APPLICATIONS OF MAGNETIC RESONANCE IMAGING TO STUDY BRAIN PATHOLOGY AND FUNCTION

A Thesis in
Integrative Biosciences

by

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ABSTRACT

Magnetic resonance imaging utilizes magnetic fields to non-invasively manipulate atomic nuclei to allow creation of images from regions within a body otherwise obscured from sight. Here we use the power of magnetic resonance imaging to gain new insights into perinatal hypoxia-ischemia (H/I), the leading cause of morbidity and mortality in infants and children, adult H/I, the third leading cause of mortality and leading cause of disability in adults and visceral pain associated with irritable bowel syndrome (IBS), afflicting an estimated 10-22% of adults.

To complete part of this work, it was necessary to develop a novel hexagonal coil for simultaneous imaging of multiple small animals. The hexagonal coil design is based on a coaxial cavity, and utilizes the magnetic field formed between two coaxial conductors with hexagonal cross-sections. The construction scheme of the coil, simple methods for tuning and matching, the neonatal animal holder, and the anesthesia manifold for equal delivery of anesthetics are described. The design is intended for in vivo animal studies, and in the case of drug studies it allows 3 control and 3 treated animals to undergo identical experimental conditions. In vivo spin echo images of six 7-days old rat pups acquired simultaneously demonstrate uncompromised signal-to-noise ratio required for microimaging. The described hexagonal coil and MR imaging was used to study the evolution of brain injury following hypoxia-ischemia (H/I) in the neonatal rat before and after a pharmacological interventions. We tested the hypothesis that repetitive administration of aminoguanidine (inducible nitric oxide synthase inhibitor) and minocycline (interleukin 1β (IL-1β) converting enzyme, iNOS inhibitor,
p38 kinase inhibitor, Ca\(^{2+}\) chelator and caspase 3) will provide significant neuroprotection, when administer following hypoxia-ischemia. The volume of the hyperintense diffusion weighted (DW) signal (cytotoxic edema) before administration of the drug was compared to the T\(_2\)-weighted hyperintense signal (vasogenic edema) volume at 7 days following the recovery from H/I. A significantly reduced hyperintense signal volume was observed on T\(_2\)-weighted images compared to initial hyperintense signal volume on DW images (p<0.0005) for both drug-treated and control (saline and PBS injected) animals. When the amount of hyperintense signal reduction was compared among different treatment groups the minocycline had tendency toward maximal hyperintense signal reduction, although this tendency was not statistically significant. The neuroprotective mechanism of minocycline is hypothesized to be due to its anti-inflammatory effects and inhibition of inducible nitric oxide synthase (iNOS), and independent of its antimicrobial actions.

To further investigate the role of inflammation in neurodegeneration following hypoxia-ischemia, we focused on IL-1 signaling in the adult mouse model. Signaling through the IL-1 type 1 receptor (IL-1R1) is necessary for microglial activation and based on previous studies, which have implicated microglia as effectors of neurodegeneration, we hypothesized that inactivating the IL-1R1 would decrease the induction of pro-inflammatory cytokines and chemokines caused by a hypoxic-ischemic insult. We further hypothesize that abrogated IL-1R1 signaling will result in failure to recruit macrophages, resulting in lower levels of inducible nitric oxide synthase (iNOS) and consequently decreased free radical damage following H/I. Magnetic resonance imaging was used to determine infarct size post H/I in wild type (WT) and IL-1R1 null mice. The
IL-1R1 null mice had significantly reduced infarct size at 24 h and 48 h post HI. The induction of multiple pro-inflammatory cytokines and chemokines was significantly reduced in the ipsilateral hemisphere of the IL-1R1 null mice compared to wild-type mice at 72 h following H/I. Levels of iNOS protein and iNOS mRNA were severely curtailed in the IL-1R1 null mice. The induction of glutamate aspartate transporter GLAST at 48 h following H/I, was similar for both mice types, indicating independence of IL-1R1 signaling. The sensory motor function of IL-1R1 null mice was normal at one month following H/I. The present findings incriminate the IL-1R1 as a master pro-inflammatory cytokine receptor, and suggest that IL-1R1 signaling contribute to neurodegeneration following mild H/I via recruitment of inflammatory cells, as well as iNOS mediated free radical damage. In conclusion we demonstrate that reducing the inflammation consequent to a mild hypoxic/ischemic insult will prevent brain damage and preserve neurological function.

Functional magnetic resonance imaging (fMRI) was applied to study brain areas activated in response to the colorectal distention (CRD) in adult anesthetized rats. The aim of the study was to determine and compare the areas of brain activated in response to the noxious visceral stimuli using fMRI and c-Fos protein expression. For the fMRI study, visceral pain was simulated with rectal balloon inflated to different pressures (40, 60 and 80 mm Hg) in a phasic manner: 90 s deflation period followed by 30 s inflation period for 16 min. The anesthetized rats underwent phasic colorectal distention, synchronized with the acquisition of fMRI images. For c-Fos experiments the stimulus was a rectal balloon distended to 80 mm Hg in a phasic manner with 30 s inflation and 90 s deflation, for 2 h. fMRI imaging revealed activation in the amygdala, hypothalamus,
thalamus, cerebellum, hippocampus, and trigeminal nucleus. In a smaller number of animals, activation was also observed in the nucleus of the solitary tract, parabrachial nucleus, superior colliculus, striatum and the insular, piriform, retrosplenial, sensory association, and perirhinal cortices. Significant increases in c-Fos expression compared to control animals, (p=0.05), was observed in the pontine parabrachial nucleus and the paraventricular nucleus of hypothalamus. The correlation of increased fMRI signal and c-Fos expression indicates involvement of the spino-parabrachio-amygdaloid pathway in response to visceral nociception.
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**LIST OF ABBREVIATIONS**

ACC – anterior cingulate cortex  
ADP – adenosine diphosphate  
AEBSF – aminoethyl benzylsufonyl fluoride  
AMP – adenosine monophosphate  
ATP – adenosine triphosphate  
BOLD – blood oxygenation level dependent  
BSA – bovine serum albumin  
CBF – cerebral blood flow  
CNS – central nervous system  
Cox-2 – cyclooxygenase two  
CRD – colorectal distention  
DW – diffusion imaging  
DWI – diffusion weighted imaging  
EDTA – ethylenediaminetetraacetic acid  
eNOS – endothelial NOS  
EPI – echo planar imaging  
FDA – food and drug administration  
FDTD - finite difference time domain  
fMRI – functional magnetic resonance imaging  
GABA – gamma aminobutyric acid  
GLAST - glutamate aspartate transporter
GLT – glutamate trasporter
GRE – gradient recalled echo
H/I – hypoxia-ischemia
IBS – irritable bowel syndrome
IFN-γ – interferon gama
IL – interleukin
IL-1 – interleukin one
IL-1 R1 – interleukin one receptor type one
IL-1 RII – interleukin one receptor type two
iNOS – inducible NOS
ip – intraperitoneal
KO – knock-out
Ltn- lymphotactin
MCA – middle cerebral artery
MCAO – middle cerebral artery occlusion
M-CSF- macophaje colony stimulating factor
MIF – macophaje migration inhibitory factor
MIP - macophaje inflammatory peptide
MR – magnetic resonance
MRI – magnetic resonance imaging
NA- number of averages
NEX – number of excitations
nNOS – neuronal NOS
NO – nitric oxide

NOS – nitric oxide synthase

NTS – nucleus of the solitary tract

PBN – pontine parabrachial nuclei

PBS – phosphate buffered saline

PET – positron emission tomography

PVN – paraventricular nucleus of hypothalamus

PVP – paraventricular nucleus of thalamus

RANTES – regulated on activation normal T-cell expressed and probably secreted

RF – radio frequency

RPA – RNAse protection assay

RT-PCR – reverse transcriptase polymerase chain reaction

SE – spin echo

SNR – signal to noise ratio

TE – echo time

TNFα - tumor necrosis factor alpha

TR – repetition time

V-CAM – vascular cell adhesion molecule

WT – wild type
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Chapter 1

General Theory of Nuclear Magnetic Resonance and Magnetic Resonance Imaging

1.1 Introduction

Nuclear Magnetic Resonance (NMR) refers to the phenomenon where the nuclei that possess nuclear spin when placed in a magnetic field will absorb electromagnetic radiation of a certain frequency (resonance). This phenomenon was first observed in “condensed” (non-gaseous) matter by Bloch and Purcell (1946) [Bloch 1946; Purcell et al. 1946], the work for which they shared the Nobel prize in 1952. The application of NMR in medicine began when Damadian in 1971 [Damadian 1971] discovered that protons (\(^{1}\text{H}\) nuclei in the water molecules) in healthy and tumor tissues have different NMR relaxation times, and two years later when Lauterbur [Lauterbur 1973] was able to obtain an image of the proton distribution (Magnetic Resonance Imaging, MRI). In 1990, Ogawa [Ogawa et al. 1990] utilized the different magnetic properties of oxyhemoglobin versus deoxyhemoglobin to develop the basis for functional magnetic resonance imaging (fMRI) and blood oxygenation level dependent contrast (BOLD).

This chapter will introduce the theoretical foundations of nuclear magnetic resonance and magnetic resonance imaging. The practical implementation of MRI is detailed, including the fundamentals of experimental MRI sequence design, and methods for contrast generation and functional MRI. In addition, detailed explanation is given
regarding the radio-frequency (RF) probe design, and strategies that are utilized for more efficient design.

1.2 Quantum mechanics approach to nuclear magnetic resonance

The relativistic quantum mechanics theory predicts an intrinsic property of the electron and nuclei known as the spin angular momentum, theory first described by Dirac [Dirac 1928]. The total angular momentum for the electron is defined as the sum of electron spin and electron orbital angular momentum. Although consisting of protons and neutrons, atomic nuclei often act as single entities with intrinsic angular momentum. It is therefore common in practice to represent total angular momentum of a nucleus by symbol \( I \) (italic letters in general indicate variables) and call it “nuclear spin”. The nuclear spin angular momentum, characterized by the spin quantum number \( I \), represents the sum of all orbital angular and spin momentums of all of the nucleons in the given state of the nuclei. It is manifested as a unique total angular momentum of the nuclei as a single entity. The most abundant nuclei in the universe, hydrogen nuclei \(^1\text{H}\) have a nuclear spin quantum number \( I=1/2 \). To exhibit the magnetic resonance behavior, nuclei must have a non-zero value of \( I \). The nuclei with spin quantum number \( I \) has magnetic moment \( \mu=gI\mu_N \), where \( g \) is constant called g-factor or nuclear factor, and is a property of a particular nucleus (for protons \( g_p=5.585694675 \)), and \( \mu_N \) is constant called nuclear magneton \( (\mu_N=5.05084\times10^{-27}\text{ J/T}) \). In general, the nuclei placed in the magnetic field, \( \mathbf{B} \) (boldface letters in general indicate vector entities), can be described by the Hamiltonian operator \( \hat{\mathbf{H}} \) (the symbol “\(^{\wedge}\)” is used to indicate an operator), \( \hat{\mathbf{H}}=-\hbar\gamma\mathbf{B}\cdot\mathbf{I}=2\mu\mathbf{B}\cdot\mathbf{I} \),
where \( \gamma \) is the gyromagnetic constant (for proton \( \gamma_p = \frac{2 \mu_p}{\hbar} \)). The solution for the Hamiltonian represents the energy of the nuclei in the magnetic field. The same nuclei without an external magnetic field will have \( 2I+1 \) so called degenerative eigen values for the Hamiltonian (meaning that the energy will be the same for different values of \( I \)).

The degeneration comes from substates characterized by the magnetic quantum number \( m \) that can take values \( m = -I, -I+1, \ldots, I-1, I, \) but these substates will not be observable unless the nuclei experience the magnetic field. Therefore the degeneration can be removed (it will be possible to observe states with different quantum number \( m \)) by interaction of the nuclei with external magnetic field. Notice that for nuclei with \( I=1/2 \), \( m \) can take only two values \( m = -1/2, +1/2 \). Once in the external magnetic field, the nuclei will have the energy \( E = -2m\mu B \), characterized now by the quantum number \( m \). Nuclei with nuclear spin \( I=1/2 \) placed in the external magnetic field, \( B_0 \), will experience an energy shift compared to the non-split levels (degenerative states). There will be an energy difference between two levels (substates) characterized by two consecutive quantum number \( m \ (m=-1/2, 1/2) \) equal to, (Eq. 1.1):

\[
\Delta E = 2\mu B_0 = \hbar \omega_L = \hbar \gamma B_0
\]

where \( B_0 \) is the magnetic field magnitude, \( B_0 = \|B_0\| \). Because of the interaction between magnetic moment and external magnetic field, nuclear spins will experience a torque and they will start precessing around the external magnetic field, with the defined frequency called the Larmor frequency, \( \omega_L \), (Eq. 1.2):
In the absence of the external magnetic field, an ensemble of the nuclear spins (magnetic
moments), is non-oriented i.e. states characterized by the magnetic quantum number \( m \) have the same energy, and therefore they are equally populated. If we put the ensemble
in the \( B_0 \), degeneracy is removed, and the energy sub-levels are split for the amount
\[ \Delta E = 2\mu \mu \mu \mu B_0. \]
The state \( m = \pm 1/2 \), also called \( \uparrow \)-state, is lower in energy, and state \( m = -1/2 \), also called \( \downarrow \)-state is higher in energy. The population of two states \( (m = \pm 1/2) \) will
be according to the Boltzmann distribution, and for the case of thermal equilibrium the
ratio of the number of nuclei in the state \( \uparrow (n_\uparrow) \) and state \( \downarrow (n_\downarrow) \) is
\[
\frac{n_\uparrow}{n_\downarrow} = e^{\frac{\Delta E}{kT}} \approx 1 + \frac{2\mu \mu \mu \mu B_0}{kT}.
\]
For room temperature and 1.0 T external magnetic, the ratio
\[
\frac{n_\uparrow - n_\downarrow}{n_\uparrow + n_\downarrow}
\]
is only \( \sim 10^{-6} \), but even though this small difference, the sample does possess
net magnetization, \( M_1 \), (Fig. 1-1), that has the direction of the external magnetic field
\( B_0 \) (i.e. longitudinal magnetization). During the precession around the \( B_0 \), nuclear spins
are not coherent, and their phases are statistically distributed, i.e. non-ordered.

1.3 Classical mechanics approach to nuclear magnetic resonance

We have mentioned that nuclear spins placed in the \( B_0 \) experience a torque. The
following differential equation can be used to describe the motion of the spin
magnetization vector \( \mathbf{M} \) (classical analog to spin angular momentum) placed in the external magnetic field, (Eq. 1.3):

\[
\frac{d\mathbf{M}}{dt} = \gamma \mathbf{M} \times \mathbf{B}.
\]  
1.3

Figure 1-1. Illustration of the spins precessing in the external magnetic field, \( B_0 \). There are more spins aligned in the direction of the external magnetic field, thus the sample possesses net magnetization along the \( B_0 \) direction, \( M_1 \).

In the case of magnetic resonance imaging (MRI) experiments \( B_0 \) is a static (time independent) magnetic field oriented along \( z \)-axis, \( B_0 = B_0 \hat{e}_z \), thus for each projection of vector \( \mathbf{M} \) along the \( x, y \) and \( z \) axes, we can write three differential equations (Eqs. 1.4):

\[
\frac{dM_x}{dt} = \gamma M_y B_0, \quad \frac{dM_y}{dt} = \gamma M_z B_0, \quad \frac{dM_z}{dt} = 0.
\]  
1.4
These are the standard differential equations, where the solution can be written in the form (Eq. 1.5, Eq. 1.6, Eq. 1.7):

\[ M_x(t) = M_x(0) \cos \omega_0 t + M_y(0) \sin \omega_0 t \]

\[ M_y(t) = -M_x(0) \sin \omega_0 t + M_y(0) \cos \omega_0 t \]

\[ M_z(t) = M_z(0) \]

where \( \omega_0 = \gamma B_0 \) was substituted, and the constants \( M_x(0) = M_x(t=0) \), \( M_y(0) = M_y(t=0) \) and \( M_z(0) = M_z(t=0) \) can be determined from the initial conditions. These are the classical equations to describe the precession of the magnetization vector, \( \mathbf{M} \), about \( z \)-axis with precession frequency, \( \omega_0 \).

We can introduce a time varying magnetic field \( \mathbf{B}_1 \equiv \mathbf{B}_1(t) \), in addition to and perpendicular to \( \mathbf{B}_0 \), oscillating with frequency \( \omega_0 \). In general a time varying electromagnetic field can be described as a combination of circularly polarized fields. A circularly polarized field may be resolved into two rotating linear components orthogonal to each other. For MRI purposes only the circularly polarized \( \mathbf{B}_1 \) field that rotates in the same direction as the precessing magnetization is of interested, and can be described as (Eq. 1.8):

\[ \mathbf{B}_1(t) = B_x \cos \omega_0 t \mathbf{x} - B_y \sin \omega_0 t \mathbf{y}, \]
where $B_1$ is the $B_1$ field magnitude, $B_1 = \|B_1\|$. With addition of the $B_1$ field, the equations that describe the precession of spin magnetization vector $\mathbf{M}$ become (Eq. 1.9, Eq. 1.10, Eq. 1.11):

\[
\frac{dM_x}{dt} = \gamma (M_y B_0 + M_z B_1 \sin \omega_0 t) \tag{1.9}
\]

\[
\frac{dM_y}{dt} = \gamma (M_z B_1 \cos \omega_0 t - M_x B_0) \tag{1.10}
\]

\[
\frac{dM_z}{dt} = \gamma (-M_x B_1 \sin \omega_0 t - M_y B_1 \cos \omega_0 t). \tag{1.11}
\]

In most MRI experiments a short pulse of magnetic $B_1$ field is applied. This is accomplished with the probe specially designed to emit the electromagnetic field having a frequency equal to $\omega_0$ (resonant frequency). The magnetic field for the purpose of MRI is called the radiofrequency (RF) field or RF pulse, since it is in the radiofrequency domain. If the duration of the RF pulse is $\tau$, then the magnetization $\mathbf{M}$ will rotate by angle $\alpha = \gamma B_1 \tau$ away from the $z$-axis. The most frequently utilized angles for MRI are $90^\circ$ and $180^\circ$ angles, called frequently $90^\circ$ or $180^\circ$ pulse. In a typical MRI experiment a $90^\circ$ pulse tips the magnetization vector from the longitudinal (along $z$-axis) into the transverse plane ($x$-$y$ plane). The magnetization in the transverse plane is detectible as MRI signal.
1.4 Relaxation mechanisms, classical approach

After the short application of the 90° RF pulse system will possess zero longitudinal magnetization (along z-axis), and will tend to reach the equilibrium state (magnetization along z-axis). Thus we can describe the evolution of magnetization along z-axis from \( M_0 \equiv M_z(0) \) to \( M_z \) or (Eq. 1.12):

\[
\frac{dM_z}{dt} = -A(M_z - M_0)
\]

The inverse of the constant \( A \), \( 1/A \) has units of time, and it is interpreted as the time required to restore initial longitudinal magnetization. In practice it is called spin-lattice or longitudinal relaxation time, \( T_1 \), since restoring the longitudinal magnetization involves exchange of energy between the spin system and its surroundings (the lattice). The equation for the evolution of longitudinal magnetization, after replacement \( C = 1/T_1 \) becomes (Eq. 1.13):

\[
\frac{dM_z}{dt} = -\frac{1}{T_1}(M_z - M_0)
\]

The spins exchange the energy between the lattice and among themselves as well. Once the magnetization is brought into the x-y plane, the components \( M_x \) and \( M_y \) will decay with the time due to spin-spin interactions. The rate at which \( M_x \) and \( M_y \) decay to zero (zero transverse magnetization) is characterized by the constant called \( T_2 \), or transverse relaxation time, or spin-spin relaxation time. The following equations describe the evolution of \( M_x \) and \( M_y \), (Eq. 1.14):

\[
\frac{dM_x}{dt} = -\frac{1}{T_2}(M_x - M_0)
\]

\[
\frac{dM_y}{dt} = -\frac{1}{T_2}(M_y - M_0)
\]
When we combine these equations with earlier set of equations, a set known as the Bloch equations can be written, (Eq. 1.15, Eq. 1.16, Eq. 1.17):

\[
\frac{dM_x}{dt} = -\frac{M_z}{T_2}, \quad \frac{dM_y}{dt} = -\frac{M_z}{T_2}. \tag{1.14}
\]

\[
\frac{dM_x}{dt} = \gamma(M_yB_0 - \frac{\omega}{\gamma})\frac{M_x}{T_2}. \tag{1.15}
\]

\[
\frac{dM_y}{dt} = \gamma M_z B_1 - \gamma M_x (B_0 - \frac{\omega}{\gamma})\frac{M_y}{T_2}. \tag{1.16}
\]

\[
\frac{dM_z}{dt} = -\gamma M_y B_1 - \frac{M_z - M_0}{T_1}. \tag{1.17}
\]

in the rotating frame of reference. The concept of rotating frame of reference is useful since it allows us to simplify the equations by substitution

\[
\frac{dM}{dt} = \gamma M \times \mathbf{B}_{\text{eff}},
\]

where \( \mathbf{B}_{\text{eff}} \) is the net magnetic field experienced by spins in the rotating frame of reference

\[
\mathbf{B}_{\text{eff}} = (B_0 - \frac{\omega}{\gamma})\mathbf{z} + B_1\mathbf{x},
\]

and \( B_1 \) is applied along the x-axis.

We can write the initial values for the components of the vector \( M \), immediately following the application of the RF pulse along x-axis, and arbitrary angle \( \alpha \), since the initial magnetization vector is along z-axis, \( \|M(0)\| = M(0) = M_0 \), (Eq. 1.18, Eq. 1.19, Eq. 1.20):

\[
M_z(0) = 0 \tag{1.18}
\]
When placed in the Bloch equations we have a set of equations that describe the evolution of magnetization vector in MRI experiments, (Eq. 1.21, Eq. 1.22, Eq. 1.23):

\[ M_y(0) = M_0 \sin \alpha \]  \hspace{1cm} 1.19

\[ M_z(0) = M_0 \cos \alpha . \]  \hspace{1cm} 1.20

When placed in the Bloch equations we have a set of equations that describe the evolution of magnetization vector in MRI experiments, (Eq. 1.21, Eq. 1.22, Eq. 1.23):

\[ M_x(t) = M_0 \sin \alpha \sin(\omega_0 t) \exp\left(-\frac{t}{T_2}\right) \]  \hspace{1cm} 1.21

\[ M_y(t) = M_0 \sin \alpha \cos(\omega_0 t) \exp\left(-\frac{t}{T_2}\right) \]  \hspace{1cm} 1.22

\[ M_z(t) = M_0 \left[1-(1-\cos \alpha) \exp\left(-\frac{t}{T_1}\right)\right] , \]  \hspace{1cm} 1.23

which is of extreme importance since its value in the \(x\)-\(y\) plane is directly proportional to the observed signal.

1.5 Relaxation mechanisms, quantum mechanics approach

As already mentioned, there are two main mechanisms that will maintain the thermal equilibrium, spin-lattice relaxation interaction and spin-spin relaxation interaction. The basis for spin-lattice relaxation interaction is electromagnetic interaction between individual magnetic moments with other neighboring magnetic moments. This interaction has the Brownian fluctuation character and therefore there will be photons that will have energy \((E = \omega_\gamma h)\) that corresponds to the Larmor frequency for the given
external magnetic field, capable of inducing the transitions between $\alpha$ and $\beta$ states in both directions. At the equilibrium, the number of transitions from higher into the lower energy state has to be equal to the number of transitions from lower to higher energy state. This process is characterized by equation $P_1 n_\alpha = P_2 n_\beta$, where $P_1$ is the probability of transition from lower to higher energy state, and $P_2$ is probability of transition from higher to lower energy state.

If we now remember that $\frac{n_\uparrow}{n_\downarrow} = e^{\frac{\Delta E}{kT}} = \frac{P_2}{P_1}$, we can conclude that the probability for spontaneous radiation transitions from a higher energy state into a lower one with emission of a photon that has energy equal to $\Delta E$ and frequency $\omega_\gamma$ is very small, since the $\Delta E$ is very small, and the mean life of the nucleon in the higher energy level is $\sim 10^{18}$ years ($\tau \sim \Delta E^3$). If we now bring photons from the outside that have energy $\Delta E$, and the correct polarization, they are going to induce radiation transitions between states $\uparrow$ and $\downarrow$, with equal number of transitions to higher (absorption of photon) and to lower (emission of photon) energy states. If both processes have equal probability $P_3$, there will be a change in the number of nuclei in the state $\uparrow (n_\uparrow)$ and state $\downarrow (n_\downarrow)$ and a new equilibrium state characterized with $\frac{n_\uparrow'}{n_\downarrow'} = \frac{P_2 + P_3}{P_1 + P_3} = e^{\frac{\Delta E}{kT'}}$, where $T'$ is the new spin temperature that is not equal to the thermal, since $\frac{n_\uparrow}{n_\downarrow} \neq \frac{n_\uparrow'}{n_\downarrow'}$. Since $\frac{P_2}{P_1} > 1$ adding the constant $P_3 = const$, will reduce this ratio and bring it closer to 1, therefore $T' > T$ and
\[
\frac{n_{↑'}}{n_{↑'}} < \frac{n_{↓'}}{n_{↓'}}, 
\]
i.e. there is an increase in the number of nuclei in the upper state and a
decrease in the number of nuclei in the lower state, and an increase in the spin
temperature in relation to the temperature of the environment.

If we now discontinue the external source of the photons, will find that the
number of spins in state \(↑\) is equal to the number of spins in \(↓\), which means that the net
magnetization has almost dropped to zero. Since the photons are longwave they act
coherently on the spins, so that after the photon field is turned off, all the spins are
precessing coherently with Larmor frequency. This means that the net magnetization
\(\mathbf{M}_2\) has just formed in the direction perpendicular to the main magnetic field \(\mathbf{B}_0\), which
is precessing around \(\mathbf{B}_0\) with \(\omega_L\), \textbf{Fig. 1-2}. Without the external photon field, the system
will spontaneously tend to transit into the initial state (i.e. the one prior to the application
of the photons). This means there will be “cooling” of the spin system at temperature
\(T'\), back to the initial temperature \(T\). There are two mechanisms that will facilitate this,
as we have already pointed. The first one is spin-lattice relaxation, and will facilitate the
restoration of initial population of spins in states \(↑\) and \(↓\), and restoration of the
longitudinal magnetization \(\mathbf{M}_1\). This process occurs with characteristic time \(T_1\), the
longitudinal or spin-lattice relaxation time. The second mechanism, which facilitates the
dephasing of the spin precession, and therefore disappearance of the transverse
magnetization \(\mathbf{M}_2\), is spin-spin relaxation, and it is due to slightly different Larmor’s
frequencies from spin to spin, and is characterized by the transverse relaxation or spin-
spin relaxation time. For each spin ensemble the transverse relaxation time is always shorter than the longitudinal relaxation time, $T_2 \leq T_1$.

---

**Figure 1-2.** Illustration of the freely precessing spins in the external magnetic field $B_0$ (A). The same spins, after the application of the $B_1$ field (B). The $B_1$ field will act coherently on the spins, and thus the sample will have the net magnetization, $M_2$, along $B_1$ direction ($90^\circ$ pulse).

The kinetics of the longitudinal relaxation is given by the pair of differential equations, (Eq. 1.24, Eq. 1.25):

$$\frac{dn_{\uparrow}}{dt} = n_{\downarrow}P_2 - n_{\uparrow}P_1\tag{1.24}$$

$$\frac{dn_{\downarrow}}{dt} = n_{\uparrow}P_1 - n_{\downarrow}P_2\tag{1.25}$$
or when combined together (Eq. 1.26):

\[
\frac{dn}{dt} = \frac{dn_{\uparrow}}{dt} - \frac{dn_{\downarrow}}{dt} = 2n_{\downarrow}P_2 - 2n_{\uparrow}P_1 = N(P_2 - P_1) - n(P_1 + P_2) = \frac{n_{\uparrow} - n_{\downarrow}}{T_i},
\]

where \( N = n_{\uparrow} + n_{\downarrow} \), \( n = n_{\uparrow} - n_{\downarrow} \) is the difference in the number of spins in the thermal equilibrium (initial state \( \uparrow \) and state \( \downarrow \)), \( n_{\pm} = N \frac{P_2 - P_1}{P_2 + P_1} = NT_i(P_2 - P_1) \) and \( T_i = \frac{1}{P_1 + P_2} \) is the longitudinal relaxation time.

The solution of the differential equation Eq. 1.26 is (Eq. 1.27):

\[
n - n_{\pm} = (n - n_{\pm})_{t=0} e^{-\frac{t}{T_i}}, \text{ where } n - n_{\pm} = n_{\uparrow}' - n_{\downarrow}' - (n_{\uparrow} - n_{\downarrow}),
\]

is the deviation from the thermal equilibrium, \( n_{\uparrow}' \) and \( n_{\downarrow}' \) are new numbers of nuclei in the state \( \uparrow (n_{\uparrow}') \) and state \( \downarrow (n_{\downarrow}') \), and \( (n - n_{\pm})_{t=0} \) is the initial deviation (after the photon field) from the thermal equilibrium.

Therefore, if \( T_i \) is large, i.e. if the probabilities for the relaxation processes \( P_1 \) and \( P_2 \) are small (weak spin-lattice interaction), populations \( n_{\uparrow} \) and \( n_{\downarrow} \) will remain unchanged very long after the initial pulse (photon field), and another pulse (additional application of the photon field) will not have any observable effect. In the solids, \( T_i \) is typically long, while for the liquids is few seconds or shorter.
1.6 Signal detection

The Faraday law of electromagnetic induction states that changing magnetic flux will induce a current in a conducting wire placed so it intercepts the magnetic flux lines. This is very useful for signal detection in MRI or nuclear magnetic resonance (NMR) since the rotating spin magnetization creates a changing magnetic flux, and we only need a conductive wire to detect changes in the induced current. The same RF coil used for excitation of the spins can be used for signal detection. In practice there is sometimes an advantage to using different coils for spin excitation and signal detection. The work presented here includes different coil designs, with each coil designed for excitation and detection. The signal detected in the RF coil will be proportional to $M_x$. The $T_2$ time is easier to observe directly than the $T_1$ time. Since the magnetization $M_x$ is precessing with frequency $\omega_L$, placed in the coil, it will induce the voltage of frequency $\omega_L$, which will decay with the time with constant $T_2$, which is the rate at which the amplitude of $M_x$ will change due to the dephasing of the spins. The dephasing of the spins occurs due to the finite range of the precession frequencies $\Delta \omega_L$. The received signal is first transformed to the rotating frame of reference by phase sensitive detection. In practice this is done by mixing the received signal separately with two reference signals, both oscillating at the Larmor frequency, but 90° out of phase with each other. The signal $S(t)$ detected in the coil has the form (Eq. 1.28):

$$S(t) = S_0 \exp\left(-\frac{t}{T_2}\right) \cos(\omega t),$$  \hspace{1cm} \text{1.28}
and after the phase sensitive detection, it can be separated into “real” $S_{\text{Re}}$ and “imaginary” $S_{\text{Im}}$ parts (Eq. 1.29, Eq. 1.30):

$$S_{\text{Re}} = S_0 \exp\left(-\frac{t}{T_2}\right)\cos(\Delta \omega_c t) \quad 1.29$$

$$S_{\text{Im}} = S_0 \exp\left(-\frac{t}{T_2}\right)\sin(\Delta \omega_c t) \quad 1.30$$

where $S_0$ is the signal amplitude at the time $t=0$.

This signal, $I(\omega)$, after the phase sensitive detection is known as free induction decay (FID) and its Fourier transform has the Lorentzian shape (Eq. 1.31):

$$I(\omega) = S_0 \frac{T_2}{1 + (\omega - \omega_c)^2 T_2^2} \quad 1.31$$

the full-width half-maximum (FMHM) is equal to $\Delta \omega_L = \frac{2}{T_2}$. The time of the transverse relaxation, therefore, determines the frequency (or energy) range within which the photons can induce transitions between spins in states $\uparrow$ and $\downarrow$ (spin-flip). In practice this time depends more on the local magnetic field inhomogeneities $B_{\text{local}}^\dagger$ (which leads to broadening of the FMHM $\Delta \omega_L$, and shortening of $T_2$) than on the spin-spin relaxation. We define the effective relaxation time, $T_2^\star$, to include both the effects of the field inhomogeneities and spin-spin interactions (Eq. 1.32):

$$\frac{1}{T_2^\star} = \frac{1}{T_2} + \gamma \|B_{\text{local}}^\dagger\| \quad 1.32$$
In solids, $T_2$ is usually orders of magnitude shorter than $T_1$, but for the liquids these times are more comparable, although $T_2$ is still significantly shorter.

1.7 RF coil design

There are many different coil designs that can be divided into two general categories: surface coils and volume coils. The surface coil design is probably the simplest one, and as the name suggests it rests on the surface of the object being imaged. It can be as simple as a loop of conducting wire with a capacitor across the ends. This loop of wire has inductance, which together with capacitance makes a resonant circuit, called the $\pi$-circuit. The resonant frequency can be adjusted by choosing different capacitor values, or using a variable capacitor. The resonant frequency of the coil is tuned to the Larmor frequency of the spins to be imaged. In practice the coil is connected to an RF amplifier, which serves to provide the driving voltage during excitation. The impedance of the amplifier is 50 $\Omega$, therefore in order to minimize the signal loss, and minimize reflected current, coil impedance is set to be 50 $\Omega$ as well. This is done with an additional capacitor (matching capacitor) placed between the voltage source and the coil, Fig. 1-3. In practice fine adjustment of the frequency (tuning) is done with the variable “tuning capacitor” $C_T$, and matching is done with the variable “matching capacitor” $C_M$, together they represent tuning and matching network, Fig. 1-3.

The main disadvantage of surface coils is inhomogeneous magnetic field, with the depth of penetration depending on the size of the coil. It is very useful for imaging areas
that lie close to the surface, as it has good signal-to-noise ratio (SNR), mainly achieved by excluding the noise from outside the region of interest. For the imaging of regions that are far from the surface, volume coils are used. In this thesis work, different types of volume coils have been used.

![Diagram of a birdcage coil with capacitors and an external magnetic field.]

**Figure 1-4.** The surface RF coil with its capacitor $C$, tuning capacitor $C_T$, matching capacitor $C_M$, and the voltage source. Capacitors $C_M$ and $C_T$, together represent tuning and matching network. The magnetic field $B_1$, generated by the surface coil is shown, as is the direction of the main magnetic field $B_0$.

The most common volume coil design is the birdcage coil, **Fig. 1-4.** Birdcage coils are widely used due to their excellent homogeneity and good SNR. A typical birdcage coil consists of two circular elements (“end rings”) and number of wires (“legs”). In order to achieve resonant modes, capacitors can be placed either on the end rings (“high pass” birdcage coil), or on the “legs” (“low pass” birdcage coil), or both (“band pass” birdcage coil), **Fig. 1-4.** The radio frequency supply to the coil is generated...
by an oscillator and amplified through the RF amplifier. The sinusoidal current
distribution through the legs will produce very a homogeneous field [Hayes et al. 1985].

Figure 1-4. The birdcage coil (shown as “high-pass” configuration) with its tuning and
matching network. The direction of magnetic field depends on the position of the voltage
source (current distribution), and it is always in the plane parallel to the end rings.

The next commonly used coil is a “slotted” tube resonator, Fig. 1-5. This coil has
simpler design than the birdcage coil. It consists of two copper plates, Fig. 1-5, arranged
to form a 90° window, connected with four capacitors, two at each end. The modification
of this coil, with two parallel plates, Fig. 1-6, has better homogeneity, but lower filling
factor for the imaging of cylindrical objects. Slotted tube resonators have very good
SNR, however, they produce a less homogeneous magnetic field than do birdcage coils.
This coil has currents flowing in opposite directions in the two copper conductors. The
magnetic field generated from currents in the opposite direction will add in the center of the coil.

Figure 1-5. The slotted tube resonator with $90^\circ$ window. The magnetic field $B_1$ is generated by the current flow $I$ (in opposite directions) in the two copper plates.

1.7.1 Coaxial cavity coil

The coaxial cavity coil [Purcell et al. 1946] consists of two coaxial cylinders Fig. 1-7, and has inherently high sensitivity. Coaxial cavities have been used for imaging previously [Vaughan 2003], including methods using the zero mode and the space between inner and outer conductors [Purcell et al. 1946]. A traditional, cylindrical
coaxial cavity coil in the zero mode is associated with a strong $B_1$ field gradient in the radial direction between the conductors as a consequence of the coaxial geometry [Woelk et al. 1994; Woelk 2000]. Strong $B_1$ field gradients lead to nonuniform signal intensity and contrast in magnetic resonance images, therefore this coil has been used mainly for spectroscopy [Woelk et al. 1994] and small sample imaging [Blaimer et al. 2001]. The improved $B_1$ field homogeneity is accomplished by replacing the cylindrical with the hexagonal geometry and additionally by replacing the continuous outer cylinder with six discontinuous plates. The hexagonal design approach is original to the thesis work.

**Figure 1-6.** The modified slotted tube resonator, with parallel plates, and with the tuning and matching network.
1.8 Image contrast in biological imaging

Signal contrast in MRI is strongly dependent upon the methods and parameters used to acquire the image. By manipulating the RF field, applying the magnetic field gradients and carefully choosing the timings, it is possible to highlight different entities of the object to be imaged. The paragraphs that follow will introduce some of the basic imaging sequences used for this thesis work, and provide the explanation of the concepts.
for the contrast formation. The basis of the contrast is the spin density throughout the object. If there are no spins (or nuclei with non-zero magnetic moment) present in the object it is not possible to acquire an MRI signal. Clinical MRI derives its signal from protons ($^1$H), and has spin densities proportional to the concentration of hydrogen atoms, primarily in water and lipid. The low density of mobile protons in the bone and short $T_2$ makes it difficult to visualize with MRI. Most soft tissues in the body have similar proton density, making the distinction between different tissues hard to notice on the proton density-weighted images. Fortunately, differences in relaxation mechanisms $T_1$, $T_2$, and $T_2^*$ provide tissue contrast, as do flow and motion.

1.8.1 Spin echo (SE) imaging sequence

The spin echo sequence [Hahn 1950; Carr et al. 1954; Meiboom et al. 1958] is a very powerful method to determine $T_2$, $T_1$ and proton density values within the imaged objects, and it was extensively used in this thesis work to determine $T_2$ values. This section is devoted to describing spin-echo sequence and how it works.

For the purpose of illustration, assume, the object of interest is placed along z-axis, and the slice of interest will be an axial slice (x-y imaging plane), Fig. 1-8, (the following explanations could be modified for any other slice orientation or object orientation).

For axial slice selection it is necessary to apply a slice selective magnetic field gradient $G_z$, along the z-axis, Fig. 1-9. In the absence of the gradient, all spin nuclei will precess with same Larmor frequency determined by the value of the external magnetic
field $B_0$. The slice selection is achieved due to the fact that the gradient field along $z$-axis will cause the nuclei along $z$-axis to precess with different Larmor frequencies. The effective magnetic field will be a sum of the static magnetic field and the applied magnetic field gradient. Because the magnetic field gradient varies linearly with the $z$-axis the effective Larmor frequency will also vary linearly with respect to $z$-axis. Only nuclei in the certain position along $z$-axis, i.e. within desired slice selection, will precess with the frequency equal to the $B_1$ field frequency (RF pulse), and can be manipulated by the RF pulse. Nuclei with a Larmor frequency outside of the bandwidth of the RF pulse will not change their orientation with respect to $B_0$. The rotation of magnetization for $90^\circ$ (90° flip angle, $\alpha$) as already mentioned is determined by the strength and duration of the RF pulse $\tau$: $\alpha = \gamma B_1 \tau$.

![Diagram](image)

**Figure 1-8.** Object to be imaged with selected axial slice, positioned within a Cartesian coordinate system.

After the application of the 90° RF pulse the magnetization is tilted into the $x$-$y$ plane and it will precess with the Larmor frequency. Due to the spin-spin interactions and local magnetic field inhomogeneities, the coherent nuclear spins will start dephasing, which
will decay the magnitude of the \( x-y \) magnetization. In order to eliminate the effect of the local magnetic field inhomogeneities on the magnetization magnitude decay, we can apply a 180° RF pulse, aimed toward reversing the spin dephasing, so the maximal signal can be observed (acquisition) at the time equal two times the time between the 90° and

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**Figure 1-9.** Illustration of the nuclear spins in the rotating frame during a spin-echo experiment.

1) Application of the 90° RF-pulse. The pulse rotates nuclear spins form \( z \)-axis into the \( x-y \) plane.

2) The spins begin to precess, dephase, and accumulate the phase.

3) Application of the 180° RF-pulse. This is equivalent to the transformation \( \phi \rightarrow -\phi \), i.e. the phase reversal occurs, and spin begin to rephase.

4) At this time all the spins are back to the initial position in \( x-y \) plane, forming an echo.
180° pulses. Gradient along $y$-axis is applied between 90° and signal acquisition (echo formation) in order to encode the phase information of the nuclei along $y$-axis. Notice that the phase-encoding gradient, $G_y$, can be placed either before or after 180° pulse. One of the advantages for placing the phase-encoding gradient after 180° pulse is to minimize motion artifacts since that way the phase encoding takes place closer to the sampling of the data. In order to encode the frequency information, additional gradient along $x$-axis is applied at the time of acquisition. The time between 90° and echo is traditionally called echo time (TE). After the 180° pulse, the maximal signal will be observed at $t=TE$. The gradient $G_z$, (Fig. 1-10), has negative one half of the area of the frequency-encoding pulse in order to ensure that nuclear spins are not dephased due to the presence of $G_z$. The negative part of the $G_z$ gradient serves to eliminate dephasing due to different frequencies in the slice because of different positions along the $z$-axis during excitation. In order to reconstruct a 2D image of the object of interest, the spin-echo sequence must be repeated with incremental increase in the amplitude of the phase encoding gradient amplitude. For standard spin-echo imaging sequence, the number of phase encoding increments is equal to the phase encoding dimension of the image matrix.
1.8.2 $T_2$ Contrast

The loss of signal due to spin-spin relaxation will not be refocused in the spin echo. Therefore, tissues with longer $T_2$ values (slower spin-spin relaxation) will have more signal than the tissues with shorter $T_2$ values, forming the basis for the $T_2$ contrast. Images created with certain repetition time (TR) and TE time aimed to enhance $T_2$ contrast are referred to as $T_2$-weighted images. The $T_2$-weighting of the signal intensity depends on the time between excitation and acquisition (TE time). With longer...
TE time more signal will be lost due to spin-spin relaxation in nuclei with rapid $T_2$ relaxation, thereby accumulating the difference between the tissues with shorter vs. longer $T_2$ time. From the acquired signal using Bloch equations (Eq. 1.21 and Eq. 1.22) it is possible to calculate the $T_2$ values. Typical curves of the transverse magnetization ($M_x$) recovery following 90°-180° pulses, for two tissues with different $T_2$ times are shown in Fig. 1-11. In practice, $T_2$ maps can be calculated from images acquired with different TE times, and by fitting an exponential decay.

1.8.2.1 Application of $T_2$-weighted imaging to ischemic injury

Free water has a very long $T_2$ time (usually a few seconds) and consequently appears bright on $T_2$-weighted images. This can be advantageous in discriminating the water content of the tissue. A few hours following ischemia there is an increase in tissue water content (vasogenic edema) in the areas undergoing ischemia, due to compromised cell membrane integrity. Hours and days following ischemia, the increase in tissue water content was found to correlate well with an increase in $T_2$ relaxation times [Kato et al. 1986]. In the early stage of cerebral ischemia occurring before there is a change in tissue water content, there will be little change observed on $T_2$-weighted images. Therefore $T_2$-weighted images are relatively insensitive for early detection of ischemic stroke. However, the applications of $T_2$-weighted imaging are very valuable for evaluation of the extent of vasogenic edema in follow up studies. Elevated $T_2$ values days after a stroke are indication of gliosis, and highly elevated $T_2$ values are indication that a cystic encephalomalacia is formed in place of brain tissue. Since elevated $T_2$ values indicate the
onset of cell death, increased signal intensity on T2-weighted images between 24-48 h following hypoxia-ischemia is usually non-reversible. In addition it has been demonstrated that the size of the infarct does not increase between 24 and 72 hours [Quast et al. 1993], so that the volume of increased signal intensity at 24 h can be considered the final lesion size.

For animal models of ischemia, it has been demonstrated that the area encompassing increased $T_2$ values (hyperintense signal) matches the infarcted areas determined by histological and enzymatic techniques, such as staining with cresyl violet, hematoxylin-eosin, and 2,3,5-triphenyltetrazolium chloride [Allegrini et al. 1992; Quast et al. 1993; Gill et al. 1995] at 1 day to 3 months after occlusion.

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**Figure 1-11.** Transverse magnetization recovery for spins with different values of $T_2$, following $180^\circ$ pulse. Two different curves shown, represent the tissue with two different $T_2$ values, where a tissue represented with a dotted line has longer $T_2$ values.
1.8.3 Gradient echo imaging sequence

The gradient echo sequence [Haase et al. 1986] is one example where imaging time is reduced compared to the spin echo sequence, and the refocusing 180° pulse is removed leaving only the gradient echo. The spin refocusing in the gradient echo sequence occurs due to the presence of the read gradient, $G_x$, Fig. 1-12, in the position where 180° pulse used to be in spin-echo sequence. The gradient echo is accomplished by two rapid and sequential gradients. The first serves to dephase the spins, and the second to rephase them forming a “gradient-echo” Fig. 1-12. During the time between the initial 90° pulse and application of two gradients there will be a signal loss associated with spin-lattice relaxation time, a small signal loss due to spin-spin relaxation time and additional signal loss due to local field inhomogeneities. The contribution from magnetic field inhomogeneities together with spin-spin relaxation is characterized by $T_2^*$ time, establishing the basis for $T_2^*$ contrast.
1.8.4 $T_2^*$ Contrast

In the gradient echo sequence, there is no $180^\circ$ refocusing pulse. Therefore, signal from neighboring spins that have the same $T_2$ values may decay at different rates depending on local magnetic field inhomogeneities, i.e. the dephasing effects due to local field inhomogeneities are not reversed. As a consequence, signal decays exponentially with increasing TE time with a decay constant $T_2^*$ rather than $T_2$, and therefore the images are $T_2^*$-weighted rather than $T_2$-weighted. Gradient echo images with a long TE time usually show reduced signal in the areas where local magnetic field inhomogeneities...
are present. This can be valuable for visualizing the brain iron, since the iron is ferromagnetic, and iron deposits will cause local magnetic field inhomogeneities and consequently appear darker on the $T_2^*$-weighted images than the rest of the brain. Following brain trauma and hemorrhage, the hemosiderin deposits can be visualized on $T_2^*$-weighted images. One disadvantage of gradient echo imaging with long TE time is the “susceptibility artifact”, the artifactual signal loss due to local magnetic field variations near tissue-air interfaces (sinus cavities).

The Blood Oxygenation Level Dependent (BOLD) contrast used for fMRI is based on $T_2^*$-weighted imaging. The goal of functional imaging is to detect small changes associated with neuronal activation. In the case of BOLD imaging small local changes in $T_2^*$ values can be observed as a consequence of changes in blood oxygenation levels following the neuronal activity. The BOLD contrast and fMRI will be discussed in more detail in section 1.9.

In order to accomplish rapid acquisition and good $T_2^*$ contrast, echo planar imaging sequences are often employed in practice for fMRI rather than conventional gradient echo.

### 1.8.5 Echo planar imaging (EPI)

The echo-planar imaging (EPI) method is a rapid acquisition technique capable of collecting all data lines for a complete image following a single RF excitation pulse [Mansfield 1977]. The main advantages of fast data acquisition are minimized motion artifacts and capability of tracking rapid changes that occur during neuronal activation.
which are the basis for fMRI. In order to minimize the acquisition time, TR and TE are shortened as much as possible. In order to further reduce the imaging time, instead of repeating the frequency encoding step during the different phase gradients, a combination of rapid switching of both read and phase-encoding gradients is employed, Fig. 1-13. Since a single excitation RF pulse is used, the signal losses associated with reduced TR time are eliminated. The sequence is characterized by application of phase-encode gradient, $G_y$, with a different strength after each read gradient, $G_x$, enabling us to cover all phase-encode steps after a single excitation pulse, Fig. 1-13. With our 3 T magnet echo planar images can be acquired in a little as 50 ms, with 33 image slices per second.

\[ S = S_0 e^{-\frac{j}{T_2}} \]

**Figure 1-13.** Illustration of the echo planar imaging sequence.
1.8.6 $T_1$ Contrast

As mentioned earlier, spin-lattice relaxation time, $T_1$, is the time required to restore the longitudinal magnetization. The simplest way to obtain $T_1$-weighted images is to apply two $90^\circ$ RF pulses within the period of $T_1$ time. The first RF pulse is applied in order to saturate the longitudinal magnetization (reduce longitudinal magnetization to zero). After the applied pulse, spins that have shorter $T_1$ time will recover longitudinal magnetization more rapidly. If the second pulse is applied within a TR time shorter than $T_1$ time for the imaging object, the longitudinal magnetization will not be able to fully recover. The acquired signal will reflect $T_1$ differences in tissues because of different amounts of longitudinal recovery for different tissues during the TR period. The tissue that has shorter $T_1$ time will appear brighter on the final image (more signal recovered), than the tissue with the longer $T_1$ time. Therefore, water, having a very long $T_1$ time, appears dark on $T_1$-weighted images. In practice, if $T_1$ maps are required, it is more convenient to excite the longitudinal magnetization by applying a $180^\circ$ pulse (inversion) prior to the second RF pulse ($90^\circ$). The $180^\circ$ pulse will invert the longitudinal magnetization, and more importantly, it will not produce any transverse magnetization (this will avoid $T_2$ relaxation effects). We can write the Bloch equation, which will describe the recovery of longitudinal magnetization.

For $\alpha = 180^\circ$ ($\cos(180^\circ) = -1$) we have (Eq. 1.33):

$$M_z(t) = M_0[1 - (1 - \cos \alpha)\exp\left(-\frac{t}{T_1}\right)] = M_0[1 - 2\exp\left(-\frac{t}{T_1}\right)], \quad 1.33$$
and we can see that signal proportional to magnetization will entirely depend on $T_1$. The time between 180° and 90° pulse is called inversion recovery time (TI), **Fig. 1-14**.

![Inversion recovery experiment to obtain $T_1$ values.](image)

**Figure 1-14.** Inversion recovery experiment to obtain $T_1$ values.

### 1.8.7 Diffusion-weighted spin-echo sequence

Diffusion refers to the random motion of the molecules, resulting from the Brownian motion. In the diffusion-weighted image, the degree of the motion (random diffusion) of the nuclear spins is used to produce contrast. The effects of diffusion can be highlighted by incorporating two “diffusion” gradients into the described spin-echo sequence [Stejskal et al. 1965]. The illustration of the diffusion-weighted spin echo sequence with diffusion gradients along $x$-axis is given in **Fig. 1-15**. In practice the direction of the diffusion gradients can be chosen to emphasize direction of diffusion or
absence of diffusion. Stationary spins will undergo dephasing during the initial application of the diffusion gradient before the 180° pulse, and rephasing afterwards, during the application of the second diffusion gradient. The stationary spins, following the second gradient will end up in phase at the time of acquisition. Spins that participate in molecular diffusion, during and after initial diffusion gradient and dephasing, will randomly move into regions of different gradient strengths, resulting in incomplete rephasing at the time of acquisition and signal attenuation. The received spin-echo signal is now attenuated due to the diffusion by a factor (Eq. 1.34):

\[ A = e^{-bD} \]  \hspace{1cm} 1.34

where D is the diffusion coefficient, and b factor (called b-value) has units of seconds per square meter and represents the sensitivity of the sequence to motion [Neeman et al. 1991; Mattiello et al. 1994]. For the sequence shown in Fig. 1-15, b-value is related to the applied diffusion gradient strength \( G_D \), duration of the diffusion gradients \( \delta \) and time interval \( \Delta \) between leading edges of the diffusion gradients, and can be calculated from the Eq. 1.35:

\[ b = \gamma^2 \delta^2 G_D^2 (\Delta - \frac{1}{3} \delta) \]  \hspace{1cm} 1.35

where \( \gamma \) is the gyromagnetic ratio.
1.8.7.1 Application of diffusion-weighted imaging to ischemic injury

The imaging sequence described in Fig. 1-15, was initially applied to biological systems in order to study the effects of biological membranes on water motion. The observation of signal hyperintensity in ischemic regions 45 minutes following ischemia [Moseley et al. 1990] without apparent signal increase on T2-weighted images, laid the foundation for application of diffusion-weighted imaging for detection of early ischemia. The observed increased signal of the ischemic tissue results from the water protons that have migrated from a more diffusible extracellular space into a more diffusion-restricted intracellular compartment. This migration of water protons reflects the cellular swelling (cytotoxic edema), one of the earliest events following hypoxia-ischemia associated with failure of Na⁺/K⁺ ATPase, as opposed to vasogenic edema which often develops hours or days following ischemic injury [Weinstein et al. 1986]. In the areas of ischemic stroke, an increase in intracellular water and a decrease in extracellular water are observed as hyperintense signal on diffusion-weighted images. The diffusion-associated changes can be visualized a few minutes after the onset of ischemia, making this sequence valuable for clinical application. It has been established [Marks et al. 1996] that increased signal intensity on diffusion weighted images is reversible and the affected tissue may recover, depending on many factor yet to be resolved.

Diffusion-weighted imaging sequences, very sensitive to molecular motion and diffusion, are highly sensitive to any other motion as well (gross, cardiac, or respiratory in the case of in vivo imaging), or other mechanisms where small movements occur.
Therefore, as a part of this thesis work considerable effort has been made toward the development of a neonatal animal holder for minimizing motion artifacts.

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Figure 1-15. Illustration of the diffusion-weighted spin-echo pulse sequence.
1.9 Functional Magnetic Resonance Imaging (fMRI)

1.9.1 Metabolism, blood flow and basis for the blood oxygenation level dependent (BOLD) contrast

The brain, as any other organ in the body, requires energy in order to maintain its function. In biological systems, energy is primarily stored in the three phosphorylated forms of adenosine: adenosine triphosphate (ATP), adenosine diphosphate (ADP), and adenosine monophosphate (AMP). The conversion of ATP to ADP involves release of energy. In order to meet high-energy demands of the brain, the oxidation of glucose is necessary to provide sufficient ATP. The oxygen consumption by the brain is 20% of the total body consumption, which is very high, considering the brain weighs about 2% of the total body weight. Therefore, the brain relies heavily on the oxygen supplied by the blood. Since oxygen is not soluble in the blood, in order to be transported to the different organs it is bound to the hemoglobin. The hemoglobin molecule consists of four polypeptide chains called “globin”, and four heme groups containing single Fe$^{2+}$ ion in the center. When the oxygen molecule is bound to the hemoglobin it is called oxyhemoglobin, and without bound oxygen it is called deoxyhemoglobin. Due to the iron ions, deoxyhemoglobin has paramagnetic properties. Binding the oxygen, hemoglobin looses its paramagnetic properties and becomes diamagnetic. The paramagnetic properties of deoxyhemoglobin will shorten T$_2^*$ in the blood vessel. This is due to local magnetic field inhomogeneities, caused by the susceptibility difference of deoxyhemoglobin and surrounding tissue. The diamagnetic oxyhemoglobin has similar magnetic properties as the surrounding tissue, and will not cause shortening of T$_2^*$. 
Measuring T$_2$ can monitor the changes of oxygen level in the blood*. This is the basis for the blood oxygen level dependent (BOLD) contrast used for functional magnetic resonance imaging (fMRI) [Ogawa et al. 1990; Ogawa et al. 1992].

It would be expected that neuronal activation followed by an increase in oxygen consumption will lead to an increase in deoxyhemoglobin and signal decrease. The opposite is observed, however, i.e. an increase in signal in the areas of activation, implying a decrease in deoxyhemoglobin. This is mainly due to small initial increase in deoxyhemoglobin, followed by a larger increase in cerebral blood flow and oxyhemoglobin to the areas of neuronal activation. The bulk effect upon neuronal activation is therefore increase in signal. The support of these mechanisms comes from positron emission tomography (PET), and near-infrared spectroscopy. Near-infrared spectroscopy studies have shown an increase in oxyhemoglobin and a decrease in deoxyhemoglobin upon activation. An increase in the total hemoglobin has been observed as well, reflecting an increase in blood volume following the activation [Villringer et al. 1993]. PET studies demonstrated no increase in oxygen consumption (reflecting no increase in deoxyhemoglobin) by the activated areas [Fox et al. 1988]. An initial small signal decrease “initial dip” has been observed in the areas of activation, perhaps reflecting a transient increase in deoxyhemoglobin. The origin of initial dip is not well understood, and the presence of the dip is controversial. The onset of the increased signal is delayed a few seconds following a neuronal activation, reflecting a “hemodynamic” autoregulation (response to stimulus).
1.9.2 Paradigm design

In order to correlate a certain stimulus to certain areas of the brain it is necessary to image the brain while the subject is presented with the stimulus or is performing a task (“on” period), and while the subject is at rest (“off” period). The paradigm design, together with the sequence used and data analysis is most important part of the fMRI. The stimulus design has to be carefully tailored in order to elicit the strongest and meaningful results. The duration of the stimulus has to be long enough in order to accommodate the hemodynamic response, i.e. at least 8 seconds. The most common paradigm design is repetition of “on” and “off” periods. The more repetitions performed the better the correlation will be among the activation during the “on” period vs. no-activation during the “off” period. A compromise has to be made between the level of statistical significance and duration of the experiment (more repetition will take longer time, but the statistical significance will be easier to reach). With more repetition, it will be easier to distinguish between the “false positive” activation not connected to the performed task or stimulus. Ideally a stimulus should be chosen so that there is only one well defined difference between the “on” and the “off” period.

For the sequence design in fMRI it is important to be $T_2^*$ weighted, since $T_2^*$ reflects the blood oxygen level. Gradient echo or echo planar imaging (EPI) can be used for this purpose. EPI is faster, less sensitive to the motion artifacts and allows for detection of response to short stimuli. In this thesis research, echo planar imaging (EPI) was used to acquire fMRI data. Some of the problems due to subject movement can be
resolved by restraining the head of the subject and by using a post-processing image co-registration algorithm.

1.9.3 Data processing

The images acquired during the designed paradigm must be analyzed in order to find statistically significant signal increase in the areas of activation, and to exclude the possibility of signal increase purely by chance. The simplest way to analyze data is to subtract the mean “off” images from the mean “on” images on pixel by pixel basis, and to perform the t-test (or non-parametric statistical analysis) on a pixel by pixel basis, to determine the significant difference in the signal intensity of the subtracted image. The pixels that have large variability due to the small movement artifacts can be eliminated by statistical tests since, for example, the t-test will reject the pixels with high variability. An image where each pixel is assigned a value based on the statistical test output is called a statistical parametric map.

Another common method to analyze functional data is correlation coefficient mapping. The time course of the response is predicted based on the designed paradigm and assumed hemodynamic response and it represents the reference function. The correlation coefficient is calculated for the signal intensity changes of each pixel with the time and the predicted reference function. In the case of good correlation (correlation coefficient \( r \) is between 0.5 and 0.7), the pixel is said to be activated in response to the applied stimulus.
1.10 The overall thesis concept

The work consists of three main parts. Each part deals with a different problem, where magnetic resonance imaging of the brain is common for all three. Part I (Chapters 2 and 3) describes the design and implementation of a novel RF coil for imaging of six small animals simultaneously. The power of magnetic resonance imaging and the application of the multi-animal probe are combined to evaluate pharmacological interventions intended to provide neuroprotection following neonatal hypoxia-ischemia. Part II (Chapters 4 and 5) investigates the role of diminished interleukin one receptor type one signaling in neuroprotection following adult hypoxia-ischemia in mice. Part III (Chapter 6) utilizes the combination of functional magnetic resonance imaging and c-Fos expression to gain insight into which brain areas are activated in response to visceral pain in an adult rat model.

Encompassing the common theme of the entire thesis, magnetic resonance imaging, the overall hypothesis is:

*Magnetic resonance imaging will facilitate acquisition of novel information regarding brain’s pathology and function.*

Part I, short summary

Perinatal hypoxia-ischemia is the leading cause of morbidity and mortality in infants and children. The FDA has not approved any treatment following perinatal H/I
because experimental pharmacological treatments shown to significantly reduce infarction volume in animal models usually fail to provide the same benefits when applied to humans. The conclusion of typical animal trials is based on statistical evidence that the treated group had a smaller infarct volume than the control group, without assessment of initial infarct volume (early cytotoxic edema) prior to the application of a therapeutic agent. In order to more effectively evaluate the efficacy of potential neuroprotective agents we propose to design and manufacture an RF-probe capable of imaging six small animals simultaneously. The proposed RF-probe will facilitate imaging of three treated and three control animals under almost identical conditions. We further propose to non-invasively determine the volume of cytotoxic edema prior to administration of the therapeutic agent with MRI, and to compare to the final infarction volume, determined by MRI as well. This procedure allows for more direct evaluation of drug effectiveness by allowing longitudinal studies in each animal. Major processes that lead to neuronal cell death in the post H/I period include calcium influx, glutamate mediated excitotoxicity, free radical generation and inflammation. Extant data suggest that blocking these neuronal death processes will potentially provide a therapeutic intervention. Given that cell death occurs over a period of time, there is an opportunity for treatment during some time period after the H/I insult.

The following specific aims and hypotheses are proposed:

Specific Aim #1. To design, manufacture and test the multi-animal probe intended for simultaneous imaging of six small animals. The following hypothesis will be tested:
The hexagonal RF-coil design based on the coaxial cavity will provide homogeneous magnetic field and uncompromised signal-to-noise ratio required for microimaging and facilitating longitudinal studies in several animals.

Specific Aim #2. To compare the volumes of hypoxic-ischemic damage prior to and after administration of the specific iNOS inhibitor, aminoguanidine, in the neonatal rat. The following hypothesis will be tested:

Repetitive administration of aminoguanidine following H/I will lead to smaller infarct volume ($T_2$-weighted hyperintensity) compared to the volume of cytotoxic edema (diffusion-weighted hyperintensity) determined in vivo prior to its administration.

Specific Aim #3. To compare the volumes of hypoxic-ischemic damage prior to and after administration of minocycline (p38 kinase inhibitor, Ca$^{2+}$ chelator, inhibitor of IL-1β converting enzyme, caspase 3 and iNOS) in the neonatal rat. The following hypothesis will be tested:

Repetitive administration of minocycline following H/I will lead to smaller infarct volume compared to the volume of cytotoxic edema determined in vivo prior to its administration.

Summary of results

The hexagonal coil design utilizes a single receiver and allows for simultaneous imaging of 6 small animals with no great compromise in SNR. Both experimental results and the analytical calculations show a variation in $B_1$ field within the imaging region of less than 10% compared to 45% of the coaxial cavity coil design.
Contrary to our expectation and previous studies, pups treated with minocycline or aminoguanidine did not have a significantly greater reduction in the infarct volume than the saline or phosphate buffered saline (PBS) treated animals. Pups that received minocycline treatment had 30% (mean±standard deviation) reduction in the infarct volume compared to 26% with saline, 25% with PBS and 25% with aminoguanidine. When the initial infarction volumes were separated into the two groups: severe (50% or more of the ipsilateral hemisphere having abnormal signal intensity on diffusion weighted-images) vs. moderate-mild (less than 50% of the ipsilateral hemisphere having abnormal DWI-signal), there was still no statistical significance between the treatment groups. Pups that had mild-moderate initial infarct volume had no statistically different reduction in the final infarct volume than the pups with severe initial infarction. This was independent of treatment.

Part II, short summary

Members of the IL-1 cytokine family are potent mediators of inflammation that are rapidly induced and secreted in response to insults such as cerebral ischemia, head injury, CNS infections, and seizures. Their levels are elevated in neurodegenerative diseases such as Alzheimer’s disease and Parkinson’s disease. IL-1 ligands can cause neurodegeneration by activating microglial cells that can produce free radicals such as NO as well as glutamate, which can activate NMDA receptors resulting in microglia-induced cytotoxicity. Two types of IL-1 receptors have been identified. The IL-1 type I
receptor (IL-1R1) is believed to be responsible for intracellular signaling, whereas the IL-1 type II receptor (IL-1RII) binds IL-1 but is incapable of signal transduction.

The following specific aims and hypotheses are proposed:

Specific Aim #1. To determine the size of hypoxic-ischemic damage in IL-1R1 null and wild type mice subjected to mild hypoxia ischemia at various time points (30 min, 24 h, 48 h, 7 days, 1 month and 2 months post H/I). The following hypothesis will be tested:

*Mice lacking the IL-1R1 receptor will have smaller volume of hypoxic-ischemic damage compared to wild type mice, due to abrogated IL-1R1 signaling.*

Specific Aim #2. To determine levels of iNOS and eNOS mRNA and iNOS protein levels in IL-1R1 null and wild type mice following hypoxia-ischemia. The following hypothesis will be tested:

*Mice lacking the IL-1R1 receptor will have lower levels of iNOS mRNA, lower protein levels of iNOS following H/I due to abrogated IL-1R1 signaling, and unchanged levels of eNOS mRNA.*

Specific Aim #3. To determine levels of proinflammatory chemokines and cytokines in IL-1R1 null and wild type mice following hypoxia-ischemia. The following hypothesis will be tested:

*Mice lacking the IL-1R1 receptor will have lower levels proinflammatory chemokines and cytokines following H/I, due to abrogated IL-1R1 signaling.*
Specific Aim #4. To determine if sensory motor function is preserved in IL-1R1 null and wild type mice following hypoxia-ischemia. The following hypothesis will be tested:

*Mice lacking the IL-1R1 receptor will have preserved sensory motor function following H/I, due to abrogated IL-1R1 signaling.*

**Summary of results**

The IL-1R1 null mice exhibited about 45% (p<0.05) reduced total infarct volume as well as significantly reduced cortical infarct volume (p<0.01) at 24 h post HI when compared to the wt mice. Longitudinal studies demonstrated that T₂ values in wt mice continued to increase with time (in 6 out of 11 damaged animals), while T₂ values in the null mice returned to nearly normal after 2 months (in 8 out of 8 damaged animals). The induction of multiple pro-inflammatory cytokines and chemokines was significantly lower in the ipsilateral hemisphere of the IL-1R1 null mice than in wild-type mice at 72 h following H/I. Levels of iNOS protein and iNOS mRNA were significantly lower in the IL-1R1 null mice, and levels of eNOS mRNA were not different from those in wild type mice. The sensory motor function of IL-1R1 null mice was normal one month after H/I.

**Part III, short summary**

One of the syndromes characterized by visceral pain is irritable bowel syndrome (IBS). It is a common gastrointestinal disorder characterized by abdominal pain and
alteration in bowel movement. It is estimated that about 10-22% of adults, mostly women, suffer from IBS. Despite this predominance little is known about its pathogenesis. Previous studies in humans (using PET and fMRI) suggest alteration in central nervous system (CNS) processing of noxious rectal stimuli. Currently, there is no accepted animal model mostly because the cardinal feature of this syndrome is abdominal pain. The lack of an appropriate animal model has limited the study of the pathophysiology of IBS and possibilities for new pharmacological approaches to the treatment of IBS. Widely used c-Fos protein expression has helped identify many brain structures involved in visceral pain processing in animals, but due to its invasiveness it cannot be applied to humans. Non-invasive methods such as fMRI and PET are available to study brain activation in humans. It has not been yet determined if the activation detected by c-Fos protein expression corresponds to the activation detected by fMRI, which is a prerequisite to correlate animal models of IBS based on c-Fos expression with fMRI findings in humans with IBS.

The following specific aim and hypothesis are proposed:

Specific Aim 1#. To determine brain activation in response to rectal balloon stimulation in adult anesthetized rats using c-Fos expression and fMRI. The following hypothesis will be tested:

Both c-Fos expression and fMRI will reveal activation of similar brain areas in response to visceral pain.
Summary of results

No fMRI activation in the brain was observed when 40 mm Hg balloon pressure was used. A similar pattern of activation was present in the brain during 60 and 80 mm Hg of balloon pressure, with the magnitude of the response being greater at the higher pressure. The number of activated pixels at 60 mm Hg, however, was not statistically different from number of activated pixels at 80 mm Hg. All rats undergoing colorectal distention (CRD) with 60 and 80 mm Hg pressure exhibited activation in the amygdala and paraventricular nucleus of hypothalamus (PVN). Additionally, in 4 rats treated with 60 mmHg CRD and 8 of the 9 rats treated with CRD at 80 mm Hg pressure, activation occurred in the hippocampus, cerebellum and trigeminal nerve. Thalamus was activated in 3 of 4 rats treated with 60 mm Hg and 7 of 9 rats treated with 80 mm Hg. In a smaller number of animals (4-5 of 9 animals), the caudate, putamen, periaqueductal grey, and the insular, retrosplenial, entorhinal, perirhinal and sensory association cortices were active. The parabrachial nucleus was activated only in 3 out of 9 animals.

Neurons positive for c-Fos were counted in the amygdala, paraventricular nucleus of thalamus (PVP), pontine parabrachial nuclei (PBN), the paraventricular nucleus of hypothalamus (PVN) and the nucleus of the solitary tract (NTS), based on known CNS visceral afferent projections and on our own fMRI data. Statistically significant differences in the c-Fos expression between control and experimental rats were observed in the parabrachial nuclei (PBN) 83±6.9 vs. 123.5±9.1 (p=0.05) and in the paraventricular nucleus of hypothalamus (PVN) 513.22 ±127.3 vs. 917.66 ± 148.0 (p=0.05), control versus experimental, respectively. In the other brain areas that had shown activation in the fMRI images, such the amygdala, thalamus, and NTS, c-Fos activation was not
significantly different between experimental and control rats. In the amygdala, intense activation was seen in both control and experimental animals. Similar activation was also seen in both control and experimental rats in the thalamic paraventricular nucleus (PVP), supraoptic nucleus, piriform cortex, cortical amygdaloid area, insular cortex, habenula, and the thalamic mediodorsal nucleus.

1.11 REFERENCES


Chapter 2

Hexagonal Zero Mode TEM Coil: A Single Channel Coil Design for Multiple Small Animal Imaging

ABSTRACT

A novel hexagonal coil design for simultaneous imaging of multiple small animals is presented. The design is based on a coaxial cavity, and utilizes the magnetic field formed between two coaxial conductors with hexagonal cross-sections. An analytical solution describing the $B_1$ field between conductors of the hexagonal coil is found from the Biot-Savart law. Both experimental results and the analytical calculations show a variation in $B_1$ field magnitude within the imaging region of less than 10%. Numerical calculations predict ~35% improvement in $B_1$ field homogeneity with the hexagonal coil design compared to a cylindrical coaxial cavity design. Experimentally measured signal-to-noise ratio (SNR) of the hexagonal coil loaded with six phantoms was only 4-5% lower than SNR of a single parallel plate resonator loaded with 1 phantom. In vivo spin echo images of six 7-days old rat pups acquired simultaneously demonstrate uncompromised SNR required for microimaging. The construction scheme of the coil, simple methods for tuning and matching, and an anesthesia device and animal holder designed for the coil are described. The hexagonal coil design utilizes a single receiver and allows for simultaneous imaging of 6 small animals with no significant compromise in SNR.
2.1 INTRODUCTION

There is a growing interest in imaging multiple animals simultaneously to increase throughput and significantly reduce imaging time. Multiple animal imaging can particularly reduce total imaging time during the kinetics studies that require repetitive experiments and include multiple animals. Fitting multiple animals into a single volume coil can require averaging in order to provide sufficient SNR for the voxel size required for micro-imaging [Xu et al. 2003]. Previous designs for multiple animal imaging have been based on separate electrically isolated radiofrequency (RF) coils and use of separate receivers [Bock et al. 2001; Bock et al. 2003]. The proximity of multiple RF coils and separate receivers inevitably leads to complicated couplings and image artifacts [Bock et al. 2001; Bock et al. 2003]. The goal here was to develop a single-channel coil capable of multiple animal imaging with good SNR. We chose a design based on a coaxial geometry [Purcell et al. 1946; Vaughan 2003] since the coaxial cavity coil has inherently high sensitivity. Coaxial cavities have been used for imaging previously [Vaughan 2003], including methods using the zero mode and the space between inner and outer conductors [Purcell et al. 1946]. A traditional, cylindrical coaxial cavity coil in the zero mode is associated with a strong $B_1$ field gradient in the radial direction between the conductors as a consequence of the coaxial geometry [Woelk et al. 1994; Woelk 2000]. Strong $B_1$ field gradients are not desirable since they lead to nonuniform signal intensity and contrast in magnetic resonance images. Ideally, a homogeneous magnetic field is produced between two parallel, infinite planes having current flow in opposite directions. Pairs of parallel plates can be connected, and connecting the first and the last pair
together they can be conformed into a coaxial structure. This conformation into a coaxial structure allows the pairs of parallel plates to behave as a single entity resonant cavity with a unique mode of resonance. The magnetic flux is preserved within the cavity and it is mutually shared among different pairs of parallel plates. The improved $B_1$ field homogeneity is accomplished by replacing the cylindrical with the hexagonal geometry and additionally by replacing the continuous outer cylinder with six discontinuous plates.

The hexagonal coil design is intended for $in$ vivo animal studies, and for that purpose we developed an anesthesia manifold to be used with the coil for equal distribution of anesthetics. For example, imaging with the hexagonal coil allows 3 control and 3 treated rat pups to undergo identical experimental conditions. An illustration of an animal holder that enables easy positioning and minimizes animal motion during acquisition is included. While the coil size employed here precludes imaging of rat pups older than 9 days, the proposed design can be utilized for construction of larger coils that will accommodate larger animals.

2.2 METHODS

2.2.1 The Hexagonal Coil

The main structural features of the hexagonal coil are shown in Fig. 1. The coil consists of 6 pairs of parallel copper plates (4.6 X 2 cm, 2.3 cm distance between two plates) conformed into a hexagonal coaxial cavity, Fig. 2-1. A hexagonal coil former was obtained by cutting 7 hexagonal pieces from 6.57 mm delrin sheet and stacking them
A 100 Watt computer-guided laser, Universal Laser System, Scottsdale AZ, was used to cut the delrin former. Copper foil (0.04 mm thickness) was cut and attached to the delrin former. The inner conductor is made of a continuous copper sheet, while the outer conductor is made of separate plates connected with wires at each end forming a complete end ring. Each inner plate is connected to the corresponding outer plate with 4 capacitors at the four corners, making the total number of capacitors 24 for the 6 pairs of copper plates. Capacitor values (~24 pF) were chosen so that the coil could be tuned to resonate at 125 MHz. The tuning and matching network (52H02 Johanson capacitors, 1.5-10 pF) can be placed across any of the 24 capacitors. The space between each pair of the parallel plates is then an imaging cell with a high filling factor. All six coils are inductively coupled and in the cyclotron mode of resonance (m=0) the B1 field is

**Figure 2-1.** Schematic drawing of the hexagonal coil discussed in the paper. Tuning and matching circuit can be placed across any capacitor. One imaging cell and its dimensions are shown on the left.
mutually shared among them. The hexagonal coil can be viewed as a coaxial cavity coil, but with a hexagonal cross-section and with the outer conductor replaced by 6 plates in parallel to strategically concentrate magnetic flux and reduce inhomogeneity in the radial direction.

The current distribution in the conductor plates is determined by the resonance mode of the coaxial cavity with the current flowing longitudinally in the opposite direction in the inner and outer plates. The cylindrical coaxial cavity, although with inherent high sensitivity, is accompanied by a strong B1 field gradient along the radial direction between the inner and outer conductors [Woelk 2000]. The hexagonal coil is designed to overcome this problem.

### 2.2.2 Analytical solution for the magnetic field

The magnetic field $\mathbf{B}(\mathbf{r})$ between the plates of the hexagonal coil can be predicted from the Biot-Savart law for the static case [Jackson 1975]. At an arbitrary location determined by radius vector $\mathbf{r}$ ($\mathbf{r} \neq \mathbf{r}'$), the magnetic field is given by (Eq. 2.1):

$$\mathbf{B}(\mathbf{r}) =\frac{\mu_0}{4\pi} \int_{V_{\text{hexagon}}} \mathbf{j}(\mathbf{r}') \times \frac{\mathbf{r} - \mathbf{r}'}{|\mathbf{r} - \mathbf{r}'|} \, d^3 \mathbf{r}' , \quad 2.1$$

where $\mathbf{j}(\mathbf{r}')$ is the current density estimated as (Eq. 2.2):

$$\mathbf{j}(\mathbf{r}') = \begin{cases} 
0 , & \text{if } \mathbf{r}' \notin \partial V_{\text{hexagon}} \\
\pm \frac{I}{2b} \hat{z}, & \text{if } \mathbf{r}' \in \partial V_{\text{hexagon}}
\end{cases} \quad 2.2$$
where \( \partial V_{\text{hexagon}} \) represents the surface of hexagonal coil, \( V_{\text{hexagon}} \) is volume of the hexagonal coil, \( I \) is the current through the hexagon, positive for outer and negative for inner plates, \( 2b \) is the width of the plate and \( \hat{z} \) is the unity vector oriented along \( z \) axis. Here we assume uniform current density in each plate, which is not expected to be exactly accurate at 125 MHz [Li et al. 1994], but should still give a reasonably accurate result. The magnetic field \( \mathbf{B}(\mathbf{r}) \) at the arbitrary location \( (\mathbf{r}) \) is the sum of magnetic fields created by current flow in each of six outer and six inner plates (Eq. 2.3):

\[
\mathbf{B}(\mathbf{r}) = \sum_{j=1}^{6} \mathbf{B}_{j,\text{in}}(\mathbf{r}) + \mathbf{B}_{j,\text{out}}(\mathbf{r}).
\]

For example, current flowing through outer plate No.1, Fig.1, will contribute to the magnetic field at the arbitrary point \( (\mathbf{r}) \), with the assumption that one side of the hexagon has a width of 2 cm (Eq. 2.4):

\[
\mathbf{B}_{1,\text{out}}(\mathbf{r}) = \frac{\mu_0}{4\pi} \int_{-b}^{b} dx \int_{-L}^{L} dz \frac{I}{2b} \hat{z} \times \frac{\mathbf{r} - \mathbf{r}'(x,z)}{|\mathbf{r} - \mathbf{r}'(x,z)|^3}, \quad \mathbf{r}'(x,z) = \left( \begin{array}{c} x \\ 2.3 + \sqrt{3} \end{array} \right) \text{[cm]}, \quad 2.4
\]

while current flow through the inner plate No.1 of the same imaging cell, will create magnetic field (Eq. 2.5):

\[
\mathbf{B}_{1,\text{in}}(\mathbf{r}) = \frac{\mu_0}{4\pi} \int_{-b}^{b} dx \int_{-L}^{L} dz \frac{-I}{2b} \hat{z} \times \frac{\mathbf{r} - \mathbf{r}'(x,z)}{|\mathbf{r} - \mathbf{r}'(x,z)|^3}, \quad \mathbf{r}'(x,z) = \left( \begin{array}{c} x \\ \sqrt{3} \end{array} \right) \text{[cm]} \quad 2.5
\]

at the arbitrary location \( (\mathbf{r}) \).

Integrals in the Eqs. 2.3-2.5, can be solved numerically for the given coil dimensions (coil length \( 2L=4.6 \) cm, \( b=1 \) cm), using MATLAB software (The Math Works, Natick, MA). The contributions to the magnetic field from the end ring wires and wires that
attach capacitors to the coil are not considered by this approach. Also, the Biot-Savart law does not account for the eddy currents generated in the conductive elements of the coil. A solution for the magnetic field generated by the source currents and eddy currents as well as the connecting wires is discussed next using the full set of Maxwell’s equations, but with some compromise in geometrical accuracy due to the method of discretization used.

### 2.2.3 Numerical solution

The magnetic field magnitude and current distribution were numerically calculated from Maxwell’s equations using the Finite Difference Time Domain method (FDTD) and commercially available software, XFDTD (REMCOM Inc., State College, PA). The coil geometry and size were modeled as closely as possible using a $160^3 \text{ mm}^3$ 3D grid with a 2 mm cell size. A second order Liao outer radiation boundary condition [Liao et al. 1984] was applied at the boundaries of the problem region. Capacitors were modeled as two parallel plates ($2 \times 2 \text{ mm}^2$) with dielectric material in between. The coil was first excited with a Gaussian pulse and from a Fourier transform of the time domain response [Kunz et al. 1993] it was found to resonate at 135 MHz. The coil was iteratively tuned to 125 MHz by changing the permittivity of the dielectric material in the capacitors. Then the coil was excited with a sinusoidal 125 MHz excitation across one capacitor as in the experiment. The numerical calculations were performed for the empty coil because at 125 MHz a sample as small as a rat pup (body dimensions of a few cm) is not expected to have a significant effect on the field distribution.
2.2.4 Numerical evaluation for factors that contribute to $B_1$ homogeneity

Four different geometry coils were modeled in order to determine the relative contributions of the hexagonal geometry and the discontinuity of the outer conductive element to the improved homogeneity of the hexagonal coil: 1) two coaxial cylinders, 2) coaxial cylinder with six arcs (30° length) in place of the outer continuous cylinder, 3) two coaxial hexagons, and 4) coaxial hexagons with six plates in place of the outer hexagon, Fig. 4. All coils were modeled with equal length and with the same distance between outer and inner conductors. Instead of tuning coils to 125 MHz resonant frequency we used twelve voltage sources and a 125 MHz sinusoidal excitation in all four cases.

2.2.5 Coil length optimization

To determine the optimal length of the coil for future reference, we performed a series of numerical calculations of the $B_1$ field produced by hexagonal coils of different lengths, evaluating the $B_1$ homogeneity of each one. The homogeneity of each coil was assessed by analysis of the standard deviation of the $B_1$ field at all grid locations in a 2 cm-diameter sphere at the center of one of the compartments.

2.2.6 Experimental $B_1$ mapping

The experimental evaluation of the coil was performed on a 3.0 T human imaging system (Medspec S300, Bruker Instruments, Ettlingen, Germany) with a head gradient
coil (50 mT/m gradient strength). For the experimental $B_1$ mapping, six 1.7 cm diameter cylindrical vegetable oil phantoms were imaged using the Gradient Recalled Echo (GRE) sequence with $TE=6$ ms, $TR=500$ ms, single 4 mm thick axial slice. The flip angle ($\alpha$) across imaging cells was calculated from Eq. 2.6, using the signal intensity ratio of scans with two different flip angles, $\alpha$ and $2\alpha$ [Insko et al. 1993], (Eq. 2.6):

$$\alpha = \arccos\left(\frac{SI(2\alpha)}{2 SI(\alpha)}\right).$$  

2.2.7 Animal procedure

All procedures were approved by the Institutional Animal Care and Use Committee of the Penn State College of Medicine. We developed the anesthesia manifold, Fig. 2-2, which evenly distributes the gas anesthesia to six animals. A neonatal rat holder that allows easy positioning and restricts free movement of the animals is shown in Fig. 2-2 as well. Animals are first placed in the holder and then subjected to 4% isoflurane for 2 min. After 2 min and during the imaging anesthesia was kept at 2%.

2.2.8 Imaging

A three-dimensional (3D) GRE sequence was used to acquire $T_1$-weighted images of the six rat pup heads in vivo. The parameters were: $TE=10$ ms, $TR=40$ ms, $512 \times 512 \times 32$ matrix, FOV=$8.06 \times 8.06 \times 2\ cm^3$, $157 \times 157 \times 625\ \mu m^3$ voxel size, NEX=1, 13
min total acquisition time, receiver bandwidth=57 kHz and with 2000 µs 3-lobe sinc-shaped pulses for excitation.

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**Figure 2-2.** Anesthesia manifold (1) and rat pup holder device (2) developed for easier positioning of a 7-day old rats. The lines represent the tubing between the anesthesia vaporizer and the manifold and between the manifold and the rat holder. The arrows represent the gas anesthesia flow. The enlarged image of the hexagonal coil (3).

A multi-slice, multi-echo spin-echo imaging sequence was used to acquire $T_2$-weighted anatomical images of the six rat pup heads in vivo, with the following parameters: 11 echoes with TE: 19.4 - 213.51 ms, TR=3000 ms, FOV=8.20 X 8.20 cm$^2$, 1 mm slice thickness, 512 X 256 matrix, 320 X 160 µm$^2$ resolution, NEX=1, 13 min total acquisition time, receiver bandwidth=50 kHz and with 3200 µs Gaussian-shaped pulses for excitation and refocusing.
2.2.9 Comparison with the single parallel plate resonator

A coil made from two parallel plates 2 cm X 4.6 cm, 2.3 cm distance between the plates; identical dimensions as any single imaging cell of the hexagonal coil was constructed for SNR comparison purposes. Six 1 mM CuSO₄ doped water phantoms (1.7 cm in diameter, 10 cm long) and six 1 mM CuSO₄ doped 50 mM saline phantoms (same dimensions as water phantoms) were used for the SNR comparison purpose. Imaging was done using a single-slice single-echo spin-echo sequence (TE=18.3 ms, TR=3000 ms, FOV=8.20 X 8.20 cm², 1mm slice thickness, 512 X 256 matrix, NEX=1, 13 min total acquisition time, receiver bandwidth=50 kHz and with 3200 µs Gaussian-shaped pulses for excitation and refocusing) that had identical parameters for imaging with both coils. In order to estimate sample noise and coil noise contributions to SNR we imaged six saline phantoms with the hexagonal coil and compared the SNR of the image with SNR of the image obtained with a single saline phantom imaged with the parallel plate coil. To calculate SNR we used the mean signal in the phantom divided by the standard deviation of the noise in the background [Haacke et al. 1999] for a region of interest approximately the size of the phantoms. In order to further estimate the sample noise contribution to SNR we compared SNR of the image where one saline phantom was imaged using the hexagonal coil to the SNR of the image where six identical saline phantoms were imaged simultaneously using the same coil.
2.3 RESULTS

2.3.1 B₁ Field Homogeneity

The improved B₁ field homogeneity of the hexagonal coil is demonstrated by the analytical calculations, the numerical calculations, and the experimental measurements. The numerical solution predicts 10% difference between maximal and minimal B₁ field across imaging cell in the hexagonal coil. The analytical solution found from the Biot-Savart law for B₁(r) field distribution across the center slice of the coil (z=0) is given in Fig. 2-3. The plot represents the results of Eqs. 2.3-2.5 for magnetic field B(r) between two plates of the hexagonal coil and magnetic field magnitude |B(r)|. From Fig. 2-3 it can be concluded that the imaging space of the hexagonal coil has homogeneity within 10%. Similar results were found from the numerical solution of Maxwell’s equation, Fig. 2-4a. The experimental B₁ map, acquired with six 1.7 cm oil phantoms, Fig. 2-4b, shows that the flip angle distribution across each imaging cell varies by less than 10 degrees. From both numerical calculations and experimental measurements we found that the B₁ field gradient between inner and outer plates along a line through the center of the imaging cell is approximately 10%.
From further numerical calculations we found that 4 coils with different geometries have different $B_1$ field homogeneity, Fig. 2-5. The biggest difference between maximal and minimal $B_1$ field magnitude is in the case of the coaxial cylindrical cavity coil, found to be 45% along a radial line through the center of an imaging cell, Fig. 2-6. Significant improvements in homogeneity were achieved when either the outer continuous cylinder was replaced with 6 arcs (each 30° long) equally spaced around the inner cylinder, or when the cylindrical geometry was replaced with hexagonal, with
greater homogeneity in the latter case. The continuous coaxial hexagonal coil had \(\sim 19\%\) difference between maximal and minimal \(B_1\) field magnitude along a radial line through the center of an imaging cell. Finally, the best homogeneity was achieved when the outer hexagon was replaced with 6 plates, arranged so they resemble hexagonal structure, achieving a difference of only \(\sim 10\%\) between maximal and minimal \(B_1\) field magnitude along a radial line through the center of an imaging cell. The presence of this \(B_1\) field gradient is due to coaxial design.

**Figure 2-4.** Numerically calculated magnetic field between the inner and outer elements of the hexagonal coil magnitude using Maxwell’s equations, (a). The location of a single sinusoidal 125 MHz excitation iz shown. The scale represents percentage of maximum magnetic field strength (0-100%). Experimental \(B_1\) map (b). Scale represents degrees of flip angle across the imaging cell. Imaging was done using six 1.7 cm diameter cylindrical phantoms filled with vegetable oil. The location of the voltage source is same as in the model. From the experimental \(B_1\) map we can conclude that flip angle distribution across the imaging cell is within 10 degrees of flip angle.
Figure 2-5. The magnetic field magnitude distribution across the coaxial cylindrical coil (a), coaxial cylinder with six 30° long arcs instead of outer cylinder (b), coaxial solid hexagonal coil (c) and hexagonal coil (d) (where outer solid hexagon is replaced with six plates, arranged so they resemble hexagonal structure). All coils were excited with twelve sinusoidal voltage sources. The magnetic field magnitude scale is shown in percentage of maximum magnetic field strength (0-100%).
Figure 2-6.  B₁ field homogeneity comparison graph. The magnetic field magnitudes across the imaging cell in the case the coaxial cylindrical coil (solid circle), coaxial cylinder with six 30° long arcs instead of outer cylinder (hollow circle), coaxial solid hexagonal coil (solid triangle) and hexagon with 6 plates (hollow triangle). The x-axis of the plot represents distance orthogonal to the planes of inner and outer element, where zero distance is the inner wall, and eighteen millimeter is the position of the outer wall. Y-axis is normalized magnetic field magnitude ranging from zero to one. From the graph we can conclude that in the case of the hexagon with 6 plates there is 10% decrease in B₁ field magnitude as we go from inner toward the outer wall, where there in the case of the cylindrical coil the B₁ gradient is more prominent and there is 45% decrease in B₁ field magnitude across the imaging cell.
From the coil length optimization calculation it was found that homogeneity decreased rapidly as length dropped below about 3.5 cm, and remained good at lengths greater than about 3.5 cm, with a local optimum in homogeneity (standard deviation in a 2 cm-diameter sphere at the center of the imaging cells of only 4.30% of the mean $B_1$ magnitude in the sphere) at a length of 4.2 cm.

### 2.3.2 Power requirements

The hexagonal coil, when loaded with the 6 cylindrical 50 mM saline phantoms required 0.5 dB less power than the single-cell equivalent rectangular coil loaded with 1 cylindrical 50 mM saline phantom to accomplish the 90° pulse during the spin echo experiments.

### 2.3.3 Bench Measurements

The hexagonal coil produced good $B_1$ field homogeneity (less than 1dB difference) across the imaging cell in all 6 cells, measured by using the HP spectrum analyzer (HP 4195A) and a small (3mm diameter) pick up coil. The unloaded quality factor (Q), measured on the same spectrum analyzer was 530 ($S_{11}$ measurement mode), while the coil loaded with six rat pups had a Q of 290.
2.3.4 Imaging

An axial T1-weighted image of six 7-day old anesthetized rat pups is shown in Fig. 2-7a, and one enlarged rat pup head is shown in Fig. 2-7b. A spin echo T2-weighted image of six 7-day old anesthetized rat pups is shown in Fig. 2-8a, and a representative enlarged image of one rat pup head is shown in Fig. 2-8b.

2.3.5 Signal to noise ratio

The summary of the SNR experiment is given in Table 2-1.

<table>
<thead>
<tr>
<th>SNR</th>
<th>1 mM CuSO4 water</th>
<th>50 mM saline + 1 mM CuSO4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rectangular coil</td>
<td>263</td>
<td>260</td>
</tr>
<tr>
<td>(single phantom load)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexagonal coil</td>
<td>255</td>
<td>253</td>
</tr>
<tr>
<td>(single phantom load)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hexagonal coil</td>
<td>255</td>
<td>249</td>
</tr>
<tr>
<td>(six phantom load)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

As expected, the lowest SNR ratio is in the case of the hexagonal coil loaded with six 50 mM saline phantoms. The SNR of the parallel plate coil loaded with one 50 mM saline phantom is only ~5% higher than this worst case.

The SNR of the hexagonal coil loaded with the single saline phantom is only 3% higher than SNR of the hexagonal coil loaded with the six saline phantoms. There was
no difference in the SNR between the single non-conductive load and six non-conductive loads when imaged with hexagonal coil.

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**Figure 2-7.** An axial T1-weighted image of six anesthetized 7-day old rat pup heads (a) and enlarged view of a single rat pup head (b), acquired with a 3D gradient recalled echo method.

---

### 2.4 DISCUSSION

With the proposed coil design we were able to achieve better $B_1$ field homogeneity than in earlier designs based on the coaxial cavity [Woelk et al. 1994; Woelk 2000]. Experimental and calculated results show good agreement. Easy tuning
and matching can allow quick examination of 6 small animals at the same time, and good SNR can allow for less than 200 µm resolution in a short period of time. The coil can fit small-aperture gradient coils and the space in the middle can be utilized for accommodation of anesthesia and temperature controls.

From numerical calculations for $B_1$ magnitude across 4 different coil geometries we can conclude that hexagonal geometry together with discontinuity of the outer conductor is responsible for the 35% improvement in homogeneity of the hexagonal coil compared to the cylindrical coaxial cavity coil.

To further evaluate the efficiency of increasing the imaging throughput with the hexagonal coil, we made a comparison of the SNR between the hexagonal coil and single coil having the same dimension as one of the imaging cells of the hexagonal coil. The

**Figure 2-8.** An axial $T_2$-weighted image of six anesthetized 7-day old rat pup heads (a) and enlarged view of a single rat pup head (b) acquired with a 2D spin echo method.
SNR obtained with the single parallel plate coil was only 4-5% higher than SNR obtained imaging six times more samples with the hexagonal coil. There is little difference in SNR when the hexagonal coil is loaded with one or six samples, even when the samples are conductive Table 1. This may be because at 125 MHz in a coil as small as this, the noise is likely to be dominated by the coil, rather than the sample [Edelstein et al. 1986]. When coil noise is much larger than sample noise, SNR is proportional to the $B_1$ field magnitude in the sample divided by the square root of power dissipated in the coil. Because the coil noise remains constant regardless of the number of samples and because the same amount of signal is available from each sample, the SNR in each sample is fairly independent of the number of samples.

The hexagonal coil can be considered as six parallel plates connected in parallel, but since there is non-zero current in the end rings they are connected partially in series as well. Assuming the coil noise dominance, the SNR measurements indicate that the coil resistance in the case of the hexagonal coil is slightly higher than in the case of single parallel plate coil. In the case of sample-dominated noise, SNR is proportional to the $B_1$ field magnitude in the sample divided by the square root of power dissipated in the sample [Edelstein et al. 1986]. Six times more sample load in the case of hexagonal imaging would expect to decrease SNR by $1/\sqrt{6}$ [Edelstein et al. 1986], but SNR is only 3% lower than imaging single load with the hexagonal coil using conductive sample, Table 1, further confirming that we are in the coil-dominated noise domain. The small contributions in noise from the samples along with any subtle inconsistencies in manufacture (including soldering) results in a situation where SNR is ~4-5% lower for the hexagonal coil than the single-cell coil.
2.5 CONCLUSIONS

In this work a novel geometry coil designed for multiple animal imaging is presented. The hexagonal geometry provides better homogeneity than previously described cylindrical cavity coils. The theoretical analysis was validated with experimental data and in vivo rat pup imaging. The discontinuity of the outer hexagonal element did not interfere with the SNR, but did improve the homogeneity of the coil. The proposed hexagonal coil design can be utilized for multiple animal imaging with high signal-to-noise ratio, where only a single receiver is required.

2.6 REFERENCES


Chapter 3

A Spontaneously Reversible Diffusion-Weighted Signal Intensity Changes Following Neonatal Hypoxia-Ischemia

ABSTRACT

MR imaging was utilized to study the evolution of brain injury following hypoxia-ischemia (H/I) in the neonatal rat before and after a drug treatment. Two drugs, minocycline and aminoguanidine, were given as neuroprotective agents following hypoxia-ischemia. The volume of the hyperintense diffusion weighted (DW) signal before administration of the drug was compared to the T2-weighted hyperintense signal volume at 7 days following the recovery from H/I. There was a significant difference (p<0.0005) in the hyperintense signal volume reduction for both drug-treated and control (saline and PBS injected) animals.

3.1 INTRODUCTION

Hypoxic-ischemic brain injury remains the third leading cause of death in the United States among adults and perinatal hypoxia-ischemia (H/I) is the leading cause of morbidity and mortality in infants and children. One of four surviving infants will exhibit severe permanent neuropsychological disabilities like cerebral palsy, learning disabilities, mental retardation and epilepsy. An enormous amount of research has been focused toward development of neuroprotective agents that will ameliorate brain damage
following hypoxia-ischemia and improve neurological outcome. The FDA has not yet approved any treatment following perinatal H/I because pharmacological treatments shown to significantly reduce infarction volume in animal models usually fail to provide the same benefits when applied to humans [Levene et al. 1985; Goldberg et al. 1986; Yrjanheikki et al. 1999; Whitelaw et al. 2002]. The failures of clinical trials were attributed to the toxic effects that have overcome the neuroprotective effect and the limited time window for the application. The most important difference between clinical and animal trials is that infants at high risk for birth asphyxia are examined and monitored carefully, with usually some sort of neurological assessment done prior to the treatment. In contrast, in the typical animal study the brain volume undergoing hypoxic/ischemic changes is usually not known prior to the application of therapeutic agents, leading to a conclusion based on statistical difference between treated groups and not the real therapeutic agent effect.

In this work the volume of hyperintense signal on diffusion weighted (DW) images was assessed prior to the administration of therapeutic agent and then compared to the volume of high signal intensity on T2-weighted images 7-days post H/I. This procedure allows the study of reversible DW signal intensity changes and determination of whether this is a spontaneously occurring process during the 7-day recovery period from H/I, or it is a consequence of the drug administration. We tested two hypotheses that repetitive administration of aminoguanidine [Iadecola et al. 1995; Zhang et al. 1998; Tsuji et al. 2000] or minocycline [Yrjanheikki et al. 1998; Yrjanheikki et al. 1999; Arvin et al. 2002] will provide significant reduction of the hyperintense DW signal volume following neonatal H/I. Aminoguanidine is known as a relatively selective inducible
nitric oxide synthase (iNOS) inhibitor [Iadecola et al. 1995]. Minocycline belongs to the second-generation tetracycline bacteriostatic agents, with a plethora of biological effects distinct from its antimicrobial action. It is a known inhibitor of p38 kinase, IL-1β converting enzyme, caspase 3, matrix metalloproteases, iNOS, and as Ca\(^{2+}\) chelator [Zhu et al. 2002]. It also inhibits tumor-induced angiogenesis [Yrjanheikki et al. 1998]. It is rapidly absorbed, with superior tissue penetration into the brain and cerebrospinal fluid (CSF) [Klein et al. 1995; O'Neil et al. 1999].

3.1.1 Hypoxic-ischemic brain injury

Hypoxia is oxygen deprivation, leading to cessation of aerobic oxidative respiration, cell injury and death. Ischemia is a loss of blood supply resulting from impeded arterial flow or reduced venous drainage in tissue. While anaerobic glycolysis can still proceed during hypoxia, ischemia compromises the availability of metabolic substrates including the glucose, so that injury to the tissue during ischemia occurs faster than during hypoxia alone. Depending on the severity of hypoxia, cells may undergo injury, adapt or die. Reversible ischemic injury occurs when cells are able to recover upon the reperfusion, and when normal blood and oxygen supply are reestablished following hypoxic-ischemic event. With extension of ischemia, cells continue to deteriorate and with time the mitochondrial oxidative machinery and the cell membrane become irreparably damaged. Common to all ischemic diseases is cellular depletion of ATP. An inability to generate high-energy compounds can be considered as a point of no
return, beyond which reperfusion cannot rescue the damaged cell. This represents irreversible cell injury.

In the event when blood flow is reestablished after a cell has experienced hypoxic-ischemic injury but has not yet died, injury to the cell can paradoxically be exacerbated by the reestablished blood flow. This type of injury is termed ischemia/reperfusion injury, and represents an important process that contributes to the brain tissue damage following hypoxia-ischemia.

Brain is more vulnerable to ischemic injury than are other tissues due to its high metabolic rate, limited intrinsic energy supply and critical dependence on aerobic metabolism of glucose. Different neuronal populations like the one found in the CA1 subfield of the hippocampus, the pyramidal layer of the cortex, the Purkinje cells and subpopulations in the striatum, thalamus, amygdala and brain stem are highly vulnerable following hypoxia-ischemia. The fact that these neurons are juxtaposed to more resistant neuronal population, and that two have common blood supply, suggests that intrinsic factors contribute heavily to neuronal sensitivity mediated by this type of injury.

### 3.1.2 Reversible cell injury during hypoxia-ischemia

The first event that occurs during the hypoxia is the drop of oxygen delivery to the cell leading to loss of oxidative phosphorylation and a decrease in ATP generation. The resulting depletion of ATP has widespread consequences on many cellular processes. Among the first cellular process affected is decreased activity of plasma-membrane ATP-dependent sodium-potassium pumps. Subsequent failure of this active transport system
due to depleted ATP levels leads to influx of sodium and efflux of potassium and in the case of neuron to cell membrane depolarization. The net gain of solutes into the cell will osmotically drive water into the cell, causing cell swelling followed by dilatation of the endoplasmic reticulum. Secondary to the gain of solutes into the cell, there is accumulation of inorganic phosphate, lactate and other products of catabolism that will further increase the intracellular osmosis leading to additional cell swelling.

The decrease in cellular ATP and oxygen availability leads to activation of glycolytic enzymes, in particular phosphofructokinase, and to rapid depletion of glycogen stores. The increased glycolysis and impaired oxidative phosphorylation lead to accumulation of lactic acid and inorganic phosphates from the hydrolysis of phosphate esters reducing the intracellular pH. The next process to occur is disruption in the protein synthesis machinery manifested as detachment of ribosomes from the granular endoplasmic reticulum with subsequent reduction in the protein synthesis.

If the ATP is not restored, the cytoskeleton disperses, resulting in the loss of ultrastructural features such as microvilli and formation of “blebs” at the cell surface. Dissociation of lipoproteins results in “Myelin figures” that can be seen within the cytoplasm or extracellularly. At this stage the entire cell is markedly swollen with increased concentration of sodium and chloride ions and decreased intracellular potassium. Due to the loss of volume control the mitochondria are swollen and the endoplasmic reticulum is still dilated. At this point if the oxygen and blood supply are restored all of these changes are reversible.
3.1.3 Irreversible cell injury during hypoxia-ischemia

If oxygen and blood supply are not restored, severe irreversibly injury takes place. The complex metabolic and vascular processes ultimately resulting in infarction and irreversible neuronal injury are not fully understood. The region of ischemia where these events take place is called the ischemic core. Surrounding the core is a hypoperfused area known as the penumbra. The penumbra is believed to be the region that can evolve toward viability or toward infarction. A number of mechanisms have been proposed to explain the circumstances that lead to development of infarction. These include formation of free radicals, excess of glutamate and glutamate mediated excitotoxicity, Ca\(^{2+}\) release, formation of vasoconstrictor elements and activation of the coagulation cascade, and inflammation. Therefore, the inhibition of free radicals production, suppression of inflammatory mediators, application of glutamate receptor antagonists, GABA agonists, Ca\(^{2+}\) chelators and anticoagulation agents represent the major strategies for therapeutic intervention.

3.2 METHODS

3.2.1 The model of hypoxia-ischemia

Hypoxic-ischemic brain damage was induced according to the Rice-Vannucci model [Rice et al. 1981; Vannucci et al. 1997] (based on the Levine preparation in the adult rat [Levine 1960]) of neonatal hypoxia-ischemia. This immature rat model has
proved valid and has been utilized by many investigators throughout the United States and abroad.

The Rice-Vannucci model utilizes 7-day old rats, and consists of unilateral common carotid artery ligation followed by systemic hypoxia produced by the inhalation of 8% O₂/balance N₂. A significant blood pressure decrease by 25-30% is present during the hypoxic period. The rat pups are capable of surviving under this severe hypoxic condition for 3 or more hours before significant mortality occurs. The 7-day postnatal rat was originally chosen since the animal’s brain development is histologically similar to that of a 32- to 34-week gestation human fetus or newborn infant. The cortical layer is complete, myelination is yet to be complete, and the germinal matrix is involuting. During the course of hypoxia, hypocapnia (deficiency of carbon dioxide in the blood) occurs in combination with hypoxemia (reduced oxygen in the blood) due to hyperventilation. The hypocapnia leads to alkalosis, however there is adjustment in the blood pH levels since the build up of lactic acid leads to metabolic acidosis.

The Rice-Vannucci model produces brain damage in the hemisphere ipsilateral to the ligation in immature rats exposed to 2-3 h of systemic hypoxia. The vasogenic and cytotoxic edema sometimes extends into the contralateral brain hemisphere, as observed during first 0-24 h following hypoxia-ischemia on diffusion-weighted and T₂-weighted images respectively, but it almost never progresses into the infarction. The affected brain structures include the cerebral cortex (mostly layers 3 and 5+6), hippocampus, striatum and subcortical and periventricular white matter. The tissue injury includes selective neuronal death leaving a gliotic scar, or infarction leaving a cystic cavitation. Altered cellular energy metabolism usually results in necrotic cell death, although there is
growing evidence for a role of apoptosis leading to brain cell death following hypoxia-ischemia.

3.2.2 Animals and surgical procedure

Male and female 7-day old Wistar rats were anesthetized with halothane (4% induction, 1.5% maintenance in 30% O₂/balance N₂) and the right carotid artery was double ligated. To induce hypoxia, each animal was exposed to a gas mixture of 8% O₂/balance N₂ for 2 h, in 37°C water bath. All rats were maintained at the Penn State College of Medicine by the Department of Comparative Medicine, an Association for Assessment and Accreditation of Laboratory Animal Care accredited facility. Animal experimentation was in accordance with research guidelines set forth by Pennsylvania State University and the Society for Neuroscience Policy on the use of animals in Neuroscience research.

3.2.3 Drug administration and treatment groups

All injection volumes were 10 µl/g of vehicle or drug dissolved in vehicle. Injections were performed with a 29-gauge needle. Animals were injected with minocycline (Sigma, St. Louis, MO), 45 mg/kg dissolved in phosphate buffered saline (PBS) pH 7.4, or PBS or saline at 1 h 30 min following H/I, and then every 24 h for 72 h. In another experiment animals received aminoguanidine (Sigma, St. Louis, MO), 100 mg/kg dissolved in saline, or saline at 4 h following H/I and then every 12 h for 72 h.
Rectal temperature was measured 1 h 30 min post injection. The dose of the minocycline and aminoguanidine were chosen according to previously published studies [Iadecola et al. 1995; Zhang et al. 1998] for aminoguanidine and [Yrjanheikki et al. 1998; Yrjanheikki et al. 1999; Arvin et al. 2002] for minocycline, which determined these doses to have the maximal protective effect.

3.2.4 Imaging

MR imaging was performed on a 3.0 T MRI spectrometer (Medspec S300, Bruker Instruments, Ettlingen, Germany) with a human head gradient coil (50 mT/m gradient strength). Groups of six 7-day old pups were imaged at the same time using a multiple animal probe [Lazovic-Stojkovic et al. 2002]. In order to minimize the motion artifact animals were placed in the specially designed animal holder described in 2.2.1. All animals were imaged 30 min following H/I using a diffusion-weighted imaging sequence (TR/TE=1500/68.8 ms, Δ=35.22, δ=20 ms, b-value=730, 128X128 matrix, 625X625 μm resolution, 1 mm slice thickness, 0.5 mm slice separation, number of averages NEX=1, 3 min 12 s total imaging time) and isoflurane anesthesia (2%). Seven days following H/I each animal was imaged individually using a T2-weighted spin echo sequence (TR/TE=3000/10.13-151.95 ms, 256 X 128 matrix size, field of view 2 x 2 cm², N=15 echoes, NEX=2, 10 slices, 0.5 mm slice thickness in 13 min) in a 2 cm slotted tube resonator and small aperture gradient coil (9.5 cm aperture and 1.1 T/m gradient strength). During imaging, animals were kept under isoflurane anesthesia (2%), and isothermal with the heating pad at 37 °C.
3.2.5 Infarct volume measurements and statistical analysis

The initial hyperintense signal volume was assessed from 4 coronal slices and DW images, using semi-automated routine and the CCHIPS/IDL software [Schmithorst et al. 2001] by an investigator blinded to the treatment groups. The ipsilateral areas that had signal intensity 25% above the matched areas on the contralateral side were included in the calculation of the initial hyperintense signal volume. These volumes were corrected for brain edema as described previously [Swanson et al. 1990] and calculated as percent of the contralateral hemisphere. The infarct volume (final hyperintense signal volume) at 7 days post H/I was determined from 8 slices and T2-weighted images using the CCHIPS/IDL software [Schmithorst et al. 2001]. The ipsilateral areas that had T2 values 25% above matching areas on the contralateral side were included in the calculation of the final hyperintense signal volume (final infarct volume). A paired t-test was used to assess the difference in the initial vs. final hyperintense signal volumes. The difference in the initial vs. final hyperintense signal volume was calculated for each animal. We used analysis of variance to test the null hypothesis that the mean differences between initial and final hyperintense signal volume were equal among different treatment groups. The results are presented as mean ± standard deviation (SD).
3.3 RESULTS

3.3.1 Temperature measurements

Pups injected with minocycline or aminoguanidine did not have significantly different rectal temperatures from the pups injected with saline or PBS at 1 h 30 min following the injections.

3.3.2 Infarct volume measurements

We were first interested whether the volume of the initially observed increased signal intensity on DW images at 30 min post H/I is the same as the volume of increased signal intensity on T2-weighted images at 7 days post H/I for all treatment groups. The well characterized Rice-Vannucci model of neonatal H/I was used, in which combination of unilateral carotid artery ligation is followed by exposure to hypoxia. This combination of moderate hypoxia and ischemia provides the reproducible pattern of injury, including neuronal loss and gliosis ipsilateral to the carotid ligation [Johnston et al. 1987; Ferriero et al. 1988; Cheng et al. 1997]. Animals that did not have any increased signal intensity on DW images, did not exhibit any increased signal intensity on T2-weighted images and were excluded from the further analysis.

Interestingly, there was a significant reduction of the volume of initially observed elevated DW signal when compared to the volume of elevated T2 signal intensity at 7 days following H/I for all treatment groups (p<0.0005 for the minocycline (N=13) and aminoguanidine (N=10) treated group, p<0.0001 for the saline (N=16 for injections
staring at 1h 30 min post H/I, and N=10 for injections starting at 4h post H/I) and PBS (N=18) treated groups). Pups that received minocycline treatment had (mean ± SD) 30±13.7% reduction in the infarct volume compared to 26±15.8% with saline, 25±12.7% with PBS and 25±13.1% with aminoguanidine, Fig. 3-1. The mean differences between initial and final infarct volume were not significantly different among different treatment groups.

Then the initial infarction volumes were separated into the two sub-groups: severe (50% or more of the ipsilateral hemisphere has abnormal signal intensity on DW images) vs. moderate-mild (less than 50% of the ipsilateral hemisphere has abnormal DW images signal). Interestingly, pups that had mild-moderate initial infarct volume had no statistically different reduction in the final infarct volume than the pups with severe initial infarction. This was independent of treatment.

When the effect of treatment was examined in the mild-moderate group, no statistically significant difference was found for the mean reduction of the infarct volume among different treatment groups. No statistically significant difference for the mean reduction of the infarct volume was found among different treatment groups in the case of initially severe H/I insult.

Among different treatment groups, however, some of the animals exhibited remarkable reduction of the initially increased signal intensity on diffusion weighted images and had normal T2 values at 7 days post H/I. An example of a minocycline treated animal that had ~40% infarct volume reduction is show in Fig. 3-2. An example of a PBS treated animal that had ~40% infarct volume reduction is show in Fig. 3-3.
Both animals were from the same litter and imaged at the same time (30 min following H/I), using the multi animal probe [Lazovic-Stojkovic et al. 2002].

Figure 3-1. Mean and standard deviation of the hyperintense signal volume reduction over period of 7 days (expressed as mean ± standard deviation of the hyperintense signal volume reduction over period of 7 days (expressed as the percentage of the contralateral hemisphere), for minocycline, PBS, aminoguanidine, and saline treated animals. There was a trend for minocycline treated animals toward the highest reduction in hyperintense signal volume, although there was no significant difference among different treatment groups was not statistically significant.
3.4 DISCUSSION

DW imaging, highly sensitive to cytotoxic edema, was used to evaluate the increased signal intensity volume before attempting drug intervention. This allows us to study reversibility of initial early DW signal intensity changes in the treated and control animals. Imaging six animals at the same time allows animals from the same litter to be studied minimizing the differences in the genetic background on the outcome and the recovery from H/I, and further reduces variations due to the different environmental
conditions during H/I and recovery. Since control animals had the same spontaneous recovery as drug treated animals, it is difficult to discern any protective effect of the administered drugs. Both minocycline and aminoguanidine failed to show any neuroprotective effect different than the control saline or PBS injected animals. It appears that the mechanism of recovery is independent of drug action. Some of the animals in each treatment group exhibited remarkable reduction of the initially increased signal intensity on diffusion weighted images, Fig. 3-2 and Fig. 3-3, and had normal T2-weighted signals at 7 days post H/I. Further elucidation of the mechanism behind the

Figure 3-3. DW-images (top row) of PBS treated animal that had remarkable recovery and ~40% reduction in the infarct volumes following hypoxia-ischemia. Images were order from the most rostral on the left to the most caudal on the right. T2-weighted images (bottom row) of the same animal 7-days following hypoxia-ischemia. Images were order from the most rostral on the left to the most caudal on the right.
reversible diffusion weighted signal changes may provide clinically beneficial therapy following neonatal H/I.

3.5 REFERENCES


Chapter 4

Interleukin-1 and The Interleukin-1 Type 1 Receptor are Essential for the Progressive Neurodegeneration That Ensues Subsequent to a Mild Hypoxic/Ischemic Injury

ABSTRACT

Background and Purpose: There are indications that inflammation causes progressive neurodegeneration in multiple neurological diseases, including cerebral ischemia; however, to date there is no consensus regarding which inflammatory mediators represent key targets for pharmacological intervention. As we have previously demonstrated that signaling through the IL-1 type 1 receptor (IL-1R1) is necessary for microglial activation and as results from other studies have implicated microglia as effectors of neurodegeneration, we hypothesized that inactivating the IL-1R1 would decrease the extent of damage caused by a hypoxic-ischemic insult. Results: We demonstrate that a mild hypoxic/ischemic insult initiates progressive neurodegeneration that leads to cystic infarcts, which can be prevented by inactivating the IL-1R1. The mild insult induces multiple pro-inflammatory cytokines and activates microglia, and these responses are dramatically curtailed in mice lacking the IL-1R1. Importantly, the IL-1R1 null mice show normal sensorimotor function at one month’s recovery. Conclusions: These findings demonstrate that abrogating the inflammation consequent to a mild hypoxic/ischemic insult will prevent brain damage and preserve neurological function.
Additionally, these data incriminate the IL-1R1 as a master pro-inflammatory cytokine receptor.

4.1 INTRODUCTION

Hypoxic/ischemic (H/I) brain damage is the leading cause of adult death and disability in developed countries. Despite substantial research on this injury few treatments are available to preserve brain cells after H/I. There is accumulating evidence that neuroinflammation contributes to the wave of secondary damage that follows H/I [Tan et al. 2003], which develops as a consequence of the initial activation of endothelial cells, endogenous microglia and resident perivascular/parenchymal macrophages followed closely by the mobilization and recruitment of peripheral inflammatory cells to the site of damage [Barone et al. 1991; Feuerstein et al. 1998; Stoll et al. 1998]. Activated microglia are known to produce a number of proinflammatory mediators, including IL-1, that increase the competence of the cerebral endothelium to recruit peripheral leukocytes into the damaged brain and activated microglia have been imaged within the damaged human brain from 5 days to 7 months after cerebral ischemia [del Zoppo et al. 2000; Gerhard et al. 2000; Pappata et al. 2000].

The rapid release of the pro-inflammatory cytokine, IL-1, in and around areas of damage is a common sequellae subsequent to a wide range of CNS injuries, including cerebral ischemia [Griffin et al. 1989; Minami et al. 1992; McGuinness et al. 1997; Touzani et al. 1999; Legos et al. 2000; Basu et al. 2002; Touzani et al. 2002]. A large body of research has shown that increased IL-1 exacerbates the damage due to injury,
while factors that diminish the IL-1 response limit the damage [Yamasaki et al. 1995; Loddick et al. 1996b]. A more detailed knowledge of IL-1 signaling in the CNS could, therefore, lead to better, more efficacious treatments for cerebral ischemia and a number of other neurodegenerative diseases.

Two ligands, IL-1α and IL-1β (cumulatively referred to as IL-1) stimulate an inflammatory response by binding to the type I IL-1 receptor, (IL-1R1), and possibly to other incompletely characterized receptors. There also is a second receptor, the Type II receptor, that can bind these ligands but does not transduce a response and is thought to act as a decoy, or sink, to inhibit IL-1 activity [Sims 2002; Boutin et al. 2003]. Signaling by IL-1 ligands also is regulated by the naturally occurring antagonist, IL-1ra, which competitively inhibits ligand binding to the IL-1R1. IL-1α and IL-1β have similar biologic activities, with IL-1β being the predominant form expressed in the CNS. Binding of IL-1α or IL-1β to the IL-1R1 leads to a signaling cascade that involves multiple second messengers, [O'Neill et al. 1998]. Inhibiting IL-1 activity, either through neutralizing antibodies, exogenously added IL-1ra, or inhibiting caspase 1 ameliorates the damage in a number of animal models of neurodegenerative diseases, including H/I, brain trauma, Multiple Sclerosis and Alzheimer’s disease [Jacobs et al. 1991; Relton et al. 1992; Yamasaki et al. 1995; Loddick et al. 1996a]. The clearly beneficial aspects of exogenously added IL-1ra suggest that therapies designed to interfere with IL-1 signaling may have clinical relevance in limiting the damage caused by these CNS injuries. However, IL-1ra does not gain easy access to the CNS and it also antagonizes binding of the IL-1 ligands to the human type 2 receptor [Arend 1993]; therefore, there is a clear
need to identify the IL-1 receptor that is responsible for exacerbating damage after cerebral ischemia and to develop inhibitors for that receptor.

4.2 METHODS

4.2.1 Mice and genotyping

Four to 6 month old male IL-1R1 (-/-) mice that had been backcrossed 9 times onto a C57BL/6 background were interbred as well as wild-type C57BL/6 mice. IL-1R1 null mice were originally provided by the Immunex corporation (Seattle, WA). All mice were bred and maintained at the Penn State College of Medicine by the Department of Comparative Medicine, an Association for Assessment and Accreditation of Laboratory Animal Care accredited facility. Animal experimentation was in accordance with research guidelines set forth by Pennsylvania State University and the Society for Neuroscience Policy on the use of animals in Neuroscience research.

PCR analysis of tail DNA was used to confirm the identity of the mice used for these studies. DNA was isolated from the mice by phenol chloroform extraction. Three different sets of primers were used for the PCR reaction: 5’ GAGTTACCCGAGGTCCAG 3’; 5’ GAAGAAGCTCACGTTGTC 3’; 5’ GCGAATGGGCTGACCGCT 3’. The PCR reaction was carried out under the following conditions: 94°C for 30 sec, 53.5°C for 1 min, and 72°C for 1.5 min. 35 cycles were performed. The PCR products were resolved and visualized on a 1% agarose gel containing ethidium bromide and run in 1x TAE buffer. The expected finding for a type I
IL-1R WT (+/+), is a single band at 1150 bp. A heterozygote (+/-) had two bands present at 1150 bp and 860 bp. An IL-1R1 null mouse (-/-) had a single band at 860 bp. Experiments were performed either with homozygous WT (+/+), or with IL-1R1 null (-/-) mice.

### 4.2.2 Induction of unilateral cerebral hypoxia-ischemia

Hypoxia-ischemia was induced in adult mice by a modification of a procedure developed in the immature rat [Levine 1960; Rice et al. 1981; Vannucci et al. 1996] and as described previously in adult mice [O'Donnell et al. 2002]. On the morning of the experiment, animals were anesthetized with halothane (4% induction, 1.5% maintenance in room air), and the right common carotid artery was isolated and double ligated with 4-0 surgical silk. The incision was sutured, and the animals were allowed to recover with access to food and water for 3 h. To induce hypoxia, each animal was placed in a 500 ml glass jar partially submerged in a temperature controlled water bath and exposed to 8% O₂/balance N₂ for 22 min. The water bath temperature was maintained at 35.5°C, which in previous experiments was shown to maintain the animals core body temperature at 37.5-37.7°C throughout the hypoxic interval. Animals were allowed to recover in room air for 30 min and then returned to their cages with free access to food and water.
4.2.3 MR imaging and infarct volume measurements

MR imaging was performed at 24 h post H/I with 33 WT mice and 28 IL-1R1 null mice. Imaging was also done at intervals including 7 days, 1 month and 2 months following HI on 14 WT and 11 knockout animals. MR imaging was performed on a 3.0 T MRI spectrometer (Medspec S300, Bruker Instruments, Ettlingen, Germany) with a gradient coil (9.5 cm aperture and gradient strength 1.1 T/m). Each mouse was imaged using a 2.5 cm slotted tube resonator. Prior to imaging, mice were anesthetized with xylazine and ketamine (2 mg/kg of xylazine, 15 mg/kg of ketamine, intraperitoneal (ip.)). For the damage assessment ten continuous coronal slices, 0.5 mm thick were acquired (effective TE_{eff}=69.4 ms, TR=3000 ms, slice thickness 0.5 mm, field of view 2 X 2 cm², matrix 256 x 256 and 8 averages) in 6 min, using the rapid acquisition with relaxation enhancement (RARE) imaging sequence. The first slice was positioned 2 mm posterior to the rhinal fissure. To quantify transverse relaxation time (T₂) changes for the longitudinal studies, each mouse was imaged with a T₂-weighted multi echo spin echo sequence in addition to RARE. The T₂-weighted spin echo sequence (TE=10-152 ms, N=15 echoes, TR=3000 ms, 128 X 128 matrix size, 13 min acquisition time and 2 averages) had the same slice thickness and position as in the case of RARE imaging. Image analysis was accomplished using the CCHIPS/IDL software [Schmithorst et al. 2001]. Slices were segmented by setting an intensity threshold and infarction areas were determined using a semi-automated routine on the RARE scans. Infarct volume was calculated based on the damaged area in each slice times the distance between the slices and corrected for brain edema as described previously [Swanson et al. 1990]. Infarct
volumes were calculated as percent of the hemisphere. $T_2$ maps were calculated on a pixel-by-pixel basis from the multi echo images.

### 4.2.4 Immunohistochemistry and in situ hybridization

Animals used for immunohistochemistry for lectin and thionin staining were perfused with culture medium containing 7 U/ml heparin followed by a fixative containing 2.5% (cryostat section) or 4% (paraffin section) paraformaldehyde in phosphate buffer, pH 7.35. Brains were processed for cryostat and paraffin sectioning and processed for lectin histochemistry and in situ hybridization as previously described [Basu et al. 2002]. The plasmid for ionized calcium binding adaptor molecule-1 (Iba-1) was generously provided by Dr Yoshinori Imai (National Institute of Neuroscience, Japan).

### 4.2.5 Cerebrovascular assessment

The cerebrovasculature was examined using intracardiac injection of carbon black ink. Wt and IL-1R1 null mice were anesthetized with xylazine/ketamine and the left cardiac ventricle was perfused with 4% paraformaldehyde for 5 min, followed by perfusion with carbon black ink diluted in 4% paraformaldehyde. Brains were carefully removed, left in fixative, and the Circle of Willis and major arteries were carefully examined under a dissecting microscope.
Animals used for protein isolation were euthanized at day 2 and perfused with culture medium containing 7 U/ml heparin to remove circulating leukocytes. Cortical regions from both hemispheres (contralateral control and H/I side) were dissected and placed in 1.5 ml microfuge tubes with 150 µl of homogenization buffer (20 mM Tris, 1 mM EDTA, 255 mM sucrose with aprotinin, leupeptin, pepstatin and AEBSF). Samples were homogenized with teflon-glass homogenizer and centrifuged at 1000Xg for 10 min. Supernatant was further sonicated and then protein concentrations were determined using the Pierce BCA Protein Assay Kit according to the manufactures instructions. All tissue samples were stored at -80°C until needed. 10 µg of each sample was electrophoresed on a NuPAGE 3-8% Tris-Acetate gradient gel (Invitrogen, Carlsbad, CA) and transferred from to a nitrocellulose membrane. The membrane was then blocked in 2% nonfat dry milk in 1 X PBS-Tween-20 for 1 h at room temperature with gentle agitation. Following blocking, the blots were incubated with anti-Cox-2, purchased from Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1/1000 in 1% BSA diluent (diluent composed of 1 mg/mL BSA dissolved in 1 X PBS-Tween-20) overnight at 4°C with gentle agitation. Following extensive washes in 1 X PBS-Tween-20, blots were incubated with donkey anti-goat horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA) at a dilution of 1/20,000 in 1% BSA diluent for 1 h, with agitation. The blots were rinsed again in 1 X PBS-Tween-20. The Renaissance™ chemiluminecence reagent from New England Nuclear (Boston, MA) was used according to the manufacturer's instructions. The blots were exposed to film for 20-30 seconds. Blots were stripped (30
min at 50°C in 62.5 mM Tris-HCl pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol) and re-probed with anti-β-tubulin to determine whether the samples were loaded equivalently. The dilution of anti-β-tubulin (Santa Cruz Biotechnology) was 1/1000 in 1% BSA diluent. Optical density measurements were made using a UVP Chemi-Imaging system (Upland, CA). We had previously optimized the antibody concentration for Cox-2 so that the band intensities produced for Cox-2 were within the linear response range for the chemiluminescence method [Basu et al. 2002].

4.2.7 RNase protection assay

Animals used for RNA isolation were euthanized at 1 and 3 days after H/I, perfused with media containing 7 U/ml heparin, and then with DEPC treated PBS. Cortical regions from both hemispheres (contralateral control and H/I side) were dissected and snap frozen in 1.5 ml microfuge tube. Total RNA was purified from homogenized tissue using Trizol reagent (Life Technologies) following the manufacturer's instructions. Multiprobe DNA templates for cytokines (TNFα, IL-1β, IL-12 p40, IL-10, IL-1α, M-CSF, IL-18, IL-6, IFN-γ, MIF) and the housekeeping genes, L32 and GAPDH, were all purchased from BD Biosciences (San Diego, CA). RPA was performed according to the manufacturer's protocol. Briefly, the DNA templates used to synthesize antisense riboprobes were labeled with [α-32 P] UTP (Perkin Elmer Research Inc, Boston, MA) using T7 polymerase. Labeled probes were hybridized with 5 µg of total RNA at 56°C for 16 h. Samples were then digested with RNase A and T1, and treated with proteinase K. The remaining RNase-protected RNA duplexes were extracted
with phenol/chloroform/isoamyl alcohol (25:24:1) and resolved on 5% denaturing polyacrylamide gels. Undigested labeled probes were loaded in the gels to serve as size markers. Mouse control RNA (provided by manufacturer) and yeast tRNA were loaded in the gels to serve as positive and negative controls for the assay. Dried gels were visualized by autoradiography and quantified using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA) after an exposure of 15-20 h. The exposed phosphor screen was then captured by laser scan, and individual bands were quantified using the ImageQuant software program supplied with the scanner. In addition, steps were taken to limit experimental variability, including the running of duplicate reactions.

4.2.8 Behavioral test

The corner test was carried out before and after 7 and 30 days of recovery from H/I on both WT and IL-1R1 null mice. In the home cage, a mouse was placed between two boards each with dimension of 30 X 20 X 1 cm³ attached at a 30° angle with a small opening along the joint between the two boards. The mouse was placed halfway between the two angled boards facing the corner. The turns in one versus the other direction were recorded from twenty trials for each test. Turning movements that were not part of a rearing movement were not scored. Scoring was performed in a blinded manner to avoid bias toward any one group of animals. The corner test has been shown to be sensitive to chronic sensorimotor and postural symmetries and is highly predictive for infarct volume measured from 1 week to 90 days after H/I [Zhang et al. 2002]. The non-ischemic mouse turns either right or left randomly, while the ischemic mouse preferentially turns toward
the injured, ipsilateral side. WT and IL-1R1 null mice were tested prior to H/I insult to obtain a baseline measure of performance and then tested 1 week and 1 month following insult.

4.2.9 Statistical analyses

Statistical significance of group differences between wild-type and knockout mice with respect to cell counts was assessed using the two-sample t-test. Group differences between wild-type and knockout mice with and without H/I insult with respect to densitometric analyses was assessed using two-way analysis of variance including multiple comparison adjusted post hoc analyses based on the Tukey approach. Data from the sensorimotor test were analyzed using repeated measures analysis of variance to assess differences between wild-type and knockout mice following hypoxic/ischemic insult. The validity of all these analyses was assessed visually using box-plots. All analyses were carried out using the SAS statistical software system version 8.2 (SAS Institute Inc. Cary, NC).

4.3 RESULTS

To assess the role of IL-1 signaling in the outcome from ischemic brain damage we used mice lacking the gene for the IL-1R1 and compared their vulnerability to WT mice. We assessed damage in the two mouse populations to a mild H/I insult at the global level using MR imaging and a sensorimotor behavioral test, at the cellular level
using immunohistochemical and in situ hybridization analyses and at the molecular level using RNase protection assays and Western blot analyses to measure mRNA and protein changes for a number of inflammatory mediators. In agreement with a previous study on these mice, an analysis of the gross anatomy of the cerebrovasculature did not reveal any differences between WT and IL-1R1 null mice [Boutin et al. 2001].

4.3.1 Mice lacking the interleukin type I receptor show reduced damage in response to hypoxia/ischemic insult

To determine whether decreased IL-1 signaling through the IL-1R1 would decrease the extent of brain damage following H/I, adult WT mice and mice lacking the IL-1R1 were compared using MRI for damage following 22 minutes of H/I. Hypoxia/ischemia was induced by ligating the right carotid artery, allowing the animals to recover for 2 hours, then exposing the animals to 8% oxygen at 35.5°C for 22 minutes. The rationale for using this paradigm is that it reproducibly produces mild damage in the region of the middle cerebral artery in the hemisphere ipsilateral to the ligation [O'Donnell et al. 2002]. An analysis of core body temperature using a rectal probe did not reveal any differences in core temperature between the WT and null mice prior to insult (37.0 ± 0.05; 37.1 ± 0.41, respectively) and at 3 h (36.8 ± 0.5; 37.1 ± 0.06, respectively) and 10 h (36.5 ± 0.7; 37.0 ± 0.6, respectively) of recovery.

MRI allowed the extent of damage to be repeatedly assessed in individual mice over 2 months of recovery. A total of 61 mice were analyzed by MR imaging at 24 h of recovery. An additional 25 mice were analyzed at intervals including 1, 7, 30 and 60 days of recovery. Fig. 4-1 shows a representative comparison of three WT and three IL-
IR1 null mice 24 h following the H/I insult. The area of hyper-intense signal on T2 weighted MRI, which represents vasogenic edema, is clearly increased in the cerebral cortex and in the striatum of WT mice compared to the IL-1R1 null mice.

RARE images were used to calculate infarct volume at the times examined. **Fig. 4-2** depicts the differences in infarct volume 24 h following insult for both groups of animals. Infarct volume was reduced by approximately 60% overall in the IL-1R1 null vs. WT and was reduced by approximately 50% when the cerebral cortex was analyzed separately. These animals were then followed at longer recovery intervals to examine whether the protection was temporary or long-lasting. **Fig. 4-3** shows T2 weighted MRI images for 3 mice in each group at 24 hours, 7 days and 1 month. The early protection from H/I seen in the receptor null mice was maintained with time. Strikingly, the size of the core infarct expanded over time in 8/11 of the WT mice, whereas the area of hyper-intense T2 weighted signal decreased in size over time in the null animals. In 8/8 IL-1R1 null animals assessed repeatedly over 2 months, infarcts did not develop.

**4.3.2 Sensorimotor function is preserved in mice lacking the interleukin type I receptor following hypoxic/ischemic insult.**

The functional recovery of the brain following H/I induced damaged was assessed in WT and IL-1R1 null mice using the corner test. **Fig. 4-4** shows the results of their test scores. The IL-1R1 null mice did not show a preference in their turning at any time point examined, suggesting relatively little neurological functional deficits as a result of the H/I insult. WT mice, however, showed a statistically significant increase in turning behavior
Figure 4-1. Vasogenic edema in WT vs. IL-1R1 null mice at 24 h post hypoxia/ischemia. Panels A and B depict representative RARE images from 3 WT mice and 3 IL-1R1 null mice, respectively. H/I was induced in adult mice by a combination of common carotid ligation and exposure to 8% O2/ balance N2 for 22 min at 35.5°C. MR imaging was performed at 24 h post H/I on a 3.0 T MRI spectrometer with a gradient coil (9.5 cm aperture and gradient strength 1.1 T/m). Each mouse was imaged using a 2.5 cm slotted tube resonator. Ten continuous coronal slices, 0.5 mm thick were acquired with effective TE=69.4 ms, TR=3000 ms, slice thickness 0.5 mm, field of view 2 x 2 cm^2, matrix 256 x 256 and 8 averages in 6 min, using the rapid acquisition with relaxation enhancement (RARE) imaging sequence.
Figure 4-2. Total and cortical infarct volume for WT and IL-1R1 null mice 24 h after hypoxia/ischemia. Mean total and cortical infarct volumes were calculated from the T2-weighted MR images at 24 h post H/I by the summation of lesion areas multiplied by slice-to-slice distance. Infarct volumes were corrected for brain edema and expressed as the percentage of the contralateral hemisphere. Compared to WT animals (N=33), total infarct volumes were ~ 60% smaller in IL-1R1 null mice (n = 28) p<0.0005 (**) at 24 h post HI. IL-1R1 null mice had significantly reduced, p<0.01 (*), cortical infarct volumes at 24 h post HI. Statistical significance was assessed using a two sample t-test.
Figure 4-3

A

24 h

7 days

1 month

2 months

WT

B

24 h

7 days

1 month

2 months

IL-1R1 Null

T2 [ms]

0-48
49-64
65-80
81-96
97-112
113-128
129-255
>256
toward the damaged side at the 1 month testing time, indicating a deficit in sensorimotor function.

4.3.3 Mice lacking IL-1R1 show increased neuronal survival 2 months following H/I.

To confirm the MRI analysis we assessed the extent of damage at the cellular level. Two months following H/I, brains from WT and receptor null mice were processed for histology. Thionin staining was used to assess the degree of neuronal survival in the damaged hemisphere. Fig. 4-5 shows a representative field from both WT and IL-1R1 null mice. Neurons present in the territory affected by the insult in the IL-1R1 null mice are more numerous as well as healthier in appearance than those in the affected hemisphere of WT mice indicating that the lack of the IL-1R1 preserves neurons after a H/I insult.

Figure 4-3. Evolution of brain damage in WT vs. IL-1R1 null mice from 24 h to 2 months post H/I. $T_2$ maps of brain tissue at 24 h, 7 days, 1 month and 2 months with corresponding RARE images in one WT mouse (A) and one IL-1R1 null mouse (B). $T_2$ maps were calculated on pixel-by pixel basis from multi echo spin echo images. Color scale represents different $T_2$ values. At 2 months of recovery, none of the 8 IL-1R1 null mice exhibited any magnetic resonance image visible lesion in either cortical or subcortical areas. In contrast, 6 out of 11 WT mice exhibited cystic cavities.
Figure 4-4. Sensorimotor function is preserved in IL-1R1 null mice following H/I. The corner test was used to assess sensorimotor function. WT and IL-1R1 null mice were tested prior to H/I to obtain a baseline measure of performance and then tested 1 week and 1 month following H/I. WT mice, however, showed a statistically significant increase in turning behavior toward the damaged side at the 1 month testing time, indicating a deficit in neurological function. * $p < 0.05$ by repeated measures ANOVA.
Figure 4-5. Cortical neurons are preserved at 2 months following H/I. Thionin staining was performed after 2 months of H/I in both WT (B and D) and IL-1R1 nulls (A and C). WT mice sustain larger infarcts after 2 months of recovery when compared to IL-1R1 null mice. Neurons present in the null mice are more numerous as well as healthier in appearance than those in the damaged hemisphere of WT mice, where extensive gliosis is observed.
4.3.4 Mice lacking IL-1R1 show reduced levels of phagocytes following H/I

Microglia and invading leukocytes are a major source of IL-1 in the CNS following cerebral ischemia. Therefore, we determined whether the lack of the IL-1R1 affected the number and activational state of the microglia/macrophages in the damaged CNS. Following H/I, brains from WT and IL-1R1 null mice were stained using the GSA-IB₄ lectin. Using this histochemical stain, resting or quiescent cells exhibit long thin processes, whereas the processes shorten and thicken as the microglia become activated towards amoeboid phagocytes. As illustrated in Fig. 4-6 WT and IL-1R1 null mice had similar numbers of quiescent and activated microglia; however, IL-1R1 null mice had substantially fewer phagocytes, suggesting a lower level of neuronal death in these animals following H/I insult, that there was a reduction in macrophage infiltration, or both. In situ hybridization for Iba-1 also was performed. Iba-1 is a calcium binding protein specific for microglia, and its expression increases during microglial activation [Ito et al. 1998]. As illustrated in Fig. 4-6 (panels D and E) Iba-1 expression clearly increases in the affected hemisphere of the WT mouse while little increase is seen in the affected hemisphere of the IL-1R1 null mouse, again suggesting that microglial activation is reduced in the IL-1R1 null mice following H/I insult.

4.3.5 Mice lacking IL-1R1 show reduced mRNA levels of inflammatory mediators following H/I

To determine whether the absence of the IL-1R1 would reduce the subsequent expression of pro-inflammatory mediators, the mRNA levels for several key pro-
Figure 4-6
inflammatory cytokines were examined by RNase protection assay. WT and IL-1R1 null mice were subjected to H/I and at 18 h or 72 h following the insult, the animals were sacrificed, their brains removed and RNA was isolated from both the contralateral and ipsilateral hemispheres. As illustrated in Fig. 4-7, the damaged hemisphere of WT mice contained induced levels of IL-1β, IL-1α, IL-6 and TNFα. These cytokines also were increased in IL-1R1 null mice as a result of H/I; however, their levels rarely reach levels higher than those seen in the undamaged WT brain. Interestingly, the basal levels of these cytokines are lower in the knockout animals, suggesting that IL-1 signaling influences normal levels of a number of inflammatory cytokines. The reduced levels of these cytokines in the damaged hemisphere of receptor null mice indicate reduced neuroinflammation in response to the H/I insult. Decreased levels of cytokines was not

**Figure 4-6.** Neocortical microglia are less activated in the IL-1R1 null mice after H/I. Panels A-C depict representative examples of microglia; (A) Resting, (B) Activated and (C) Phagocytic, as revealed by tomato lectin histochemistry. (D), (E). Representative examples of sections processed for in situ hybridization for Iba1 mRNA from WT (D) and IL-1R1 null mice (E). In WT mice increased hybridization was observed in the ipsilateral side when compared with contralateral side of the same animal. In contrast, in the IL-1R1 null mice the level of hybridization was equal or less than that seen in the contralateral hemisphere of WT mice. (F) Results of cell counts for different stages of microglia at 48 h of recovery in WT and IL-1R1 null mice. *p < 0.05 by two sample t-test.
Figure 4-7

A

B

C

D

E

F

Arbitrary Units

Arbitrary Units

Arbitrary Units

Arbitrary Units

Arbitrary Units

WT-CC  WT-H/I  KO-CC  KO-H/I

WT-CC  WT-H/I  KO-CC  KO-H/I

WT-CC  WT-H/I  KO-CC  KO-H/I

WT-CC  WT-H/I  KO-CC  KO-H/I

WT-CC  WT-H/I  KO-CC  KO-H/I

WT-CC  WT-H/I  KO-CC  KO-H/I
observed uniformly as levels of MIF and M-CSF showed little or no increase as a result of H/I and also did not differ significantly between WT and IL-1R1 null mice.

**4.3.6 Mice lacking IL-1R1 show reduced levels of Cox-2 protein following H/I.**

Cox-2 is an enzyme involved in prostaglandin synthesis and has been used by numerous investigators as an index of inflammation. Cox-2 protein levels were assessed using western blot analysis on protein homogenates isolated from damaged and undamaged hemispheres of mice following an H/I insult. As depicted in Fig. 4-8, Cox-2 protein levels mirrored the changes obtained from the analysis of cytokine mRNA levels. Cox-2 was dramatically increased in the WT brain as a result of H/I. In the IL-1R1 receptor nulls the basal levels of Cox-2 were lower than those of the WT animals and although Cox-2 levels increased as a result of H/I, they did not rise substantially higher than the levels seen in the undamaged WT brain.
Figure 4-8

(A) Western blot analysis showing Cox-2 and B-Tub expression in WT and IL-1R1−/− mice under control (CC) and ischemia/reperfusion (H/I) conditions.

(B) Bar graph depicting the quantification of Cox-2 expression. Asterisks indicate statistically significant differences (p < 0.05, **p < 0.01).
4.4 DISCUSSION

Recent studies have implicated neuroinflammation in the etiology of brain damage subsequent to cerebral ischemia, however, to date there is no consensus regarding which inflammatory mediators represent key targets for pharmacological intervention. The results reported here demonstrate that mild H/I initiates a slow and progressive neurodegenerative process that leads to cystic infarcts, which can be prevented by inactivating the IL-1 type 1 receptor. We show that inactivating the gene for IL-1R1 decreases the production of numerous downstream pro-inflammatory mediators that significantly decreases the amount of neuronal damage observed following

Figure 4-8. Abrogated response of Cyclooxygenase-2 in IL-1R1 null mice after H/I. (A) Cerebral cortex from WT and IL-1R1 null (KO) mice were analyzed by immunoblot for Cox-2. Blots were reprobed for β-tubulin to establish equal protein loading. The top band in each lane represents Cox-2 ($M_r = 80$ kDa), whereas the bottom band represents β-tubulin ($M_r = 57$ kDa). Samples from 3 WT and 3 IL-1R1 null mice are depicted. For each mouse, tissue from the H/I hemisphere as well as non-damaged hemisphere (CC) were analyzed. (B) Densitometric analysis of immunoblot after H/I. Measurements of the optical densities for Cox-2 for each sample were normalized to the levels of β-tubulin. Levels of Cox-2 were significantly lower in IL-1R1 null mice under basal conditions (* $p < 0.005$) and after the insult (** $p < 0.005$) by two sample $t$-test.
an H/I insult. The observation that the perifocal area enlarges in WT mice but does not increase in the IL-1R1 null mice directly implicates IL-1R1 as a cause of the progressive neurodegeneration that ensues after a mild H/I insult. Thus, therapies designed to block signaling by this receptor will likely prevent secondary neuronal damage subsequent to cerebral ischemia. Furthermore, since IL-1R1 null mice are essentially normal, and since IL-1 is not essential for maintaining normal brain functions, therapeutic interventions targeting the IL-1R1 will likely have few serious side effects or complications. Unfortunately, there are no small molecule inhibitors presently available to antagonize this receptor.

Neuroinflammation in cerebral ischemia is thought to develop as a consequence of two sequential processes. The first process is the activation of endothelial cells, microglia and resident perivascular/parenchymal macrophages followed closely by the mobilization and recruitment of peripheral inflammatory cells into the site of damage [Barone et al. 1991; Feuerstein et al. 1998; Stoll et al. 1998]. The activated microglia are known to produce a number of pro-inflammatory mediators that increase the competence of the cerebral endothelium to recruit peripheral leukocytes, primarily polymorphonuclear leukocytes and monocyte/macrophages, into the damaged brain [del Zoppo 1994]. This inflammatory response was originally believed to be delayed and a reaction to necrosis; however, recent studies in animal models have identified leukocytes in microvessels as early as 30 minutes following a middle cerebral artery (MCA) occlusion [Garcia et al. 1994]. In the same animal model necrosis was not observed until 72 to 96 h following the MCA occlusion [Garcia et al. 1993; Garcia et al. 1995].
A number of mechanisms have been postulated to explain how leukocytes may damage the brain. These include: 1) obstructing microvessels and thus, hindering cerebral blood flow to the ischemic region following reperfusion [del Zoppo et al. 1991]; 2) production of vasoconstrictive mediators, such as superoxide anions, thromboxane A₂, endothelin-1, and prostaglandins that can alter cerebral artery vasoreactivity [Hamann et al. 1994; Hartl et al. 1996]; 3) release of cytotoxic enzymes, free radicals, and nitric oxide (NO) [Hartl et al. 1996]; and 4) release of proteolytic enzymes that can damage endothelial membranes and the basal lamina, thus altering the blood brain barrier and contributing to the formation of edema [Hamann et al. 1995; Hamann et al. 1996]. Support for the view that leukocytes can damage viable tissue during acute cerebral ischemia comes from data showing that infarct volume is reduced in neutropenic animals compared to normal controls [Bednar et al. 1991; Matsuo et al. 1994; Connolly et al. 1996]. Furthermore, in humans, a correlation between the degree of leukocyte accumulation and infarct volume has been observed by CT studies [Akopov et al. 1996]. The infiltration of leukocytes into the ischemic brain is a multi-step process [Furie et al. 1995] whereby they first marginate in the venules, adhere to endothelial cells and finally migrate into the parenchyma. At each one of these steps, their functions are regulated by inflammatory mediators such as cytokines and chemokines that are produced soon after the onset of cerebral ischemia.

IL-1 actions in ischemia and other acute brain damage have been well documented in rodents. Numerous studies have shown early increases in IL-1β expression in response to ischemia [Rothwell et al. 1993; Szafarski et al. 1995; Yamasaki et al. 1995; Bhat et al. 1996; Hagberg et al. 1996; Liu et al. 1999; Legos et al.
Furthermore, administering exogenous IL-1β exacerbates ischemic damage [Yamasaki et al. 1995], while neutralizing antibodies to IL-1β or administering the natural antagonist IL-1ra have been shown to block or reduce ischemic brain damage [Relton et al. 1992; Yamasaki et al. 1995; Loddick et al. 1996b]. The receptor responsible for the damaging effects of IL-1 following cerebral ischemia has to date, been unresolved. In this report we show that mice lacking the IL-1R1 have a reduced level of damage following an H/I insult. Our results further show that IL-1R1 null mice have: 1) a diminished activation of microglia in response to H/I, 2) an attenuated level of Cox-2 expression in response to H/I, 3) attenuated levels of pro-inflammatory cytokines, 4) preservation of sensorimotor dysfunction after H/I, and 5) protection from progressive neurodegeneration subsequent to H/I. Based on these results, we conclude that IL-1 signaling through the IL-1R1 is critical for initiating and propagating neuroinflammation and subsequent neurodegeneration observed following an ischemic insult.

The protection obtained is likely a result of diminishing the production of a number of inflammatory mediators rather than inhibiting any one signal. IL-1 increases the expression of endothelial cell adhesion proteins and the subsequent infiltration of leukocytes to sites of neurodegeneration. In an earlier study we demonstrated that the induction of V-CAM is significantly reduced in the IL-1R1 null mice compared to WT mice after a traumatic brain injury [Basu et al. 2002]. The absence of this receptor on the endothelium would decrease the extent of inflammation. Furthermore, we observed reduced recruitment of leukocytes into the damaged brain in the IL-1R1 null mice. IL-1 is also known to increase the expression of other pro-inflammatory mediators such as IL-6, TNFα and Cox-2 from microglia and other cell types. This suggests that IL-1 is at the
apex of the pyramid of cytokine signaling in the CNS. While H/I damage still induced expression of these mediators in the receptor null mice, their levels rarely reached the basal level seen in WT animals. These results suggest that a critical level of expression of some or all of these proinflammatory mediators may be necessary to induce neuroinflammation and that in the absence of IL-1 signaling these levels are not reached.

The protection obtained is not likely due to differences in cerebral blood flow (CBF). Boutin et al (2001) demonstrated that mice lacking both forms of IL-1 (α and β) exhibited dramatically reduced ischemic infarct volumes (70% reduction) compared with wild type mice, and in that communication the investigators used laser Doppler flowmetry to monitor CBF during and after insult. Doppler monitoring showed that changes in CBF during or subsequent to transient forebrain ischemia were similar in all strains. Additionally, Touzani et al., (2002,) used laser Doppler flowmetry to measure CBF during and after ischemia in IL-1R1 null mice. They did not find any significant differences in CBF between WT and IL-1R1 null mice during or after transient forebrain ischemia. These studies provide evidence that the absence or presence of IL-1 or the IL-1R1 receptor does not have a measurable effect on CBF and, hence, on the outcome of insult. Moreover, we have previously documented decreased neuroinflammation subsequent to a stab wound injury, where there should be no contribution of CBF to the extent of injury and here, we demonstrate that there is progressive neurological damage in the WT mice that correlates with the neuroinflammation. This progressive degeneration does not occur in the IL-1R1 nulls. Thus, while we did not directly measure CBF herein, there are multiple lines of evidence to reject the hypothesis that increased CBF is responsible for the neuroprotection observed.
Studies have suggested that receptors other than the type 1 receptor exist within the brain and that these other receptors are responsible for exacerbating damage after cerebral ischemia. Using MCA occlusion on IL-1R1 null mice Touzani et al., (2002) failed to observe any difference in infarct size between WT and null mice [Touzani et al. 2002]. Furthermore, addition of exogenous IL-1 to the IL-1R1 null mice exacerbated the damage seen following experimental cerebral ischemia. Taken together their results suggested that other receptors for IL-1 exist within the brain. There are two likely explanations for the discrepant results between their study and ours: 1) We assessed neuroprotection after a more mild insult than was used by Touzani et al., (2002), thus as a consequence of a more severe insult other tissue damaging mechanisms may have masked the protection afforded by deleting the IL-1R1; 2) Touzani et al., (2002) used IL-1R1 null mice on a C57BL/6/SV129 mixed background whereas we used mice that had been backcrossed 8 generations onto a C57BL/6 background. As the SV129 mouse strain is known to be one of the most resistant strains of mice to ischemic damage, achieving damage in this strain likely required a more severe insult, which, again, could have masked the contribution of IL-1β to the insult [Fujii 1997].

Progressive neurodegeneration after cerebral ischemia is not a new phenomenon, however, our data strongly implicate neuroinflammation in the second wave of cell death. Du et al., (1996) used a mild focal adult ischemia model and reported that the infarct volume increased over time. Similar results have been reported by using a perinatal model of H/I [Du et al. 1996; Geddes et al. 2001]. In neither study was the cause of the progressive degeneration identified. Supporting a role for IL-1 in this progressive degenerative process is the observation that levels of caspase 1 and of IL-1β increase for
weeks subsequent to a H/I insult [Bhat et al. 1996; Hedtjarn et al. 2002]. The observation
that the perifocal area enlarges in WT mice but does not increase in the IL-1R1 null mice
directly implicates IL-1R1 signaling as a cause of progressive neurodegeneration after
cerebral ischemia.

To determine whether the preservation of brain tissue correlated with a
preservation of neurological function we used the corner test as a measure of
sensorimotor function. We observed significant differences in the behavior of WT versus
IL-1R1 null mice in this test following an H/I insult. While no differences were observed
at early times following insult, clear differences were seen one month after insult and
these differences correlated with the increase in infarct size observed over time. These
data correlate well with those of others who noted that significant differences could only
be seen 60-90 days following the insult [Zhang et al. 2002]. By linking the extent of
damage seen by MR imaging to a behavioral test that measures sensorimotor function we
were able to demonstrate that the disruption of IL-1 signaling through the IL-1R1 led to a
functionally relevant sparing of neuronal damage following H/I insult.

The critical role of IL-1 in a number of neurodegenerative diseases including
cerebral ischemia is well documented. While the exact nature of IL-1’s actions in these
disorders is not clearly understood, most of the disorders include a neuroinflammatory
component. The results of our studies show that disrupting IL-1 signaling through the
IL-1R1 down-regulates a number of pro-inflammatory mediators, limits microglial
activation and reduces the neuronal damage observed following a mild H/I insult. Therapies
designed to inhibit signaling by this receptor will likely prove efficacious in
preventing secondary neuronal damage subsequent to cerebral ischemia. Due to the
neuroinflammatory component in a number of other neurodegenerative diseases, it is likely that such therapies also would prove useful in these disorders.

4.5 REFERENCES


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Chapter 5

Neuroprotection Following Hypoxia-Ischemia in IL-1 Type I Receptor Deficient Mice: Suppression of Inflammation and Inducible Nitric Oxide Synthase (iNOS) Production

ABSTRACT

Background and Purpose: Interleukin 1 (IL-1) is a potent pro-inflammatory cytokine implicated in multiple aspects of neurodegeneration. In the present study we investigate whether signaling through IL-1 type I receptor (IL-1R1) is essential for IL-1 neurodegenerative action in brain following mild hypoxia-ischemia (HI). We hypothesize that abrogated IL-1R1 signaling will result in failure to recruit macrophages, resulting in lower levels of inducible nitric oxide synthase (iNOS) and consequently decreased free radical damage following H/I. Methods: IL-1R1 null mice and WT mice were subjected to a mild hypoxia-ischemia insult. MRI was used to determine infarct size post H/I in both groups of mice. The quantitative RT-PCR was used to assess levels of iNOS mRNA, and Western Blot analysis was used to assess protein levels of iNOS and the glutamate transporter protein GLAST. An RNase protection assay was used to evaluate changes in chemokine mRNA expression following H/I in the two mouse groups. Results: The IL-1R1 null mice had significantly reduced infarct size at 48 h post HI. The induction of mRNA expression for several chemokines including macrophage inflammatory peptide-1 alpha (MIP-1α), macrophage inflammatory peptide-1 beta (MIP-1β), lymphotactin (Ltn), regulated upon activation normally T-expressed and presumably...
secreted (RANTES) and macrophage chemotactic peptide (MCP), was significantly reduced in the ipsilateral hemisphere of the IL-1R1 null mice compared to wild-type mice at 72 h following HI. Levels of iNOS protein and iNOS mRNA were significantly reduced in the IL-1R1 null mice. The induction of GLAST at 48 h following H/I was similar for both mice types, indicating independence of IL-1R1 signaling.

**Conclusions:** The present findings suggest that IL-1R1 signaling contributes to neurodegeneration following mild H/I via recruitment of inflammatory cells, as well as iNOS mediated free radical damage.

### 5.1 INTRODUCTION

It is well established that both acute and chronic inflammation contribute to neuronal damage in insults including cerebral ischemia, traumatic head injury, CNS infections and neurodegenerative disorders such as multiple sclerosis, Alzheimer’s and Parkinson’s diseases [Natale et al. 2003; Tan et al. 2003; Blasko et al. 2004; McGeer et al. 2004; Minagar et al. 2004]. Recruitment and infiltration of peripheral inflammatory cells as well as activation of resident microglia and perivascular macrophages initiate neuroinflammation. Proinflammatory cytokines, mainly IL-1\(\beta\) and TNF-\(\alpha\), are responsible for activation of endothelial cells, enabling them to recruit and interact with monocytes/macrophages, neutrophils and T-cells across the blood-brain barrier [Anthony et al. 1997; Glabinski et al. 1998; Bernardes-Silva et al. 2001].

The IL-1 cytokine family members include IL-1\(\alpha\), IL-1\(\beta\) and naturally occurring IL-1 receptor antagonist (IL-1ra). IL-1\(\beta\) and IL-1ra are rapidly induced in response to
experimental ischemia [Lu et al. 2003], and IL-1α and IL-1β are potent mediators of inflammation [Dinarello et al. 1993; Rothwell 1999]. IL-1 ligands, not neurotoxic alone can cause neurodegeneration via activation of microglial cells. Activated microglia have been found to secrete cytotoxic molecules such as reactive oxygen species, and glutamate, the latter interacting with N-methyl-D-aspartate (NMDA) receptors resulting in microglia-induced cytotoxicity [Piani et al. 1992; Basu et al. 2002]. IL-1 binding cites have been identified throughout the brain [French et al. 1999], and two types of IL-1 receptors have been identified so far. The IL-1 type I receptor (IL-1R1) is believed to be responsible for intracellular signaling, whereas the IL-1 type II receptor (IL-1RII) binds IL-1 but is incapable of signal transduction [McMahan et al. 1991; Labow et al. 1997].

There is accumulating evidence suggesting that nitric oxide (NO) may play a role as a mediator of ischemic brain injury depending on the cell type and nitric oxide synthase isoform by which it is produced [Sugimoto et al. 2002; Moro et al. 2004]. There are three isoforms of nitric oxide synthase (NOS): endothelial (eNOS), neuronal (nNOS) and inducible (iNOS). Among them eNOS and nNOS are constitutively expressed, while iNOS is transcribed in response to immunological stimuli [Nathan 1997]. The NO produced by eNOS and synthesized within endothelial cells is proposed to be protective due to a beneficial vasodilatory effect on the blood vessels following H/I. In contrast, NO produced by iNOS and synthesized by activated macrophages and astrocytes has been proposed to contribute to the free radical damage seen following H/I [Loihl et al. 1999]. Proposed mechanisms that connect iNOS derived NO to delayed ischemic injury include the reaction of NO with superoxide anion (O$_2^-$) to form highly reactive
peroxynitrite (ONOO⁻) [Fukuyama et al. 1998], and the inhibition of the enzymes involved in mitochondrial respiration and ATP synthesis, exacerbating the already compromised energy state of the ischemic brain [Iadecola et al. 1997; Nomura 2004].

The purpose of this study is to investigate the induction of chemokines and iNOS, both known to contribute to hypoxic-ischemic damage, and to determine whether they are regulated in response to the IL-1R1 signaling. We used IL-1R1 knock-out (KO) mice [Glaccum et al. 1997], to test the hypothesis that loss of the IL-1R1 would decrease the induction of inflammatory chemokines and iNOS, consequently providing neuroprotection following hypoxia-ischemia. The results presented in this work contribute to an understanding of the mechanism of neuroprotection afforded by abrogated IL-1R1 signaling.

5.2 METHODS

5.2.1 Animals

Adult male IL-1R1 null mice of C57BL/6 background and wild type C57BL/6 mice between 2 and 6 months of age were bred and maintained at the Hershey Medical Center by the Department of Comparative Medicine, an AAALAC accredited facility. IL-1R1 null mice were originally provided by Amgen Inc (Seattle, WA). The experiments were in accordance with research guidelines set forth by Penn State University and the Society for Neuroscience Policy on the Use of Animals in Neuroscience Research.
PCR analysis of tail DNA was used to identify mice carrying the mutant allele. DNA was isolated from each mouse by phenol chloroform extraction. Three different sets of primers were used for the PCR reaction: 5’ GAGTTACCCGAGGTCCAG 3’; 5’ GAAGAAGCTCACGTTGTC 3’; 5’ GCGAATGGGCTGACCGCT 3’. The PCR reaction was carried out under the following conditions: 94°C for 30 s, 53.5°C for 1 min, and 72°C for 1.5 min. 35 cycles were performed. The PCR products were resolved and visualized on a 1% agarose gel containing ethidium bromide and run in 1X TAE buffer. The expected finding for a type I IL-1R WT (+/+) is a single band at 1150 bp. A heterozygote (+/-) had two bands present at 1150 bp and 860 bp. An IL-1R1 null mouse (-/-) had a single band at 860 bp.

5.2.2 Induction of unilateral cerebral hypoxia-ischemia

Hypoxia-ischemia was induced in adult mice by a Levine procedure [Levine 1960] and as described previously in adult mice [O'Donnell et al. 2002]. On the morning of the experiment, animals were anesthetized with isoflurane (4% induction, 1.5% maintenance in room air), and the right common carotid artery was isolated, separated from the vagus nerve and double ligated with 4-0 surgical silk. The incision was sutured, and the animals were allowed to recover with access to food and water for 3 h. To induce hypoxia, each animal was placed in a 500 ml glass jar partially submerged in a temperature controlled water bath at 35.5 °C and exposed to 8% O₂/balance N₂ for 22 min. The water bath temperature at 35.5 °C was shown to maintain the animals core body temperature at 37.5-37.7 °C throughout the hypoxic interval in previous
experiments. Animals were allowed to recover in room air for 30 min and then returned to their cages with free access to food and water.

5.2.3 MR Imaging and infarct volume measurements

MR imaging was performed at 30 min post HI with 7 WT mice and 6 IL-1R1 KO mice. Imaging was repeated at 48 h with same animals for damage volume assessment. MR imaging was performed on a 3.0 T MRI spectrometer (Medspec S300, Bruker Instruments, Ettlingen, Germany) with a custom built gradient coil (9.5 cm aperture and gradient strength 1.1 T/m). Each mouse was imaged using a 2.5 cm slotted tube resonator. Initially, mice were imaged 30 min following H/I using a diffusion-weighted imaging sequence, four 1 mm thick slices (TR/TE=1500/68 ms, Δ=30 ms, δ=15 ms, b-value=1088.5, field of view 2 X 2 cm², 96 X 96 matrix, 0.5 mm slice separation, NEX=1, 2 min 24 s total imaging time) and isoflurane anesthesia (2%). The first slice was positioned 2 mm posterior to the rhinal fissure. Prior to imaging at 48 h, mice were anesthetized with xylazine and ketamine (2 mg/kg of xylazine, 15 mg/kg of ketamine, ip.).

For the damage assessment ten continuous coronal slices, 0.5 mm thick were acquired with effective TE=69.4 ms, TR=3000 ms, slice thickness 0.5 mm, field of view 2 X 2 cm³, matrix 256 X 256 and 8 averages in 6 min, using the rapid acquisition with relaxation enhancement (RARE) imaging sequence. To quantify T₂ weighted changes, each mouse was imaged with a T₂-weighted multi echo spin echo sequence in addition to RARE. The T₂-weighted spin echo sequence had TE=10 -152 ms and TR=3000 ms, 128 X 128 matrix size and N=15 echoes, 2 averages in 13 min, with the same slice thickness and position as
the RARE images. Image analysis was accomplished using the CCHIPS/IDL software [Schmithorst et al. 2001]. Slices were segmented by setting an intensity threshold and infarction areas were determined using a semi-automated routine on diffusion-weighted and RARE scans. Infarct volume was calculated based on the damaged area in each slice times the distance between the slices and corrected for brain edema as described previously [Swanson et al. 1990]. Infarct volumes were calculated as percent of the hemisphere. T2 maps were calculated on a pixel-by-pixel basis from the multi echo images.

5.2.4 Reverse transcriptase polymerase chain reaction

The WT C57BL/6 and IL-1R1 KO mice were subjected to hypoxia-ischemia as described above. Three WT and three IL-1R1 KO mice were sacrificed at 18 h and 72 h following injury. Cortical regions from both hemispheres (contralateral control and H/I side) were dissected and tissue samples were homogenized in Trizol reagent (Molecular Research, Bethesda, MD) (1 ml/50-100 mg tissue) using a glass-Teflon homogenizer. Total cellular RNA was isolated according to the manufacturer’s instructions. Isolated total RNA (1.0 µg) was reverse transcribed using oligo-dT and random nonamers.

Oligonucleotide primer pairs against mouse iNOS, e-NOS and housekeeping gene cyclophilin were designed, checked for specificity using the BLAST database (http://www.ncbi.nlm.nih.gov/BLAST/), and prepared in the Molecular Core Facility of the Pennsylvania State College of Medicine. Primer sequences are shown in Table 5-1. PCR parameters were established for each primer set to determine the optimal annealing
temperature and cycle number for evaluation within the linear range of amplification. Reactions were prepared and cycled on a Hybaid gradient thermal cycler using standard reaction times and temperatures for the denaturing, annealing, and extension steps. Briefly, a master mix was created containing 10X PCR buffer, dNTPs, \textit{Taq} polymerase, and \textsuperscript{32}P-labeled dCTP. PCR products were then mixed with 2X formamide running dye and heat denatured at 95 °C for 3 min; next, 7 µl samples per well were loaded onto precast 5% urea/acrylamide gels (Bio-Rad, Hercules, CA) and electrophoresed at 104 V for ~1 h. Gels were dried, placed onto a blanked PhosphorImager (Molecular Dynamics Sunnyvale, CA) screen cassette, and exposed overnight. The exposed phosphor screen was then captured by laser scan (Molecular Dynamics), and individual bands were quantified using the ImageQuant software program (Molecular Dynamics) supplied with the scanner. In addition, steps were taken to limit experimental variability, including the running of duplicate reactions and/or duplicate gel lanes.

**Table 5-1**: Oligonucleotide primers used for RT-PCR

<table>
<thead>
<tr>
<th></th>
<th>Sense</th>
<th>Antisense</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS</td>
<td>5'-CCCTTCCGAAGTTTCTGGCAGCAGC-3'</td>
<td>5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3'</td>
</tr>
<tr>
<td>eNOS</td>
<td>5'-TTCCGGCTGCCACCTGATCCTAA-3'</td>
<td>5'-AACATATGTCCTTGCTCAAGGCA-3'</td>
</tr>
<tr>
<td>cyclophilin</td>
<td>5'-CCATCGTGTACATCAAGGACTTCAT-3'</td>
<td>5'-TTGCCATCCAGGCAAGGTCTC-3'</td>
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5.2.5 RNase protection assay

Animals used for RNA isolation were euthanized at 1 and 3 days after H/I, perfused with media containing 7 U/ml heparin, and then with DEPC treated PBS. Cortical regions from both hemispheres (contralateral control and H/I side) were dissected and snap frozen in 1.5 ml microfuge tube. Total RNA was purified from homogenized tissue using Trizol reagent (Life Technologies) following the manufacturer's instructions. Multiprobe DNA templates for cytokines (MIP-1 α, MIP-1 β, RANTES, MCP, Ltn) and the housekeeping genes, L32 and GAPDH, were all purchased from Pharmingen (mCK-5b). RPA was performed according to the manufacturer's protocol. Briefly, the DNA templates used to synthesize antisense riboprobes were labeled with [α-32 P] UTP (Perkin Elmer Research Inc, Boston, MA) using T7 polymerase. Labeled probes were hybridized with 5µg of total RNA at 56°C for 16 h. Samples were then digested with RNase A and T1, and treated with proteinase K. The remaining RNase-protected RNA duplexes were extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and resolved on 5% denaturing polyacrylamide gels. Undigested labeled probes were loaded in the gels to serve as size markers. Mouse control RNA (provided by manufacturer) and yeast tRNA were loaded in the gels to serve as positive and negative controls for the assay. Dried gels were visualized by autoradiography and quantified using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA) after an exposure of 15-20 hrs. The exposed phosphor screen was then captured by laser scan, and individual bands were quantified using the
ImageQuant software program supplied with the scanner. In addition, steps were taken to limit experimental variability, including the running of duplicate reactions.

5.2.6 Western blotting

Adult WT C57BL/6 and IL-1R1 KO mice were subjected to hypoxia-ischemia as described above. Mice were sacrificed at 48 following injury. Tissue samples from each hemisphere were placed in 1.5 ml microcentrifuge tubes with 150 µl of homogenization buffer (20 mM Tris, 1 mM EDTA, 255 mM sucrose with protease inhibitor cocktail (aprotinin, leupeptin, pepstatin and AEBSF) from Sigma, 1 ml of cocktail per 20 g cells wet). Samples were homogenized and then sonicated for 10 pulses 2 X each. Protein concentrations were determined using the Pierce BCA Protein Assay Kit according to the manufacturer's instructions. All tissue samples were stored at -80°C until needed.

For Western blotting for Glutamate Aspartate Transporter (GLAST) and iNOS, 10 µg of each protein sample was resolved on a separate Nupage precast gels from Invitrogen (Carlsbad, CA). Proteins were transferred from the gel to a nitrocellulose membrane. The membrane was then blocked in 2% nonfat dry milk in 1 X PBS-Tween 20 for 1 h at room temperature with gentle agitation. After blocking, the blots for GLAST were incubated with rabbit anti – GLAST antibodies, 1:1000 (Alpha Diagnostic International, San Antonio, TX), and the blots for iNOS were incubated with rabbit anti - iNOS, 5 µg/mL (Chemicon International, Temecula, CA) diluted in 1% BSA diluent overnight at 4 °C with gentle agitation.
Following extensive washes in 1X PBS-Tween 20, blots were incubated with goat anti-rabbit horseradish peroxidase (Jackson Immunoresearch, West Grove, PA) diluted 1/10000 in 1% BSA diluent for 1 h at RT with agitation. The blots were rinsed again in 1XPBS-Tween 20. The Renaissance chemiluminescence reagent from New England Nuclear was used according to the manufacturer’s instructions. The blots were exposed to film for 20-30 s.

After the exposure, blots were stripped (30 min at 50 °C in 62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol) and reprobed with anti-β-tubulin to determine whether the samples were loaded equivalently. The dilution of anti-β-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA) was 1/1000 in 1% BSA diluent. Optical density measurements were obtained using NIH image 1.62a.

5.2.7 Statistical analysis

The choice of statistical analysis was based on normal distribution of the samples. In the case of normal distribution ANOVA with Fisher LSD post-hoc test was performed where p<0.05 was considered statistically significant. In the case of non-normal data distribution Kruskal-Wallis one way analysis of variance on ranks was performed with Student-Neuman-Keuls post hoc test, where p<0.05 was considered statistically significant. For the damage volume comparison between WT and IL-1RII null mice Wilcoxon two sample test was used. The PC-based software SigmaStat (Jandel Scientific, San Rafael, CA) was used for statistical analysis.
5.3 RESULTS

Damage from the H/I insult was initially evaluated using diffusion-weighted and quantitatively assessed using T₂-weighted imaging at 30 min and 48 h respectively. The expression of iNOS, eNOS, chemokines and GLAST in response to mild H/I insult was examined in the wild type and IL-1R1 null mice to determine whether it is dependent on IL-1R1 signaling. The mRNA levels of iNOS, eNOS and chemokine were examined using RT-PCR and RNase protection assays in the two mouse populations. Protein levels of iNOS and GLAST were determined by Western blot analyses. The specific cell types that express iNOS were determined by double labeling immunohistochemistry. Our previous analysis of the gross anatomy of the cerebrovasculature did not reveal any differences between WT and IL-1RI null mice [Basu et al. 2002], [Boutin et al. 2001].

5.3.1 Mice lacking the interleukin type I receptor show reduced damage in response to hypoxia/ischemic insult as revealed by MRI at 48 h

To determine whether decreased IL-1 signaling through the IL-1R1 would have the impact on the early changes, during the recovery period, 7 adult WT mice and 6 mice lacking the IL-1R1 were initially compared using diffusion-weighted imaging for cytotoxic edema 30 min following H/I. To further determine whether decreased IL-1R1 signaling can have an impact on the evolution of the initial cytotoxic edema into vasogenic edema, the same animals were imaged at 48 h following H/I. The initial hyperintense signal on diffusion-weighted images, representing cytotoxic edema, was observed in 5 out of 7 WT mice and in 1 out of 6 IL1-R1 null mice.
Interestingly, in some WT animals (2 out of 7) the initial cytotoxic edema was restricted mainly to the cortex, but evolved at 48 h into vasogenic edema encompassing both the cortex and the striatum, Fig. 5-1. In the rest WT mice, initial cytotoxic edema included both cortex and the striatum, and progressed into vasogenic edema encompassing both brain areas. From six IL-1R1 null mice, only one exhibited cytotoxic edema that included both the cortex and the striatum, Fig. 5-1. At 48 h post HI, however the vasogenic edema in this animal was mainly restricted to the striatum, and the cortex appeared to have normal $T_2$ values, Fig. 5-1. In the animals that did not have any hyperintense signal present on diffusion-weighted images, $T_2$ values appeared normal at 48 h following HI, indicating that initially observed protection was not followed by delayed injury.

RARE images were used to calculate infarct volume at 48 h. The IL-1R1 null mice had significantly smaller (p<0.05) total and cortical infarct volumes at 48 h post HI, Fig. 5-2. The mean infarct volumes at 48 h following insult are shown in Fig. 5-2 for both groups of animals.
Figure 5-1. Evolution of brain damage in WT vs. IL-1R1 null mice from 30 min to 48 h post HI. Diffusion-weighted images (DWI), T2 maps and T2-weighted images of one WT mouse and one IL-1R1 null mouse. Coronal brain slices from rostral to caudal area are shown from bottom to top. Hyperintense areas on DWI represent the initial cytotoxic edema. T2 maps taken at 48 h are shown in the middle, color-coded in red is the significant increase in T2-values (97-112 ms) representing vasogenic edema. T2 maps were calculated on pixel-by-pixel basis from a multi echo spin echo images. Color scale represents different T2 values. T2-weighted images used for calculation of the damaged area are shown on the right.
5.3.2 iNOS mRNA and protein levels are significantly decreased in the IL-1R1 null mice at 18 and 72 h following HI

The iNOS enzyme is involved in NO production as a part of a host defense mechanism. It is induced following ischemia, and it contributes to the pathogenesis of neuronal ischemic injury [Kader et al. 1993; Iadecola et al. 1997]. To determine whether the absence of IL-1R1 would reduce the inducible nitric oxide synthase, the mRNA levels for iNOS were examined by RT-PCR, and iNOS protein levels were assessed using Western blot analysis.

The basal levels (levels in the hemisphere contralateral to the ligation) of iNOS mRNA were significantly lower in the IL-1R1 null mice than in WT mice (p<0.05 or p<0.01) at both 18 and 72 h. In both groups of mice, levels of iNOS mRNA in the ipsilateral (damaged) hemisphere were significantly increased compared to their basal levels. Levels of iNOS mRNA induced in the damaged hemisphere in the IL-1R1 null mice were not statistically different from the levels observed in the undamaged hemisphere of the wt mice at both 18 and 72 h, Fig. 5-3. In the wild type mice there was a tendency (not statistically significant) toward increase in iNOS mRNA expression at 72 h compared to 18 h in the ipsilateral hemisphere, however in the IL-1R1 null mice there was tendency toward decrease in iNOS mRNA at 72 h compared to 18 h in the ipsilateral hemisphere.
Figure 5-2. Total and cortical infarct volumes for WT and IL-1R1 null mice at 48 h after hypoxia-ischemia. Mean total and cortical infarct volumes were calculated from the T2-weighted MR images at 48 h post H/I by the summation of lesion areas multiplied by slice-to-slice distance. Infarct volumes were corrected for brain edema and expressed as the percentage of the contralateral hemisphere. Compared to WT animals (N=7), total infarct volumes were ~ 90% smaller in IL-1R1 null mice (N=6) p<0.05 (*) at 48 h post HI, by Wilcoxon two sample test. IL-1R1 null mice had significantly reduced, p<0.05 (*), cortical infarct volumes at 48 h post HI, by Wilcoxon two sample test.
Protein levels of iNOS at 18 h in the ipsilateral hemisphere of WT mice were significantly higher, p<0.01 with a 4 fold increase, compared to the levels in the same

**Figure 5-3.** iNOS mRNA induction is depressed in IL-1R1 null mice, and eNOS induction is unchanged in IL-1R1 null mice compare to wild type. $^{32}$P-labeled RT-PCR analysis of iNOS (A) and eNOS (B) mRNA transcripts at 18 h and 72 h after hypoxia-ischemia in IL-1R1 null mice and WT mice. Samples from three wild-type (WT) and three IL-1R1 null mice are depicted. For each mouse, tissue from the nondamaged hemisphere (contralateral cortex (CC)) as well as the hypoxic-ischemic hemisphere (hypoxia-ischemia cortex (HI)) were analyzed by RT-PCR. Quantification of $^{32}$P-labeled PCR product was performed using the ImageQuant software program supplied with the PhosphorImager. Values of iNOS and eNOS mRNA transcripts were normalized to cyclophilin from the same PCR, and each value is shown as the mean ± S.E.. Statistical analysis was performed using ANOVA followed by Fisher’s LSD post-hoc test. Levels of iNOS mRNA were significantly lower in IL-1R1 null mice at 18 h in the HI hemisphere compared to the WT mice (* p<0.05), and significantly lower at 72 h (**) $p$ <0.01. There was no significant difference in e-NOS expression between IL-1R1 null mice and WT mice, and there was no significant difference between e-NOS mRNA levels in the CC vs. HI hemisphere of the same mice type.

Protein levels of iNOS at 18 h in the ipsilateral hemisphere of WT mice were significantly higher, p<0.01 with a 4 fold increase, compared to the levels in the same
hemisphere of IL-1R1 null mice, Fig. 5-4. In the IL-1R1 null mice the basal levels of iNOS were lower than those of the WT animals and although iNOS levels increased as a result of H/I, there was no significant difference from the levels seen in the undamaged WT brain.

5.3.3 eNOS mRNA levels are unchanged in the IL-1R1 null mice at 18h following H/I

The expression of eNOS following hypoxia-ischemia is thought to be beneficial, since it promotes collateral circulation and improves microvascular blood flow [Iadecola et al. 1997]. To determine whether the absence of the IL-1R1 would have any effect on the eNOS induction, the mRNA levels for eNOS were examined by RT-PCR.

There was no significant difference in the eNOS mRNA levels between the two mice types at 18 h following H/I, Fig. 5-3. There was a trend toward higher eNOS mRNA levels in the ipsilateral hemisphere in both mice types compared to the contralateral hemisphere, although this trend was not statistically significant, Fig. 5-3.

5.3.4 Mice lacking IL-1R1 show reduced mRNA levels of inflammatory chemokines at 18 and 72 h following H/I

To determine whether the absence of IL-1R1 would reduce the subsequent expression of pro-inflammatory mediators, the mRNA levels for several key pro-
Figure 5-4. Western blot analysis of iNOS and GLAST expression in the ipsilateral and contralateral cortex of WT and IL-1R1 null mice following hypoxia-ischemia. Densitometric analysis of immunoblot after stroke. Cerebral cortex from WT and IL-1R1 null (KO) mice were analyzed by immunoblot for (A) iNOS and (B) Glutamate-aspartate transporter GLAST. Blots were reprobed for β-tubulin to establish equal protein loading. Samples from 3 WT and 3 IL-1R1 null mice are depicted. For each mouse, tissue from the HI hemisphere as well as non-damaged hemisphere (CC) were analyzed, and each value is shown as the mean ± S.E.. Measurements of the optical densities for iNOS and GLAST for each sample were normalized to the levels of β-tubulin. Levels of iNOS were significantly lower in HI hemisphere of IL-1R1 null mice after the insult (* p < 0.01) and levels of GLAST were increased in the HI hemisphere of both mice type equally (* p < 0.01) using ANOVA followed by Fisher’s LSD post hoc test.
inflammatory chemokines were examined by RNase protection assay. WT and IL-1RI null mice were subjected to HI and at 18 h or 72 h following the insult, the animals were sacrificed, their brains removed, and RNA was isolated from both the contralateral and ipsilateral hemispheres. As illustrated in Fig. 5-5, the damaged hemisphere of WT mice exhibited increased levels of mRNA for MIP-1α, MIP-1β, MCP, RANTES and Ltn. The mRNA for these chemokines were also increased in IL-1RI null mice as a result of H/I, however their levels in the damaged hemisphere were significantly lower than those in the wild type animals. Interestingly, the basal levels of these chemokines are also lower in the knockout animals, suggesting that IL-1 signaling influences basal expression of these chemokines. The reduced mRNA levels of these chemokines in the damaged hemisphere of receptor null mice compared to their WT counterparts indicate reduced recruitment of inflammatory cells in response to the H/I insult.

5.3.5 Glutamate-aspartate transporter (GLAST) protein levels are unchanged in the IL-1R1 null mice at 18 h following H/I

Following cerebral ischemia, a massive increase in glutamate release has been well documented [Rothman et al. 1986]. One of the cellular adaptive responses to increased glutamate concentration, observed in glutamate treated astrocyte cultures [Duan et al. 1999], are increased levels of glutamate transporter GLAST. To determine whether lack of IL-1 receptor signaling can influence GLAST induction, GLAST protein levels were assessed using Western blot analysis at 18 h following H/I.
Figure 5-5

**MIP-1α**

- WT 18 h
- KO 18 h
- WT 72 h
- KO 72 h

**MIP-1β**

- WT 18 h
- KO 18 h
- WT 72 h
- KO 72 h

**MCP**

- WT 18 h
- KO 18 h
- WT 72 h
- KO 72 h

**RANTES**

- WT 18 h
- KO 18 h
- WT 72 h
- KO 72 h

**Ltn**

- WT 18 h
- KO 18 h
In both mice types there was a significant increase in GLAST protein levels in the ipsilateral hemisphere compared to the contralateral at 18 h following H/I, Fig. 5-4. There was a trend toward higher GLAST levels in the ipsilateral hemisphere of WT animals compared to receptor null mice, although this was not statistically significant, Fig. 5-4.

5.3.6 Immunostaining

To visually determine levels of iNOS protein and the cell type expressing it in response to hypoxic/ischemic insult, immunostaining was performed in WT and IL-1R1
null mice. Using double-labeled immunofluorescence it was found that macrophages and neurons are the predominant cell type expressing the iNOS in both mice types, Fig. 6.

5.4 DISCUSSION

This work has demonstrated significantly reduced brain injury following mild H/I as a consequence of deficient IL-1 receptor type 1 signaling. While the immune response is quick and the peak of microglial activation is around 72 h post H/I, iNOS has been shown to remain elevated in the ipsilateral hemisphere for at least one week after H/I [Mizushima et al. 2002] contributing to delayed neuronal damage [Sugimoto et al. 2002]. A number of reports have implicated IL-1 cytokines in neurodegeneration, and it was previously shown that double knockout mice deficient in IL-1α/IL-1β [Boutin et al. 2001] appear to be neuroprotected following ischemic insult. The receptor responsible for IL-1 signaling leading to neurodegeneration, as well as the mechanism underlying observed neuroprotection remained unclear. In this study, we demonstrated significantly lower levels of iNOS and chemokines as a consequence of abrogated IL-1R1 signaling. This implicates a role of IL-1R1 as an upstream signaling element involved in direct or indirect upregulation of both chemokines and iNOS. We cannot rule out the possibility that lower levels of iNOS in the IL-1R1 null mice might be a consequence of reduced chemokine mRNA expression. Our data suggest that lower chemokines levels in IL-1R1 null mice are the consequence of abolished IL-1R1 signaling, since the iNOS -/- null mice had elevated mRNA levels of chemokines (MIP-1α, MIP-1β, MCP) following MCAO despite loss of iNOS expression [Loihl et al. 1999]. It is interesting to note that
the iNOS -/- mice have lower infarct volumes compared to the WT, however the neuroprotection is only partially observed in the cortex [Iadecola et al. 1997]. Our results using the IL-1R1 null mice, as well as previous reports using IL-1α/IL-1β double knockout mice [Boutin et al. 2001], indicate significant neuroprotection in the cortex. This discrepancy suggests additional mechanisms beyond reduced iNOS mediated injury to be involved in observed neuroprotection in IL-1 and IL-1R1 null mice. The acute inflammation is one of the possible mechanisms, from the evidence that iNOS knockout mice had increased expression of chemokines involved in macrophage recruitment, and other mechanisms are yet to be determined.

The baseline expression of iNOS is significantly lower in the IL-1R1 null mice, indicating regulation dependent on IL-1R1 stimulation. Following H/I, there are probably other pathways independent of IL-1R1 signaling that contribute to iNOS induction. Similar reduction in iNOS mRNA levels was observed in IL-1α/IL-1β double knockout mice compared to WT following MCAO, where Ohtaki et al. proposed that IL-1 contributes to peroxinitrate formation after transient ischemia. Similar expression of eNOS in both mice type following H/I, rules out the possibility of downregulated eNOS levels, and consequentially increased injury as seen in mice deficient in eNOS following middle cerebral artery occlusion (MCAO) [Huang et al. 1996]. This result is expected since endothelial cells responsible for eNOS production do not express IL-1R1 [French et al. 1999], and moreover it excludes the possibility for eNOS downregulation through indirect IL-1R1 signaling.

Touzani et al. [Touzani et al. 2002], observed similarities in the infarct volumes between WT and IL-1R1 KO mice, following middle carotid artery occlusion (MCAO).
They hypothesized involvement of other putative mediators of neuronal death such as glutamate, nitric oxide, tumor necrosis factor-α and other free radicals or existence of additional IL-1 receptor. In this study we determined that IL-1R1 null mice have lower levels of iNOS protein compared to the WT, ruling out the possibility that nitric oxide is significantly contributing to neuronal death in IL-1R1 null mice. It is unlikely that glutamate mediated neuronal death is increased in IL-1R1 null since the GLAST protein levels are the same for both mice types following HI, and glutamate uptake is enhanced with iNOS blocking [Ye et al. 1996; Hu et al. 2000]. Reduced glutamate uptake as a consequence of increased levels of IL-1β and tumor necrosis factor-α (TNF-α) have been demonstrated previously [Ye et al. 1996; Hu et al. 2000; Liao et al. 2001]. The mRNA levels of tumor necrosis factor-α following H/I are significantly decreased in IL-1R1 KO mice [Basu, to be published in JCBFM, 2004], ruling out the possibility that TNF-α significantly contributes to neuronal death in IL-1R1 mice.

The existence of additional receptors for IL-1 in the brain [Touzani et al. 2002], was postulated based on the exacerbated ischemic brain damage in IL-1R1 KO mice following the intracerebroventricular injections of IL-1β and also the relatively restricted IL-1R1 distribution in the brain. The exacerbation of brain damage following IL-1 injections was based on statistical evidence, without assessment of damage prior to the second IL-1β injections and direct comparison of damage progression following IL-1β injections. The careful assessment of ischemic brain damage in vivo using MRI prior to and following IL-1β intracerebroventricular injections following mild H/I (our unpublished observation) indicate the opposite, i.e. that the ischemic brain damage is
unchanged following IL-1β intracerebroventricular injections at 48 h in the IL1-1R1 KO mice and significantly increased in WT mice.

The IL-1 type I receptor is expressed in discrete brain regions such as the hippocampus and the choroid plexus (ependymal cells but not endothelial cells) under the normal conditions, suggesting a physiologic role in these areas. It is already known that the expression of IL-1R1 under the pathological conditions is elevated [Gabellec et al. 1996], and recently Ohtake et al. [Ohtaki et al. 2003] shown progressive increase in IL-1R1 immunoreactivity in microvasculature and neuron-like cells, following transient MCAO.

5.5 CONCLUSIONS

Based on data presented here we can conclude that abrogated IL-1 type I receptor signaling is responsible for decreased hypoxic/ischemic brain damage. Observed neuroprotection is afforded by reduced levels of inflammatory chemokines and iNOS. The initial deficiency in chemokines has long-term impact through the failure of macrophage and inflammatory cell recruitment, and deficient IL-1R1 signaling disrupts the link between neuroinflammation and iNOS mediated damage. Taken together the results presented here strongly suggest that therapies targeted to antagonize IL-1R1 signaling will likely prove more efficacious than inhibiting iNOS alone in preventing secondary neuronal damage subsequent to stroke.
5.6 REFERENCES


Chapter 6

Regional Activation in the Rat Brain During Visceral Stimulation Detected by c-Fos Expression and fMRI

ABSTRACT

Brain areas activated in response to the colorectal distention (CRD) were examined in adult anesthetized rats. The aim of the study was to determine and compare the areas of brain activated in response to the noxious visceral stimuli using c-Fos protein expression and functional magnetic resonance imaging (fMRI). Methods: For the fMRI study (3.0 T magnet), anesthetized rats underwent phasic colorectal distention, synchronized with fMRI acquisition. Stimulation consisted of eight cycles of balloon inflation (30 s) and deflation (90 s). Inflation pressures of 40, 60, or 80 mm Hg were used. For c-Fos experiments the stimulus was a rectal balloon, distended to 80 mm Hg in a phasic manner with 30 s inflation and 90 s deflation, for 2 h. Results: fMRI imaging revealed activation in the amygdala, hypothalamus, thalamus, cerebellum, hippocampus, and trigeminal nuclei. In a smaller number of animals, fMRI- detected activation was also observed in the nucleus of the solitary tract, parabrachial nucleus, superior colliculus, striatum, and the insular, piriform, retrosplenial, sensory association, and perirhinal cortices. Compared with control animals, significant increases in c-Fos expression ($p=0.05$) were observed in the pontine parabrachial nucleus and the paraventricular nucleus of hypothalamus.
6.1 INTRODUCTION

Irritable bowel syndrome (IBS) is a common gastrointestinal disorder characterized by abdominal (visceral) pain and alteration in bowel habits. It is estimated that 10-22% of adults, predominantly women, suffer from IBS or other forms of functional bowel disease. Despite the high morbidity, its pathogenesis remains poorly understood. Contributing to this is the lack of a generally accepted animal model of IBS, the likely multifactorial etiology of the condition, and the fact that functional disorders of the gut are classified according to the patient symptoms rather than by biological markers [Al-Chaer et al. 2000; Mayer et al. 2002; Camilleri et al. 2004].

Visceral pain appears to be conveyed by the spinal nociceptive visceral sensory neurons located in the laminae I, V and X of the spinal cord. Information is then conveyed to higher brain structures via spino-parabrachio-amygdaloid and -hypothalamic pathways [Bernard et al. 1996]. Although not directly involved in conveying the painful stimuli, vagal afferent relays modulate nociceptive processing in the spinal cord [Mayer et al. 1994]. There is some evidence that parabrachial nuclei (PBN) along with the spino-parabrachio-amygdaloid pathway play an important role for somatic and visceral pain processing [Cechetto et al. 1985; Randich et al. 1992]. The PBN projects to the amygdala, the hypothalamus and intralaminar thalamic nuclei, an important relay to the prefrontal cortex. How activation of these afferent pathways becomes perceived as painful or non-painful sensations is not well understood [Rainville et al. 1997; Monnikes et al. 2003].
Studies in human volunteers using positron emission tomography (PET) [Silverman et al. 1997] and fMRI [Mertz et al. 2000] indicated changes in the central nervous system (CNS) processing of noxious rectal stimuli in IBS. One study demonstrated anterior cingulate cortex (ACC) activation in response to painful rectal distention in healthy but not in IBS subjects, using PET imaging. Another study, using fMRI, also showed activation of ACC in healthy subjects, but even more activation in the ACC of IBS patients [Mertz et al. 2000]. Other areas commonly reported to be activated in response to painful rectal balloon stimulation were the insular cortex, prefrontal cortex and the thalamus [Silverman et al. 1997; Mertz et al. 2000], but the results were inconsistent.

It is generally accepted that the symptoms of IBS result from a response to a visceral stimulus. The mechanisms and pathways involved in the sensation of visceral pain remain poorly understood [Ness et al. 1987; Ness et al. 1988; Traub et al. 1993; Traub et al. 1996; Silverman et al. 1997; Mertz et al. 2000; Mayer et al. 2002]. C-Fos protein expression has been widely used to identify many brain structures involved in the visceral pain processing in animals but cannot be applied to humans. Non-invasive methods such as fMRI and PET are available to study brain activation in humans. It has not yet been determined if the activation detected by c-Fos protein expression corresponds to the activation detected by fMRI. Development of an animal model that enables the study of the brain–gut alterations is important for the study of pathophysiology of the IBS, and will allow testing of novel pharmacological approaches.
The aim of this study was to examine brain activation in response to noxious visceral stimuli using fMRI and c-Fos immunohistochemistry, and to compare the results obtained by both methods. We tested the hypothesis that c-Fos expression and fMRI brain activation detect similar brain nuclei in response to colorectal distention in the anesthetized rats.

Some preliminary results have been presented previously [Wrzos et al. 2001; Lazovic-Stojkovic et al. 2002; Wrzos et al. 2002].

6.2 MATERIALS AND METHODS

6.2.1 Procedure

All procedures were approved by the Institutional Animal Care and Use Committee of the Penn State College of Medicine. Male Sprague-Dawley rats (Charles River) weighing 200-450 g were kept in groups of 5 in stainless steel cages on a 12:12 light/dark cycle at 22-25°C with 60% humidity. Water and rodent pellets were available ad libitum.

6.2.2 Anesthesia

For each experiment, the animals were anesthetized with chloral hydrate (400 mg/kg, ip.). The rats received additional ip injections of chloral hydrate (32 mg) to maintain a level of anesthesia that eliminated the hind limb pinch-withdrawal reflex. Chlortal hydrate was selected because it has an intermediate effect on c-Fos expression,
less that several other anesthetics used for MRI studies (e.g. halothane, urethane, a-chloralose, pentobarbital [Takayama et al. 1994; Rocha et al. 1997] and it has been used for fMRI studies in children [Altman et al. 2001].

After the induction of anesthesia, a 4 – 5 cm long lubricated, latex balloon was inserted in the rectum and descending colon, 1-2 cm proximal to the anal verge. The tubing leading to the balloon was taped to the base of the tail.

### 6.2.3 fMRI study

After balloon insertion, anesthetized rats were placed in a stereotaxic frame and in the imaging coil. The balloon was attached to a barostat with tygon tubing (5/16” outer diameter and 1/16” wall thickness). The barostat was interfaced to the MRI system console to synchronize distention and deflation cycles with image acquisition. The fMRI paradigm consisted of eight inflation and deflation cycles consisting of 90 s baseline period with the balloon deflated, during which time 9 images were acquired, followed by a 30 s activation or inflation period (during which 3 images were obtained), for a total of 16 min for each of 3 pressure levels. The desired pressure levels of colorectal distention were achieved by rapidly inflating the balloon controlled by the barostat. The pressure levels studies were 40 mm Hg (4 studies), 60 mm Hg (4 studies), and 80 mm Hg (9 studies), with some of the rats being subjected to more than one pressure level. Neuronal responses specific to inflation were determined by subtracting the effects of the control (balloon insertion without inflation).
To minimize signal loss due to susceptibility artifacts at air-tissue interfaces (sinus cavities), the axial plane was chosen for the fMRI study. Ten axial (0.5 mm thick) slices were positioned relative to the bregma. Anatomical $T_2$-weighted images (effective TE/TR=147.2/2700 ms, field of view 3 x 3 cm$^2$, matrix size 256 X 256, 8 averages) were acquired in 6 min using the RARE imaging sequence. Functional images were obtained using a $T_2^*$-weighted echo planar imaging sequence (effective TE/TR=35/1250 ms, 128 X 92 matrix size zero-filled to 128 X 128, 8 averages) 10 s for one image, and with the same slice position, slice thickness, field of view as the anatomical images.

Post processing and analysis of fMRI data were performed using the CCHIPS software [Schmithorst et al. 2001]. Image co-registration and motion corrections were achieved with a pyramid co-registration algorithm [Thevenaz et al. 1998]. Signal intensity acquired during “off” cycles and “on” cycles was cross-correlated with the stimulation paradigm. The cross-correlation coefficient ($r$) value of the pixels between $r=0.4$ and $r=0.7$ is considered statistically significant, and is shown as color-coded on the image. Colored areas (pixels) represent the activated regions. Total numbers of activated pixels for brain structures that extended over more than one slice was determined by adding activated pixels per each slice.

### 6.2.4 c-Fos expression study

Anesthetized rats were kept unrestrained. Two sets of experiments were performed. In the first set, “A”, 5 rats without balloon insertion, control (A) no balloon were compared with 5 rats who underwent insertion of the balloon, without inflation.
After 2 h, rats from both groups were euthanized with Nembutal (100 mg/kg, \textit{ip}) and perfused transcardially with 4% paraformaldehyde. The brains were removed, and processed for c-Fos protein detection by immunohistochemistry (see below).

In the second set of experiments, “B”, 10 rats with balloon insertion without inflation, control (B) balloon were compared with 10 rats with balloons that were inserted and inflated using the phasic distention paradigm, experimental (B). The distention paradigm consisted of 2 h of colorectal distention (CRD) during which the balloon was inflated and then deflated with the cycle of 30 s inflation to a pressure of 80 mm Hg and 90 s deflation, using a computer controlled barostat (Distender, G & J Electronics, Inc). At the end of two hours of either colorectal distention or non-distention, all rats were euthanized with Nembutal (100 mg/kg, \textit{ip}) and perfused transcardially with 4% paraformaldehyde. The brains were removed, and processed for c-Fos protein detection by immunohistochemistry. The brains were postfixed in 4% paraformaldehyde for 4 – 24 h at 4°C and cryoprotected in 30% sucrose in phosphate buffer overnight. The brains were sectioned into 50 μ coronal sections, which were incubated with rabbit polyclonal anti-c-Fos antibody (1 mg/ml, SC 52, Santa Cruz, CA), followed by biotinylated goat anti-rabbit IgG (Zymed, CA) and avidin-biotin horseradish peroxidase complex (ABC, Vector Labs, CA). Reaction product was shown by using 3,3'-diaminobenzidine (Sigma) enhanced by nickel ammonium sulfate and cobalt. The anatomy was confirmed using cresyl violet staining, and control tissue was processed without using anti-c-Fos antibody.
6.2.5 Quantification of the c-Fos positive nuclei [Traub et al. 1992]

Brain sections of interest were digitized from light microscopic images using a Color Digital Camera (Hitachi Instruments), and c-Fos positive neurons were counted (Optimas 6.2). Brain areas and cytoarchitectural subdivisions were identified based on the atlas of Paxinos and Watson [Paxinos et al. 1986]. Brain sections with the highest number of c-Fos positive cells for each respective nucleus, or nucleus subdivisions, were chosen for counting. Cell nuclei were automatically counted as c-Fos positive if the pixel density reached 200% of the background. The sections were re-counted by hand to include overlapping c-Fos positive nuclei. Values were presented as mean ± SEM (standard error of the mean) for the control and experimental rats for the specific nuclei. For statistical analysis the number of positive nuclei in the control and experimental animals were compared using t-tests at the \( p \leq 0.05 \) as the level of significance.

6.3 RESULTS

6.3.1 fMRI

No activation in the brain was observed when 40 mm Hg balloon pressure was used (Fig. 6-1A). The total number of activated pixels in response to a distention pressure of 60 mm Hg, for each region for each animal, is shown in Table 6-1, and in response to an 80 mm Hg distention stimulus in Table 6-2. All brain areas activated during 60 mm Hg distention (Fig. 6-1B, Fig. 6-2A) were also activated during 80 mm Hg
distention (Fig. 6-1C, Fig. 6-2B), with greater number of pixels activated during 80 mm Hg distention. The difference between the numbers of activated pixels at 60 and

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**Figure 6-1.** Axial fMRI images of the rat brain with no activation during 40 mm Hg CRD (A) and with activated hypothalamus (yellow circle) and the amygdala (red circles) during the rectal balloon stimulation at the pressures of 60 mm Hg (B) and 80 mm Hg (C), of the same animal. Activation of the nucleus of the solitary tract during the 80 mm Hg pressure is also shown (blue circle) and activation of the trigeminal nucleus during 60 and 80 mm Hg (white circles). Signal intensity acquired during “off” cycles and “on” cycles at corresponding areas is cross-correlated with the stimulation paradigm. When the cross correlation coefficient of particular pixel ($r$) reaches statistical significance ($0.4 < r < 0.7$), pixel appears as color-coded on the image. Colored pixels represent the activated regions. The color scale, shown on the right represents different values of the cross correlation coefficient.
80 mm Hg was not statistically significant. In individual rats, there were areas that were activated at 80 mm Hg but not at 60 mm Hg distention, but this was not a consistent finding for all of the rats. Different colors shown on Fig. 6-1 and Fig. 6-2 represent different values of the cross correlation coefficient (see Methods). All rats undergoing

**Figure 6-2.** Axial fMRI images of the rat brain with activated thalamus (yellow circle), periaqueductal gray (green circle) and superior colliculus (red circle) during the rectal balloon stimulation at the pressure of 60 mm Hg (A) and 80 mm Hg (B), of the same animal. During 80 mm Hg pressure there was additional activation in the cerebellum (pink circle), hippocampus (white circle) and prelimbic and infralimbic structures (blue circle) for the particular animal (B). The color scale, shown on the right represents different values of the cross correlation coefficient.
CRD with 60 and 80 mm Hg pressure exhibited activation in the amygdala and paraventricular nucleus of hypothalamus (PVN) (Table 6-1 and Table 6-2, Fig. 6-1B and Fig. 6-1C). Additionally,

**Table 6-1:** Number of pixels in the rat brain areas activated during CRD at the level of 60 mm Hg observed in fMRI.

<table>
<thead>
<tr>
<th>Brain Area</th>
<th>Number of pixels activated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat No.</td>
<td>1</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>37</td>
</tr>
<tr>
<td>Thalamus</td>
<td>15</td>
</tr>
<tr>
<td>Amygdala</td>
<td>40</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>9</td>
</tr>
<tr>
<td>Insular cortex</td>
<td>5</td>
</tr>
<tr>
<td>Piriform cortex</td>
<td>3</td>
</tr>
<tr>
<td>Retrosplenial cortex</td>
<td>6</td>
</tr>
<tr>
<td>Sensory cortex</td>
<td>4</td>
</tr>
<tr>
<td>Perirhinal cortex</td>
<td>2</td>
</tr>
<tr>
<td>Entorhinal cortex</td>
<td>5</td>
</tr>
<tr>
<td>Temporal lobe</td>
<td>6</td>
</tr>
<tr>
<td>Caudate/Putamen</td>
<td>4</td>
</tr>
<tr>
<td>Globus pallidus</td>
<td>3</td>
</tr>
<tr>
<td>Superior colliculus</td>
<td>7</td>
</tr>
<tr>
<td>Trigeminal n.</td>
<td>43</td>
</tr>
<tr>
<td>Parabrachial n.</td>
<td>2</td>
</tr>
<tr>
<td>Solitary n.</td>
<td>8</td>
</tr>
<tr>
<td>Periaqueductal grey</td>
<td>4</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>30</td>
</tr>
</tbody>
</table>
in 4 of 4 rats treated with 60 mm Hg CRD and 8 of the 9 rats treated with CRD at 80 mm 
Hg pressure, activation occurred in the hippocampus, cerebellum and trigeminal nerve 
(Fig. 6-1 and Fig. 6-2, Fig. 6-1 and Table 6-2).

Table 6-2: Number of pixels in the rat brain areas activated during CRD at the level of 80 
mm Hg observed in fMRI.

<table>
<thead>
<tr>
<th>Brain Area</th>
<th>Number of pixels activated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat No.</td>
<td>1  2  3  4  5  6  7  8  9</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>75 20 19 6 3 16 15 34 35</td>
</tr>
<tr>
<td>Thalamus</td>
<td>53 12 17 10 2 7 2</td>
</tr>
<tr>
<td>Amygdala</td>
<td>100 19 25 17 8 41 52 67</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>24 14 25 7 6 15 17</td>
</tr>
<tr>
<td>Insular cortex</td>
<td>13 4 4 8</td>
</tr>
<tr>
<td>Piriform cortex</td>
<td>2 4 5</td>
</tr>
<tr>
<td>Retrosplenic cortex</td>
<td>15 3 5 15 6 3</td>
</tr>
<tr>
<td>Sensory cortex</td>
<td>5 17 2 10</td>
</tr>
<tr>
<td>Perirhinal cortex</td>
<td>20 8 3</td>
</tr>
<tr>
<td>Entorhinal cortex</td>
<td>6 26 7 8 4 3 7</td>
</tr>
<tr>
<td>Temporal lobe</td>
<td>7 2</td>
</tr>
<tr>
<td>Caudate/Putamen</td>
<td>8 22 10 25</td>
</tr>
<tr>
<td>Globus pallidus</td>
<td>23 10 5</td>
</tr>
<tr>
<td>Superior colliculus</td>
<td>17 27 22 10</td>
</tr>
<tr>
<td>Trigeminal n.</td>
<td>47 35 45 40 31 39 48 4</td>
</tr>
<tr>
<td>Parabrachial n.</td>
<td>5 3 3 4 7</td>
</tr>
<tr>
<td>Solitary n.</td>
<td>15 10 15 18 2 4</td>
</tr>
<tr>
<td>Periaqueductal grey</td>
<td>16 3</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>87 91 41 12 57 23 7 4</td>
</tr>
</tbody>
</table>

The thalamus was activated in 3 of 4 rats treated with 60 mm Hg and 7 of 9 rats treated 
with 80 mm Hg (Fig. 6-2, Table 6-1 and Table 6-2). In the 4-5 of the 9 animals, 
subjected to the 80 mm Hg, the caudate, putamen, periaqueductal gray, and the insular,
retrosplenial, entorhinal, perirhinal, and sensory association cortices were activated (Table 6-1 and Table 6-2). The parabrachial nucleus was activated in 5 out of 9 animals subjected to 80 mm Hg (Table 6-2).

A typical time-curve of fMRI signal intensity of all activated areas is shown in Fig. 6-3.

**Figure 6-3.** A typical time-curve of fMRI signal intensity changes during the rectal balloon stimulation, plotted as the averaged signal intensity of all activated areas. The x-axis represents the image number (96 total number of images) and y-axis represents the averaged signal changes during the rectal balloon stimulation. The balloon was inflated during the images: 10-12, 22-24, 34-36, 46-48, 58-60, 70-72, 82-84, and 94-96.
6.3.2 c-Fos Expression

6.3.2.1 Experiment A

Specific nuclei examined and counted, included the amygdala, paraventricular nucleus of the thalamus (PVP), pontine parabrachial nuclei (PBN), the paraventricular nucleus of hypothalamus (PVN) and the nucleus of the solitary tract (NTS). These nuclei were chosen based on our own fMRI data and on published CNS visceral afferent projections [Traub et al. 1996]. Numbers of activated c-Fos neurons in rats without balloon inserted, control (A), compared with the rats with balloon inserted, but not inflated (first c-Fos experiment), are shown in Table 6-3. Statistically significant differences were observed in the paraventricular nucleus of thalamus (PVP), 69.2±12.2 vs 169.4±20.2 (p=0.003, N=5); and the central nucleus of amygdala on the right side (CeAR), 63.8±6.3 vs 136±26.8 (p=0.05, N=5). On the left side, at the CeAL, the difference was not statistically significant [91.8±29.4 vs 120.7±35.7, p=0.5, N=5]. There was also no significant difference at the PVN, PBN or area postrema (AP). Other areas with neurons expressing c-Fos were also observed (PVN, PBN), but the differences between controls without balloon and the rats with a balloon inserted but not inflated were not significant.
6.3.2.2 Experiment B

Numbers of activated c-Fos neurons in the rats with an inflated balloon compared with the control rats with a control (B), un-inflated balloon, are shown in Table 6-4. Statistically significant differences in c-Fos expression between control (B) and experimental rats were observed in the left parabrachial nuclei (PBN L) 93.1±11.8 vs.
132.3±14.4 (p=0.05, N=10) and in the right paraventricular nucleus of hypothalamus (PVN R) 264.1±66.9 vs. 487.2±85.5 (p=0.05, N=10), control (B) versus experimental, respectively.

**Table 6-4:** Number of c-Fos LI in the rat brain areas activated during CRD: control rats with the inserted balloon (no distention) vs. distended balloon at the pressure of 80 mm Hg.

<table>
<thead>
<tr>
<th>Brain Area</th>
<th>Control (B) Balloon not distended</th>
<th>Experimental Distention</th>
<th>p -value (N=10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVN L</td>
<td>250.6 ± 61.7</td>
<td>412.2 ± 66.5</td>
<td>0.09</td>
</tr>
<tr>
<td>PVN R</td>
<td>264.1 ± 66.9</td>
<td>487.2 ± 85.5</td>
<td>0.05</td>
</tr>
<tr>
<td>CeA L</td>
<td>89.8 ± 24.5</td>
<td>93.6 ± 24.7</td>
<td>0.8</td>
</tr>
<tr>
<td>CeA R</td>
<td>93.9 ± 24.6</td>
<td>88.9 ± 20.6</td>
<td>0.9</td>
</tr>
<tr>
<td>PVP</td>
<td>110.1 ± 20.1</td>
<td>128.8 ± 18</td>
<td>0.5</td>
</tr>
<tr>
<td>PBN L</td>
<td>93.1 ± 11.8</td>
<td>132.3±14.4</td>
<td>0.05</td>
</tr>
<tr>
<td>PBN R</td>
<td>72.9 ± 12.2</td>
<td>99.2±9.4</td>
<td>0.1</td>
</tr>
<tr>
<td>AP</td>
<td>161.5 ± 28.4</td>
<td>200 ± 32.8</td>
<td>0.4</td>
</tr>
</tbody>
</table>
An example showing c-Fos expression indicating activated neurons in PBN and PVN in control (B) rats (balloon inserted, not inflated) compared with experimental rats (balloon inserted and inflated) is shown on Fig. 6-4.

In the amygdala, thalamus, and NTS, c-Fos activation was not significantly different between experimental and control (B) rats. In the amygdala, intense activation was seen in both control and experimental animals (Table 6-4). Similar activation seen in

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**Figure 6-4.** Representative digital micrographs of the brain sections immuno-labeled for c-Fos protein. Examples of the neurons labeled for the Fos are indicated by arrows. Shown: paraventricular nucleus of the hypothalamus (left and right sides) of control animal (A) (100 X magnification), and of an experimental animal (B) and parabrachial nucleus (left and right sides) of control animal (C), and of the experimental animal (D) (100 X magnification).
both control (B) and experimental rats in the thalamic paraventricular nucleus (PVP), supraoptic nucleus, piriform cortex, cortical amygdaloid area, insular cortex, habenula, and the thalamic mediodorsal nucleus.

6.4 DISCUSSION

Our study compared two different markers of neuronal activation, fMRI and c-Fos expression, in the brain of rats subjected to noxious visceral stimulation. The results support partially the initial hypothesis that both methods should detect similar brain nuclei in response to the same stimulus pressure. Nevertheless, certain differences were observed. The stimulus could not be identical because the two techniques required different time course for the markers of activation to be detected. fMRI studies permit the detection of a response to more than one stimulus pressure in an individual animal.

The methods detect different aspects of neuronal activation. With c-Fos method, a specific nuclear protein is detected immunohistochemically and presumed to reflect somal electrophysiological activity. More than an hour is needed for the protein expression of c-Fos protein to reach the levels necessary for immunohistochemical detection [Traub et al. 1992], whereas in fMRI, the time course for changes in blood oxygenation levels is a matter of seconds. In fMRI, indirect assessment of neuronal activity is measured as changes in blood oxygenation levels dependent on regional blood flow, without specifically assigning that activity to somas or terminal arborizations. Another possible explanation for observed differences in the number of activated areas is
that anesthesia may influence c-Fos expression and brain hemodynamics coupled to the neuronal activation in different way.

Using c-Fos, significant differential activation in response to control (B) balloon and experimental balloon conditions was restricted to the paraventricular nucleus of hypothalamus and parabrachial nuclei. Activation in the thalamic paraventricular nucleus (PVP), supraoptic nucleus, piriform cortex, cortical amygdaloid area, insular cortex, habenula, and the thalamic mediodorsal nucleus was similar in both control and experimental balloon rats. It is unclear if this is a result of anesthesia or stress prior to the induction of anesthesia.

In our fMRI studies, an increased number of pixels (larger activation area) was consistently observed with increasing distention pressure in the activated areas, suggesting an increase in the number of neurons involved in visceral nociception. This observation is in accord with the study of Sengupta and Gebhart [Sengupta et al. 1994], where an increase in mechanoreceptor discharge rate in the gut occurred with an increase in colorectal distention pressure. “High-threshold” nociceptive receptors responded to mechanical stimuli (colorectal distention) within the noxious range, while “low-threshold” receptors encoded the stimulus from innocuous up to the noxious range. When the number of c-Fos positive nuclei was determined [Monnikes et al. 2003] in response to different pressures of colorectal distention, an increasing number of c-Fos positive nuclei was observed with increasing pressures (from 10 mm Hg to 40 mm Hg and 70 mm Hg) in NTS, rostral ventrolateral medulla, nucleus cuneiformis (NC), periaqueductal gray, and the amygdala.
In this fMRI study, the 40 mm Hg pressure did not produce a response, while a response was observed following a stimulus of 60 mm Hg and greater. In the study by Bernard et al [Bernard et al. 1994], in non-anesthetized rats, the threshold for the neuronal response was between 25 and 100 mm Hg with a mean pressure threshold of 56±24 mm Hg. Our threshold value was similar, suggesting that chloral hydrate anesthesia used in our experiments had minimal effect on the neuronal threshold. No differential activation was observed during the 40 mm Hg stimulus. That allows us to conclude that the “lower threshold” receptors might be inhibited by the chloral hydrate anesthesia. Even though rats were anesthetized, activation of the amygdala revealed by fMRI might indicate an emotional-affective aspect of visceral stimulus, as extensively demonstrated with somatic stimuli [Bernard et al. 1992]. In our c-Fos studies, control animals that underwent balloon insertion without distention had shown many activated neurons in the amygdala. If balloon insertion is highly stressful for the animal, it may produce a “ceiling effect”, precluding the detection of a statistically significant difference in response to the balloon distention [Bohus et al. 1996]. Stam et al [Stam et al. 2002] found significantly more c-Fos labeled nuclei in the amygdala of rats that had their first stressful experience when exposed to CRD compared with the rats that have been exposed to foot shock stress for two weeks (under anesthesia) prior to CRD. They concluded that the differences observed under anesthesia indicate that long-lasting stress-induced alterations in cortico-limbic response to colonic distention do not necessarily require conscious affective responses.

Significant activation of the NTS was observed when detected using fMRI [Hylden et al. 1989; Esteves et al. 1993], whereas the difference between the number of
c-Fos-positive cells found in the central NTS in experimental rats was not statistically significant compared with control rats. This may be due to the high within-group variance. In contrast, significant activation in the PBN was noted in the c-Fos studies, but only in few rats by fMRI. The limitations of fMRI spatial resolution may have hindered observing PBN activation in more animals, especially because that analysis was performed in the axial plane in which the cross-sectional area of the PBN is the smallest. The resolution limits the ability to detect very small nuclei and is one advantage for the c-Fos method.

Similar studies performed by others in non-anesthetized rats identified c-Fos activation in infralimbic and prelimbic cortices, the mediodorsal thalamic nucleus, the central amygdaloid nucleus, dorsomedial and ventromedial nuclei of the hypothalamus (DMH and VMH respectively), NTS, rostral ventrolateral medulla, NC and periaqueductal gray [Traub et al. 1996; Monnikes et al. 2003]. We did not observe consistent cortical activation with c-Fos technique. This probably resulted from the use of chloral hydrate anesthesia that has been reported to hinder the cortical activation [West 1998].

The observed cortical fMRI activation included insula, retrosplenial, piriform, and perirhinal cortex previously identified to play a part in the emotional component of a painful stimulus [Strigo et al. 2003]. We also observed activation of the cerebellum by fMRI in rats. Activation of cerebellum in response to visceral pain has been reported previously in human fMRI studies [Ladabaum et al. 2000; Lotze et al. 2001; Strigo et al. 2003].
These studies demonstrate advantages and disadvantages of two methods of detecting neuronal activation. FMRI is non-invasive, and applicable for human studies. FMRI studies in animals require use of anesthesia, however, hindering activation of certain areas. The superior spatial resolution of c-Fos over fMRI facilitates detection of activation of very small nuclei and single neurons involved, but is not applicable to humans.

6.5 CONCLUSIONS

We conclude that the activation observed by fMRI included brain areas detected by c-Fos expression in addition to brain areas that did not stain differentially for c-Fos compared with controls. These findings suggest that fMRI is able to detect a larger number of brain areas activated, therefore making it a more sensitive method under the given anesthesia conditions. Some similarities of regional brain activation in response to gut stimuli do exist when assessed by fMRI and c-Fos expression, despite differences in the resolution, methodology and physiology of the mechanisms behind the detection by these methods. The superior resolution of c-Fos combined with fMRI sensitivity would be the best method to evaluate whether an animal model show CNS activation that parallels the altered CNS processing of visceral stimuli in functional disorders such as IBS. The c-Fos and fMRI data taken together imply the importance of the spino-parabrachio-amygdaloid pathway in visceral nociception, indicating that the inhibition of the specific steps of the pathway may have potential in the visceral pain management.
6.6 REFERENCES


Chapter 7

Discussion

This thesis research approaches three problems: multiple animal imaging, changes following hypoxia-ischemia and visceral pain processing, using different components of magnetic resonance imaging. Original to this thesis work, a novel coil design has enabled an efficient approach to study the effectiveness of pharmaceutical agents following neonatal hypoxia-ischemia. This technology advance made it possible 1) to determine initial changes under the same experimental conditions, which is extremely important following H/I, and 2) to significantly reduce the experimental time, enabling the study of two different agents in a short period of time. The newly developed technology was used to investigate several questions. The first one was to study early changes, 30 min following neonatal H/I, the second one was to determine the neuroprotective effect of two drugs: minocycline and aminoguanidine, and the third one was to study early changes, 30 min following adult H/I. Despite our initial hypothesis that both minocycline and aminoguanidine should be neuroprotective and significantly reduce the volume of vasogenic edema following H/I, both drugs have not shown any significant neuroprotective effect in comparison to control animals treated with saline and PBS. The finding of the study, even though negative for the drug efficacy, is valuable since it contributes to understanding the mechanism behind the neuroprotection observed independent of the drug action. The other possibility for the results that we cannot rule out is animal-to-animal variability, including the subtle differences in the blood vessels
and consequently the blood flow, which could easily account for the different outcome following H/I. In the future a combination of MRI and RNA microarray analysis may be a powerful combination to first determine the initial changes following H/I non-invasively, and then to determine potential gene candidates involved in neuroprotection, in animals where spontaneous neuroprotection is observed. The greater complexity of the human genome than that of rodents, and differences between animal models and actual neonatal H/I in human newborns make advances in neuroprotective strategies a very challenging task.

In the second part of the thesis research we gained new insights into the role of IL-1 type 1 receptor signaling. We have demonstrated for the first time that the IL-1 receptor is responsible for numerous inflammatory actions following adult H/I. Additionally, this work has demonstrated reduced cytotoxic edema and significantly reduced ischemic brain injury at 48 h following mild H/I as a consequence of deficient IL-1 receptor type 1 signaling. Following H/I, ATP depletion results in Na⁺/K⁺ ATPase failure and water influx into the cell causing the cytotoxic edema. The precise mechanism by which disrupted IL-1R1 signaling contributes to preservation of brain energy metabolism remains to be determined. The preserved function of Na⁺/K⁺ ATPase in IL-1R1 animals following H/I may point toward a new mechanism independent of the inflammatory role of IL-1 cytokines involved. The results of in vitro work [Vega et al. 2002], point toward the increase in glucose utilization as a consequence of IL-1α and TNF-α cytokine treatment and involvement of Na⁺/K⁺ ATPase as a potential target. The reduced cytotoxic edema in the IL-1R1 null mice is difficult to explain as a consequence of reduced iNOS production and reduced inflammation since the onset for both events is
at least a few hours following H/I. The discrepancy between neuroprotection observed in iNOS -/- mice and IL-1R1 -/- mice suggests that additional mechanisms beyond reduced iNOS mediated injury are involved in observed neuroprotection in IL-1 and IL-1R1 null mice. Extensive cortical injury can occur as a consequence of enhanced glutamate excitotoxicity by injections of IL-1β into the striatum or hypothalamus [Allan et al. 2000]. We propose that due to the diminished IL-1R1 signaling, these distant actions of IL-1β are disrupted providing the protection in the cortex. The increase in damage observed in some wild type animals in the period between 30 min and 48 h following H/I suggests that the inflammatory response and NO-mediated free radical damage are responsible for increasing the severity of brain damage after H/I. The main conclusion from the second part of the thesis is that therapies based on IL-1R1 antagonism may prove more efficient than inhibiting iNOS alone. The further insights into the mechanism by which disrupted IL-1R1 signaling contributes to reduced cytotoxic edema, may represent the link between the inflammatory role of IL-1 cytokines and their role in maintaining the homeostasis.

Visceral pain processing still remains to be understood. In the third part of this thesis we have focused on the visceral response in anesthetized animals using two techniques. The high sensitivity of fMRI to motion has precluded study of the visceral pain response in non-anesthetized animals. This creates a severe limitation in the ability to extend the conclusions beyond unconscious visceral pain perception. Even though we are not able to hypothesize about conscious visceral pain perception, several brain areas that may serve as potential therapeutic targets have been identified. Among them is the amygdala, involved in emotional-affective aspect of the visceral pain, as extensively
demonstrated with somatic pain [Bernard et al. 1992] in humans. There is increasing evidence that stressful life events may contribute to IBS etiology [Chang 2004; Lea et al. 2004], and for a role of the amygdala in the stress induced development of IBS [Greenwood-Van Meerveld et al. 2001; Monnikes et al. 2001]. The activation of the amygdala (c-Fos expression) observed after balloon insertion itself, indicates this is a highly stressful event, but also points out the role of the central amygdaloid nucleus as an important structure for mediating coping processes [Bohus et al. 1996]. There is also evidence for long-lasting alterations in higher-order central nervous system responsivity to colonic distention after a brief but intense stressful experience even in the absence of conscious affective responses. This suggests a role of CeA in the alternation of the neural pathways in the presence of stress [Stam et al. 2002]. The significant activation of the parabrachial nucleus observed with c-Fos expression implicates this nucleus as an important relay in visceral pain perception. The low spatial resolution of fMRI has limited observation of such small nuclei, but with further advances in technology, it is expected that spatial resolution will improve significantly. The c-Fos and fMRI data taken together imply the importance of the spino-parabrachio-amygdaloid pathway in visceral nociception, indicating the inhibition of each component to have a potential role in the visceral pain management.

In conclusion we provided evidence to support the overall hypothesis of the thesis work that MRI technology will enable the acquisition of novel information regarding brain function and pathology. The first aim of the thesis: to design and manufacture the multi-animal probe was accomplished successfully. This probe has enabled the second and third aim of the study, and despite the initial hypothesis that both aminoguanidine
and minocycline will be neuroprotective following neonatal H/I there was no significant protection. In the second part of the thesis using MRI we supported the initial hypothesis that IL-1R1 null mice will have lower volume of cytotoxic edema, lower volume of vasogenic edema, and additionally that the initially observed neuroprotection is long-lasting. Complementary to the MRI findings we provided support for the hypothesis that IL-1R1 null mice have lower levels of iNOS and lower levels of chemokines and cytokines following H/I, which may contribute to the observed neuroprotection. As a result of neuroprotection the final hypothesis that IL-1R1 null mice will have preserved sensory motor function was also supported. For the last part of the study we have partially supported the hypothesis that both fMRI and c-Fos expression will detect similar brain areas in response to visceral pain. The conclusion from the differences in brain activation determined using both methods is that fMRI can be viewed as a complementary technique to the c-Fos expression, and that the combination of both techniques will contribute to a better understanding of visceral pain response.

7.1 REFERENCES


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Attended eight years Elementary and four years High school in Belgrade Yugoslavia, with GPA 5.0 (5.0 is the highest) through the high school. Awarded October Prize for the outstanding sport results, Belgrade 1988. Member of the National Swimming Team during the period of 1988-1995. Awarded National Fellowship for advance results in sport in the period of 1988-1995. National record holder in the discipline of 50 m butterfly (1994). Received the degree of bachelor of Science in Physics, University of Belgrade, Yugoslavia in 1999. Graduated with GPA 9.6 (10.0 is the highest) and the thesis title: "Quantum correction for Schwarschild black hole".

Attending Pennsylvania State University from 1999, option Molecular Medicine toward the degree of doctor of Philosophy. Since June 2000 to this day, full time research assistant in the Center for the Nuclear Magnetic Resonance Research. The research includes application of magnetic resonance imaging and functional magnetic resonance imaging, and RF-Coil development. Special interests are focused on neurodegenerative changes after cerebral ischemia.