



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



Jaakko Paasonen<sup>a,\*</sup>, Petteri Stenroos<sup>a</sup>, Raimo A. Salo<sup>a</sup>, Vesa Kiviniemi<sup>b</sup>, Olli Gröhn<sup>a</sup>

<sup>a</sup> A.I.V. Institute for Molecular Sciences, University of Eastern Finland, Kuopio, Finland

<sup>b</sup> Department of Radiology, Oulu University Hospital, Oulu, Finland

### ARTICLE INFO

#### Keywords:

Anesthetics

Awake

Functional connectivity

Magnetic resonance imaging

Functional

Rats

### ABSTRACT

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

**Abbreviations:** AC,  $\alpha$ -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.

\* Corresponding author. A.I.V. Institute for Molecular Sciences, University of Eastern Finland, P.O. Box 1627, FI-70211, Kuopio, Finland.

E-mail address: [jaakko.paasonen@uef.fi](mailto:jaakko.paasonen@uef.fi) (J. Paasonen).

<https://doi.org/10.1016/j.neuroimage.2018.01.014>

Received 17 October 2017; Accepted 8 January 2018

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  $22 \pm 2$  °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:  $\alpha$ -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; Pirttimäki 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 N<sub>2</sub>/O<sub>2</sub> 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, Södertälje, 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 pCO<sub>2</sub>, pO<sub>2</sub>, sO<sub>2</sub>, 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 complex-network 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 N<sub>2</sub>/O<sub>2</sub> 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 ( $8 \times 12 \times 15 \text{ mm}^3$  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  $5.0 \times 5.0 \text{ cm}$ , matrix size  $512 \times 512$ , 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  $2.5 \text{ cm} \times 2.5 \text{ cm}$ , matrix size  $64 \times 64$ , 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 ( $2 \times 2$  voxel full-width at half-maximum Gaussian kernel), and co-registration were applied using SPM8 ([www.fil.ion.ucl.ac.uk/spm](http://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](http://aedes.uef.fi)), and SPM8 ([www.fil.ion.ucl.ac.uk/spm](http://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  $>0.15 \text{ Hz}$  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  $\pm$  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 ( $\text{pCO}_2$ ,  $\text{pO}_2$ , and  $\text{sO}_2$ ) 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 ( $126 \pm 18 \text{ ng/ml}$ ) and second ( $98 \pm 10 \text{ ng/ml}$ ) MRI measurement did not differ significantly from the pre-habituation samples ( $95 \pm 15 \text{ ng/ml}$ ;  $p = .25$  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  $2.8 \pm 0.2\%$ , corresponding to an estimated value in humans of 4–10% (Power et al., 2012, 2014). Maximal single head movement in the awake group was  $123 \pm 23 \mu\text{m}$ , which is roughly 1/3 of the voxel size. No head motion was observed in the anesthetized rats.

### Functional connectivity matrices and mean connectivity

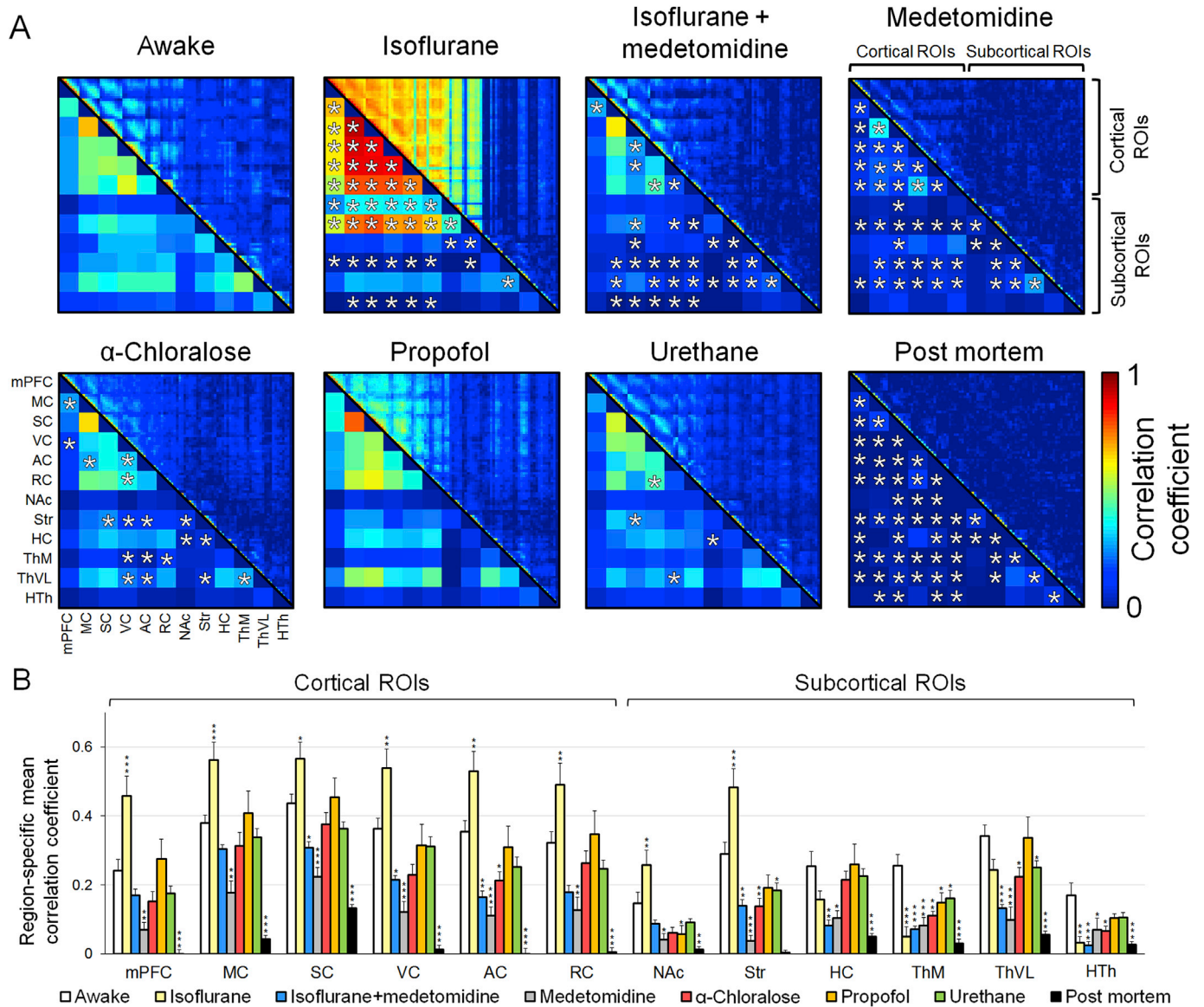
The group-level FC matrices are shown in Fig. 1A, and the region-specific mean CCs are shown in Fig. 1B. The mean FC pattern obtained from awake animals comprised mainly moderate to strong CCs (e.g., cortico-cortical CCs  $0.43 \pm 0.03$  in a 12 ROI matrix). Only connections originating from the nucleus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

**Table 1**  
Physiologic parameters obtained from rats that underwent resting-state functional magnetic resonance imaging.

	$\text{pCO}_2$ (mmHg)	$\text{pO}_2$ (mmHg)	pH	$\text{sO}_2$ (%)	Heart rate (bpm)	Weight (g)
Awake	–	–	–	–	$406 \pm 8$	$320 \pm 4$
$\alpha$ -Chloralose	$37.6 \pm 2.2$	$141 \pm 8$	$7.48 \pm 0.02$	$99.0 \pm 0.3$	$403 \pm 9$	$342 \pm 4$
Isoflurane	$45.1 \pm 2.0$	$133 \pm 8$	$7.39 \pm 0.02$	$98.9 \pm 0.1$	$427 \pm 13$	$317 \pm 12$
Isoflurane + medetomidine	$38.2 \pm 1.5$	$133 \pm 4$	$7.42 \pm 0.02$	$98.9 \pm 0.1$	$272 \pm 4$	$310 \pm 16$
Medetomidine	$40.8 \pm 2.4$	$149 \pm 5$	$7.42 \pm 0.01$	$98.6 \pm 0.6$	$273 \pm 10$	$333 \pm 12$
Propofol	$40.9 \pm 2.3$	$137 \pm 5$	$7.42 \pm 0.02$	$99.0 \pm 0.0$	$412 \pm 11$	$323 \pm 24$
Urethane	$39.0 \pm 1.5$	$144 \pm 5$	$7.38 \pm 0.01$	$99.0 \pm 0.1$	$457 \pm 7$	$349 \pm 8$

bpm, beats per minute.



**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; HTh, hypothalamus; MC, motor cortex; mPFC, medial prefrontal cortex; NAc, nucleus accumbens; RC, retrosplenial cortex; ROI, region of interest; SC, somatosensory cortex; Str, striatum; ThM, medial thalamus; ThVL, ventrolateral thalamus; VC, visual cortex.

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).

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 thalamo-cortical 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  $0.06 \pm 0.03$ ; awake  $0.14 \pm 0.02$ ) and medial thalamus (PRO  $0.15 \pm 0.03$ ; awake  $0.26 \pm 0.03$ ).

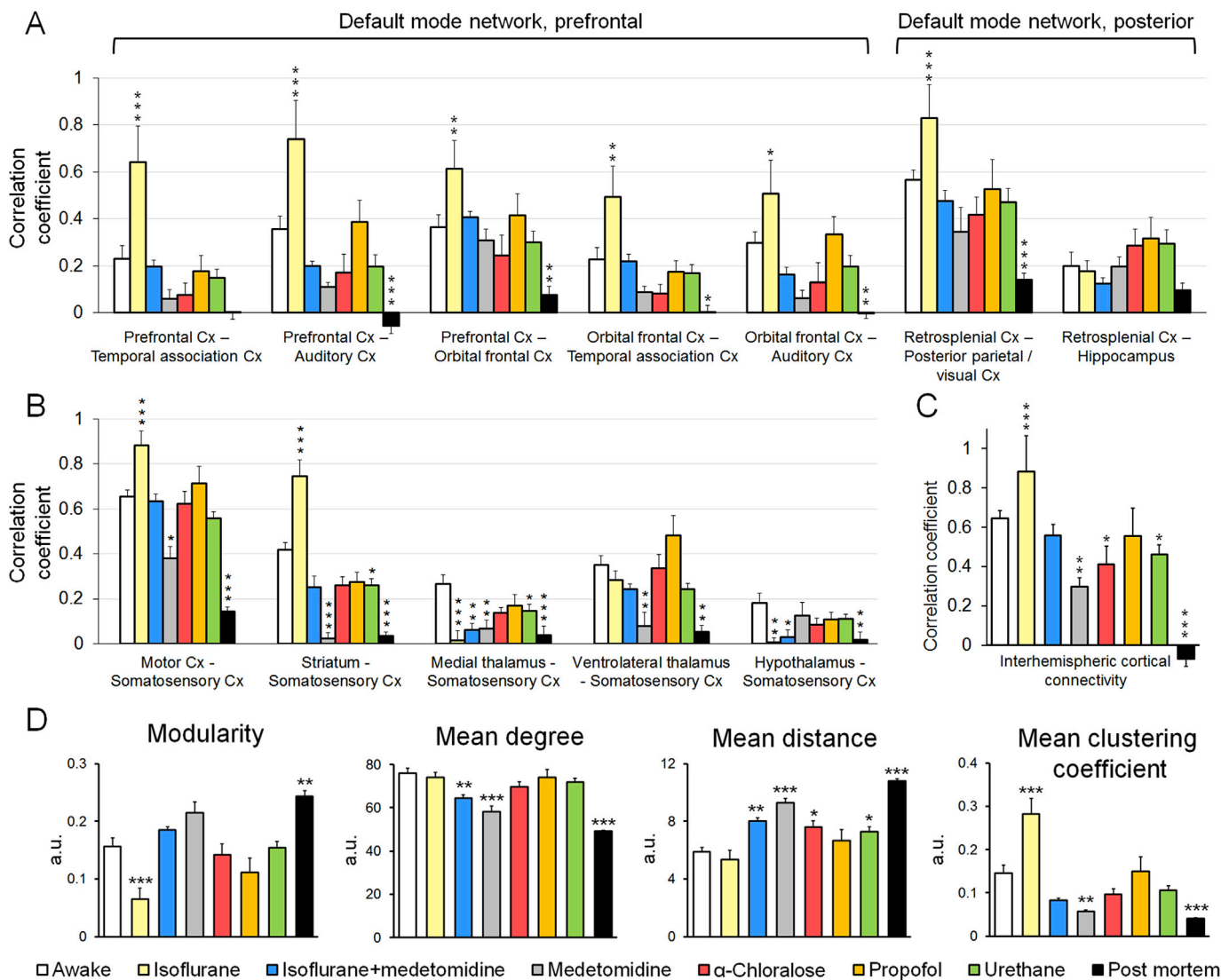
The FC pattern under URE anesthesia was close to that in the awake group; only 6% (4/66) of all connections were significantly suppressed. The 92 ROI matrix appeared visually similar to that in the awake group, although with slightly lower CCs (URE  $0.11 \pm 0.001$ ; awake  $0.15 \pm 0.001$ ). In the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

In the FC matrices of post-mortem data, only negligible CCs ( $0.02 \pm 0.01$ ) 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  $0.18 \pm 0.04$ ; awake  $0.20 \pm 0.06$ ). 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



**Fig. 2. Functional connectivity of default mode network (A), somatosensory cortex (B), and interhemispheric cortices (C), and complex-network parameters (D).** Interhemispheric connectivity was obtained from motor cortex. Statistical tests were performed with one-way ANOVA and Dunnett's multiple comparison against the awake group (\* $p < .05$ , \*\* $p < .01$ , \*\*\* $p < .001$ ). a.u., arbitrary unit; Cx, cortex.

low CCs observed in the ISO (ISO  $0.88 \pm 0.06$ ; awake  $0.65 \pm 0.03$ ) and MED (MED  $0.38 \pm 0.05$ ; awake  $0.65 \pm 0.03$ ) 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*

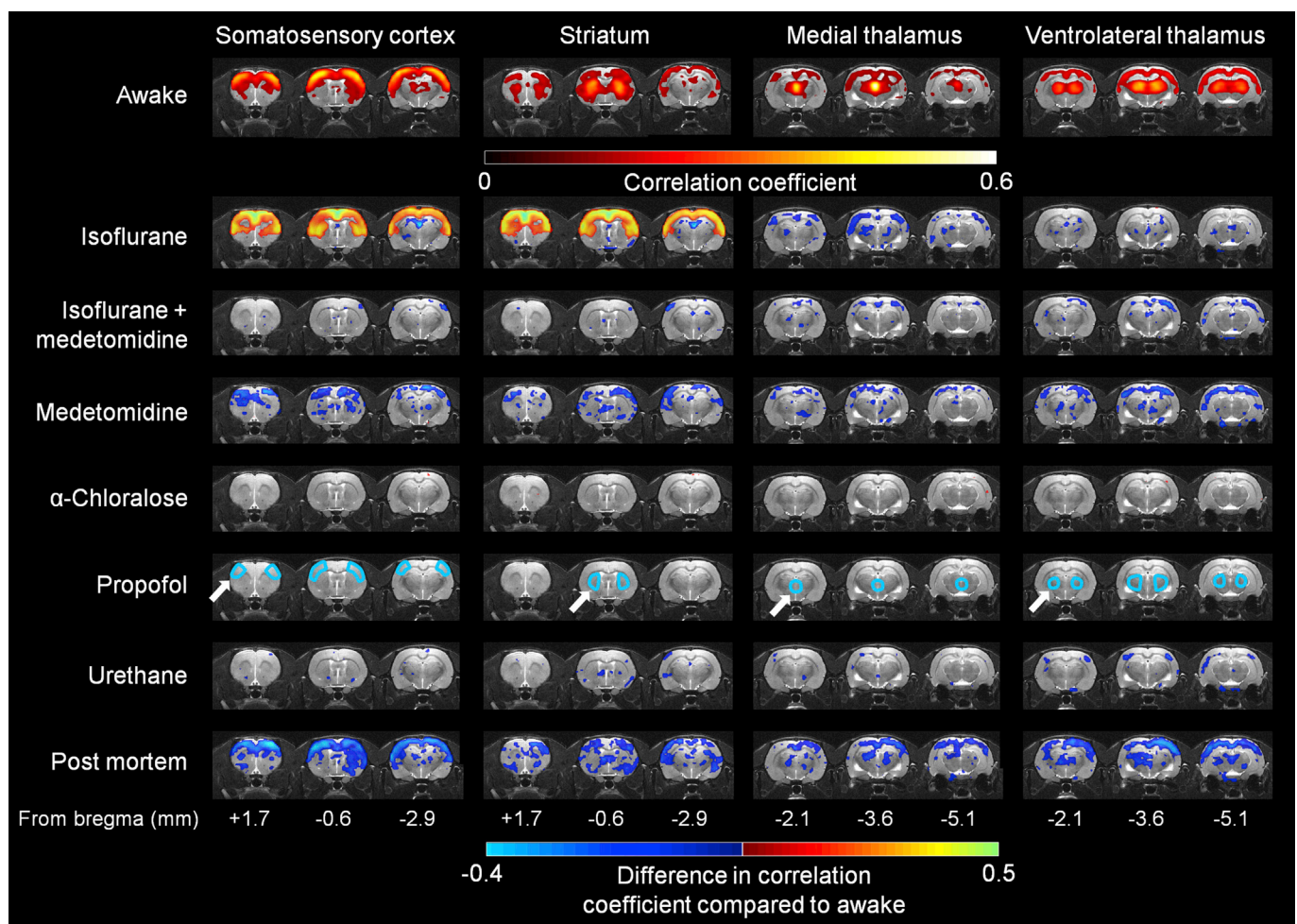
The representative seed-based connectivity maps obtained from the awake animals, and differences between the awake and anesthetized animals are shown in Fig. 3. In the ISO group, the cortical and striatal connections were merged into one uniform network, while thalamo-

cortical 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.

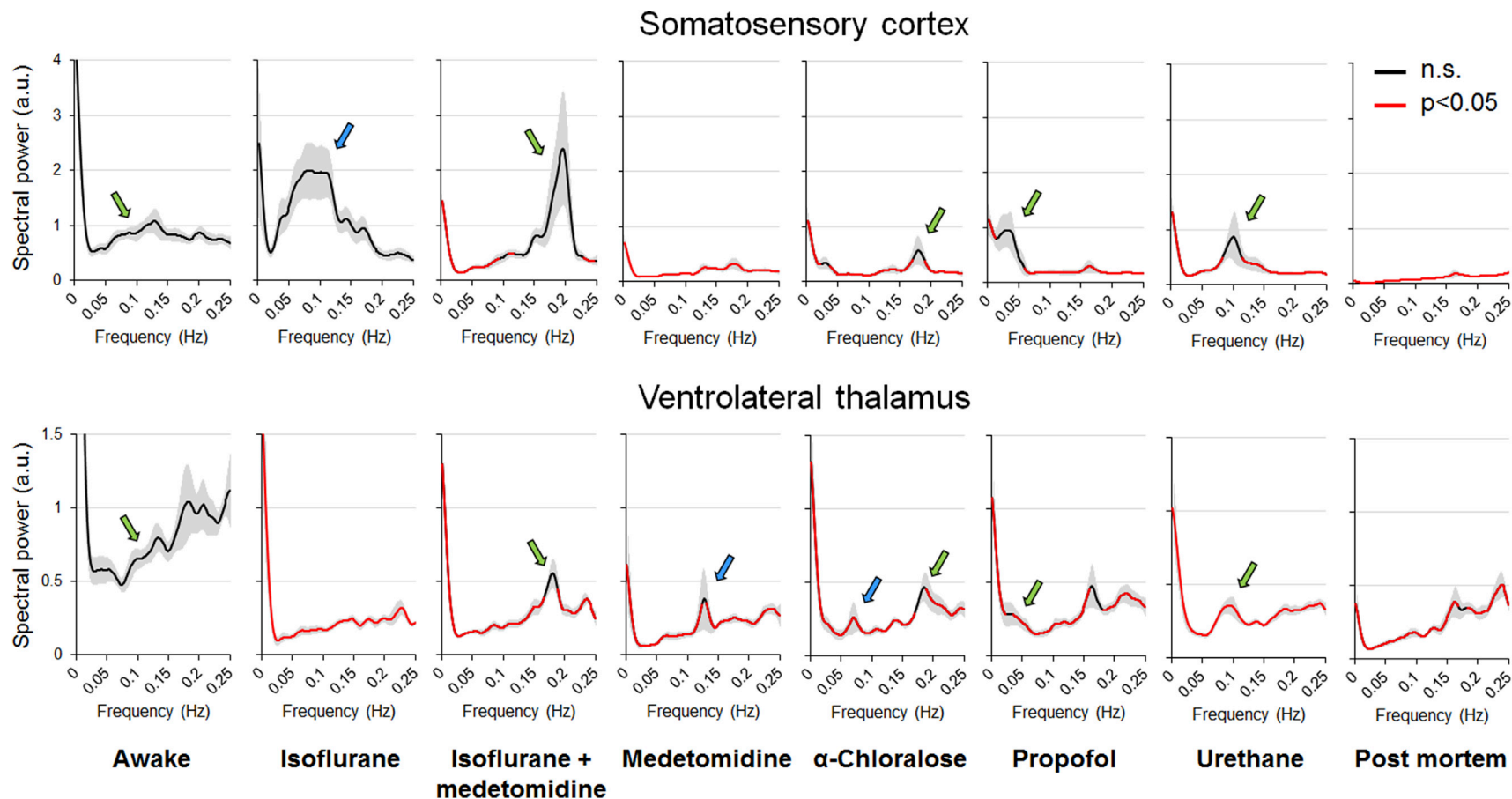
*Spectral power of BOLD signals*

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 T<sub>2</sub>-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 concentrated at low frequencies (0.02–0.04 Hz) in both regions, while the rest of the frequencies appeared not to differ from those in the post mortem group. 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 thalamo-cortical 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 thalamo-cortical 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 in detail in the cited studies, we focus on the findings related to FC.

***α-Chloralose (60 mg/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 fronto-cortical regions, the majority of the thalamo-cortical and intra-subcortical 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 disconnectivity 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 (0.1 mg/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 (0.06 mg/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 (Bryndtsen 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 thalamo-cortical 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 (1250 mg/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, thalamo-cortical 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 whole-brain 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  $37 \pm 2$  min and the time between the cessation of initial ISO anesthesia and initiating rsfMRI scan was  $38 \pm 2$  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.

#### Acknowledgements

This work was supported by the Finnish Funding Agency for Technology and Innovation (Tekes, 70036/11) and the Academy of Finland (#298007). In addition, we thank Maarit Pulkkinen for technical assistance with the animal preparation.

#### 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

- Baek, K., Shim, W.H., Jeong, J., Radhakrishnan, H., Rosen, B.R., Boas, D., Franceschini, M., Biswal, B.B., Kim, Y.R., 2016. Layer-specific interhemispheric functional connectivity in the somatosensory cortex of rats: resting state electrophysiology and fMRI studies. *Brain Struct. Funct.* 221 (5), 2801–2815.
- Barttfeld, P., Uhrig, L., Sitt, J.D., Sigman, M., Jarraya, B., Dehaene, S., 2015. Signature of consciousness in the dynamics of resting-state brain activity. *Proc. Natl. Acad. Sci. U.S.A.* 112 (3), 887–892.
- Belcher, A.M., Yen, C.C., Stepp, H., Gu, H., Lu, H., Yang, Y., Silva, A.C., Stein, E.A., 2013. Large-scale brain networks in the awake, truly resting marmoset monkey. *J. Neurosci.: Off. J. Soc. Neurosci.* 33 (42), 16796–16804.
- Biswal, B., Yetkin, F.Z., Haughton, V.M., Hyde, J.S., 1995. Functional connectivity in the motor cortex of resting human brain using echo-planar MRI. *Magn. Reson. Med. : Off. J. Soc. Magn. Reson. Med.* 34 (4), 537–541.
- Brynmildsen, J.K., Hsu, L.M., Ross, T.J., Stein, E.A., Yang, Y., Lu, H., 2017. Physiological characterization of a robust survival rodent fMRI method. *Magn. Reson. Imag.* 35, 54–60.
- Fox, M.D., Raichle, M.E., 2007. Spontaneous fluctuations in brain activity observed with functional magnetic resonance imaging. *Nature Reviews. Neuroscience* 8 (9), 700–711.
- Franks, N.P., 2008. General anaesthesia: from molecular targets to neuronal pathways of sleep and arousal. *Nature Reviews. Neuroscience* 9 (5), 370–386.
- Fransson, P., 2006. How default is the default mode of brain function? further evidence from intrinsic BOLD signal fluctuations. *Neuropsychologia* 44 (14), 2836–2845.
- Fukuda, M., Vazquez, A.L., Zong, X., Kim, S.G., 2013. Effects of the alpha(2)-adrenergic receptor agonist dexmedetomidine on neural, vascular and BOLD fMRI responses in the somatosensory cortex. *Eur. J. Neurosci.* 37 (1), 80–95.
- Gao, Y.R., Ma, Y., Zhang, Q., Winder, A.T., Liang, Z., Antinori, L., Drew, P.J., Zhang, N., 2016. Time to wake up: studying neurovascular coupling and brain-wide circuit function in the un-anesthetized animal. *Neuroimage*.
- Gotman, J., 2008. Epileptic networks studied with EEG-fMRI. *Epilepsia* 49 (Suppl. 3), 42–51.
- Grandjean, J., Schroeter, A., Batata, I., Rudin, M., 2014. Optimization of anesthesia protocol for resting-state fMRI in mice based on differential effects of anesthetics on functional connectivity patterns. *Neuroimage* 102 (Pt 2), 838–847.
- Greicius, M.D., Kiviniemi, V., Tervonen, O., Vainionpaa, V., Alahuhta, S., Reiss, A.L., Menon, V., 2008. Persistent default-mode network connectivity during light sedation. *Hum. Brain Mapp.* 29 (7), 839–847.
- Griffin, K.M., Blau, C.W., Kelly, M.E., O'Herlihy, C., O'Connell, P.R., Jones, J.F., Kerskens, C.M., 2010. Propofol allows precise quantitative arterial spin labelling functional magnetic resonance imaging in the rat. *Neuroimage* 51 (4), 1395–1404.
- Hamilton, C., Ma, Y., Zhang, N., 2017 Sep. Global reduction of information exchange during anesthetic-induced unconsciousness. *Brain Struct. Funct.* 222 (7), 3205–3216.
- Hudetz, A.G., Liu, X., Pillay, S., 2015. Dynamic repertoire of intrinsic brain states is reduced in propofol-induced unconsciousness. *Brain Connect.* 5 (1), 10–22.
- Jonckers, E., Delgado y Palacios, R., Shah, D., Guglielmetti, C., Verhoye, M., Van der Linden, A., 2014. Different anesthesia regimes modulate the functional connectivity outcome in mice. *Magn. Reson. Med.* 72 (4), 1103–1112.
- Kalthoff, D., Po, C., Wiedermann, D., Hoehn, M., 2013. Reliability and spatial specificity of rat brain sensorimotor functional connectivity networks are superior under sedation compared with general anesthesia. *NMR Biomed.* 26 (6), 638–650.
- Kalthoff, D., Seehafer, J.U., Po, C., Wiedermann, D., Hoehn, M., 2011. Functional connectivity in the rat at 11.7T: impact of physiological noise in resting state fMRI. *Neuroimage* 54 (4), 2828–2839.
- Khatamian, Y.B., Golestani, A.M., Ragot, D.M., Chen, J.J., 2016. Spin-echo resting-state functional connectivity in high-susceptibility regions: accuracy, reliability, and the impact of physiological noise. *Brain Connect.* 6 (4), 283–297.
- King, J.A., Garelick, T.S., Brevard, M.E., Chen, W., Messenger, T.L., Duong, T.Q., Ferris, C.F., 2005. Procedure for minimizing stress for fMRI studies in conscious rats. *J. Neurosci. Meth.* 148 (2), 154–160.
- Kiviniemi, V.J., Haanpaa, H., Kantola, J.H., Jauhiainen, J., Vainionpaa, V., Alahuhta, S., Tervonen, O., 2005. Midazolam sedation increases fluctuation and synchrony of the resting brain BOLD signal. *Magn. Reson. Imag.* 23 (4), 531–537.

- Lahti, K.M., Ferris, C.F., Li, F., Sotak, C.H., King, J.A., 1998. Imaging brain activity in conscious animals using functional MRI. *J. Neurosci. Meth.* 82 (1), 75–83.
- Lee, S.P., Silva, A.C., Ugurbil, K., Kim, S.G., 1999. Diffusion-weighted spin-echo fMRI at 9.4 T: Microvascular/tissue contribution to BOLD signal changes. *Magn. Reson. Med.* 42 (5), 919–928.
- Liang, Z., Liu, X., Zhang, N., 2015. Dynamic resting state functional connectivity in awake and anesthetized rodents. *Neuroimage* 104, 89–99.
- Liu, X., Zhu, X.H., Zhang, Y., Chen, W., 2013a. The change of functional connectivity specificity in rats under various anesthesia levels and its neural origin. *Brain Topogr.* 26 (3), 363–377.
- Liu, X., Zhu, X.H., Zhang, Y., Chen, W., 2011. Neural origin of spontaneous hemodynamic fluctuations in rats under burst-suppression anesthesia condition. *Cerebr. Cortex* 21 (2), 374–384 (New York, N.Y.: 1991).
- Liu, X., Pillay, S., Li, R., Vizuet, J.A., Pechman, K.R., Schmainda, K.M., Hudetz, A.G., 2013b. Multiphasic modification of intrinsic functional connectivity of the rat brain during increasing levels of propofol. *Neuroimage* 83, 581–592.
- Lu, H., Stein, E.A., 2014. Resting state functional connectivity: its physiological basis and application in neuropharmacology. *Neuropharmacology* 84, 79–89.
- Lu, H., Zou, Q., Gu, H., Raichle, M.E., Stein, E.A., Yang, Y., 2012. Rat brains also have a default mode network. *Proc. Natl. Acad. Sci. U.S.A.* 109 (10), 3979–3984.
- Lu, H., Zuo, Y., Gu, H., Waltz, J.A., Zhan, W., Scholl, C.A., Rea, W., Yang, Y., Stein, E.A., 2007. Synchronized delta oscillations correlate with the resting-state functional MRI signal. *Proc. Natl. Acad. Sci. U.S.A.* 104 (46), 18265–18269.
- Lukasik, V.M., Gillies, R.J., 2003. Animal anaesthesia for in vivo magnetic resonance. *NMR Biomed.* 16 (8), 459–467.
- Ma, Y., Hamilton, C., Zhang, N., 2017. Dynamic connectivity patterns in conscious and unconscious brain. *Brain Connect.* 7 (1), 1–12.
- Magnuson, M.E., Thompson, G.J., Pan, W.J., Keilholz, S.D., 2014. Time-dependent effects of isoflurane and dexmedetomidine on functional connectivity, spectral characteristics, and spatial distribution of spontaneous BOLD fluctuations. *NMR Biomed.* 27 (3), 291–303.
- Majeed, W., Magnuson, M., Keilholz, S.D., 2009. Spatiotemporal dynamics of low frequency fluctuations in BOLD fMRI of the rat. *J. Magn. Reson. Imag.: JMIR* 30 (2), 384–393.
- Masamoto, K., Kanno, I., 2012. Anesthesia and the quantitative evaluation of neurovascular coupling. *J. Cerebr. Blood Flow Metabol. : Off. J. Int. Soc. Cerebr. Blood Flow Metabol.* 32 (7), 1233–1247.
- Nallasamy, N., Tsao, D.Y., 2011. Functional connectivity in the brain: effects of anesthesia. *Neuroscientist: Rev. J. Bringing Neurobiol. Neurol. Psychiat.* 17 (1), 94–106.
- Nasrallah, F.A., Tan, J., Chuang, K.H., 2012. Pharmacological modulation of functional connectivity: alpha2-adrenergic receptor agonist alters synchrony but not neural activation. *Neuroimage* 60 (1), 436–446.
- Paasonen, J., Salo, R.A., Huttunen, J.K., Grohn, O., 2017 Sep. Resting-state functional MRI as a tool for evaluating brain hemodynamic responsiveness to external stimuli in rats. *Magn. Reson. Med.* 78 (3), 1136–1146.
- Paasonen, J., Salo, R.A., Shatillo, A., Forsberg, M.M., Narvainen, J., Huttunen, J.K., Grohn, O., 2016. Comparison of seven different anesthesia protocols for nicotine pharmacologic magnetic resonance imaging in rat. *Eur. Neuropsychopharmacol. J. Eur. Coll. Neuropsychopharmacol.* 26 (3), 518–531.
- Pawela, C.P., Biswal, B.B., Hudetz, A.G., Schulte, M.L., Li, R., Jones, S.R., Cho, Y.R., Matloub, H.S., Hyde, J.S., 2009. A protocol for use of medetomidine anesthesia in rats for extended studies using task-induced BOLD contrast and resting-state functional connectivity. *Neuroimage* 46 (4), 1137–1147.
- Pawela, C.P., Biswal, B.B., Cho, Y.R., Kao, D.S., Li, R., Jones, S.R., Schulte, M.L., Matloub, H.S., Hudetz, A.G., Hyde, J.S., 2008. Resting-state functional connectivity of the rat brain. *Magn. Reson. Med.* 59 (5), 1021–1029.
- Paxinos, G., Watson, C., 1998. *The Rat Brain in Stereotaxic Coordinates*, fourth ed. Academic Press, San Diego.
- Peltier, S.J., Kerssens, C., Hamann, S.B., Sebel, P.S., Byas-Smith, M., Hu, X., 2005. Functional connectivity changes with concentration of sevoflurane anesthesia. *Neuroreport* 16 (3), 285–288.
- Pirttimäki, T., Salo, R.A., Shatillo, A., Kettunen, M.I., Paasonen, J., Sierra, A., Jokivarsi, K., Leinonen, V., Andrade, P., Quittek, S., et al., 2016. Implantable RF-coil with multiple electrodes for long-term EEG-fMRI monitoring in rodents. *J. Neurosci. Meth.* 274, 154–163.
- Power, J.D., Barnes, K.A., Snyder, A.Z., Schlaggar, B.L., Petersen, S.E., 2012. Spurious but systematic correlations in functional connectivity MRI networks arise from subject motion. *Neuroimage* 59 (3), 2142–2154.
- Power, J.D., Mitra, A., Laumann, T.O., Snyder, A.Z., Schlaggar, B.L., Petersen, S.E., 2014. Methods to detect, characterize, and remove motion artifact in resting state fMRI. *Neuroimage* 84, 320–341.
- Raichle, M.E., 2015. The brain's default mode network. *Annu. Rev. Neurosci.* 38, 433–447.
- Rubinov, M., Sporns, O., 2010. Complex network measures of brain connectivity: uses and interpretations. *Neuroimage* 52 (3), 1059–1069.
- Shannon, B.J., Dosenbach, R.A., Su, Y., Vlessenko, A.G., Larson-Prior, L.J., Nolan, T.S., Snyder, A.Z., Raichle, M.E., 2013. Morning-evening variation in human brain metabolism and memory circuits. *J. Neurophysiol.* 109 (5), 1444–1456.
- Shin, T.J., Cho, D., Ham, J., Choi, D.H., Kim, S., Jeong, S., Kim, H.I., Kim, J.G., Lee, B., 2016. Changes in thalamo-frontal interaction under different levels of anesthesia in rats. *Neurosci. Lett.* 627, 18–23.
- Smith, J.B., Liang, Z., Watson, G.D., Alloway, K.D., Zhang, N., 2017 Jul. Interhemispheric resting-state functional connectivity of the claustrum in the awake and anesthetized states. *Brain Struct. Funct.* 222 (5), 2041–2058.
- Smucny, J., Wylie, K.P., Tregellas, J.R., 2014. Functional magnetic resonance imaging of intrinsic brain networks for translational drug discovery. *Trends Pharmacol. Sci.* 35 (8), 397–403.
- Thompson, G.J., Pan, W.J., Magnuson, M.E., Jaeger, D., Keilholz, S.D., 2014. Quasi-periodic patterns (QPP): large-scale dynamics in resting state fMRI that correlate with local infraslow electrical activity. *Neuroimage* 84, 1018–1031.
- Tu, Y., Yu, T., Fu, X.Y., Xie, P., Lu, S., Huang, X.Q., Gong, Q.Y., 2011. Altered thalamocortical functional connectivity by propofol anesthesia in rats. *Pharmacology* 88 (5–6), 322–326.
- Upadhyay, J., Baker, S.J., Chandran, P., Miller, L., Lee, Y., Marek, G.J., Sakoglu, U., Chin, C.L., Luo, F., Fox, G.B., et al., 2011. Default-mode-like network activation in awake rodents. *PLoS One* 6 (11), e27839.
- Vincent, J.L., Patel, G.H., Fox, M.D., Snyder, A.Z., Baker, J.T., Van Essen, D.C., Zempel, J.M., Snyder, L.H., Corbetta, M., Raichle, M.E., 2007. Intrinsic functional architecture in the anesthetized monkey brain. *Nature* 447 (7140), 83–86.
- Williams, K.A., Magnuson, M., Majeed, W., LaConte, S.M., Peltier, S.J., Hu, X., Keilholz, S.D., 2010. Comparison of alpha-chloralose, medetomidine and isoflurane anesthesia for functional connectivity mapping in the rat. *Magn. Reson. Imag.* 28 (7), 995–1003.
- Wilson, D.A., Hoptman, M.J., Gerum, S.V., Guilfoyle, D.N., 2011. State-dependent functional connectivity of rat olfactory system assessed by fMRI. *Neurosci. Lett.* 497 (2), 69–73.
- Zhao, F., Zhao, T., Zhou, L., Wu, Q., Hu, X., 2008. BOLD study of stimulation-induced neural activity and resting-state connectivity in medetomidine-sedated rat. *Neuroimage* 39 (1), 248–260.
- Zhurakovskaya, E., Paasonen, J., Shatillo, A., Lipponen, A., Salo, R., Aliev, R., Tanila, H., Grohn, O., 2016. Global functional connectivity differences between sleep-like states in urethane anesthetized rats measured by fMRI. *PLoS One* 11 (5), e0155343.