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Brain intracellular metabolites are freely diffusing along cell fibers in grey and white matter, as measured by diffusion-weighted MR spectroscopy in the human brain at 7 T

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Abstract

Due to the specific compartmentation of brain metabolites, diffusion-weighted magnetic resonance spectroscopy opens unique insight into neuronal and astrocytic microstructures. The apparent diffusion coefficient (ADC) of brain metabolites depends on various intracellular parameters including cytosol viscosity and molecular crowding. When diffusion time (t_d) is long enough, the size and geometry of the compartment in which the metabolites diffuse strongly influence metabolites ADC. In a previous study, performed in the macaque brain, we measured neuronal and astrocytic metabolites ADC at long t_d (from 86 ms to 1011 ms) in a large voxel enclosing an equal proportion of white and grey matter. We showed that metabolites apparently diffuse freely along the axis of dendrites, axons and astrocytic processes. To assess potential differences between these two tissue types, here we measured for the first time in the Human brain the t_d -dependency of metabolites trace/3 ADC at 7 teslas using a localized diffusion-weighted STEAM sequence, in parietal and occipital voxels respectively containing mainly white and grey matter. We show that, in both tissues and over the observed timescale (t_d varying from 92 to 712 ms) metabolite ADC reaches a non-zero plateau, suggesting that metabolites are not confined inside subcellular regions such as cell bodies, or inside subcellular compartments such as organelles, but are rather free to diffuse in the whole fiber-like structure of neurons and astrocytes. Beyond the fundamental insights into intracellular compartmentation of metabolites, this work also provides a new framework for interpreting results of neuroimaging techniques based on molecular diffusion, such as diffusion-weighted magnetic resonance spectroscopy and imaging.

Keywords

Magnetic resonance spectroscopy; diffusion; metabolites; human brain; microstructure; intracellular compartmentation.

Introduction

Diffusion-weighted NMR spectroscopy (DW-MRS) offers the unique ability to noninvasively quantify the translational displacement of endogenous metabolites *in vivo* (Nicolay et al., 2001). Under normal conditions, brain metabolites hardly cross the biological membranes so that their diffusion path is dictated by intracellular parameters such as cytosol viscosity, molecular crowding, size and shape of the compartment. Furthermore, the preferential compartmentation of glutamate and N-acetyl-aspartate in neurons and of myoinositol and choline compounds in astrocytes (Urenjak et al., 1993; Choi et al., 2007) opens a unique insight into different cellular environments. The impact of various pathologies (global ischemia, multiple sclerosis or brain tumors) on intracellular diffusion has been studied (Harada et al., 2002; Budde and Frank, 2010; Wood et al., 2012; Zheng et al., 2012), and observed changes were suggested to be related to microstructural alterations, such as axonopathy and alterations in cellular viscosity or restriction. However, the relative contribution of each of these parameters to NMR-measured diffusion is unclear and the interpretation of experimental data remains complex and additional studies are still required to better characterize the main determinants of intracellular diffusion.

The apparent diffusion coefficient (ADC) as measured by DW-MRS is related to the average quadratic displacement $\langle x^2 \rangle$ of molecules within the time during which the displacement is observed (the diffusion time t_d), with $ADC \sim \langle x^2 \rangle / (2t_d)$ in the approximation of the Gaussian phase distribution, valid at low diffusion-weighting factors. Performing measurements at different diffusion times, and observing the time dependence of $\langle x^2 \rangle$ or of the ADC may allow untangling and quantifying the different parameters governing molecular displacement. Recently, the use of oscillating gradients allowed us to reach ultra-short diffusion times (< 1 ms) and probe short-range restriction and cytosol viscosity in the rat brain (Marchadour et al., 2012). On the opposite end of the diffusion time range, if diffusion time is long enough, restriction by cell walls is expected to have a more and more significant impact on the ADC, with metabolite ADC decreasing towards an asymptotic value in a manner that depends on cell geometry and size. In this context, we have recently shown that, in a large voxel enclosing an equal proportion of white and grey matter in the monkey brain, ADC values of both neuronal and astrocytic metabolites barely depended on t_d at long t_d values, varying from 86 ms to 1 s (Najac et al., 2014). The observed metabolite ADC stability is not consistent with the idea of metabolites being confined in subcellular regions such as cell bodies, or in small subcellular compartments such as organelles (nucleus, mitochondria, etc...), in which case metabolite ADC would tend toward zero as t_d is increased, since $\langle x^2 \rangle$ would reach an upper limit imposed by compartment size. This behavior is rather characteristic of “unrestricted” diffusion, as it occurs in the direction parallel to the fibers both in neurons (axons, dendrites) and in astrocytes (astrocytic processes), while restriction in the transverse directions is extremely strong, i.e. the ADC perpendicular to the fibers ($ADC_{\perp}$) is approximately zero in the range of measured t_d (the fact that $ADC_{\perp}$ is approximately equal to zero in the range of measured t_d has been recently corroborated by measurements in the corpus callosum in the Human brain (Ronen et al., 2014)). In these conditions and at long t_d , ADC equals $D_{intra}/3$ for isotropically oriented fibers, D_{intra} being the intracellular diffusion coefficient including effects of cytosol

viscosity, molecular crowding and intracellular tortuosity (Najac et al., 2014). Although it provided new insights into diffusion and compartmentation, a limitation of our previous work in the monkey brain was that the spectroscopic voxel had to be big enough to reach sufficient signal-to-noise ratio, and thus contained equal proportions of grey and white matter. However, grey and white matter are very different in terms of cellular organization; thus a logical question is whether there are potentially different diffusion behaviors in these two tissue types. In particular, grey matter is sometimes represented as a collection of neuronal cell bodies (which is indeed a striking characteristic seen on histology data), suggesting that fiber-like diffusion may not be characteristic of grey matter neurons, while it is well known that white matter is essentially made of myelinated axons and contains very few neuronal soma. The potential subcellular compartmentation of metabolites in organelles may also be different in both tissues, since grey matter contains most of the neuronal nuclei, as well as a larger number of mitochondria to meet its superior energy needs. In the present study, in order to assess tissue differences in metabolite compartment shape, we explored metabolites diffusion in the human brain at 7 teslas, allowing us to measure metabolite diffusion at very long t_d in two voxels containing significantly different proportions of white and grey matter.

Materials and Methods

This study adhered to the LUMC Institutional Review Board guidelines. A total of sixteen healthy volunteers (age 25 ± 5 years, 9 females and 7 males) participated in this study and gave informed consent prior to the session.

Data acquisition

Experiments were performed on a 7 T Philips Achieva whole-body MRI scanner (Philips Healthcare, Best, The Netherlands) equipped with gradient coils reaching a maximal strength of 40 mT/m with a slew rate of 200 T/m/s. A head RF coil consisting of a quadrature birdcage for transmission and 32-channel phased array for reception (Nova Medical Ins., Wilmington, MA) was used for all measurements.

Anatomical Images—A short survey scan and a sensitivity encoding (SENSE) reference scan were followed by a 3D T_1 -weighted gradient echo acquisition to allow for accurate planning of the experiment. Imaging parameters were: field of view (AP, FH, RL) $246.4 \times 246.4 \times 174.0$ mm³, resolution $1 \times 1 \times 1$ mm³, TR/TE=4.7/2.1 ms (total scan time: 3 min).

DW-MRS—Two sets of experiments were carried out in a 12 mL voxel ($20 \times 20 \times 30$ mm) positioned in the parietal lobe and in a 12 mL voxel ($20 \times 20 \times 30$ mm) positioned in the occipital lobe (Figure 1). DW-MRS data were acquired using a diffusion-weighted stimulated echo acquisition mode (STEAM) sequence (echo time TE=50 ms and variable mixing time TM, see below). Cardiac gating on every 3rd cardiac cycle was achieved using a pulse-oximeter resulting in a TR of about 3 s. As shown previously, this allowed avoiding strong fluctuations in signal intensities due to cardiac pulsation (Posse et al., 1993; Upadhyay et al., 2007). Shimming was performed using a pencil beam method employing

second order shims and resulting in tNAA linewidth of ~18 Hz. Full water suppression was applied for non-diffusion-weighted spectra and only partial suppression for diffusion-weighted spectra to allow for a sufficient water signal residue for individual spectral phasing and frequency drift corrections of each spectrum during post-processing (Kan et al., 2012). Diffusion-weighting was applied successively in three orthogonal directions ([1 1 -0.5], [-0.5 1 1] and [1 -0.5 1]) yielding maximal effective gradient strength (Gudbjartsson et al., 1996). The diffusion gradient duration δ was set to 24 ms and the separation time between gradients was 100, 550 and 720 ms (obtained varying mixing time TM). Two b -values were chosen (a null b -value and a high b -value at 2828, 2899 and 2856 s/mm² at t_d =92, 542 and 712 ms respectively) resulting in a different gradient strength at each diffusion time (maximum G_{diff} =35, 15 and 13 mT/m respectively). A bipolar gradient scheme was employed to minimize eddy currents. Since at long diffusion time, cross-terms between diffusion gradients and other gradients (crusher, slice-selective and background gradients) may become significant, possibly leading to biased ADC measurement (Neeman et al., 1990; Zhong et al., 1991), additional DW-spectra were acquired with diffusion gradients of opposite polarity for each gradient direction and each t_d (Neeman et al., 1991; Jara and Wehrli, 1994; Najac et al., 2014). Spectra were acquired with an increased number of averages to maintain similar SNR for each diffusion time (32 averages at t_d =92/542 ms and 48 averages at t_d =712 ms). Non-water suppressed spectra (4 averages) were also acquired for eddy current correction. Data at two different diffusion times in one voxel-of-interest could be acquired during each session resulting in 4 data sets acquired at t_d =92/712 ms and 3 sets acquired at t_d =542 ms in parietal lobe, and 5 data sets acquired at t_d =92/542 ms and 6 sets at t_d =712 ms in occipital lobe.

Data processing

Anatomical Images—3D T₁-weighted images were segmented using FSL (Brain Extraction Tool (Smith, 2002), FMRIB's Automated Segmentation Tool (Zhang et al., 2001) and an in-house Matlab routine to determine the volume fraction of white matter (WM), grey matter (GM) and cerebrospinal fluid (CSF) in the volumes-of interest.

DW-MRS—Post-processing was performed with in-house Matlab routines (The Mathworks, Natick, MA, USA) and consisted of scan-to-scan phasing, frequency drift correction and eddy current correction using non-water suppressed reference spectra. Resulting spectra were then analyzed with LCModel (Provencher, 1993) with basis-sets generated by home-written density matrix simulation programs, tailored for each t_d used in the experiments. Only metabolites with Cramér-Rao lower bound (CRLB) lower than 10% at all b -values were retained for the analysis: total NAA (tNAA=NAA+NAAG), total creatine (tCr=Cr+PCr) and choline compounds (tCho=Cho+PCho+GPC). For each direction, ADCs were calculated as the logarithm of the geometric mean of the signal obtained with both diffusion gradient polarities, divided by the reference signal, to eliminate the bias due to cross-terms (Neeman et al., 1991; Jara and Wehrli, 1994). Subsequently, the rotationally invariant metabolite trace/3 ADC was estimated by averaging the ADC values for the three directions (Basser et al., 1994). Additionally, the water trace/3 ADC was also measured using the non-water suppressed spectra. In the following, reported ADC values will refer to trace/3 ADC.

Statistical Analysis

Differences in ADC between WM and GM voxels were derived calculating the p-value using a standard unpaired Student's t-test.

Results

Cross-terms correction

To verify that cross-terms between the gradients are properly cancelled, an *in vitro* experiment was performed at two diffusion times ($t_d=92$ and 542 ms) at room temperature on a water phantom. Spectra were acquired with the same design as *in vivo* experiments but with a smaller b -value ($b=0$ and 770 s/mm², maximum $G_{diff}=17.5$ and 7.5 mT/m at $t_d=92$ and 542 ms respectively). As shown in Table 1, large differences are observed between water ADC measured with positive and negative gradient polarities. When increasing t_d , water ADC increases with positive gradient and decreases with negative gradient. When averaging the water signal obtained with both gradients polarities, the water ADC remains stable. This demonstrates that when measuring diffusion using a diffusion-weighted sequence such as the STEAM sequence used here, the effect of cross-terms at long diffusion time is significant, and that this effect can be cancelled out by measuring the geometric mean of the signal obtained with separately measured positive and negative diffusion gradients. It is important to note that potential cross-terms due to spatially varying microscopic gradients cannot be compensated for using this method.

GM, WM and CSF volume fraction

Table 2 shows the volume fraction of WM, GM and CSF in the two regions-of-interest. As expected, the fractions are significantly different in parietal voxels compared to occipital voxels. Parietal voxels predominantly reflected WM, whereas occipital voxels predominantly reflected GM.

In vivo brain metabolite ADC

Representative MR spectra at null and high b -values at the longest diffusion time are shown in Figure 1. The SNR was very similar in all spectra when increasing the diffusion time (SNR=16±3 at null b -value and SNR=11±2 at high b -value when SNR is calculated for all t_d and in both parietal and occipital voxels). The spectral linewidth (FWHM) remained also relatively stable when increasing the b -value (tNAA FWHM=17.5±1.6 Hz at null b -value and 19.8±3.8 Hz at high b -value in the parietal voxels and tNAA FWHM=15.6±2.5 Hz at null b -value and 16.8±2.8 Hz at high b -value in the occipital voxels when averaging FWHM for all t_d) indicating a good eddy current correction. Although good quality spectra could be obtained for all t_d and b -values, only the signals of tNAA, tCr and tCho could be quantified with enough precision (LCModel CRLB < 10%). Figure 2 shows the ADC for the three metabolites as a function of t_d in both volumes of interest. As can be appreciated from the figure, metabolite ADC did not depend on diffusion time in either region-of-interest. ADC for the three metabolites (tNAA, tCho, and tCr) averaged over the subjects are reported in Table 3. For tNAA and tCr, ADC was significantly lower in grey matter than in white matter ($p<0.006$), as has been previously reported (Ellegood et al., 2006; Ellegood et al., 2011; Kan

et al., 2012), whereas for tCho the difference in ADC between the two tissue types did not reach statistical significance ($p=0.064$), as also reported recently (Ercan et al., 2014).

***In vivo* water ADC**

As shown in Figure 2 and Table 3, water ADC remained stable in parietal white matter and a small decrease was observed in occipital grey matter. To the opposite of metabolites, water ADC in white matter was significantly lower than in grey matter, as observed previously, although ADC values reported here are lower (Ellegood et al., 2005). This difference might be ascribed to the higher b -value used in our studies. Comparison with one previous study, performed at 7 teslas using a similar b -value and nearly identical white matter voxel, reveals no strong difference in water ADC (Branzoli et al., 2014).

Discussion

Data quality

The good spectral quality achieved in the experiments enabled us to accurately estimate the ADC of three brain metabolites (tNAA, tCr, tCho) at long diffusion times (from 92 ms up to 712 ms) in two very different regions of the human brain: a parietal voxel containing predominantly white matter, and an occipital voxel that comprised mostly grey matter. Cross-terms between diffusion and other gradients were efficiently minimized by acquiring DW-spectra with positive and negative diffusion gradient polarity, contributing to the accuracy of the subsequent ADC calculations.

No dependence of metabolite ADC on the diffusion time

In the present study, no dependence of metabolite ADC on the diffusion time was observed in either predominantly white matter or in predominantly grey matter voxels. This result is similar to our former study performed in the monkey brain at 7 T in a voxel that contained a mixture of white and grey matter (Najac et al., 2014). It suggests that metabolite ADC at long diffusion time is predominantly determined by a “free” diffusion component, whereby the metabolites diffuse parallel to the axis of fiber-like cellular constituents such as axons and dendritic processes in both white and grey matter, rather than in close-structure cells bodies or in organelles, where the diffusion, in absence of inter-compartmental exchange, is isotropically restricted over relatively short distances, rapidly leading to $ADC \sim 0$. Figure 3 illustrates how these different situations would affect metabolite ADC, and how the comparison with experimental data rules out the possibility that metabolites are confined in subcellular regions or organelles (see figure legend for details, in particular for the numerical simulations used). Although it cannot bring specific information about the intracellular compartment shape/size, the stability of water ADC with diffusion time is consistent with the idea that water diffusion can only be less restricted than for metabolites, as water is also present in the extracellular space and can diffuse more easily through biological membranes.

Fiber-like cell structure of both neurons and astrocytes

The cell-specific compartmentation of metabolites (tNAA in neurons, tCho in astrocytes, and tCr in all cells) provides a unique insight into the different cellular architectures in both white and grey matter (Urenjak et al., 1993; Choi et al., 2007). The human cortex has 1.4

astrocytes for every neuron (Nedergaard et al. 2003; Ribeiro et al., 2013). Astrocytes are defined by many branching processes radiating from the cell body (stellate cells). Astrocytes are usually called protoplasmic astrocytes in grey matter and fibrous astrocytes in white matter. Protoplasmic astrocytes have profuse short processes radiating uniformly from the cell body and are distributed homogeneously within grey matter (Nedergaard et al., 2003; Oberheim et al., 2012). To the opposite, fibrous astrocytes have an elongated form with fewer but longer processes (Oberheim et al., 2012). In white matter, astrocytes are oriented longitudinally in the plane of fibers bundles (Oberheim et al., 2009; Budde et al., 2011; Lundgaard et al., 2013). In contrast, neurons consist of a soma (cell body), a long projection called an axon and one or more branching dendrites (Purves et al., 2004; Lopez-Munoz et al., 2006). Neuronal soma and dendrites are primarily localized in grey matter whereas myelinated axons (bundles) are mainly running through a long distance in white matter (Zhang et al., 2000; Assaf and Pasternak, 2008; Filley, 2010). In this context, the stability of ADC in white matter largely confirms the hypothesis that metabolite diffusion in white matter reflects a fiber-like cell structure (metabolites diffusing in elongated astrocytes and myelinated axons). It is a more surprising finding that also in grey matter all metabolites ADC reach a non-zero plateau, synonym of diffusion along the axes of fibers. Although comparisons with former studies are subject to caution due to species and methodological differences, our results are consistent with quantitative measurements of astrocytic and neuronal fibers volume found in the literature. The neuropil volume fraction (unmyelinated axons, dendrites and astrocytic processes) was estimated to be 65% on average in different regions in white matter and cortical and subcortical grey matter of fixed baboon brain using diffusion-weighted MRI and sophisticated modeling (Jespersen et al., 2007). In another study, performed in the mouse grey matter using electron micrography, the neuropil volume was estimated to occupy 80% of the total intracellular volume (Chklovskii et al., 2002).

Metabolites are not confined in small subcellular structures

Importantly, our data suggest that metabolites are not significantly confined in small subcellular regions like cell bodies, or in intracellular compartments such as organelles, in which case the metabolite ADC would decrease towards zero with increasing t_d . This is for example illustrated on Figure 3 by the numerical simulations of ADC in 5- μ m and 20- μ m diameter spheres. Instead, they are apparently free to diffuse in the whole fiber-like cell internum, both in white matter as well as in grey matter. This type of information can be hardly addressed by microscopy techniques, and stress the unique type of information that DW-MRS can provide on localization of metabolites in cellular compartments.

Comparison with the literature

All three metabolites ADC were slightly lower in comparison to other studies performed in Humans in both regions (Posse et al., 1993; Harada et al., 2002; Kroenke et al., 2004; Ellegood et al., 2005; Ellegood et al., 2006; Upadhyay et al., 2007; Upadhyay et al., 2008; Ellegood et al., 2011; Kan et al., 2012; Wood et al., 2012; Branzoli et al., 2013; Ronen et al., 2013). Reasons for this discrepancy may include longer diffusion gradient duration ($\delta=30$ ms) leading to a non-instantaneous diffusion during gradient application (Upadhyay et al., 2007; Upadhyay et al., 2008) and shorter gradient separation time ($\tau=57.5$ ms) resulting in shorter diffusion time (Kan et al., 2012). A possible explanation is also a lower sensitivity in

most former studies which were performed at 1.5 T (Posse et al., 1993; Harada et al., 2002; Kroenke et al., 2004) or 3 T (Ellegood et al., 2005; Ellegood et al., 2006; Upadhyay et al., 2007; Upadhyay et al., 2008; Ellegood et al., 2011). Another reason for the lower ADC values is the difference in white and grey matter proportions in the voxels-of-interest which are positioned in different brain regions (corpus callosum, primary visual cortex, frontal lobe and arcuate fasciculus) among studies (Kroenke et al., 2004; Ellegood et al., 2005; Upadhyay et al., 2007; Upadhyay et al., 2008; Wood et al., 2012; Branzoli et al., 2013; Ronen et al., 2013). In our opinion, the most relevant explanation for this discrepancy is the absence of cross-terms correction in past studies, as we have shown that using diffusion gradients with a positive polarity, i.e. same polarity as slice selection and spoiler gradients, lead to an overestimated ADC, although cross-terms were shown to be not significant in one previous study performed using the PRESS sequence (Kan et al., 2012). The absence of significant cross-terms in the PRESS sequence is due to the fact that spoiler gradients are positioned symmetrically around the 180° pulses and are almost immediately refocused, unlike in STEAM.

Differences in ADC between white and grey matter

As in previous studies (Ellegood et al., 2011; Kan et al., 2012), we found that metabolite ADC is lower in grey matter compared to white matter, although for tCho this difference is not significant. In the light of the present work, where ADC is found to essentially reflect diffusion along fibers (i.e. $ADC \sim D_{intra}/3$ with D_{intra} being the intracellular diffusion coefficient including effects of cytosol viscosity, molecular crowding and intracellular tortuosity), differences in ADC between GM and WM should primarily be interpreted in terms of different D_{intra} rather than different cell shapes. For example, echoing a previous work where an inverse relationship was found between oxidative metabolism and glutamate ADC using ^{13}C labeling combined with diffusion-weighting in the monkey brain (Valette et al., 2008), a larger density of mitochondria associated with the higher oxidative metabolism of GM neurons would result in a larger tortuosity and hence decreased D_{intra} in GM for metabolites having a large neuronal fraction (tNAA and tCr). The absence of significant difference in between WM and GM tCho ADC suggests that D_{intra} is the same in fibrous astrocytes (located in WM) and in protoplasmic astrocytes (distributed mainly in GM).

Conclusion

In this study, we performed DW-MRS measurements at long diffusion times in Humans at 7 T in two volumes of interest that consisted of widely different proportions of white matter and grey matter. The stability of ADC with respect to the diffusion time in both regions suggests that astrocytic and neuronal intracellular metabolites freely diffuse along the axes of cell fibers and are not confined into small subcellular domains and compartments. The absence of significant apparent restriction suggests that metabolite ADC may be very sensitive to the intracellular diffusion coefficient D_{intra} along fiber direction. For example, our work supports the idea that neurite beading, by decreasing D_{intra} , may be the main mechanism of intracellular ADC decrease in the context of ischemic stroke (Budde and Frank, 2010). Other factors potentially affecting D_{intra} include intracellular viscosity, molecular crowding and intracellular tortuosity. Our work implies that metabolite ADC

measured on clinical scanners, even at relatively long diffusion times (up to hundreds of ms) characteristic of these scanners, may provide a unique biomarker for disease processes that early and subtly affect these intracellular factors. Potential candidates are Alzheimer's disease (AD) and Huntington's disease (HD). Histopathologically, AD and HD are respectively characterized by the presence of intracellular neurofibrillary tangles (hyperphosphorylation of the microtubule-associated protein tau) and aggregates of mutant Huntingtin (Htt) that may increase viscosity and tortuosity inside the cytosol (Arrasate et al., 2012; Meraz-Rios et al., 2013). In contrast, variations in cell size (e.g. neuronal atrophy or astrocytic hypertrophy in Alzheimer's disease) may be more difficult to detect within the diffusion times achievable on clinical scanners: in particular, sensitivity to fiber diameter would require ultra-short t_d of a few ms, e.g. see (Marchadour et al., 2012), while sensitivity to fiber length would require much longer t_d values, even than those used in the present study.

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Figure 1.

Position of the 12 mL spectroscopy voxels either in the parietal lobe or the occipital lobe of the human brain. Voxels locations are shown for NAA (red box) and water (white box). On the right, corresponding spectra acquired at $t_F=712$ ms with null b -value (left) and high b -value (right) in one direction during a single experiment. As shown here, good quality spectra were obtained even at ultra-long diffusion times, allowing for the measurement of $tNAA$, tCr and $tCho$ signals.

Figure 2.

Water and brain metabolite ADC was measured at three different diffusion times (92, 542, 712 ms) and averaged over the experiments ($n=4$ at $t_d=92/712$ ms and $n=3$ at $t_d=542$ ms in parietal white matter and $n=5$ at $t_d=92/542$ ms and $n=6$ at $t_d=712$ ms in occipital grey matter). No strong dependence of ADC on t_d was observed in white matter and in grey matter.

Figure 3.

Effect of metabolite confinement on ADC time-dependency. A) Diffusion path (in blue) of a metabolite diffusing in a long fiber; B) Diffusion path (in red) of a metabolite being confined in the cell body, due to difficult access to cellular processes; C) Diffusion path (in green) of a metabolite being confined in a mitochondria; D) Comparison between measured ADC (here for tNAA in GM) and theoretical curves expected in the three situations described above, demonstrating that only diffusion along fibers is compatible with experimental data.

Theoretical curves were simulated in Matlab using the short-gradient-pulse approximation (Balinov et al., 1993; Linse and Söderman, 1995), as we have detailed in (Najac et al. 2014), in 5- μm diameter infinite cylinders (blue curve), 20- μm diameter spheres (red curve) and 5- μm diameter spheres (green curve). D_{intra} was set to 0.37 $\mu\text{m}^2/\text{ms}$, which is the mean of the

estimate for tNAA in WM reported in two published studies (Kroenke et al., 2004; Ronen et al., 2013).

Table 1

The effects of cross terms were quantified *in vitro*. Trace ADC of water ($\mu\text{m}^2/\text{ms}$) is increasing with t_d with positive gradients whereas it is decreasing with t_d with negative gradients. When averaging the signal acquired with both polarities, the trace ADC is independent of t_d .

	$t_d=92\text{ ms}$	$t_d=542\text{ ms}$
Positive diffusion gradients	2.9	3.9
Negative diffusion gradients	1.3	0.2
Geometric mean	2.1	2.1

Table 2

Estimated volume fraction of WM, GM and CSF in the parietal and occipital voxels obtained segmenting 3D T1-weighted images using FSL (Brain Extraction Tool (Smith, 2002), FMRIB's Automated Segmentation Tool (Zhang et al., 2001) and an in-house Matlab routine. The error corresponds to standard error. P-values are derived applying a standard unpaired Student's t-test to the volume fraction obtained in the two voxels.

	Parietal voxel	Occipital voxel
WM (%)	86.0±4.4	23.9±4.8
GM (%)	12.7±3.9	65.1±7.1
CSF (%)	1.3±0.8	11.0±4.9

Table 3

ADC of metabolite and water determined *in vivo* $\pm$ s.d. ($\mu\text{m}^2/\text{ms}$). Two different diffusion times could be acquired during each session resulting in $n=4$ at $t_d=92/712$ ms and $n=3$ at $t_d=542$ ms in parietal white matter and $n=5$ at $t_d=92/542$ ms and $n=6$ at $t_d=712$ ms in occipital grey matter. Time-average values are measured by averaging ADC over all subjects and all diffusion times. P-values are derived applying a standard unpaired Student's t-test to assess the ADC difference between GM and WM.

tNAA	$t_d=92$ ms	$t_d=542$ ms	$t_d=712$ ms	Time-average
Parietal WM voxel	0.17 ± 0.02	0.18 ± 0.02	0.15 ± 0.02	0.17 ± 0.02
Occipital GM voxel	0.12 ± 0.01	0.11 ± 0.02	0.12 ± 0.03	0.12 ± 0.02
p-value				$3e-6$

tCr	$t_d=92$ ms	$t_d=542$ ms	$t_d=712$ ms	Time-average
Parietal WM voxel	0.17 ± 0.02	0.15 ± 0.02	0.13 ± 0.03	0.15 ± 0.03
Occipital GM voxel	0.13 ± 0.01	0.11 ± 0.02	0.13 ± 0.02	0.12 ± 0.02
p-value				0.006

tCho	$t_d=92$ ms	$t_d=542$ ms	$t_d=712$ ms	Time-average
Parietal WM voxel	0.13 ± 0.02	0.13 ± 0.02	0.10 ± 0.01	0.12 ± 0.02
Occipital GM voxel	0.10 ± 0.02	0.10 ± 0.02	0.10 ± 0.02	0.10 ± 0.02
p-value				0.064

Water	$t_d=92$ ms	$t_d=542$ ms	$t_d=712$ ms	Time-average
Parietal WM voxel	0.47 ± 0.02	0.43 ± 0.03	0.45 ± 0.04	0.45 ± 0.03
Occipital GM voxel	0.69 ± 0.06	0.64 ± 0.03	0.62 ± 0.05	0.65 ± 0.06
p-value				$2e-11$