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In vivo detection of carnosine and its derivatives using chemical exchange saturation transfer

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Purpose: To detect carnosine, anserine and homocarnosine in vivo with chemical exchange saturation transfer (CEST) at 17.2 T.

Methods: CEST MR acquisitions were performed using a CEST-linescan sequence developed in-house and optimized for carnosine detection. In vivo CEST data were collected from three different regions of interest (the lower leg muscle, the olfactory bulb and the neocortex) of eight rats.

Results: The CEST effect for carnosine, anserine and homocarnosine was characterized in phantoms, demonstrating the possibility to separate individual contributions by employing high spectral resolution (0.005 ppm) and low CEST saturation power (0.15 μ T). The CEST signature of these peptides was evidenced, in vivo, in the rat brain and skeletal muscle. The presence of carnosine and anserine in the muscle was corroborated by in vivo localized spectroscopy (MRS). However, the sensitivity of MRS was insufficient for carnosine and homocarnosine detection in the brain. The absolute amounts of carnosine and derivatives in the investigated tissues were determined by liquid chromatography–electrospray ionization–tandem mass spectrometry using isotopic dilution standard methods and were in agreement with the CEST results.

Conclusion: The robustness of the CEST-linescan approach and the favorable conditions for CEST at ultra-high magnetic field allowed the in vivo CEST MR detection of carnosine and related peptides. This approach could be useful to investigate noninvasively the (patho)-physiological roles of these molecules.

KEYWORDS

carnosine, chemical exchange saturation transfer, histidine dipeptides, in vivo MRS, mass spectrometry, ultra-high field

1 | INTRODUCTION

Chemical Exchange Saturation Transfer (CEST)¹ is a noninvasive method that has proved useful to detect low-concentrated endogenous metabolites with labile protons. Based on the exchange of protons between a metabolite of interest with bulk water, CEST provides an indirect detection of the considered metabolite with enhanced sensitivity compared to its direct detection using ¹H-NMR spectroscopy. In the last 10 years, CEST techniques have been widely applied to detect and map important metabolites such as glucose (glucoCEST),² glutamate (gluCEST),³ or creatine (CrCEST),⁴ as well as, less specifically, compounds possessing amide moieties (APT-CEST).⁵ Yet, it remains challenging to investigate weaker CEST effects from molecules with low concentrations and slow exchange rates. In this study, we focus our attention on carnosine, for which the CEST detection has only been reported *in vitro*. Bodet et al.⁶ have studied the CEST effect of carnosine in aqueous solutions (at a concentration of 50 mmol/L) and demonstrated that at 7 T, under physiological conditions ($T = 37^{\circ}\text{C}$, $\text{pH} = 7.1$), carnosine can induce a CEST effect of about 1% (asymmetric magnetization transfer ratio) at approximately 3.1 ppm away from water.

Carnosine (β -alanyl-L-histidine) is a small endogenous dipeptide present in relatively high concentrations in several excitable tissues in mammals.⁷ In particular, carnosine can be found in skeletal muscles (from 5 to 7 mmol/L in humans⁸ and 3 to 5 mmol/L in rats⁹). In rodents, carnosine is present in the muscles together with a methylated derivative called anserine (β -alanyl-3-methylhistidine) whose concentration can reach even higher values.⁹ Relatively large amounts of carnosine have also been reported in the olfactory bulb.¹⁰ In the rest of the brain, the concentration of carnosine decreases below 0.2 mmol/L but another carnosine derivative is also present, homocarnosine (γ -amino-butyryl-histidine) at slightly higher concentrations (≈ 0.5 mmol/L in the rat brain¹¹ and ≈ 0.3 – 0.4 mmol/L in the human brain¹²).

As carnosine, both anserine and homocarnosine have a labile proton capable of generating CEST effect (the amide proton of the peptide bond). Compared to other metabolites, such as glutamate or glucose, the exchange rate of carnosine is one order of magnitude lower,⁶ which limits the attainable signal amplification through CEST and makes its indirect detection *in vivo* more challenging.

A conventional CEST acquisition consists in the recording of the water signal following the application of saturation pulses at varying saturation offsets (the so-called Z-spectrum). Such acquisitions are time-consuming and therefore prone to frequency drifts

and temperature fluctuations, resulting in poor signal stability over time, and compromised detection sensitivity. For this reason, in this study, we chose to use a fast acquisition approach which we call CEST-linescan (CEST-LS), that enables the acquisition of an entire Z-spectrum in only two scans. The CEST-LS pulse sequence was developed according to the ultrafast Z-spectrum (UFZ) methodology introduced by Xu et al.^{13,14} and it is based on the application of magnetic field gradients during the saturation module resulting in the spatial encoding of the saturation frequency. Because it allows a dramatic reduction of the acquisition time, CEST-LS results in increased signal stability while maintaining a contrast-to-noise ratio similar to that of standard CEST sequences, which is particularly useful for *in vivo* applications.

Using the CEST-LS approach in combination with an ultra-high magnetic field (17.2 T), we demonstrate the detection of carnosine and its derivatives *in vivo*, in the brain and the skeletal muscle of healthy adult rats.

2 | METHODS

All phantom and *in vivo* CEST acquisitions were performed on a 17.2 T (¹H Larmor frequency = 730.2 MHz) Bruker Biospec preclinical scanner equipped with a Bruker Avance III console running Paravision 6.0.1 (Bruker BioSpec) and a gradient system allowing a maximum gradient strength of 1 T/m. Phantom images were acquired using a 25-mm diameter quadrature bird-cage volume coil (Rapid Biomedical) while *in vivo* images were acquired using a receive surface coil (Rapid Biomedical) and a 45-mm diameter birdcage transmit volume coil (Rapid Biomedical).

2.1 | CEST-linescan pulse sequence

CEST data were acquired using a CEST-LS sequence developed for Paravision 6.0.1 starting from a standard gradient echo pulse sequence in which we deactivated the phase encoding gradient and added a CEST saturation module. Compared to the simplest implementation of the UFZ technique,¹³ the CEST-LS sequence used here includes a slice selection gradient and outer-volume saturation bands (Figure 1), in order to limit the CEST acquisition to a given region-of-interest (ROI). The saturation module consists in saturation pulses applied on-resonance in the presence of a gradient. As a result, spins that are located along the direction of the gradient at a distance d from the isocenter will experience an irradiation at saturation offsets given by $\gamma \times G_{\text{sat}} \times d$, where γ is the gyromagnetic ratio and G_{sat} is the amplitude of the gradient. During the acquisition,

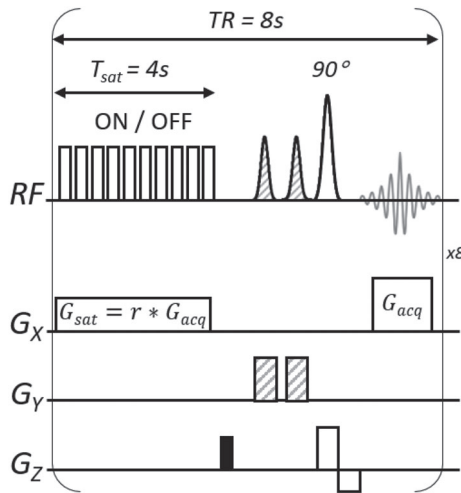


FIGURE 1 The chemical exchange saturation transfer (CEST) saturation module was placed in front of a standard gradient echo sequence in which the phase encoding gradient was removed. Gradient spoilers were applied on the z direction (solid black rectangle). Field of view saturation bands were added to eliminate the signal outside the desired region-of-interest (hatch pattern). The CEST saturation pulses are switched ON and OFF (amplitude at zero) to generate saturated or reference profiles, respectively.

a gradient G_{acq} is applied along the same direction as the saturation gradient. The saturation range, BW_{sat} , is dictated by the acquisition bandwidth, BW_{acq} , and the ratio between the saturation gradient G_{sat} and the acquisition gradient G_{acq} :

$$r = \frac{G_{sat}}{G_{acq}} = \frac{BW_{sat}}{BW_{acq}}$$

It follows that, for an acquisition of N points, the apparent spectral discrimination, equivalent to the saturation frequency increment in conventional Z-spectra and referred to as CEST spectral resolution, is given by $(r \times BW_{acq})/N$.

To compensate for the irregular one-dimensional profile along the encoding direction, a second profile is acquired, with the saturation pulse turned off (corresponding to the proton density distribution along the line). The ratio between the saturated profile over the unsaturated profile gives a normalized Z-spectrum.

2.2 | CEST phantom experiments

The phantom studied was a 2.5 ml syringe filled with a mixture of 10 mmol/L carnosine, 10 mmol/L anserine and 20 mmol/L homocarnosine in 0.01 mol/L phosphate-buffered saline with the pH adjusted to 7.2. Carnosine and anserine were purchased from Sigma-Aldrich and homocarnosine from Cayman Chemical. During MR

acquisitions the syringe was maintained at 37°C using a feedback controlled air heating system.

For all CEST acquisitions, the saturation module consisted in 10 rectangular pulses of 400 ms duration, separated by a 0.01 ms delay. Z-spectra were acquired in a $8 \times 9 \times 5 \text{ mm}^3$ ROI using saturation $B_{1,sat}$ powers ranging between 0.15 and 1 μT . Two different spectral resolutions were used: 0.03 ppm and 0.005 ppm, corresponding to ratios r of 0.05 and 0.01, respectively.

2.3 | In vivo experiments

Eight male adult Dark Agouti rats (200-300 g, Janvier Labs) were used in this study. This strain has been selected because Agouti rats are known to present higher amounts of carnosine and anserine.⁷ The animals were housed by pair under a 12-h night/12-h daylight cycle with water and food available ad libitum. All animal procedures were approved by the Comité d'Éthique en Expérimentation Animale, Commissariat à l'Énergie Atomique et aux Énergies Alternatives, and by the Ministère de l'Éducation Nationale, de l'Enseignement Supérieur et de la Recherche (France) under reference A15 – 40 and were conducted in strict accordance with the recommendations and guidelines of the European Union (Directive 2010/63/EU) and the French National Committee (Décret 2013–118).

During all MRI exams, an air–oxygen mixture (2:1) was continuously delivered to the animals through a nose-cone mask. For induction, isoflurane was maintained at 3% for 3 min, then lowered to 1.5–2% and adjusted throughout the experiment to maintain the respiration rate at around 60 breaths/min. The body temperature was kept between 36 and 38°C using a heated water circulation system. The respiration and body temperature were monitored using a small animal monitoring system (SA Instruments Inc.).

For each exam, an anatomical T_2 -weighted reference image was acquired to help positioning the CEST-LS ROI (Figure 3A). Local B_0 field homogenization was performed prior to the CEST-LS acquisitions using an automatic shimming routine. All CEST-LS acquisitions were performed with $TE/TR = 3/8000 \text{ ms}$ (with negligible T_1 or T_2^* weightings) and a saturation power of 1 μT . Other parameters varied depending on the investigated region and are detailed in Table 1. Eighty repetitions (pairs of CEST-LS datasets with and without the saturation module) were acquired to increase the SNR for a total acquisition time of 21m20s.

Additionally, single-voxel $^1\text{H-NMR}$ spectra were acquired using a LASER sequence¹⁵ ($TE/TR = 25/3000 \text{ ms}$, 256 averages, 8 kHz acquisition bandwidth, 1024

TABLE 1 Parameters used for in vivo Z-spectra generated with CEST-LS. r is the ratio between the saturation gradient G_{sat} and the acquisition gradient G_{acq} . Please note that the spectral resolution is given after zero-filling with a factor of 2. In the manuscript, only zero-filled data are shown, and zero-filled spectral resolutions are given.

Investigated region	Muscle		Olfactory bulb		Neocortex	
CEST-LS ROI dimension (mm ³)	9 × 4 × 2		4 × 1.8 × 2.5		7 × 3 × 2	
Z-spectrum	Full	Zoom	Full	Zoom	Full	Zoom
Ratio r	0.15	0.05	0.15	0.07	0.15	0.07
Saturation range (ppm)	10.3	3.4	10.3	4.8	10.3	4.8
Spectral resolution after zero-filling (ppm)	0.08	0.03	0.08	0.04	0.08	0.04

points) from a $3 \times 1.8 \times 1.5$ mm³ voxel placed at the center of the CEST-LS ROI (Figure 3A). Water suppression was achieved using VAPOR.¹⁶

2.4 | LC-ESI-MS/MS

Olfactory bulb, brain (devoid of olfactory bulb and cerebellum) and skeletal leg muscle (gastrocnemius) tissues were weighted and homogenized at a wet weight:volume ratio of 100 mg tissue:ml of HCOONH₄ at 100 mM, pH 4 at 4°C with a Bead Bug tissue homogenizer (Benchmark Scientific) for 2 min at 4000 rpm. The samples obtained were centrifuged at 4°C for 10 min at 14 000 rpm and the supernatant stored at −80°C until the analysis. Samples for LC-MS analyses were prepared by diluting 20 μl of tissue supernatants with 180 μl of acetonitrile for olfactory bulb and muscle and 80 μl for brain; acetonitrile was spiked with [¹³C3]-carnosine used as internal standard (IS), synthesized as described by Maspero et al.,¹⁷ to reach a 1 μM final concentration. The samples were kept in ice for 20 min and centrifuged at 4°C for 10 min at 14 000 rpm. Aliquots of 100 μl of the supernatant were transferred to vials containing 100 μl of acetonitrile spiked with increasing concentration of carnosine, anserine (Flamma s.p.a., Chignolo D'isola) and homocarnosine (Sigma) to reach the following final concentrations; 0, 1, 2, 3, and 4 μM. Before transfer in vials for the analysis the samples were vortexed for 30 s. Each sample was prepared in triplicate and injected once in a randomized way.

The analyses were performed on an ExionLC-100 coupled to an API4000 equipped with a Turbo-V ESI source (AB-Sciex) using the same chromatographic conditions as reported by Maspero et al.¹⁷ with the following modification: mobile phase A was HCOONH₄ 100 mM at pH = 4 instead of pH = 3. The mass spectrometer was set to acquire in multiple reaction monitoring mode using the transitions reported in Supporting Information Table S1 and identical source parameters as in Maspero et al.¹⁷ The instrument control was carried out with Analyst (version 1.6.3; AB-Sciex).

2.5 | Data analysis

2.5.1 | CEST-LS

All CEST-LS data were analyzed using in-house written MATLAB scripts (Mathworks, version 9.7, R2019b). To obtain the Z-spectra, CEST data were processed as follows: after a zero-filling which doubled the number of FID data points (from 64 to 128), the ratio between the Fourier transform of the signal acquired with and without saturation was plotted against the saturation frequency offsets. The chemical shifts of the Z-spectrum were corrected using a cubic spline data interpolation in order to center the water peak on 0 ppm. For higher resolution Z-spectra, not covering the water peak, the chemical shift correction was performed using other known, major peaks: phosphocreatine at 2.6 ppm in the muscle and creatine at 2 ppm in the olfactory bulb and neocortex.

The in vivo Z-spectra were analyzed using a Python-based algorithm (PEAKIT)¹⁸ able to detect, calculate heights and areas and evaluate the significance of CEST peaks. For the skeletal muscle, only the significance of the carnosine + anserine peak was assessed while for the olfactory bulb and cortex, the areas of carnosine, homocarnosine and APT peaks were also determined. The area of the APT peak was calculated over a range of 2.8 ppm and centered on the peak maximum, as found by PEAKIT. For the carnosine and homocarnosine peaks, the range for area calculation was automatically selected by PEAKIT. PEAKIT is freely available from <https://github.com/SKMikee/PeakIt>.

2.5.2 | MRS

Following removal of the residual water signal using the Hankel Lanczos singular value decomposition (HLSVD) algorithm, in vivo ¹H-NMR spectra were processed over the [0.5–9] ppm range using LCModel 6.2.¹⁹ The basis set of metabolite spectra was simulated using a spin

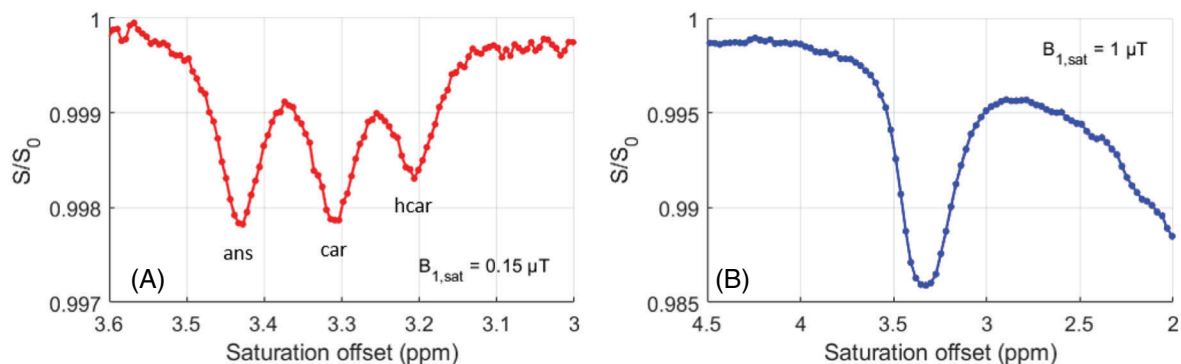


FIGURE 2 (A) CEST-LS Z-spectrum obtained with $B_{1,sat}$ 0.15 μ T and a resolution of 0.005 ppm. Homocarnosine, carnosine and anserine peaks are clearly visible and distinct. (B) CEST-LS Z-spectrum obtained with $B_{1,sat}$ 1 μ T and a resolution of 0.03 ppm. The Z-spectra in A and B were averaged over 10 and 18 repetitions, respectively. We note that in,⁶ the CEST effect of carnosine was reported at 3.1 ppm which is slightly different from our results. This is most likely due to slight differences in pH.

simulation software implemented in Matlab and based on the density matrix formalism.

2.5.3 | LC-ESI-MS/MS

The calibration curves were built for each analyzed sample by using a standard addition method. The peak area ratios of analyte/IS were plotted versus the nominal concentrations of genuine carnosine, anserine and homocarnosine spiked in the sample to reach the following final concentrations: 0, 1, 2, 3, and 4 μ M. Weighted ($1/\times 2$) least-squares linear regressions were calculated by using the software GraphPad Prism. The calculated analyte concentration was then reported as moles per gram of wet tissue on the basis of the weight of the wet tissue and serial dilutions.

3 | RESULTS

3.1 | In vitro study

Figure 2 shows representative Z-spectra acquired on the mixture sample with $B_{1,sat}$ of 0.15 μ T and spectral resolution 0.005 ppm (Figure 2A) and $B_{1,sat}$ of 1 μ T and spectral resolution 0.03 ppm (Figure 2B). The very low $B_{1,sat}$ of 0.15 μ T coupled with a very high spectral resolution (0.005 ppm) allowed to accurately separate and detect the CEST effects of homocarnosine (hcar), carnosine (car) and anserine (ans) at 3.20 ± 0.01 ppm, 3.31 ± 0.01 ppm and 3.43 ± 0.01 ppm, respectively. These parameters did not, however, give satisfactory results in vivo due to the low contrast-to-noise ratio. Moreover, we aimed at covering a range of frequencies sufficiently large to encompass reference peaks such as creatine or phosphocreatine, which set

a higher bound for the resolution at 0.03 ppm (see next section). We therefore used $B_{1,sat}$ of 1 μ T and a spectral resolution equal or lower than 0.03 ppm for all in vivo acquisitions. Figure 2B shows the CEST-LS Z-spectrum obtained on the mixture sample with $B_{1,sat}$ of 1 μ T and spectral resolution of 0.03 ppm. The larger bandwidth corresponding to the higher $B_{1,sat}$ power did not allow the separation of the three peptides. We conclude therefore that in vivo one cannot distinguish between carnosine, anserine and homocarnosine.

3.2 | In vivo study

3.2.1 | Skeletal muscle

The position of the CEST-LS acquisition is depicted by the orange ROI shown on the anatomical image of a rat lower-leg in Figure 3A. Figure 3B shows an example of a Z-spectrum (spectral resolution 0.08 ppm) acquired in the orange rectangle in Figure 3A, in which we can recognize the creatine (Cr) and phosphocreatine (PCr) CEST peaks on the left, as well as a broad peak corresponding to the NOE effect from aliphatic protons (Nuclear Overhauser Enhancement) on the right. Figure 3C shows a higher spectral resolution Z-spectrum (0.03 ppm resolution) which was acquired using a weaker CEST saturation gradient ($r = 0.05$ vs. 0.15). The Cr and PCr peaks are more evident at higher spectral resolution. Moreover, two additional peaks corresponding to the combination of carnosine and anserine at 3.32 ± 0.06 ppm and APT at 3.5 ± 0.06 ppm are also visible.

Similar results were obtained on four different animals. The analyses performed with PEAKIT confirmed the significance of all carnosine + anserine peaks ($p < 0.05$).

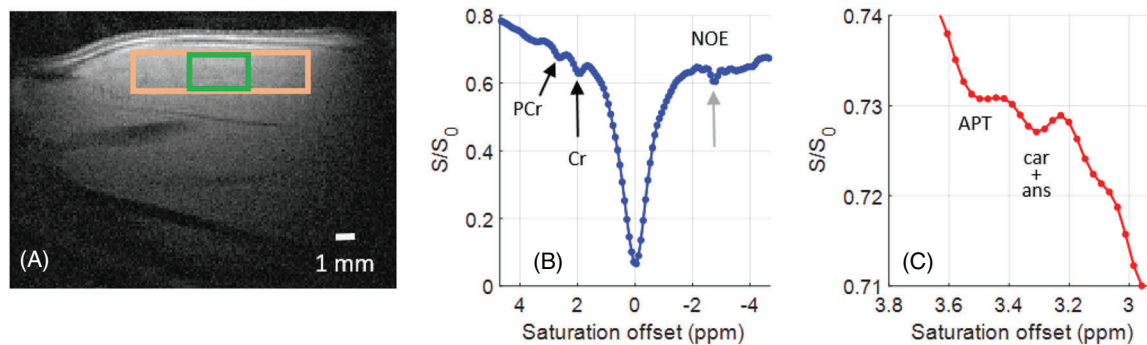


FIGURE 3 (A) Anatomical image of a rat lower-leg muscle showing the ROI used for CEST-LS in orange and the voxel for $^1\text{H-NMR}$ spectroscopy in green. (B) Low resolution Z-spectrum showing the Creatine (Cr) and PhosphoCreatine (PCr). The sharp peak indicated by a gray arrow at -2.7 ppm corresponds to fat²⁰ (C) Zoom showing combined car + ans and APT peaks. All Z-spectra were acquired with $B_{1,\text{sat}} = 1 \mu\text{T}$.

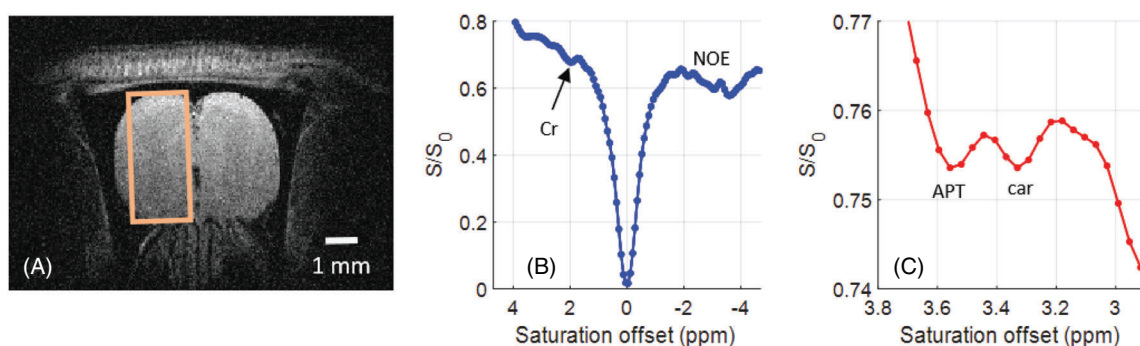


FIGURE 4 (A) Anatomical image of a rat olfactory bulb showing the region-of-interest used for the CEST-LS acquisitions; (B) Low-resolution Z-spectrum showing the Cr peak (C) High resolution Z-spectrum showing car and APT peaks. All Z-spectra were acquired with $B_{1,\text{sat}} = 1 \mu\text{T}$.

3.2.2 | Olfactory bulb

As the olfactory bulb is a relatively small region, the ROIs chosen for the CEST-LS acquisitions were smaller than in the leg (Figure 4A and Table 1). The Z-spectrum in Figure 4B shows clearly a peak at 2 ppm, as well as a shoulder at 2.6 ppm corresponding to Cr and PCr, respectively. By increasing the spectral resolution to 0.04 ppm (Figure 4C), we put in evidence the APT peak at 3.5 ppm. Close to it, the carnosine peak is recognizable. Note that the spectral resolution for the Z-spectrum in Figure 4C is slightly lower than that for the leg muscle to compensate for the lower signal-to-noise ratio due to the smaller ROI size. Similar results were obtained in four animals with the significance for the carnosine peak confirmed by PEAKIT ($p < 0.05$). The average ratio of the carnosine to the APT peak areas was found 0.51 ± 0.31 .

3.2.3 | Neocortex

Finally, the last set of experiments was performed in the neocortex. Despite a lower histidine dipeptides expected

concentration,⁹ the larger size of this brain structure and the better B_0 homogeneity compared to the olfactory bulb were beneficial to the SNR of these experiments. On the low resolution Z-spectrum (Figure 5B) acquired in the orange ROI in Figure 5A we recognize the 2 ppm Cr peak.²¹ The zoom on the high resolution Z-spectrum (Figure 5B) shows a broad APT peak as well as a local decrease around 3.2 ppm which corresponds to the CEST contribution of homocarnosine. The homocarnosine peaks were detected with a p-value below 0.05 using PEAKIT for all four animals scanned. The average ratio of the homocarnosine to the APT peak areas was found 0.21 ± 0.06 , which is 2.4 times lower than the carnosine/APT ratio found in the olfactory bulb.

3.2.4 | In vivo direct spectroscopy

The $^1\text{H-NMR}$ spectra of carnosine and carnosine derivatives samples show two distinct resonances downfield from water at chemical shifts 7.08 and 8.08 ppm, corresponding to the C4 and C2 histidine-imidazole protons, respectively. These two resonances are easily identifiable

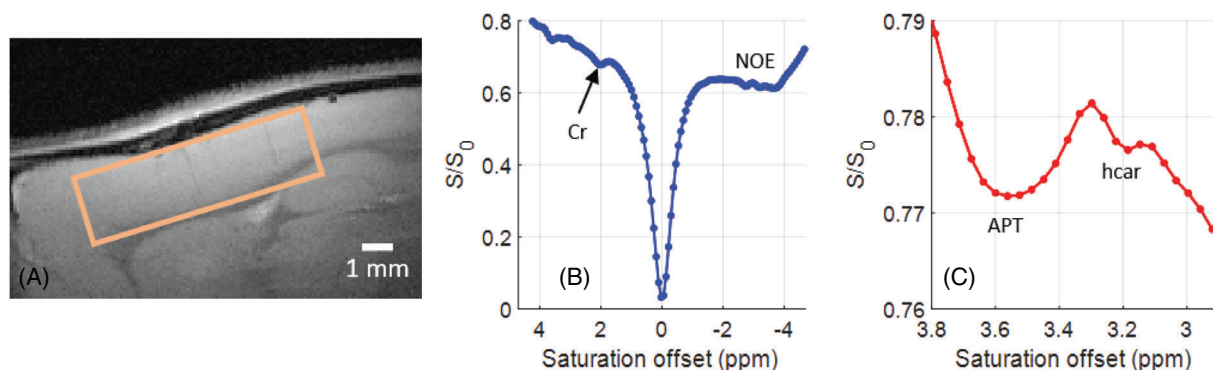


FIGURE 5 (A) Anatomical image of a rat neocortex showing the region-of-interest used for the CEST-LS acquisitions. (B) Low-resolution Z-spectrum showing the Cr peak. (C) High-resolution Z-spectrum showing the hcar and APT peaks. All Z-spectra were acquired with $B_{1,sat}=1 \mu T$.

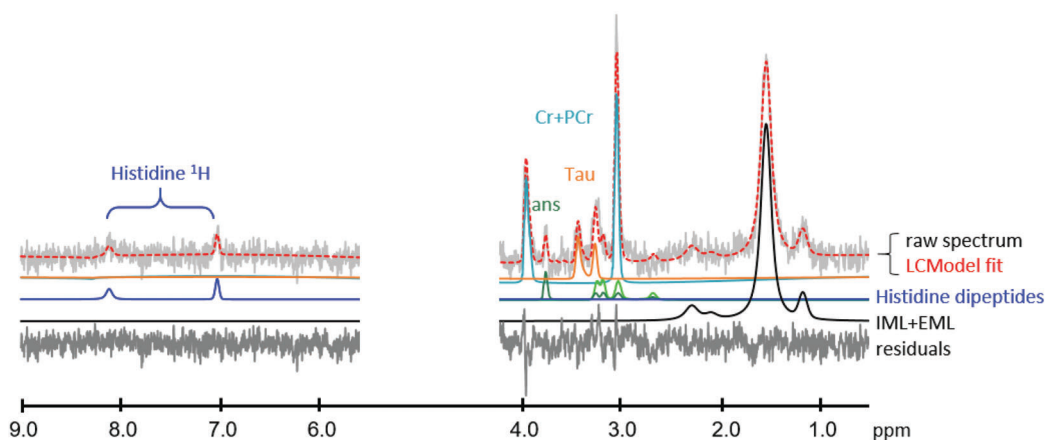


FIGURE 6 In vivo NMR spectrum acquired on a $3 \times 1.8 \times 1.5 \text{ mm}^3$ voxel in the leg skeletal muscle (green box in Figure 3A). Peaks at approximately 7.08 and 8.08 ppm are the signature of both carnosine and anserine (labeled Histidine dipeptides and represented in blue) while the peak at 3.7 ppm is proper to anserine (dark green). The light green line on the right represents carnosine.

on the spectrum in Figure 6, which was obtained in the green voxel showed in Figure 3A, confirming the presence of carnosine and anserine in the leg skeletal muscle. From the decomposition of the ^1H -NMR spectra using the LCModel,¹⁹ we estimate an average ($N = 4$ animals) of total carnosine concentration of $(8.4 \pm 0.5) \text{ mmol/L}$ considering a Cr+PCr concentration of 38 mmol/L.

For the olfactory bulb and the neocortex, ^1H -MRS did not allow the detection of neither carnosine nor homocarnosine. The heterogeneity and the small size of the olfactory bulb as well as the low concentration expected in the neocortex may explain why we were below the limit of detection in both cases.

3.2.5 | LC-ESI-MS/MS

The calibration curves built for each sample were linear for all the three analytes (carnosine, anserine, and

homocarnosine) with R^2 from 0.8990 to 0.9689 for the olfactory bulb, 0.8821 to 0.9636 for the muscle, and 0.9535 to 0.9845 for the brain. The extrapolated concentrations of the three dipeptides were calculated for each sample, and the results expressed as nmoles of dipeptide contained in 1 g of wet tissue. Table 2 reports the mean \pm SD of carnosine, anserine and homocarnosine in the rat tissues. Anserine is the most abundant histidine dipeptide in skeletal muscle, with a concentration of almost 36% higher than carnosine while homocarnosine was not detected. The olfactory bulb contains only carnosine while anserine and homocarnosine were both undetectable. Homocarnosine was the most abundant peptide in the brain with a content almost three time higher than that of carnosine while anserine was not detected. Table 2 also reports the total amount of histidine dipeptides calculated as the sum of carnosine + anserine + homocarnosine. The skeletal muscle is the tissue characterized by the highest amount of histidine dipeptides with a content almost

TABLE 2 Carnosine, anserine, homocarnosine and total histidine dipeptides concentrations found in the muscle, olfactory bulb and brain. The values are averaged over four rats and reported as nmoles of dipeptide per gram of wet tissue \pm SD.

	Concentration (nmoles/g of wet tissue)			
	Carnosine	Anserine	Homocarnosine	Histidine dipeptides
Muscle	2262.2 \pm 1254.7	3518.5 \pm 774.6	N.D.	5780
Olfactory bulb	447.5 \pm 116.0	N.D.	N.D.	447.5
Brain	55.7 \pm 18.4	N.D.	168.9 \pm 39.9	224.6

Abbreviation: N.D., not detected.

13 and 25 folds higher than that of the bulb and brain, respectively.

4 | DISCUSSION AND CONCLUSIONS

In this study, we demonstrate the *in vivo* detection of carnosine and its derivatives in rats using CEST. This was accomplished by using a custom designed fast CEST acquisition, CEST-LS, in combination with an ultra-high magnetic field (17.2 T) imaging system. The characterization of the CEST effect was performed using the PEAKIT software,¹⁸ which makes a local estimation of the baseline and therefore reduces the contamination from other broad CEST signals (e.g. taurine, glutamine, glutamate in the brain²²) improving the detection selectivity of the technique. We cannot however, completely exclude any signal contamination from other metabolites or peptides if present. The presence of carnosine and derivatives was validated in the leg with direct NMR spectroscopy *in vivo* but was not possible for the olfactory bulb and the neocortex. This confirms that, under our experimental conditions, CEST is a more sensitive approach than localized MRS.

Moreover, by optimizing the CEST-LS acquisition parameters we were able to disentangle the contributions of carnosine, anserine and homocarnosine in a phantom sample. This was, however, not possible *in vivo* due to the low CEST effect achieved with the saturation powers employed for *in vitro* acquisitions (0.15 μ T) and the presence of other CEST contributions.

The LC-ESI-MS/MS quantification of carnosine, anserine and homocarnosine reports a content of histidine dipeptides of more than 13 and 25 times higher in the skeletal muscle than in the olfactory bulb and brain, respectively. These data explain well the ¹H-MRS results and in particular the fact that the technique is not able to detect these peptides in the olfactory bulb and in the neocortex. According to the LC-ESI-MS/MS analysis the concentration of carnosine in the olfactory bulb is approximately 2.6 times higher than the homocarnosine concentration found

in the rest of the brain. These results are in agreement with CEST-LS data, which, taking into account the area ratio carnosine/APT for the olfactory bulb and homocarnosine/APT for the neocortex as an index of the relative histidine dipeptide content indicate a 2.4 times higher concentration in the olfactory bulb.

Although some of its physiological functions are still being investigated, it has been clearly established that carnosine presents direct and indirect antioxidant activity through its ability to scavenge free radicals,^{23,24} inhibit protein carbonylation and glycoxidation,^{25,26} and chelate metallic ions.²⁷ Carnosine was suggested to be involved in various biochemical processes, including pH-buffering in the muscle²⁸ and Ca²⁺ regulation.²⁹ Tiedje et al.³⁰ have also presented carnosine as an important neurotransmitter in the olfactory bulb. Homocarnosine is seen in long range projecting GABAergic neurons³¹ and appears to possess anticonvulsant properties as well as to play a role in modulating cortical excitability.^{32,33} Despite this, there are very few human study reports of *in vivo* homocarnosine/carnosine detection using MRS^{12,34-36} and, to our knowledge, none using CEST. This is nevertheless due to the low concentrations at which these metabolites are present in the brain. CEST approaches as the one presented in this manuscript could potentially be extended to human studies. The optimal B_{1,sat} power for *in vivo* carnosine detection is relatively low at 1 μ T, and therefore compatible with the specific absorption rate constraints imposed for human brain investigations. Carnosine presents a CEST contribution at 3.3 ppm away from water and therefore its detection is only moderately affected by direct water saturation with the considered B_{1,sat} values. Numerical simulations performed at 11.7, 7, and 3 T show a carnosine CEST effect decrease by 8, 23, and 48%, respectively, compared to that at 17.2 T (Supporting Information Figure S1). This decrease combined with the close proximity of other CEST contributions such as glutamate at 3 ppm and APT at 3.5 ppm will make the *in vivo* detection of carnosine and derivatives at lower magnetic fields more challenging. Further investigations are necessary in order to assess whether ultra high magnetic field scanners (from 7 up to 11.7 T)

available today for human imaging would allow the CEST detection of homocarnosine/carnosine in the human brain or anserine/carnosine in the skeletal muscle.

While the CEST-linescan strategy presented here offers the great advantage of being minimally affected by temporal variations in frequency, temperature, or physiological parameters, it has the local nature of the detection as main limitation. It should be noted, however, that the technique does not require a homogeneous sample along the gradient direction for accurate local measurements, as the signal is normalized by the reference signal at each position acquired in the absence of saturation. This normalization procedure also makes the CEST-LS technique less susceptible to B_0 inhomogeneities compared to standard CEST approaches. In the current study, we used the water peak or other known CEST peaks to find the chemical shift offset of the peaks under study. The precision of such referencing can be affected by large B_0 fluctuations, which can however be corrected by acquiring a B_0 map in the CEST-LS ROI. The implementation of such correction step is underway. We note that a similar approach, ultrafast localized CEST-spectroscopy with PRESS,³⁷ has been previously used for performing CEST acquisitions in the human brain at 3 T. Compared to ultrafast localized CEST-spectroscopy with PRESS, our CEST-LS approach offers more flexibility in the choice of the acquisition parameters, allowing for shorter echo times.

To conclude, despite the low CEST effect exhibited by carnosine and its derivatives, we show the possibility of their *in vivo* detection, both in the skeletal muscle and in the brain using CEST-LS. We propose this approach for the detection of other low concentrated metabolites both in preclinical and clinical settings at high magnetic fields.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

Table S1 MRM transitions for carnosine, anserine, homocarnosine and L-carnosine ¹³C3.

Figure S1 Simulated MTR_{asym} for a 10 mM carnosine solution at four different B₀ magnetic fields: 3 T, 7 T, 11.7 T and 17.2 T. The Bloch-McConnell equations were solved using a twopool exchange model and employing the Matlab tool "CEST sources" available at www.cestsources.org. For simulations, the relaxation parameters of gray matter water were taken to be T1 = 1, 1.7, 2.1, 2.3 s and T2 = 0.08, 0.055, 0.035, 0.03 s for B0 = 3, 7, 11.7, 17.2 T, respectively.

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