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Comparing BOLD fMRI signal changes in the awake and anaesthetized rat during electrical forepaw stimulation

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1. Summary

The difference between awake curarized and alfa-chloralise anaesthetized animals was studied with respect to the BOLD signal response in an fMRI experiment. By studying the activation of the cortex upon electrical forepaw stimulation in the same rat, but following consecutively applied curarization and alpha-chloralose anaesthesia protocols, it was possible to compare quantitatively the effect of both immobilisation protocols on the fMRI data. The largest BOLD signal change as a result of forepaw stimulation was found in the awake condition, however the activated areas are less specific than those in the anaesthetized state leaving it more difficult to interpret.

2. Introduction

Most functional Magnetic Resonance Imaging (fMRI) studies are using Blood Oxygenation Level dependent (BOLD) contrast, which is sensitive to changes in the proton relaxation times in the neighbourhood of blood vessels[1]. BOLD fMRI allows one to study the active brain during external stimulation or upon performing specific tasks. The BOLD contrast upon neural activation displays consecutive opposite responses. The early negative response[2,3] reflecting the drop in oxygen content and the late response, which is the overshoot of oxygen supply resulting in a positive signal change. Although the signal changes of the delayed response may be higher than those of the early response (3.4% against 0.4% at 1.5T[3]), it is still necessary to have a high overall signal stability and signal to noise ratio (SNR).

Animal studies do provide an excellent tool to study both fMRI phenomena and the underlying mechanisms in brain functioning (e.g. comparing fMRI-results with more invasive techniques). While there are many differences between human and animal (rat) studies, the signal change is based on the same principle. Nevertheless it is known that for the same field strength the signal changes in humans are usually more significant. This is mainly due to the larger volume of the

activated areas in human studies as compared to rat studies. Our objective was to obtain an as high as possible signal change in animal fMRI studies. To reach this goal we used several means: 1. We made use of a high main field strength, resulting in a higher SNR and a higher BOLD signal change[4]. The precise mechanisms of the BOLD contrast and the size of the contribution of different sizes and types of blood vessels to the BOLD signal change isn't yet perfectly understood[5,6], but it is commonly assumed that the contribution of the capillaries to the BOLD effect becomes more important at higher main fields[4] . 2. Another likely reason for the different signal change in humans is the state of consciousness during the experiment. The anaesthetics used in animal studies, definitely lower both the basal brain activity and the brain activation during stimulation[7]. However not all anaesthetics will have the same effect on brain activity and metabolism. In most rat fMRI studies anaesthetics are used that have the least disadvantageous effects on the brain activity, like alpha-chloralose[8,9] and propofol[10,11]. But it is expected that a difference remains in the stimulus-evoked activation in awake, conscious animals compared to anaesthetized animals[11,12]. To investigate this, different approaches can be used. The animals can be trained not to move in the magnet while performing the fMRI experiments[13], but this is not possible in all experiments and with all animal species. Another technique is to avoid involuntary movement during the fMRI experiment[11,14] by tightly restraining the animal. However this approach also has some pitfalls, since the slightest movement is a major snare to fMRI studies the positioning of the animal has to be very tight. Immobilising the rat by paralysing the animal during the experiment is another possible solution, muscle relaxants can be administered such that the animal remains fully conscious but that no movement is observed[15].

In this study we compared the BOLD signal changes in the rat cerebral cortex as a result of a simple forepaw stimulation paradigm, observed consecutively in the conscious curarized state and in the alpha-chloralose anaesthetized state.

3. Materials and methods

3.1. Animal preparation

Adult Wistar rats (n=6, 200-300g) were initially prepared for the experiment during halothane anaesthesia. Sedation was initiated with 4 minutes exposure to 5% halothane (Fluothane, Zeneca, Belgium) in a 3/7 O_2/N_2O mixture. The animal was injected with atropine (20 µg/kg, Federa, Belgium) to avoid excessive salivation. Subsequently the animal was intubated with a plastic radiopaque catheter of 16GA (1.7X45 mm) covered with lidocaine (Xylocaine, Astra Pharmaceuticals, Belgium) to avoid irritation of the respiratory tract. Halothane anaesthesia dosage was lowered to 1.5%. The animal was catheterised through the tail vein to enable intravenous (i.v.) administration of the paralyzing agent mivacurium (Mivacron, GlaxoWellcome, Belgium) or the anaesthetic alpha-chloralose (Acros Organics, Belgium). Copper wires were wrapped round the right forepaw, the left hindpaw and the tail for ECG monitoring during the experiment (normal heartrate approx 300 bpm). A temperature probe was inserted rectally and the temperature of the rat was kept constant (at 37.5 ± 0.5 °C) with a warm water blanket connected to a heating pump (Gaymar, UK). A piezo-electric transducer strip was attached behind the rat's neck for monitoring respiration and other involuntary movement during the imaging experiment. The animal was fixed in a plexi stereotactic head holder, consisting of an incisor bar and blunt earplugs, enabling accurate positioning within the magnet and immobilization of the animal. After the insertion of the animal in the magnet, the respirator was switched on (70 breaths per minute). After tuning and shimming an initial dose of mivacurium was injected (0.5 mg/kg), and a maintenance dose of 3mg/kg/hr. was continued during the first stage of the experiment. In the second stage of the experiment mivacurium administration was stopped, and a bolus of alpha-chloralose was injected (60 mg/kg i.v.). The experimental procedures were approved by the Ethical Committee of the University of Antwerp, in accordance with Federal laws.

3.2. MRI

MR-imaging was performed at 300 MHz on a SMIS MR microscope (SMIS, Guilford England) with a horizontal 7T magnet and 8 cm aperture self-shielded gradient coils with a gradient strength of 0.1 T/m (Oxford Instruments, England). A circular RF surface antenna with integrated carbon EEG electrodes[16] of 16 mm diameter was placed on top of the skull and was used for both transmitting and receiving the MR signal. To image the cerebral cortex, the surface RF antenna was positioned with its center approximately 1mm posterior to the bregma. Twelve coronal images, with a slice thickness of 1mm, were taken from 4 mm anterior to 7 mm posterior to the bregma. All images were acquired with a multislice gradient echo (GE) sequence. After tuning and shimming (^{1}H-linewidth of approx. 40 Hz), coronal and horizontal images were taken, with a Field Of View (FOV) of 20 mm, a gradient echo time (TE) of 6 ms, a repetition time (TR) of 400 ms and an acquisition matrix of 256x128 data points which were used as the underlying anatomical images. Two averages were taken and all images were reconstructed after zerofilling of the acquisition matrix to 256x256 data points. Functional imaging was performed at the same position of the high-resolution images. The GE-sequence was T_2^*-weighted (TE = 14 ms, slice thickness = 1 mm, FOV = 20 mm, image acquisition matrix = 128x64, TR = 400 ms, 2 averages, flip angle = 90°), consisting of 6 consecutive slices, with their position chosen on the high resolution anatomic slices. The total acquisition time for one fMRI image set was 51.2 s.

3.3. EEG recording

Simultaneously to the fMRI experiment, electroencephalographic recordings (EEG) were obtained with a sample rate of 1000 Hz using a non magnetic EEG preamplifier (Schwarzer, Germany), to verify for differences in brain activity between the awake and the anaesthetized state of the rat during the fMRI aqcuisitions. We made use of a special RF surface antenna with integrated EEG carbon electrodes[16]. The obtained EEG signals were processed with Brainlab software (OSG, Belgium).

3.4. Stimulation protocol

Forepaw stimulation was accomplished by inserting two needle electrodes between two toes of the forepaw. Square electrical pulses (WPI stimulator, Sarasota Florida USA) were delivered with a frequency of 2.5 Hz and a duration of 0.5 ms, resulting in a duty cycle of 0.125 %. The current amplitude was set at 0.6 mA. The fMRI protocol for all the animals consisted of 20 imagesets in the following cycle: 2 dummy images, 6 images pre-stimulation, 6 images with stimulation of the left forepaw, 6 images post-stimulation, resulting in a total acquisition time of 17 min. 40 sec.. This fMRI protocol (2-6-6-6) was used for both the mivacurium curarized and the alpha-chloralose anaesthetized state of the rat.

3.5. Data analysis

fMRI data

The fMRI data were analysed off-line using routines in MEDx (Version 3.0, Sensor Systems, Inc, Sterling, USA) and custom developed routines and procedures in IDL (Interactive Data Language, RSI, Boulder Colorado, USA). The following steps were taken to obtain the activation maps: Motion detection, the motion occurring in the time series was generally found to be at subpixel level (<1/4 pixel). If the motion was more severe the dataset was discarded. Filtering the data with a 3 x 3 pixel Gaussian convolution filter for noise reduction of the images. An unpaired t-test comparison of the “stimulation” and “non stimulation” images resulting in statistical t-value maps. The final activation maps were obtained by overlaying the thresholded ($t = 2$, $v=16$, $p=0.05$) t-value maps on the corresponding high resolution images, resulting in images displaying in colour those regions for which the signal intensities are significantly different between the datasets acquired with and without stimulation.

Different regions of interest (ROI’s) were marked on the high resolution maps and are shown in the drawing of figure 6.1. The following three ROI’s were taken: ROI 1 is the specific contralateral forepaw area as displayed by Paxinos and Watson[17], ROI 2 is the entire contralateral somatosensory cortex including ROI 1 and ROI 3 is the ipsilateral somatosensory cortex. The

volume of the activated areas in these ROI's was measured by counting the number of activated pixels in the different slices and multiplying this number with the voxel size.

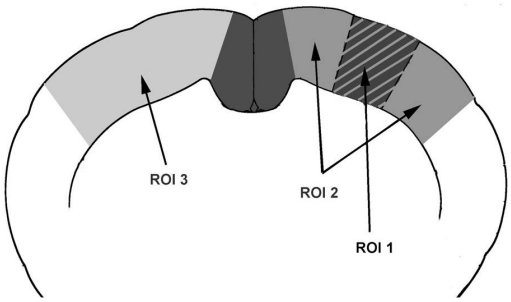

Figure 6.1: Schematic drawing of the different regions of interest which are used for volume determination of the activated areas displayed in table 1. ROI 1: contralateral forepaw area of the somatosensory cortex, ROI 2: contralateral side of the somatosensory cortex, ROI 3: ipsilateral side of the somatosensory cortex.

EEG data

To reduce artefacts in the EEG trace due to the switching of the magnetic field gradients during an MRI acquisition the EEG data obtained were filtered using a special designed adaptive filtering scheme[18]. After filtering, differences in EEG data acquired at different states of consciousness of the rat were visually evaluated.

4. Results

4.1. Overall signal change

Brain activation was observed in both the anaesthetized and curarized state of the rat, with the BOLD signal change the largest at the contralateral side of the somatosensory cortex after electrical stimulation of the left forepaw. Figure 6.2a displays coronal activation images of a rat curarized with mivacurium while figure 6.2b demonstrates activated regions of the same rat anaesthetized with alpha-chloralose. The curarized state is providing more pronounced BOLD signal changes and larger areas of activation than the anaesthetized state. Comparing both conditions it is observed that higher signal changes are observed in both the contralateral (including the specific forepaw area[17,19]), as in the ipsilateral somatosensory cortex. But the differences of activated areas between both states of consciousness is especially large in the non-specific areas (mainly the ipsilateral side of the somatosensory cortex) of the rat brain.

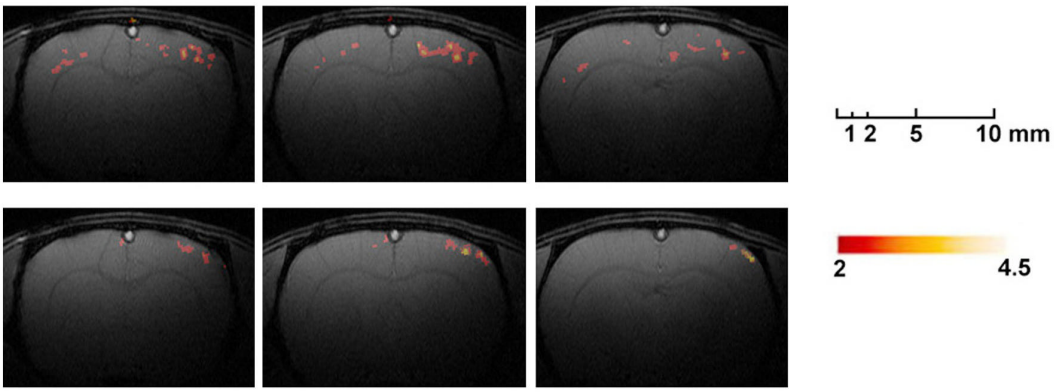

Figure 6.2: Cerebral activation maps of three consecutive coronal slices resulting from electrical stimulation of the left forepaw of the rat in the curarized (a) and alpha-chloralose anaesthetized (b) state. Activation is shown as coloured spots superposed on the corresponding anatomical gradient echo images. All activations were computed from a functional imageset of 20 images and are shown in a colour code representing t-values ranging from 2.0 (red) to 4.5 (yellow).

4.2. Specific signal changes

When comparing the volumes of the activated areas in different regions of interest in Table 6.1 (ROI's are shown in figure 6.1) in all 6 rats in both states of consciousness the following is observed: the curarized state displays an overall higher volume of activated areas as compared to the anaesthetized state. Moreover, the interanimal variation is also higher for the curarized than for the anaesthetized state. A large variation of the activated volumes between the different rats was observed in the different ROI's in the curarized state, whereas the variation of the activated volumes in the anaesthetized state is much smaller. The data in the table clearly shows that the mean activation area is larger in the curarized animal than in the anaesthetized animal, both for the specific and non-specific areas.

	Awake			**Anaesthetized**		
	ROI 1	**ROI 2**	**ROI 3**	**ROI 1**	**ROI 2**	**ROI 3**
Rat 1	3.100591	9.326186	2.392582	1.879886	2.734379	1.48926
Rat 2	0.830079	1.831058	1.977542	1.782229	2.783207	1.049806
Rat 3	1.464846	2.685551	0.976564	1.855472	2.31934	0.53711
Rat 4	6.323252	9.790054	4.028327	2.758793	4.541023	1.635745
Rat 5	4.125983	5.493173	5.786142	1.782229	2.905278	1.513674
Rat 6	1.48926	2.416996	2.709965	0.781251	1.538088	0.219727
Mean	2.889002	5.25717	2.97852	1.806643	2.803552	1.07422
SE	0.848442	1.455767	0.693062	0.256057	0.402915	0.238043

Table 1: Individual and mean activated volumes as calculated for the three different ROI's shown in figure 1 for 6 different rats.

4.3. EEG during the fMRI protocol

Two EEG traces of the same rat obtained during the fMRI experiment are shown in the curarized (figure 6.3a) and the anaesthetized state (figure 6.3b). The spontaneous EEG of the curarized animal (figure 6.3a) is comparable to the normal EEG of an awake rat, i.e. having characteristics of both an active and a resting animal. When a rat is exploring, the EEG shows desynchronization consisting of small amplitude and theta activity. In the wakeful resting state the theta activity is not present. The EEG trace of alpha-chloralose anaesthetized animals contains high-amplitude transients superimposed on apparently normal background activity and possibly indicative of alpha-chloralose evoked irritation. These traces with the high amplitude transients shown in figure 6.3b are specific for alpha-chloralose anaesthetized animals and reported in other studies[20]. Except for these alfa chloralose specific differences the overall EEG signal displays the same normal background activity of a rat being asleep, which EEG pattern is characterised by the signal changing from small amplitude fast activity into large amplitude slow activity. No difference is observed in the EEG traces between the activated and the resting state in both states of consciousness.

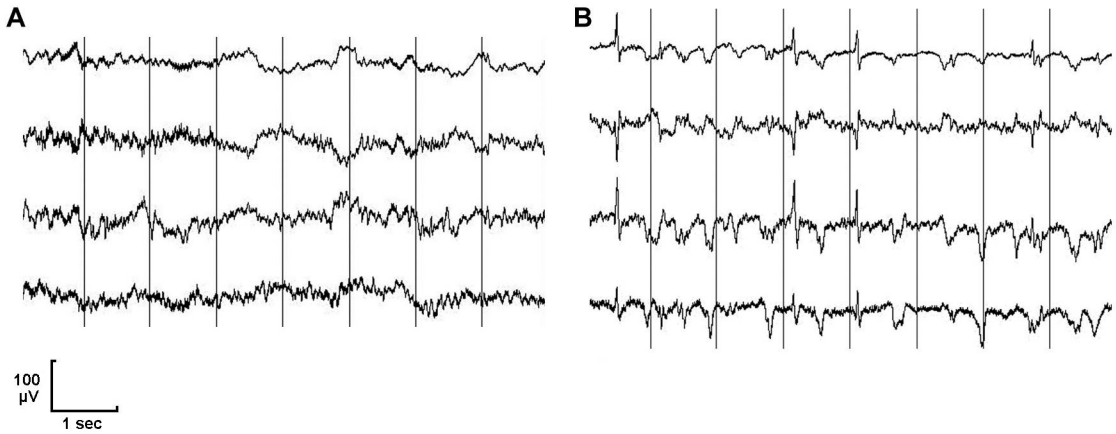

Figure 6.3: Filtered EEG traces acquired during fMRI acquisition of the curarized (a) and the alpha-chloralose anaesthetized (b) state. The EEG signals resort from 4 non-invasive carbon electrodes positioned on the head of the rat.

5. Discussion:

The observed regions of signal elevation in the cortex as a result of electrical stimulation of the left forepaw corresponded to the front regions in the rat cortex[19] and to areas of activation described in other fMRI studies[8,21]. Simultaneously acquired EEG data were used to discern the differences in overall brain activity between the curarized and anaesthetized states. The EEG signal in the anaesthetized state resembled typically EEG traces measured in alpha-chloralose anaesthetized rats[20], while the signal in the awake state was similar to the underlying signal in the anaesthetized state.

In both the curarized and the anaesthetized states of the animal, a change in signal intensity was found in the contralateral somatosensory cortex as a result of forepaw stimulation. Although the BOLD signal change in the curarized state was generally higher than in the alpha-chloralose anaesthetized state, it was also clear that more non-specific areas were activated in the curarized state as the activation was more widespread. Using a different fMRI paradigm and a higher stimulation current, we still observed the same pattern of higher and more widespread areas of activation in the curarized animals in comparison to anaesthetized animals (data not shown). The results of these experiments were in accordance with Lathi and coworkers[11] who also found a higher and more widespread signal change in the awake animal in contrast to the propofol anaesthetized animal.

Observe also the difference in position (minimal overlap) in the activated areas between both states, where the anaesthetized animal shows a more superficial activation. It is shown by Grune and coworkers[21] that the position of the observed activated areas depends on the echo time used in the BOLD experiment, the maximal signal change is observed at a TE which equals the T_2* time of the tissue. The T_2* value of the tissue is not homogeneous in the entire rat cortex, it is smaller at the surface and larger in the deeper layers. This effect is a result of the larger susceptibility effects at the surface (air-skull-brain passage) and the venous organisation in the cortex. If the animal is anaesthetized the CBF and CBV-values are lower in comparison to an awake animal, resulting in a higher T_2* value of the cortex[22]. Performing fMRI experiments in both conditions with the same echo time can result in different positions of the observed activated areas.

Because cortical activation was studied in the same rat using consecutively applied anaesthesia protocols, we can exclude other causes than the type of anaesthesia for the difference in activated areas. The pharmacological effects of mivacurium and atropine will have no large influence on the observed signal changes in the awake state. Rat fMRI studies have been conducted using a combination of alfa-chloralose anaesthesia and mechanically ventilated paralyzed animals[21,23], while other fMRI studies use only anaesthetised rats[8]. No differences in results have been reported between both types of protocols. Atropine which is a potent parasympatholytic with a medical half-life of 2.5 h. was used in a very small dose. At small doses it inhibits salivary secretions and has no clear effect on the vagal system or nervous system. In spectroscopic imaging studies atropine is also used with no reported effects on CBF changes[24].

Being conscious the rat perceives additional stimuli and impressions beyond the stimulus applied to its forepaw. The signal change observed in the awake state may therefore have contributions of effects secondary to the experiment like stress, discomfort and a higher state of arousal. Although great care was taken to make the experimental conditions as comfortable as possible for the rat, we suspect that the firm stereotaxic positioning, which prevents the movement of the rat, may cause the awake rat discomfort and stress. The loss of voluntary muscle control is also a very significant source of stress, as is observed in humans[25], therefore we expect the paralyzation induced by the mivacurium to be an important extra stressor. Moreover the repetitive noise of the gradient switching during the functional experiment, which generates noise strength up to about 110 dB[26] in the middle of the magnet bore, undoubtedly will affect the awake animal much more than the anaesthetized animal. It has been shown in several human fMRI studies that the noise of the gradients during an fMRI experiment can result in effects on the auditory cortex[27] and on other areas such as the motor and visual systems[28,29]. Even in rat fMRI experiments acoustic noise has been observed to increase the signal intensity change in the somatosensory cortex[30].

In table 6.1 and figure 6.2 we also observed small ipsilateral activation especially in the awake state. Although bilateral activation following forepaw stimulation is not expected, it is also reported in other fMRI studies[11,23], addressing the signal change to partial ipsilateral activation following electrical stimulation. FMRI studies of painful stimuli reported both bilateral activation in human brain and in the rat somatosensory cortex following noxious stimuli[31]. In the awake state the electrical stimulation will result in a higher feeling of discomfort or pain from the

stimulated paw, therefore giving rise to a higher bilateral activation compared to the anaesthetised case.

All these previously described factors can play an important role in the fMRI response of the rat brain in the awake state resulting in a more complex activation pattern, while in the anaesthetized animal the role of these factors will be significantly lower. Lathi et al.[11,14] also suggested a higher complexity of the signal change in awake rats. But the difference between both studies is that in their experiments, the rats were immobilised by firm stereotactical restraining instead of being curarized. As a consequence there is the extra factor of the pain and stress induced by the restrainment for their animals, resulting in an even more complex activation signal.

6. Conclusion

Awake but curarized animals have a higher, more widespread BOLD signal change following forepaw stimulation, but the more widespread, non-specific activation makes the interpretation much more difficult. Definitely the problems and difficulties for interpretation that will be encountered using awake animals will mostly outweigh the advantages of the higher signal change.

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