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TITLE:

Functional Magnetic Resonance Spectroscopy at 7 T in the Rat Barrel Cortex During Whisker Activation

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KEYWORDS:

Cerebral activation, whisker stimulation, brain metabolism, *in vivo* ^{1}H-MRS, LCModel, rat, lactate, BOLD fMRI

SUMMARY:

After checking by blood-oxygen-level-dependent functional magnetic resonance imaging (BOLD fMRI) that the corresponding somatosensory barrel field cortex area (called S1BF) is correctly activated, the main goal of this study is to quantify lactate content fluctuations in the activated rat brains by localized proton magnetic resonance spectroscopy (^{1}H-MRS) at 7 T.

ABSTRACT:

Nuclear magnetic resonance (NMR) spectroscopy offers the opportunity to measure cerebral metabolite contents *in vivo* and noninvasively. Thanks to technological developments over the last decade and the increase in magnetic field strength, it is now possible to obtain good resolution spectra *in vivo* in the rat brain. Neuroenergetics (*i.e.*, the study of brain metabolism) and, especially, metabolic interactions between the different cell types have attracted more and more interest in recent years. Among these metabolic interactions, the existence of a lactate shuttle between neurons and astrocytes is still debated. It is, thus, of great interest to perform functional proton magnetic resonance spectroscopy (^{1}H-MRS) in a rat model of brain activation and monitor lactate. However, the methyl lactate peak overlaps lipid resonance peaks and is

difficult to quantify. The protocol described below allows metabolic and lactate fluctuations to be monitored in an activated brain area. Cerebral activation is obtained by whisker stimulation and ^{1}H-MRS is performed in the corresponding activated barrel cortex, whose area is detected using blood-oxygen-level-dependent functional magnetic resonance imaging (BOLD fMRI). All steps are fully described: the choice of anesthetics, coils, and sequences, achieving efficient whisker stimulation directly in the magnet, and data processing.

INTRODUCTION:

The brain possesses intrinsic mechanisms that allow the regulation of its major substrate (*i.e.*, glucose), both for its contribution and its utilization, depending on variations in local cerebral activity. Although glucose is the main energy substrate for the brain, experiments performed in recent years have shown that lactate, which is produced by the astrocytes, could be an efficient energy substrate for the neurons. This raises the hypothesis of a lactate shuttle between astrocytes and neurons[1]. Known as ANLS, for astrocyte-neuron lactate shuttle[2], the theory is still highly debated but has led to the proposal that glucose, rather than going directly into neurons, may enter the astrocytes, where it is metabolized into lactate, a metabolite that is, then, transferred to the neurons, which use it as efficient energy substrate. If such a shuttle exists *in vivo*, it would have several important consequences, both for the understanding of basic techniques in functional cerebral imaging (positron emission tomography [PET]) and for deciphering the metabolic alterations observed in brain pathologies.

To study brain metabolism and, particularly, metabolic interactions between neurons and astrocytes, four main techniques are available (not including micro-/nanosensors): autoradiography, PET, two-photon fluorescent confocal microscopy, and MRS. Autoradiography was one of the first methods proposed and provides images of the regional accumulation of radioactive ^{14}C-2-deoxyglucose in brain slices, while PET yields *in vivo* images of the regional uptake of radioactive ^{18}F-deoxyglucose. They both have the disadvantage of using irradiative molecules while producing low-spatial resolution images. Two-photon microscopy provides cellular resolution of fluorescent probes, but light scattering by tissue limits the imaging depth. These three techniques have been used previously to study neuroenergetics in rodents during whisker stimulation[3-6]. *In vivo* MRS has the dual advantage of being noninvasive and nonradioactive, and any brain structure can be explored. Moreover, MRS can be performed during neuronal activation, a technique called functional MRS (fMRS), which has been developed very recently in rodents[7]. Therefore, a protocol to monitor brain metabolism during cerebral activity by ^{1}H-MRS *in vivo* and noninvasively is proposed. The procedure is described in adult healthy rats with brain activation obtained by an air-puff whisker stimulation performed directly in a 7 T magnetic resonance (MR) imager but may be adapted in genetically modified animals, as well as in any pathological condition.

PROTOCOL:

All animal procedures were conducted in accordance with the Animal Experimentation Guidelines of the European Communities Council Directive of November 24, 1986 (86/609/EEC). The protocol met the ethical guidelines of the French Ministry of Agriculture and Forests and was

approved by the local ethics committees (Comité d'éthique pour L'expérimentation Animale Bordeaux n°50112090-A).

NOTE: During the MR measurements, an adequate level of anesthesia and physiological monitoring (body temperature, respiratory rate) are indispensable requirements.

1. Animals

1.1. Use male Wistar rats weighing between 350 and 450 g.

1.2. Keep them on a 12:12 h light:dark cycle and provide food and water *ad libitum*.

2. Anesthesia

2.1. Prepare the equipment needed for anesthesia (**Figure 1A,B**, see **Table of Materials**): a 5 mL syringe containing medetomidine in physiological saline solution (240 µg/kg/h, with a perfusion rate of 20 µL/min), a 0.5 mL syringe containing atipamezole (20 µL, in 0.5 mL of saline solution), and eye ointment.

NOTE: Keep all equipment under the extractor hood, except the 5 mL syringe containing medetomidine, which is placed in the syringe pump near the magnet for anesthesia during MR acquisitions.

2.2. Place the rat in the induction chamber, start the anesthesia by delivering 4% isoflurane, and adjust the oxygen flow rate to 1.5 L/min.

2.3. Evaluate the depth of anesthesia by assessing the withdrawal of paw reflexes.

2.4. When the rat does not respond to stimulation, take it out of the anesthesia box, place it on the bench with its nose in the isoflurane mask, and maintain anesthesia by delivering 2.5% in oxygen at 1.5 L/min.

2.5. Gently massage the tail and place the tourniquet (**Figure 1C**).

NOTE: The massage can be performed in warm water, with a temperature between 38 and 42 °C, to obtain better vasodilation of the veins.

2.6. Insert the peripheral intravenous catheter (22 G), previously heparinized, in the left or right tail vein. Note that a venous return is observed (a drop of blood is visible at the distal part of the needle) when the catheter is correctly inserted (**Figure 1D**).

2.7. Blow out any air bubbles present in the catheter dead space volume using the 2 mL syringe containing physiological saline solution and heparin.

2.8. Apply the eye ointment and prepare the syringe containing atipamezole (17 µg/mL) to awaken the rat at the end of the experiment.

3. Rat Placement in Magnet for Whisker Stimulation

3.1. Place a breathing sensor on the magnet bed and then transfer the rat from the bench to the magnet bed. Place it in the prone position with its nose in the isoflurane mask, with the breathing sensor located between the ribcage and the magnet bed.

NOTE: All equipment that enters the MRI room should be MRI-safe.

3.2. Decrease the isoflurane (from 4% to 1.5%–2%) during rat placement and switch the anesthesia to medetomidine at the end of this procedure. Ensure that the right whiskers are free, having cut the right edge at the front of the rat MRI bed beforehand to allow movement of the whiskers.

3.3. Hold the rat in position with tape and monitor its breathing which must be between 60 and 80 breaths per minute.

3.4. Make a sail that traps all right whiskers in the paper tape (**Figure 2**). Align the flexible outlet pipe of the air-puff system along the rat MRI bed so that the part exiting the tube is perpendicular and at around 1.5 cm from the sail. Fix it with paper tape.

4. Whisker Stimulation

4.1. Connect the flexible inlet pipe from a compressed air source (1 bar) to a solenoid control valve input and the outlet pipe to the solenoid control valve output (**Figure 3**). Ensure that the solenoid control valve stays outside the magnet room.

4.2. Plug the pulsing device into the solenoid valve and into the magnet using the transistor-transistor logic (TTL)-port. Configure it so that the pulsing frequency = 8 Hz, the pulsing time = 20 s, and the resting time = 10 s.

NOTE: These parameters, visualized on the small liquid crystal display (LCD) screen, are adjustable *via* the three dedicated analogical potentiometers. The electronic pulsing device, which controls the paradigm, must be composed of high-quality electronic components to avoid any drift in time parameters (for correct postprocessing).

5. BOLD fMRI Acquisition

5.1. Place the rat brain so that it is in an upright position and use the ear bars to maintain it. Place the volume array coil above the rat's head (**Figure 4A**) and fix it using tape. Check that the sail is moving correctly (anteroposterior movement, no rotation, and no friction of sail) when the air-puff system is turned **On**; then, switch it **Off**.

5.2. Insert the bed and the coil in the center of the magnet. Check that the sail is still moving correctly once the bed is inside the magnet when the air-puff system is **On**; then, switch it **Off**. Switch completely from isoflurane to medetomidine (perfusion rate: 20 µL/min).

5.3. Check that the rat is well located using a localization sequence (TE = 2.5 ms; TR = 100 ms; average = 1; repetition = 1; slice = 1 mm; image size = 256 x 256; field of view (FOV) = 50 x 50 mm; scan time = 12 s 800 ms). Drag the **Localizer** sequence tab into the **Instruction name** and click on **Continue**.

5.4. Drag the **T2_Star_FID_EPI** sequence tab into the **Instruction name**, center the FOV on the middle of the brain, and click on the **Adjustment platform** tab to open the edited scan instruction. Record a B_0 map and proceed to a scan shim.

NOTE: For a B_0 map, use the following parameters: first echo time = 1.65 ms; TR = 20 ms, average = 1; flip angle = 30°; echo spacing = 3.805 ms; slice = 58 mm; image size = 64 x 64 x 64; FOV = 58 x 58 x 58 mm; scan time = 1 m 24 s 920 ms. For scan shim, use the following parameters: voxel selective excitation = STEAM Gaussian pulse; TE = 5 ms; mixing time = 10 ms; acquisition duration = 204.8 ms; bandwidth = 10,000 Hz; dwell time = 50 µs).

5.5. Start the **T2 Star_FID_EPI** sequence (TE = 16.096 ms; TR = 500 ms; average = 1; repetition = 600; slice = 1 mm; four consecutive slices; image size = 128 x 128; FOV = 20 x 20 mm; bandwidth = 333,333.3 Hz; scan time = 5 min 00 s).

NOTE: Due to the TTL port, an external trigger signal will start the air-puff system at the same time. The paradigm = [20 s activation + 10 s rest] x 10, for the total duration of the 600 scans, 500 ms per scan. The slices are centered on the middle of the barrel field area.

5.6. Acquire another localization sequence (same as the one described in step 5.3) to compare with the first one and check whether the rat has moved during the T2_Star_FID_EPI sequence.

5.7. Bring the bed to its initial position, remove the volume array coil, and connect the surface coil.

6. BOLD Processing

6.1. Open the T2 Star_FID_EPI file and read the T2 Star_FID_EPI image in **Image Display**. Open the start-up window of the functional controller, called **FunController**.

6.2. In this **Processing** tab, select the functional imaging window and define the stimulation protocol (duration and alternation of **On**/**Off** periods, corresponding to the paradigm used).

6.2.1. Select the protocol window (dataset with 600 frames) and insert the value of 40 in the **On Period** tab and 20 in the **Off period** tab. Click on the **Invert Attribution** tab and drag the **Stimulation States** slider to the left to select the value **1**.

6.3. In the preprocessing window, click on the **Median filter in plane** for preprocessing and on the **Median filter (2D, 3D)** for postprocessing.

6.4. Click on the **Execute** tab and drag the cursors to adjust the overlay lookup table. Visualize the activated brain area (**Figure 4B**).

7. Proton MRS Acquisitions

7.1. To correctly position the surface coil, modify the position of the rat head. Rotate the head (approximately 30° clockwise) so that the surface coil (**Figure 5A**) can be placed just above the left barrel cortex while being horizontal and located at the magnet center when inside the magnet.

7.2. Plug the surface coil, fix it on the rat brain using tape (**Figure 5B**), and check that the sail is moving correctly (anteroposterior movement, no rotation, and no friction of the sail) when the air-puff system is turned **On**; then, switch it **Off** at the main switch.

7.3. Check that the sail is moving correctly once the bed is inside the magnet when the air-puff system is **On**. Then, switch it **Off**.

7.4. Check the rat is positioned correctly using a localization sequence. Set parameters as follows: TE = 2.5 ms; TR = 100 ms; average = 1; repetition = 1; slice = 1 mm; image size = 256 x 256; FOV = 50 x 50 mm; scan time = 12 s 800 ms.

7.4.1. Drag the **Localizer** sequence tab into the **Instruction name** window and click on the **Continue** tab to execute the scan program.

7.5. When the brain localization is correct, drag the **T2_TurboRARE** sequence tab in the **Instruction name** window and click on **Continue** to execute the scan program. These anatomical images, together with the previous BOLD fMRI acquisition, will allow the correct localization of the voxel in the S1BF for MRS.

NOTE: The T2_TurboRARE parameters are 14 slices, 2 mm per slice, FOV = 2.5 x 2.5 cm, TE = 100 ms, TR = 5,000 ms, matrix = 128 x 128, sequence time = 2 min 40 s.

7.6. Drag the **LASER** sequence tab into the **Instruction name** window, place the voxel (2 mm high, 2.5 mm long, 3 mm deep) at the center of the S1BF area.

7.6.1. Use a rat brain atlas and the BOLD fMRI enhancement to localize the zone on the T2 images (**Figure 6**). Click on the **Adjustment platform** tab to open the edited scan instruction. Click on the

Wobble tab and change the impedance (electronic loading) of the receive coil slightly to tune it. Click on the **Apply** tab when the tuning is finished to close the instruction editor and apply the changes in the edited instruction.

7.7. Record a B_0 map and proceed to scan shim and, then, perform a local shim.

NOTE: For the B_0 map, use the following parameters: first echo time = 1.65 ms; TR = 20 ms, average = 1; flip angle = 30°; echo spacing = 3.805 ms; slice = 58 mm; image size = 64 x 64 x 64; FOV = 58 x 58 x 58 mm; scan time = 1 m 24 s 920 ms. For the scan shim, use the following parameters: voxel selective excitation STEAM Gaussian pulse; TE = 5 ms; mixing time = 10 ms; acquisition duration = 204.8 ms; bandwidth = 10,000 Hz; dwell time = 50 µs. For the local shim, use the following parameters: water suppression, VAPOR acquisition duration = 1,363.15 ms; points = 4,096; bandwidth in Hz = 3,004.81 Hz; bandwidth in ppm = 10 ppm; dwell time = 166.40 µs; spectral resolution = 0.37 Hz/points. The LASER parameters are: echo time = 19.26 ms; TR = 2,500 ms; averages = 128 or 32; scan time = 5 min 20 s or 1 min 20 s; acquisition points = 4,096.

7.8. Perform ^{1}H-MRS.

7.8.1. Start the ^{1}H-MRS acquisition first during a resting period (4 x 32 LASER scans + 128 LASER scans; 2,500 ms per scan).

7.8.2. Acquire another localization sequence (same as the one described in step 5.3) to compare with the first one recorded and ensure that the rat has not moved during the LASER acquisition.

7.8.3. Perform ^{1}H-MRS during whisker activation using the LASER sequence (4 x 32 LASER scans + 128 LASER scans; 2,500 ms per scan) with the air-puff system **On** (paradigm = 20 s of activation and 10 s of rest).

7.8.4. Once again, perform a localization sequence to check whether the rat has moved.

NOTE: The number of scans and resting/activated periods can be adapted and modified, but always ensure that the rat is not moving by regularly performing a localization sequence.

7.9. Bring the bed to its initial position, remove the surface coil, and move the rat back to the bench. Inject atipamezole into a skin fold made in the rat's back to reverse the anesthesia and awaken it.

8. Proton MRS Processing

8.1. Open the LCModel software and click on the appropriate tab to select the right data type (**Free Induction Decay** file) and choose the right file. Click on the **OK** tab when this is done.

8.2. Optimize the quantification control parameters step by step.

8.2.1. In the **Title** section, manually enter a title and define an adequate ppm range (*e.g.*, 0.2 to 4.0 ppm) by manually typing in the necessary value in the respective fields.

8.2.2. In the **Basis file** section, select and download the required file to fit the macromolecule baseline correctly (it can be provided by the software provider).

8.2.3. Define and load the input control parameters. Prepare the save process of all useful file types beforehand (TABLE = compact tables; PS = necessary PostScript output; CSV = format for spreadsheets; COORD = coordinates for plots). Click on the **RunLCModel** tab to start the LCModel quantification.

8.3. Define selected metabolites to generate statistics.

NOTE: LCModel provides metabolite quantification and estimates errors by a value termed Cramér-Rao lower bound (CRLB). A value with a CRLB < 15 is considered as an optimal quantification. A CRLB > 25 indicates an unreliable value.

REPRESENTATIVE RESULTS:

This protocol allows the quantification of metabolite fluctuations during cerebral activation, which is obtained by right whisker stimulation directly in the magnet.

BOLD fMRI In this study, the overall goal of BOLD fMRI was to check that the whisker stimulation was efficient, to visualize the activated S1BF area, and to correctly locate the voxel for ^{1}H-fMRS. The device built for whisker activation is efficient. Indeed, when right whiskers were stimulated using the homemade air-puff system, a positive BOLD signal was detected in the left barrel cortex (**Figure 4B**), also called the S1BF, for the somatosensory barrel field (n = 8). A positive signal enhancement was detected in the left barrel cortex in eight out of eight rats, whereas only background was detected in the right hemispheres. When BOLD fMRI was performed without whisker stimulation, no signal enhancement was observed either in the left or in the right S1BF.

***In vivo* localized spectroscopy**In a comparison between anatomical MR images and rat brain atlas schemes[8], the activated brain area visualized by BOLD fMRI allows the voxel to be placed in the S1BF area, which is activated during whisker stimulation. This voxel is located on three consecutive slides (1 mm thick) since the barrel cortex is 3 mm long. When the brain slide is virtually separated into four quarters, the voxel is located in the upper left quarter at an approximately 45° angle (**Figure 6**).

When the paradigm for whisker stimulation was turned on, an increase in lactate content was observed in the left S1BF (**Figure 6**, typical spectra obtained in one rat). To better visualize metabolic fluctuations between resting and activated periods, a spectral subtraction was performed (**Figure 6**). From this subtracted spectrum, the increase in lactate content with brain activation was visualized much more easily, while in this rat, the N-acetylaspartate (NAA) signal was slightly decreased. Lactate increase during neuronal stimulation was also observed on the spectral deconvolution (**Figure 7A,B**). While the lactate peak was hardly detected on the *in vivo*

spectrum at rest, LCModel was able to quantify it (**Figure 7A**) with accuracy and good CRLB values. Indeed, out of 23 rats, only one spectrum had a CRLB value for lactate quantification equal to 24. None were > 25. For all other spectra, the values ranged between 3 and 19.

The variations in lactate content in all 23 rats are presented in **Figure 8**. Out of 23 rats, a decrease in lactate content was observed only in one rat. There was a statistically significant difference in lactate content between resting and activated periods (0.132 ± 0.012 and 0.163 ± 0.011, respectively, values relatives to PCr + Cr content, paired *t*-test, $p = 0.0005$ [parametric, two-tailed], $n = 23$). Therefore, a 31.6% ± 7.8% increase in lactate content was measured during neuronal stimulation.

A slight decrease in NAA content can be observed in **Figure 6**, which represents typical spectra obtained in one animal. However, this NAA variation was not significant (a 1.2% ± 1.2% decrease was measured, $n = 23$).

FIGURE LEGENDS:

Figure 1: Equipment and steps for anesthesia. (**A**) Picture of the equipment to be prepared before starting anesthesia. (**B**) Isoflurane pump and induction chamber. (**C**) Tourniquet placement. (**D**) Picture shows the catheter has been correctly inserted; note the drop of blood in the catheter needle, which is a positive sign of a correct location in the vein.

Figure 2: Whisker stimulation. All right whiskers are trapped in a sail made with paper tape. The sail allows all right whiskers to be stimulated at the same time with the air-puff system and, therefore, maximizes the neuronal activation of the barrel cortex. The outlet of the air-puff system (black tube) should be located around 1.5 cm and perpendicular to the sail. Check outside the magnet to make sure the sail is moving correctly by turning the air-puff system on. The sail must move at 8 Hz in an anteroposterior direction (no rotation).

Figure 3: Air-puff system for whisker stimulation. (**A**) A flexible pipe connects the compressed air to (**B**) the solenoid control valve. A second flexible pipe brings pulsed air from the solenoid control valve output to the sail. The solenoid control valve is plugged into the pulsing device, which controls the paradigm.

Figure 4: BOLD fMRI. (**A**) Volume array coil placement. The rat head is in a horizontal position and blocked by ear bars. Check that the sail is moving freely and is not blocked by the coil or by the MRI bed. (**B**) A typical BOLD signal in the activated left barrel cortex (red arrow). No signal is detected in the contralateral right hemisphere (blue arrow). The threshold is set at 76.5% of the maximum of the intensity value.

Figure 5: Surface coil. (**A**) Picture of the surface coil used in this study. (**B**) Surface coil placement. The rat head must be slightly turned so that the left barrel cortex and, therefore, the surface coil are located in the center of the MRI bed (the head is turned at an angle of around 30°, a good

compromise between the correct location of the left barrel cortex for the surface coil and free movements of the sail of the right whiskers, which should not be blocked by the MRI bed).

Figure 6: Typical localized ^{1}H-MRS at rest (blue spectrum) and during whisker activation (red spectrum). The voxel (green square) is located in the left S1BF on the anatomic T2_TurboRARE images using rat brain atlas schemes and signal enhancement on BOLD fMRI images. The spectral subtraction is plotted in black. Lactate and N-acetylaspartate (NAA) peaks are indicated at 1.32 and 2.02 ppm, respectively.

Figure 7: Typical spectral deconvolution of MRS spectra. (**A**) Deconvolution of a 128-scan rest spectrum. (**B**) Deconvolution of a 128-scan activated spectrum. Residue, subtraction between experimental spectrum (raw data), and the LCModel fit; MM = macromolecule; Cr = creatine + phosphocreatine; PCho + GPC = phosphocholine + glycerophosphocholine; NAA = N-acetylaspartate; Lac = lactate; GABA = γ-aminobutyric acid; Gln = glutamine; Glu = glutamate.

Figure 8: Variations in lactate content during brain stimulation. Blue dot: lactate content at rest, determined by LCModel and relative to the creatine + phosphocreatine content. Red dot: lactate content during whisker stimulation, determined by LCModel and relative to the creatine + phosphocreatine content. The difference between activated and rest, p = 0.0005, paired t-test (parametric, two-tailed), n = 23.

DISCUSSION:

The barrel cortex, also called S1BF for the somatosensory cortex or barrel field, is a region within the cortical layer IV that can be observed using cytochrome c oxidase staining[9], and its organization is well known since it has been largely described[10,11]. One vibrissa is connected to one barrel, in which around 19,000 neurons are organized in a column[12]. The whisker-to-barrel cortex pathway has several advantages. First, it can be activated inside the magnet by using an MRI-compatible air-puff system, which can be easily homemade (to ensure that in the largest part of the S1BF area, which corresponds approximately to the size of the voxel in which MRS is performed, all whiskers are squeezed in a sail that allows the stimulation of a maximum of vibrissa). Second, right whisker activation leads to left barrel cortex activation, and this brain area is located in the somatosensory cortex, which allows the use of a high-sensitive surface coil. Third, this method of activating the somatosensory cortex is noninvasive compared to electrical paw stimulation, the latter having the disadvantage of stimulating other brain structures, including some in the right hemisphere[13]. Therefore, the protocol used here is the most suitable to perform an *in vivo,* noninvasive, and longitudinal study of brain metabolism under cerebral activation.

The choice of anesthetic is crucial, as many of anesthetics induce changes in neurovascular coupling, brain metabolism, and/or brain activity[14,15]. For example, isoflurane, the most common anesthetic used for MRI, leads to a three- to sixfold increase in brain lactate content[15,16] and, therefore, should not be used in brain metabolic studies. Medetomidine is an α2-adrenoreceptor agonist, which induces reliable sedation, analgesia, muscle relaxation, and anxiolysis[17]. These effects can be quickly reversed using atipamezole, an α2-antagonist. Medetomidine is the best

candidate to perform functional studies in rodents[18] since it has a very low impact on the BOLD signal and the lowest modifications in brain metabolite contents.

It is also important to follow the whisker activation paradigm correctly. Since NMR acquisitions last several minutes, the use of successive activation/rest periods is essential to limit the desensitization of neurons in the activated brain area. The parameters of this paradigm (20 s of activation followed by a rest period of 10 s) were chosen to obtain the highest BOLD fMRI signal in the corresponding barrel cortex. Much care must be taken to respect these time windows since it is crucial to determine the activated/rest period for BOLD treatment, even if it is controlled by the TTL port. To obtain a high level of barrel cortex activation, the sail that groups the whiskers together is also important since it allows the largest portion of the S1BF area to be stimulated. Much care must be taken to place the outlet air tube in front of this sail so that it can move on an anteroposterior plane. The frequency has to be carefully calibrated since it has been shown that neurons in the barrel cortex are activated when whisker stimulation frequency is between 5 and 15 Hz[19]. Using a lower or higher frequency will not lead to the activation of the S1BF area.

The protocol used in this study makes it possible to compare spectra acquired in the same brain area at rest and during brain stimulation and, therefore, to monitor metabolic changes linked to cerebral activation. It is important to perform a localization sequence at the beginning and at the end of the NMR spectroscopy protocol, to ensure that that animal has not moved and that the differences in metabolic contents measured between the resting and activated states are due to brain stimulation and not to movement artifacts.

Using the protocol described herein, an increase in lactate content was measured between resting and activated periods. Lactate increase using *in vivo* NMR spectroscopy during brain activation was first observed in humans in the early 1990s[20,21]. However, most measurements were performed in humans rather than rodents, in which the signal-to-noise ratio is much lower. In the rat, *ex vivo* NMR quantification of lactate during rat brain activation was performed by Mazuel *et al.*[22], who observed an increase in brain lactate content with neuronal activation. The results presented here show that lactate was increased during whisker activation. However, since localized MRS does not allow cellular resolution, it is still unknown from which cellular compartment lactate is coming (neurons or astrocytes). To go further in the understanding of cerebral metabolic exchanges, such as the still debated ANLSH (astrocyte-neuron lactate shuttle hypothesis), this protocol has to be applied to genetically modified animals for the key components in this shuttle, such as the monocarboxylate transporters.

In the study described here, no statistically significant difference in NAA content was observed. A decrease in NAA content during visual stimulation was previously found in humans[23-25], but not confirmed by Mangia and Tkac[26]. In the current study, we observed an increase in NAA content during brain activation in 50% of the rats and a decrease in the other half. Therefore, NAA should be avoided as the internal reference for quantification during functional MRS. No other variation in metabolite content was detected.

Both lactate and NAA variations during neuronal activation have led to controversies[23,26-29]. To further our understanding of these metabolic fluctuations linked to brain activity, it would be interesting to apply this protocol to transgenic animals. This would provide further information about the underlying process. Overall, localized ^{1}H-MRS during a task, or functional MRS[29], is an emerging technique in rodents, relevant to the study of regional dynamic changes in metabolites, in normal or pathological brains.

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DISCLOSURES:

The authors have nothing to disclose.

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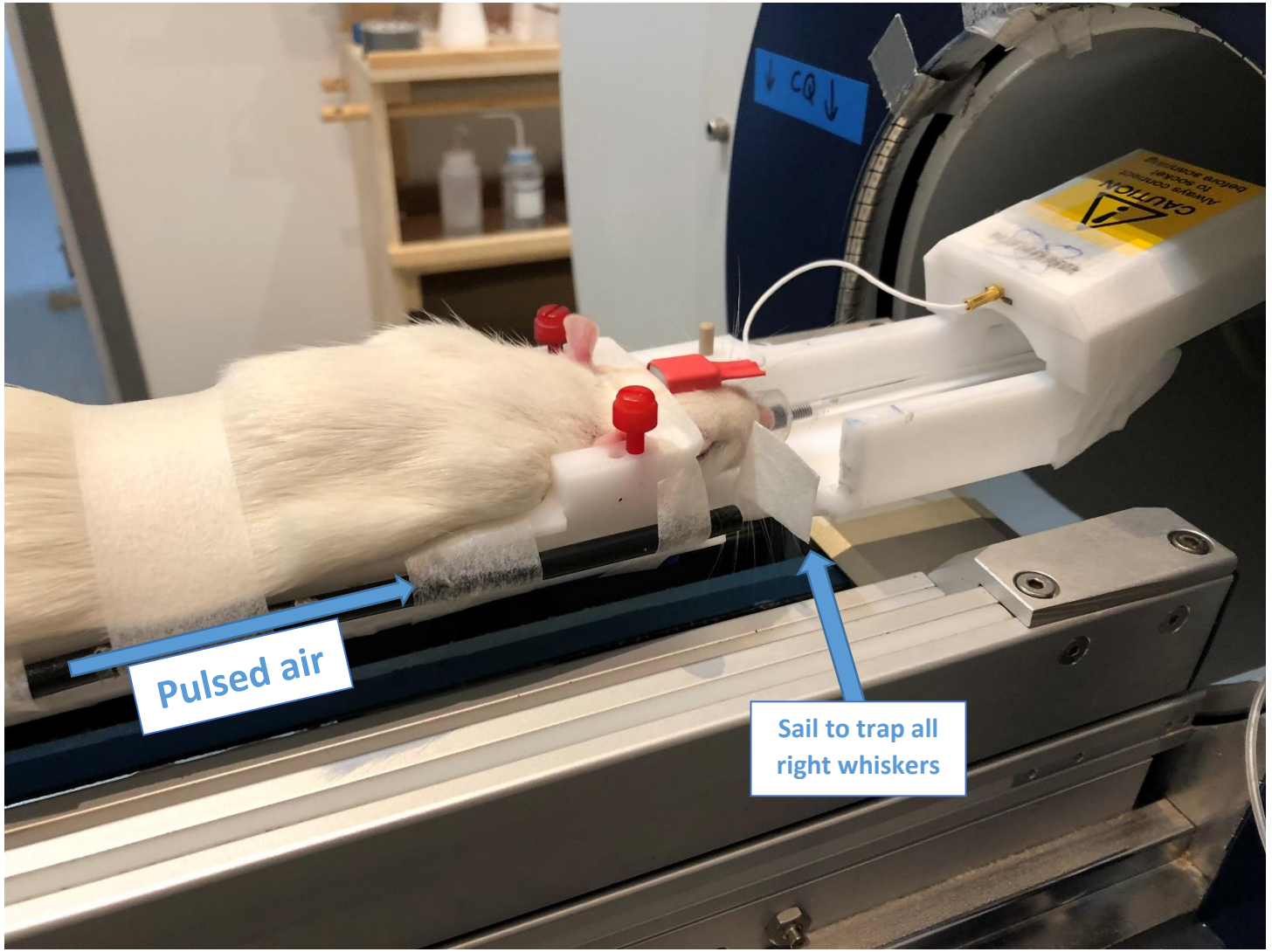

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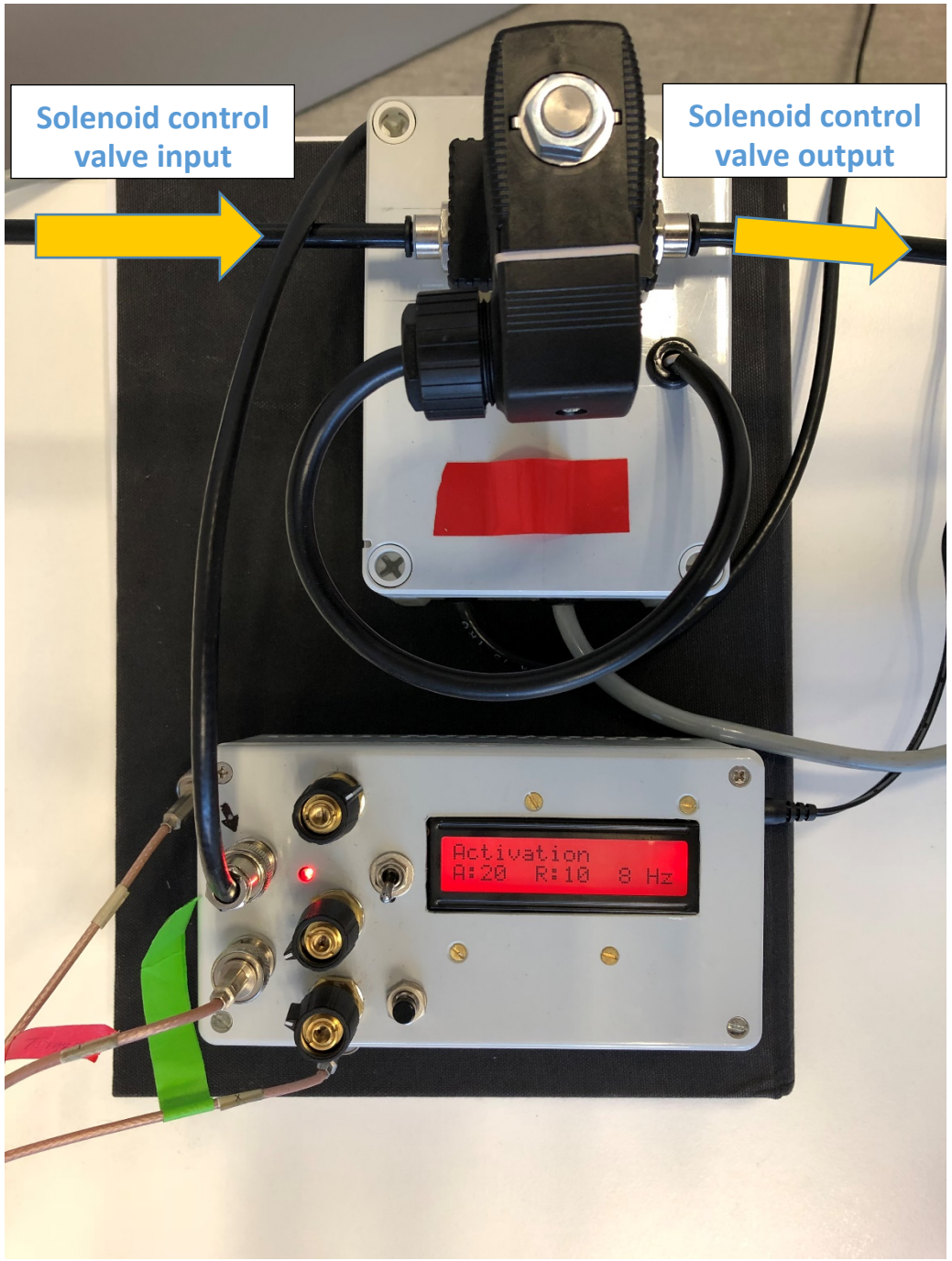

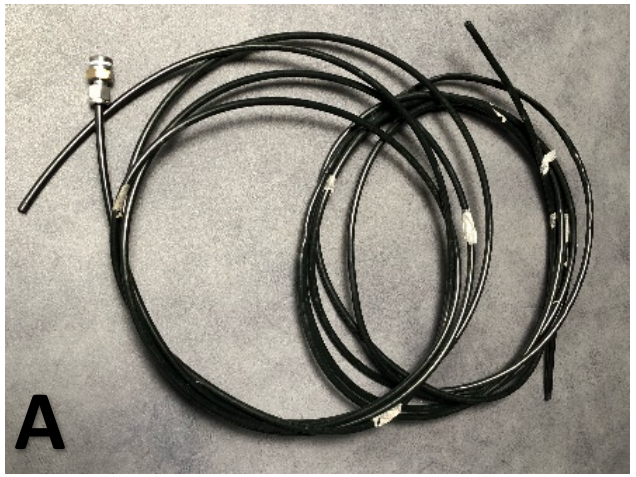

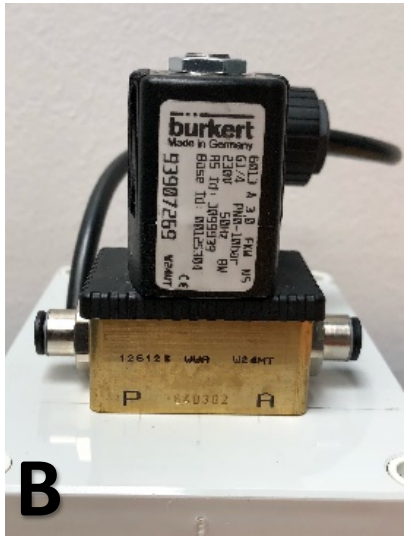

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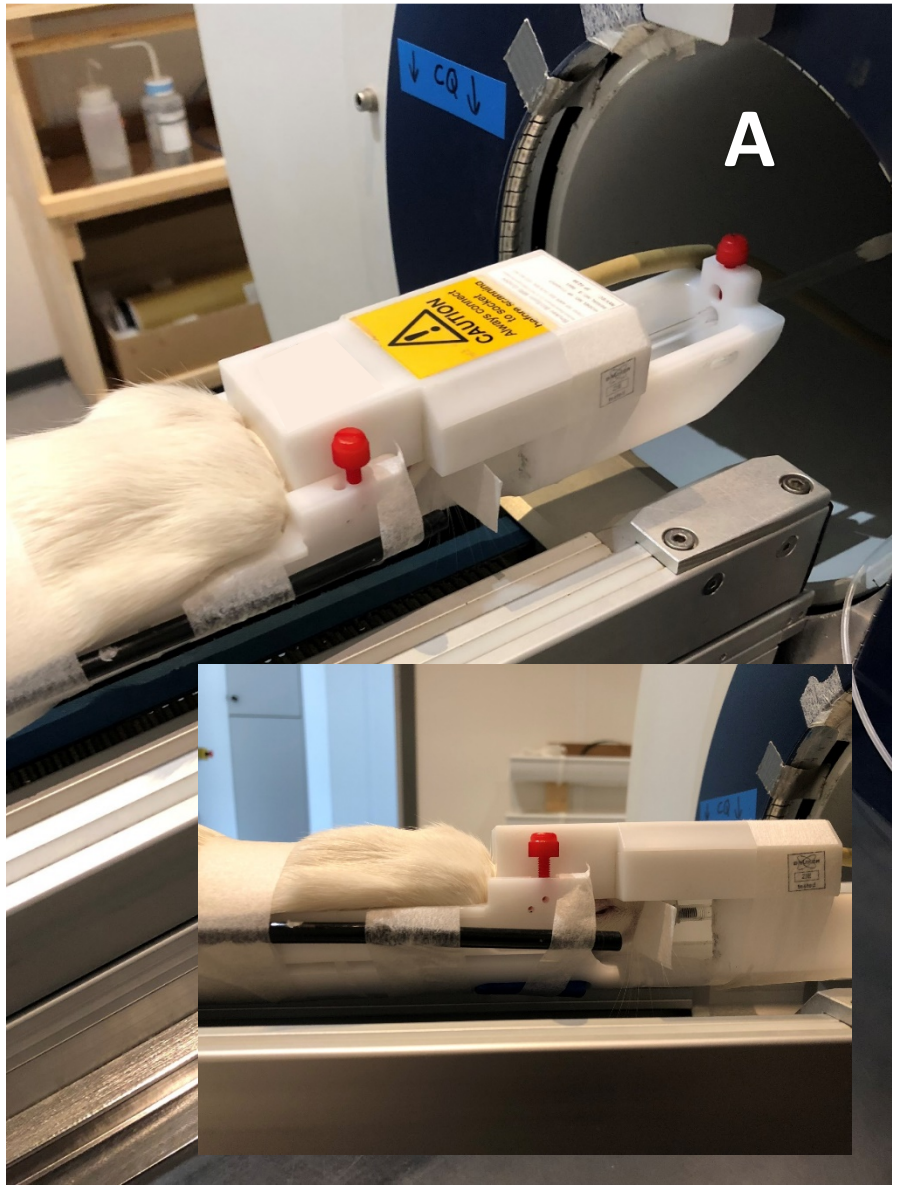

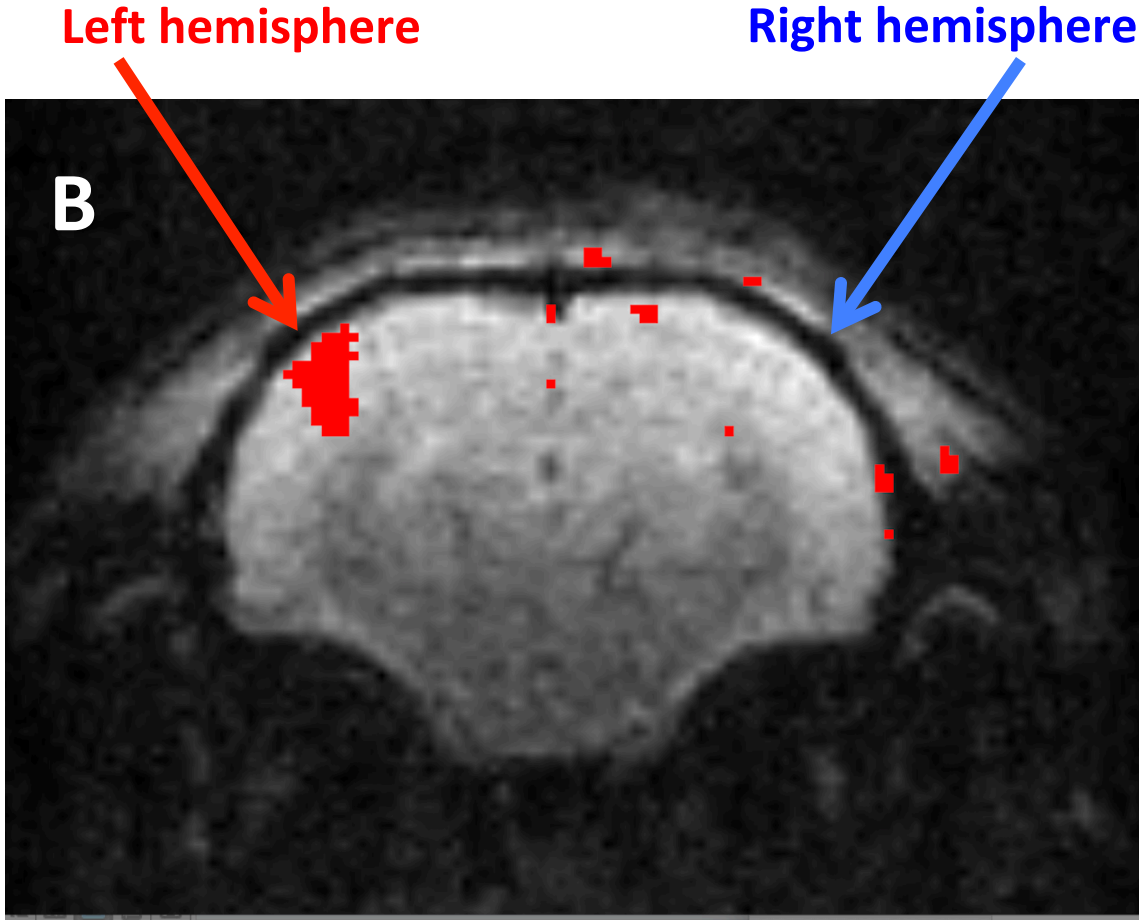

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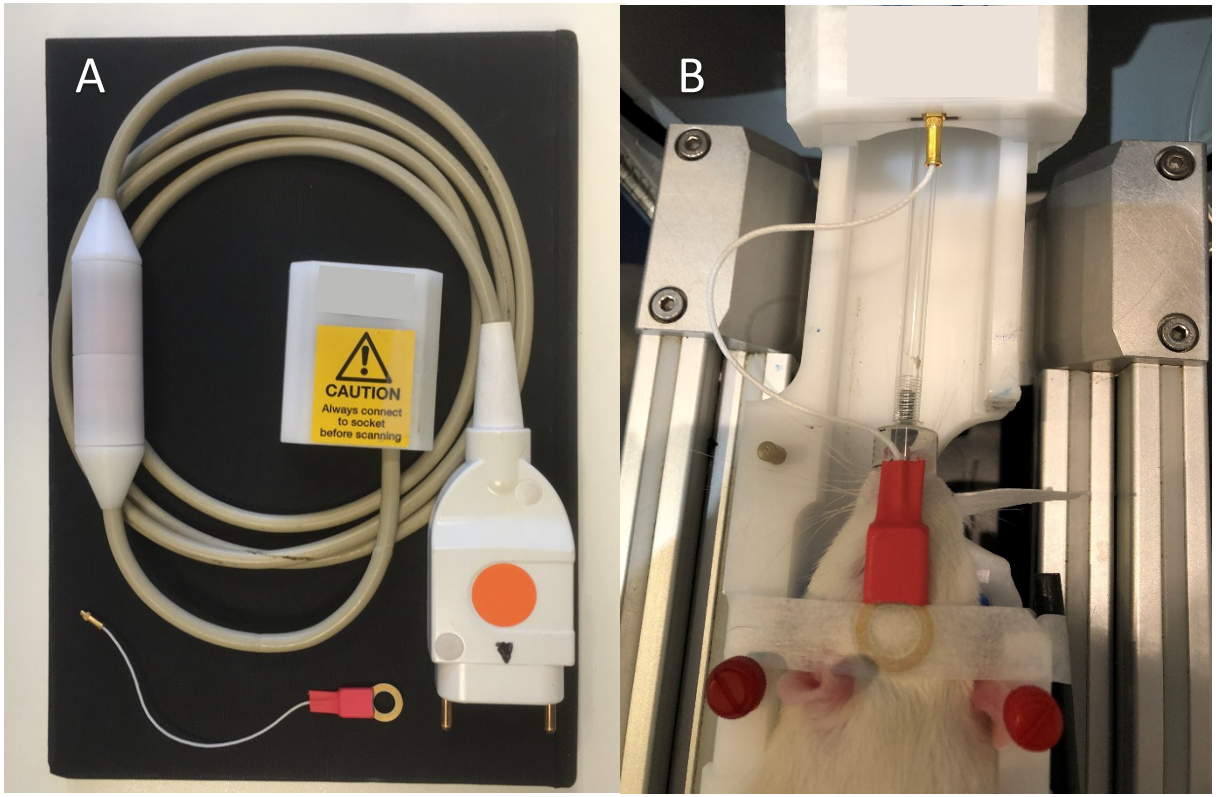
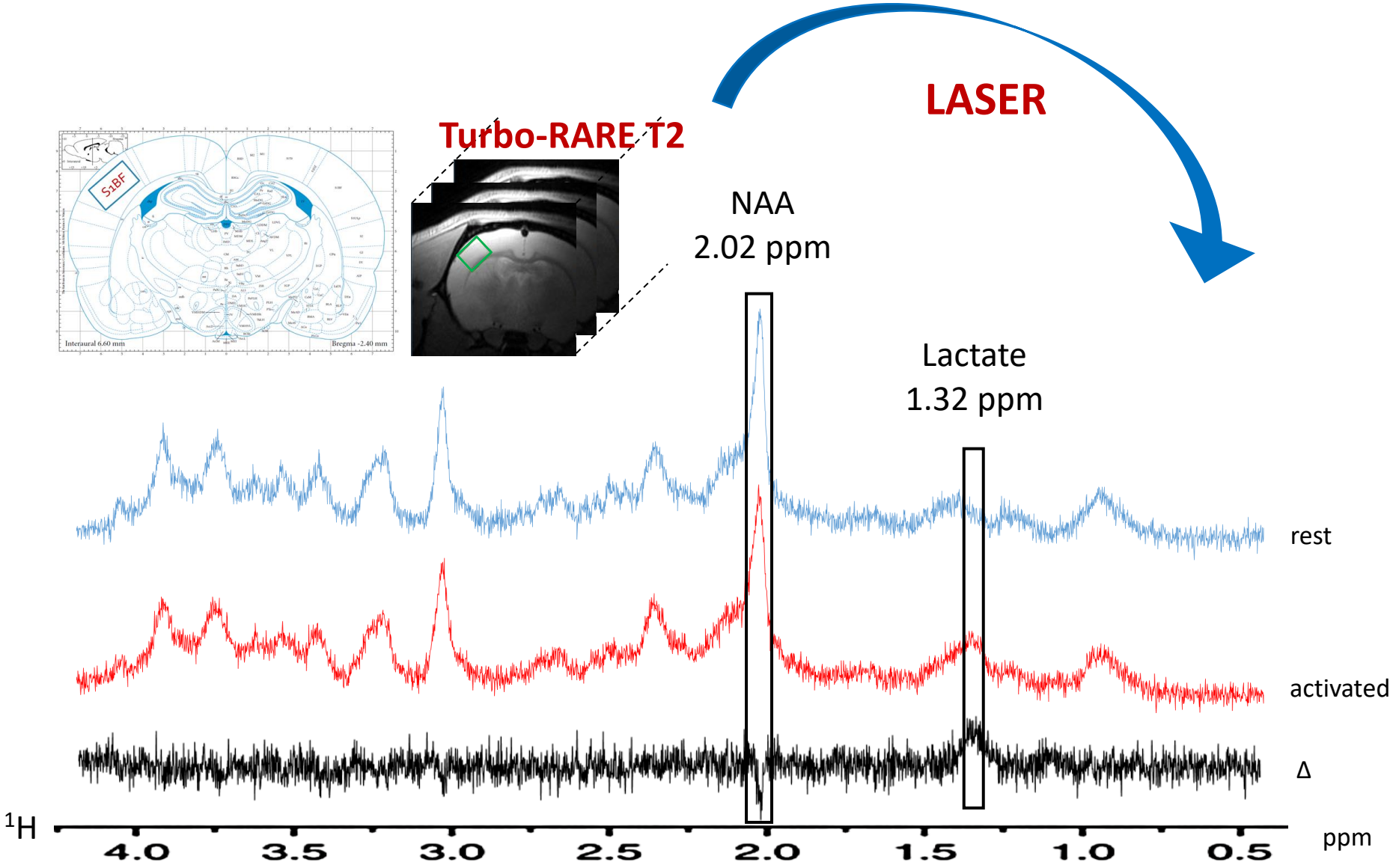


Figure 6



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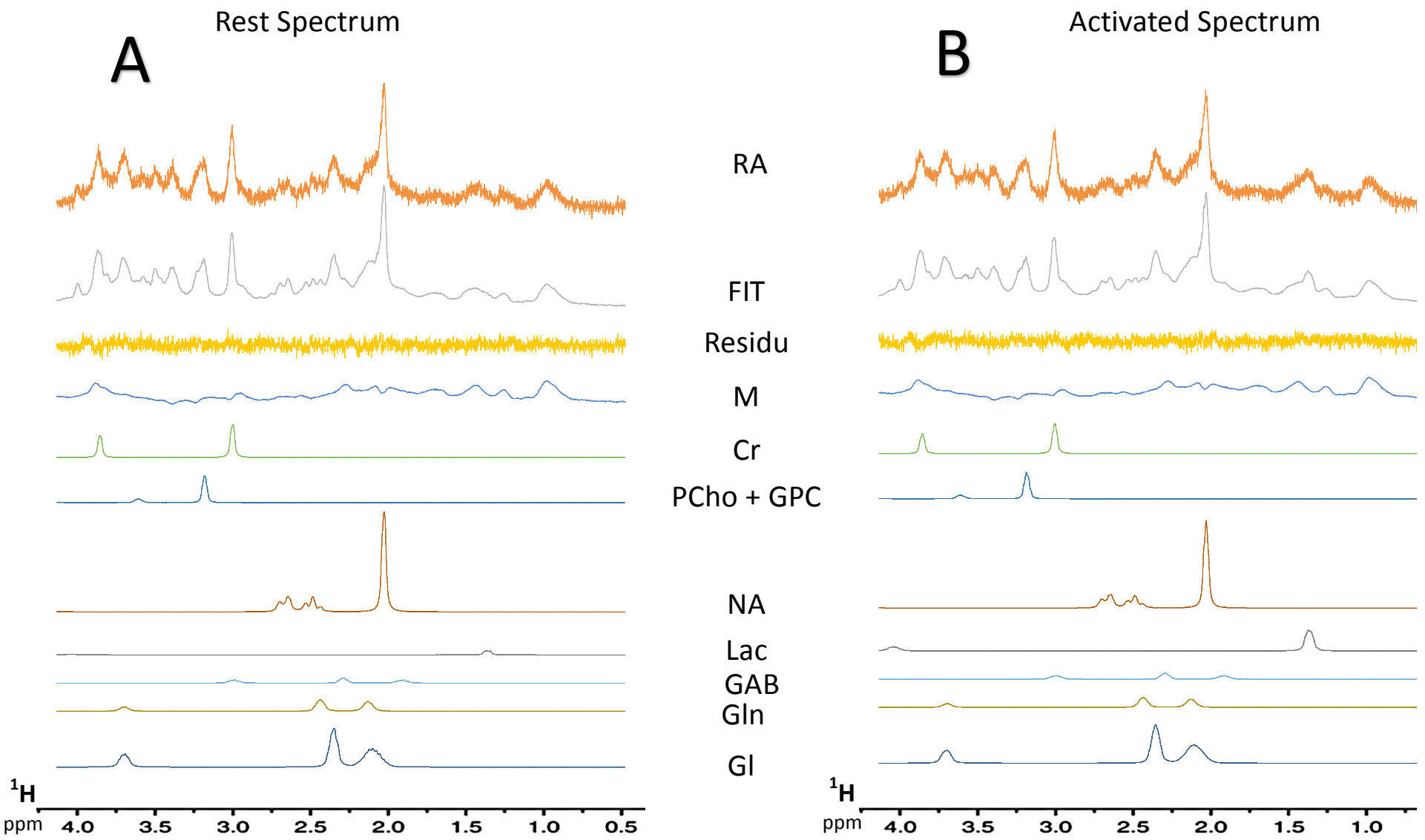

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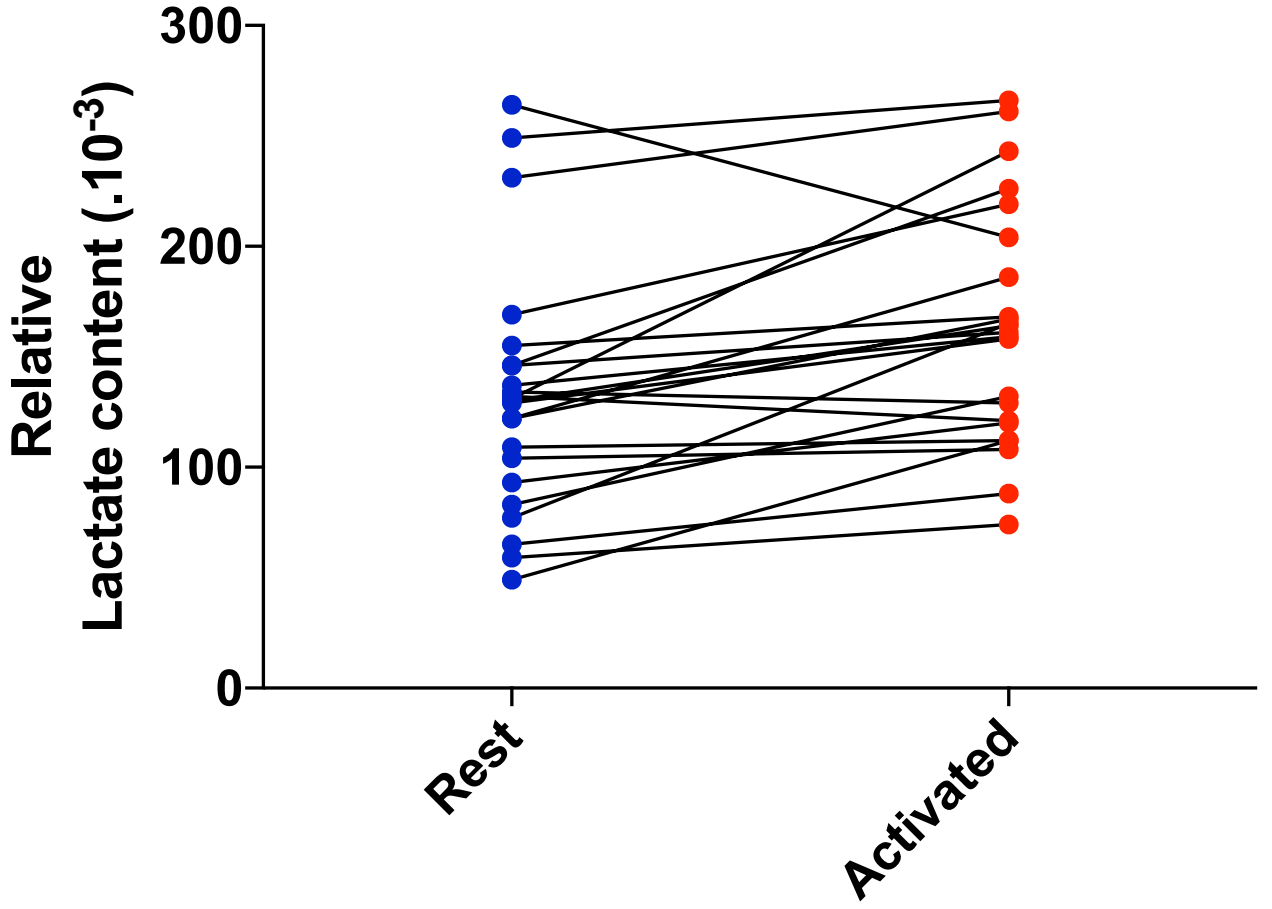

Name of Material/ Equipment	Company
0.5 mL syringe with needle	Becton, Dickinson and Company, USA
^{1}H spectroscopy surface coil	Bruker, Ettlingen, Germany
7T Bruker Biospec system	Bruker, Ettlingen, Germany
Arduino Uno based pulsing device	custom made
Atipamezole	Vétoquinol, S.A., France
Breathing mask	custom made
Eye ointment	TVM laboratoire, France
Induction chamber	custom made
Inlet flexible pipe	Gardena, Germany
Isoflurane pump, Model 100 series vaporizer, classic T3	Surgivet, Harvard Apparatus
Isoflurane, liquid for inhalation	Vertflurane, Virbac, France
KD Scientific syringe pump	KD sientific, Holliston, USA
LCModel software	LCModel Inc., Ontario, Canada
Medetomidine hydrochloride	Vétoquinol, S.A., France
Micropore roll of adhesive plaster	3M micropore, Minnesota, United States
Micropore roll of adhesive plaster	3M micropore, Minnesota, United States
Monitoring system of physiologic parameter	SA Instruments, Inc, Stony Brook, NY, USA
NaCl	Fresenius Kabi, Germany
Outlet flexible pipe	Gardena, Germany
Paravision software	Bruker, Ettlingen, Germany
Peripheral intravenous catheter	Terumo, Shibuya, Tokyo, Japon
Rat head coil	Bruker, Ettlingen, Germany
Sodic heparin, injectable solution	Choai, Sanofi, Paris, France
Solenoid control valves, plunger valve 2/2 way direct-acting	Burkert, Germany
Terumo 2 ml syringe	Terumo, Shibuya, Tokyo, Japon
Terumo 5 mL syringe	Terumo, Shibuya, Tokyo, Japon
Wistar RJ-Han rats	Janvier Laboratories, France

Catalog Number	Comments/Description
2020-10	0.33 mm (29 G) x 12.7 mm
T116344	
70/20 USR	
V8335602	Antisedan, 4.28 mg
40365	Ocry gel 10 g 30x17x15 cm
1348-20	4.6-mm diameter, 3m long
WWV90TT	from OH 43017, U.S.A
QN01AB06	1000 mg/mL
Legato 110	
6.2	
QN05CM91	Domitor, 1 mg/mL
MI912	
MI925	
Model 1025	
B05XA03	0.9 % 250 mL
1348-20	4.6-mm diameter, 4m long
6.0.1	
SP500930S	22 G x 1", 0.85x25 mm, 35 mL/min
B01AB01	5000 IU/mL
3099939	Model type 6013
SY243	with 21 g x 5/8" needle
05SE1	

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Author(s): Jordy BLANC, Hélène ROUMES, Leslie MAZUEL, Philippe MASSOT, Gérard RAFFARD, Marc BIRAN and Anne-Karine BOUZIER-SORE

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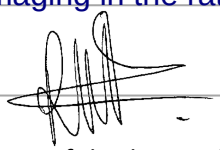
Signature: 

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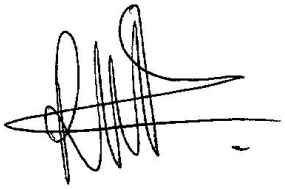

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4. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

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5. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

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9. 6.7: Please specify the dose of atipamezole given to the rat.

Dose is now specified l.101 and 116.

10. Steps 7, 8, and their sub-steps: Software must have a GUI (graphical user interface) and software steps must be more explicitly explained ('click', 'select', etc.). Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc.).

This has been done (5.4, 6.2, 7.3,7.4, 7.5, 8.2).

11. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

This has been done (3.1, 5.1, 7.1).

12. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Essential steps (corresponding to 2.5 pages) are highlighted in yellow.

13. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

Highlighted steps are complete sentences.

14. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

All relevant details required for an essential step are also highlighted in yellow, the ones that are not relevant were not highlighted.

15. Figure 5: Please add 1H to the ppm scale. The green square can hardly be seen. Please increase contrast.

This has been done.

16. Figure 7: Please add 1H to the ppm scale.

This has been done.

17. Table of Materials: Please use SI abbreviations for all units (L, mL, µL, h, min, s, etc.) and include a space between all numerical values and their corresponding units (15 mL, 37 °C, 60 s; etc.). Please use the period symbol (.) for the decimal separator.

We carefully check all SI abbreviations.

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18. References: Please do not abbreviate journal titles.

Journal titles have been modified.

Reviewers' comments:

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please thoroughly address each concern by revising the manuscript or addressing the comment in your rebuttal letter.

Reviewer #1:

Manuscript Summary:

The authors present a protocol for localized MR spectroscopy (MRS) and fMRI during whisker stimulation in rat. MRS and fMRI are performed in different settings. The same anesthetized animal is used, but it needs to be repositioned and the detector is changed.

Overall, the experiments are well motivated in the introduction. However, some relevant literature on MRS in rat, in particular on fMRS, has not been cited and should be included. Regarding the described data analysis of fMRI it is also not clear why fMRI scan proved added value compared to fMRS alone.

*The reference "Mullins, P. G. Towards a theory of functional magnetic resonance spectroscopy (fMRS): A meta-analysis and discussion of using MRS to measure changes in neurotransmitters in real time. Scand J Psychol. **59**, 91-103 (2018)" and "Stanley, A. J. and Raz, N. Functional magnetic resonance spectroscopy: The "New" MRS for cognitive neuroscience and psychiatry research. Front Psychiatry **9**, 16, (2018)" were added. These articles present all studies that have performed fMRS. One study from Mangia and Tkac was also added.*

In our study, fMRI was performed only to check that the home-build whisker activator is efficient. This point was not clear enough and the entire manuscript was modified such as the main objective of the study, fMRS, is now clearly stated.

Major Concerns:

1. For fMRI it is described that the air puff system must be switched on at the same time. This sounds as if this could be done manually. However, manual synchronization does not yield scientifically sound results. Fluctuations in timing will make the data useless for analysis. Usually the stimulator is connected to a TTL-port of the Biospec. This must be explained in more detail.

The stimulator is in fact connected to the Biospec via a TTL-port. This point is now clarified.

2. For analysis of fMRI data the tool in Paravision 6 is suggested. This is not adequate. I am not familiar with this tool, but to my knowledge it simply performs a simple statistical test. This may serve to get a first, very rough idea if responses may be detected. For a scientifically sound analysis more complex strategies must be used (eg SPM, AFNI, etc), which is standard in the community.

We agree with the reviewer. As the main objective of fMRI in our study was to check if the barrel cortex was efficiently activated by the whisker stimulation, we used the tool in Paravision 6 (FUNController tool) only to visualize the BOLD signal. This point is now much more clear in the manuscript.

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3. A more complex fMRI analysis will also allow for detecting network activations and not just S1BF, as insinuated by the authors. Such activation will be essential to justify performing fMRI in addition to fMRS.

Yes, we agree with the referee. However, the overall goal of the paper was to describe how to perform MRS in an activated brain area. This is the reason why we focused on the S1BF area, which is the most suitable place to perform fMRS in an activated zone with whisker stimulation using a surface coil.

Minor Concerns:
1. Most crucial step to obtain high quality spectra is shimming. This is mentioned but not described in detail. Since this is the most important step, shimming must be described in far more detail.

A much more described section for each MR settings has been added.

2. The LASER sequence it mentioned, but not explained. For the non-expert this may be helpful. Further, the experimental parameters for LASER must be given.

LASER sequence parameters are now added.

3. The magnet does not have a 12-cm bore - it is 20 cm.

We would like to apologize for this mistake. It has been corrected.

4. In Fig 2. arrows pointing at the sail and the tube will be helpful.

Arrows have been added.

Reviewer #2:

Manuscript Summary:

This seems to be largely a proof-of-concept study that demonstrates the ability to measure brain metabolism and BOLD activation resulting from the same stimulus, unilateral whisker stimulation. While the overall demonstration requires implementation of several advanced techniques, it is unclear what new information is gained by the study, from the perspective of the neurobiology of sensation/perception or the perspective of applications of NMR in biomedicine. A potential strength may be the visual demonstration of the equipment and procedures, however the study has not yet reached this stage in the review process. Otherwise, it is simply another demonstration of using MRS to measure in vivo changes in brain metabolism following a stimulus whose neurobiological sequelae are largely already known (i.e. whisker stimulation and barrel cortex activation). Even if the visual demonstration is impressive enough to warrant publication in this journal (whose purpose is largely to visualize experiments), several issues must be addressed.

Major Concerns:

The choice of stimulation is appropriate and the rationale is described adequately. However, it is unclear why a sail needs to be present. The authors state that this "regroups" the whiskers and that this increases the signal. Please explain further.

The barrel cortex, also called S1BF for somatosensory cortex or barrel field, is a region within cortical layer IV that can be observed using cytochrome c oxidase staining (Wong-Riley

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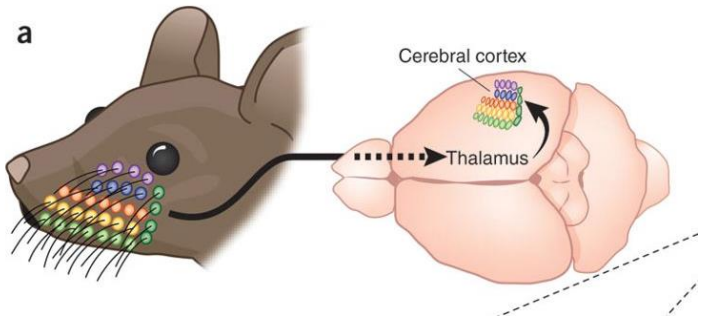
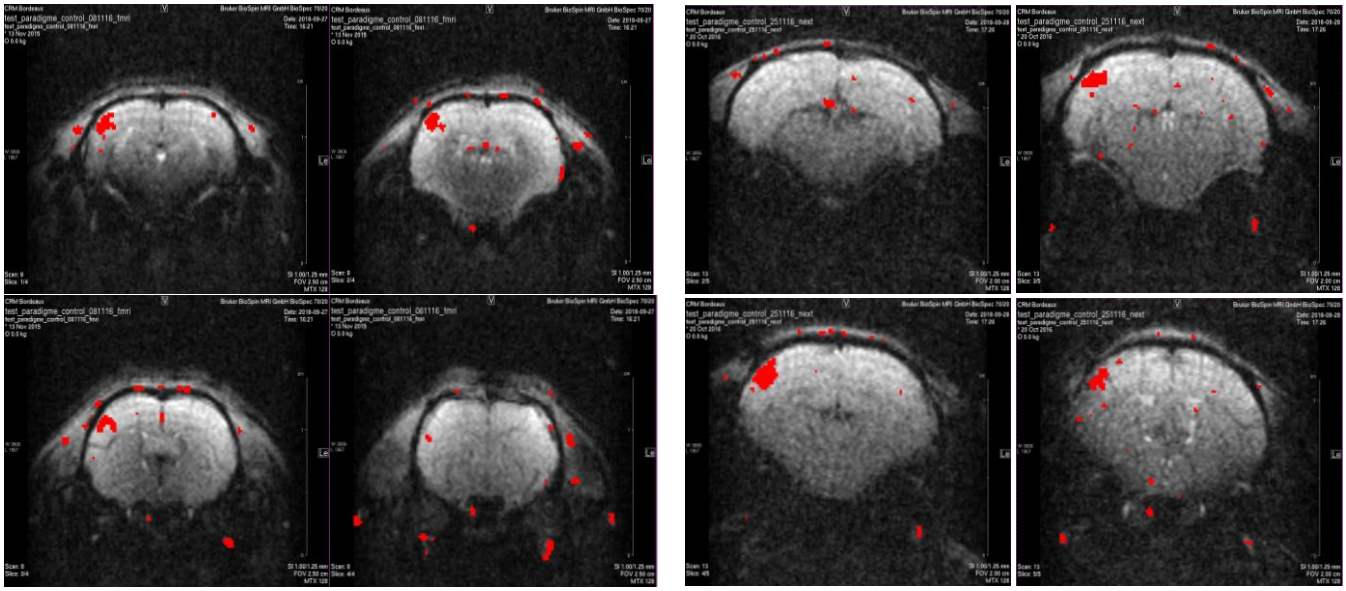
and Welt, PNAS 1980). One vibrissa is connected to one barrel (as shown in the figure) in which around 19,000 neurons are organized in a column (Felmeyer, Front in Neuroanat 2012). Using a sail allows to stimulate a maximum of vibrissa, and therefore the largest part of the S1BF area, corresponding to approximately the size of the voxel in which MRS is performed.



A paragraph explaining why a sail is needed has been added in the text, which explains that if only one or few whiskers are activated, the metabolic changes will occur only in few barrels and therefore the metabolic changes would not be detectable. This text can by found l.326-333 and l.358-359. Using this sail, the entire barrel cortex was activated, as demonstrated by the two following figures. Indeed, S1BF area is around 3-mm depth, and a signal enhancement on the BOLD fMRI was observed on 3 consecutives slices (1-mm thickness), and NOT on the fourth one.



Presentation of results needs considerable work. Statistical analysis seems completely absent from the paper which is unacceptable. Analyses should be presented in the text and also in figures. *For example, figure 7 shows increase in lactate and decrease in NAA, but it is unclear whether this is representative data from one animal. Please include the data from the entire pool of subjects, along with appropriate statistical analyses.

Presentation of results has been completely changed. Values for 5 additional rats that were scanned recently (only for fMRS) have been also added. Rather than presenting the ratio of lactate during activation/lactate at rest, contents of lactate at rest and during brain activation (quantification relative to phosphocreatine + creatine content) are presented. Paired t-test was performed on these values and the statistical analysis has been added. It is now clearly stated

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that fMRS was performed in 23 rats, and that Figure 6 presents typical spectra obtained in one rat. Figure 8 has also been added, in which results for each individual rat can be visualized, and which shows that lactate is increasing with brain activation in 22 out of 23 rats.
Here are the details of the statistics that have been performed using the Prism software:

Col Stats		A	B	C
		Rest	Activated	A/R
		Y	Y	Y
1	Number of values	23	23	23
2				
3	Minimum	49	74	0.7727
4	25% Percentile	93	120	1.068
5	Median	130	161	1.254
6	75% Percentile	146	204	1.525
7	Maximum	264	266	2.286
8				
9	Mean	132	162.7	1.316
10	Std. Deviation	55.76	53.73	0.3734
11	Std. Error of Mean	11.63	11.2	0.07785
12				
13	Lower 95% CI of mean	107.9	139.5	1.154
14	Upper 95% CI of mean	156.1	186	1.477
15				
16	Sum	3036	3743	30.26

Paired t test		
1	Table Analyzed	Lactate n=23
2		
3	Column B	Activated
4	vs.	vs.
5	Column A	Rest
6		
7	Paired t test	
8	P value	0.0005
9	P value summary	***
10	Significantly different (P < 0.05)?	Yes
11	One- or two-tailed P value?	Two-tailed
12	t, df	t=4.042 df=22
13	Number of pairs	23
14		
15	How big is the difference?	
16	Mean of differences	30.74
17	SD of differences	36.47
18	SEM of differences	7.605
19	95% confidence interval	14.97 to 46.51
20	R squared (partial eta squared)	0.4262
21		
22	How effective was the pairing?	
23	Correlation coefficient (r)	0.7787
24	P value (one tailed)	<0.0001
25	P value summary	****
26	Was the pairing significantly effective?	Yes

*While figure 6 shows BOLD activation in the contralateral barrel cortex, it seems as if this is data from only 1 animal. Please show all subjects and provide statistical analyses. Also, labels indicating left vs right are needed.

It is now clearly stated in the manuscript that BOLD fMRI was performed in 8 rats and that a signal enhancement was observed in all animals. In our study, fMRI was performed only to check that the home-build whisker activator is efficient. This point was not clear enough and the entire manuscript was modified according to referee's 1 comments such as the main objective of the study, fMRS, is now clearly stated. Therefore, the fMRI section has been shortened. However, to reply to reviewer 2, all BOLD fMRI and statistics are shown in the following figure in this rebuttal letter (ROI1 was located in the activated left S1BF, ROI2 in the contralateral S1BF (at rest) and ROI3 was placed in a non-activated cortex zone, to measure the noise).

Arrows have been added to indicate left and right hemispheres in Figure 6.

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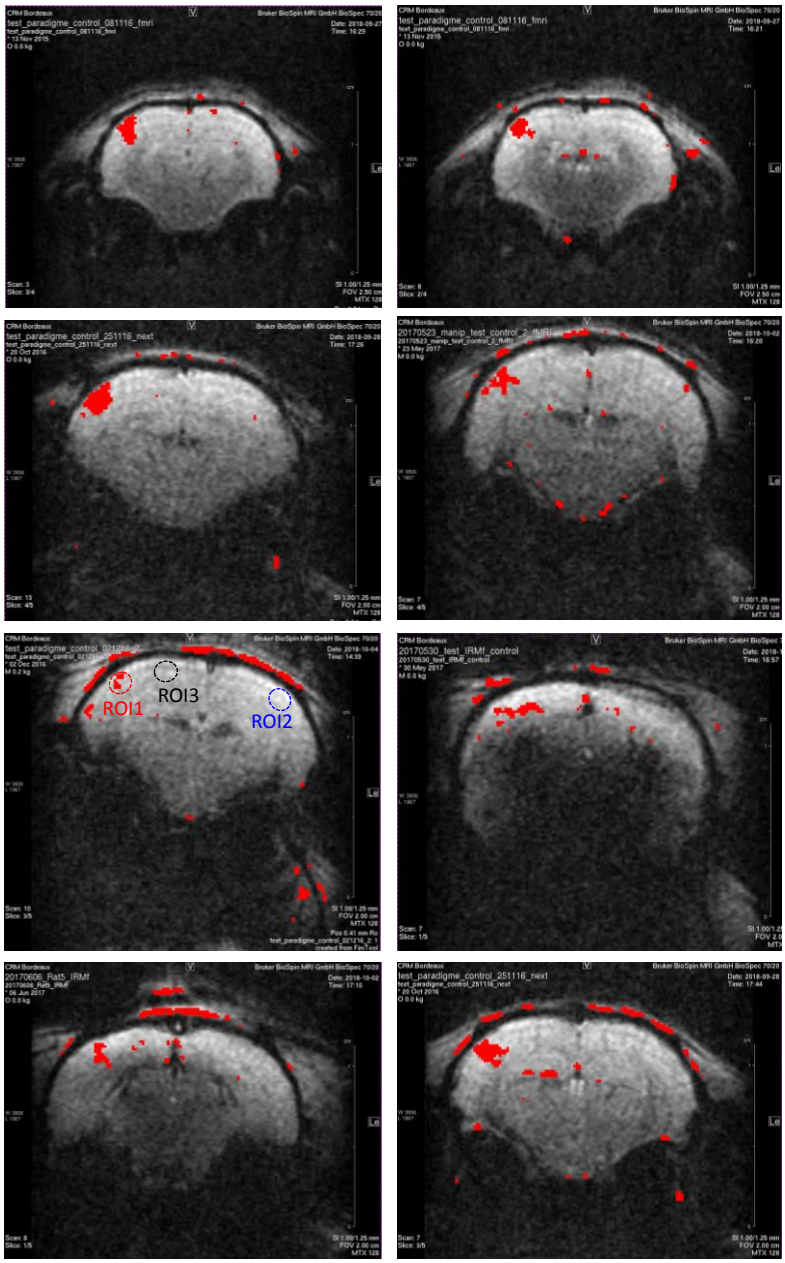

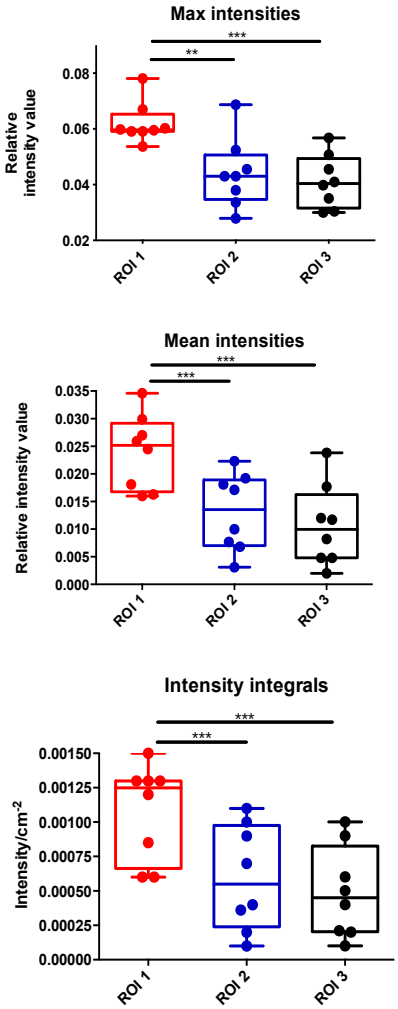

Page 10, line 360: the paragraph is incomplete; the last line trails off with no ending.

We apologize for this mistake. It has been removed.

The authors spend a great deal of the introduction talking about the controversial astrocyte to neuron lactate shuttle, but there is no further discussion of the results and how they contribute to this controversy. This must be addressed.

This point is now discussed l.372-383.

The authors talk about lactate but not about NAA. Please introduce and discuss the changes in NAA.

In the results section, NAA values are presented in more details. It's now clearly stated that NAA decrease was observed on Figure 6, which shows spectra for one rat, but that this

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variation was not found in all animals and that no statistical variation was found. In the discussion, this point has also been extended l.385-390.

The language/writing needs considerable improvement. As a native English speaker it was difficult to get through the whole manuscript. For example, in the abstract I doubt the authors meant to say that "brain activation is controlled by BOLD fMRI" as BOLD fMRI is simply a tool to measure one proxy of brain activation.

The English throughout the article has been copyedited by a professional copyeditor very familiar with scientific English. We also modified the text and the title. It's now clearly explained that the main goal of the article was to present a way to perform functional MRS and that BOLD fMRI was used only to check that the whisker stimulator was efficient. We hope that the overall interest to perform fMRS is now much more clear in this revised manuscript.

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