Functional connectivity in the rat at 11.7 T: Impact of physiological noise in resting state fMRI

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ARTICLE INFO

Abstract

Resting state functional MRI (rs-fMRI) of the brain has the potential to elicit networks of functional connectivity and to reveal changes thereof in animal models of neurological disorders. In the present study, we investigate the contribution of physiological noise and its impact on assessment of functional connectivity in rs-fMRI of medetomidine sedated, spontaneously breathing rats at ultrahigh field of 11.7 Tesla. We employed gradient echo planar imaging (EPI) with repetition times of 3 s and used simultaneous recordings of physiological parameters. A model of linear regression was applied to quantify the amount of BOLD fMRI signal fluctuations attributable to physiological sources. Our results indicate that physiological noise – mainly originating from the respiratory cycle – dominates the rs-fMRI time course in the form of spatially complex correlation patterns. As a consequence, these physiological fluctuations introduce severe artifacts into seed-based correlation maps and lead to misinterpretation of corresponding connectivity measures. We demonstrate that a scheme of motion correction and linear regression can significantly reduce physiological noise in the rs-fMRI time course, remove artifacts, and hence improve the reproducibility of functional connectivity assessment.

In conclusion, physiological noise can severely compromise functional connectivity MRI (fcMRI) of the rodent at high fields and must be carefully considered in design and interpretation of future studies. Motion correction should be considered the primary strategy for reduction of apparent motion related to respiratory fluctuations. Combined with subsequent regression of physiological confounders, this strategy has proven successful in reducing physiological noise and related artifacts affecting functional connectivity analysis. The proposed new and rigorous protocol now opens the potential of fcMRI to elicit the role of brain connectivity in pathological processes without concerns of confounding contributions from physiological noise.

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Introduction

Functional magnetic resonance imaging (fMRI) of the brain's “resting state” (rs-fMRI) has gained considerable interest during the last 15 years after its first application to probe functional connectivity (Biswal et al., 1995). More recently, it has found its way into animal research (Lu et al., 2007; Pawela et al., 2008, 2009a; Zhao et al., 2008) and has since evolved into a unique tool to investigate functional connectivity in animal models of various pathologies, for example recently demonstrated in stroke (van Meer et al., 2010). In this context, functional connectivity MRI (fcMRI) could complement studies of fMRI and extend the scope to brain networks that are not accessible through sensory stimulation. In addition, fMRI as well as fcMRI can profit from the ultrahigh fields available in animal MRI regarding the gain in signal-to-noise, sensitivity and resolution.

The terms rs-fMRI and fcMRI are often used interchangeably, although having a slightly different meaning. To avoid confusions, we will use the term rs-fMRI when emphasizing the conditions under which data was acquired; fcMRI is used in a more general context focusing on the application as a tool to reveal connectivity networks. As assessment of functional connectivity relies on spontaneous rs-fMRI signal fluctuations, it is important to know all contributions to these fluctuations. Apart from inherent thermal noise and hardware imperfections (Weisskoff, 1996), the rs-fMRI signal time course is affected by physiological noise that scales linearly with the signal intensity (Kruger and Glover, 2001). As a consequence, the significance of physiological noise increases with field strength and can be the dominating source of temporal noise in human fMRI at high field (Triantafyllou et al., 2005). Although physiological noise comprises spontaneous fluctuations of potentially neuronal origin, there are various interfering sources of non-neuronal fluctuations, most prominently related to the cardio-respiratory cycle (Glover et al., 2000). Contributions of these sources are substantial and have

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1053-8119/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.neuroimage.2010.10.053

Please cite this article as: Kalthoff, D., et al., Functional connectivity in the rat at 11.7 T: Impact of physiological noise in resting state fMRI, Neuroimage (2010), doi:10.1016/j.neuroimage.2010.10.053
recently been quantified for fMRI in the human brain at 7.0 Tesla (Bianciardi et al., 2009). Particularly the respiration related signal fluctuations are known to increase with field strength and to depend on the geometric dimensions (Raj et al., 2000). In translation to high field fMRI and fcMRI of the rodent, a significant and potentially confounding contribution of physiological noise can thus be expected. Therefore, the sensitivity to detect changes of functional connectivity in pathological processes could be compromised in the presence of physiological noise. Moreover, the applicability of temporal filtering to remove physiological fluctuations is limited, because the high respiratory and cardiac rates are usually undersampled.

With this motivation we conducted BOLD rs-fMRI experiments in healthy, spontaneously breathing rats under Medetomidine sedation at ultrahigh field of 11.7 Tesla with simultaneous physiological recordings in order to identify and quantify the contribution of non-neuronal sources of physiological noise to the rs-fMRI signal. We applied a scheme of motion correction and subsequent linear regression with the physiological parameters to correct for these contributions. Finally we compared the original and corrected data regarding functional connectivity analysis to assess the impact of physiological noise on connectivity measures.

Material & methods

Experimental protocol

Animal experimental protocol

MRI experiments were performed on spontaneously breathing, male Wistar rats (n = 22, 300-350 g) using Medetomidine sedation with slight modifications to a previously described protocol (Weber et al., 2006). All experiments were conducted in accordance with the German Laws for Animal Protection and were approved by the local animal care committee and regional governmental body (Bezirksregierung Köln).

Data used for this work was acquired in two studies (n = 5 / n = 17) that also involved BOLD fMRI using electrical forepaw stimulation. In both studies, animals were initially anaesthetized using Isoflurane in a mixture of N2O (70%) and oxygen (30%). Animals were placed in a plastic cradle in prone position. The head was carefully fixed using a bitebar and earbars.

After positioning, anaesthesia was maintained at ~1.5% Isoflurane for adjustments and additional imaging experiments. After completion of these, a bolus of 0.5 ml Medetomidine solution (Domitor®, Pfizer; 1 ml/kg bodyweight added to 10 ml of saline solution) was injected subcutaneously. Onset of the agent's effect was observed by a slowly discontinued within the next 5-10 minutes. 15 to 20 minutes after positioning, anaesthesia was maintained at ~1.5% Isoflurane for adjustments and additional imaging experiments. After completion of these, a bolus of 0.5 ml Medetomidine solution (Domitor®, Pfizer; 1 ml/kg bodyweight added to 10 ml of saline solution) was injected subcutaneously. Onset of the agent's effect was observed by a decrease of respiratory and cardiac rates after which Isoflurane was slowly discontinued within the next 5-10 minutes. 15 to 20 minutes after the bolus, continuous infusion of Medetomidine solution was started at 1 ml/h and N2O was replaced by N2. After completion of the imaging session, Atipamezol (Antisedan®, Pfizer; 1 ml/kg bodyweight) was injected subcutaneously together with ~2 ml of saline to reverse the sedative effect and substitute for fluid loss during the experiment.

Physiological monitoring & recording

To observe physiological parameters during MRI experiments, an MR compatible monitoring system (Small Animal Instruments Inc., NY, USA) was used. Via breakout module the system was connected to a custom-made data acquisition system based on DASYLab (measX, Mönchengladbach, Germany) that allowed continuous recording of physiological parameters and, moreover, received the MRI trigger channels.

The monitoring system was operated using a fiber optic temperature probe, respiration pad and fiber optic pulse oxymeter to monitor body temperature (T), oxygen saturation (SO2), respiratory and cardiac rates (rBPM / cBPM) as well as their corresponding waveforms. The acquisition system logged the slow parameters (T, SO2, rBPM, cBPM) every 10 s over the whole session while MRI triggers, respiratory and cardiac waves were recorded during rs-fMRI data acquisition with a temporal resolution of 1 kHz.

To maintain body temperature at 37 °C during the imaging session, a feedback-controlled water circulation system (medres, Cologne, Germany) was used to supply the base of the cradle and an additional heating pad on the back of the animal.

MR imaging protocol

Experiments were conducted on a 117/16 BioSpec system (Bruker BioSpin, Ettlingen, Germany) with Avance II hardware and a BGA9s gradient system with maximum strength 750 mT/m and a minimum ramp time of 125 μs. Transmission was achieved with a quadrature volume resonator (inner diameter 72 mm) and a standard rat brain quadrature surface coil (~30 × 30 mm2) was used for signal reception (Bruker BioSpin, Ettlingen, Germany). MRI experiments were executed with ParaVision 5 software.

Adjustments. Animals were positioned in the bore to align the forelimb region of the primary somatosensory cortex (S1ff) to the magnet isocenter, determined by its distance caudal to the rhinal fissure (~5.4 mm).

To optimize field homogeneity, an implementation of MAPSHIM in ParaVision 5 was used for shimming. After acquisition of a fieldmap, a local shim was performed on a 8.0 × 6.5 × 6.0 mm³ voxel containing the cortical region of interest.

EPI imaging parameters. For acquisition of the actual rs-fMRI scans, gradient echo planar imaging (gEPI) was performed using a 96 × 96 imaging matrix with an in-plane resolution of 300 × 300 μm² at a bandwidth of 250 kHz. The k-space center was sampled at an echo time (TE) of 17.5 ms with an asymmetric echo at 25% echo position. Five consecutive slices of 1.2 mm thickness were acquired and a separate trigger pulse was played out for each. Each scan consisted of 100 volumes and five additional dummy scans at the start. The repetition time (TR) was chosen to TR = 3000 ms (n = 5) and TR = 2840 ms (n = 17), respectively. In both cases, a volume delay of 2500 ms was used, meaning that the five slices were acquired within ~500 ms. Within each imaging session, one (n = 17) or two (n = 5) of these rs-fMRI scans were conducted and used for analysis. To enable determination of the raw noise present in the EPI time series, a separate scan with five frames at 0° flip angle was acquired in each session (Triantafyllou et al., 2005) containing noise only.

Data analysis

Unless noted otherwise, all image based processing was performed with ImageJ (Version 1.42q; National Institutes of Health, Bethesda, USA; http://rsbweb.nih.gov/ij) using custom-made plug-ins and macros that utilize the Apache Commons Maths Library (Version 2.1; The Apache Software Foundation; http://www.apache.org). Results of ROI analyses were handled and further processed using Excel (Microsoft Corporation, Redmond, USA). Processing of physiological monitoring data was performed using IDL (ITT Visual Information Solutions, Boulder, USA).

Prior to any further processing, native ParaVision EPI datasets were converted to 32-bit NIFTI format (Neuroimaging Informatics Technology Initiative; http://nifti.nimh.nih.gov). Image intensity was mapped back to the original raw data range to ensure comparability of scans within a session and with respect to the noise determination. To make the datasets suitable for motion correction and brain extraction using FSL tools (FMRI Software Library; http://www. fmrib.ox.ac.uk/fsl), voxel size was scaled up in the NIFTI header by a factor of 10.

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Processing of physiological recordings

Pre-processing. Processing of respiratory waveforms involved detection of peak inspiration and subsequent baseline correction using the median values (representing expiration) within the respective respiratory cycle. The resulting waveforms were smoothed using a sliding window of 50 ms. Based on this, the respiratory derivative was calculated and smoothed likewise. Processing of the cardiac waveform is less complex due to its more regular shape. Cardiac waveforms were temporally filtered (band-pass filter, 2.5 – 10.0 Hz) and smoothed (sliding window, 50 ms). Based on this, the cardiac derivative was calculated. Peak detection was used to calculate the cardiac and respiratory rates that were median filtered (3 s window) to remove outliers or detection errors.

Based on the temporal information stored with the waveforms, corresponding values of body temperature and oxygen saturation were extracted from the session recordings. All these data were merged into a multichannel file covering the entire scan duration with a temporal resolution of 10 ms including trigger information.

Creation of MRI regressors. These pre-processed physiological recordings were sampled at the time points of EPI data acquisition, indicated by the slice-specific trigger pulses. Samples were rearranged according to the interleaved slice acquisition scheme. In order to account for latencies of physiological parameters affecting the MRI signal and to compensate for synchronization imperfections, physiological recordings were sampled at various time lags from the actual MRI data acquisition. The time lags were ±200 ms in 20 ms steps for the fast waveforms (respiratory, cardiac and their derivatives) and ±45 s in 5 s steps for the slowly changing parameters.

Complemented by motion parameters (x-/y-translation, z-rotation) and drift terms (linear to 4th order), these resampled physiological recordings formed a set of potential regressors that were subsequently correlated with the rs-fMRI signal.

Processing of rs-fMRI data

Determination of raw noise level. To determine the contribution of intrinsic noise, including thermal noise of sample and electronics, referred to as the raw noise σfi, the standard deviation of signal intensity σfl within the noise scans (flip angle 0°) was calculated and corrected for the Rician distribution of magnitude noise using the relation σfl = 1.527 σfi (Gudbjartsson and Patz, 1995).

Motion correction. Motion correction was performed using FSLs mcfilt tool. Transformations were restricted to in-plane translation and rotation and performed separately for each slice in the imaging volume, as this increased the image stability in pre-tests. Transformation parameters were stored for later use in the regression process.

Effect of motion correction on raw noise level. Due to bilinear interpolation involved in the motion correction process, the raw noise present in a voxel time course is reduced as the (statistically independent) noise of neighboring voxels is averaged to some degree. The level of this reduction depends on the specific transformation parameters and may spatially not be homogeneous over all voxels within a slice. To quantify this effect, we created artificial datasets containing Gaussian noise at the previously determined level of σfi. These datasets then underwent motion correction with the exact same parameters as the corresponding original datasets.

From the transformed datasets we calculated the temporal standard deviation σfi,MC voxelwise. The resulting maps were blurred using a 3×3 pixel Gaussian kernel to reduce statistical errors due to the limited number of samples in the artificial noise data.

For variance analysis of motion corrected and all further processed data, these maps of σfi,MC were used while the single number of σfi was used as a homogeneous raw noise level for the original datasets.

Regressor analysis, correlation and selection

Power spectra and cross-correlation of regressors. In a first step, potential regressors underwent power spectral analysis to gain information on the frequency distribution of the individual regressors. Furthermore, regressors were cross-correlated (at zero time lag) with each other to form a cross-correlation matrix and identify interdependencies between them. Power spectra and matrices were first inspected individually. Matrices were then averaged for all rs-fMRI scans; power spectra were averaged separately with respect to the underlying scan repetition time (3000 ms vs. 2840 ms).

Correlation of regressors and rs-fMRI signal. Secondly, potential regressors were cross-correlated with their corresponding motion corrected rs-fMRI time series on a voxelwise basis to form corresponding correlation maps. These maps were then color coded and visually inspected for common patterns of correlation with respect to strong positive or negative correlation and their distribution over the brain.

In a subsequent ROI analysis the relative number of significantly correlated voxels (|CC|>0.3) within the brain was determined for each of the potential regressors and plotted over its time lag if applicable.

Selected regressors. Based on the results of this process, a set of regressors was selected for the subsequent process of linear regression. The selected regressors consisted of the motion parameters and drift terms as well as the respiratory and cardiac waves with their corresponding derivatives at a time lag of 0 ms and ±60 ms respectively (Table 1).

Linear regression and variance explained

We have assumed a plain linear relation between the different recorded regressors ri(t) and the rs-fMRI signal S(t), i.e.

\[ S(t) = S_0(t) + \sum \alpha_i r_i(t) \]

Table 1

Overview of potential and selected regressors with their respective correlation to the rs-fMRI signal.

<table>
<thead>
<tr>
<th>Group</th>
<th>Potential Regressors</th>
<th>Timelag/Step</th>
<th>Correlation to rs-fMRI Signal</th>
<th>Selected Regressors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Motion Parameters</td>
<td>n = 3 ( (Δx, Δy, Δz) )</td>
<td>-</td>
<td>8% / 10% / 29%</td>
<td>-</td>
</tr>
<tr>
<td>Drift Terms</td>
<td>n = 4 ( (1.-4. order) )</td>
<td>-</td>
<td>11% / 2% / 9% / 3%</td>
<td>x</td>
</tr>
<tr>
<td>Body Temperature</td>
<td>-</td>
<td>± 45/5 s</td>
<td>4%</td>
<td>x</td>
</tr>
<tr>
<td>Oxygen Saturation</td>
<td>-</td>
<td>± 45/5 s</td>
<td>2%</td>
<td>-</td>
</tr>
<tr>
<td>Respiration Rate</td>
<td>-</td>
<td>± 45/5 s</td>
<td>2%</td>
<td>x</td>
</tr>
<tr>
<td>Cardiac Rate</td>
<td>-</td>
<td>± 45/5 s</td>
<td>2%</td>
<td>x</td>
</tr>
<tr>
<td>Respiratory Wave</td>
<td>n = 2 ( (Orig./Deriv.) )</td>
<td>± 200/20 ms</td>
<td>21% / 26% (Fig. 3A)</td>
<td>± 0 ms</td>
</tr>
<tr>
<td>Wave</td>
<td>n = 2 ( (Orig./Deriv.) )</td>
<td>± 200/20 ms</td>
<td>2% / 2% (Fig. 3B)</td>
<td>-60 ms</td>
</tr>
</tbody>
</table>

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with $S_0(t)$ representing the actual residual signal “cleaned” from the influence of all regressors.

Having selected the regressors of interest, a stepwise linear regression was performed on the motion corrected data in the following order:

- motion parameters
- drift terms 1st to 4th order
- respiratory wave + derivative (time lag 0 ms)
- cardiac wave + derivative (time lag -60 ms)

The residuals of the corresponding regression were used as the input data for the respective next regression step.

**Estimation of variance explained.** For every dataset after each regression step, maps of temporal statistics were calculated from the residuals yielding mean signal intensity $S$, temporal variance $\sigma^2$, raw noise level $\sigma_0$ and temporal and image signal-to-noise ratio (tSNR and SNR$_0$). Determination of the respective $\sigma_0$ and SNR$_0$ was performed as described above. Maps of these statistical parameters subsequently were summarized in a region-of-interest (ROI), to yield single numbers for the whole brain (as covered by the five 1.2 mm slices). Respective ROIs were generated using FSL’s brain extraction tool. Results of this ROI analysis were then taken to determine the variance explained.

Within the linear model assumed, the variance explained after the $i$th regression step was determined using the relation (Montgomery et al., 2006)

$$R^2_{\text{adj}} = 1 - \frac{\sigma^2_i}{\sigma^2_0} \cdot \frac{n - 1}{n - ndof - 1}$$

where $\sigma^2_i$ is the residual variance after the $i$th regression step, $n$ reflects the number of time points in the dataset (i.e. NR = 100) and $ndof$ is the number of degrees of freedom (i.e. number of regressors) involved in the regression process up to and including the $i$th step. This way, the parameter is adjusted for the reduction of variance that would be expected from a corresponding number of random regressors. To quantify the isolated contribution of the $i$th regression step, the variance explained by all the previous regression steps was subtracted yielding

$$\Delta R^2_{\text{adj}} = R^2_{\text{adj}} - R^2_{i-1, \text{adj}}$$

**Variance explained by motion correction and raw noise.** The contribution of motion correction to the reduction of variance was determined via the ratio of temporal variance before and after motion correction:

$$\Delta R^2_{\text{MC}} = 1 - \frac{\sigma^2_{\text{MC}}}{\sigma^2_{\text{orig}}}$$

This variance reduction is partially due to a decreased raw noise level by interpolation. We accounted for this by applying motion correction to simulated noise data as described in a previous section. We were therefore able to quantify this contribution using

$$\Delta R^2_{\text{MC}, \text{adj}} = R^2_{\text{MC}} - R^2_{\text{MC}, \text{orig}}$$

and defining the adjusted variance explained by motion correction (i.e. cleared of the effect of interpolation) as

The variance contributed by raw noise was defined accordingly as

$$R^2 = \frac{\sigma^2_0}{\sigma^2_{\text{orig}}} R^2_{\text{MC}} = \frac{\sigma^2_{\text{MC}}}{\sigma^2_{\text{orig}}}$$

before and after motion correction, respectively.

**Connectivity analysis**

To estimate the impact of physiological noise on measures of functional connectivity drawn from rs-fMRI data, a seed based connectivity analysis was performed on original and corrected data.

**Selection of seed regions.** Seed regions for connectivity analysis were manually drawn bilaterally on the EPI images corresponding to the forelimb region of the primary and the secondary somatosensory cortices (S1fl / S2), caudate putamen (CPu) and thalamus (Th), focused on the ventral nuclei. Determination of these regions was based on the Paxinos & Watson Rat Brain Atlas (Paxinos and Watson, 1998) overlaid on the EPI images, supported by anatomical images and BOLD fMRI activation maps obtained in the same session.

**Functional connectivity maps and matrices.** The average voxel time course was extracted from each seed region and cross-correlated to all voxels across the brain to form functional connectivity maps (fcMaps) for each seed region. Furthermore, the averaged seed region time courses were cross-correlated with each other to form a connectivity matrix (fcMatrix). These connectivity maps and matrices were created from the original rs-fMRI data as well as from the motion corrected and regressed data.

**Results**

Screening of the 27 rs-fMRI scans collected from $n=22$ animals did not show noticeable irregularities or acquisition problems. The image signal-to-noise ratio (SNR$_0$) ranged from about 80 to 120 with a mean and SD of 96 (±11). The corresponding voxel-based temporal SNR (tSNR) ranged from about 50 to 80 with a mean and SD of 67 (±9) which corresponds to average signal fluctuations of 1.5% in the voxel time course. No actual subject motion was observable within the individual datasets or in comparison of overview scans taken at the beginning and end of a respective study.

Recording and processing of physiological parameters was successful in all except two sessions, in which pulse oxymetry failed and hence $S_{O_2}$, cBPM and the cardiac wave could not be recorded.

**Regressor correlation and selection**

The physiological recordings were sampled down to the temporal resolution of EPI data acquisition to form regressors that were then analyzed and correlated with the rs-fMRI data.

**Interaction of regressors and power spectra**

To reveal interaction and interrelation of different regressors, cross-correlation matrices of regressors were calculated for each dataset (Fig. 1A). As a first important observation, the rare and marginal changes occurring in the slow physiological parameters (body temperature, oxygen saturation, respiratory and cardiac rates) are usually correlated to the 1st to 4th order drift terms. As a consequence, a linear effect of these slow parameters on the rs-fMRI signal could equally well be compensated for by regression of the drift terms so that an additional regression of the slow parameters would be redundant. We therefore decided to reject the slow parameters for the subsequent regression process. Furthermore, in 80% of the scans, either the respiratory wave or its derivative showed significant (|CC|>0.3) correlation to one of the
motion parameters (most prominently y-translation). As a consequence, part of the respiration related MRI signal changes will already be compensated for by motion correction and additional regression of the resulting motion parameters.

Regarding power spectra of the regressors, the most striking observation is a pronounced peak in the spectrum of the y-translation motion parameter (Fig. 1B). In data acquired at TR = 3000 ms the peak forms at around 0.08 Hz and is present in all of the datasets. To elicit its origin, experiments were repeated ex-vivo (data not shown) and were found to also show this peak. We therefore concluded that it has a non-physiological origin and is related to some undersampled periodic hardware process. This is confirmed by the fact that the peak is shifted towards 0.00 Hz in scans acquired with slightly different sampling at TR = 2840 ms.

As expected, spectra of the slow physiological parameters are mostly limited to the low frequency range below 0.05 Hz in accordance with their correlation to drift terms. Spectra of the respiratory and cardiac waveforms each show a broad peak corresponding to their respective aliased respiratory or cardiac frequency (Fig. 1C). Due to the variation in respiratory and (less pronounced) cardiac rates and in combination with the strong undersampling, the respective peaks are found at variable positions in the spectrum when comparing data from different imaging sessions.

Correlation with rs-fMRI data

Having completed this characterization, regressors were correlated to their corresponding rs-fMRI data on a voxelwise basis to form correlation maps.

Inspection of these correlation maps revealed strong correlations of the respiratory regressors with the rs-fMRI signal that form complex patterns of positive and negative correlation in all regions of the brain (Fig. 2). Strong correlations are also observed with the motion parameters, most of all with the y-translation parameter. They appear as rims of positive and negative correlation along horizontal edges in the EPI images (Fig. 2A). Correlations with the cardiac regressors (wave and derivative) are less pronounced and limited to regions in proximity to larger vessels, at the brain base and occasionally around the sagittal sinus.

To quantify the correlation of the individual regressors with the rs-fMRI fluctuations, we determined the number of correlated voxels p_CCVx (positive and negative, i.e. |CC| > 0.3) in a whole brain ROI in the correlation maps (see Table 1). This treatment prevented a cancellation of positively and negatively correlated voxels within the same ROI. For the respiratory and cardiac regressors we examined p_CCVx for the various time lags to identify a suitable choice for the time lag in the subsequent regression process (Fig. 3). We eventually selected time lags of Δt = 0 ms for the respiratory and Δt = -60 ms for the cardiac regressors, as these showed maximal correlations.

Linear regression & variance explained

Using the selected regressors, stepwise linear regression was performed on the rs-fMRI data as described. Completion of the four regression steps took about 2.5 min for a single dataset on our system (Intel Xeon L5410 QuadCore).

We subsequently determined the variance explained by motion correction and the following regression steps for each dataset (Table 2). The results show a pronounced inter-individual / inter-session variation, especially regarding the explained variance by motion correction and motion regression. In summary, however, we found that motion correction - the initial processing step - already reduces the overall variance by 15% to 35%. An additional reduction of 10% (relative to the variance of the original data) is gained by regression of the obtained motion parameters. The further reductions of variance by respiratory regressors average to ~5%. Drift terms and cardiac regressors account only for about 1% of the signal variance, respectively. A summary of the contributions of motion correction and different regressors is visualized as a pie chart in Fig. 4. Care must be taken, however, when interpreting these results, as they are dependent on the processing order with respect to interdependency, especially of motion and respiration regressors.

Applying the proposed correction strategy of motion correction and subsequent regression resulted in an average tSNR gain of 30%.

Impact on rs-fMRI connectivity analysis

Functional connectivity maps (fcMaps) were calculated from the original and corrected data using seed region correlation as described.
We firstly compared fcMaps obtained from original and processed data by visual inspection to assess the impact of the correction process. Secondly, we generated functional connectivity matrices (fcMatrix) for all datasets to be able to investigate the effect of the correction in terms of reproducibility of functional connectivity.

**Functional connectivity maps (fcMaps)**

The comparison of fcMaps obtained from original and corrected data reveals a visible reduction of physiological artifacts in fcMaps subject to correction. Only in four of the 27 datasets analyzed, the effect could be considered negligible. In all other datasets, the correction process substantially improved the spatial specificity of the connectivity maps. This was particularly true for cortical seed regions, which – in the original data – in many cases appeared to have unspecific cortical connections and were also prone to motion artifacts (Fig. 5A). For the subcortical regions, the thalamus seeds profited from the correction in several cases (Fig. 5C). The caudate putamen showed a strong and robust interhemispheric connectivity in most of the original datasets, only in few cases the correction contributed to a further improvement here (Fig. 5C).

A more detailed investigation of the different correction and regression steps revealed that in most of the cases, motion correction and regression of motion parameters contributed the major improvement of specificity. There were some cases in which respiratory regression was required to further enhance the specificity by reducing artifacts particularly in the posterior slices. The effect of the drift regression was noticeable only in few datasets, the effect of the cardiac regression virtually negligible in all. These observations are in line with the contribution of the regressors as estimated using the analysis of explained variance.

**Functional connectivity matrices (fcMatrices)**

The fcMatrices reflect the effects of the correction as observed in the fcMaps. Removal of physiological artifacts clearly enhanced the matrix specificity, e.g. in the S1–S2 connectivity (Fig. 5B) and the thalamic connectivity (Fig. 5D). An examination of partial correlations corrected for the global brain signal fluctuations (depicted lower left of the matrix diagonal in Figs. 5B and D) in the original data reveals that regression of the global signal alone does not improve the specificity of functional connectivity. This is because the physiological artifacts exhibit complex correlation patterns with positive and negative contributions that cannot be removed by a global signal regression.

The significance of the corrections effect on the fcMatrices varies from subject to subject, just as observed in the fcMaps. In summary,
However, the correction strongly increases the stability of the matrices when comparing different subjects or sessions. When averaging all fcMatrices obtained from the original data (Fig. 6), the inter-subject standard deviation of the correlation coefficients (averaged over all of the possible connections) is 0.212 (0.036). This deviation is reduced by 25% to 0.158 (0.041) when using corrected data instead. This improvement of stability appears to be especially pronounced in the cortical regions (see Fig. 6, SD Matrix).

Discussion

We have acquired rs-fMRI data in spontaneously breathing Wistar rats under Medetomidine sedation with a TR of ~3 s and simultaneously recorded physiological data. After motion correction, the rs-fMRI signal exhibited strong correlations to motion parameters and the respiratory waveform, being significantly correlated to each other. Correlations to low order fluctuations and to the cardiac waveform were of less significance. We performed stepwise linear regression on the rs-fMRI data to remove these effects and fed the corrected data into a seed based connectivity analysis. A comparison of these results to those obtained from the original data revealed that motion correction and subsequent regression significantly reduced physiological artifacts, enhanced the specificity of seed based functional connectivity maps and increased the stability of the obtained connectivity measures.

The idea of correcting fMRI data based on external physiological recordings has evolved since its first application in k-space (Hu et al., 1995; Wovk et al., 1997) and image based approaches (Glover et al., 2000). In the human field, it has developed into sophisticated methods correcting for secondary effects like fluctuations in respiratory volume (Birn et al., 2006, 2008) or cardiac rate (Shmueli et al., 2007).

This study is – to the best of our knowledge – the first study that systematically investigates the contributions of physiological noise sources to the rs-fMRI BOLD signal in the rat.

Motion and respiration related fluctuations

We found in our data strong correlations of the rs-fMRI signal to the respiratory time course as well as to the motion parameters extracted from the preceding motion correction. Moreover, cross-correlation of the regressors revealed a tight coupling of the phase-encoding displacement in the motion parameters to the respiratory time course as well as to the motion parameters. A comparison of these results to those obtained from the original data revealed that motion correction and subsequent regression significantly reduced physiological artifacts, enhanced the specificity of seed based functional connectivity maps and increased the stability of the obtained connectivity measures.
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these artifacts are increasing with field strength and proximity of the imaging volume to the chest (Raj et al., 2000), this mechanism is especially important for high-field fMRI in the rat and may explain our findings.

In addition, we observed a motion component with a very distinct frequency behaviour that we could eventually assign to the aliased cycle of the cryo cooler system of the magnet. We suspect that the recoil of the helium pump leads to subtle vibrations of the magnet which translate into apparent motion in phase-encoding direction of the cryo cooler system of the magnet. We therefore conclude that regression of these parameters for physiological noise removal in the rat is not essential. Comparable results can be obtained by regression of low order drift terms, which can be implemented without additional physiological recordings.

The effects of cardiac pulsatility and heart rate on fMRI in the human brain have been described along with appropriate correction strategies (Dagli et al., 1999; Glover et al., 2000; Shmueli et al., 2007). We found that cardiac related signal fluctuations played only a minor role in our data, the more so as they merely affected the very base of the brain which is of less interest for connectivity studies.

Linear regression

For the sake of simplicity we chose to test for a strictly linear relation between the considered physiological waveforms and the rs-fMRI signal. It might, however, be beneficial for the noise reduction to process these waveforms prior to regression using a specific transfer function, e.g. histogram equalization as employed in the RETROICOR approach (Glover et al., 2000).

The fact that regression of the respiratory waveforms – despite their high correlation to the rs-fMRI signal – only marginally contributes to the explained variance can be addressed to the order of the regression process. Due to the tight coupling of (apparent) motion and respiration, the gross of respiratory related fluctuations will have already been removed in the preceding regression of the motion parameters.

Impact of physiological noise on connectivity analysis

The observed signal fluctuations related to motion and respiration severely affect the results of the functional connectivity analysis. They produce apparent connectivity – positive or negative – between regions that share common physiological noise behavior instead of true functional connectivity.

Fig. 5. Individual Impact of Physiological Noise on Functional Connectivity Results. T2 weighted anatomical and EPI images with indicated seed regions (A) and corresponding position of acquired slices relative to Bregma (B). Seed based functional connectivity maps of S1 / S2 cortical regions (C, different datasets), Thalamus (Th) and Caudate Putamen (CPu) regions (E, same dataset) and their corresponding pairwise correlation matrices (D, F) obtained from the original data and after correction. Seed regions and connectivity maps are overlaid on the original EPI images. Connectivity matrices show plain correlations top right and partial correlations (whole brain signal removed) bottom left of the diagonal; numbers reflect the actual correlation coefficient (sign skipped for readability). Removal of physiological artifacts (false positive correlations) in connectivity maps, e.g. in cortex (C) or thalamus (E), corresponds to an improved specificity in connectivity matrices (D, F). Only minor improvement was observed for interhemispheric connectivity of CPu regions (F), which exhibited the most pronounced coupling of all the regions.

Fig. 6. Overall Impact of Physiological Noise on Functional Connectivity Results. Average and standard deviation of all 27 individual connectivity matrices (Fig. 5) obtained from original (top) and corrected data (bottom). Although no pronounced effect is observed for the average over all connectivity matrices, their stability is significantly improved by applying the correction scheme as visible in comparison of the corresponding standard deviations.

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The presented correction strategy was able to significantly reduce physiological fluctuations in the rs-fMRI time course and thereby remove related artifacts in the obtained connectivity maps, which - post correction – reveal the typical patterns of homotopic, interhemispheric connectivity. These were initially reported by Biswal et al. (Biswal et al., 1995) and were observed in recent studies of rs-fMRI in the rodent (Majeed et al., 2009; Pawela et al., 2008; Zhao et al., 2008). However, as the severity of the described artifacts strongly varied between subjects and sessions, we believe that the removal of these physiological confounders is a prerequisite to detect subtle changes in connectivity as an effect of pathological processes. In our data, correction increased specificity as well as reproducibility of connectivity obtained, opening new approaches to analyze also group-averaged connectivity conditions with high reliability.

Global signal regression and temporal filtering

Many studies of functional connectivity employ a regression of the average signal within the brain to increase specificity of the obtained connectivity measures. It has been shown, however, that global signal regression introduces negative correlation to fcMRI data (Murphy et al., 2009; Weissenbacher et al., 2009). We have – without global signal removal - observed negative correlations only in individual cases (e.g. between thalamus and caudate putamen; see Fig. 5) that disappear in the group average. Partial correlations between somatosensory cortex and caudate putamen in contrast clearly remain negative also in the group average.

As this study was focused on physiological confounders rather than the actual connectivity networks, a more detailed discussion of this issue is beyond the present scope and it could only contribute little to the ongoing debate on whether negative correlations can be congruously interpreted as anticorrelated neuronal networks. It should be pointed out, however, that animal studies do have plenty of potential to shed light on this issue by allowing e.g. pharmacological perturbations or invasive methods.

Looking at global signal regression as a strategy for physiological noise removal, one must acknowledge that the spatial correlation patterns we observed for respiratory and motion regressors are highly inhomogeneous, containing likewise positive and negative correlations. This finding implies that global signal regression on its own is not capable of removing these crucial physiological artifacts.

Another post-processing strategy frequently applied in functional connectivity analysis is low pass or band pass temporal filtering of the rs-fMRI signal. It removes high frequency Gaussian noise and in turn emphasizes low frequency fluctuations around 0.1 Hz. We found that the frequency bands contained in our respiratory and cardiac regressors are relatively broad. Due to variations and high undersampling of the respiratory rate, especially the position of the respiratory band varies from subject to subject and may interfere with the low frequency fluctuations of interest. Hence, it is clear that plain low pass or band pass filtering cannot remove these physiological fluctuations, a conclusion earlier reached also for fMRI of the macaque (Teichert et al., 2009). Some approaches using band stop filters selective to frequencies of physiological relevance have been successfully applied (Biswal et al., 1996; Buonocore and Maddock, 1997; Deckers et al., 2006). These techniques are computationally less demanding compared to linear regression, but require comparable physiological data (and may still interfere with the low frequency spectrum).

None of the two techniques – global signal regression or temporal filtering – is appropriate to remove physiological fluctuations in our undersampled rs-fMRI data. We therefore decided (except for the complementary partial correlation in connectivity matrices) not to include these steps in the present study in order to show results “as raw as possible”. When focus is more on the actual connectivity results, however, these steps could be easily integrated to complement the presented correction scheme.

Animal experimental protocol

For our study of resting-state fMRI we have chosen a protocol employing Medetomidine sedation (Weber et al., 2006), an α2-adrenoceptor agonist that is becoming increasingly popular in fMRI of the rodent (Adamczak et al., 2010; Pawela et al., 2009b; Zhao et al., 2008). It allows for non-invasive experiments producing light sedation and robust activation and it has several advantages compared to α-Chloralose. In particular it is suitable for longitudinal studies, which is an advantage in studies of disease progression, recovery or therapy. It is evident that choice and depth of anesthesia directly affect functional connectivity as has been shown in humans (Peltier et al., 2005) and rats (Austin et al., 2005) and very recently been studied in a comparison of Isoflurane, Medetomidine and α-Chloralose (Williams et al., 2010). The choice of anesthetic has thus to be carefully considered in the design of studies and interpretation of their results.

Based on the sources of physiological noise identified, we speculate that the choice of anesthetic does not have a direct effect on the rs-fMRI signal sensitivity to physiological noise. However, through its effect on physiological parameters, e.g. shape, depth and rate of respiration, the anesthetic may influence to which extent physiological noise is present in the data. The contributions and characteristics of physiological noise may also depend on other experimental conditions such as the subject’s bedding (prone vs. supine), positioning and fixation.

Conclusions drawn from our results are based on data acquired in spontaneously breathing animals, whereas in fMRI studies employing α-Chloralose and some using Medetomidine (Pawela et al., 2008, 2009a,b), animals are artificially ventilated and paralyzed using muscle relaxants. The biophysical origin of respiration related signal changes, however, remains valid and it can thus be expected that – due to the absolutely constant ventilation rate and shape – affected frequencies in the rs-fMRI signal spectrum are more narrow and predictable. Subject-to-subject variations in the contribution of respiratory physiological noise might be decreased in mechanically ventilated animals, if ventilation rate and volume are comparable. Artificial ventilation does also enable synchronization of MR acquisition to the ventilator, in order to reduce respiration related fluctuations and limit them to certain frequency bands.

Successful BOLD activation studies critically depend on a physiologically well-controlled partial pressure of CO2 (pCO2) in the blood. As CO2 is a potent vasodilator, hypercapnia alters the hemodynamic response to electrical activity and can thereby not only diminish the stimulus-related BOLD activation but potentially affect the detectability of synchronized resting state fluctuations. The employed protocol of Medetomidine sedation has been carefully validated regarding stability of blood gas parameters in the past (Ramos-Cabr et al., 2005; Weber et al., 2006) and can also be adapted for long experiments (Pawela et al., 2009b). Even though we did not measure pCO2, we therefore assume that pCO2 was in a reasonable range throughout our experiments. This is supported by the fact, that BOLD activation was observed in all animals (electrical forepaw stimulation performed in all sessions; data not shown) and that blood oxygen saturation was stable throughout the experiments indicating normal respiratory function.

Apart from this, fluctuations in pCO2 are generally slow and – except through their effect on respiratory regulation – unlikely to affect the fMRI signals’ sensitivity to physiological noise.

It should be noted that the oxygen concentration in the supplied gas mixture, in contrast, might have an impact on the aforementioned sensitivity. Since oxygen is paramagnetic, its intrapulmonary concentration determines the level of respiratory related susceptibility.
changes. They, in turn, are responsible for the majority of observed signal fluctuations.

EPI acquisition

Most BOLD fMRI studies in the rodent – even at high fields – employ gradient recalled EPI which has a higher sensitivity, though lower specificity, to functional activation. It is likely that the use of spin echo EPI will exhibit different, probably lower, susceptibility to physiological fluctuations. Especially the observed respiratory contributions may be reduced in SE-EPI, assumed that they mainly stem from field variations related to thorax motion in the respiratory cycle (Raj et al., 2000). A transition from single-shot to multi-shot EPI can be expected to change the characteristics of physiological noise in rs-fMRI time course and would require more sophisticated preferably k-space based correction strategies (Hu et al., 1995; Wokk et al., 1997).

An important point that was not covered in this study is the use of navigator echoes (Hu and Kim, 1994). These techniques employ a phase correction based on a data snippet collected shortly before the actual EPI acquisition. As the majority of fluctuations most likely originates from frequency and phase changes, the use of navigator echoes can reduce physiological noise and some of the hardware related fluctuations in the fMRI signal (Barr et al., 2008). We therefore conducted first pilot experiments employing an EPI navigator in resting state acquisition. We found, however, that it did not distinctly reduce the respiration related fluctuations in single-shot GRE EPI. Nevertheless, the potential of this technique in application to fMRI and rs-fMRI of the rodent has to be further investigated.

Temporal sampling

Most functional connectivity studies in the rodent utilize BOLD EPI at repetition times of 1.5 s (Lu et al., 2007) to 2 s (Biswal and Kannrppati, 2009; Pawela et al., 2008; Zhao et al., 2008) to acquire rs-fMRI data, which is comparable to repetition times commonly used in human studies. There are, however, large differences in the respective physiological cycles: Respiratory and cardiac rate in the rat are ~70 / 300 per minute compared to ~10 / 60 in human adults. This implies a strong undersampling of the respiratory and cardiac cycles in the rat, and signal fluctuations related to these sources will therefore be aliased into spectral bands whose frequency and width delicately depend on the exact physiological frequency and its stability.

Recently, there have been approaches using very short repetition times of 100 ms (Majeed et al., 2009; Williams et al., 2010) in spontaneously breathing rats, therefore being able to resolve the respiratory and even cardiac frequencies. This enables removal of related fluctuations by simple temporal filtering, however with the drawback of low signal to noise and high demands on the hardware that also limits the number of acquirable slices.

For studies conducted in spontaneously breathing animals, approaches based on respiratory gating may also be considered in order to reduce respiratory related physiological noise.

Field strength

It has been shown that the contribution of physiological noise to the rs-fMRI signal fluctuations increases with magnetic field strength B₀, just as the signal increases (Triantafylloff et al., 2005). This includes spontaneous fluctuations due to neuronal activity on the one hand, but, on the other hand, non-neuronal physiological fluctuations increase as well (Raj et al., 2000). We have recently compared fMRI in the rat at 7.0 and 11.7 Tesla with identical protocols and were not able to verify the theoretically expected increase in BOLD activation (Seehafer et al., 2010). In this context and with respect to the results presented here it is worthwhile to investigate how field strength affects the individual noise components and under which conditions the gain in sensitivity from even higher fields can be maximized in view of the increasing contribution of physiological noise (Bodurka et al., 2007).

Conclusion

In the present study we have investigated physiological noise in rs-fMRI of the rat at ultrahigh field and its potential impact on functional connectivity analysis. Our results demonstrate that respiration related signal fluctuations are grave and can seriously compromise functional connectivity results. The proposed correction scheme of motion correction and subsequent regression of physiological recordings was successful in removing the majority of the underlying physiological artifacts and thus significantly increased specificity and reproducibility of functional connectivity measures obtained from rs-fMRI data. Furthermore, our results indicate that – due to coupling of respiration to apparent motion – standard motion correction and subsequent regression of the motion parameters are capable of removing the gross of respiratory noise from the data. Implementation of these steps does not even require separate physiological recordings and is therefore recommended as a minimum.

We conclude that the awareness for and appropriate treatment of physiological noise in high field rs-fMRI of the rodent is essential to prevent misinterpretation and to increase reproducibility of the corresponding functional connectivity measures. Strategies to reduce the compromising contribution of physiological noise – both on the acquisition and post-processing side – must be explored and applied to fully exploit the potential of fcMRI, which only then can reliably grant insight into physiological and pathological processes affecting brain connectivity.

Acknowledgments

This work was supported in part by grants from the EU 7th framework program (ENCITE; HEALTH-F5-2008-201842) and from the German Ministry of Education and Research (BMBF Biomarkers of Ageing 0314104).

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