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The use of MR-detectable reporter molecules and ions to evaluate diffusion in normal and ischemic brain

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Abstract

As a result of the technical challenges associated with distinguishing the MR signals arising from intracellular and extracellular water, a variety of endogenous and exogenous MR-detectable molecules and ions have been employed as compartment-specific reporters of water motion. Although these reporter molecules do not have the same apparent diffusion coefficients (ADCs) as water, their ADCs are assumed to be directly related to the ADC of the water in which they are solvated. This approach has been used to probe motion in the intra- and extracellular space of cultured cells and intact tissue. Despite potential interpretative challenges with the use of reporter molecules or ions and the wide variety used, the following conclusions are consistent considering all studies: (i) the apparent free diffusive motion in the intracellular space is approximately one-half of that in dilute aqueous solution; (ii) ADCs for intracellular and extracellular water are similar; (iii) the intracellular ADC decreases in association with brain injury. These findings provide support for the hypothesis that the overall brain water ADC decrease that accompanies brain injury is driven primarily by a decrease in the ADC of intracellular water. We review the studies supporting these conclusions, and interpret them in the context of explaining the decrease in overall brain water ADC that accompanies brain injury.

Keywords

diffusion; intracellular reporter molecule; brain; biophysics; stroke

INTRODUCTION

Diffusion MRI is a well-established means to detect brain injury. This is because the apparent diffusion coefficient (ADC) of brain water decreases within minutes of injury (1–4), providing contrast between normal and injured tissue unavailable by T₁- and T₂-weighted MRI. This decrease in ADC follows a variety of injuries, including stroke (3,5), trauma (6), status epilepticus (7–9), excitotoxic injury (10,11) and spreading depression (12–15). Despite the widespread use of diffusion MRI for the early detection of brain injury in clinical practice, the mechanism(s) underlying this contrast remains, surprisingly, poorly understood and controversial. Motivated by the extraordinary diffusion behavior of water in

brain, this article focuses on the diffusion of intracellular water as investigated through the use of MR-detectable reporter molecules.

The evaluation of the diffusion characteristics of intracellular water in brain requires the discrimination between the MR signals for intra- and extracellular water, which is a challenging task. Some research groups have discriminated between these two compartments on the basis of their diffusion characteristics, assuming that water in the intracellular compartment has a much smaller ADC than that in the extracellular compartment. This approach can be useful for cell culture systems in which the diffusion behavior of extracellular water is well characterized and extracellular water motion is essentially unrestricted (conditions requiring careful validation) (16,17). However, it presents problems when applied to intact systems, as ADCs for water in the two compartments are probably similar (18).

It is also difficult to distinguish between the ^1H MR signals from intra- and extracellular water on the basis of spin–spin relaxation, spin–lattice relaxation or chemical shift, because these characteristics are very similar for the two populations. One means of circumventing this difficulty is to manipulate the MR characteristics of one water compartment (typically extracellular water) to make them substantially different from those of the other compartment. For example, relaxation agents can be used to shorten the spin–spin and/or spin–lattice relaxation time constants (19–21) or change the magnetic susceptibility (22,23), providing a means of compartment discrimination based on these MR properties. However, water exchange between the two compartments must be taken into account in experiments designed to separate them. Reliable data concerning exchange lifetimes are sparse, although the available data suggest that the exchange lifetime for intra-cellular water in mammalian systems can range from 10 ms for red blood cells (24) and rat hippocampal slices (25) to 1000 ms for intact brain and other cell types (20,26–28).

A third approach, and the main topic of this article, is the use of endogenous or exogenous reporter molecules or ions (hereafter ‘molecules’) which are relatively concentrated in either the intracellular or extracellular space (18,29–34). Although the molecules used for the studies described below undoubtedly have different diffusion properties from water, it is assumed that they are present in the aqueous portion of the relevant compartment, where they are surrounded by water. As a result, changes in the diffusion characteristics of their surrounding aqueous environment are reflected by changes in the diffusion characteristics of the reporter molecules. It is well known that the diffusion of various molecules in biological tissues is influenced by a myriad of additional factors, such as noncovalent bonding to macromolecules and charge (35). Despite these issues and the complexity of the intracellular milieu, displacements of nonwater molecules within the cytoplasm seem relatively well behaved (36). Further, the results obtained from a variety of molecules – large, small, charged, neutral – provide a consistent picture. The use of reporter molecules has the advantage that, a given molecule, being relatively restricted to a single compartment, typically does not undergo compartmental exchange on the timescale of the MR measurement, simplifying the analysis.

Herein, we summarize currently accessible research on intraand extracellular water diffusion employing MR-detectable reporter molecules. We also discuss the implications of this research on our understanding of the physiologic mechanism(s) behind the reduction of tissue water ADC associated with brain injury.

Before proceeding, we provide some brief comments regarding ADC, a central parameter derived and reported in a multitude of MR diffusion studies *in vivo*. It is well understood from decades of MR research into diffusion in porous media, where displacement motion is

hindered and/or restricted, that the MR diffusion signal – the plot of the pulsed-field-gradient echo attenuation vs. b value – is not monoexponential, except in the case of free (unhindered, unrestricted) diffusion as characterized by a true Gaussian displacement probability [see, for example, the classic text by Callaghan (37) and the review article by Yablonskiy^{Q3} and Sukstanskii (38)]. Nevertheless, despite recognition that mammalian tissue presents a highly complex structure of hindrances and restrictions to displacement, the *in vivo* MR diffusion signal is often modeled as a monoexponential function with decay rate constant ADC. Thus, the use of the term ‘apparent’ as a modifier in ‘ADC’ reminds us that the mathematical model does not correspond to an accurate representation of the true physical model, and that the derived ADC is a summary parameter that depends on both tissue microstructure and MR acquisition parameters (39). The diffusion time (t_{diff}) is one such MR acquisition parameter. It represents the time during which the MR diffusion experiment is sensitive to water displacements – typically the time between the application of diffusion-sensitizing magnetic field gradient pulses. Typical diffusion times for clinical systems are on the order of 50 ms. The longer the diffusion time, the more time for exchange between compartments (e.g. water exchange between intra- and extracellular spaces). Further, longer diffusion times are associated with greater molecular displacements, and make it more likely that molecules will encounter and interact with diffusion barriers in their environment. Conversely, the ADC measured at the limit of short diffusion times, during which molecules theoretically do not encounter local barriers to displacement, is referred to as the ‘free’ diffusion coefficient. In complex systems, the ‘free’ diffusion coefficient is often greater than that obtained at longer diffusion times. When comparing diffusion measurements between studies, it is important that the diffusion time be borne in mind.

A discussion of the many ongoing efforts to develop mathematical MR data models that more closely approximate diffusion within the highly complex physical structure of mammalian tissue is beyond the scope of this article, and the reader is referred to a recent review by Mulkern *et al.* (40) on the subject. Nevertheless, the quantification of the nonmonoexponential nature of the diffusion signal requires the acquisition of data at high b values for which the signal-to-noise ratio is limited. Most MR diffusion studies (particularly clinical studies) are limited to moderate b values [i.e. $(b)(\text{ADC}) \sim 1$] for which the diffusion signal is reasonably approximated, in an empirical sense, as a monoexponential decay. If one examines a semilogarithmic plot of the diffusion signal attenuation curve corresponding to the low to moderate b values typically employed in clinical studies, the curve roughly approximates a straight line (i.e. a mono-exponential function) despite the fact that the relationship between the signal intensity and b value is not monoexponential.

Nonmonoexponential diffusion signals obtained by the inclusion of measurements at high b values are often well modeled by biexponential and other concise mathematical functions (40–44). It is tempting to ascribe simple compartment-based physical models to such data, e.g. two compartments corresponding to a biexponential analysis (41). However, because the MR diffusion signal *in vivo* is relatively uninformative, generally characterized by a monotonic decay, of relatively low signal-to-noise ratio and obtained using a limited b value range, such interpretations may not be justified in the absence of strong, defining, prior information and careful validation (42,43). MR-detectable reporter molecules may employ nuclides with relatively low magnetogyric ratios relative to ^1H in $^1\text{H}_2\text{O}$, making it difficult to achieve high b values when measuring their diffusion, because b values are proportional to the square of the magnetogyric ratio. Further, reporter molecules are typically present in millimolar concentrations, as compared with ~ 100 M for ^1H in $^1\text{H}_2\text{O}$. This makes it comparatively demanding to obtain data at high b values for reporter molecules, because the signal-to-noise ratio at high b values is reduced by signal attenuation as a result of diffusion. Thus, detailed analysis of signal attenuation curves is typically not performed using reporter molecules.

GENERAL CONSIDERATIONS

It is important to bear in mind the potential pitfalls associated with the interpretation of studies in which compartment-specific water diffusion characteristics are inferred from the diffusive behavior of reporter molecules. First, in practice, reporter molecules are generally not perfectly confined to a given target compartment, although control tissue culture studies are often performed to show that they are at least compartment selective, in the sense that the majority population fraction of a particular molecule is confined to the compartment of interest (18,45). Thus, despite long compartmental exchange lifetimes (as compared with water) for compartment-selective reporter molecules, data interpretation must take into consideration some degree of cross-compartment contamination. Second, it is possible that reporter molecules are restricted to subcompartments within the compartment of interest, particularly in the intracellular space. This has been proposed for $^{133}\text{Cs}^+$, for example, for which different chemical shifts have been associated with different compartment assignments (46). Third, it is possible that reporter molecules bind to slowly moving cellular constituents rather than remaining associated only with the water of hydration. (Of course, this last consideration affects the diffusive behavior of water as well, which can bind macromolecules.) Despite these caveats, the studies described below demonstrate notable consistency across various reporter molecules.

The ADCs for various reporter molecules are summarized in Table 1. Table 1 also includes the ADCs for the same compounds in dilute aqueous solution at the relevant temperature and in brain following injury. Many of the complexities of diffusive behavior in living systems are simplified in Table 1. For example, there is strong anisotropy of ADCs in white matter tracts (32); however, the value shown in Table 1 is a directionally averaged value. In addition, as noted above, the measurements from reporter molecules do not take into account the nonmonoexponential nature of the diffusion signal attenuation curve, and the values reported in Table 1 are typically derived from studies in which the diffusion signal attenuation curve was modeled as a monoexponential function [the studies of Zhao *et al.* (47) and Kroenke *et al.* (32) described below are exceptions]. Nevertheless, there is good agreement among the results. For example, the values for $\text{ADC}_{\text{in}}/\text{ADC}_{\text{free}}$ range from 0.11 to 0.59, suggesting that intracellular diffusive motion is on the order of one-half of that of dilute aqueous solution. This is supported by diffusion measurements of water in various systems (21,29,47,47).

ENDOGENOUS INTRACELLULAR REPORTER MOLECULES

A number of endogenous, compartment-specific, MR-active reporter molecules have been employed to assess water diffusion. ^1H MRS has been used to measure the diffusion of choline-containing compounds (Cho), creatine and phosphocreatine (Cre), and *N*-acetylaspartate (NAA) in rat brain (30–32). For these studies, it was assumed that these compounds are restricted to the intracellular space during the MR diffusion measurement. NAA, for example, has an intracellular pre-exchange lifetime of hours (49). Typical ADCs for these reporter molecules ranged from 0.15 to 0.22 $\mu\text{m}^2/\text{ms}$, significantly lower than the values of 0.94 $\mu\text{m}^2/\text{ms}$ for Cho and 0.78 $\mu\text{m}^2/\text{ms}$ for NAA in aqueous solution (30,32). Similarly, values measured in human brain are on the order of 0.17 $\mu\text{m}^2/\text{ms}$ for Cho, 0.14 $\mu\text{m}^2/\text{ms}$ for Cre and 0.20 $\mu\text{m}^2/\text{ms}$ for NAA (50–52).

Measurements in rat brain have been used to estimate the apparent intracellular free diffusion coefficient for NAA. The diffusion of NAA was modeled as diffusion within randomly oriented, long, narrow cylinders (32). Because NAA is an intraneuronal constituent (49) and neuronal processes, such as dendrites, are stochastically oriented when considered over millimeter-scale brain regions, the diffusion of NAA appears to be isotropic

in gray matter on a macroscopic scale (at the spatial resolution of a typical voxel). However, on a microscopic scale (well below the spatial resolution of a typical voxel), NAA diffusion would be expected to be highly anisotropic, with displacements perpendicular to dendrites being markedly hindered and parallel displacements ideally unhindered. A model originally developed by Yablonskiy *et al.* (53) for ^3He diffusion in the lung was applied to the analysis of the nonmonoexponential, multiple b value NAA diffusion signals from a large voxel comprised primarily of gray matter within the brain of rat *in vivo*. This simple, two-parameter model describes the macroscopic diffusion signal in terms of ADCs for microscopic displacement parallel and perpendicular to the local fiber axis, $\text{ADC}_{\parallel}$ and $\text{ADC}_{\perp}$, respectively. Accounting for the microscopic anisotropy, with diffusion times ranging from 50 to 100 ms, the model represented the NAA diffusion data well. NAA displacements occurred exclusively parallel to the local fiber axis: $\text{ADC}_{\parallel}$ 0.36 and $\text{ADC}_{\perp} \sim 0 \mu\text{m}^2/\text{ms}$. Assuming that $\text{ADC}_{\parallel}$ reflects diffusion unrestricted/unhindered by cell boundaries (root-mean-square displacement small relative to the length of the neuron), it can be thought of as the apparent intracellular free diffusion coefficient for NAA. From this derived parameter, the apparent viscosity of the neuron cytoplasm is estimated to be twice as large as that of a temperature-matched dilute aqueous solution, where the free diffusion coefficient of NAA is $0.78 \mu\text{m}^2/\text{ms}$ at 37°C (52). Similar, although more qualitative, results were found for white matter of human brain *in vivo* (32). When diffusion sensitizing gradients were aligned parallel vs. orthogonal to the axonal fibers of the corpus callosum, NAA $\text{ADC}_{\parallel}$ was two- to three-fold greater than $\text{ADC}_{\perp}$.

EXOGENOUS INTRACELLULAR REPORTER MOLECULES

The diffusion characteristics of a variety of exogenous and endogenous molecules in the intracellular space of *Xenopus* oocytes have been used to evaluate the role of cytoplasmic streaming in intracellular molecular displacements (29). Because of their large size (diameter on the order of 1 mm, which is much larger than the typical root-mean-square diffusion displacements), oocytes provide a means by which to obtain direct measurement of ADC for intracellular water and spectroscopic measurements of ADC for other intracellular reporter molecules. In this case, because of the larger cell dimensions, it is expected that the derived ADCs reflect diffusion unrestricted/unhindered by cell boundaries. The exogenous reporter molecules employed in the study were polyethylene glycol (PEG) (molecular weight ranging from 1000 to 4600 Da), tetramethylammonium ion (TMA) and Cs^+ . Endogenous reporter molecules were Na^+ , H_2O and ATP. A plot of ADC in the intracellular space vs. diffusion in dilute aqueous solution (ADC_{free}) for these compounds was linear with an intercept near zero (Fig. 1). This indicates that intracellular motion, at least in the *Xenopus* oocyte, is predominantly a result of Brownian motion. Had cytoplasmic streaming been a significant factor, the plot would have had an intercept offset from zero, corresponding to the contribution of cytoplasmic streaming to the overall ADC. In addition, intra-cellular water ADC showed no dependence on diffusion time over a broad range (3.4–100 ms), suggesting that barriers to displacement are finely spaced ($\leq 2\text{--}3 \mu\text{m}$). The calculated intracellular apparent viscosity, which reflects the combined effects of barriers to motion, intermolecular binding and fluid phase viscosity, was $2.07 \pm 0.09 \text{ cP}$, twice that of a dilute aqueous solution, a finding consistent with that of Kroenke *et al.* (32). One of the first MR-active, exogenously administered reporter molecules used to study rat brain was ^{133}Cs , which was added to the drinking water of rats (33,54). ^{133}Cs is the naturally occurring, nonradioactive, 100% abundant isotope of cesium. It is an alkali metal found in the same group in the Periodic Table as lithium, sodium, potassium and rubidium. Being a K^+ analog, Cs^+ accumulates in the intracellular space and shows very slow exchange with the extracellular space (55). As the Cs^+ ion electronic structure is highly polarizable, its ^{133}Cs chemical shift is very sensitive to local environmental factors, making it possible to distinguish intra- and extracellular $^{133}\text{Cs}^+$ resonances on the basis of chemical shift,

particularly for tissue culture studies. However, the relative concentration of extracellular Cs^+ *in vivo* is too low to make its routine detection by ^{133}Cs MR practical with current technology. Thus, *in vivo* studies have focused on Cs^+ in the intracellular space. The ADC for Cs^+ in brain is approximately $1.0 \mu\text{m}^2/\text{ms}$ (33), as compared with $2.0 \mu\text{m}^2/\text{ms}$ for Cs^+ in aqueous solution at body temperature (Author^{Q5} X, unpublished). (The HeLa cell Cs^+ intracellular ADC is only $0.50 \mu\text{m}^2/\text{ms}$ at 37°C with $t_{\text{diff}} = 14 \text{ ms}$; Author^{Q6} X, unpublished.)

A second exogenous reporter molecule is 2-fluorodeoxyglucose (2FDG). The MR-active ^{19}F isotope is the 100% naturally abundant, nonradioactive form of fluorine, and the MR sensitivity of ^{19}F is very close to that of ^1H . This reporter molecule can be confined to either the intra- or extracellular space. Following intravenous administration, it enters cells, where it is phosphorylated and trapped as 2-fluorodeoxyglucose-6-phosphate (FDG-6-P), thus serving as a reporter molecule for the intracellular space. Alternatively, FDG-6-P can be infused directly into the lateral cerebral ventricles, from which it diffuses throughout the extracellular space, thereby serving as a reporter molecule for the extracellular space. This allows the comparison of FDG-6-P ADCs for the intra- and extracellular compartments. (Note that, because of the complexity of separating the ^{19}F FDG-6-P MR signal from the intra- and extracellular compartments, studies in which the label is confined to either the intra- or extracellular compartment are performed on different animals.) In rat brain, FDG-6-P ADCs measured at diffusion times of 26–29 ms are approximately $0.16 \mu\text{m}^2/\text{ms}$ for the intracellular space, as compared with $0.14 \mu\text{m}^2/\text{ms}$ for the extracellular space and $0.70 \mu\text{m}^2/\text{ms}$ in aqueous solution at body temperature (18).

EXTRACELLULAR REPORTER MOLECULES

In addition to ^{19}F FDG-6-P, other molecules have been used to evaluate diffusion in the extracellular space. For example, the MR-detectable, 100% naturally abundant form of sodium, ^{23}Na , is present in both the intra- and extracellular space as Na^+ , but the tissue content ratio is 8 : 2 in favor of the extracellular space. Thus, the ^{23}Na MR signal detected from normal brain arises primarily from extracellular Na^+ , and its diffusion characteristics reflect those of extracellular water. The ADC for Na^+ in brain is $1.15 \mu\text{m}^2/\text{ms}$, as compared with $1.9 \mu\text{m}^2/\text{ms}$ for Na^+ in aqueous solution at body temperature (34). Further, a variety of exogenous reporter molecules – mannitol, phenylphosphonate and PEGs – have been infused into the extracellular space of rat brain. ADCs measured at a diffusion time of 22 ms range from $0.06 \mu\text{m}^2/\text{ms}$ for the highest molecular weight PEG compound tested to $0.22 \mu\text{m}^2/\text{ms}$ for phenylphosphonate, the lowest molecular weight compound tested (45).

Kroenke *et al.* (56) examined the MR diffusion characteristics of TMA, an exogenous reporter molecule of the extracellular space and a much used ionophoresis probe (35). Although the root-mean-square displacements in MR diffusion studies are typically on the order of 1–10 μm , displacements in ionophoresis experiments are $\geq 100 \mu\text{m}$. Monoexponential analysis of TMA diffusion data obtained from rat brain *in vivo* with localized ^1H MRS yielded a 3.6-fold lower ADC compared with ionophoresis measurements. However, a processing scheme that considered individual displacement distance subpopulations of TMA molecules yielded ADCs that approached the ionophoresis-derived value as the elapsed displacement distance increased to 15 μm . It was concluded that MR and ionophoresis studies provide complementary information, and that ADC estimates obtained from the two techniques are sensitive to different biophysical determinants.

CHANGES IN ADC FOLLOWING BRAIN INJURY

Although their ADCs are usually substantially smaller than that of water, endogenous and exogenous intracellular reporter molecules and ions have generally shown a significant ADC

decrease with brain injury concomitant with that of the water ADC decrease [see, for example, (18,30,31,33,45)]. The markedly lower MR signal-to-noise ratio and spatial resolution achievable with reporter molecules in comparison with water confounds a truly quantitative comparative analysis. However, the evidence supports the proposition that intracellular incoherent displacement motion decreases significantly in brain injury. A recent study highlights this conclusion. Goodman *et al.* (33) examined the ^{133}Cs MR diffusion signal from Cs^+ , an exogenous intracellular reporter molecule, in both brain and muscle of rat. The ADC of Cs^+ in brain decreased from $1.0 \mu\text{m}^2/\text{ms}$ in healthy tissue to $0.24 \mu\text{m}^2/\text{ms}$ following global ischemia, whereas there was no significant change in the ADC of Cs^+ in temporalis muscle after injury. Not only is the decrease in brain intracellular Cs^+ ADC remarkable for its magnitude ($\sim 75\%$), neither Cs^+ nor water showed a substantial ADC decrease in temporalis muscle with injury. MR diffusion analysis at higher signal-to-noise ratio with rat leg muscle gave results similar to those from temporalis muscle: a minimal (10%) decrease in Cs^+ ADC over 1 h post-mortem (33), consistent with other studies showing a similar minimal reduction of muscle water ADC in response to injury (57). The strong concordance between Cs^+ and water diffusion behavior in rat brain and muscle in the face of injury suggests that water ADC changes are dominated by and reflective of displacement motion within the intracellular space. Further, the findings suggest that the reduction in ADC associated with injury is tissue specific, being much greater in brain than muscle.

Changes in diffusion in association with injury have also been studied with FDG-6-P. As described above, it is possible to load FDG-6-P into either the intra- or extracellular space depending on the route of administration. Following global ischemia, the ADC of FDG-6-P in the intracellular space falls from 0.16 to $0.10 \mu\text{m}^2/\text{ms}$. Similarly, the ADC of FDG-6-P in the extracellular space fell from 0.14 to $0.09 \mu\text{m}^2/\text{ms}$ (18). These findings suggest that water ADC decreases in both the intracellular and extracellular compartments following injury.

There have also been studies in which compartment-selective measurements of water have been made in rat brain in association with stroke. By infusing a Gd-containing contrast agent into the extracellular space, Silva *et al.* (21) were able to separate the intra- and extracellular water signal on the basis of reductions in the T_1 and T_2 relaxation time constants of extracellular water. Consistent with the reporter molecular studies described above, they found that the ADC for intracellular water decreased over 30%.

An interesting observation was made by Wick *et al.* (31), who acquired ^1H spectroscopy data in a 'diffusion-weighted' fashion so that an increase in signal intensity reflected a reduction in ADC. In rat forebrain ischemia, the signal intensity for NAA increased $12 \pm 4\%$, whereas that of myo-inositol increased $46 \pm 9\%$. As NAA is primarily localized to neurons and myo-inositol to glia, these results suggest that the effects of injury on diffusion vary according to cell type. In this case, the data can be interpreted as implying that glia undergo a greater ADC reduction than neurons in response to ischemia. This is consistent with physiologic studies suggesting that glia and neurons have different responses to injury (58). The study mentioned above, in which skeletal muscle water ADC did not decrease significantly following injury, provides another example of variation in water ADC response to injury according to cell type. Overall, differences in the ADC response of different brain cell types to injury have not been evaluated carefully.

The initial reduction of brain water ADC following injury has attracted considerable interest because of its widespread clinical utility for the early identification of brain injury. However, the initial ADC reduction is transient, as the change in ADC following stroke is a dynamic process. Studies in animals (59) and humans (60,61) show that ADC gradually increases, reaching normal values at approximately 1 week after injury, a process known as

pseudonormalization. Thereafter, ADC increases further and remains higher than normal. The precise cause of this gradual increase in ADC is not known, but may reflect changes in tissue structure, such as the removal of dead cells and debris following injury, with increased spacing between remaining cellular elements.

MECHANISM FOR THE WATER ADC DECREASE THAT ACCOMPANIES BRAIN INJURY

A variety of hypotheses have been proposed to explain the water ADC decrease that accompanies brain injury. A simple one is related to changes in ADC of intracellular water. The data outlined above suggest that the ADCs for intracellular reporter molecules – and, by inference, intracellular water – decrease significantly following brain injury. As intracellular water comprises 80% of total brain water (62), a sufficiently large decrease in intracellular water ADC (as suggested by the Cs^+ and FDG-6-P studies) could explain the decrease in overall water ADC. This could be augmented, of course, by a decrease in the ADC of extracellular water, as suggested by ^{19}F FDG-6-P (18) studies.

The mechanism(s) by which intracellular water ADC decreases in response to injury is not clear, but, whatever the mechanism, it must explain the change in ADC that occurs over the course of minutes (and, in the case of reversible injury, a rapid return of ADC to normal). Further, the majority of triggers identified for the reduction of brain water ADC are associated with metabolic stress (e.g. excitotoxic injury, seizure, stroke). One model system in which intracellular water diffusion can be assessed directly under a variety of conditions is the *Xenopus* oocyte. Short-term reduction of ATP levels to approximately 20% of normal in this model have little effect on intracellular water ADC, as do changes in microtubule polymerization, which might occur as a consequence of rapid changes in intracellular pH associated with injury (63). Simple cell swelling in this model system, caused by placing the cells in hypotonic medium, leads to an increase in intracellular water ADC, possibly by diluting intracellular contents (63). In contrast, measures of intracellular water ADC in *Aplysia* gastric neurons under conditions causing cell swelling did not change (64), suggesting that these cells compensated osmotically for the increase in intracellular water content. Despite their differences, both studies indicate that cell swelling alone does not explain the reduction in ADC associated with injury. Another potential mechanism for a reduction in intracellular water ADC following injury is related to cytoplasmic streaming (65). As cytoplasmic streaming is energy dependent, metabolic stress could lead to a reduction in streaming, causing a reduction in intracellular water ADC. However, data from *Xenopus* oocytes suggest that intracellular motion is Brownian in character, with no evidence of significant molecular displacement caused by active processes (29). In assessing these results, it is important to bear in mind that *Xenopus* oocytes and *Aplysia* gastric neurons, although living cells, differ significantly from neurons and glia. They are more robust than typical mammalian cells, making it difficult to injure them with simple hypoxia (i.e. mimic a stroke). *Xenopus* oocytes also contain significant energy stores in the form of yolk platelets. Further research on cell systems more closely approximating brain will be necessary to identify the mechanism(s) underlying the reduction in brain water ADC associated with injury.

Although a model dependent on a reduction in ADC for intracellular water has the virtue of simplicity, a more sophisticated version would take water exchange into account. Water exchange between the intra- and extracellular compartments during the diffusion measurement will contribute to overall displacement. As noted in the Introduction, accurate values for the brain intracellular water pre-exchange lifetime are not known, although the available data suggest that the exchange lifetime for intracellular water is on the order of 100–1000 ms (20,26–28). Another means by which to infer the compartmental localization

of water during a typical diffusion experiment is to estimate the root-mean-square displacement of water molecules based on ADC. Using a tissue water ADC of $1.0 \mu\text{m}^2/\text{ms}$, a diffusion time of 50 ms corresponds to a root-mean-square displacement of 7 μm . This displacement is on the order of the size of a single cell body (5–10 μm) and much greater than the radial dimension of a dendrite or glial process (1 μm).

One possible explanation for such substantial inferred displacement distances is that a considerable fraction of water molecules exchange between the intra- and extracellular spaces during a typical diffusion measurement. If, in fact, the pre-exchange lifetime is short relative to the diffusion time (fast exchange regime), the brain could be viewed as an 'effective medium' comprising intra- and extracellular water with an ADC that is a weighted average of ADCs in the two compartments. Fast exchange across cell boundaries could explain the significant displacements observed during the diffusion time of the MR measurement. In addition, it is conceivable that the reduction in overall water ADC associated with injury is a result of a reduction in cell membrane permeability in injured cells, possibly related to changes in aquaporin function. In support of this idea, there is indirect evidence that changes in membrane water permeability caused by alterations in aquaporin expression are associated with changes in water ADC (66,67). However, the Cs^+ studies described above suggest an ADC for intracellular Cs^+ on the order of $1.0 \mu\text{m}^2/\text{ms}$, and it is very likely that Cs^+ is confined to the intracellular space. Further, Cs^+ ADC decreases with brain injury (33). Explanations for this reduction based on a reduction in membrane permeability to Cs^+ are implausible, as the membrane permeability to Cs^+ is expected to be low prior to injury; thus, decreasing it further would probably have little effect on Cs^+ ADC.

The model we favor incorporates the assumptions that: (i) the brain compartmental structure locally restricts diffusion in only one or two dimensions; and (ii) the pre-exchange lifetime is long relative to the diffusion time (slow to intermediate exchange regime). For example, consider a dendrite or glial process approximated as a narrow but long cylinder that restricts diffusion to only one dimension locally, i.e. along the long axis of each cylinder. Likewise, consider the space between cells approximated as a series of thin, but broad, flat sheets that restrict diffusion to only two dimensions locally, i.e. across the broad plane of each sheet. (Indeed, the myelin of oligodendrocytes might, in some cases, be approximated in this fashion.) Recalling that the MR diffusion experiment monitors diffusion only along one axis (the direction of the applied diffusion sensitizing gradient), a collection of randomly distributed one-dimensional cylinders or two-dimensional sheets would allow for significant displacement distances, but yield an ADC significantly reduced from that expected of isotropic free diffusion. In the limit of diffusion confined to one or two dimensions only, the MR-measured ADC is one-third or two-thirds, respectively, of the ADC for isotropic free diffusion (37). This model could explain how Cs^+ in brain can have a relatively high ADC (on the order of $1.0 \mu\text{m}^2/\text{ms}$, or half that of Cs^+ in dilute aqueous solution) despite the fact that it is probably confined to the intracellular space during the entirety of the diffusion measurement. This model is also consistent with the findings of Kroenke *et al.* (32), who found relatively high ADCs for NAA for diffusion parallel to the fibers of the corpus callosum in rat and human. In this scenario, the explanation proposed above to explain the overall reduction in water ADC associated with injury – that it is caused by a reduction in intracellular water ADC – remains tenable.

Compartmental water diffusion anisotropy and compartmental water exchange both potentially contribute to the ADC observed in mammalian brain. A number of laboratories are exploring diffusion models of the central nervous system that incorporate well-defined, but simplified, structural and exchange characteristics [for example, see (68–74)]. In

addition, multidimensional MR diffusion experiments can provide deeper insight into local structural anisotropy and compartmental exchange [(75,76) and references therein].

Another potential influence on the diffusion measurement in tissue is the effect of T_2 relaxation time constants for water in different compartments. Diffusion measurements are typically made using pulse sequences that incorporate a spin echo with echo times on the order of 50 ms. As a result, the diffusion data obtained in these studies have some degree of T_2 weighting, with compartments having longer T_2 relaxation time constants contributing more to the measurement than those with shorter values. Models for diffusion measurement that incorporate T_2 relaxation times have been developed (77). Further, there is evidence that T_2 relaxation time constants, particularly for intracellular water, increase following injury in isolated *Aplysia* neurons (48). Unfortunately, accurate measures of intra- and extracellular water T_2 values, and their relative changes following injury, are not available for brain.

It is worth pointing out that the ‘compartment shift hypothesis’ was once the most common theory proposed to explain the reduction in brain water ADC following injury. This theory incorporates the following assumptions. First, intracellular water ADC is significantly less than extracellular water ADC. Second, total tissue water ADC is a weighted average of ADCs for intra- and extracellular water. Third, ADCs for intra- and extracellular water do not change significantly in association with injury. According to this theory, a shift of water from the faster diffusing extracellular space to the slower diffusing intracellular space, as a result of injury-related cytotoxic edema, leads to a decrease in overall water ADC by weighting the value towards slower diffusing, intracellular water (3,5,8,10,78). One problem with this explanation lies in the estimated ADCs for intra- and extracellular water. The overall tissue water ADC decreases by roughly 30% following brain injury, and the cytotoxic edema induced by stroke is associated with a compartment shift (extra- to intracellular) of approximately 10% of total brain water (62). Thus, as illustrated in the two examples below, the ADCs for intra- and extracellular water would have to be markedly different for a volume shift of only 10% to cause a 30% decrease in ADC.

Consider a simplified biophysical model in which: (i) the intracellular space has a fractional spin population (f_{in}) of 0.8 and the extracellular space a fractional population (f_{ex}) of 0.2; (ii) in healthy brain tissue, the tissue water ADC is approximately $1.0 \mu\text{m}^2/\text{ms}$; (iii) in ischemic brain tissue, edema causes these compartmental fractions to change to $f_{in} = 0.9$ and $f_{ex} = 0.1$; and (iv) in ischemic brain tissue, the tissue water ADC decreases by 30%, to approximately $0.7 \mu\text{m}^2/\text{ms}$.

In the first example, let us assume that water molecules are in slow exchange between compartments. For simplicity, let us also consider that the ADC measurement employs two b values – 0 and $1 \text{ ms}/\text{mm}^2$. It is common in clinical studies to use only two b values. As noted in the Introduction, the relationship between the diffusion signal intensity and b value is not monoexponential, but a monoexponential function is a reasonable approximation of the signal attenuation curve at moderate b values. For this example, we represent the signal intensity as the sum of two monoexponential functions corresponding to intra- and extracellular water. To perform the calculation, it is also necessary to assume a value for the extracellular water ADC in healthy brain tissue. We use $3.0 \mu\text{m}^2/\text{ms}$, approximately the free diffusion constant for water at 37°C , which is the maximum reasonable extracellular ADC (assuming only Brownian motion). Under these circumstances, a four- to five-fold difference in intra- and extracellular ADCs is necessary to account for the 30% ADC decrease associated with injury.

In the second example, let us assume that water molecules are in well-mixed fast exchange between the intra- and extracellular compartments. Here, the overall ADC will be the

arithmetic mean (population weighted) of the ADCs from contributing compartments. It is straightforward to derive the compartment-specific ADCs required to obtain a 30% decrease in ADC following injury: $ADC_{in} = 0.4 \mu m^2/ms$ and $ADC_{ex} = 3.4 \mu m^2/ms$, an eight-fold compartmental ADC difference.

Although, as noted above, the true values for intra- and extracellular water pre-exchange lifetimes are not known, the above examples encompass the limiting conditions of fast and slow exchange. The free diffusion coefficient of water at 37° C is $2.9 \mu m^2/ms$ (79) and it is unrealistic to consider that diffusion in the crowded and tortuous extracellular space approaches or exceeds this value. Further, the reporter molecule studies cited above consistently indicate that intra- and extracellular water ADCs are within a factor of two of each other, which is far too small a difference for the compartment shift (edema) hypothesis to explain a 30% reduction in ADC with injury.

CONCLUSION

MR diffusion reporter molecules provide a means to simplify the analysis of a structurally complex system by focusing measurements on, in principle, individual components rather than the whole. The compartment-specific/selective data provided by MR diffusion reporter molecules provide boundary conditions and guideposts that will serve to inform and challenge complex multiparameter models of water diffusion in the central nervous system. As noted earlier, the biophysics governing the displacement motion of MR reporter molecules will not be identical to that governing water displacement in the same compartment. Nevertheless, it is likely that the qualitative findings are reflective of water diffusion characteristics. For example, evidence from MR reporter molecules suggests the following: (i) the intracellular 'diffusional' viscosity is only twice that of a dilute aqueous solution; (ii) the ADCs of the intra- and extracellular space are similar; and (iii) the intracellular ADC decreases significantly following stroke (central nervous system ischemia). Ongoing experiments with MR reporter molecules are now poised to delve more deeply into the underlying mechanisms governing this clinically important, scientifically challenging problem.

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Abbreviations used

ADC	apparent diffusion coefficient
ADC_{ex}	ADC for extracellular reporter molecules
ADC_{free}	diffusion in dilute aqueous solution
ADC_{in}	ADC for intracellular reporter molecules
Cho	choline-containing compounds

Cre	creatine and phosphocreatine
FDG	fluorodeoxyglucose
2FDG	fluorodeoxyglucose
FDG-6-P	2-fluorodeoxyglucose-6-phosphate
NAA	N-acetylaspartate
PEG	polyethylene glycol
TMA	tetramethylammonium ion

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Figure 1.

Plot of intracellular apparent diffusion coefficient (ADC) vs. free ADC in dilute aqueous solution for several reporter molecules at a diffusion time of 8.6 ms. The water ADC representative of the entire cell was calculated from imaging experiments. All other ADCs were calculated from MRS data. The data were well modeled ($R^2 = 0.983$) by a least-squares fit to a straight line with a slope of 0.45 ± 0.02 and y-intercept of $2 \pm 2 \mu\text{m}^2/\text{ms}$. FDG-6-P, 2-fluorodeoxyglucose-6-phosphate; PEG, polyethylene glycol; TMA, tetramethylammonium ion. [Reproduced with permission from (29).]

Table 1

Apparent diffusion coefficients (ADCs, $\mu\text{m}^2/\text{ms}$) for various reporter molecules

System	Reporter molecule	Molecular weight (Da)	Intracellular ADC	Calculated intracellular ADC _{free}	Dilute aqueous solution ADC	ADC _{in} /dilute aqueous ADC	Change after injury	Reference
<i>Xenopus</i> oocyte	Water	18	0.94 ^a		2.12 ^a	0.44	—	(29)
	TMA	74	0.59 ^a		1.07 ^a	0.55	—	(29)
	PEG	2000	0.06 ^a		0.12 ^a	0.50	—	(29)
	FDG-6-P	182	0.32 ^a		0.54 ^a	0.59	—	(29)
	Cs ⁺	133	0.80 ^a		1.87 ^a	0.43	—	(29)
	Na ⁺	23	0.62 ^a		1.32 ^a	0.47	—	(29)
HeLa cells	Water	18	0.60 ^{b,c}	2.0	2.92 ^c	0.21	—	(18,46)
<i>Aplysia</i> gastric neuron	Water	18	0.28 ^d		2.58 ^d	0.11	—	(47)
	Water	18	0.69 ^c		2.92 ^c	0.24	−31%	(18,21)
Rat brain	FDG-6-P	182	0.16 ^c		0.70 ^c	0.23	−40%	(18)
	Cs ⁺	133	1.0 ^c		2.00 ^e	0.51	−75%	(29,33)
	NAA	175	0.16 ^{c,f}	0.36	0.78 ^c	0.21	−36%	(30)
	Creatine	113	0.17 ^c		0.85 ^g	0.20	−27%	(30,32)
Human brain	Choline	69	0.15 ^c		0.99 ^g	0.15	−36%	(30)
	NAA	175	0.14 ^c		0.72 ^g	0.19	—	(51)
	Creatine	113	0.17 ^c		0.84 ^g	0.20	—	(51)
	Choline	69	0.18 ^c		1.03 ^g	0.17	—	(51)

FDG-6-P, 2-fluorodeoxyglucose-6-phosphate; NAA, *N*-acetylaspartate; PEG, polyethylene glycol; TMA, tetramethylammonium ion.

^a 23°C.

^b For diffusion time 24 ms.

^c 37°C.

^d 27°C.

$\epsilon_{37^\circ\text{C}}$ (Zhao L, unpublished^{Q4}),

f Directionally averaged value.

g Corrected to 37°C using the Stokes–Einstein equation, for which ADC varies linearly with temperature (in K).