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Is There A Path Beyond BOLD? Molecular Imaging of Brain Function

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Abstract

The dependence of BOLD on neuro-vascular coupling leaves it steps removed from direct monitoring of neural function. MRI based approaches have been developed aimed at reporting more directly on brain function. These include: manganese enhanced MRI as a surrogate for calcium ion influx; agents responsive to calcium concentrations; approaches to measure membrane potential; agents to measure neurotransmittors; and strategies to measure gene expression. This work has led to clever design of molecular imaging tools and many contributions to studies of brain function in animal models. However, a robust approach that has potential to get MRI closer to neurons in the human brain has not yet emerged.

Keywords

Manganese enhanced MRI; MRI calcium indicators; MRI gene expression reporters; MRI detection of neurotransmittors; MRI detection of membrane potential

Developing MR for Measuring Brain Function Prior to BOLD

It can be argued that 1990 was a landmark year for MRI of the brain. For this Neuroimage issue on the 20th anniversary of fMRI, Seiji Ogawa's 1990 publications describing BOLD are most important (Ogawa et al, 1990a, Ogawa and Lee, 1990b, Ogawa et al, 1990c). That same year Moseley and colleagues published the observation that the apparent diffusion coefficient of water decreased during stroke (Moseley et al, 1990a) and that the apparent diffusion coefficient of water is anisotropic in white matter (Moseley et al, 1990b), the basis of diffusion tensor imaging. However, throughout the 1980's there was growing interest in measuring parameters with magnetic resonance that had relevance to brain function. This included the pioneering spectroscopy studies on brain from the Chance (Schnall et al, 1987), Radda (Radda, 1992), and Shulman (Pritchard and Shulman, 1986) laboratories. This work primarily focused on phosphorus energy metabolism, ^{13}C measurements of glucose metabolism, and ^{1}H studies of metabolites. In 1983, Smith et al published a paper describing a ^{19}F labeled calcium chelator for MR measurements of calcium concentration (Smith et al, 1983) that was used to monitor function in brain slices (Bader-Goffer, et al, 1990). This agent was inspired by the early demonstration of fluorescent based calcium indicators (Tsien, 1980). Fluorescent calcium indicators have grown into an important tool for measuring neural function. The recent success of genetically encoded, fluorescent calcium indicators will help sustain increasingly sophisticated studies of neural function (Looger and Griesbeck, 2011; Miyawaka et al, 1997). Prior to 1990, it was also being recognized that ^{19}F

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perfluorocarbon emulsions could be used to measure oxygen with MRI in vivo (Clark et al, 1984). Ackerman and colleagues explicitly set out to measure tissue function in analogy to PET measurements of deoxyglucose and regional blood flow (Ackerman et al, 1987; Deuel et al, 1985). Interest had also been developing to use oxygen-17 to measure blood flow and oxygen consumption (Arai et al, 1990).

In 1986, the discovery that small iron oxide particles were potent MRI contrast agents was made independently in the Lauterbur and Leigh laboratories (Mendoca-Dias and Lauterbur, 1986; Renshaw et al, 1986). Renshaw working with Leigh demonstrated that antibodies could be coupled to iron oxide particles for targeted MRI contrast and Lauterbur was interested in the ability to track cells in liver, spleen, and brain with iron oxide particles (Hawrylak et al, 1993; Mendoca-Dias and Lauterbur, 1986). Cell tracking using iron oxide contrast has grown into a robust area of investigation in MRI (Bulte, 2009; Ho and Hitchens, 2004). Magnetic resonance was also beginning to be integrated with emerging transgenic mouse technology to study problems in physiology (Koretsky, 1995). Early papers demonstrated that expression of creatine kinase could be used as a reporter strategy by detecting the metabolic product phosphocreatine with magnetic resonance in *E.coli* (Koretsky and Traxler, 1989), yeast (Brindle et al, 1990) and liver in transgenic mice (Koretsky et al, 1990). Developing reporter protein strategies that enable detection of gene expression with MRI is now an active research area (Gilad et al, 2007). Measurement of gene expression of immediate early genes such as cfos has a long history of use for studying brain function (Barth, 2007), and MRI has the potential to do this non-invasively. Thus, prior to the first human BOLD fMRI results there was a body of work aimed at using MRI to monitor brain function including metabolic correlates of brain function, oxygenation, blood flow, calcium concentrations, cell tracking and gene expression. Indeed, these studies helped lay the foundation for the area we now call molecular imaging with MRI. Despite the success of BOLD fMRI there continues to be work attempting to find MRI techniques to measure brain function more directly than relying on vascular responses to infer neural activity. The goal of this contribution is to highlight some of the attempts to get closer to neurons using MRI with emphasis on those approaches that are now part of the toolbox of molecular imaging.

Direct Measurement of Membrane Potential: The Holy Grail

Neurons require propagation of action potentials to transmit information. Direct measurement of membrane potential changes can be accomplished with patch clamp techniques (Nauen, 2011) and fast responding, membrane potential sensing optical dyes (Chavane et al, 2011; Peterka et al, 2011; Waggoner, 1979). Patch clamp techniques are limited to a small number of sites and, although widely used in brain slice work, remain a challenge to perform in vivo (Peterson, 2009). Optical membrane potential dyes have a small dynamic range and can only be imaged from the surface of the brain. Exciting new developments in fluorescent proteins that sense membrane potential look promising to enable two-photon fluorescence techniques to make multi-site measurement of membrane potential from deeper tissue and allow targeting to specific cell types (Kralj et al, 2011). Other electrophysiological tools such as MEG, EEG and extracellular electrodes measure extracellular currents or potentials generated by action potentials and are thus less direct then monitoring membrane potential changes. A MRI technique that can either directly measure membrane potentials or the effects of action potential propagation would enable more direct monitoring of neural function than is possible with BOLD.

There is a literature that attempts to measure the effects of fluctuating magnetic fields generated from action potential propagation without the need to resort to any contrast agents. There have been attempts to measure the effects of fluctuating magnetic fields

associated with neural activity on the phase or apparent relaxation of MRI signals (Bandettini et al, 2005). No attempt will be made to summarize this field. It remains an active area of investigation but none of the proposed measurements have yet proven to be widely adopted. There are also recent attempts to monitor the swelling of cells known for many years to occur during action potential propagation using diffusion based MRI techniques (Le Bihan, 2007). This also remains an active area of investigation but with no consensus yet on whether such approaches will be broadly applicable. The prediction that there is a significant Lorentz force on neurons due to propagating action potentials in a magnetic field causing small motion of neurons that may be detected by MRI has also been considered with contradictory conclusions at this stage (Song and Takahashi, 2001; Roth and Basser, 2009).

A number of approaches to making MRI agents directly sensitive to membrane potential can be envisioned. MRI contrast agents with properties similar to the optical membrane potential sensitive dyes can be imagined. Indeed, lipophilic cations have long been used to measure slow membrane potential changes using fluorescence with particular emphasis on mitochondrial membrane potential (Waggoner, 1979). There is a large literature in nuclear medicine using radiolabeled derivatives of lipophilic cations to measure delivery of agent but the fact that they distribute intracellularly based on membrane potential in not exploited (Liu, 2007; Madar et al., 2006). The membrane permeant triphenyl phosphonium ion has been used to measure slow potential changes and can be directly detected with ^{31}P NMR, although this has not been used in MRI. A complex of a DO3A-conjugated triphenylphosphonium cation with a paramagnetic metal chelated has been reported (Yang et al, 2007). However, to the best of my knowledge no work using MRI has yet been done in this direction to use these agents to attempt to measure neural potential changes.

More attractive for studying neural activity are the fast responding class of membrane potential optical dyes (Chavane et al, 2011; Peterka et al, 2011; Waggoner, 1979). A new class of fluorescent based protein indicators of fast membrane potential are being developed that show much promise (Kralj et al. 2011). The ideas used may be amenable to adaptation to an MRI based sensor. However, little work has been done to develop an MRI agent that can monitor fast membrane potentials. Paul Lauterbur's group did propose an idea aimed at measuring membrane potentials (Frank and Lauterbur, 1993). They embedded iron oxide particles in a gel matrix that would be expected to shrink and swell do to changes in electric field. Altering the distribution of particles caused changes in T_2 enabling a probe of membrane potential. This class of agent is based on T_2 changes allowing potential changes to be measured on fast time scales. Challenges of this approach are to find ways to embed the particles in the portion of the membrane where potentials are actually changing, as well as to make gels that are specific for membrane potential changes and not affected by other changes such as pH or Ca^{2+}. The idea to control the aggregation of iron oxide particles to make sensors has grown (Atanasijevic, 2006; Colomb et al, 2011; Haun et al, 2011; Perez et al, 2002), however, no further work on sensing membrane potential has been performed. While there would be many uses of MRI based ways to monitor membrane potential changes directly, it remains a holy grail in need of new maps.

Calcium Dynamics Using MRI

Measurement of Intracellular Calcium

When a neurotransmitter binds to its receptor on the postsynaptic neuron and triggers an action potential, Ca^{2+} enters the neuron eventually leading to release of neurotransmitter at the neuron's pre-synaptic terminal. Thus, measurement of Ca^{2+} influx or changes in intracellular Ca^{2+} concentrations is an excellent surrogate for membrane potential changes. As mentioned in the introduction, the development of robust fluorescent indicators of Ca^{2+}

(Tsien, 1980) and the recent generation of fluorescent proteins sensitive to Ca^{2+} (Looger and Griesbeck, 2011; Miyawaka et al, 1997) have been very important for studies of neural function in brain slices and in animal brains.

There have been a number of MRI based agents to measure Ca^{2+}. Based on the first generation of fluorescent Ca^{2+} indicators, ^{19}F based Ca^{2+} indicators were developed and applied to brain slice work (Bader-Goffer et al. 1990; Smith et al, 1983,). It was demonstrated that a chelator with a well positioned ^{19}F could have a large chemical shift upon binding Ca^{2+}. The large concentrations needed compared to the free Ca^{2+} concentration raised concerns about buffering and techniques to deliver the agent efficiently to the intact brain have hampered work with these agents. There is now a growing literature developing Gadolinium (Gd) chelates as Ca^{2+} sensors (Angelovski et al, 2008; Li et al, 1999; Li et al, 2002; Mamedov et al., 2011; Henig et al. 2011). The basic idea is to use a dual chelator one to hold the Gd and another to bind Ca^{2+}. The trick is to engineer the molecule so that water exposure to Gd in the chelate changes when Ca^{2+} binds. This enables changes in T_1 relaxivity due to changing Ca^{2+} concentrations by this class of agent. Ca^{2+} binding can vary accessibility to the first or second coordination sphere of water or change the exchange rate in order to alter T_1 relaxivity. Proof of principle, a growing list of possible agents, details of the mechanism for relaxivity changes, and potential problems such as aggregation have been studied (Li et al 2002; Henig et al, 2011; Mamedov, 2011, Mishra et al, 2011). There has also been the suggestion to use Ca^{2+} induced oligomerization of Gd dota-bisphosphonate conjugates as an indicator (Kubicek et al, 2010). Extremely large changes in relaxivity (200–500%) were reported on binding Ca^{2+}. Unfortunately this agent was not specific for Ca^{2+} over other divalent metal ions such as Mg^{2+} and Zn^{2+}. A problem with agents that change T_1 relaxivity is that typically calcium transients are very rapid (~ 10 msec) inside cells to keep up with spiking neurons. It is not clear how agents that change relaxivity on the T_1 time scale (~ 1 sec) can keep up. However, as was the case with many of the early generation fluorescent indicators, they slow responsing MRI agents may be useful for monitoring average calcium changes from a series of many action potentials. The trick will be to engineer the affinity and the off rates to enable this type of integration of calcium transients.

Rather than use changes in T_1 relaxivity, there has been some initial work using chemical exchange dependent saturation transfer (CEST agents) to monitor Ca^{2+} (Angelovski et al, 2011). CEST agents rely on measuring chemical exchange between a chemically shifted resonance on the agent and water (Ward et al, 2000). Rather than the fast exchange that Gd based agents rely on, these agents rely on slow chemical exchange between a proton on the CEST agent and water protons. Development of CEST agents made a major step forward when it was demonstrated that binding of water to a paramagnetic ion on a chelate could be used to induce a large chemical shift and have exchange rates useful for detection via CEST strategies. This class of agents are known as paraCEST agents (Zang et al. 2001). There are now a growing number of CEST agents that measure enzymes, metabolites, zinc, pH and temperature (Viswanathan, 2010). A major advantage of CEST agents is that the exchange effect can be readily turned on and off with appropriate MRI sequences. The Ca^{2+} paraCEST agent relied on synthesizing calcium chelators to the paraCEST agents and optimizing for changes induced by changing Ca^{2+} (Angelovski et al, 2011). The chemical exchange effect of CEST agents builds up on a T_1 time scale and so these agents will be limited to following average Ca^{2+} levels.

There has not been any work in neural preparations using either the T_1 relaxivity based agents or the paraCEST based agents. An interesting suggestion is to use these paramagnetic based agents to monitor changes in extracellular calcium with neural activity (Angelovski et al, 2008, Mishra et al, 2011). Decreases in extracellular Ca^{2+} as large as 0.2mM have been

reported in brain slice work due to influx of Ca^{2+} into cells during neural activity (Fedirko et al, 2007). An extracellular MRI agent would need to have low affinity for Ca^{2+} to respond to the changes in the mM levels of extracellular Ca^{2+}, making it difficult to engineer specificity for Ca^{2+} over other ions. However, if successful this would bypass problems with loading the agents to the intracellular space and problems associated with the relatively high concentrations needed for MRI causing buffering of intracellular Ca^{2+}.

Building on the idea to use density or aggregation state of iron oxide particles as a sensor, the Jasanoff lab has engineered iron oxide particles to enable Ca^{2+} to control their aggregation state (Atanasijevic et al, 2006). This builds on similar ideas from Josephson and Weissleder who have developed a number of sensors by controlling the aggregation of iron oxide particles (Perez et al, 2002; Haun et al, 2011). The Ca^{2+} indicator used the protein calmodulin as the calcium sensor on one set of particles and a peptide known to bind only to Ca^{2+} bound calmodulin on the other set of particles. An increase in calcium causes the particles to aggregate and altered the apparent T_2 of water (Atanasijevic et al, 2006). It is possible to use site directed mutagenesis to optimize the properties of this indicator (Green et al, 2006). Problems with this approach include delivering agents to the cytoplasm and the need to set the concentration of agent just right to get desired T_2 changes. Assembly to the aggregated state can be quite slow even in vitro and it is not clear how much the cytoskeleton may further slow the process. There has been recent work modeling the processes that control MRI relaxivity and the aggregation of iron oxide nanoparticles including ferritin (Bennett et al, 2008; Matsumoto and Jasanoff, 2008; Shapiro et al, 2006). Shapiro et al also discuss time resolution limitations associated with the entire range of MRI based Ca^{2+} agents (Shapiro et al, 2006).

There has been recent interest in altering the expression of ferritin as a gene expression reporter strategy (see discussion below; Cohen et al, 2005; Genove et al, 2005). It may be possible to engineer chimeras of ferritin with Ca^{2+} binding proteins to enable neurons to express their own calcium indicators as is done with Ca^{2+} sensitive fluorescent proteins (Looger and Griesbeck, 2011; Miyawaka et al, 1997). This strategy would be a great step forward for animal studies.

Manganese Enhanced MRI (MEMRI) as a Surrogate for Calcium Influx

Mn^{2+} was the first paramagnetic ion used to alter contrast in MRI (Lauterbur, 1973). Early work demonstrated that it was transported intracellular, however, toxicity concerns hindered its development. With the advent of more sensitive MRI scanners the use of Mn^{2+} as a useful contrast agent has had a renaissance especially for brain imaging in animal models. Mn^{2+} is an essential heavy metal for life and as such there are many interesting biological processes to transport and sequester Mn^{2+}. One such mechanism is that Mn^{2+} can enter cells on voltage gated calcium channels. Therefore, the influx of Mn^{2+} into excitable tissue is a surrogate for Ca^{2+} influx. This idea was used to make functional maps of somatosensory areas in brain by Lin et al in the rat brain (Lin et al, 1997). There is now a significant body of work that has used Mn^{2+} as a versatile contrast agent to study the brain (Boretius and Frahm, 2011; Inoue et al. 2011). There have been a few studies that have demonstrated excellent agreement between MEMRI maps and fMRI maps of neural representations (see Silva in this issue of Neuroimage). Recent work in the olfactory bulb (Chuang et al, 2009a), hypothalamus (Kuo et al, 2006; Parkinson et al, 2009), retina (Berkowitz et al, 2006; Berkowitz et al, 2009), motor cortex (Eschenko et al, 2010), nociceptive pathways (Yang et al, 2011), and the auditory midbrain in mice (Yu et al. 2005; Yu et al. 2007) demonstrates that MEMRI has specificity to image small neural representations as well as laminar activity and even an individual olfactory glomerulus. Many of the areas mapped would be too small for BOLD based fMRI studies. For most areas of the brain, Mn^{2+} entry is rate limiting and so requires breaking the blood brain barrier (BBB). Novel approaches using focused

ultrasound and BBB breaking antibodies have been used with MEMRI (Howles et al, 2010; Lu et al, 2010).

Mn^{2+} has been used to monitor activity in a few brain areas without breaking the BBB in areas that transport Mn^{2+} relatively quickly from the blood, such as the hypothalamus (Kuo et al, 2006) and the retina (Berkowitz et al, 2006). Another approach to avoid breaking the BBB has been to use very long periods of stimulation in the presence of Mn^{2+} (Eschenko et al, 2010; Yu et al, 2005). There are a number of limitations to using MEMRI. Mn^{2+} leaves the brain on the time scale of weeks (Chuang et al, 2009b) limiting its applicability. It is not yet clear what are the minimum number of action potentials required to enable enough accumulation of Mn^{2+} to enable detection but it is likely that many are required. Nor is it clear what the distribution of Mn^{2+} is between neurons and glial, since Mn^{2+} can accumulate in both cell tyoes in an active brain area. The slow efflux enables MEMRI to make very high resolution maps and allows brain areas to be stimulated outside the magnet of MRI systems and imaged (Eschenko et al, 2010; Lin et al, 1997; Yu et al, 2005). The idea to use Mn^{2+} accumulation to map active brain areas has not yet been attempted in the human brain. Access through the BBB and concerns about toxicity are the main barriers (Eschenko et al. 2010; Jackson et al, 2011). However, work demonstrates that the FDA approved Mn^{2+} based contrast, MnDPDP, can deliver Mn^{2+} to the pituitary and choroid plexus (Wang et al. 1997), offering the intriguing possibility of using MEMRI to study human brain function.

Two other unique properties of Mn^{2+} are finding widespread use in animal models. Mn^{2+} is transported in brain in an anterograde enabling direct monitoring of brain connectivity with laminar specificity (Pautler et al, 1998; Tucciarone et al. 2009; Inoue et al, 2011; Zhang et al, 2010). The tracing occurs on a much slower time scale than uptake used to map neural activity. The second use property is that once accumulated in the brain, Mn^{2+} gives contrast that enables cytoarchitectural information to be obtained from MRI (Aoki et al, 2004, Bock et al, 2009; Silva et al, 2008; Watanabe et al, 2001). Indeed structures as small as individual olfactory glomeruli have been resolved using the different types of MEMRI experiments (Chaung et al, 2009a; Chuang et al, 2010).

MRI Agents to Measure Neurotransmitter Release

Another area that would enable MRI to get closer to neurons is monitoring neurotransmitter release. Spectroscopic techniques have enabled average glutamate recycling and GABA recycling rates to be measured (Novotny et al, 2003). While very useful for studying pathophysiology of the brain, the time scale of the experiments and the resolution do not lend themselves readily to monitoring functional brain states. Spectroscopic detection of GABA levels has been associated with neural activity and may represent changes in the “inhibitory” state of a brain area (Chen et al, 2005; Muthukumaraswamy et al, 2009).

Recently there has been exciting work using advanced laboratory protein evolution techniques to make a MRI sensor of dopamine (Shapiro et al, 2010). The idea was to use a heme based protein whose ligand binding alters water access to the metal in the heme. In this case bacterial cytochrome P450-BM3, which has a paramagnetic iron in its heme site and is accessible to water in its unliganded state was used. Addition of the natural ligand arachidonic acid to BM3 led to a decrease in T_1 relaxivity. Directed evolution was used and mutants with an increased affinity to dopamine were selected for further rounds of mutatgenesis. After a number of rounds of mutagenesis, a protein that had high affinity for dopamine rather than arachidonic acid was produced creating an MRI sensor of dopamine. Preliminary results indicated that this agent will be useful for monitoring extracellular dopamine in the rat brain (Shapiro, 2010). Optimization of the metal binding has enabled increasing the dynamic range (Lelyveld et al. 2011). Of course, one can imagine evolving

protein sensors for other neurotransmitters and the resulting protein can be expressed and secreted by the neurons themselves, bypassing delivery issues. It will be exciting to see if these uses of advanced protein evolution approaches can be widely adapted for making agents suitable for studies in animals. Application to the human brain will have to await toxicity studies and efficient ways to deliver these protein constructs through the BBB. An advantage is that these agents detect extracellular levels of neurotransmitter so there is no need to deliver the agents to the intracellular space.

Rather than monitor neurotransmitters directly it might be useful to monitor other constituents of neurotransmitter containing vesicles. In particular, many neurotransmitter vesicles are enriched in zinc. There have been a number of MRI agents designed to measure zinc. These are primarily based on approaches similar to those used for calcium sensitive agents. T_1 paramagnetic chelates or paraCEST chelates have been modified to contain zinc chelating groups that modulate the relaxivity or exchange upon binding zinc (Lee et al, 2010; Major et al, 2007; Mishra et al, 2011; Trowski et al, 2005; Zhang et al, 2007). As with the calcium indicators these have been primarily proof of principle studies. Recently, a MRI based zinc indicator has been used to measure extracellular zinc release from the pancreas in rodent in response to elevated glucose (Lubag et al, 2011). Insulin vesicles are high in zinc and the ability to have a surrogate marker of insulin release would be very important for following the progression of diabetes. In this case, a Gd based chelator (Gd DOTA-diBPEN) binds zinc which in turn imparts a high affinity of the chelate for albumin. The binding of the low molecular weight Gd chelate to albumin increases the T_1 relaxivity due to a change in correlation time (Esqueda et al, 2009). It would be very exciting if this approach proved to be sensitive enough to extend to monitoring release of zinc for central nervous system synapses!

MRI Reporters of Gene Expression

A number of genes increase expression in response to neural activity. Monitoring the expression of these genes has been a surrogate for monitoring neural activity. Most important has been cfos and other early neural response genes such as Arc (Barth, 2007). The advent of fluorescent proteins has enabled rodent models that enable direct monitoring of increased gene expression due to brain activity (Barth, 2007; Wang et al, 2006). A similar strategy could be accomplished with MRI. There has been a long hunt for a robust MRI reporter strategy for monitoring gene expression. All gene reporter strategies rely on detecting the expression of a detectable protein or RNA. The MRI approaches all rely on expressing a protein that can alter magnetic resonance properties. Starting with work using creatine kinase (and subsequently the related arginine kinase) to alter metabolites that could be detected by magnetic resonance (Auricchio et al, 2001; Brindle, 1990; Koretsky and Traxler, 1989; Koretsky et al, 1990; Landis et al, 2006; Li et al, 2005; Walter et al. 2000), there has now been development of a large number of strategies (Gilad et al. 2007).

The enzyme beta-galactosidase has been used since the birth of molecular biology as a reporter protein and so it has been a target for development of a number of MRI agents that can detect this enzyme. A Gd chelate where the Gd is protected from water until the enzyme beta-galactosidase is expressed to cleave the chelate and an increase in T_1 relaxivity has been demonstrated (Louie et al, 2000). A number of similar Gd chelate strategies have now been reported (Chang et al, 2007; Hanaoka et al, 2008; Keliris et al, 2011). Shortcomings have been the relatively small change in relaxivity associated with enzymatic activation of these agents as well as the problem of distinquishing the amount of agent from the amount of activation. A recent study reports on a Gd chelate that was designed to polymerize in the presence of beta-galactosidase and melanin (Arena et al, 2012). Fluorinated substrates have been designed so that the fluorine chemical shift changes when the substrate is cleaved by

beta-galactosidase enabling 19F NMR to detect gene expression (Cui et al, 2004; Liu et al, 2007). These agents are limited by the need for very high concentrations required. Colormetric agents have been used for functional assays of beta-galactosidase for a long time in isolated cells which rely on enzymatic activity to form a precipitate which is high in iron. Recently. it has been demonstrated that these agents can be used in vivo for MRI (Bengstrom et al, 2010; Cui W et al, 2010). In order to apply any of the gene expression reporter protein strategies that require exogenous contrast agent to the brain will require them to be permeable through the BBB.

Approaches to increase stored iron in a cell have the potential to be the most sensitive by altering T_2 relaxivity. There have been a number of proteins whose expression was controlled in an attempt to increase cellular iron, such as expressing the transferrin receptor that increases iron transport into cells (Koretsky et al, 1996). Increasing tyrosinase to produce melanin which binds iron has also been proposed as a potential reporter protein strategy to increase cellular iron (Weissleder et al, 1997). There has been growing interest in expressing ferritin, which is an iron storage protein. Changes in T_2 have been reported in mouse models that overexpress ferritin (Cohen et al, 2005; Cohen et al, 2007, Choi et al, 2011; Genove et al, 2005; Ziv et al, 2010). Ferritin has low MRI relaxivity and so large levels of expression are required. Dual expression of both transferrin receptor and ferritin has been reported, but cells needed to be pre-incubated with high iron to get significant changes in relaxivity compared to controls (Deans et al., 2006). Recent work have reported only small changes in MRI signals due to ferritin overexpression (Aung et al, 2009; Kim et al, 2010; Ono et al, 2009; Van de Velde G et al, 2011). A transgenic mouse expressing human ferritin heavy chain in brain did not accumulate iron indicating that very high levels of expression may be required (Hasegawa et al, 2011). The high levels of expression and small signal changes detected with expression of the transferrin receptor or ferritin indicate that the idea to cause iron accumulation in cells via gene expression needs to be optimized. Recently, a more optimal ferritin with higher iron loading and a higher T_2 relaxivity has been reported and used to track neural precursors in the rodent brain (Iordanova et al, 2010; Iordanova and Ehrens, 2012). There is a large potential for optimizing ferritin since similar sized iron oxide particles have about 100 times higher relaxivity. Maybe the advanced protein evolution techniques used to make the MRI dopamine sensor can be applied successfully to improve ferritin. In addition to using the normal mammalian mechanisms to increase cell iron, there is some evidence that expression of the bacterial iron transporter MagA may be sufficient for producing magnetic nanoparticles in mammalian cells making it a potential reporter protein (Zurkiya, 2008, Goldhawk et al, 2009).

Other approaches to altering MRI contrast via expression of a gene have involved expressing peptides that enable efficient exchange of magnetization from lysine or arginine protons to water protons and applying CEST techniques (Gilad et al, 2007, Liu et al, 2011). Expressing cell surface proteins such as the transferrin receptor or the majorhistocompatibility class 1 antigen that can be targeted with antibodies carrying exogenous MRI contrast is yet another strategy that has been demonstrated (Moore et al, 1998; So et al, 2005). Expressing enzymes that can degrade the coating on iron oxide particles taken up in the endosomal pathway and leading to loss of MRI contrast has been suggested to detect gene expression with MRI (Granot and Shapiro, 2011). It should be possible to make manganese based agents that will increase contrast when degraded by specific enzymes (Shapiro and Koretsky, 2008). Finally, there has been work that targets MRI contrast directly to expressed RNA (Liu et al, 2009; Liu et al 2011). Oligonucleotides specific for transcribed RNAs from a number of interesting brain genes were complexed to iron oxide particles which are retained longer than control iron oxide particles. Since iorn oxide particles usually are taken up into lysosomes that added oligonucleotide probes must help the particle enter the cytoplasm in order to be able to hybridize to the target RNA. If

similar results can be achieved by other groups this would represent an exciting approach to monitor gene expression.

A large variety of novel approaches are being developed to try and find a sensitive and specific approach that can be used as a gene expression reporter for MRI. In most of the cases demonstrated to date very strong gene expression promoters have been used to get enough protein to lead to detectable MRI signal or cells were first transfected to produce high levels of the reporter protein in vitro prior to transplanting into an animal. Even under these optimal circumstances the contrast changes reported have been small. The majority of the studies have been proof of principal with few applications yet. Thus, it is not clear that any of these strategies can be robustly used to monitor changes in normal cellular promoters such as found on the genes that respond to neural activity. It is also difficult to envision how these techniques will translate to work on the human brain. However, when a widely applicable approach is developed it will be very useful for rodent and non-human primate studies. The important use of the multiple colors available from fluorescent proteins make it likely that different MRI reporter strategies that make use of T_1, T_2 and CEST contrast will be important.

Conclusion

In the early 1990's, I had the good fortune of having an excellent graduate student at Carnegie Mellon University, Yi-Jen Lin. Yi-Jen joined my lab and we were worried that the new MRI hemodynamic based strategies for mapping the brain might have limited utility. This led to a hunt for alternative strategies and the development of MEMRI (Lin and Koretsky, 1997) as the well as the thought to make gene expression reporters that might eventually be used for mapping brain activity (Koretsky et al, 1996). On the 20th anniversary of BOLD fMRI in humans, it has become clear that there was no reason for Yi-Jen and I to have worried! BOLD has developed beyond anyone's wildest imagination to study all aspects of brain function, open up the field of resting state connectivity, and decode what is on our minds! This has raised the bar for any new MRI tools that hope to measure brain function.

Despite this tremendous success of BOLD fMRI, there remains a very active field of inquiry aiming to develop approaches to move MRI closer to directly measuring neural activity. The dependence of fMRI on vascular responses still lead to ambiguities related to localization of neural activity, timing of neural activity, defining the neurotransmitter systems responsible for neural activity, and defining the cellular source of the neural activity. The dream of molecular imaging techniques would be to add specificity to fMRI studies that would circumvent the problems associated with relying on vascular responses to detect neural responses indirectly. There has been some progress and a number of very interesting ways to control MRI contrast to sense a growing list of biological molecules and biological processes. The impact so far has been in defining new modes of MRI contrast and studying brain function in animal models. In this growing field of molecular imaging, animal studies are where the exciting MRI development originates, as was the case for BOLD fMRI in 1990 (Ogawa et al, 1990a, Ogawa and Lee, 1990b, Ogawa et al, 1990c). Translation of these new molecular imaging strategies to the human brain seems a long way off, but our hope is that when we look back twenty years from now a range of MRI approaches will be available to get us closer to the neurons.

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