NeuroImage 90 (2014) 374–380

Contents lists available at ScienceDirect

NeuroImage

journal homepage: www.elsevier.com/locate/ynimg

Intracellular metabolites in the primate brain are primarily localized in long fibers rather than in cell bodies, as shown by diffusion-weighted magnetic resonance spectroscopy

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ARTICLE INFO

Article history:
Accepted 15 December 2013
Available online 29 December 2013

Keywords:
Magnetic resonance spectroscopy
Diffusion
Metabolites
Brain
Monkey
Cell geometry

ABSTRACT

Due to their pure intracellular compartmentation, the translational diffusion of brain metabolites *in vivo* depends on the intracellular environment, including viscosity, molecular crowding and subcellular structures. However, as the diffusion time is increased, metabolites have enough time to significantly encounter cell boundaries, so that cell size and geometry are expected to strongly determine metabolite diffusion path. In the present work, diffusion-weighted nuclear magnetic resonance spectroscopy was used to investigate brain metabolite diffusion *in vivo*, at long and ultra-long diffusion times (from ~80 ms to more than 1 s), in a voxel with equal proportions of white and grey matter in macaque monkeys. No dramatic dependence of the ADC on the diffusion time was observed, suggesting that metabolites' apparent diffusion is largely unrestricted over these time-scales. In an attempt to explain this stability and relate it to plausible cell geometries, data were analyzed with two simple geometrical models describing diffusion either in fibers such as axons, dendrites and astrocytic processes, or in closed cell bodies. Results support the idea that DW-MRS is sensitive to cell shape, and that a vast fraction of brain metabolites is diffusing in long fibers rather than being confined in cell bodies.

Introduction

Diffusion-weighted (DW) ^{1}H magnetic resonance spectroscopy (MRS) is a neuroimaging tool to investigate the motion of brain metabolites *in vivo* (see (Nicolay et al., 2001) for review). In contrast to water molecules, which are present everywhere, brain metabolites are confined into the intracellular space and hardly cross biological membranes, so that the interpretation and modeling of metabolite diffusion are much more direct and require less assumptions compared to water diffusion. In addition, some metabolites have a preferential cellular compartmentation, and can therefore be considered as cell-specific endogenous tracers of the intracellular space: N-acetyl-aspartate and glutamate reside essentially in neurons whereas myo-inositol and choline are glial markers whose concentrations increase during astrogliosis, and are preferentially compartmentalized in astrocytes (Choi et al., 2007). In this context, DW-MRS offers the unique perspective of probing the intracellular environment non-invasively under normal and pathological conditions. Changes in endogenous metabolite diffusivity have already been detected in pathologies. Alterations in the diffusion of metabolites are observed after global ischemia (Abe et al., 2000; Dijkhuizen et al., 1999; Dreher et al., 2001; Van der Toorn et al., 1996; Zheng et al., 2012), in multiple sclerosis (Wood et al., 2012), in brain tumor (Hakumäki et al., 1998; Harada et al., 2002; Valette et al., 2012) or even during neuronal activation (Branzoli et al., 2013) and are often associated to tissue structural damage (Budde and Frank, 2010; Song et al., 2003; Wick et al., 1995). Different approaches to quantitatively evaluate microstructural features such as axonal diameter or axonal angular dispersions using DW-MRI and DW-MRS approaches have been developed (Assaf and Cohen, 1998; Assaf et al., 2008; Ronen et al., 2013). However, the influence of cellular parameters (viscosity, molecular crowding, cell size and geometry…) on DW-MRS measurement remains still unclear and requires further investigation.

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). In the limit of low diffusion-weighting b, where the Gaussian phase distribution approximation holds (Stepisnik, 1999), we have:

$$ADC \approx \frac{\langle x^2 \rangle}{2t_d} \quad (1)$$

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http://dx.doi.org/10.1016/j.neuroimage.2013.12.045

In vivo, geometrical constraints due to cell membranes and subcellular structures may hinder the translational displacement of metabolites, so that $\langle x^2 \rangle$ may increase less than linearly with time, as opposed to free diffusion. This would result in the ADC decreasing towards an asymptotic value when t_d increases (Tanner and Stejskal, 1968). The time-dependency of the ADC is related to intracellular structures such as organelles but also, as t_d is increased, to long-range geometrical constraints of the intracellular compartment. It can therefore be expected that ADC measured at "long" t_d, where "long" means enough for metabolites to have time to encounter cell walls, may allow the determination of cell shape and size. However, the actual ability of DW-MRS to address these questions remains to be proven. To date, *in vivo* DW-MRS studies have been performed in rodents (Dreher et al., 2001; Hakumäki et al., 1998; Merboldt et al., 1993; Pfeuffer et al., 1998; Valette et al., 2012; Van der Toorn et al., 1996; Wick et al., 1995), monkeys (Valette et al., 2005, 2007) and humans (Ellegood et al., 2005, 2006, 2011; Harada et al., 2002; Kan et al., 2012; Kroenke et al., 2004; Posse et al., 1993; Ronen et al., 2013; Upadhyay et al., 2007, 2008; Zheng et al., 2012) with diffusion times ranging between 13 ms and 240 ms, but always at a single t_d for each study. When combining all published studies, no strong dependence of the ADC on t_d is observed. This is surprising in the context of restricted diffusion, although comparisons between studies are always subject to caution due to inter-species and methodological differences. In contrast, *in vivo* DW-MRS performed at ultra-short t_d (from 1 ms to 13 ms) in rodents demonstrated a significant decrease of brain metabolite ADC as t_d increases, as we have recently reported (Marchadour et al., 2012). In that work, the relative insensitivity of ADC at long t_d and its dependency at ultra-short t_d could be quantitatively explained by two very different, but both biologically relevant cell geometries. The first model consisted in long fibers, representing dendrites, axons and astrocytic processes, isotropically distributed as expected in a large spectroscopic voxel. The second model consisted in large spherical cell bodies filled with organelles. The fact that both models could account for data published in the past (Marchadour et al., 2012), and conversely that no model could be declared superior to the other, suggests that the range of t_d used in the literature was not large enough to assess brain cells shape and size, and appeals to perform measurements at longer t_d to better characterize how DW-MRS can address these questions.

In the present study, diffusion of neuronal and astrocytic metabolites is investigated at long and ultra-long diffusion times in the primate brain, with t_d varying from ~80 ms to more than 1 s. Experiments show that the ADC of all metabolites barely depends on t_d and reaches a non-zero plateau, suggesting that no additional restriction barriers appear over these time-scales, i.e. there are either diffusing in long and narrow fibers, with restriction being free along fibers direction and totally restricted in the transverse directions, or in large cell bodies with distant cell walls. Data are then modeled using analytical models to mimic diffusion either in spherical cell bodies, or in fibers. Results suggest that the cell body model cannot realistically account for the data, and that the ADC stability is characteristic of "unrestricted" diffusion as occurring in the direction parallel to fibers in both neurons (axons, dendrites) and astrocytes (astrocytic processes), while restriction in the two transverse directions is already extremely strong, i.e. the ADC perpendicular to fibers (ADC$_\perp$) is approximately equal to 0 in the range of observed t_d.

Materials and methods

NMR setup

Experiments were performed on a 7 Tesla scanner from Agilent (formerly Varian) equipped with a gradient coil reaching 100 mT/m in 325 μs along each axis, running with VnmrJ (Palo Alto, CA, USA). A ^{1}H quadrature surface coil was used for radiofrequency emission and reception. Anatomical localization was achieved with a scout image. An $18 \times 18 \times 18$ mm^3 (5.8 mL) voxel was positioned in the region of interest. Shimming was performed with Fastmap (Gruetter, 1993). Spectra were acquired using a STEAM (Stimulated Echo Acquisition Mode) sequence with echo time TE = 18 ms and repetition time TR = 2800 ms, modified for diffusion-weighting by inserting one diffusion gradient pulse of duration $\delta = 5$ ms during each half of the echo time (e.g. see (Valette et al., 2005)). In a large spectroscopic voxel, the distribution of fiber orientation (in particular white matter fiber bundles) is large and expected to lead to a quasi-isotropic fiber distribution, so that diffusion is expected to be the same along any axis. Therefore, in our study, diffusion gradients were applied along the three axes (x, y, and z) simultaneously, which allowed reaching stronger b. Different diffusion times t_d were obtained by varying the mixing time TM, while keeping b constant by adjusting diffusion gradient strength. Water suppression was performed with an optimized 8-pulses VAPOR scheme (Tkác et al., 1999). Outer volume suppression was applied to improve localization and avoid extra-voxel contamination.

Cross-term suppression

The brain metabolite ADC can be estimated from the signal attenuation S/S_0 measured when diffusion-weighted gradients G_d are applied relative to reference signal (acquired at $G_d = 0$):

$$ADC = -\frac{1}{b} \ln\left(\frac{S}{S_0}\right) \quad (2)$$

In the above equation, the diffusion-weighting factor b is defined as:

$$b = \gamma^2 G_d{}^2 \delta^2 \left(\Delta - \frac{\delta}{3}\right) \quad (3)$$

Here we have $t_d = \Delta - \delta/3$, where Δ is the delay between the two diffusion gradients. In the above equation, it is assumed that cross-terms, i.e. the time-integral of the product of diffusion gradients with other gradients such as crusher, slice-selective and background gradients (i.e. magnetic field inhomogeneities) are all zero. This is generally not the case with STEAM (Tanner, 1970). The cross-terms may become important as t_d is increased (when b is kept constant, i.e. when G_d is decreased while other gradients remain constant) leading to a modified echo decay rate that may severely bias the measurement of ADC at long and ultra-long t_d (Neeman et al., 1990; Zhong et al., 1991). To cancel out cross-terms, DW-spectra can be acquired with positive and negative diffusion gradients for each t_d, the geometric mean over both conditions being then calculated (Jara and Wehrli, 1994; Neeman et al., 1991).

Validity of the approach was assessed *in vitro* using an agarose gel phantom ($c_{agar} = 3\%$). Water DW-spectra were acquired at $b = 0$ s/mm^2 and $b = 1000$ s/mm^2 with both gradient polarities using five different mixing times (75, 250, 500, 750, 1000 ms). For each condition, the water signal was simply derived from the area under the water resonance. The ADC was estimated using Eq. (2), taking for S either the signal obtained with positive diffusion gradients, or negative diffusion gradients, or the geometric mean of the signal obtained with both gradient polarities.

In practice, only cross-terms generated by spatially constant gradients such as crusher, slice-selective and constant background gradients are suppressed. Cross-terms generated by spatially varying microscopic background gradients cannot be compensated for by the geometric mean method, and may lead to biased ADC measurement. Therefore, the robustness of the approach to spatially varying gradients was also tested in an agarose gel phantom ($c_{agar} = 3\%$) containing iron particles ($c_{iron} = 0.1$ mM) to create local micro-inhomogeneities.