functional magnetic resonance imaging (fMRI) is a powerful noninvasive tool for studying spontaneous resting state functional connectivity (RSFC) in laboratory animals. Brain function can be significantly affected by generally used anesthetics, however, rendering the need for awake imaging. Only a few different awake animal habituation protocols have been presented, and there is a critical need for practical and improved low-stress techniques. Here we demonstrate a novel restraint approach for awake rat RSFC studies. Our custom-made 3D printed restraint kit is compatible with a standard Bruker Biospin MRI rat bed, rat brain receiver coil, and volume transmitter coil. We also implemented a progressive habituation protocol aiming to minimize the stress experienced by the rats, and compared RSFC between awake, lightly sedated, and isoflurane-anesthetized rats. Our results demonstrated that the 3D printed restraint kit was suitable for RSFC studies of awake rats. During the short 4-day habituation period, the plasma corticosterone concentration, movement, and heart rate, which were measured as stress indicators, decreased significantly, indicating adaptation to the restraint protocol. Additionally, 10 days after the awake MRI session, rats exhibited no signs of depression or anxiety based on open-field and sucrose preference behavioral tests. The RSFC data revealed significant changes in the thalamo-cortical and cortico-cortical networks between the awake, lightly sedated, and anesthetized groups, emphasizing the need for awake imaging. The present work demonstrates the feasibility of our custom-made 3D printed restraint kit.
INTRODUCTION

Functional magnetic resonance imaging (fMRI) is a modern imaging modality enabling noninvasive measurements of neuronal activity. The detection is typically based on the blood oxygenation-level dependent (BOLD) contrast (Ogawa et al., 1990), which exploits the neurovascular coupling cascade to provide an indirect measure of brain activity. Traditionally, fMRI is used to detect local activation changes induced by a precise stimulus, but advanced fMRI techniques, such as resting state fMRI (Biswal et al., 1995), have enabled studies of resting state functional connectivity in large-scale brain networks.

Despite the fMRI method being fully compatible with clinical settings, experimental rodent studies are still irreplaceable, as they not only provide a means to scrutinize the neurovascular coupling mechanisms, but also allow for controlled genetic, electrical, and pharmacologic manipulations, and more invasive monitoring that enables mechanistic studies of normal and abnormal brain functions. In the majority of preclinical fMRI studies, however, anesthetics complicate interpretations of the results. Anesthesia is required to prevent the animals from moving as well as to decrease discomfort and stress in the animals, but it also has significant undesirable effects on fMRI responses to stimuli or drugs (Paasonen et al., 2016, 2017) and resting state activity (Hamilton et al., 2017; Paasonen et al., 2018) by interfering with normal brain function and neurovascular coupling (Franks and Lieb, 1994; Martin et al., 2006; Zhao et al., 2007; Ferron et al., 2009; Gao et al., 2016).

To avoid the disrupting effects of anesthetics, several groups have implemented awake animal fMRI imaging protocols (King et al., 2005; Zhang et al., 2010; Becerra et al., 2011; Febo, 2011; Tsurugizawa et al., 2012; Brydges et al., 2013; Chang et al., 2016; Kenkel et al., 2016). Awake animals are likely to move and experience stress during fMRI, which raises the demand for carefully optimized restraint and habituation protocols. Several studies have demonstrated, based on physiologic markers of stress, that rats are able to adapt to the level of restraint required for fMRI (King et al., 2005; Tsurugizawa et al., 2010; Reed et al., 2013; Chang et al., 2016).

Despite the advantages provided by awake animal imaging, the method is still relatively rarely exploited, partly due to several methodologic issues. First, there are only a few readily available solutions for restraint implementation, leading to a demand for custom-made restraint kits and beds. Second, the custom-made restraint parts are typically not compatible with standard radiofrequency coils. If large volume coils are used for signal reception, decreases in the signal-to-noise ratio hinder the detection of fMRI signal changes. Third, the habituation protocols can last up to 8 days (King et al., 2005) making the approach very time-consuming. Therefore, there is a clear demand for fast, easily implemented, low-stress, and low-cost approaches that could be performed using standard hardware available in the preclinical MRI laboratory.

To address some of the essential methodologic limitations in awake rat imaging, the first aim of this study was to construct an open-access 3D printable restraint kit that is fully compatible with a standard Bruker rat bed and surface coil. The second aim of this study was to investigate the possibility of performing a habituation protocol under very light sedation.
that would minimally affect brain function, but at the same time allow for a short habituation period. The developed restraint kit and habituation protocol were implemented into fMRI studies to obtain RSFC data from awake and lightly sedated (0.5% isoflurane) rats, and the RSFC data were subsequently compared with corresponding data obtained from anesthetized (1.3% isoflurane) rats.

**MATERIALS AND METHODS RESTRAINT PARTS**

The custom-made 3D printed (with Zmorph 2.0 SX) restraint kit (Figure 1), compatible with an existing Bruker Pharmascan rat bed and quadrature surface receiver coil (Bruker Biospin, Ettlingen, Germany), was designed for rat brain imaging. The restraint kit, printed from acrylonitrile styrene acrylate (other materials e.g., acrylonitrile butadiene styrene can be also used), comprises a foam-padded arc-shaped shoulder and neck support, padded cheek supports, and a sliding sledge with a padded nose cone, which functions as a gas mask. The sliding sledge also includes a bite bar. Thus, the contact points for the restraint are neck area, shoulders, muzzle and cheeks of the rat. The RF-coil is attached on top of the head making little physical contact.

The 3D printed restraint kit was designed with CAD software (Autodesk 123D Design), and the kit requires no additional modifications (e.g., screw threads) to the animal bed. The 3D printing files (file format: STL), excluding the MRI bed, are available in the electronic Supplementary Material. For the cheek supports, we provide a 3D printable model for the whole support and separately for a curved plate (presses against the cheek) but we recommend to use a tougher carbon fiber bar that can be fixed to the plastic plate. In 3D printing, we used layer thickness of 200 µm for all parts except for the cheek support bars 50–100 µm layer thickness is recommended. Also, we advise to use support material in 3D printing. In addition to 3D printed parts, plastic screws (diameter 3.8 mm, length 20 mm) and compatible plastic nuts (outer diameter 7 mm) were used for attaching the restraint parts, and foam pad was used to soften points of restraint.
FIGURE 1 | A custom-made 3D printed rat restraint kit (A), a rat restrained in the bed with the developed restraint kit (B), and a restrained rat in the bed with a Bruker quadrature receiver coil attached (C). The rat restraint kit (A) consists of a standard Bruker rat bed (A1), a padded shoulder/neck support (A2), a sliding sledge including a bite bar (A3), padded cheek supports (A4), and a padded nose cone (A5). A replicate of Bruker bed was used in mock scanner.

ANIMALS

All animal procedures were approved by the Animal Experiment Board in Finland, and conducted in accordance with the guidelines set by the European Commission Directive 2010/63/EU.

A total of 23 adult male rats were used (RccHan Wistar, 320 ± 25 g, purchased from Laboratory Animal Centre, University of Eastern Finland). Rats were divided into three groups: awake (n = 8), lightly sedated (isoflurane 0.5%, n = 7), or anesthetized (isoflurane 1.3%, n = 8). The rats in the 1.3% isoflurane group were group-housed in cages, while rats in the awake and lightly sedated groups were individually housed because the rats were also individually habituated. All animals were maintained on a 12/12 h light-dark cycle at room temperature of 22 ± 2°C with humidity of 50–60%. Food and tap water were available ad libitum.

HABITUATION AND IMAGING SCHEDULE FOR AWAKE AND LIGHTLY SEDATED RATS

Habituation sessions were gradually increased from 15 to 45 min (Figure 2). Rats in the lightly sedated group were habituated for 4 consecutive days prior to fMRI. Rats in the awake group were habituated and imaged in two 5-day periods. During the first week, we used a protocol
identical to that for the lightly sedated group. In the following week, training was continued for another 4 days with fixed 45-min sessions, followed by a second fMRI session. Rats were habituated daily and imaged (two rats sequentially at one session) at the same time of day (9:00–12:00 a.m.) to control for circadian rhythm variations.

HABITUATION PROTOCOL FOR AWAKE AND LIGHTLY SEDATED RATS

Rats were habituated for imaging in a mock scanner mimicking the real MRI environment. Located in a separate habituation room, the mock scanner comprised a plastic tube (ID = 72 mm) mimicking a magnet bore, a rat bed, a standard platform for the rat bed, a web camera for recording movement, and a speaker (JBL LSR308, Harman International Industries, Stamford, CT, USA). We measured the sound level using a microphone (MT830R, Audio-Technica, Leeds, England) positioned inside the real MRI bore and played the sounds of the MRI at the same sound pressure as that inside an actual animal scanner.

At the beginning of each habituation session, the rats were first anesthetized with 5% isoflurane (Baxter, Lessines, Belgium; in a carrier gas mixture of N₂/O₂ [70/30]), weighed, and transferred to a hood where anesthesia was maintained with 1.5–2.0% isoflurane. The restraint protocol described below is also available in video format (Supplementary Video 1). A piezoelectric pneumatic sensor (M2M, Cleveland, OH, USA) was taped onto the belly and adhesive electrocardiography (ECG) electrodes (3M, St. Paul, MN, USA) were secured to the limbs. The forepaws were attached with masking tape along the sides of the rat without allowing the adhesive surface of the tape to come into contact with the rat fur. The hind paws were tied together with the tail with masking tape.

A sheet of foam plastic was wrapped around the rat from the shoulders to the hind paws to restrain body movement. Foam plastic assured a tight, but flexible and warm environment that allowed the rat to breathe normally. Foam plastic was secured with tape around the shoulders and hind paws. Silicone plugs were used to protect hearing from the MRI scanner sounds, thus increasing comfort.

Next, the rat was transferred to the animal bed in the mock scanner. Isoflurane was maintained at 1.5–2.0%. The head was placed on the sliding sledge and the upper teeth were secured behind the bite bar. The nose cone was tightened to the sledge to prevent muzzle movement in the vertical direction. The padded cheek supports, positioned on the both sides of the head, were gently tightened below the ears to prevent lateral movement of the head. The head was further secured by adjusting the shoulder and neck support; this also reduces the effects of lower body movements on the head. The nose cone, cheek supports, and shoulder/neck support also suppressed rotational movement of the head. To minimize body movement, tape was placed around the rear part of the body and attached to the bed.

ECG electrodes and a breathing sensor were connected to an amplifier (Biovet CTI system, m2m imaging, Corp., USA). A heating pad was placed on top of the rat and connected to a Biovet CTI temperature regulation unit to keep the body warm. To minimize discomfort, a rectal probe was not used but thermometer was placed under the animal to monitor temperature.

Finally, a Bruker rat surface quadrature receiver coil was placed on top of the head, and the animal bed was pushed inside the mock MRI tube. The receiver coil used in the habituation sessions was similar to the one used during the actual fMRI session.

The pre-habituation preparations lasted approximately 8 min per rat, excluding the induction of anesthesia. After the preparations, isoflurane was decreased to either 0.5% in the lightly sedated group or turned off in the awake group.
MRI sounds, starting with the sounds of pilot imaging and shimming, and then continuing with continuous EPI scanning, were played for a given time (15–45 min, see Figure 2), while simultaneously monitoring the physiologic parameters, i.e., the respiratory rate and heart rate. Movement was monitored and recorded through a web camera. If the rat was moving excessively, defined as continuous movement >30 s, or more than a third of the time used for the session, the habituation protocol was ceased and continued the next day.

After reaching the desired habituation session length (see Figure 2), isoflurane was raised to 2%. When physiologic parameters indicated a sufficient depth of anesthesia, the rat was released from the kit and transferred back to the hood where the plastic foam and tape were removed. Blood samples (total of 150 µl) were obtained from the lateral tail vein. Next, the rat was returned to the cage and chocolate cereal balls were given as a reward. Blood samples were centrifuged at 3,500 RPM for 10 min, after which plasma was collected and samples were stored at −70°C for corticosterone analysis.

**FIGURE 2 |** Rat habituation and imaging schedule. D, days, W/E, weekend.

**FMRI PROTOCOL**

MRI was performed with a 7T/16cm horizontal Bruker Pharmascan system and ParaVision 5.1 software. A standard Bruker quadrature resonator volume coil (ID = 72 mm) and a rat brain quadrature surface coil were used. A 3D field-map based shimming method was used to optimize the field homogeneity.

Functional imaging was performed with single-shot spin-echo echo planar imaging sequence with the following parameters: repetition time 2,000 ms, echo time 45 ms, matrix size 64 × 64, field-of-view 2.5 × 2.5 cm, 9–11 slices of 1.5 mm thickness, and a bandwidth of 250 kHz. The same imaging parameters were used with all animals, except that 300 fMRI volumes (10 min) were acquired from anesthetized rats and 600–750 fMRI (20–25 min) volumes were acquired from awake and lightly sedated rats. Despite habituation, awake animals tend to move slightly. Therefore, a longer period was obtained from awake and lightly sedated rats to ensure a continuous motion-free 10-min period for analysis.

Anatomic images were acquired after the fMRI scans with fast spin-echo sequence with the following parameters: repetition time 4,680 ms, echo spacing 16.1 ms, 8 echoes, effective echo time 48.4 ms, matrix size 512 × 512, field-of-view 5.0 × 5.0 cm, 30 slices of 0.75 mm thickness, and bandwidth of 46.875 kHz. The restraint preparations for the awake and lightly sedated rats were similar to those for the habituation protocol, except that heart rate was measured using a pulse oximetry sensor.
Physiologic parameters (heart rate, respiration, and temperature) were monitored continuously during the imaging, and movement was estimated from the real-time reconstructed EPI images. Rats were kept warm using a water circulation heated animal bed. After measurements, blood samples for corticosterone level analysis were collected as in the habituation sessions.

The protocol for imaging anesthetized rats (isoflurane 1.3%) was described earlier (Paasonen et al., 2018). Briefly, small cannulas (BD Intramedic™ PE-10, Franklin Lakes, NJ, USA) were inserted into the femoral artery and vein, and tracheostomy was performed under 2% isoflurane anesthesia. As isoflurane anesthesia suppresses respiratory function and easily leads to hypercapnia, mechanical ventilation (Inspira, Harvard Apparatus) was used to maintain normal blood gas values (pCO$_2$ 45.1 ± 2.0; i-STAT Model 300, Abbott Point of Care Inc., Princeton, NJ, USA) measured from the arterial blood sample (150 µl). Muscle relaxant (~1 mg/kg/h i.v., pancuronium bromide, Pavulon (R), Actavis) was given while connecting the animal to the ventilator. Rats were killed immediately after the measurements.

**FMRI ANALYSIS**

First, a 10-min motion-free fMRI data period was selected from each subject. Next, the fMRI data were converted to NIfTI (http://aedes.uef.fi), slice-timing corrected, motion-corrected, spatially smoothed, and co-registered to a reference brain (SPM8).

Regions of interest (ROIs) were drawn according to an anatomic atlas (Paxinos and Watson, 1998). The 12 ROIs for whole brain analysis were the medial prefrontal cortex (mPFC), motor cortex (MC), somatosensory cortex (SC), visual cortex (VC), auditory cortex (AC), retrosplenial cortex (RC), nucleus accumbens (NAc), striatum (Str), hippocampus (HC), medial thalamus (ThM), ventrolateral thalamus (ThVL), and hypothalamus (HTH) (Supplementary Figure 1A).

Additional ROIs were drawn for the default mode network (DMN) analysis, based on the article by Lu et al. (2012): medial frontal cortex (including prelimbic cortex and anterior cingulate cortex), temporal association cortex, orbitofrontal cortex, retrosplenial cortex, posterior parietal/visual cortex and hippocampus (Supplementary Figure 1B).

RSFC was estimated with ROI-based Pearson correlation (r) analysis. Prior to correlation calculations, data were bandpass filtered at 0.01–0.15 Hz. Subsequently, the r-values were transformed to Z-scores prior to calculating group averages and statistical comparisons. After performing the calculations, the mean Z-scores were returned to r-values. Correlation coefficients between the 12 ROIs were used to form functional connectivity (FC) matrices (Figure 4) and the same ROIs were used as seed regions in correlation maps. Voxel-wise correlation maps for the awake group were calculated by using somatosensory cortex, striatum, and medial thalamus as seed regions (Figure 5A). Subsequently, difference maps between the awake and 0.5 and 1.3% isoflurane groups were calculated (Figure 5B). Mean correlation values (Supplementary Figure 2) from each of the 12 ROIs were additionally calculated to obtain mean connectivity of the brain regions. Correlation coefficients within the rat DMN were also calculated (Figure 6). Moreover, we calculated partial correlation coefficients by using mass center displacement values (x, y, z and roll, pitch, yaw) as a regression for motion.

Statistical group-level differences (p < 0.05) in DMN and mean functional connectivity were calculated using a one-way ANOVA and Tukey’s multiple comparison post-hoc test. FDR-corrected t-tests (p < 0.05) were applied to FC matrices. FDR-corrected, one-sample t-test
was performed to obtain functional connectivity map from the awake rats. Two-sample \( t \)-test \( (p < 0.05) \) was performed to compare 0.5 and 1.3% isoflurane groups to the awake rats. Statistical testing was performed with GraphPad Prism (Version 5.03, GraphPad Software Inc., La Jolla, CA, USA) or Matlab (R2011a), Natick, MA, USA.

ANALYSIS OF MOVEMENT DURING FMRI

In addition to visually estimated movement incidents, motion correction parameters, obtained from SPM8, were analyzed to evaluate the movement of the head during the fMRI session. Translational (x-y-z-directions) and rotational (roll, pitch, and yaw) movements were calculated.

First, co-registered translational and rotational mass center displacement values, given by SPM8, were detrended and normalized. Movement was evaluated from these values during the awake fMRI session by subtracting every subsequent time-point from the preceding value. After removing visible movement incidents (sharp peaks in mass center displacement) from a selected 10-min fMRI period, we preprocessed the data again to obtain 10-min motion-free fMRI periods. In these periods the motion was substantially lower in relation to voxel size (Supplementary Figure 3A). Of 16 awake datasets, 2 (2/16) were excluded from the analysis due to excessive movement.

Each subtracted translational and rotational movement was then converted to absolute values. These absolute values were summated over 10-min MRI sessions to obtain summated translational and rotational movement (Supplementary Figure 3B). Additionally, absolute maximum translational displacement in the y-direction, in which most of the movement happened, was calculated to estimate overall displacement of the animal head in the restraint kit (Supplementary Figure 4). This displacement value represents maximum displacement of the animal head from the starting position and does not inform of the separate movement incidents.

ESTIMATION OF STRESS

Physiologic values (plasma corticosterone level, respiratory rate, heart rate, movement, and weight) were monitored during the habituation and fMRI sessions to estimate stress. Plasma corticosterone concentrations were quantified by a corticosterone immunoassay (Corticosterone rat/mouse ELISA Cat. No.: RTC002R, Demeditec Diagnostics, Kiel, Germany). Corticosterone concentrations were measured with a microtiter plate reader (Labsystems Multiskan MS, Vantaa, Finland) at an absorbance of 405 nm. Average respiratory and heart rates were calculated from values during habituation and fMRI sessions. Only values from 6 min onwards were included to minimize the possible influence of anesthesia on arousal. If the interval between two visually estimated movement incidents was \( \geq 4 \) s (two fMRI volumes), they were considered separate incidents.

Additionally, we estimated the potential habituation-induced depression and anxiety by conducting open-field and sucrose preference behavioral tests with a small subgroup of awake habituated rats \( (n = 5) \). Control rats \( (n = 5) \) were anesthetized daily with isoflurane for the same amount of time that the habituated rats were anesthetized.

In the open-field test we used a circular plastic arena \( (ID = 100 \) cm) placed in a quiet room. Color of the arena was black to distinguish white Wistar rats from the environment. We used a lux meter (TR-710, Trifitek Finland Oy, Alajärvi, Finland) to confirm homogenous lighting conditions \( (19.3 \pm 1.1 \) lx, 9 measurement points) across the test arena. Test was performed 10 days after the last procedure. In the test, rat was placed on the arena edge having its nose toward the wall. Movement and other activities were video-recorded for 10
min. Data were analyzed with Ethovision XT 7.1 (Noldus, Wageningen, The Netherlands), where the arena was divided into center (circle with 40 cm diameter) and edge (up to 10 cm from borders) zones. Distance traveled, activities (rearing and grooming), and time spent in different zones were subsequently calculated.

In the sucrose preference test, rats were first habituated to 1% sucrose water, which was made available ad libitum for 2 days next to regular tap water. During the next 2 days, the bottles with sweetened and regular water were weighed to calculate consumption during each day. The positioning of the bottles was switched each day.

The plasma corticosterone level, respiratory rate, heart rate, movement, and weight measurements were analyzed with ANOVA for repeated measures and Dunnett’s post-hoc test in GraphPad Prism. Statistical comparison of corticosterone levels between days was done by comparing each day to baseline level. Comparison of respiratory rate, heart rate, movement and weight between days were done by comparing each day to habituation day 1. Statistical testing for the open field and sucrose preference tests was performed with a two-sample, two-tailed t-test assuming equal variances.

RESULTS

STRESS LEVEL INDICATORS

The measured values for stress level indicators (plasma corticosterone level, heart rate, respiratory rate, and movement) obtained from awake and lightly anesthetized rats during the habituation protocol are summarized in Figure 3.

Plasma corticosterone concentrations on the MRI day 1 did not differ statistically from the baseline concentrations in the awake or lightly sedated groups ($p = 0.25$ and $p = 0.36$, respectively, paired t-test). In the awake group, corticosterone concentrations significantly increased from the baseline level on habituation day 1 ($p = 0.014$, paired, t-test). However, concentrations returned to baseline level on the habituation day 4 (baseline: 95 ± 43 ng/ml, day 4: 102 ± 30 ng/ml, $p = 0.75$, paired, t-test). Also corticosterone concentration did not appear to differ statistically on MRI day 2 from MRI day 1 ($p = 0.064$,
FIGURE 3 | Stress level indicators of plasma corticosterone (A), movement (B), respiratory rate (C), and heart rate (D) of awake and lightly sedated rats during habituation and fMRI. Mean values ± SEM of corticosterone (ng/ml), movement (counts/min), respiration (breaths/min) and heart rate (beats/min) are presented (n = 7–8 in each group). Statistical testing between days was done with ANOVA for repeated measures and Dunnett’s post-hoc test, and between groups with a two-sample t-test. Significant differences of each time point are compared to either BL (A) or D1 (B–D) values. One symbol equals p < 0.05, two symbols equal p < 0.01, three symbols equal p < 0.001.
FIGURE 4 | The group-level functional connectivity (FC) obtained from the 1st (n = 8) and 2nd (n = 6) awake time points, lightly sedated rats (n = 7), and anesthetized rats (n = 8). The connectivity data were analyzed from 300 volumes from 12 regions of interests. Statistical testing was done by using FDR-corrected t-test, *p < 0.05, **p < 0.01, ***p < 0.001. Significant differences against 1st awake time point are presented. AC, auditory cortex; HC, hippocampus; HTH, hypothalamus; mPFC, medial prefrontal cortex; ThM, medial thalamus; MC, motor cortex; Nacc, nucleus accumbens; RSC, retrosplenial cortex; SC, somatosensory cortex; Str, striatum; ThVL, ventrolateral thalamus; VC, visual cortex.

FIGURE 5 | The group-level seed-based statistical correlation maps obtained from the awake group (A) and group-level differences compared to the awake group (B) obtained from three representative brain regions. Brain regions of somatosensory cortex, striatum, and medial thalamus were selected. Statistically significant voxels were obtained with one-sample t-test (A) (p < 0.001) and two-sample t-test (B) (p < 0.05).

paired t-test). Noticeably, in the lightly sedated group, there was no clear difference between the baseline (117 ± 20 ng/ml) or the 1st habituation day (168 ± 27 ng/ml) (p = 0.066, paired t-test).
FIGURE 6 | The group level mean correlation coefficients (± SEM) between the seven pathways in the rat DMN. The 300-volume data (10min) were obtained under two awake time points, lightly sedated and anesthetized conditions. Statistical testing was done by one-way ANOVA and Tukey’s multiple comparison post-hoc test, *p < 0.05, **p < 0.01, ***p < 0.001. Cx, cortex.
Movement of awake rats decreased significantly already during habituation day 2 ($p = 0.002$, paired t-test) and, from thereon, differed significantly in each habituation and MRI days from habituation day 1. Importantly, there was no further decrease in movement on MRI day 2 (0.77 ± 0.18 counts/min) compared with MRI day 1 (1.0 ± 0.20 counts/min) ($p = 0.20$, paired t-test). In the lightly sedated group, movement did not differ significantly between days but rats moved significantly less during MRI day 1 compared to awake rats ($p < 0.001$, two-sample t-test).

The heart rate of awake rats was significantly lower during habituation day 4 and MRI day 1 compared with habituation day 1 ($p = 0.018$ and $p < 0.001$, respectively, paired t-test). Heart rates during MRI day 2 did not differ significantly from MRI day 1 ($p = 0.67$, paired t-test). In the lightly sedated group, there was no significant difference in the heart rates between habituation day 1 and MRI day 1 ($p = 0.49$, paired t-test).

The breathing rate was significantly lower during MRI day 1 in lightly sedated group compared with the awake group ($p < 0.001$, two-sample t-test) but the levels did not change significantly in either group between the habituation days. Additionally, average body weight of animals remained stable during the habituation (varied between 316 to 325 ± 4.2 g in awake rats and 319 to 320 ± 5.1 g in lightly sedated rats) (data not shown).

Results from the open field and sucrose preference tests are illustrated in the Supplementary Figures 5, 6. There were no differences in locomotor activity, time spent in different zones, rearing activity, or sucrose preference between the groups ($p > 0.05$, two-sample t-test). However, habituated rats spent more time grooming than control rats (20.2 ± 3.6 s vs. 4.9 ± 2.8 s, $p = 0.01$, two-sample t-test).

**MOVEMENT DURING FMRI**

Generally, the awake rats moved more compared with the other groups. For example, there was a significant difference in the summated movement in $X$- (1st awake time-point 988 µm, 1.3% group 339 µm, $p < 0.001$, two-sample t-test) and $Y$- (1st awake time-point 3,330 µm, 1.3% group 423 µm, $p < 0.001$, two-sample t-test) directions, and in rotational directions of roll (1st awake time-point 18.3°, 1.3% group 11.0°, $p < 0.01$, two-sample t-test) and yaw (1st awake timepoint 9.11°, 1.3% group 3.40°, $p < 0.001$, two-sample t-test) between the 1st awake time-point and 1.3% isoflurane groups. Maximum displacement from the mass center was also significantly higher in the 1st (253 µm) and 2nd (271 µm) awake time-points compared with the 1.3% isoflurane group (51 µm; $p < 0.001$ and $p < 0.01$, respectively, two-sample t-test). Notably, lightly sedated rats did not move significantly more than anesthetized rats based on summated movement or displacement values. Movement incidents, summated movement and displacement values during 10-min fMRI sessions are illustrated in Supplementary Figures 3 and 4.

**RESTING STATE FUNCTIONAL CONNECTIVITY**

**FUNCTIONAL CONNECTIVITY MATRICES**

Functional connectivity matrices are shown in Figure 4. FC matrices obtained from the awake animals after the first and second habituation week were almost identical with no significant difference between them ($p > 0.99$, t-test, FDR adjusted). In the lightly sedated group, connectivity was significantly weaker in the three pathways compared with that in the first awake group: between medial thalamus and motor, somatosensory, and visual cortex.
In the anesthetized group, correlation values were significantly higher in several (21/66) cortico-cortical and cortico-striatal connections compared with the first awake group ($p < 0.05$, $t$-test, FDR adjusted). In addition, statistically stronger connectivity in anesthetized rats was observed in mean connectivity values in the medial frontal cortex (0.41), motor (0.61), somatosensory (0.60), visual (0.59), auditory (0.57) cortices, and in the striatum (0.49) compared with the first awake group (medial frontal cortex [0.20], motor [0.37], somatosensory [0.37], visual [0.37], auditory [0.33] cortices, and striatum [0.26]) ($p < 0.05$, Supplementary Figure 2).

In contrast, the correlation was significantly lower in several subcortical-cortical (22/66) connections in anesthetized rats compared with the awake rats ($p < 0.05$, $t$-test, FDR adjusted). Further, the mean correlation was significantly lower in the medial thalamus ($p < 0.01$) in anesthetized rats than in awake rats.

Seed-based partial correlation, with motion as a regression, did not differ statistically from correlation values calculated without motion regression ($p > 0.05$ $t$-test, FDR adjusted). Therefore, the possible remaining motion artifacts were not affecting the results obtained with functional connectivity analyses.

**VOXEL-WISE CORRELATION MAPS**

The seed-based mean correlation map and group difference maps obtained from three representative ROIs are shown in Figure 5. In the 1.3% isoflurane group, the connectivity of the somatosensory cortex and the striatum was more widespread and stronger compared to the awake group, while thalamocortical connectivity was clearly suppressed. In the lightly sedated group, we did not find statistically significant voxel wise correlation differences compared to the awake group after multiple comparison correction.

**DEFAULT MODE NETWORK**

The functional connectivity of seven pathways in the rat DMN is illustrated in Figure 6. In the 1.3% isoflurane group, connectivity was stronger in almost all DMN connections ($p < 0.05$), excluding medial frontal–orbitofrontal and retrosplenial cortex–hippocampus connections, when compared with the first awake time-point. In the lightly sedated group, the DMN connectivity did not differ statistically from those in the awake groups.

**DISCUSSION**

It has become increasingly recognized that anesthesia is a major confounding factor in animal fMRI studies, especially when assessing functional connectivity in the brain (Liang et al., 2012, 2015; Liu and Duyn, 2013; Paasonen et al., 2018). Awake animal imaging offers the possibility of avoiding the drawbacks of anesthesia, and robust, practical, and low-stress awake imaging methods are in high demand. Indeed, a number of different approaches are available for awake fMRI (Table 1).

The existing awake imaging methods, however, have several shortcomings with regard to both technical feasibility and animal welfare. Many of these approaches require dedicated beds and/or receiver coils, with poor filling factors, making the replication of protocols by others practically impossible. Additionally, the use of possibly painful ear bars, the lack of ear protection, the lack of soft padding materials, and the use of long non-progressive habituation times (Table 1) may induce major acute pain or long-term stress.
In the present work, we aimed to avoid several of these shortcomings by implementing novel ideas and adopting known best practices to create a significantly improved approach for awake fMRI. We designed a 3D printable restraint kit compatible with a common rat bed type and standard RF-coil with good

<table>
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<th>Publications</th>
<th>Species</th>
<th>Type of MRI receiver coil</th>
<th>Restraint of the head</th>
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<th>Habituation protocol</th>
<th>Padded holder parts</th>
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<tr>
<td>Current study</td>
<td>Winter</td>
<td>Quadrature surface coil</td>
<td>Cylindrical head holder</td>
<td>Body tube</td>
<td>15-45 min with increments of 10 min/day, 4-8 days</td>
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<td>No</td>
<td>Yes</td>
<td>Decrease in corticosterone, 120 ± 160 ng/ml after fMRI (28)</td>
<td>Resting state fMRI</td>
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<td>Single loop, surface coil</td>
<td>Cylindrical head holder</td>
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<td>20 min to a maximum of 30 min, 5 days</td>
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<td>Yes</td>
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<td>Quadrature surface coil (transmitter/receiver)</td>
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<td>Surface coil compatible to head fixation mount</td>
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<td>Body tube</td>
<td>90 min/day, 3 days</td>
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<td>Snuggle sack</td>
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<td>No</td>
<td>Decrease in corticosterone &gt;1,300 ng/ml after fMRI</td>
<td>Resting state fMRI, stimulus fMRI (air puff)</td>
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filling factor. In the kit design, we aimed to take the comfort of animal into account, to reduce the amount stress experienced by the animal, and to minimize the required habituation period, while still maintaining robust fixation of the head. Indeed, the physiologic markers of stress indicate that fully awake rats were able to adapt to the measurement environment in 4 days, showing very low peak concentrations of plasma corticosterone throughout the habituation period. Additionally, we show that by using light sedation, the habituation protocol could be shortened to 2 days. After the 4-day habituation period, the RSFC pattern of lightly sedated rats better resembled the pattern obtained under the awake state than that under an anesthetized state. Thalamo-cortical connectivity, however, was significantly suppressed in the lightly sedated state compared with the awake state.

3D PRINTED RESTRAINT KIT

The 3D printed restraint kit and protocol described here was robust and easy to use during the in vivo experiments. The initial preparations and fixation of the rat to the bed typically took less than 10 min under isoflurane anesthesia. The restraint kit was confirmed to be suitable for a wide range of rat sizes, as rats weighting from 200 to 480 g were tested in the pilot experiments. Because no dedicated surface coil or animal bed is required, our protocol provides an affordable option for any awake rat fMRI study design. We provide the restraint kit template (STLfile, Supplementary Material) for public use and for further modifications.

MOVEMENT DURING FMRI

In fMRI studies, it is crucial that patients or animals remain motionless during the imaging for acquisition of artifact-free data, as movement can compromise the fMRI analysis (Power et al., 2012, 2014). In our habituation protocol, rats adapted quickly to the restraint environment and movement decreased rapidly. Importantly, the occasional lower body movement did not typically affect the head position, indicating robust fixation achieved by our restraint kit.

Furthermore, we assume the 3D-printed kit can be helpful to reduce head motion also in lightly sedated animals, as our 3Dprinted kit has more fixation points compared to a standard rat head fixation setting and we do not use ear bars causing possible pain in ear canals.

Our analyses indicated that the awake rats, as expected, tended to move more frequently than the lightly sedated and anesthetized rats. Typically, head movement of the awake rats occurred in vertical Y-direction, which seemed to be due to shifts in the teeth positioning. Nevertheless, the amount of movement of awake rats in the present study was relatively equal to that of human movement in a clinical fMRI (Power et al., 2012, 2014). Based on the results reported by Power et al. (2012, 2014) we estimated the mean position change relative to voxel size (4 × 4 × 4 mm) to be 4–10% in humans, while in our rat experiments position change was 2.8 ± 0.2% relative to voxel size (0.391 mm shortest dimension).

STRESS

Based on the observed decreases in the stress indicators (plasma corticosterone level, movement, and heart rate), the custom-made restraint kit with the applied habituation protocol was well-suited for the acclimatization of both awake and lightly sedated rats.
The plasma corticosterone concentration, perhaps the most reliable stress marker, returned to the baseline level by habituation day 4 in the awake group. Similar trend was observed already by habituation day 2 in the lightly sedated group. Generally, corticosterone concentrations remained surprisingly low even after habituation day 1, as several groups report concentrations in the range of 240–550 ng/ml on the first habituation day (King et al., 2005; Chang et al., 2016). In our study, the corresponding value was 162 ng/ml in the awake group. This observation strongly indicates that the animals were more gently introduced to the measurement environment compared with several previous reports.

Moreover, movement, as an indicator of adaptation, decreased rapidly during the habituation period to very low levels compared with previous reports (King et al., 2005; Chang et al., 2016). Movement of the brain in our study was in the range of 0–350 µm, while previous studies reported movement in the range of 0–550 µm or 0–1,300 µm (King et al., 2005; Chang et al., 2016).

Heart rate decreased to close to 400 beats per minute in awake animals after the 4-day habituation period. This is considered to be within the normal heart rate range in adult Wistar rats during the active day phase (Zhang and Sannajust, 2000).

Among the awake and lightly sedated groups, body weight did not decrease during the habituation weeks, which is in line with other observations, suggesting only minor stress induced by the habituation protocol. In the awake group, weight increased in a normal manner already over the weekend, further supporting the normal behavior.

Breathing rate and movement pointed to clear differences in the brain state between rats in the awake and lightly sedated groups. Breathing rates of lightly sedated rats were notably lower than those of awake rats on the MRI day 1. Movement was already minimal in lightly sedated rats from the starting day and also differed from that of awake rats on the MRI day 1.

In both animal groups, plasma corticosterone concentrations and movement levels tended to increase slightly on the imaging day compared with the 4th habituation session, indicating a small difference between the habituation and scanning conditions. Certain properties of the MRI device, such as possible sounds that may mimic ultrasonic communication between rats (>20 kHz), could not be reproduced in our mock scanner. Furthermore, small mechanical vibrations coming from the gradient set could not be mimicked in the habituation environment. Nevertheless, the slight increases in the stress indicators did not reach statistical significance.

In addition to acute stress, rats can develop chronic stress after the habituation protocol. Low et al. (2016) demonstrated that long-lasting elevated plasma corticosterone concentrations, reduced nociceptive behavior, and increased activation of the central amygdala are observed following commonly applied habituation protocols (30 min, 3 days). Therefore, chronic stress can mask or compromise fMRI results, especially those obtained at later time-points. To address this issue, we evaluated depression- and anxiety-like behavior in habituated rats with well-known open-field and sucrose preference behavioral tests (Brenes Sáenz et al., 2006). Our results suggested no differences between habituated and control rats in locomotor activity, rearing, or center time in the open-field, or in the sucrose preference test, which speaks against major anxiety or depression-like behavior due to the habituation. In contrast, the habituated rats groomed more often than the control rats. This could indicate that the habituated rats felt more comfortable, as they were used to handling and experiencing new environments, thus making their adaptation faster. However, we recognize that the relatively small group sizes in behavioral tests may have hindered the detection of potential differences.
HABITUATION TIME

The shortest required training period for awake and lightly sedated rats based on the measured indicators for stress was estimated to be 4 and 2 days for the awake and lightly sedated rats, respectively, to achieve a low amount of movement and low corticosterone concentrations. Additionally, heart rate decreased significantly in the awake group during the 4-day period. Importantly, our RSFC data show similar connectivity at the end of the first and second week of habituation. Thus, the additional week of habituation yielded no additional benefit for connectivity analysis.

A protocol in which rats are completely restrained for a prolonged time, i.e., several hours, is widely used to study chronic stress (Chiba et al., 2012; Stepanichev et al., 2014). To avoid chronic stress, habituation sessions in different awake MRI studies typically last only in the range of 30–90 min. To elaborate this protocol further, in our study, the restraint session times started as a very short period of 15 min and progressively increased up to a maximum of 45 min on the day 4.

RESTING STATE CONNECTIVITY

Overall, the measured functional connectivity differed significantly between brain states. Even with a sub-anesthetic dose of isoflurane, the connectivity was significantly modulated compared with awake rats.

Strong connectivity across the cortical regions in the 1.3% isoflurane group is likely due to the anesthesia-induced burst suppression activity, which is characterized by alternation of silent brain states with almost no electrical activity (suppression) and active states with high-amplitude activity (bursts) (Derbysheir et al., 1936). Isoflurane induces burst suppression activity at a wide range of doses (e.g., 1.25–2.0%) (Hudetz and Imas, 2007; Vincent et al., 2007; Liu et al., 2013). The cycling between silent and active states is directly reflected by the hemodynamics and therefore is also visible in the fMRI BOLD signal (Liu et al., 2011, 2013). This cycling is likely to induce strong cortico-cortical and cortico-striatal correlations. Similar strong cortico-cortical or cortico-striatal connectivity in rats under high isoflurane anesthesia has been also observed previously (Williams et al., 2010; Liu et al., 2011, 2013; Kalthoff et al., 2013). When the cortical and cortico-striatal burst suppression synchronization is the most dominant feature in the brain, it is evident that the intrinsic connectivity is heavily masked.

The effect of burst suppression was also evident in the DMN in the 1.3% isoflurane group. While DMN is generally suppressed under anesthesia (Deshpande et al., 2010; Liu et al., 2015), brain regions associated with the DMN exhibited increased connectivity under 1.3% isoflurane anesthesia compared with awake rats. By contrast, isoflurane anesthesia induced heavy disruption of cortico-subcortical connectivity, mainly affecting connections between the cortex and medial thalamus. As the thalamus works as a key hub for controlling sensory information flow to the cortex, disrupted thalamo-cortical connectivity is considered to be the central mechanism regulating consciousness (Nallasamy and Tsao, 2011).

In the lightly sedated group, there were no abnormally high cortico-striatal correlations indicating burst suppression activity, which is consistent with previous reports (Liu et al., 2013), enabling more detailed analysis of intrinsic connectivity. Indeed, the FC matrices and maps in the cortex and striatum were fairly similar between the lightly sedated and awake rats, although global connectivity tended to be slightly suppressed. Suppression of connectivity most likely originates from reduced excitatory activities or increased inhibitory activities (Franks, 2008) induced by the low isoflurane dose. Additionally, we observed significantly suppressed connectivity in three medial thalamo-cortical connections in the
seed-based connectivity analysis. In the voxel-wise analysis, however, this phenomenon was not detected after correcting for multiple comparisons. In summary, light sedation protocol allows two-times faster habituation protocol, but its drawbacks are the anesthesia induced effects on RSFC that appear to be emphasized in the thalamo-cortical connectivity.

**EFFECT OF PHYSIOLOGY AND METHODOLOGIC CONSIDERATIONS**

In this study, we used a spin echo EPI sequence to study resting state networks. We have used spin echo EPI (SE-EPI) to have comparable results obtained from our previous EEG/fMRI studies which are conducted using SE-EPI to reduce susceptibility artifacts caused by electrodes. However, most of the fMRI studies today are conducted with a gradient echo EPI. While fMRI BOLD contrast in spin echo EPI has more specificity to small capillaries, gradient echo EPI is more sensitive to larger venules giving more detectable fMRI contrast (Lee et al., 1999). This can potentially influence comparison of rsfMRI maps obtained from studies implementing gradient echo EPI sequences. It is also worth noting that the current study was conducted with a 7T magnet, and with the higher magnetic fields, motion related artifacts can increase.

Because of local availability, Wistar rats were used in this study instead of Sprague-Dawley rats, despite SD rats are known to be less anxious and easier to habituate (Rex et al., 2004). Moreover, despite from results indicating female rats being more adaptable to restraint (Albonetti and Farabollini, 1992), we used male Wistar rats instead of female rats as the majority of biomedical research is conducted using male rats to avoid possible bias caused by hormonal rhythm variations.

As physiologic parameters such as breathing and heart rates differ between awake, lightly sedated, and anesthetized animals, they must be taken into account in the connectivity analysis. For this purpose, both high-pass and low-pass filters were used for the BOLD signals. A low-pass filter at 0.15 Hz was used to minimize the effect of physiologic noise, like respiratory and heart rate artifacts, on the BOLD signal. The high-pass filter at 0.01 Hz reduces the possible false-positive correlation of extremely low fluctuations of the BOLD signal originating from, e.g., hardware-related drifts. Nevertheless, the low sampling rate (0.5 Hz) does not allow for the complete removal of physiologic noise, and the effect on the connectivity analysis remains unclear.

Spontaneous behavioral changes, such as changes in eye state (open vs. closed), variations in respiratory and olfactory activity of awake animals can influence dynamic connectivity (Liu and Duyn, 2013; Di and Biswal, 2015). During the habituation sessions, rats kept their eyes open and blinked normally. Once rats woke up from the initial anesthesia, breathing rate remained mostly stable during the habituation and fMRI sessions. In addition, based on respiratory rates, rats did not spontaneously fall asleep (possible due to slight stress and noise) during the scanning. Spontaneous olfactory stimulation was controlled by cleaning the animal beds after imaging each rat, and the scanner room was kept clean from other animal odors. In this study, we used standard EPI sequence causing significant sound pressure. The current experimental setup can be clearly improved by using pulse sequences with sound optimized gradient wave form design (Hutter et al., 2018) or radial acquisition (Lehto et al., 2017).

As rats were initially anesthetized and prepared under isoflurane, we cannot completely ignore the possible residual effects of isoflurane on functional connectivity in awake animals. The effect of isoflurane is short, however, and it is eliminated rather quickly from the body as
rats usually wake within 2–4 min after the cessation of isoflurane, depending on the dose and duration. Also, isoflurane was turned off already during the shimming stage. Therefore, animals had several minutes to wake up from the anesthesia before the functional scanning started. Furthermore, the first minute from each functional dataset was excluded from analysis.

Finally, in the sub-cohort open field and sucrose preference studies, the relatively small animal numbers could possibly prevent the detection of small differences between groups, however, it rules out possible robust long-term anxiety or depression caused by stress.

SUMMARY AND CONCLUSION

We present a novel and easily implemented approach for awake rat fMRI. Our method introduces a 3D printed rat restraint kit compatible with a standard Bruker rat MRI bed, and quadrature surface receiver and transmitter coils. By using this kit and a modified habituation protocol, we were able to perform low stress RSFC studies in fully awake rats. Based on measured physiologic markers for stress and the amount of movement, a 4-day acclimatization period was sufficient for awake rats, and the habituation protocol can be shortened to 2 days when 0.5% isoflurane is used. Additionally, we demonstrated that 1.3% isoflurane anesthesia in rats markedly affected brain connectivity compared with that in the awake state, and that the effect was significant, albeit less marked, for cortico-thalamic connections when 0.5% isoflurane was used. Overall, the proposed approach is likely to make awake fMRI studies in rat more common in future, and thus increase the reliability and translatability of the results from rat fMRI studies.

AUTHOR CONTRIBUTIONS

PS and JP contributed in data collection and analysis. RS contributed in data analysis. KJ contributed in the 3D printing process. HT was consulted in the behavioral tests. OG contributed in the study design. PS contributed in writing of the manuscript and all authors took part in reading and revision of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2018.00548/full#supplementary-material
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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.
Functional connectivity under six anesthesia protocols and the awake condition in rat brain

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Functional connectivity under six anesthesia protocols and the awake condition in rat brain

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A B S T R A C T

Resting-state functional magnetic resonance imaging (rsfMRI) is a translational imaging method with great potential in several neurobiologic applications. Most preclinical rsfMRI studies are performed in anesthetized animals, but the confounding effects of anesthesia on the measured functional connectivity (FC) are poorly understood. Therefore, we measured FC under six commonly used anesthesia protocols and compared the findings with data obtained from awake rats. The results demonstrated that each anesthesia protocol uniquely modulated FC. Connectivity patterns obtained under propofol and urethane anesthesia were most similar to that observed in awake rats. FC patterns in the α-chloralose and isoflurane-medetomidine combination groups had moderate to good correspondence with that in the awake group. The FC patterns in the isoflurane and medetomidine groups differed most from that in the awake rats. These results can be directly exploited in rsfMRI study designs to improve the data quality, comparability, and interpretation.
INTRODUCTION

Advances in functional magnetic resonance imaging (fMRI) methods have enabled noninvasive investigations of functional brain networks (Biswal et al., 1995). Task-free resting-state fMRI (rsfMRI) studies have shown that functional connectivity (FC) is modulated in various central nervous system (CNS) diseases, such as Alzheimer's disease, bipolar disorder, depression, autism, epilepsy, multiple sclerosis, and schizophrenia (Fox and Raichle, 2007; Lu and Stein, 2014; Smucny et al., 2014), and in different arousal states, such as during sleep and anesthesia (Nallasamy and Tsao, 2011). Importantly, FC network structures are observed across species (Belcher et al., 2013; Lu et al., 2012; Upadhyay et al., 2011; Vincent et al., 2007). Therefore, FC can be exploited in controlled preclinical experiments investigating normal brain function, pathophysiologic mechanisms of complex CNS diseases, or in the search for new biomarkers as diagnostics and targets for novel treatments. Because animals do not need to perform any tasks during the measurements, and several pharmacologically-, surgically-, or genetically-induced disease models are readily available, investigation of FC is a highly attractive option for a wide range of neurobiologic study designs. Furthermore, the combination of FC with more invasive techniques, such as electrophysiologic recordings, electrical or optogenetic brain stimulation, and histopathology, can provide insights into the mechanisms of normal and disease-modified FC.

The vast majority of preclinical experiments studying FC in disease models or during drug-induced modulation are conducted under general anesthesia to prevent motion artifacts and stress in the animals (Lukasik and Gillies, 2003). Anesthetics, however, are likely to disturb neuronal activity, brain metabolism, neurovascular coupling, and FC (Gao et al., 2016). Some functional networks may be preserved under anesthesia while others are suppressed (Nallasamy and Tsao, 2011), making it more difficult to determine the effects of disease or treatment on FC. Moreover, emerging data indicate that FC is modulated in an anesthetic- and/or dose-dependent manner (Grandjean et al., 2014; Hamilton et al., 2017; Jonckers et al., 2014; Kiviniemi et al., 2005; Liu et al., 2013a, 2013b; Lu et al., 2007; Pawela et al., 2009; Peltier et al., 2005; Williams et al., 2010), which hinders generalization and meta-analysis of the results as several different

Abbreviations: AC, α-chloralose; BOLD, blood oxygenation level dependent; CC, correlation coefficient; CNS, central nervous system; DMN, default mode network; FC, functional connectivity; FDR, false discovery rate; fMRI, functional magnetic resonance imaging; ISO, isoflurane; MED, medetomidine; PRO, propofol; rsfMRI, resting-state functional magnetic resonance imaging; URE, urethane.

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anesthesia protocols are currently used in the preclinical MRI field (Lukasik and Gillies, 2003).

To overcome the confounding effects of anesthetics, fMRI protocols for imaging awake animals have been introduced (King et al., 2005; Lahti et al., 1998). Although preclinical fMRI with awake subjects appears to provide a robust platform for more reliable detection of neuronal activity (Gao et al., 2016), the approach has its own limitations. To minimize stress and motion artifacts, the animals typically undergo a habituation protocol in an environment mimicking that during the actual measurement. As these training protocols may last up to 8 days (King et al., 2005), however, the approach is clearly more labor- and time-intensive than the protocols performed under anesthesia. Furthermore, spontaneous movement and stress responses are still likely to be present during the imaging despite acclimatization. Additionally, certain study designs requiring strong analgesia or muscle relaxation, e.g., investigations of the effects of acute stroke or seizures, are inappropriate or not possible with awake animals.

Because both approaches – imaging of either anesthetized or awake subjects – have their advantages and limitations, it is likely that both will remain in use. Despite the possible effect of residual stress, the FC pattern of awake subjects may better represent the normal physiologic state than the FC pattern obtained from a deeply anesthetized animal. Therefore, if the use of anesthesia is unavoidable, it is crucial to characterize the effects of different anesthetics on FC for comparison with the awake condition.

Despite the rapidly increasing amount of preclinical rsfMRI studies, only a few groups have extensively compared FC under different anesthetics (Grandjean et al., 2014; Williams et al., 2010), or more importantly, between different anesthetics and the awake state (Jonckers et al., 2014). To our knowledge, no studies to date have compared FC between awake rats and rats in various anesthetized states. Therefore, we acquired blood oxygenation level dependent (BOLD) rsfMRI data from naïve rats under six anesthesia protocols and during the awake state and analyzed the characteristics of cortical, subcortical, and cortico-subcortical (e.g., thalamo-cortical) connectivity under each condition. Additionally, we investigated the connectivity of central regions within the rat default mode network (DMN).

MATERIALS AND METHODS

Animal preparations

All animal procedures were approved by the Animal Health and Welfare committee of the Regional State Administrative Agency, and conducted in accordance with the guidelines set by the European Commission Directive 2010/63/EU. Adult male Wistar rats (RccHan:WIST, Kuopio Laboratory Animal Centre, Kuopio, Finland; n = 70, 235-440 g) were used in the fMRI experiments. The animals were maintained on a 12/12 h light-dark cycle at 22±2 C with 50%-60% humidity. Food and water were available ad libitum.

Anesthetized rats

The present study partly exploits unpublished data obtained in our previous pharmacologic fMRI study (Paasonen et al., 2016). Here, the anesthesia groups were the following: α-chloralose (AC, n = 9; 60 mg/kg, i.v., Sigma-Aldrich, St. Louis, MO, USA), isoflurane (ISO, n = 8; 1.3%, Baxter, Lessines, Belgium), medetomidine (MED, n = 8; 0.1 mg/kg/h, i.v., Orion Pharma, Espoo, Finland), combined ISO and MED (ISO + MED, n = 8; 0.06 mg/kg/h i.v. MED and 0.5-0.6% ISO), propofol (PRO, n = 8; 7.5 mg/kg bolus and 45 mg/kg/h, i.v., Norbrook Laboratories Limited, Newry, Northern Ireland), and urethane (URE, n = 21; 1.25 g/kg, i.p., Sigma-Aldrich, Helsinki, Finland).
Protocols for AC, ISO, MED, and URE are described in detail in our previous report (Paasonen et al., 2016), and protocols for PRO (Griffin et al., 2010; Liu et al., 2013b) and ISO + MED (Brynildsen et al., 2017; Fukuda et al., 2013; Lu et al., 2012; Pirttimaki et al., 2016) were adapted from the literature. There is, however, no established protocol for the ISO + MED anesthesia, and thus the protocol in the present work was compiled based on the literature. All anesthesia protocols in the present study are reported to preserve moderate to good interhemispheric FC.

All rats were first anesthetized with ISO (5% induction and 2% maintenance) in a N₂/O₂ 70/30 mixture with a calibrated vaporizer. Small cannulas were inserted into the femoral artery and vein for blood sampling and drug injections, respectively. Subsequently, a tracheal tube was inserted for mechanical ventilation (MA1-7061, Harvard Apparatus Inc.). After surgical preparations, lidocaine (Xylocain™, AstraZeneca, Sodertälje, Sweden) was applied to the wounds, and the anesthesia switched to one of the protocols described above.

The rat heads were tightly fixed in a water-circulation heated rat holder with ear pins and a bite bar. Muscle relaxant, pancuronium bromide (~1 mg/kg/h, i.v., Pavulon™, Organon, Oss, Netherlands), was given to rats while connecting tracheal tube to the mechanical ventilator. Body temperature was maintained at ~37 C. A small animal monitoring system (Model 1025, Small Animal Instruments Inc., New York, NY, USA), including a rectal temperature probe, respiration pneumatic sensor, capnograph, and fiber optic oximetry sensor or cardiogram electrodes, was used for real-time monitoring. Arterial blood samples (~0.15 ml) were analyzed (i-STAT Model 300, Abbott Point of Care Inc., Princeton, NJ, USA) for pCO₂, pO₂, sO₂, and pH values.

After the measurements, the rats were killed using 5% ISO for ~5 min, following an intravenous injection of concentrated potassium chloride into the femoral vein while the rat was still inside the magnet. Additionally, cervical dislocation was performed outside the magnet. To estimate the influence of hardware- and mechanical ventilation-induced noise and drift on FC data, and to provide a proper reference for complexnetwork analyses, the rsfMRI measurement was repeated in 8 rats 5-10 min after the potassium chloride-induced cardiac arrest, without discontinuing the ventilation.

Awake rats

The detailed protocol for habituation is provided in a separate report (Stenroos et al., in preparation). Briefly, the rats were habituated in a mock scanner. Rats (n = 8) were first anesthetized with ISO (5% induction and 2% maintenance in the same N₂/O₂ 70/30 mixture). Forepaws were secured along the sides, and hindpaws with tail were secured using masking tape. The body was wrapped with plastic foam sheet, allowing for normal breathing motion. Silicone plugs were inserted into the ear cavities to protect the rat from the MRI scanner noise.

After the initial preparations, the rats were transferred to a standard Bruker rat holder. The head was secured with a custom-built restraint kit including a cushioned nose cone, and cheek, neck, and shoulder supports. A standard rat brain radio-frequency quadrature receiver coil (Bruker Biospin, Ettlingen, Germany) was placed on top of the head, and the holder was pushed inside a plastic tube mimicking the magnet bore, after which the ISO was decreased to zero. The preparation steps took typically 8-10 min. The sounds of the full MRI protocol were then played through a loudspeaker producing similar sound pressure to that measured from the MRI bore. The original MRI sounds were recorded with a MT830R microphone (Audio-Technica, Leeds, England).
During the first week of training, the rats were habituated for 4 days in the mock scanner and imaged on the day 5 in the MRI. After a weekend pause, the same procedure was repeated. The length of the habituation session was increased in 10-min increments, starting from 15 min on the first day, up to 45 min on day 4. The subsequent habituation sessions lasted 45 min.

After the habituation or imaging, the ISO was increased back to 2%, and the animal was released from restraint. Subsequently, a blood sample (~0.15 ml) was obtained from a tail vein for corticosterone analysis (Corticosterone Mouse/Rat ELISA immunoassay kit RTC002R, Demeditec Diagnostics, Kiel, Germany), and then the animal was returned back to its cage.

Magnetic resonance imaging

The MRI measurements were performed using the 7 T Bruker Pharmascan system, operated with ParaVision 5.1 software (Bruker Biospin). The same standard coils, a rat brain quadrature surface coil and a quadrature resonator volume coil, were used with all animals. Shimming was optimized for the cerebrum (8x12x15 mm³ voxel) using a three-dimensional fieldmap-based automatic shimming method.

Anatomic images were acquired with fast spin-echo sequence (TurboRARE, repetition time 4.7 s, echo spacing 16 ms, effective echo time 48 ms, echo-train length 8, field-of-view 5.0 x 5.0 cm, matrix size 512x512, and 30 slices with a thickness of 0.75 mm). Functional BOLD images were acquired with single-shot spin-echo echo planar imaging sequence (repetition time 2 s, echo time 45 ms, field-of-view 2.5 cm x 2.5 cm, matrix size 64x64, and 9-11 slices with a thickness of 1.5 mm).

The rsfMRI acquisition comprised 300 vol (10 min) and 600-750 vol (20-25 min) with anesthetized and awake animals, respectively. Despite the habituation, awake animals tended to move slightly during imaging, which can induce a bias in the data analysis between awake and anesthetized animals. To ensure comparable datasets, a longer acquisition was used for the awake animals, from which a 10-min motion-free period was used in the subsequent analyses.

Data preprocessing and analysis

The data were converted from Bruker format to NIfTI using Aedes (http://aedes.uef.fi). Next, slice-timing correction, head motion correction, spatial smoothing (2x2 voxel full-width at half-maximum Gaussian kernel), and co-registration were applied using SPM8 (www.fil.ion.ucl.ac.uk/spm) and Matlab (Version 2011a, The Mathworks Inc., Natick, MA, USA). No artifacts or subject motion were detected in anesthetized rats. In awake rats, two datasets (2/16) were excluded due to a missing continuous 10-min motion-free period during the rsfMRI data acquisitions.

The FC analyses were performed using an in-house Matlab code, Aedes (aedes.uef.fi), and SPM8 (www.fil.ion.ucl.ac.uk/spm). For whole brain analysis, 12 regions of interest (ROIs) were drawn according to an anatomic atlas (Paxinos and Watson, 1998) to a reference brain (Figure S1A). These 12 ROIs were further divided into 92 smaller ROIs, which were used in graph theory-based complex-network analyses (Brain Connectivity Toolbox, https://sites.google.com/site/bctnet/).

Additional ROIs (Figure S1B) were drawn according to Lu et al. (2012) to evaluate the connectivities of the central hubs (the prefrontal cortex [including anterior cingulate and prelimbic cortices], orbital frontal cortex, and retrosplenial cortex) in the rat DMN.

Prior to the FC analyses, the data were band-pass filtered at 0.01-0.15 Hz as spontaneous BOLD fluctuations correlate well with electrical activity up to 0.159 Hz under ISO and MED anesthesia (Thompson et al., 2014). Additionally, our power spectrum data indicated increased non-
neuronal noise >0.15 Hz measured from the post mortem group. The Pearson’s correlation coefficients (CCs) were calculated using Matlab. As the strongest negative correlation in mean correlation matrices was only 0.03, analyses were focused on positive correlations. Spectral powers for BOLD signals were calculated by fast-Fourier transform, using a linear trend removal instead of a band-pass filter. Subsequently, the group-level spectral power data were smoothed with an Aedes trend estimation function.

Prior to calculating the mean values or statistical comparisons, the CCs were transformed to Z-scores using the Fisher transformation. Statistical comparisons were performed with either Matlab or GraphPad Prism (Version 5.03, GraphPad Software Inc., La Jolla, CA, USA). All group-level values are represented as mean ± standard errors of mean (SEM).

RESULTS

Spontaneous BOLD fluctuations were measured in 70 rats under awake and six anesthetized conditions. Additionally, post mortem data were obtained to estimate the effects of hardware- and mechanical ventilation-induced noise on the FC data.

Physiology

The physiology of anesthetized animals was carefully controlled, and all measured physiologic parameters (Table 1) were in the normal range. Statistical comparison (one-way analysis of variance [ANOVA] and Tukey’s multiple comparison test) indicated no differences in body weight or blood gas values (pCO2, pO2, and sO2) among groups. The heart rates differed among the groups, however, as protocols including medetomidine induced lower values compared with the other anesthetics. Bradycardia is a known side effect of medetomidine (Lukasik and Gillies, 2003), and is thus considered a characteristic feature. Additionally, the pH in the AC group was slightly higher than that in the ISO and URE groups, but within normal range.

The blood corticosterone levels measured in the awake rats after the first (126±18 ng/ml) and second (98±10 ng/ml) MRI measurement did not differ significantly from the pre-habituation samples (95±15 ng/ ml; p < .25 against week 1 data, and p < .88 against week 2 data; paired t-test), indicating successful habituation to the MRI environment. As there were no differences in corticosterone levels or FC matrices (paired t-test, data not shown) between the two time points, all FC awake data were pooled together to increase the statistical power.

The head motion in the awake group was in acceptable range; average relative motion to voxel size was 2.8±0.2%, corresponding to an estimated value in humans of 4-10% (Power et al., 2012, 2014). Maximal single head movement in the awake group was 123±23 μm, which is roughly 1/3 of the voxel size. No head motion was observed in the anesthetized rats.
Table 1. Physiologic parameters obtained from rats that underwent resting-state functional magnetic resonance imaging. bpm, beats per minute.

<table>
<thead>
<tr>
<th></th>
<th>pCO₂ (mmHg)</th>
<th>pO₂ (mmHg)</th>
<th>pH</th>
<th>sO₂ (%)</th>
<th>Heart rate (bpm)</th>
<th>Weight (g)</th>
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<tbody>
<tr>
<td>Awake</td>
<td>–</td>
<td>37.6±2.2</td>
<td>141±8</td>
<td>7.48±0.02</td>
<td>99.0±0.3</td>
<td>320±4</td>
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<tr>
<td>α-Chloralose</td>
<td>45.1±2.0</td>
<td>133±8</td>
<td>7.39±0.02</td>
<td>98.9±0.1</td>
<td>427±13</td>
<td>317±12</td>
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<td>Isoflurane</td>
<td>38.2±1.5</td>
<td>133±4</td>
<td>7.42±0.02</td>
<td>98.9±0.1</td>
<td>272±4</td>
<td>310±16</td>
</tr>
<tr>
<td>Isoflurane + medetomidine</td>
<td>40.8±2.4</td>
<td>149±5</td>
<td>7.42±0.01</td>
<td>98.6±0.6</td>
<td>273±10</td>
<td>333±12</td>
</tr>
<tr>
<td>Medetomidine</td>
<td>40.9±2.3</td>
<td>137±5</td>
<td>7.42±0.02</td>
<td>99.0±0.0</td>
<td>412±11</td>
<td>323±24</td>
</tr>
<tr>
<td>Propofol</td>
<td>39.0±1.5</td>
<td>144±5</td>
<td>7.38±0.01</td>
<td>99.0±0.1</td>
<td>457±7</td>
<td>349±8</td>
</tr>
<tr>
<td>Urethane</td>
<td>40.8±2.4</td>
<td>149±5</td>
<td>7.42±0.01</td>
<td>98.6±0.6</td>
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<td>273±10</td>
<td>333±12</td>
</tr>
<tr>
<td>Medetomidine</td>
<td>40.9±2.3</td>
<td>137±5</td>
<td>7.42±0.02</td>
<td>99.0±0.0</td>
<td>412±11</td>
<td>323±24</td>
</tr>
<tr>
<td>Propofol</td>
<td>39.0±1.5</td>
<td>144±5</td>
<td>7.38±0.01</td>
<td>99.0±0.1</td>
<td>457±7</td>
<td>349±8</td>
</tr>
<tr>
<td>Urethane</td>
<td>40.8±2.4</td>
<td>149±5</td>
<td>7.42±0.01</td>
<td>98.6±0.6</td>
<td>273±10</td>
<td>333±12</td>
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Functional connectivity matrices and mean connectivity

The group-level FC matrices are shown in Fig. 1A, and the region specific mean CCs are shown in Fig. 1B. The mean FC pattern obtained from awake animals comprised mainly moderate to strong CCs (e.g., cortico-cortical CCs 0.43±0.03 in a 12 ROI matrix). Only connections originating from the nucleus accumbens and hypothalamus showed consistently low CCs (0.14±0.02 and 0.17±0.02 in a 12 ROI matrix, respectively).

Under ISO anesthesia, connectivity was heavily modulated compared with the awake group. All cortico-cortical CCs (15/15) were significantly stronger (p < .05, t-test, false discovery rate [FDR] corrected), while 64% (23/36) of cortico-subcortical and 33% (5/15) subcortico-subcortical CCs were affected. The region-specific mean CCs were high throughout the cortex and striatum (ISO 0.46±0.57; awake 0.24±0.44), but heavily decreased in the rest of the regions, especially the medial thalamus (ISO 0.05±0.03; awake 0.26±0.03) and hypothalamus (ISO 0.03±0.02; awake 0.17±0.04).

Under MED anesthesia, the FC was globally suppressed compared with the awake condition (mean CCs in 12 ROI matrices: MED 0.10±0.01 and awake 0.29±0.02). If the nucleus accumbens and hypothalamus were not considered, 89% (40/45) of the remaining connections were suppressed, including 92% (11/12) of the thalamo-cortical connections. The region-specific mean CCs were also low, particularly in the cortex (MED 0.07±0.22; awake 0.24±0.44) and striatum (MED 0.04±0.02; awake 0.29±0.03).

When ISO and MED were used together (ISO + MED), only 33% (5/15) of the cortico-cortical CCs were decreased; the corresponding value was 53% (8/15) for subcortico-subcortical CCs. A significant amount (50%, 18/36) of the cortico-subcortical CCs, however, were weakened by ISO + MED, including 75% (9/12) of the thalamo-cortical connections. The region-specific mean CCs were generally lower (0.02-0.31) than those in the awake group (0.15-0.44), especially in the posterior cortical regions (e.g., auditory cortex ISO + MED 0.16±0.02; awake 0.35±0.03), medial thalamus (ISO + MED 0.07±0.01; awake 0.26±0.03), and hypothalamus (ISO + MED 0.02±0.01; awake 0.17±0.04).
Fig. 1. Group-level functional connectivity (FC) matrices obtained under awake, anesthetized, and post mortem conditions (A), and corresponding region-specific mean correlation coefficients (B). In panel A, the lower triangular parts of the matrices show the FC results obtained in 12 regions of interest (ROIs), while the upper triangular parts show the results obtained in 92 ROIs. Stars in lower triangular parts indicate a statistical difference compared with the awake group (t-test, \( *p < .05 \), false discovery rate corrected). Statistical testing in the panel B was performed using a one-way ANOVA and Dunnett’s multiple comparison against the awake group (\( *p < .05, **p < .01, ***p < .001 \)). AC, auditory cortex; HC, hippocampus; ThM, medial thalamus; ThVL, ventrolateral thalamus; VC, somatosensory cortex; Str, striatum; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; RC, retrosplenial cortex; ROI, region of interest; SC, somatosensory cortex; ThM, medial thalamus; ThVL, ventrolateral thalamus; VC, visual cortex.
Similarly, to the ISO + MED group, 33% (5/15) of the cortico-cortical connections were weakened in the AC group. In contrast, only 22% (8/36) of the cortico-subcortical and 33% (5/15) of subcortico-subcortical connections were suppressed. Roughly half (7/12) of the thalamocortical CCs were comparable to those in the awake group. While the cortical region-specific mean CCs were close (0.15-0.38) to those of the awake group (0.24-0.44), the coefficients were clearly lower in the subcortical regions (AC 0.06-0.22; awake 0.15-0.34).

In the PRO group, there were no statistical differences in 12 ROI matrices compared with the awake group after the FDR correction. The CC values in the cortical regions, however, appeared to be slightly higher than those in the awake group (see 92 ROI matrices in Fig. 1). The significantly higher variance observed in the PRO group (e.g., in motor cortex p < .05, t-test with Welch’s correction) may have hindered the detection power. Nevertheless, region-specific FC was suppressed in connections originating from the nucleus accumbens (PRO 0.06±0.03; awake 0.14±0.02) and medial thalamus (PRO 0.15±0.03; awake 0.26±0.03).

The FC pattern under URE anesthesia was close to that in the awake group; only 6% (4/66) of all connections were significantly suppressed. The 92 ROI matrix appeared visually similar to that in the awake group, although with slightly lower CCs (URE 0.11±0.001; awake 0.15±0.001). In the region-specific mean FC analysis, the connectivity of the striatum (URE 0.18±0.02; awake 0.29±0.03), medial thalamus (URE 0.16±0.02; awake 0.26±0.03), and ventrolateral thalamus (URE 0.25±0.02; awake 0.34±0.03) was weakened by URE anesthesia.

In the FC matrices of post-mortem data, only negligible CCs (0.02±0.01) were observed, indicating that hardware-induced noise, mechanical ventilation-related motion, and data processing-induced artifacts did not influence our findings.

Default mode network hubs, cortical connectivity, and complex-network structures

The FC of the prefrontal and posterior parts of the rat DMN (Lu et al., 2012) is shown in Fig. 2A. The CCs were remarkably high throughout the DMN in the ISO group (ISO 0.49-0.83; awake 0.23-0.57), except between the retrosplenial cortex and hippocampus (ISO 0.18±0.04; awake 0.20±0.06). Except for ISO, the anesthetics appeared to suppress the prefrontal parts of the DMN, especially the long-distance connections. In contrast, the CCs of the posterior parts of the DMN were closer, or even higher in the anesthetized rats compared with the awake rats. In the PRO group, the CCs of the DMN were similar (0.17-0.53) to those in the awake group (0.20-0.57).

The strength of five representative connections from the somatosensory cortex is shown in Fig. 2B. The intracortical connectivity was comparable to the awake group in all anesthesia groups, except the high and low CCs observed in the ISO (ISO 0.88±0.06; awake 0.65±0.03) and MED (MED 0.38±0.05; awake 0.65±0.03) groups, respectively. The cortico-thalamic and cortico-hypothalamic connections, however, were generally disrupted by the anesthetics. The differences in interhemispheric cortical connectivity between the groups (Fig. 2C) closely followed the differences in the mean cortical (Fig. 1B) and intracortical connectivities (Fig. 2B).
The parameters obtained from complex-network analyses are shown in Fig. 2D (for concepts and quantities of complex-networks, see (Rubinov and Sporns, 2010)). Modularity was significantly reduced in the ISO group (58%), and the PRO group exhibited a similar decreasing trend (29%). The mean degree was decreased in the ISO + MED (15%) and MED (23%) groups, while the other groups were comparable to the awake group. Mean distance was slightly increased in the AC (30%), URE (24%), and ISO + MED (36%) groups, and considerably increased in the MED (58%) group. The mean clustering coefficient was greatly increased in the ISO (93%) group, and significantly decreased in the MED (61%) group.

Differences in correlation maps

The representative seed-based connectivity maps obtained from the awake animals, and differences between the awake and anesthetized animals are shown in Fig. 3. In the ISO group, the cortical and striatal connections were merged into one uniform network, while thalamocortical FC was suppressed. In the ISO + MED, AC, and URE groups, there were only minor differences in cortical and striatal connectivity compared with the awake group, while
varying amounts of differences existed in the thalamo-cortical connectivities. FC in the MED group was heavily suppressed, consistent with the results from other analyses. In the PRO group, no significantly different voxel-wise correlations were detected (FDR corrected) compared with the awake group. The comparison between the awake and post mortem groups confirmed that the correlations outside the ROIs had a physiologic origin.

Spectral power of BOLD signals

The group-level spectral powers of BOLD signals, obtained from representative cortical and thalamic regions, are shown in Fig. 4. In the awake group, the spontaneous signal fluctuations occurred relatively evenly throughout the observed frequency range. In contrast, the spectral power peaked at a narrow frequency range under anesthesia in a protocol dependent manner. Additionally, the spectral power profiles suggested independent (e.g., AC, ISO, and MED) or shared (e.g., AC, ISO + MED, PRO, and URE) fluctuations between the thalamus and cortex.

In the ISO group, the spectral power of the cortex was strong at 0.05-0.12 Hz, while ventrothalamic fluctuations were fully suppressed. In the ISO + MED group, the bandwidth of strong cortical fluctuations was shifted to higher frequencies (centered around 0.19 Hz) compared with the ISO group, and a spectral power peak within the corresponding
Fig. 3. Seed-based correlation coefficient maps obtained from the awake rats (top row), and statistical differences compared with the anesthetized animals (remaining rows). Seed regions (somatosensory cortex, striatum, medial thalamus, and ventrolateral thalamus) are illustrated in the propofol row (white arrows), as there were no significantly different voxels between the propofol-anesthetized and awake rats. Correlation coefficient maps are overlaid on T2-weighted anatomic images. Statistical comparison was done with voxel-wise t-tests (p < .05 with false discovery rate correction).
Fig. 4. Group-level spectral powers of blood oxygenation level-dependent signals, obtained from eight groups. Black line indicates no significant (n.s.) difference, while red line indicates difference compared with the awake group (p < .05, t-test, false discovery rate corrected). Small green arrows highlight similar peaks in the cortex and thalamus, while blue arrows indicate peaks that are present in only one of the two regions.
range was also observed in the thalamus. In contrast to the ISO + MED group, the power of the cortical fluctuations was minimal in the MED group, while a distinct peak was observed in the thalamus around 0.13 Hz.

In the AC group, the cortical fluctuations were observed at both low frequencies and higher frequencies (around 0.18 Hz). Similar increases in spectral power were also observed in the thalamus, although the thalamus expressed an additional peak around 0.07 Hz that was not visible in the cortex. In the PRO group, the spontaneous fluctuations were observed at both low frequencies and higher frequencies (around 0.18 Hz). Similarly, increases in spectral power were also observed in the thalamus, although the thalamus expressed an additional peak around 0.07 Hz that was not visible in the cortex. In the URE group, there was an increase in spectral power around 0.10 Hz in both the cortical and thalamic regions.

In all groups, an increase in cortical spectral power was apparent at low frequencies (<0.05 Hz) compared with the post mortem data. The post mortem data also suggest that an external source increased spectral power slightly, starting from ~0.16 Hz up. The noise was more emphasized in the thalamic region, possibly because of the lower signal to noise ratio.

DISCUSSION

To our knowledge, the present study provides the most extensive dataset of rat brain connectivity in both anesthetized and awake animals. The findings are in excellent agreement with prior knowledge; the FC of the rat brain is uniquely modulated by different anesthesia protocols. Importantly, we were able to use reference data obtained from awake animals under identical scanning conditions to pinpoint anesthesia specific alterations in connectivity.

Anesthesia-induced disruption of peripheral information flow

Because the thalamus is in a key position to exchange information between the periphery and cortex, many of the potential pathways associated with anesthesia-induced loss of consciousness involve the thalamic arousal nuclei (Franks, 2008); the switch in balance between arousal and inhibitory networks, controlled by the pons, thalamus, and hypothalamus, is hypothesized to be essential in anesthesia. In an awake state, the thalamo-cortical pathway allows information to flow through to the cortex. In contrast, a low-frequency bursting pattern is typically present in the thalamo-cortical pathways under anesthesia, preventing peripheral information flow and its processing at cortex. Importantly, the bursting pattern in thalamo-cortical neurons can spread extensively to bilateral cortical regions.

The results of the present study are consistent with the concepts discussed above. First, the measured FC in the awake group (Figs. 1-3) showed clear functional connectivity between the subcortical and cortical brain regions. The results of complex-network analysis (Fig. 2) indicate a high mean degree and short mean distance in the awake group, suggesting a network structure with multiple connections and effective paths, which is expected in the awake brain. Importantly, the spectral power of the BOLD signal was relatively high at a wide frequency range in both the cortex and thalamus in awake rats (Fig. 4); this suggests that neuronal activity at a diverse range of frequencies, or dynamic FC, which is also expected from awake subjects.

Second, the comparison between anesthetized and awake rats resulted in several observations supporting modulation of the thalamocortical activity by anesthetics; all anesthetics significantly suppressed the FC of either the thalamus or hypothalamus (Fig. 1B). In certain anesthesia groups, the spontaneous BOLD signal fluctuations were particularly enhanced in the cortex (Fig. 1B), which may be associated with the thalamo-cortical bursting activity. Simultaneously, the FC pattern appeared to spread to adjacent regions (Fig. 1A, AC, ISO and PRO), which also points toward low-frequency, less
specific thalamocortical bursting. Almost all anesthetics significantly modulated the complex-network structure (Fig. 2D) by, e.g., modifying the number of modules, decreasing the amount of connections, or increasing the mean path length. Lastly, all anesthetic heavily suppressed the BOLD signal spectral power (Fig. 4); a characteristic frequency profile with a relatively narrow-ranged peak was observed in each group, which could suggest either anesthesia-induced bursting activity or less dynamic FC.

Characteristic effects of different anesthesia protocols on functional connectivity

Several studies reported that the parameters of FC are dependent on either the anesthetic or its dose: for example AC (Jonckers et al., 2014; Lu et al., 2007; Williams et al., 2010), ISO (Grandjean et al., 2014; Hamilton et al., 2017; Jonckers et al., 2014; Kalthoff et al., 2013; Liu et al., 2013a; Williams et al., 2010), ISO + MED (Grandjean et al., 2014), MED (Grandjean et al., 2014; Hamilton et al., 2017; Nasrallah et al., 2012; Pawela et al., 2009; Williams et al., 2010), midazolam (Kiviniemi et al., 2005), PRO (Barttfeld et al., 2015; Grandjean et al., 2014; Hudetz et al., 2015; Liu et al., 2013b), sevoflurane (Peltier et al., 2005), and URE (Grandjean et al., 2014; Jonckers et al., 2014). Similarly, many recent preclinical studies reported differences in functional network properties between awake and anesthetized states (Barttfeld et al., 2015; Hamilton et al., 2017; Jonckers et al., 2014; Liang et al., 2015; Ma et al., 2017; Smith et al., 2017). The majority of these studies, however, evaluated only one anesthetic, making it difficult to compare between anesthetics. As the mechanisms of action and the effects of anesthetics on receptors, physiology, and neurovascular coupling are discussed anesthetic-specifically in detail in the cited studies, we focus on the findings related to FC.

α-Chloralose (60mg/kg). The FC measured from AC-anesthetized rats moderately resembled the corresponding awake data. Although the FC pattern appeared to be globally suppressed, cortical connectivity, which was similar to that in previous rat studies (Baek et al., 2016; Lu et al., 2007; Williams et al., 2010), was mainly well preserved compared with the awake group. Connectivity between the striatum and cortex, similar to our results, has also been reported (Williams et al., 2010), although with slightly lower CCs. The strength of the connections originating from the striatum, however, was clearly weaker to that in the awake group in the present work.

The cortical peak in spectral power around 0.18 Hz in the AC group is also consistent with a previous study (Williams et al., 2010), although the authors demonstrated the peak in spectral power at a slightly lower frequency range. In the present study, the BOLD spectral power increased at similar frequencies in the thalamus and cortex, except the thalamus exhibited an additional peak at 0.07 Hz not visible in the cortex. Thus, the spectral power analyses support the ROI analyses; thalamo-cortical BOLD fluctuations indicate both preserved and disconnected thalamo-cortical activity under AC anesthesia.

Taken together, our observations suggest that AC is a potential anesthetic for rsfMRI studies; despite the significantly suppressed connectivity, the cortical FC pattern has many similarities to that in the awake condition, and the thalamo-cortical coupling appears to be partially preserved.

Isoflurane (1.3%). In the ISO group, high CCs were observed throughout the cortical and striatal regions. Based on the connectivity matrices and CC maps, these regions share similar strong BOLD fluctuation profiles, indicating a widespread network structure. The affected complex-network parameters, namely low modularity (or the amount of delineated subnetworks) and high clustered connectivity, were also consistent with these observations. Similar findings are commonly reported (Kalthoff et al., 2013;
Liu et al., 2011; Williams et al., 2010), unless subanesthetic doses of ISO are used (Liu et al., 2013a). As detection of the large-scale synchronized network is robust and its properties are dose-dependent (Liu et al., 2013a), and the network structure is clearly distinct from the awake data, the phenomenon can be argued to be a characteristic feature of ISO anesthesia in rats. Previous work indicates that the synchronization of the neocortex originates from ISO-induced burst-suppression activity measured with electroencephalography techniques (Liu et al., 2011, 2013a).

In contrast to the remarkably increased FC strength in the frontocortical regions, the majority of the thalamo-cortical and intrasubcortical connections were heavily suppressed by ISO, consistent with previous studies (Hamilton et al., 2017; Shin et al., 2016). In addition, our spectral power analysis indicated negligible fluctuation powers in the thalamus, further indicating strong ISO-induced suppression of thalamic activity. In contrast, the spectral powers of the cortical fluctuations were high, even when compared with the awake rats, suggesting clear thalamo-cortical disconnection under ISO anesthesia.

The published data related to the cortical BOLD fluctuation frequencies in rat are generally more heterogenous. Kalthoff et al. (2013) reported a majority of spectral powers at low frequencies (up to 0.05 Hz), while Williams et al. (2010) reported increases around 0.10-0.15 Hz in addition to low frequencies. In the present study, the corresponding peaks in cortical spectral power were observed within a slightly broader range (0.05-0.12 Hz), which partially overlaps the range described earlier (Williams et al., 2010). Thus, the high power at low frequencies is rather consistently detected, while the peak power at higher frequencies may be absent or vary between 0.05 and 0.15 Hz. This, most likely, originates from the different level of the burst-suppression effect.

Our results, in combination with previous investigations, indicate that anesthetic doses of ISO heavily mask the naturally occurring FC of the rat brain by inducing synchronous cortico- striatal fluctuations and silencing subcortical activity, which are both uncharacteristic for awake rats. These effects, however, can be minimized by using low doses of ISO (Liu et al., 2013a).

Medetomidine (0.1mg/kg/h). The CCs in the MED group were low compared with the awake data. Similar CCs were reported earlier (Kalthoff et al., 2013; Magnuson et al., 2014; Pawela et al., 2009; Williams et al., 2010; Zhao et al., 2008), although some studies reported slightly higher values (Nasrallah et al., 2012; Pawela et al., 2008, 2009). The vast majority of the connectivity, especially intracortical, cortico-striatal, and thalamo-cortical, was significantly suppressed by MED. Consistent with these observations, Kalthoff et al. (2013) measured low cortico-striatal connectivity strength, and Williams et al. (2010) reported reduced FC between networks. The intercortical connectivity in the present work, however, was moderate under MED anesthesia, which supports the good interhemispheric specificity of networks, as reported previously (Kalthoff et al., 2013; Pawela et al., 2008; Williams et al., 2010; Zhao et al., 2008).

In the present study, the thalamo-cortical activity was almost completely absent in the MED group. Similarly, Zhao et al. (2008) did not detect any synchronous BOLD activity with the thalamus as the seed region, and Pawela et al. (2008) reported only diffuse thalamo-cortical connectivity.

The spectral power of cortical fluctuations was also remarkably low in the MED group; compared with the post mortem data, increases were observed mainly at <0.03 Hz, consistent with previous reports (Kalthoff et al., 2013; Nasrallah et al., 2012). Additionally, some studies suggest a possible increase in power around 0.10-0.18 Hz (Grandjean et al., 2014; Magnuson et al., 2014; Williams et al., 2010), which also fits
well with our findings. In contrast to the cortex, a relatively clear peak in spectral power was observed in the thalamus. No similar peak was observed in the cortex, suggesting that MED anesthesia results in separate spontaneous fluctuation profiles of the thalamus and cortex.

Taken together, the BOLD fluctuations under MED anesthesia appeared to be globally suppressed; the FC pattern was clearly distinct from that in the awake group, and only partially comparable with that under the other anesthetics.

Combination of isoflurane (0.5-0.6%) and medetomidine (0.06mg/kg/h). Compared with the results obtained with either ISO or MED alone, the overall FC pattern under ISO + MED better resembled the data measured from awake rats; the biggest contribution to the differences between the ISO + MED and awake groups originated from the partial suppression of intra-subcortical and thalamo-cortical connections. The cortical CCs were mildly affected, resulting in good interhemispheric and intracortical connectivities similar to the awake state, and previous reports (Brynildsen et al., 2017; Lu et al., 2012). The good cortical connectivity values may be a result of the combined vasodilation effect of ISO and vasoconstriction effect of MED, but they may also originate from reduced confounding effects because of the lower doses of each anesthetic; the exact interactions, however, are complex and difficult to predict.

There are only a few reports on cortico-subcortical or thalamocortical connectivity under ISO + MED anesthesia, most likely because of the novelty of the anesthesia protocol. According to Lu et al. (2012) there is moderate connectivity between the retrosplenial cortex and hippocampus, which is supported by our findings. In the present study, the connectivity between the ventrolateral thalamus and cortex was similar to that in the awake state, while the connectivity between the medial thalamus or hypothalamus and cortex appeared to be almost entirely diminished by ISO + MED anesthesia.

A peak in both cortical and thalamic spectral power was observed around 0.18 Hz in the ISO + MED group. The increase in cortical power was comparable to that in the ISO group, although the fluctuations occurred at a higher frequency range in the ISO + MED group, similar to what is reported in mice (Grandjean et al., 2014). Distinct from the ISO group, the increase in the fluctuation power was also present in the thalamus, which, in contrast, resembles the MED group. These observations indicate that in the ISO + MED group both anesthetics, ISO and MED, have their characteristic effect on BOLD signal fluctuations.

Interestingly, several of the results in the ISO + MED group appeared to be associated with one of the combined anesthetics. For example, comparison of the results between the ISO, ISO + MED, and MED groups suggested that dysconnectivity of the hypothalamus was a solely ISO driven effect, while suppression of hippocampal BOLD fluctuations was induced by MED. Therefore, in addition to developing protocols with less confounding effects on FC, the use of a combination of anesthetic agents in rsfMRI studies may also provide new insights into the mechanisms of anesthetics.

The results of the present study promote the use of ISO + MED anesthesia in longitudinal preclinical fMRI studies. The FC pattern, having good cortical and partially preserved thalamo-cortical connectivity, clearly resembled the awake condition compared with the study designs exploiting either ISO or MED alone.

Propofol (7.5 mg/kg + 45 mg/kg/h). Generally, the FC pattern in the PRO group resembled the corresponding pattern in the awake group. The significant differences observed mainly consisted of connections originating from subcortical regions. The PRO group, however, had significantly higher statistical deviation, hindering the
detection power. The deviation may originate from complex and sensitive dose-dependent changes in FC, as there is a clear shift in FC between the PRO doses of 40 and 60 mg/kg/h (Liu et al., 2013b), or from the dynamic changes occurring especially in cortex (Hudetz et al., 2015).

Compared with the awake group, the connectivity pattern in the PRO group appeared less specific. Based on correlation matrices, BOLD fluctuations were more widespread to adjacent regions especially in intracortical and cortico-subcortical connections. These observations were further supported by complex-network analysis, where a trend of decreased modularity was observed. The extent of this phenomenon was clearly smaller than that in the ISO group, but it may still indicate anesthesia-induced global modulation of cortical activity. In a previous study, the number of correlating cortical voxels was also high for a PRO dose of 60 mg/kg/h (Liu et al., 2013b), further supporting our observation.

In contrast to many other anesthetics, thalamo-cortical synchrony is consistently detected under PRO anesthesia (Liu et al., 2013b; Tu et al., 2011). Consistent with our results, Liu et al. (2013b) detected thalamic connectivity to areas such as the cingulate and retrosplenial cortex. Additionally, the subcortical fluctuations appear to be well preserved, even at high PRO doses (Liu et al., 2013b).

Similar to other anesthetics, the spectral power of BOLD fluctuations was significantly suppressed by PRO. In both the cortex and thalamus, there was a significant fluctuation power at <0.05 Hz, indicating a similar fluctuation profile of the regions and potential basis for the connectivity.

In summary, PRO anesthesia induced a surprisingly similar FC pattern to that in the awake state. Cortical connectivity was strong, although less specific, and thalamo-cortical synchrony was mainly maintained. If the optimal window and stability for the depth of anesthesia can be obtained (Liu et al., 2013b), PRO is a very promising anesthetic for preclinical rsfMRI studies.

Urethane (1250mg/kg). The FC of URE-anesthetized rats also resembled the data obtained from awake animals. The intracortical and thalamo-cortical connectivity was mainly good, and the FC patterns in the ROI matrices were perhaps the closest to those in the awake group. Nevertheless, the CCs were slightly lower than those in the awake group, and some striatal and thalamic connections were significantly affected by URE; this may suggest specific thalamo-cortical dysconnectivity and mechanisms of URE anesthesia.

Despite URE being commonly used in electrophysiologic and pharmacologic studies, FC studies in URE-anesthetized rats are scarce and lack whole brain analyses. Nevertheless, our results are supported by similar CCs obtained from cortico-hippocampal (Wilson et al., 2011), intracortical, and thalamo-cortical connections (Zhurakovskaya et al., 2016). Some studies investigated the FC in URE-anesthetized mice (Grandjean et al., 2014; Jonckers et al., 2014), but as stated by the authors (Jonckers et al., 2014), a direct comparison between species is difficult. Nevertheless, Grandjean et al. (2014) detected moderate or good cortical and thalamic connectivity under URE anesthesia, similar to that under PRO anesthesia. These observations are in good agreement with our rat data.

The BOLD fluctuation powers were widely suppressed in the URE group. The data suggest that the fluctuations occur mainly at <0.03 Hz and at 0.10 Hz in both the cortex and thalamus. These observations show significant anesthesia-induced suppression of spontaneous activity, but also thalamo-cortical synchrony.

Taken together, our results indicate that FC is only mildly modulated by URE anesthesia; cortical connectivity was good and more specific compared with the several other anesthetics. Additionally, thalamocortical connectivity was better
preserved than with several other anesthetics. The urethane-induced sleep-like activity (Zhurakovskaya et al., 2016), however, may induce additional variations in the FC data.

Exploiting and interpreting functional connectivity of awake and anesthetized rats

The brain is hypothesized to have different processing layers, which could be divided into self-referential impulsive mental activities and core features of intrinsic baseline activity (Fransson, 2006). As the unconscious brain lacks the self-referential impulsive mental activities, this indicates a fundamental difference between the awake and anesthetized brain. It could also be argued that the anesthetized brain better represents the baseline activity as functions, such as cognitive processing, pain perception, and movement, are suppressed (Nallasamy and Tsao, 2011). The results of the present study, especially from spectral analyses, support these arguments: the BOLD fluctuations under anesthesia occurred at narrow frequency ranges, possibly reflecting more homogenous baseline activity.

The FC of the anesthetized brain, however, is not similar across different anesthesia protocols. It is well known that anesthetics have unfavorable effects on neural activity and neurovascular coupling mechanisms (Masamoto and Kanno, 2012), which impede the interpretation of rsfMRI FC data. Although the present work cannot disentangle the contribution of each of these factors to FC, our results reveal crucial differences in the net effects across anesthesia groups.

As one would expect, none of the FC patterns obtained under anesthesia were the same as than in the awake group. In fact, some of the anesthesia protocols produced clearly distinct connectivity patterns compared with the awake state. Without prior knowledge, the use of such protocols can significantly hinder the detection of, or even mask, the events under investigation. In contrast, with prior knowledge one could try to avoid such pitfalls, or even exploit the characteristic features of the anesthesia protocols. For example, a pathophysiologic change in a specific neuronal pathway could be studied under such anesthetic conditions in which BOLD fluctuations are known to be enhanced by the anesthetic.

Several preclinical approaches, however, are interested in the wholebrain connectivity. Most of the anesthesia protocols in the present study preserved good cortical connectivity. Therefore, an anesthesia protocol preserving at least some degree of thalamo-cortical connectivity can be recommended. Thalamo-cortical connectivity is a timely topic in functional neuroimaging, as it plays a key role in several hypothesized disease mechanisms, such as epilepsy (Gotman, 2008).

While dynamic changes in FC were not covered in the present work, it is important to note that the dynamic properties between anesthetized and awake brains are likely different. As the awake brain is free to spontaneously initiate, maintain, and end activities (Fransson, 2006), the dynamic profile is expected to be more versatile. The FC under anesthesia, however, is not stable either, as changes in connectivity have been detected at various time scales ranging from seconds (Majeed et al., 2009) to minutes (Wilson et al., 2011; Zhurakovskaya et al., 2016) to hours (Magnuson et al., 2014; Paasonen et al., 2017; Pawela et al., 2009). It is especially important to take into account the long-term shifts in FC, which are most likely due to the varying level of anesthesia, in study designs including long-term measurements. Nevertheless, the dynamic alterations in sensory and cognitive functions occurring in awake subjects during imaging can induce more complex and uncontrollable variations, which can be relatively easily suppressed with a mild anesthesia protocol.

Default mode network
The DMN is perhaps the most widely studied functional network of the brain. Network structures resembling the human DMN are observed across species and under different levels of consciousness, suggesting that the DMN has a very fundamental role in mammalian brain baseline function (Raichle, 2015). One of the commonly reported features of the DMN is that it is “deactivated” during a task. According to Raichle (2015), however, the DMN may be more modulated than shut down during such events.

In the present work, we investigated the CCs between key regions of the rat DMN (Lu et al., 2012) under awake and anesthetized conditions. Our results support the concept that the DMN is at least partially preserved across different anesthesia protocols, but there is also a significant anesthesia-induced modulation of CCs in DMN. Importantly, the wide synchronization of the neocortex under ISO anesthesia induces abnormally high CCs across the DMN. Therefore, if such highly correlating network activity is detected under any type of anesthesia, the conclusions related to the DMN require caution.

Anesthesia groups other than ISO appeared to have lower CCs compared with the awake group, particularly in the prefrontal parts of the DMN; these regions are associated with such processes as social behavior, mood control, motivational drive, and sensory processing (Raichle, 2015). Therefore, decreased connectivity in the prefrontal regions of the DMN might be directly related to the loss of consciousness. In humans, sedation decreases DMN strength in the posterior cingulate cortex (Greicius et al., 2008), which is involved, e.g., in awareness.

In contrast to the frontal parts of the DMN, the CCs in the posterior parts of the DMN appeared similar or even more active in the anesthetized rats than in the awake group. Apparent increases were observed especially in hippocampal connectivity. Interestingly, the posterior parts of the DMN are associated with recollection functions in the memory network, which are strongly active in the evening, and possibly during the early stages of sleep (Raichle, 2015; Shannon et al., 2013). As non-rapid-eye-movement sleep and anesthesia share many similarities in neurophysiology and brain activity (Franks, 2008), our observations suggest similarities in DMN modulation.

It is important to note that the sensory processing and attention of awake animals likely led to higher variability in the DMN state compared with the anesthetized rats in the present study. The precise effect of these state changes on CCs, however, are unclear.

Methodologic considerations

The majority of our findings were in excellent agreement with those of previous studies, and some factors may explain the differences. First, fMRI contrasts across studies differ: rsfMRI measurements in the present study were obtained with spin-echo BOLD sequences, while most of the previous studies used gradient-echo BOLD. Compared with gradient echo, spin-echo has higher capillary-level specificity in high magnetic fields (Lee et al., 1999), suffers less from susceptibility-induced artifacts, and is less sensitive to physiologic noise (Khatamian et al., 2016). The sensitivity in gradient-echo sequences is better, however, which can be beneficial for the detection of weaker hemodynamic signals.

Second, animal preparations vary considerably across studies: the differences in factors, such as rat strain, anesthetic dose, administration route, use of ventilation, or preceding anesthesia, may induce different outcomes in FC. The time window for FC measurements, which is critical (Paasonen et al., 2017; Pawela et al., 2009), also varies from a half an hour to a few hours after inducing anesthesia. As the effects of anesthetics on FC appear to be dose- and time-dependent, the importance of these factors cannot be understated. Nevertheless, the findings in the present study share a
surprising number of similarities with previous findings, indicating moderate to
good reproducibility of the results between
different research settings.

Previous studies report only a minor
influence of physiologic noise on FC in rats (Kalthoff et al., 2011; Majeed et al., 2009),
and motion, breathing rate, and heart rate
did not likely interfere with our results for
the following reasons. First, anesthetized
animals were paralyzed to prevent motion,
and only motion-free data were used from
awake animals. Second, the post mortem
data obtained with ventilation showed no
clear breathing motion-induced effects on
FC. Third, the data suggest no clear
association between heart rate and FC. As
an example, the ISO + MED and MED
groups had similar heart rates but distinct
FC patterns and BOLD spectral powers.
Nevertheless, the interference of physiologic
noise, such as vague aliased pulsations
originating from heartbeat, cannot be
completely ruled out.

The lingering effect of ISO (Magnuson et
al., 2014) used during surgeries is expected
to be minimal, as sufficient time was
allowed between preparations and fMRI
measurements. The surgeries lasted 37±2
min and the time between the cessation of
initial ISO anesthesia and initiating rsfMRI
scan was 38±2 min.

Conclusions

The present study revealed that the FC
properties were uniquely modulated by the
different anesthesia protocols, and each of
the observed patterns was distinct from that
in the awake group. Connectivity
parameters obtained under PRO and URE
anesthesia exhibited the fewest differences
compared with the awake rats. FC patterns
in the AC and ISO + MED groups exhibited
moderate to good correspondence with the
awake group, while FC in the ISO and MED
groups exhibited the most differences
compared with the awake rats. These
results, combined with other prior
information related to, e.g., the suitability of

anesthetics for follow-up or pharmacologic
studies, can be directly exploited for rsfMRI
study design and data interpretation. Further studies are required, however, to
characterize more detailed dose- and time-
dependent effects of anesthetics on FC, to
further optimize this widely exploited
preclinical rsfMRI technique.

Conflicts of interest

Authors have no relations that could
lead to conflict of interest.

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**APPENDIX A. SUPPLEMENTARY DATA**

Supplementary data related to this article can be found at https://doi.org/10.1016/j.neuroimage.2018.01.014.

**REFERENCES**


Multi-band SWIFT Enables Quiet and Artefact-Free EEG-fMRI and Awake fMRI Studies in Rat


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Multi-band SWIFT enables quiet and artefact-free EEG-fMRI and awake fMRI studies in rat

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ABSTRACT

Functional magnetic resonance imaging (fMRI) studies in animal models provide invaluable information regarding normal and abnormal brain function, especially when combined with complementary stimulation and recording techniques. The echo planar imaging (EPI) pulse sequence is the most common choice for fMRI investigations, but it has several shortcomings. EPI is one of the loudest sequences and very prone to movement and susceptibility-induced artefacts, making it suboptimal for awake imaging. Additionally, the fast gradient switching of EPI induces disrupting currents in simultaneous electrophysiological recordings. Therefore, we investigated whether the unique features of Multi-Band SWEEP Imaging with Fourier Transformation (MB-SWIFT) overcome these issues at a high 9.4 T magnetic field, making it a potential alternative to EPI. MB-SWIFT had 32dB and 20-dB lower peak and average sound pressure levels, respectively, than EPI with typical fMRI parameters. Body movements had little to no effect on MB-SWIFT images or
functional connectivity analyses, whereas they severely affected EPI data. The minimal gradient steps of MB-SWIFT induced significantly lower currents in simultaneous electrophysiological recordings than EPI, and there were no electrode-induced distortions in MBSWIFT images. An independent component analysis of the awake rat functional connectivity data obtained with MB-SWIFT resulted in near whole-brain level functional parcellation, and simultaneous electrophysiological and fMRI measurements in isoflurane-anesthetized rats indicated that MB-SWIFT signal is tightly linked to neuronal resting-state activity. Therefore, we conclude that the MB-SWIFT sequence is a robust preclinical brain mapping tool that can overcome many of the drawbacks of conventional EPI fMRI at high magnetic fields.
INTRODUCTION

Functional MRI (fMRI) studies in animal models provide invaluable information regarding normal and abnormal brain function. When fMRI is combined with optical and/or electrical recording and stimulation techniques, multiple spatial and temporal scales of brain network activity can be assessed and manipulated. This allows for investigations of complex central nervous system functions and disease mechanisms, and can facilitate the search for biomarkers of disease progression and treatment response.

Combining complex experimental setups with fMRI, however, is not straightforward. Surgical manipulations of the skull and implantation of measuring or stimulation instruments can induce strong magnetic susceptibility differences in the brain, leading to severe \( B_0 \) field distortions that are difficult or impossible to compensate for or correct (In et al., 2017). As rodent fMRI studies are typically performed with the echo planar imaging (EPI) pulse sequence at high magnetic fields, inhomogeneities of the \( B_0 \) field significantly affect the image quality, and are commonly observed as geometric deformations and signal dropouts. Additionally, the rapidly switching strong gradients in the EPI sequence create strong acoustic noise and can cause severe artefacts in complementary recording techniques such as electroencephalography (EEG).

Another major issue in animal fMRI studies is anesthesia, which is used to minimize the stress and movement of the animals (Lukasik and Gillies, 2003). Anesthesia heavily modulates brain function and subsequently the outcome of both stimulation (Huttunen et al., 2008; Paasonen et al., 2016, 2017; Sommers et al., 2009) and resting-state fMRI experiments (Grandjean et al., 2014; Jonckers et al., 2014; Paasonen et al., 2018), making it perhaps the biggest confounding factor in animal fMRI experiments.

To overcome the effects of anesthesia, awake rodent imaging protocols have been introduced (King et al., 2005; Lahti et al., 1998; Stenroos et al., 2018). Despite careful habituation protocols and tight head fixation, however, completely motion-free data is rarely acquired. Part of the issue may be the EPI sequence, which is one of the loudest MRI sequences and may cause additional discomfort for the rats. The subsequent spontaneous body movement induces changes in \( B_0 \), which extend into the brain and induce deformations in the \( B_0 \)-sensitive EPI images.

Recently, we introduced Multi-band SWEEP Imaging with Fourier Transformation (MB-SWIFT) fMRI in the context of deep brain stimulation of the rat.
and acquired fMRI responses comparable to those obtained with spin-echo (SE) EPI with minimal susceptibility artefacts from a tungsten wire electrode implanted into the ventral posteromedial nucleus of the thalamus (Lehto et al., 2017). MB-SWIFT is a three-dimensional radial MRI pulse sequence with large excitation and acquisition bandwidths, practically zero echo time, and minimal gradient switching steps during data acquisition (Idiyatullin et al., 2006; Idiyatullin et al., 2015). It is worth mentioning that the multi-band in the context of MB-SWIFT does not refer to the excitation of multiple slices (Moeller et al., 2010), but to multiple side bands of the excitation profile in readout dimension (Idiyatullin et al., 2015).

Interestingly, the functional signal-to-noise ratio appears to be higher in MB-SWIFT than in SE-EPI (Lehto et al., 2017). As MB-SWIFT is not sensitive to susceptibility-induced T2(*) effects, the fMRI contrast does not rely on the traditional blood oxygenation level-dependent (BOLD) effect (Ogawa et al., 1990), and likely originates from the in-flow effect of the cerebral blood (Lehto et al., 2017). Because of the sensitivity to blood in-flow, the method is also sensitive to MRI coil geometry. Additionally, the near zero echo time leads to sensitivity to most materials with hydrogens in the range of the radio frequency coils.

As a continuation of our previous study, the aim of this work was to assess whether the unique features of MB-SWIFT can be exploited in rat fMRI experiments to overcome many of the limitations associated with traditional EPI fMRI studies. The small gradient steps of MB-SWIFT should decrease the acoustic scanner noise and minimize the artefacts in simultaneous electrophysiological recordings, while the large bandwidth and near-zero echo time should make the images insensitive to B0 distortions, originating from, e.g., movement, EEG electrodes, and air cavities. We further investigated whether the functional connectivity (FC) maps obtained from awake and isoflurane-anesthetized rats were comparable to those obtained in previous EPI studies, and how well MB-SWIFT signal correlates with neuronal activity by conducting simultaneous EEG-fMRI recordings.

**MATERIALS AND METHODS**

Animal procedures were approved by the Animal Experiment Board in Finland, and conducted in accordance with the European Commission Directive 2010/63/EU guidelines. In total 13 adult (418±23 g) male Wistar rats (RccHan®:WIST; Envigo RMS B.V., Horst, Netherlands) were used in the experiments. The animals were individually-housed and maintained on a 12/12 h light-dark cycle at 22±2°C with 50%-60% humidity. Food and water were available ad libitum.

### 1.1. MAGNETIC RESONANCE IMAGING

All MRI data were acquired in a high-field 9.4 T/31 cm bore magnet interfaced with an Agilent DirectDRIVE console (Palo Alto, CA, USA) using a custom-made (either in-house made or by Neos Biotec, Pamplona, Spain) surface transmit-receive radio frequency coil with a 22-mm inner diameter (ID). The materials of the custom-made coils (e.g., polytetrafluoroethylene) were selected so as not to be visible in the MB-SWIFT images. Animal holder parts were made of polyoxymethylene, which is slightly visible in the MB-SWIFT images.

All imaging except during the controlled body movement experiment was performed with a 12-cm ID gradient coil set. To allow more room for movement, the controlled body movement experiments were conducted in a larger 21-cm ID gradient coil set with slightly different MRI sequence parameters (values in parentheses below).

Anatomical images were acquired using a fast spin echo (SE) multislice (FSEMS) sequence with the following parameters: TR 3000 (3200) ms, echo spacing 12 ms, effective
TE 48 ms, number of echoes 8, averages 4, bandwidth 62.5 kHz, matrix size 256x256 cm², field of view (FOV) 3.5 x 3.5 (4.0 x 4.0) cm², and 20-30 slices with a 0.5-1.0 mm thickness.

A multi gradient echo sequence was used to obtain B₀ field maps. The parameters were as follows: TR 1000 ms, TE 3.3 ms, echo spacing 2.1 ms, echoes 5, flip angle 60, bandwidth 100 kHz, FOV 4.0x4.0 cm², and 5 slices with 1.0-mm thickness.

MB-SWIFT fMRI data were acquired with the following parameters: TR 0.97 ms, 2000 spokes per volume resulting in a temporal resolution of ~2 s, excitation/acquisition bandwidths 192/384 kHz, matrix size 64³, FOV 3.5 x 3.5x6.4 (4.0x4.0x6.4) cm³, and flip angle 6. SE-EPI data were acquired with the following parameters: TR 2 4 s, TE 35 ms, bandwidth 208 kHz, matrix size 64x64 (64x32), FOV 3.5x3.5 (4.0x4.0) cm², and 8 15 slices with 1.5-mm thickness.

The lengths of the fMRI scans were as follows: 750 vol (25 min) for the awake resting-state fMRI measurements, 165 vol (5.5 min) for the controlled body movement experiments, and 300 vol (10 min) for the simultaneous electrophysiological and fMRI resting-state measurements of anesthetized rats.

During MRI, a warm water circulation system (Corio CD, Julabo, Seelbach, Germany) was used to keep the animals warm (~37 C). Small animal physiology monitoring equipment (Model 1025, Small Animal Instruments Inc., New York, NY, USA) was used to follow the respiration rate. Temperature of the anesthetized rats was monitored with a rectal probe. Additionally, an MRI-compatible video camera (12M-i, MRC Systems GmbH, Heidelberg, Germany) was attached to the animal cradle to track the movement of the rats during the awake and controlled body movement experiments.

1.2. SOUND PRESSURE LEVEL MEASUREMENTS

Acoustic scanner noise levels inside the magnet bore were measured using an omnidirectional condenser microphone (MT830R, AudioTechnica Limited, Leeds, UK), attached to an audio interface (Scarlett 2i2, Focusrite Audio Engineering, High Wycombe, UK), and recorded with a PC and Audacity software (version 2.3.0, https://www.audacityteam.org/).

The system was calibrated by playing recorded MRI sounds through a loudspeaker (JBL LSR308, Harman International Industries, Stamford, CT, USA). Peak pressures ranging from 59 to 119 dB were recorded simultaneously with a sound pressure level (SPL) meter (Type 2232, Brüel & Kjær, Nærum, Denmark) and the MTR830R microphone. The SPL meter used A-weighting. The calculated SPL calibration curve for the microphone was highly linear (R²< 0.998).

In the MRI SPL measurements, the microphone was attached at a location near the rat head during in vivo measurements. The experiment was repeated 5-10 times with slightly different microphone positions (e.g., left side, right side, top, behind, front, etc.) for each pulse sequence - SE-EPI with 8 and 15 slices, and MB-SWIFT. Additionally, the background noise levels were measured.

1.3. CONTROLLED MOVEMENT DURING EPI AND MB-SWIFT

To determine the effect of body movement on fMRI images, it is necessary to prevent head-movement. Surgery was performed in one rat to attach a custom-made chronic implant directly on the skull to secure head fixation.

For surgery, the rat was anesthetized with isoflurane (Attane vet 1000 mg/g, Piramal Healthcare UK Limited, Northumberland, UK; 5% induction and 2% maintenance) in a mixture of N₂/O₂ 70%/30%, and placed into a stereotaxic frame (David Kopf Instruments, Tujunga,
The scalp was removed from top of the skull, and the skull was cleaned with sterile saline and hydrogen peroxide (3%). Small holes penetrating the dense compact bone layer were drilled throughout the skull to facilitate bone cement adhesion (Palacos R + G, Heraeus Medical, Hanau, Germany). After the bone cement dried (~10 min), a layer of dental cement (Selectraplus, DeguDent GmbH, Hanau, Germany) was applied. Two plastic pins (2 mm diameter, separated by 12 mm) aligned in left-right direction and covered with heat-shrinkable tubes were fixed on top of the dental cement layer, and final layers of dental cement were molded around the pins. After the cement dried, the pins were removed, leaving two holes in the implant for head-fixation. Buprenorphine (0.03 mg/kg s.c.; Temgesic, Indivior Europe Ltd, Dublin, Ireland) was given for postoperative pain and saline (10 ml/kg/day s.c.) was given to prevent dehydration. Injections were continued for 2 days twice daily, and the recovery and welfare of the rat were monitored closely.

After a recovery period of 1 week, the rat was anesthetized for an fMRI experiment with isoflurane (5% induction and 1.3-2.0% maintenance) to maintain the respiration rate at approximately 60 breaths per minute. A commercially available harness for rodents (TRIXIE Heimtierbedarf GmbH & Co. KG, Tarp, Germany) was placed on the anesthetized rat, and strings were attached to the posterior parts of the harness. In combination with a custom-made MRI holder including a head-fixation system compatible with an open loop radio frequency coil, the strings allowed us to remotely lift the body of the rat inside the bore. A breathing sensor was pushed inside the harness to monitor the depth of anesthesia. The head was fixed using an in-house made head-fixation system, including two pins going through the implant. Visual inspection was performed to ensure that the body movement induced by pulling the strings did not move the head and did not alter the tuning and matching of the radio frequency coil.

After the animal preparation and initial MRI adjustment steps, the controlled body movement fMRI experiments were performed. A block model paradigm consisting 30 vol (60 s) of baseline and 15 vol (30 s) of controlled movement was used. The paradigm was repeated three times within one scan, resulting in a total of 165 vol (5.5 min). The scan was repeated three times with both MB-SWIFT and SE-EPI fMRI sequences. The B0 maps were acquired from one rat in different body positions. After finishing the experiments, the rat was returned to its cage.

The whole fMRI experiment was repeated with the same rat after a 1 week rest period, resulting in a total of 12 fMRI scans (6 with each sequence).

1.4. AWAKE FUNCTIONAL MRI WITH MB-SWIFT

The awake protocol is described in our previous report (Stenroos et al., 2018). Briefly, nine rats went through a 4-day habituation protocol before the awake fMRI. Each day, the rat was first anesthetized with isoflurane (5% induction and 2% maintenance in the same N2/O2 70%/30% mixture). The paws were secured with masking tape, and the body was gently wrapped with a plastic foam sheet. Silicone plugs were inserted into the ear cavities for hearing protection. The rat was transferred to an MRI holder and the head was fixed with a custom-made padded restraint kit. The holder was pushed into a mock scanner, isoflurane was set to zero, and the sounds of the fMRI protocol were played through a loudspeaker. The audio recording/playback system was the same as described in section 2.1.

The length of the habituation session was increased in 10-min increments, starting from 15 min on the first day, up to 45 min on day 4. The length of the resting-state fMRI measurement on the day 5 was 25 min. The imaging was started when the
breathing rate reached ~110 breaths per minute, indicating a conscious condition. After habituation or imaging, isoflurane was increased gradually to 2%-3%, and the rat was released from restraint back to its cage. Chocolate cereal was given before and after the training as a reward.

1.5. SIMULTANEOUS EEG AND FMRI MEASUREMENTS

To investigate the image distortions and the magnitude of gradient switching artefacts of MB-SWIFT and SE-EPI during simultaneous intracranial EEG and fMRI measurements, three rats underwent a surgery for acute electrode implantation prior to MRI. Additionally, the correlation between EEG and MB-SWIFT fMRI signals under isoflurane anesthesia was studied.

EEG was recorded with epidural electrodes made of polytetrafluoroethylene insulated silver wire (0.2 mm bare wire diameter, nominal outer diameter of coated wire 0.27 mm; AG549511, Advent Research Materials, Oxford, UK). The electrode tip was exposed for a 2-mm length and bent into an L-shape. The ground electrode comprised 20 mm of bare wire that was placed under the skin in the neck.

The rat was initially anesthetized with isoflurane as described in section 2.3. The skull was exposed and carefully cleaned and dried. The silver wire electrodes were implanted bilaterally on the dura over the somatosensory (S1) cortex (AP 2.12; ML 2.5) through small craniotomies. Similarly, the reference electrode was placed on the dura over the cerebellum. The intracranial electrodes were attached to the skull with cyanoacrylate glue.

After finishing the electrode implantation, the functionality of the electrodes was tested, and the rat was immediately transferred to an MRI holder for imaging. EEG was measured and recorded with an MRI compatible BrainAmp MR system (5 kHz sampling rate; Brain Products GmbH, Gilching, Germany) equipped with a preamplifier (10x amplification; Multi Channel Systems, Reutlingen, Germany). The isoflurane concentration (1.3%-2.0%) during simultaneous fMRI and EEG data acquisition was adjusted to induce a clear burst-suppression state (Liu et al., 2011) monitored by EEG. After finishing the measurements, the rats were anesthetized with 5% isoflurane and then killed by cervical dislocation.

1.6. DATA PROCESSING AND ANALYSIS

The audio data were analyzed using Audacity and MATLAB (R2011a and R2017b; Mathworks Inc., Natick, MA, USA). As many hearing related parameters, such as loudness, have a logarithmic nature, linear averages for peak and mean dB levels across the measurements were calculated.

The MB-SWIFT data were first reconstructed with SWIFT package 2018 (https://www.cmrr.umn.edu/swift/index.php) using correlation, gridding, and three iterations of the FISTA algorithm (Beck and Teboulle, 2009). The reconstruction time for one 3D volume was roughly 0.5-1.0 s while using a workstation (Celsius R970, Fujitsu, Tokyo, Japan). From each MB-SWIFT fMRI data-set, the first three volumes were discarded as the signal was reaching a steady-state. Subsequently, all MRI data were written to NIFTI using Aedes (http://aedes.uef.fi).

A single loop receiver coil was used for all measurements, and thus the fMRI images had a strong B1 intensity gradient. This hampered the motion correction of MB-SWIFT awake rat fMRI data. Therefore, the intensity bias was removed from the images using an N4ITK bias correction (Tustison et al., 2010) with 4th order splines for the local reference image and 2nd order splines for the separate fMRI volumes. The bias-corrected volumes were motion corrected using Advanced Normalization Tools (ANTs, http://stnava.github.io/ANTs/) (Avants et al., 2011). The acquired motion correction transformations were then applied to the original MB-SWIFT fMRI
data sets. Three nuisance regressors for both translation and rotation were also computed from the motion correction transformation matrices.

For group-level analyses, the fMRI data were co-registered. Because MB-SWIFT fMRI images have low anatomical contrast, we used an indirect approach. The anatomical FSEMS images of each subject were first co-registered to a reference FSEMS volume with ANT's using affine and non-linear SyN registrations. Next, the acquired transformations were applied to the MB-SWIFT fMRI data sets. Finally, data were spatially smoothed (0.5 mm full-width at half-maximum Gaussian kernel) using SPM8 (www.fil.ion.ucl.ac.uk/spm).

Prior to the analysis of the awake rat MB-SWIFT fMRI data, volumes including movement were discarded. Exclusion was based on combined visual inspection of the image mass center (>0.1 voxels) and visible image displacement throughout the time series in raw data. Data periods containing less than continuous 45 motion-free volumes (1.5 min) were also excluded. The amount of movement events was calculated as described earlier (Stenroos et al., 2018).

The effect of controlled body movement on fMRI voxels in the brain was analyzed with a block-design general linear model available in Aedes. Data-driven independent component analysis (ICA; FSL MELODIC, https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/MELODIC) was used for the investigation of FC. Brain masks were drawn manually and used unless stated otherwise. Components that were located in big vessels, located only at surfaces, not bilateral, or anatomically poorly localized were excluded from the results to minimize the inclusion of non-neural components. The number of components for awake rat data (30) was chosen based on previous awake rat studies, in which 20-40 components were used, e.g., (Becerra et al., 2011; Liang et al., 2011). For isoflurane-anesthetized rats, fewer components (20) were selected because the isoflurane-induced cortical burst-suppression activity, which we aimed to detect, occurs throughout the cortex (Liu et al., 2011) and is easily detectable with low number of components.

For awake rat data, partial correlation coefficients were calculated between regions of interest defined by the obtained ICs (thresholded by MEODIC). The original MB-SWIFT signal was band-pass filtered at 0.01-0.15 Hz. Motion correction parameters and the brain volume global signal were used as nuisance regressors to minimize the residual effect of movement. For each subject, motion-free time series were divided into continuous 100 vol series, from which correlation values were averaged, and left-over data that did not fit the 100-vol limit were discarded. Fisher transformation to Z-scores was applied prior to averaging of correlation coefficients.

EEG data were inspected and analyzed using MATLAB and Spike2 (CED Ltd, Cambridge, England). Data were denoised from gradient switching artefacts using a wavelet-ICA method (Sheoran et al. 2014). Briefly, a discrete stationary wavelet decomposition was applied (MATLAB swt-function, mother wavelet sym8 at decomposition level 15) and then a fast ICA algorithm (https://research.ics.aalto.fi/ica/fastica/) was used to obtain ICs from the resulting wavelets. The components containing artefacts were manually selected and removed. Finally, an inverse discrete stationary wavelet transform was applied to recover the artefact-free data.

Subsequently, the denoised EEG data were band pass-filtered (0.05-15 Hz). The envelope of the signal was calculated using the absolute value of a Hilbert transformation. The data were then convolved with the hemodynamic response function (HRF) for anesthetized rats (Silva et al., 2007), and down-sampled to the MRI temporal resolution (0.5 Hz).

To minimize the effect of non-neuronal fluctuations in the EEG-fMRI correlation
analysis, ICA was used to extract the fMRI time series for the isoflurane-induced cortical activity (Liu et al., 2011), which was subsequently compared with the cortical HRF-convolved EEG envelopes. Both MB-SWIFT and EEG data were detrended and median normalized, and the signals were aligned by maximizing cross-correlation. In sliding window analysis, to investigate the temporal stability of the EEG-fMRI correlation, correlations were calculated with 50 vol windows and 10 vol steps.

The threshold for statistical significance was set to p < 0.05. Statistical tests were performed with either analyzing software described above or MATLAB. All group-level values in the text and figures are represented as mean standard deviation. Data and codes are available upon request.

RESULTS

1.7. ACOUSTIC MEASUREMENTS

The results of the acoustic scanner noise measurements are summarized in Fig. 1. One of the most important observations was that the peak SPL (84.9±1.7 dB) was dramatically lower (p < 10⁹, two-tailed Student’s t-test) when measured during MB-SWIFT than when measured during the 8- and 15-slice EPI sequences (116.9±0.6 dB and 116.8±0.7 dB, respectively). The average SPL of MB-SWIFT (63.6±1.7 dB) was also clearly lower than that of both the 8-slice (72.0±5.4 dB, p < 0.005) or 15-slice (83.3±4.3 dB, p < 10⁹) EPI sequences (two-tailed Student’s t-test). There was also a significant increase (11.3 dB) in the average SPL during SE-EPI when the slice number was increased from 8 to 15 (p < 0.001, two-tailed Student’s t-test).

If considering a 6-dB increase to double the sound pressure and a 10-dB increase to double the psychoacoustic loudness (Schomer, 1978), the 32.0-dB difference in peak SPLs observed between the MB-SWIFT and EPI indicates a 39.8 times higher peak sound pressure and a 9.2 times higher peak loudness during EPI compared with MB-SWIFT. Regarding the average SPLs, the 8-slice EPI (8.4 dB higher compared with MB-SWIFT) and 15-slice EPI (19.7 dB higher compared with MB-SWIFT) sequences had 2.6 and 9.7 times higher average SPLs, and were 1.8 and 3.9 times louder than MB-SWIFT, respectively.

The representative spectrograms in Fig. 1B suggest that the MBSWIFT-induced sound mainly consists of frequencies up to 15 kHz, while intense frequencies throughout the measurement range (up to 22 kHz) are present during the SE-EPI sequence. This is important as rats have the highest auditory sensitivity around 12-24 kHz (Borg, 1982), which clearly differs from the 2-5 kHz range in humans.

Representative audio samples are available in the supplementary material. The recording gain was same across the samples, allowing for direct comparison of the audible loudness across the files.

1.8. THE EFFECT OF BODY MOVEMENT ON FMRI

The effect of controlled body movements on SE-EPI and MB-SWIFT fMRI data is summarized in Fig. 2, and a representative dataset showing the body movement-induced B₀ frequency shifts is shown in Supplementary Fig. 1. Motion correction was not used at this stage, as the skull was tightly fixed and did not move physically during the experiments. During SE-EPI, body movement had a significant and widespread influence on voxels throughout the FOV (Fig. 2A). The statistical maps suggest a ventral shift and/or squeeze of roughly two voxels in each experiment, as the red-yellow clusters (Fig. 2A) form the shape of the head below the position of the head in the baseline image. Analysis of images across time confirmed that the brain is apparently moving down (~2.3 mm) in the phase encoding (vertical) direction. In contrast, a minimal number of voxels was affected during the MB-SWIFT sequence, which most likely originates from the insensitivity of MB-SWIFT to the B₀ shifts.
The few apparently activated voxels in MB-SWIFT images were mainly on the skin outside the brain. Prior to the experiments, it was observed that the skin around the head was stretching because of the body movement, which is consistent with the ostensibly activated voxels with MB-SWIFT. The average time-series obtained from the cortex (Fig. 2B) confirms that the body movement did not affect the MB-SWIFT
Fig. 1. Peak and average sound pressure levels (A), and representative sound spectrograms (B) measured inside the 9.4T MRI bore during MB-SWIFT and SE-EPI. Measurements were repeated 5-10 times per condition. Values in panel A are mean±SD. The SE-EPI sequence in panel B includes 15 slices within a repetition time of 2 s. The statistics shown in panel A were calculated using a two-tailed Student's t-test.
Fig. 2. Effect of controlled body movement on fMRI images (A) and signal intensity (B). Motion correction was not used at this stage. On the left of the panel A, unprocessed single slice fMRI images acquired with SE-EPI or MB-SWIFT sequences are shown. Statistical activation maps (block-model design general linear model, p < 0.001, false discovery rate corrected) obtained from each individual experiment (#1-#6) are shown next to the raw images. Representative group-averaged fMRI time series obtained from the cortex are shown in panel B. Blue and red arrows in raw images in panel A indicate the regions from which the time series were obtained. The gray bars in panel B indicate the time periods of controlled body movement. The values in panel B are mean±SD.
fMRI signal, while the signal variation was considerable during SE-EPI.

Because SE-EPI images were heavily affected by the body movement, the SE-EPI data were motion-corrected and compared to uncorrected SE-EPI data to investigate whether motion correction would improve the data quality. The motion correction parameters closely followed the changes observed in voxel-level time series, which confirms that apparent motion explains the majority of the signal changes. The group level time-series revealed that rigid ANTs motion correction fails to correct the movement-induced signal losses and blurring observed with the SE-EPI sequence (Supplementary Fig. 2). When affine ANTs were used and combined with the regression of the obtained 12 motion correction parameters, the group-level time-series improved considerably (Supplementary Fig. 2). The statistical tests, however, indicated that 2 of the 6 datasets were still affected by movement.

Due to the minimal influence of body movement on MB-SWIFT images, we tested whether the MB-SWIFT data quality in the body movement experiment was good enough to allow for resting-state analyses. The group-level ICA revealed a clear network (Fig. 3) commonly observed in isoflurane-anesthetized rats related to the cortical burst-suppression activity (Liu et al., 2011; Paasonen et al., 2018), suggesting that even a significant body movement during MB-SWIFT fMRI may not prevent the identification of functional networks. With SE-EPI data, controlled body movement accounted for the vast majority of the variance in ICA, and the corresponding component was not visible with either rigid or affine corrections including the regression of motion parameters (data not shown).

A video showing the actual body movement during the experiments is available in the supplementary material.

Supplementary video related to this article can be found at https://doi.org/10.1016/j.neuroimage.2019.116338.
The movement of animals during MB-SWIFT (0.48±0.23 events/min) was less frequent (p < 0.03, two-tailed Student’s t-test) than in our previous awake SE-EPI experiments (1.00±0.58 events/min) (Stenroos et al., 2018). Out of 750 measured volumes, 658±92 (87.7%) volumes per rat were included into the ICA analysis after excluding data periods affected by movement. Both parameters indicate that the measurements were successful.

1.9. AWAKE RAT FUNCTIONAL CONNECTIVITY MEASUREMENTS WITH MB-SWIFT

Fig. 4. Anatomically well-defined group-level independent components obtained with MB-SWIFT and independent component analysis from awake rats. The data-driven analysis was set to run with 30 components, of which 13 anatomically best localized bilateral components are shown. On top left, all 13 selected components are shown together, while the rest of the figure represents each component individually. The components are numbered from anterior cortical components to posterior subcortical components. The statistical maps are overlaid on anatomical T2-weighted images. The orientation of images is same as in Fig. 3. IC, independent component.
The group-level awake rat ICA results summarized in Fig. 4 show that MB-SWIFT fMRI can be used to parcellate brain resting-state activity in an awake rat at an excellent spatial level. Multiple anatomically well localized bilateral components were identified, suggesting distinct functionally connected networks. The cortical connectivity was divided into several components, including the anterior frontal (#1), medial frontal (#2), orbital frontal (#3), motor (#4), insular (#5), somatosensory (#6), auditory (#7-8), visual (#9), and retrosplenial (#10) cortices. For both striatum and thalamus well-localized components were observed (#11 and #12, respectively). The hippocampal component, however, was somewhat vague and limited to the temporal (ventral) part. Nevertheless, the observed components covered most of the cortical regions, large parts of striatum and thalamus, and some parts of hippocampus, leading to almost whole brain level functional segregation.

The correlation matrices (Fig. 5) showed strong correlation (0.6-0.7) patterns between several cortical ICs, which formed clear clusters of high correlations. This suggests that these specific components inside the squares form subnetworks or modules, such as prefrontal, lateral frontal, and parietal cortical networks, where more intensive FC is expressed within the network. Additionally, the striatal component (#11) consistently correlated with several cortical components (#1-6), indicating robust subcortico-cortical activity. The standard deviations are shown in Supplementary Fig. 3.

1.10. SIMULTANEOUS EEG AND MB-SWIFT FMRI

From one dataset out of six (10 min each), the last 3 min of EEG data were discarded due to signal artefacts originating from external noise.

The investigation of MRI-induced EEG artefacts (Fig. 6A) indicates that the SE-EPI-induced artefacts (16±6.5 mV) are roughly an order of magnitude higher in amplitude than those induced by MB-SWIFT (1.7±0.4 mV; p < 0.02, Student's t-test, one representative artefact per rat). The periodic low amplitude artefacts observed during MB-SWIFT (Fig. 6B, top row) can be effectively removed with standard artefact removal approaches (Fig. 6B, bottom row).

Fig. 6C shows that compared with SE-EPI, MB-SWIFT is rather insensitive to image distortions induced by the surgical operations and EEG electrodes. Compared with the FSEMS image, the SE-EPI image shows vertical stretching of the cortex in regions close to the measuring electrodes (white arrows). Additionally, the lateral parts of the cortex in the same image show signal pile-up artefacts (red arrows), observed as cortical thinning. Additionally, the air cavities disturb the brain shape in the SE-EPI image in the lower brain regions (yellow arrows). No similar artefacts are observed in MB-SWIFT images. Thus, it appears that while using MB-SWIFT in simultaneous EEG measurements, the whole brain, including regions close to the measuring electrodes, can be analyzed.

To demonstrate this, Fig. 7A shows representative data where the correspondence between the HRF-convolved EEG envelope and MB-
Fig. 5. Group-level (A) and individual-level (B) correlation matrices obtained between independent components of awake rats. The numbering of independent components corresponds to that in Fig. 4.
Fig. 6. Examples of the fMRI gradient switching artefact amplitudes in EEG signals (A), raw and denoised EEG signals obtained during EEG/MBSWIFT fMRI (B), and EEG-electrode-induced image distortions (C) during simultaneous EEGfMRI in an isoflurane-anesthetized rat. Panel A shows that the gradient artefacts are roughly 10 times higher during SE-EPI compared with MBSWIFT (15 mV and 1.5 mV, respectively). The voltage scale is the same for all three EEG signals in section A, while a smaller voltage scale is used for the two signals in section B. The white arrows in panel C indicate the electrode-induced signal distortions in fast spin echo multi slice (FSEMS) and spin echo echo planar imaging (SE-EPI) images. Red arrows indicate signal pile-up artefacts in SE-EPI images. Yellow arrows show typical distortions in the lower brain regions around air cavities in SE-EPI fMRI images. Compared with FSEMS and SE-EPI images, no distortions were observed in MBSWIFT images.
Fig. 7. Representative simultaneous EEG-fMRI data obtained from one isoflurane-anesthetized rat (A), and temporal partial sliding window correlations between EEG and fMRI signals across all measurements (B). EEG was measured from the somatosensory cortex. The denoised EEG signal envelope was convolved with a hemodynamic response function (HRF) of a rat and compared with the cortical component of the MB-SWIFT signal obtained with independent component analysis (ICA; component corresponding to the one shown in Fig. 3). Sliding window analysis (50 vol windows with 10 vol steps) estimates the temporal stability of the correlation between EEG and MBSWIFT fMRI signals (B). Black line in B represents the average across measurements (gray lines). Three rats were imaged, each having two cortical electrodes. EEG, electroencephalography; HRF, hemodynamic response function; ICA, independent component analysis.
SWIFT fMRI signal during moderate isoflurane-induced burst-suppression activity is very clear. Cortical bursts were well aligned between signals during both fast (first 3 min) and slow (from 4 to 10 min) bursting patterns. The group-level average correlation between measured EEG signals (n < 6) and cortical hemodynamic MB-SWIFT components was 0.57±0.04 (in all cases p < 10^-6).

As the depth of anesthesia, and thus the burst-suppression pattern and incidence, varied slightly during the measurements and across animals, the temporal stability and condition-dependency of the EEG/MB-SWIFT fMRI correlations was investigated with a sliding window analysis. Despite the varying neuronal activity, a very stable correlation of around 0.6 was observed between neuronal activity and fMRI signal throughout the measurements and subjects (Fig. 7B), indicating the robustness of the method.

**DISCUSSION**

In the present study, MB-SWIFT was used for the first time in FC studies, in awake rat experiments, and in simultaneous electrophysiological and fMRI measurements. The results indicate that MB-SWIFT is a robust functional brain mapping tool that can overcome many of the limitations associated with conventional EPI fMRI at high magnetic fields.

1.11. **ACOUSTIC SCANNER NOISE DURING FMRI**

Acoustic scanner noise is a serious issue in fMRI, as it can induce hearing loss and stress (Lauer et al., 2012) and confound the fMRI results in both stimulus evoked and resting-state applications by direct and/or indirect mechanisms (Moelker and Pattynama, 2003). As the trend in fMRI has constantly been towards higher spatial and temporal resolution in higher magnetic fields, there is an urgent need for alternative options to loud EPI sequences, especially for awake imaging. To achieve close to isotropic resolution with a traditional 2D EPI sequence, the slice number has to be quite high. In the current study, an increase from 8 to 15 slices (TR < 2 s) in SE-EPI almost quadruples the average SPL (+11.3 dB). In many studies, same number of slices is squeezed within TR = 1 s, which drastically increases SPLs. Additionally, the Lorentz forces behind the acoustic noises increase logarithmically with the main magnetic field and the gradient currents (Mansfield et al., 1998), inducing more pronounced scanner noise in ultra-high fields with small voxels.

Although comparative animal studies are limited, recent human studies indicate that the use of more silent fMRI sequences, such as EPI with a sinusoidal readout gradient and interleaved silent steady state, significantly improves the data quality, e.g., in working memory tasks (Tomasi et al., 2005), auditory stimuli experiments (Schmitter et al., 2008), and FC measurements (Andoh et al., 2017). In these studies, the ‘silent’ sequences were 7.2-20 dB quieter than the standard fMRI sequence of the group. Here, MB-SWIFT was found to have a roughly 32-dB and 20-dB lower peak and average SPL, respectively, compared with our standard fMRI sequence. This is a major improvement, as for both rat and human, the loudness grows as described by Steven’s law (Pierrel-Sorrentino and Raslear, 1980), and thus lower SPL can be considered more comfortable for both species. Indeed, the degree of animal movement during the awake rat MB-SWIFT fMRI experiments was only half of that observed during SE-EPI fMRI in our previous study (Stenroos et al., 2018), in which a similar training protocol and lower 7 T field were used. The reduced movement supports the choice of more silent sequences in awake animal studies, as a significant reduction in movement directly enhances data quality. In addition, such data possibly reflect more stress-free and normal brain function without confounding acoustic stimulus-induced modulation. We have,
however, no direct biological measures to confirm decreased stress levels during MB-SWIFT in the current study.

Although discussion related to acoustic scanner noise is typically focused on SPLs, there are other important factors to consider. A recent study reported a clear difference in the resting-state network characteristics between continuous and periodical data acquisition approaches, where periodical scanning is considered to have more disturbing effects on FC (Langers and van Dijk, 2011). This might be especially important in certain EPI studies, where a long TR is used to allow for the acquisition of a clean electrophysiological signal between fMRI images. From this viewpoint, MB-SWIFT is a particularly advantageous sequence because it allows for continuous data acquisition and relatively stable SPL with no audible pauses.

Additionally, auditory sensitivity differs across species. Compared with humans, the auditory sensitivity of rats is shifted to higher frequencies, as the highest sensitivity begins around 8-12 kHz and extends up to 38 kHz (Borg, 1982; Kelly and Masterton, 1977). In mice the highest sensitivity starts around 15 kHz (Lauer et al., 2012). Interestingly, the MB-SWIFT-induced scanner noise appears to contribute only minimally to frequencies higher than 12 kHz, while during SE-EPI frequencies up to 22 kHz and likely beyond are present (Fig. 1B). Therefore, besides lower SPL induced by MB-SWIFT, the acoustic frequency spectrum, which avoids the highest auditory sensitivity of rat, may improve the quality of the data obtained in awake rats.

As the main auditory sensitivity of rats extends up to 38 kHz, the lack of ultrasound coverage is a shortcoming in our acoustic measurements. Frequencies higher than 22 kHz, however, are aliased into the measured signal and likely contribute to the total SPL, making comparison between the sequences reasonable.

1.12. DYNAMIC MOVEMENT-INDUCED LOCAL FIELD FLUCTUATIONS DURING FMRI

Subject movement during fMRI is a major concern, as up to 90% of the signal variance may be explained by motion (Friston et al., 1996). In general, three types of movement occur during MRI: periodic involuntary movements (e.g., respiration-induced chest and lung cavity movement), sudden involuntary movements (e.g., swallowing and eye blinking), and conscious body part movements (Godenschweger et al., 2016).

Even if the movement does not occur physically inside the FOV, as in our controlled body movement experiment, it induces changes in B0-field that can extend and induce local field offsets inside the FOV. For example, in EPI fMRI, the respiratory cycle explains a major part of the physiological noise in humans (Bianciardi et al., 2014), and is tightly coupled with the phase encoding voxel shifts in rats (Kalthoff et al., 2011). EPI is particularly sensitive to any local field offsets in the phase encoding direction due to its low bandwidth, and is thus prone to spatial voxel shifts, image distortions, and signal drops (Jezeard, 2012).

Several modern approaches have been developed to correct for the motion artefacts occurring during EPI fMRI, yet none is able to fully recover the data (Zaitsev et al., 2017). Additionally, the constant shift towards ultra-high fields complicates shimming, and is likely to emphasize the field inhomogeneity and motion artefact issues in EPI as they are field-dependent (Jezeard, 2012; Raj et al., 2000).

Although the SE-EPI images were affected by body movement in our study, they induced only minor to no effects on MB-SWIFT images. This is due to the high acquisition bandwidth of MB-SWIFT in all spatial encoding directions so that dynamic local field offsets are not high enough to deform the image or shift voxels. The temporary body relocation in our controlled movement experiments was estimated to exceed the scale of normal involuntary movements, such as breathing and muscle twitching, and to be in a similar range as conscious
movements. Hence, MB-SWIFT is rather insensitive to movement-induced artefacts originating from outside the FOV. Movement occurring inside the FOV is still problematic, although radial sampling offers better resilience to movement as the center of the k-space is typically oversampled compared with Cartesian sampling (Glover and Pauly, 1992).

1.13. BRAIN FUNCTIONAL CONNECTIVITY IN AWAKE RATS MEASURED WITH MB-SWIFT

There is a growing interest in awake rodent fMRI imaging (King et al., 2005; Lahti et al., 1998) to allow for behavioral study settings and thus increased translational value of the preclinical experiments. Early awake rat FC studies demonstrated anatomically well-localized signal fluctuations between several brain regions important for emotional and cognitive functions (Zhang et al., 2010). Later it was demonstrated that such fluctuations are confounded by anesthetics (Liang et al., 2015; Paasonen et al., 2018; Smith et al., 2017). Despite the clear advantages of awake rat imaging, subject motion and stress remain among the biggest hurdles (King et al., 2005).

Comparing the exact amount of movement artefacts during awake rat fMRI studies across laboratories is difficult, as there are no established convergent methods to analyze or report these artefacts. Nevertheless, compared with our previous SE-EPI studies (Stenroos et al., 2018), the number of movements during MB-SWIFT was significantly smaller, most likely because of a different type of acoustic scanner noise, as discussed earlier. This can be considered a significant methodological improvement.

The fMRI FC studies conducted so far in awake rats have exploited EPI, which raises concern over whether the FC data obtained with MBSWIFT and those obtained with the current gold standard are comparable. To allow for comparison as generalizable as possible, simple and straightforward data-driven analyses were used to estimate FC in the current study. In general, our FC results obtained with MB-SWIFT are in excellent agreement with those obtained in previous EPI studies (Becerra et al., 2011; Liang et al., 2011; Liang et al., 2015; Ma et al., 2018; Paasonen et al., 2018; Smith et al., 2017; Zhang et al., 2010).

Zhang et al. (2010) and Liang et al. (2015) reported a moderate correlation between prefrontal cortex and parietal cortex, and retrosplenial cortex and visual cortex, similar to our results. The ICs reported by Liang et al. (2011) appear to be very close to the components shown in Fig. 4, with small differences likely originating from our stricter component exclusion criteria. Becerra et al. (2011) showed cortical and thalamic ICs similar to our results. Moderate correlations between the striatum and prefrontal cortex, and between the insular and parietal cortices were reported by Smith et al. (2017), and match our results. The low-dimensionality functional atlas suggested by Ma et al. (2018) largely resembles our functional parcellation. Lastly, we previously reported moderate to good correlations across cortical regions, between the striatum and cortical regions, and between the prefrontal cortex and parietal cortex (Paasonen et al., 2018), which also support our current findings.

Although a default mode network is reported to exist in anesthetized rats (Lu et al., 2012), the awake rat studies to date have yielded no clear and convergent results. The spatial maps of putative default mode-like activity in early awake rat reports by (Upadhyay et al., 2011) and Zhang et al. (2010) overlap only partially with the maps obtained in anesthetized rats (Lu et al., 2012). Similarly, the current work did not detect ICs corresponding specifically to the default mode network reported by Lu et al. (2012). Instead, several ICs overlapping parts of the suggested rat default mode network were observed, including medial frontal, anterior cingulate, retrosplenial, and parietal cortices. The reason for the discrepancy between the above-mentioned observations can only be speculated, but as the animals are still in an unnatural environment, one explanation could be a de-activation of the default mode due to
sensory and cognitive processing. Therefore, more thorough investigations are required to properly define the default mode network in awake rats (Becerra et al., 2011).

Taken together, the FC data obtained with MB-SWIFT in awake rats are consistent with those obtained in previous EPI studies. Despite the relatively small number of subjects (n = 9), the data-driven ICA was able to achieve near whole-brain functional parcellation, which has not been routinely achieved with even larger group sizes (typical n = 15-42) in previous awake rat EPI studies.

1.14. SIMULTANEOUS EEG AND FMRI MEASUREMENTS WITH MB-SWIFT

Simultaneous EEG and fMRI measurements provide a combination of millisecond resolution and whole-brain coverage, respectively, but also combined information related to the electrical, vascular, and metabolic activity of the brain (Mirsattari et al., 2007; Pirttimaki et al., 2016). Despite the clear advantage of concurrent measurements, recordings are often performed separately due to technical challenges.

In the current study, we show that neither silver wire electrodes on the dura nor the surgical procedures influence MB-SWIFT images, whereas they induce clear distortions in SE-EPI images. Our previous study showed that a deep brain electrode made of tungsten wire induces only minimal image distortions in MB-SWIFT compared with SE-EPI (Lehto et al., 2017). It is worth mentioning that the image distortions are even more severe in commonly used gradient echo EPI. Distortion-free MB-SWIFT images not only allow for analysis of regions immediately next to the electrodes, but also regions next to air cavities, such as the amygdala. The insensitivity to susceptibility-induced deformations and signal voids originates from the large acquisition bandwidth and zero acquisition delay of MB-SWIFT.

The main MRI-induced artefact in EEG is the gradient switching-induced current, which can heavily mask the biological EEG signal (Mirsattari et al., 2007). While the electrophysiological signal typically varies in the scale of 0.5 mV, EPI-induced currents can range from 5 mV up to more than 20 mV (Pan et al., 2011; Sumiyoshi et al., 2011), which fits well with our current findings (Fig. 6A). Modern real-time and/or post-processing protocols are able to remove the EPI-induced artefacts almost completely (Mirsattari et al., 2005; Pan et al., 2011), but only if the amplifier is not saturated by the MRI artefact. Saturated signal cannot be recovered, and it can take hundreds of milliseconds for the signal to again reach a steady state. Hence, the significantly lower currents in EEG induced by MB-SWIFT provide a safer approach for simultaneous EEG-fMRI under suboptimal experimental conditions.

In the present work, there was a clear correspondence between electrical activity and the HRF-convolved MB-SWIFT time-course in the isoflurane-anesthetized rats. The results in Fig. 7 closely resemble the findings characterizing the coupling between EEG and cerebral blood flow under isoflurane-induced burst-suppression activity (Liu et al., 2011). Liu et al. (2011) reported correlation values ~0.6 between laser Doppler-measured blood flow and EEG, while another study reported slightly lower correlation values (~0.4) between BOLD and local field potentials (Pan et al., 2011). Thus, it appears that MB-SWIFT indirectly follows the neuronal activity at least as well as laser Doppler flowmetry and EPI fMRI in isoflurane-anesthetized rats.

Simultaneous EEG/SE-EPI data acquired in the current work was not analyzed, as the cortical regions were considered to be heavily distorted (Fig. 6C). The surgical procedures were slightly different from those in our previous report, in which chronic electrodes were implanted for measurements at 7 T (Pirttimaki et al., 2016). Thus, the EPI distortions in the current study likely originate from the higher magnetic field, and from the open interface
between the air and skull. Nevertheless, these observations emphasize the insensitivity of MB-SWIFT to susceptibility-induced artefacts in complex experimental protocols.

1.15. LIMITATIONS OF MB-SWIFT

Because MB-SWIFT has a near zero echo time and no volume selection, there are some methodological limitations. First, signal is received from everything that includes protons with a T2 of tens of microseconds and longer and is in the range of the transmitter/receiver coils. As it is practically very challenging to have all materials in the setup free of protons, the FOV has to cover everything that the receiver coil typically sees.

Second, because the MB-SWIFT fMRI images have a low anatomical contrast, standard motion correction and co-registration methods fail to register the images correctly. In addition to signal intensity correction, motion correction and co-registration require adjustments to algorithm parameters or the use of indirect methods.

Third, as any pulse using high excitation bandwidth, MB-SWIFT can become a high-SAR pulse sequence. We estimated the rat head SAR to be approximately 20 W/kg at 9.4 T, which translates to approximately 2 W/kg at 3 T (Lehto et al., 2017). As this is below, e.g., the FDA head SAR limit (https://www.aapm.org/meetings/02AM/pdf/8356-48054.pdf), MB-SWIFT fMRI using such high excitation bandwidth, flip angle, and time resolution is also feasible in humans.

CONCLUSIONS

Our results demonstrate that MB-SWIFT has unique features that make it a valuable tool for preclinical fMRI studies. The 20-dB difference in the average SPL between the MB-SWIFT technique and our standard EPI pulse sequence clearly exceeds the typical drop of 12 dB with silent EPI pulse sequences (Hutter et al., 2018; Zapp et al., 2012). In addition to the low acoustic noise, the insensitivity to movement and susceptibility-induced artefacts make MB-SWIFT ideal for awake rodent studies and simultaneous EEG-fMRI measurements. Importantly, the fMRI results were of high quality and consistent with those of previous EPI studies, indicating that MB-SWIFT is a valid alternative to EPI for demanding fMRI protocols. The benefits of MB-SWIFT enable fMRI study designs involving complex experimental setups at high magnetic fields.

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APPENDIX A. SUPPLEMENTARY DATA

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Functional magnetic resonance imaging (fMRI) is a versatile non-invasive imaging tool for measuring whole-brain function in response to a stimulus or at rest. In preclinical studies, anesthetics are in common use but can extensively modulate awake resting-state networks. In this thesis, resting-state fMRI was used to study and compare brain function of anesthetized and awake animals. Optimized imaging protocols for awake animals were proposed which can increase the translational value of preclinical fMRI studies in the future.