Imaging Microvascular Changes Associated with Neurological Diseases

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy

Medical Biophysics
University of Toronto

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Abstract

Microvascular lesions of the brain are observed in numerous pathological conditions including Alzheimer’s disease (AD). Regional patterns of microvascular abnormality can be characterized using current neuroimaging technologies. When applied to mouse models of human disease, these technologies reveal cerebral vascular patterns and help uncover genotype-to-phenotype relationships. This thesis focuses on the development and testing of techniques for measuring two perfusion-related metrics in mouse brain regions, namely, cerebral blood volume (CBV) and cerebral blood flow (CBF) using micro-computed tomography (micro-CT) and arterial spin labeling (ASL), respectively. The main developments for measurement of CBV have included: refinements to micro-CT specimen preparation; registration of micro-CT images to an MRI anatomical brain atlas; and masking of major vessels to calculate small-vessel CBV (sv-CBV). The development of this micro-CT technique provided reference values of CBV over neuroanatomical brain regions in wildtype mice. A separate study was conducted to assess regional sv-CBV in a mouse model of AD; this study was motivated by the prevalence of
microvascular lesions in patients who suffer from AD. Significant regional differences in sv-CBV were found between AD-afflicted mice and controls. The main developments for measurement of CBF have included: design and implementation of accurate ASL slice positioning and optimization of inversion efficiency parameters. The development of this ASL technique provided reference values of CBF over neuroanatomical brain regions in wildtype mice. These techniques for measuring CBV and CBF over mouse brain regions could lead to improved characterization of vascularity in models of neurological diseases.
Acknowledgments

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Attributions

Beyond sources already cited in the body of the thesis, I would like to acknowledge the following specific attributions:

1- Dr. John G. Sled authored the automated vessel tracking software;

2- Dr. Jason P. Lerch authored the cortical surface plotting tools and non-linear registration pipelines;

3- Dr. Lisa X. Yu collaborated with the author of this thesis to perform the surgical procedures described in chapter 2 and 3;

4- Dr. Jian Wu, under the guidance of Dr. Yu-Qing Zhou, performed the pulsed-wave Doppler ultrasound measurements described in chapter 4;

5- All histology protocols including immunohistochemistry and slide scanning were performed by staff at the Centre for Modeling Human Disease (Pathology Core) under the supervision of Lily Morikawa;

6- The TgCRND8 mouse colony, described in chapter 3, was managed by staff at the Toronto Centre for Phenogenomics, with original mouse breeding pairs obtained from Dr. Sheena Josselyn with the permission of Dr. Peter St. George-Hyslop. Genotyping was performed by the Centre for Applied Genomics of the Hospital for Sick Children.
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<td>two-dimensional</td>
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<tr>
<td>3D</td>
<td>three-dimensional</td>
</tr>
<tr>
<td>ACA</td>
<td>anterior cerebral artery</td>
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<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
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<td>am-CASL</td>
<td>amplitude-modulated continuous arterial spin labeling</td>
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<td>ANOVA</td>
<td>analysis of variance</td>
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<td>APP</td>
<td>amyloid precursor protein</td>
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<td>ASL</td>
<td>arterial spin labeling</td>
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<td>B(_1)</td>
<td>radio-frequency amplitude</td>
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<td>BBB</td>
<td>blood brain barrier</td>
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<td>identification for a common inbred mouse strain</td>
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<td>CASL</td>
<td>continuous arterial spin labeling</td>
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<td>CBF</td>
<td>cerebral blood flow</td>
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<tr>
<td>CBV</td>
<td>cerebral blood volume</td>
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<tr>
<td>COV</td>
<td>coefficient of variation</td>
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<td>Abbreviation</td>
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<tr>
<td>CPP</td>
<td>cerebral perfusion pressure</td>
</tr>
<tr>
<td>CPV</td>
<td>cerebral plasma volume</td>
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<tr>
<td>CRCV</td>
<td>cerebral red cell volume</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>ETL</td>
<td>echo train length</td>
</tr>
<tr>
<td>( f_m )</td>
<td>modulation frequency</td>
</tr>
<tr>
<td>FOV</td>
<td>field-of-view</td>
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<tr>
<td>G</td>
<td>gradient strength</td>
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<td>Hct</td>
<td>hematocrit</td>
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<tr>
<td>IP</td>
<td>intraperitoneal</td>
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<tr>
<td>micro-CT</td>
<td>micro-computed tomography</td>
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<td>MCA</td>
<td>middle cerebral artery</td>
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<td>magnetic resonance imaging</td>
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<td>magnetization transfer</td>
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<tr>
<td>NEX</td>
<td>number of excitations</td>
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<tr>
<td>PASL</td>
<td>pulsed arterial spin labeling</td>
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<td>PBS</td>
<td>phosphate buffered saline</td>
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pCASL  pseudo-continuous arterial spin labeling

PECAM-1  platelet endothelial cell adhesion molecule-1

PET  positron emission tomography

PS  permeability surface area product

RF  radio frequency

SAR  specific absorption rate

SEM  standard error of the mean

SNR  signal-to-noise-ratio

SPECT  single photon emission computed tomography

SSS  superior sagittal sinus

sv-CBV  small-vessel cerebral blood volume

T  unit of magnetic field strength: tesla

TE  echo time

TR  repetition time

$T_1$  time constant for longitudinal relaxation

$T_{1\text{SAT}}$  time constant for longitudinal relaxation, enhanced by magnetization transfer

$T_2$  time constant for transverse relaxation
$T_2^*$ time constant for decay of transverse magnetization, including both spin-spin relaxation and relaxation due to magnetic field inhomogeneity

TgCRND8 identification code for a mouse model of Alzheimer’s disease
Chapter 1
Introduction and Overview

This thesis focuses on the development and testing of techniques for measuring two perfusion-related metrics to quantify regional patterns of cerebral microvascular abnormalities of genetically modified mice. The proposed metrics, namely, cerebral blood volume (CBV) and cerebral blood flow (CBF), are measured using state-of-the-art imaging technologies. The background to this work is introduced here, while description of the experimental design and findings are detailed in the main body of the thesis, comprising three manuscripts.

There are two main goals of this introduction, namely, 1-to place the techniques developed in this thesis within the broader context of the available methods for characterizing microvascular abnormalities; and 2-to provide a rationale for the developments and applications described in the body of this thesis.

For ease of discussion, this introduction has been divided into seven sections each of which deals with a relevant theme. We start by considering the benefits of using mouse models in our study. In the context of mouse imaging, the principles for measuring CBV and CBF are outlined. The normal state of CBV and CBF is discussed, before considering cerebro-vascular abnormalities in Alzheimer’s disease. This is followed by outlining the structure and organization of this thesis.

1.1 Advantages of studying mouse models

There has been rapid growth in our knowledge of the 20,000-25,000 protein-coding genes(1) that comprise the human genome and in our technological ability to manipulate homologous genes in genetically engineered mouse models. Each of the following subsections describes a distinct advantage of using mice as mammalian models for the study of genetics.

1.1.1 Humans and mice still possess many common traits after 75 million years of evolution

The genetic pathways in mice are very similar to those of humans. There is a very high probability, approximately 99%, that a given mouse gene has a homologue in the human genome.
This leads to many similarities in the observable traits of the two species. Despite obvious differences in brain function, mice and humans have a surprisingly similar spatial arrangement of brain regions. It has long been recognized that the cerebral vasculature in the mouse has a similar anatomical arrangement to that in humans.

1.1.2 Genetically identical mice of various strains are available

Gregor Mendel published the laws of inheritance in 1866 based on observations made in pea plant hybridization experiments. Long before Mendel's work, it was known that certain strains of mice spontaneously produced coat-colours that were different than the norm. By the 1700s, mouse fanciers in Japan and China had domesticated many varieties as pets while Europeans subsequently imported favorites and bred them to local mice, thereby creating progenitors of modern laboratory mice. The laws of Mendelian inheritance were demonstrated on mouse coat colours, which extended genetic principles from pea plants to mammals. Subsequently, mating programs were established, resulting in many of the modern well-known inbred strains. These inbred strains represent an unlimited family of genetically identical individuals and provide an opportunity to perform experiments on very similar phenotypes without much intrinsic variability. Various genetic engineering techniques may be employed such as the inactivation of genes to create knock-out mouse models, introduction of genes from another species (knock-in models) and inactivation of a gene in a confined spatial or temporal region (conditional knockouts).

1.1.3 The complexity of mammalian biology can be studied from a system-wide perspective

The completion of the human genome project involved decoding a database consisting of 3.3 billion base pairs of deoxyribonucleic acid (DNA), known as the human genome. As a next step, if we can learn how the genome results in observable human characteristics, known as the phenotype, then this would lead to a vastly improved understanding of our biological makeup. Thus, a current challenge facing biologists is to uncover genotype-to-phenotype relationships. This is complicated, however, by the fact that single gene modifications seldom result in a single phenotypic change. In fact, single gene changes typically give rise to multiple and often diverse phenotypes. Furthermore, many common diseases affecting humans, including Alzheimer’s disease, involve multiple genes.
Therefore, a paradigm known as systems biology is being developed to focus on how complex properties of a system emerge from the interactions between simpler components (4). In this paradigm, genotype-to-phenotype relationships are understood from a holistic perspective by associating genetic variants with traits observed from a whole organ or whole organism system-wide perspective. Since the genomes of several strains of mice have also been sequenced, mouse models can contribute to this approach by permitting genotype-to-phenotype relationships to be uncovered. This can be achieved by genetically modifying some members of an inbred strain of mice and looking for associated changes in the phenotype relative to unmodified members.

Two of the key technologies contributing to mammalian phenotyping are magnetic resonance imaging (MRI) and micro-computed tomography (micro-CT) (4). The application of imaging technologies provides numerous benefits for the discovery of genotype-to-phenotype relationships. Imaging can go beyond simply revealing anatomical differences, as it can also provide glimpses of physiological phenotypes such as those associated with brain perfusion.

1.2 General principles for measuring CBV and CBF

The term perfusion describes the delivery of arterial blood to the capillary bed, which involves transport of oxygen, glucose and other nutrients from blood to tissue. Techniques for measuring perfusion-related parameters have and will continue to be useful in neuroscience. These techniques provide the ability to chart out microvascular structure over brain regions and to detect deviations from normal cerebral blood flow. The following sections define the principles for measurement of two of the most useful metrics for characterizing perfusion.

1.2.1 Definitions

In quantitative brain imaging, the key perfusion-related parameters describing the vascular structure and the rate of perfusion are cerebral blood volume (CBV) and cerebral blood flow (CBF), respectively.

CBV is defined as the total volume of blood per unit volume of brain tissue (5) and is conventionally expressed in percentage units. CBV values are similar between species but heterogeneous over regions, with human values ranging from 2.7% in white matter to 8.6% in
occipital cortex(6). CBV depends on both vessel density and diameter and is, thus, sensitive to the distribution of vessel types. On average and under *in vivo* conditions, the approximate breakdown of CBV is estimated as follows: 10% is associated with arterial blood, 20% with capillary blood and 70% with venous blood(7). Estimates for the compartmentalization of CBV within the microvasculature vary; for example, one report provides a breakdown of 20% arteriolar, 50% capillary and 30% venular(8) and another report provides 21% arteriolar, 33% capillary and 46% venular(9). Several techniques for measuring CBV are covered in section 1.4.

CBF is defined as the volume of arterial blood (in mL) delivered to the capillary bed of 100g of brain tissue per minute. This definition assumes that arterial blood is delivered to the capillary bed such that the constituent components of blood, including oxygen and nutrients, are deposited in the tissue element of interest; this contrasts with arterial blood flow that transits through a tissue element on route to another destination, without locally depositing its constituent components(10). CBF varies substantially between species, with healthy human CBF at about 50 mL/100g/min, while mouse CBF has been typically found to be 150 mL/100g/min or higher. The scaling of units to 100g of brain tissue is a conventional practice, though the quantity of brain tissue may also be expressed in volume units, in which case CBF would have the dimensions of a rate constant. This dimensional analysis highlights the role of CBF in delivery of metabolic substrates and clearance of metabolic products. Specifically, the rate of delivery of any substrate to the tissue is the product of CBF and the arterial concentration of the substrate(8).

There are several techniques for measuring CBF over neuroanatomical brain regions, with some of the key imaging methods discussed in section 1.2.2. CBF is heterogeneous under normal circumstances, typically varying by at least a factor of four over mouse brain regions(11). This variation may be attributed to differences in metabolic demand of tissues. In fact, CBF has been shown under normal conditions to be proportional to the measured metabolic rate(12).

This thesis focuses on the mapping of CBV and CBF over mouse brain regions, which is of particular interest for delineating phenotypes of models of neurological diseases, such as Alzheimer's disease(13). As explained in the following subsections, there are numerous considerations in mapping these metrics in the brain.
1.2.2 General tracer kinetic model

Principles for measurement of CBF may be described by the following linear systems model(8; 14), which describes the kinetics of a tracer as it travels from feeding arterial source to the receiving tissue region:

\[ C_T(t) = C_A(t) \ast [f \cdot r(t)] \]  \hspace{1cm} [1.1]

\[ \tau = \int_0^\infty r(t) = \frac{\lambda}{f}, \]  \hspace{1cm} [1.2]

where \( C_A(t) \) is the tracer concentration in the arterial blood (also known as the arterial input function), \( C_T(t) \) is the tracer concentration in tissue, \( f \) is cerebral blood flow, \( \lambda \) is the partition coefficient, \( r(t) \) is the residue function, while \( \ast \) denotes the convolution operation.

The partition coefficient is defined in a steady-state tracer experiment as the ratio of tracer concentration in tissue to that in the artery. Specifically, if \( C_A(t) \) is constant, i.e. \( C_A(t) = C_0 \), for a lengthy period of time, then \( \lambda = \frac{C_T(\infty)}{C_0} \). The partition coefficient is also known as the volume of distribution of the tracer because it is a generalization of CBV to include cases where the tracer is diffusible outside of the vasculature. Tracers can be categorized based on their \( \lambda \), with diffusible tracers having \( \lambda \sim 1 \) and intravascular tracers having \( \lambda \sim \text{CBV} \).

The residue function is the probability that a particle of the tracer that entered a volume element (i.e. voxel) of the region of interest at \( t=0 \) is still there at time \( t \). In other words, the transport and distribution of the tracer are characterized by \( r(t) \), which is a monotonically decreasing function with initial value 1. The area under the residue function, as described in equation [1.2], is referred to as the mean transit time (\( \tau \)) and represents the fundamental time constant governing the kinetics of the tracer. It is clear from equation [1.2] that the class of tracer selected, which affects \( \lambda \), can strongly affect the time constant for the experiment.

The general model described by equations [1.1] and [1.2] holds provided that two assumptions are true for the course of the experiment, namely: 1- the brain is in steady state with CBF constant; and 2-the tracer is metabolically inert(8).
1.3 Measurement of CBF

To apply the general tracer kinetic model for measurement of CBF using neuroimaging technologies, it is necessary to relate the signal associated with the tracer, as measured by the imaging modality, to the concentration of the tracer, i.e. $C_A(t)$ and $C_T(t)$. In the following sections, techniques for measuring CBF are compared based on different tracer properties and a discussion is provided on challenges of applications to mice.

1.3.1 Tracers for the measurement of CBF

Several tracers used for CBF quantification are listed in Table 1.1 along with their key properties. Tracers for CBF measurement can be labeled in various ways so as to be detectable by neuroimaging modalities. For example, water can be radioactively labeled to be detectable by positron emission tomography (PET) or can be magnetically labeled for detection by arterial spin labeling (ASL), an MRI technique. Since water is a diffusible tracer and has the same partition coefficient irrespective of the labeling method, both PET and ASL methods share many common properties. Thus, it is useful to think about CBF measurement from the point of view of the tracer selected.

The pioneering method for measuring total blood flow through the heart or lungs was the Fick principle, enunciated by Adolf Fick in 1870. This principle states that the quantity of a tracer taken up by an organ per unit time is equal to the product of blood flow through that organ and the tracer concentration difference between artery and vein\(^{15; 16}\). A breakthrough in cerebrovascular research followed with the advent of the “Kety method”, also known as the “tissue uptake” method, devised by Kety and Schmidt in 1948, which adapted Fick’s principle to measure whole brain CBF in humans using inhaled nitrous oxide, a diffusible tracer. Due to the non-availability of tomographic neuroimaging methods to provide voxel-wise tissue concentration, Kety and Schmidt invasively sampled arterial and venous blood to measure the concentration-time curves for nitrous oxide over a ten minute time period, long enough for the nitrous oxide to reach equilibrium within the brain. Whole brain CBF was estimated to be 54±12 ml/100g/min, based on data from 14 healthy young males\(^{17}\).
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<th>Class</th>
<th>Route of administration</th>
<th>Manner of labeling</th>
<th>Imaging method*</th>
<th>Measurement method</th>
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<td>water</td>
<td>diffusible</td>
<td>injection / inhalation</td>
<td>radioactive: $H_2^{15}O$</td>
<td>PET</td>
<td>tissue equilibration</td>
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<td>endogenous</td>
<td>MRI</td>
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<td>magnetic inversion of proton magnetic moment</td>
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<td>xenon</td>
<td>diffusible</td>
<td>inhalation / injection</td>
<td>radioactive: $^{133}Xe$</td>
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<td>radioopacity of stable Xe</td>
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<td>diffusible</td>
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<td>intravascular</td>
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<td>hexamethylpropylene-amine oxime</td>
<td>diffusible</td>
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<td>magnetic susceptibility of gadolinium</td>
<td>MRI</td>
<td>indicator dilution</td>
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<tr>
<td>gadolinium chelate</td>
<td>intravascular</td>
<td>injection when blood brain barrier is intact</td>
<td>magnetic susceptibility of gadolinium</td>
<td>MRI</td>
<td>indicator dilution</td>
</tr>
</tbody>
</table>

* PET=Positron Emission Tomography; MRI=Magnetic Resonance Imaging; CT=Computed Tomography; SPECT=Single Photon Emission Computed Tomography
Kety’s model can be considered a special case of the more general kinetic model described by equations [1.1] and [1.2] with an assumed residue function of \( r(t) = \exp(-t / \tau) \). This residue function is equivalent to assuming that the nitrous oxide reaches a rapid equilibrium between tissue and blood within a single well-mixed compartment(8). Several methods of CBF measurement assume this residue function and are collectively known as “tissue equilibration” techniques. As listed in Table 1.1, water, xenon and iodoantipyrine are examples of diffusible tracers that are well suited to meet the requirements of the tissue equilibration method(7; 18).

Tissue equilibration methods that measure CBF during the administration phase of the tracer, when the tissue concentration of the tracer is rising, are known as “saturation methods” or “tissue wash in” methods. One standard saturation method for measuring CBF involves rapid injection of radioactively labeled water, H\(_2\)\(^{15}\)O, followed by PET measurement of the emitted radioactivity during the initial rise in tracer concentration. In PET, the concentration of tracer as a function of time is determined by measuring the proportional curve for the number of radioactive decays per second(8; 19). This approach, which requires high signal-to-noise ratio (SNR) and accurate timing of measurements with respect to the arrival of the bolus, utilizes the concept that the early part of the tissue concentration-time curve is sensitive to CBF while being independent of \( \lambda \)(8).

Another equilibration method that utilizes PET imaging involves continuous inhalation of C\(^{15}\)O\(_2\) leading to rapid transfer of \(^{15}\)O to endogenous water within the pulmonary vasculature to form H\(_2\)\(^{15}\)O(8; 19). In this method, CBF is calculated based on the steady state tissue concentration reached when the delivery of H\(_2\)\(^{15}\)O by flow is balanced by the clearance of tracer by outflow or decay of the radiolabel. A key strength of using PET is its capability to measure a variety of physiological parameters, such as regional metabolic rate, in relation to CBF. One significant drawback of applying PET to mice, however, is the limited spatial resolution, which in most small animal PET systems is between 1 to 2.5 mm(20). The spatial resolution of small animal PET systems has been primarily limited by uncertainty in the localization of positron emitters and by challenges in reducing detector element size(21).

In reality, even diffusible tracers are incompletely extracted into the tissue space on first passage, due to limited permeability across the capillary wall. Two-compartment models which account for finite permeability-surface area product (PS) may be applied for greater accuracy and have
been seriously considered for improving the accuracy of CBF measurements using endogenous and radiolabeled water (22; 23).

The family of CBF measurement methods based on the tissue equilibration principle also include techniques that make a single time point measurement after the tracer concentration in tissue has reached equilibrium. One such technique is autoradiography, which is often considered to be the gold standard for measuring CBF over brain slices. In autoradiography, the tracer is a radio-labeled molecule such as $^{14}$C-iodoantipyrine, which is nearly completely extracted from the capillaries at CBF values below 180 mL/100g/min (18). Brain concentration of the tracer is measured by arresting the circulation and sectioning brain tissue with a cryostat, followed by placing the slices in contact with an X-ray sensitive film. This technique is considered very reliable because major sources of error have been thoroughly quantified (24). One drawback, however, is that, unlike modern tomographic methods, autoradiography measures CBF in 2D.

Those tissue equilibration methods that first load up the brain with the tracer, then stop tracer administration and finally measure CBF as the tracer clears from the tissue are known as “clearance methods”, “desaturation methods” or “tissue washout methods”. For example, using xenon as a tracer, the clearance curve is measured and fitted to a decaying exponential with the time constant for clearance $\tau = \lambda / f$. In the clearance method, measurements are sensitive to both $f$ and $\lambda$ (8). One such technique is xenon computed tomography, where stable xenon provides CT contrast by increasing x-ray attenuation. A challenge in applying this technique to small-animals, however, is the low SNR, despite administration of high concentrations of xenon (18).

CBF can also be measured using a tracer that gets trapped in the capillary bed or brain tissue after initial administration, in which case the residue function is $r(t) = 1$ and the general model becomes independent of $\lambda$. These methods, prized for their simplicity, are known as “indicator fractionation” or “microsphere methods”, since they are modeled on the microsphere trapping technique. Microsphere trapping involves arterial infusion of a bolus of carbonized or plastic microspheres with mean diameter of 15µm, radiolabeled with a gamma emitter, which after delivery to the capillary bed can be detected by differential gamma spectroscopy (7; 16; 18). As an alternative to radiolabeling, microspheres can also be fluorescently labeled. The microspheres are too large to fit through the capillaries so they remain lodged at the precapillary level within
the tissue. An integrated arterial-concentration time curve for the injected microspheres can be measured at any convenient artery. When applied to large animals, microsphere trapping is considered to be the gold standard for CBF measurement. When applied to small animals, however, the large number of infused microspheres needed to obtain sufficient measurement accuracy is believed to disturb circulatory parameters and has provided unreliable results in the past(25).

The indicator fractionation method is also utilized with single photon emission computed tomography (SPECT) when highly diffusible, short lived tracers get trapped in brain tissue after an initial capillary passage and behave as “liquid microspheres”. These tracers freely pass the blood brain barrier and undergo rapid conversion to highly bound metabolites that are retained for several minutes, long enough for tomographic imaging. The tracer radioactivity is detected by arrays of stationary sensors or by a gamma camera rotating around the head, and the detected photon count rate is scaled proportionally to tracer concentration units(8; 26). Recent advances in pin-hole camera technology provide better magnification for high resolution SPECT in mice. However, increases in resolution are traded off with limited sensitivity since a pinhole passes only a small fraction of the incident photons. Continued development of multiple pinholes(27) and improved image reconstruction is making SPECT a feasible option for phenotyping mice.

It is worth noting that the specific CBF measurement method depends more on the nature of the tracer than on the imaging modality used to detect it. Consider, for example, that using radioactive $^{133}$Xe and SPECT, CBF is measured by the tissue equilibration method, whereas using $^{123}$I- iodoamphetamine and SPECT, CBF is measured by the indicator fractionation method.

For agents that remain intravascular in the brain during the data collection period, the so-called “intravascular” or “blood pool” tracers, the time course of the experiment is governed by time constant $\tau = \frac{CBV}{f}$, substantially smaller than that for diffusible tracers. For these intravascular tracer methods, $r(t)$ has a complex form and the shape of the tissue-concentration curve is affected by both $f$ and $\lambda$. Therefore, accurately measuring CBF requires high SNR and high temporal resolution. In such cases, CBF is sometimes determined without assuming a specific residue function, based on deconvolution of equation [1.1] using a sampled $C_A(t)$. This method has been implemented using MRI bolus tracking, also known as dynamic contrast
enhanced imaging, which uses exogenous paramagnetic tracers such as gadolinium chelates. The MRI signal depends on the effects of the contrast agent on the time constants for longitudinal relaxation ($T_1$) and for transverse relaxation ($T_2$) of blood, which in turn are affected by the distribution of magnetic susceptibility and the water exchange between intra and extravascular spaces. Quantification is based on relating the MRI signal changes to $C_T(t)$, which depends on either susceptibility ($T_2$ or $T_2^*$) or relaxivity ($T_1$) contrast. In the case of susceptibility contrast, $C_T(t)$ is typically assumed to be proportional to the change in the transverse relaxation rate, $\Delta R_2^*(t)$, whereas in the case of relaxivity contrast, $C_T(t)$ is typically assumed to be proportional to change in longitudinal relaxation rate, $\Delta R_1(t)$. In reality, these linear relationships may be oversimplified as the MR signal changes depend on a complex interplay of water exchange and susceptibility-induced changes, which complicates quantitative interpretation(8; 9). Other persistent challenges of MRI bolus tracking include high sensitivity to noise and stringent requirements on knowledge of $C_A(t)$. However, this technique has been applied to species as small as rats(28).

1.3.2 Developing arterial spin labeling in mice

Arterial Spin Labeling (ASL), developed in 1992(29), is an MRI technique for measuring CBF using endogenous arterial blood water as a diffusible tracer. Due to use of water as the carrier of contrast, ASL techniques closely parallel steady-state PET studies using $H_2^{15}O$. In fact, the development of ASL was modeled on the established $H_2^{15}O$ technique after it was realized that developments in labeling water for use in MR angiography could be extended to CBF. As an MRI technique, ASL can acquire CBF images in 3D and these data can be related to other MRI data sets, such as anatomical images which provide delineation of regional boundaries. When optimized, ASL offers better spatial resolution in CBF mapping than most other neuroimaging techniques, including nuclear medicine methods like PET and SPECT(8). Since ASL uses endogenous contrast, it is completely non-invasive, providing great ease of use in conducting research on very small animals such as mice. Prior to this thesis, there had been a few studies demonstrating 2D ASL in mice. Since this thesis develops 3D ASL for use in mice, however, several complexities involved in transitioning from 2D to 3D ASL needed to be addressed, as described in chapter 4.
ASL can be implemented in several different ways, though the following five steps can be considered essential:

(i) the arterial input function, $C_A(t)$, is defined by flipping magnetization of inflowing water in arterial blood (illustrated in Fig.1.1) using 180° radiofrequency (RF) inversion pulses; (ii) after allowing enough time delay for the labeled blood to irrigate the tissue of interest (a period referred to as the post-label delay) an image of the tissue regions is acquired, which may be termed the ‘label image’; steps (i) and (ii) may be collectively termed the ‘label experiment’; (iii) a ‘control experiment’ is then performed where steps (i) and (ii) are repeated, under conditions where there is effectively no inversion of arterial blood; this leads to acquisition of a ‘control image’; (iv) the label image is then subtracted from the control image to produce an ASL difference image. This subtraction removes the signal of background water spins while preserving the signal associated with the labeled arterial blood that was delivered to the capillary bed; (v) thus, CBF is modeled from the ASL difference image.

The first step in defining the arterial input function can be accomplished in two general ways. In Pulsed ASL (PASL), the blood water magnetization is inverted in a thick slab located next to the tissue slice of interest using a brief RF pulse, whereas in Continuous ASL (CASL), the labeling is performed continuously in a plane that is distant and upstream of the imaging volume using velocity-dependent adiabatic inversion. Although it is possible to label in a truly continuous manner using separate decoupled RF coils for labeling and imaging, it is conventional to also describe as CASL those experiments which use one coil for both labeling and imaging, provided that the tissue is nearly saturated with inverted blood water at the time of measurement. It would be more technically correct to refer to these latter experiments as almost-continuous ASL, a practice followed by some researchers, although this thesis follows the more common nomenclature. PASL, which deposits less RF power than CASL, is often favored for human studies with strict regulations on RF power deposition. On the other hand, CASL, the method of choice for this thesis, is preferred in small animal research where maximum SNR is desired. To obtain high SNR, the completeness of blood water inversion, as measured by the inversion efficiency, must be optimized in CASL experiments. Thus, a major focus of chapter 4 is the development of an optimal inversion efficiency protocol for mouse ASL.

Use of a post-label delay, as described in step (ii), serves two important purposes: 1- to clear the intense signal from large arteries that can lead to CBF quantification errors, and 2- to ensure that
the labeled blood is completely delivered to all imaging voxels. For this to be effective, the length of the selected post-label delay should be larger than the measured arterial transit time\(^{31}\). Depending on the T\(_1\) of blood and arterial transit time, it may also be important to account for spin relaxation during the period of transit from inversion to measurement slices.

The quality of the control image generated in step (iii) is crucial to the accuracy of CBF measurement. For an ideal control experiment, the arterial blood enters the tissue fully relaxed and the static spins in the tissue have an identical signal to that of the label experiment. In this case, the calculated difference image, described in step (iv), only depends on the delivered arterial spins. Although the long RF pulse in CASL is off-resonance relative to the imaging slice, this pulse can still produce a small direct effect on the longitudinal magnetization of the static tissue spins and an even more significant saturation of semisolid water protons leading to the magnetization transfer (MT) effect. The so-called semisolid water protons, associated with macromolecules and membranes, are not directly detectable due to their short T\(_2\), although they influence the spin state of the liquid protons through magnetization transfer\(^{32}\). Thus, it is important that the control experiment reproduce these effects to avoid quantification errors.

Several pulse sequence methods have been devised to design a control experiment for CASL that can accurately compensate for MT effects\(^{33}\). One such method, known as amplitude-modulated CASL (am-CASL), is explored in this thesis and has been successfully applied in human multi-slice applications\(^{34}\). In am-CASL, the RF pulse used in the control experiment differs from that of the label experiment in that it is sinusoidally modulated at frequency \(f_m\), creating two closely spaced inversion planes, which leads to double inversion. Provided that the average RF power used in the control and label experiments is the same, magnetization transfer effects can be cancelled in the difference image. A major challenge in implementing am-CASL, however, is its sensitivity to arterial blood velocity. Thus, this technique must be carefully calibrated for use in mice, as discussed in chapter 4.

For the final step of modeling CBF from the ASL difference image, the magnetization of the ASL difference image is related to tracer concentration. In ASL, there are two ways for the tracer to clear from the voxel, namely, venous outflow or longitudinal relaxation. The former is described by the residue function, \(r(t)\), for water, while the latter by a relaxation term, \(m(t)\). In this case, the general kinetic model is written as:
where $\Delta M(t)$ and $\Delta m_a(t)$ represent the tissue and arterial blood magnetizations of the ASL difference image, respectively.

In the simple case of a single well-mixed compartment, the residue function which describes the washout of labeled spins from a voxel is a decaying exponential, $r(t) = \exp(-t/\tau)$. Furthermore, $m(t) = \exp(-t/T_1)$ describes the fraction of magnetization remaining in the voxel after time $t$ due to longitudinal relaxation effects (8; 23). As described in chapter 4, strongly enhanced longitudinal relaxation due to magnetization transfer is accounted for using a binary spin bath model.

A mathematically equivalent description of this single-compartment model, which dates back to work in 1992 by Detre and coworkers (29), is:

$$\frac{d\Delta M(t)}{dt} = \frac{-\Delta M(t)}{T_1} + f(\Delta m_a(t) - \Delta m_v(t)), \quad [1.4]$$

where $\Delta m_v(t)$ represents the venous blood magnetization of the ASL difference image and $\Delta m_v(t) = \frac{\Delta M(t)}{\lambda}$.

Two-compartment CBF models, which have been reviewed elsewhere (23), have been applied in human ASL to improve the accuracy of CBF quantification. It is not, however, well known whether use of these more complex models is necessary for mouse ASL. Unlike single-compartment models, two-compartment models account for restricted permeability of the vessel wall. A specific two-compartmental formulation provided by Parkes and Tofts (23) considers the magnetization of the ASL difference image for each tissue element as divided by the semipermeable endothelial membrane into extravascular and blood compartments, as follows:
\[ \Delta M(t) = v_{ew} \Delta m_e(t) + v_{bw} \Delta m_b(t), \]  

where \( v_{ew} \) and \( v_{bw} \) are the volumes of extravascular and blood water per unit tissue volume and \( \Delta m_e(t) \) and \( \Delta m_b(t) \) are the magnetization differences for the extravascular water and blood water, respectively.

Based on this formulation, the following two-compartment model equations describe the dynamics of the blood compartment and extravascular compartments, each with their own volumes and longitudinal relaxation times \( (T_{lb}, T_{le}, \text{respectively}) \):

\[
\frac{d(v_{bw} \Delta m_b(t))}{dt} = -\frac{v_{bw} \Delta m_b(t)}{T_{lb}} + f \Delta m_a(t) - f \Delta m_e(t) + PS(\Delta m_e(t) - \Delta m_b(t)),
\]

\[
\frac{d(v_{ew} \Delta m_e(t))}{dt} = -\frac{v_{ew} \Delta m_e(t)}{T_{le}} + PS(\Delta m_b(t) - \Delta m_e(t)).
\]

For slow perfusion systems, where the measurement time is less than \( \tau \), the labeled water is assumed to never leave the voxel, in which case \( \Delta m_e(t) = 0 \). On the other hand, for fast perfusion systems, where measurement time is greater than \( \tau \), the venous blood has the same magnetization as blood in the voxel, such that \( \Delta m_e(t) = \Delta m_b(t) v_w^b \) where \( v_w^b \) is the volume of water per unit volume of blood. The former assumption is known as the slow solution whereas the latter is the fast solution(23). Comparison is made between this two-compartment model using the fast solution and the traditional single-compartment model, as discussed in chapter 4.
1.4 Measurement of CBV

As with CBF, measuring CBV by neuroimaging technologies involves mathematically relating the signal associated with the tracer, as measured by the imaging modality, to the concentration of the tracer, i.e. $C_A(t)$ and $C_T(t)$. The section below compares CBV measurement techniques based on the indicator dilution method. This is followed by an introduction to *ex vivo* measurement of CBV.

1.4.1 Determining CBV using the indicator dilution method

CBV measurement utilizing intravascular tracers is commonly performed using the indicator dilution method, which does not require knowledge of the residue function. Specifically, the following expression for the partition coefficient can be deduced from equations [1.1] and [1.2]:

$$\lambda = \frac{\int_C^\infty C_T(t)dt}{\int_0^\infty C_A(t)dt} [1.8]$$
Equation [1.8] shows that λ can be determined from an experiment using any bolus of intravascular tracer by measuring the area under the tissue-concentration time curve, with a global scaling by the area under the arterial input function. This method does not require knowledge of the shape of r(t) or CBF. Since intravascular tracers have a volume of distribution $\lambda \approx CBV$, this leads to a convenient method to determine CBV.

One important correction should, however, be pointed out. CBV is divided between cerebral plasma volume (CPV) and cerebral red cell volume (CRCV) as follows (9):

$$CBV = CPV + CRCV$$  \[1.9\]

Most measurements of CBV utilize tracers that distribute within the blood plasma and are, thus, measuring CPV. If the hematocrit (Hct), namely, $Hct = CRCV / CBV$, was constant through the vasculature, CBV could be scaled by a global measurement of hematocrit as $CBV = CPV / (1 - Hct)$. However, in actuality, due to a differential in velocities of red cell and plasma components in the microvasculature, hematocrit depends on the vessel size and, thus, requires more complex corrections (9). Local microvascular hematocrits were reported to vary between 0.22 and 0.35 over 44 rat brain areas in a study that autoradiographically measured both CPV and CRCV using $^{125}$I-labeled serum albumin and $^{55}$Fe-labeled red blood cells (35). Thus, it is important that hematocrit effects are considered when using plasma tracers to measure CBV, since regions may substantially differ in their vessel size distribution.

Though equation [1.8] suggests a method for measuring CBV for a bolus with an arbitrary shape, high temporal resolution is required to accurately sample the concentration-time curves. Relative to dynamic methods, CBV images with greater SNR can be obtained by measuring the tracer concentration after a steady-state has been reached. These steady-state methods have two requirements, including: 1- the tracer is uniformly distributed throughout the vasculature, and 2- the tracer must have a long enough half-life to provide sufficient contrast after steady state is reached.

Numerous intravascular tracers have been employed for measurement of plasma blood volume using steady-state methods including radionuclides, fluorescent dyes, and ultrasmall superparamagnetic iron oxide particles.
An early application to mice of the steady-state indicator dilution method, known as the radioisotope dilution method, involved use of radioiodine as an intravascular tracer and an autogamma spectrometer for detection of radioactivity(36). This technique provided average brain CPV of 35-40μL/g, which is equivalent to a CBV of approximately 3.5%.

A higher resolution steady-state method for measuring CBV in mice utilized fluorescent dyes detected with multi-photon laser scanning microscopy; this application focused on the capillaries of the left parietal cortex of seven wildtype mice and estimated CBV to be 2.2±0.2% in “the capillary rich region of interest, avoiding large vessels”(37).

Ultrasmall superparamagnetic iron oxide particles have a long enough half-life to be useful for steady-state CBV measurements using MRI relaxivity or susceptibility contrast(9). This method, particularly useful for longitudinal studies, has been used in mice to provide regional maps proportional to CBV(38), though absolute scaling of CBV is usually not provided. Scaling of CBV requires accurate knowledge of the blood-tissue susceptibility differences when using susceptibility contrast, while for relaxivity contrast, scaling of CBV requires reference measurements in the vascular compartment(9). Another limitation of this method is the inability to differentiate microvessel contributions to CBV from that of major vessels.

### 1.4.2 Determining CBV from *ex vivo* images of vascular networks

The methodologies described in section 1.4.1 estimate CBV through measurement of CPV followed by correction using reference values of hematocrit. The main reason for taking this indirect approach is that these methods measure CBV *in vivo*, where red blood cells occupy a fraction of the lumen. Unlike CBF measurements which require that blood should circulate, however, CBV is a description of vascular structure, which, in principle, can be applied to *ex vivo* images.

If CBV is applied to the study of *ex vivo* images of suitably prepared specimens, then the distribution space of intravascular tracers can be expanded from the plasma space to include the entire lumen. This can be accomplished by purging the vasculature of its blood contents followed by infusion of an intravascular contrast agent into the vasculature. Such a procedure can be applied to an anesthetized animal and the contrast agent can be detected *ex vivo* on dissected brain specimens. In this thesis, CBV is measured *ex vivo* using micro-CT imaging, which
provides higher resolution than corresponding in vivo studies due to the ability to scan for longer durations without radiation dose limitations.

To obtain accurate CBV measurements, there are several important considerations. First, it is necessary that the infusion of the contrast agent completely fill the cerebral vasculature. Second, it is preferable that the pressure in the microvasculature replicate in vivo conditions as closely as possible. Third, a consistent method must be followed for determining concentrations of the contrast agent.

A conventional method for vascular cast preparation has been to fill the vasculature with a contrast agent by transcardial perfusion and then, after stopping the infusion pump, allow the infusate to solidify(39). When considering extending this method to quantitative studies of CBV, an outstanding challenge is that the pressure in microvessels in any given region will depend on the pressures throughout the vascular network of which it is a part. Regulatory mechanisms that act in the in vivo state can not be relied upon after the animal is sacrificed. The importance of meeting this challenge motivates the development of vascular perfusion under controlled pressure, as described in chapter 2.

Another important consideration is the choice of contrast agent, which for this thesis is particulate lead chromate and lead sulfate suspended in silicone rubber. This substance, known as Microfil, has several advantageous properties, including negligible shrinkage of the rubber during the curing process(40), curing to solid form at room temperature within a relatively short time interval of 90 minutes, hydrophobic properties which aid in clearance of residual aqueous liquids(41), lead contrast suitable for micro-CT imaging and low viscosity to facilitate perfusions. With respect to the lattermost point, the viscosity (mean±SD) of Microfil, prior to curing, is 8.3±0.4 cP, relative to mouse blood which is 1.6±0.3 cP, as determined at 37ºC at three shear rates (200, 650 and 2000 Hz) using a parallel plate rheometer (TA Instruments, AR1000).

Sections 1.3 and 1.4 have discussed biophysical methods for CBV and CBF measurement, using a subset of the available neuroimaging methods. It is recognized that there are numerous other tracer-based methods for measuring CBV and CBF, which have been reviewed elsewhere in the literature(7; 16; 18). The next section discusses how CBV and CBF are regulated and interrelated under normal conditions.
1.5 CBV and CBF in the normal state

Due to the tremendous sensitivity of vital centers to changes in oxygen levels, healthy brains – human and mice alike - are supplied with a near constant level of blood flow. The high priority given to the cerebral vasculature is underscored by the fact that, even though the brain weighs only 2% of the total body weight, it receives 15-20% of the overall blood supply. Due to its importance, self-preservation of the brain is provided by protective features of its vasculature appearing at different levels of organization. At the macroscopic level, cerebral blood vessels are not simply arranged as branching tree patterns, but instead have various collateral pathways known as anastomoses. The most prominent of the anastomoses is the Circle of Willis, residing at the base of the brain, which distributes blood from four feeding arteries, namely, the right and left internal carotid arteries and the right and left vertebral arteries. If any of these vessels becomes occluded, the Circle of Willis ensures that brain tissue remains adequately perfused by the remaining three arteries. The perfusion state of the surface of the cerebral cortex is further protected by an extensive network of anastomoses connecting arterioles of the major cerebral arteries that branch from the Circle of Willis, namely the anterior, middle and posterior cerebral arteries(16). A second protective mechanism is seen at the microscopic level, where a series of tight junctions between endothelial cells forming capillary walls, namely, the blood brain barrier (BBB), restricts passage of solutes. A third protective mechanism, called autoregulation, ensures that as pressure changes (ΔP) occur, cerebrovascular resistance (CVR) changes in a compensatory manner through dilation or constriction of arterioles to maintain relatively constant CBF, as expressed by the following relationship: \[ CBF = \frac{\Delta P}{CVR} \] (16; 42).

Blood vessels of the brain have an important role in maintaining regional perfusion. The role played by capillaries, in the long term coupling of blood flow with metabolism, has been summarized by Kuschinsky(43) in three sequential steps: first, different levels of functional activity lead to a heterogeneous distribution of regional metabolic rates; second, regional metabolism determines capillary development as shown by the correlation between local metabolic rate and capillary density; and, third, capillary density is the critical determinant of CBF as shown by its proportionality with CBF(44). Thus, the correlation between metabolic rate and CBF is maintained by long term capillary growth mechanisms that couple local blood flow in the brain to metabolic demand. These interrelationships have been experimentally demonstrated in the rat brain(12). Furthermore, the dependencies on capillary density may
extrapolate to CBV as shown by a PET study on 34 healthy volunteers, where a strict coupling of oxygen utilization (CMRO$_2$), CBF and CBV was observed(6). The coupling of CBV and CBF may represent an instance of the biological concept known as ‘form fits function’ applied to the cerebral vasculature.

Hemodynamic changes occurring in the microvasculature, which are associated with neuronal activation, are at the core of measurement principles for functional imaging technologies, including functional MRI and optical imaging of intrinsic signals(8; 45; 46). Relevant to the time scales of these functional imaging techniques, the short-term relationship between CBV and CBF is complex(8). This thesis will focus on characterizing the resting state of the microvasculature, as distinguished from these transient changes measured by functional imaging technologies. The latter is a rich field of investigation, but beyond the scope of this thesis.

1.6 Abnormal CBV and CBF in Alzheimer’s disease

A growing number of recognized heritable diseases of the cerebral vasculature can be characterized using neuroimaging technologies that measure CBV and CBF. Since the scope of this subject is vast, this introduction is restricted to describing one such example, namely, Alzheimer’s Disease (AD), which is featured in this thesis.

The defining characteristics of AD are neuronal destruction and cognitive decline, together with the presence of two families of misfolded proteins: amyloid-beta (Aβ) peptides and microtubule-associated tau proteins, which accumulate as extracellular amyloid plaques and intracellular neurofibrillary tangles, respectively. AD is also associated with abnormalities of both microvascular structure and regional patterns of CBF.

From a structural perspective, autopsies on patients with AD, show twisted, kinked and looped cerebral capillaries(13). The density of these capillaries is significantly reduced in a regional pattern that parallels neuronal loss. The hippocampus, which plays an important role in the formation of new memories, is also one of the first regions targeted by amyloid plaque burden and atrophy(47) and, under the light microscope, is observed to have thin and stringy microvessels with fewer branches(48; 49). From a flow perspective, numerous studies on AD
patients based on different imaging techniques, including H$_2$O PET and $^{133}$Xe SPECT, show CBF decreases by 10-30% in the hippocampus and cerebral cortex(50), though some recent H$_2$O PET and ASL studies have also reported paradoxical preservation of flow(51–54).

One of the key histopathological hallmarks of AD is the deposition of amyloid plaques which result from the misfolding of Aβ peptide. These peptides are secreted when the ubiquitously expressed transmembrane protein known as amyloid precursor protein (APP) is subdivided by secretase enzymes into Aβ peptide fragments. While Aβ40 is normally the dominant isoform present, increased levels of Aβ42 are a common feature of AD and are thought to contribute to amyloid plaque development. Amyloid exists in several physical states most of which are soluble forms, with the exception of insoluble mature fibrils which can aggregate and deposit in the brains of AD patients as diffuse deposits, compact deposits and vascular deposits near small to medium sized blood vessels. Dense-core plaques are known as neuritic plaques because they are frequently surrounded by dystrophic neurites and immune cells. Aβ may even be responsible for indirect damage to neurons by activating microglial immune cells. It remains unclear whether these neuritic plaques are more active at damaging neurons than soluble Aβ. Vascular amyloid deposition incites a pathological response known as cerebral amyloid angiopathy (CAA), affecting the majority of AD patients, and leading to degeneration of endothelial cells, smooth muscle cells and brain pericytes(55).

Much of the study of genetics of AD has focused on the APP gene, whose abnormal expression leads to an autosomal dominant pattern of inheritance, observed in families afflicted with an early-onset form of AD. APP mouse models that host the corresponding transgenes and overexpress mutant amyloid, replicate much of the amyloid pathology and cognitive decline present in human AD. Specifically, these mouse models have been instrumental in providing an improved understanding of mechanisms by which Aβ is cleared from the brain, which includes: degradation of Aβ by enzymes and immune cells; transport across the blood brain barrier for vascular elimination; and drainage by cerebrospinal fluid to the lymphatic system(55). Vascular elimination via the blood brain barrier is normally most efficient, which has led to considerable interest in understanding the role of the microvasculature in AD. It has been argued that impaired vascular clearance of Aβ is central to AD pathogenesis(56), though this issue remains unsettled.
Mouse models overexpressing APP replicate amyloid deposition leading to dense-core or diffuse plaques and CAA. Similar patterns of microvascular damage to that of AD patients is replicated in several of these mouse models with evidence of microvessel structure abnormalities, widespread CAA and decreased vessel density(55). Table 1.2 summarizes several representative neuroimaging studies that have revealed abnormalities in resting state CBV and CBF in APP models. Advancements in this area should help to establish these mouse models and to clarify the role played by the vasculature in the pathogenesis of AD.

Table 1.2: Abnormalities in resting state CBV and CBF in APP mouse models

<table>
<thead>
<tr>
<th>Key vascular abnormalities</th>
<th>Neuroimaging technique</th>
<th>APP mouse models</th>
<th>Publication year</th>
<th>Source Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>widespread reductions in CBF in both models:</td>
<td>autoradiography</td>
<td>Tg2123 &amp;</td>
<td>2002</td>
<td>(57)</td>
</tr>
<tr>
<td>30 to 50% in cerebral cortex, hippocampus, basal ganglia , thalamus and corpus callosum</td>
<td>($^{14}$C-iodoantipyrine administered to awake mice)</td>
<td>Tg2576</td>
<td></td>
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<td>4 to 9% reductions of relative CBV in the cerebral cortex, hippocampus and thalamus</td>
<td>MRI – steady-state susceptibility contrast</td>
<td>Tg mAPP</td>
<td>2004</td>
<td>(58)</td>
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<td>significant reductions in relative CBV in hippocampal subregions and entorhinal cortex</td>
<td>MRI – steady-state susceptibility contrast</td>
<td>J20</td>
<td>2007</td>
<td>(59)</td>
</tr>
<tr>
<td>significant reductions in relative CBV and CBF in occipital cortex</td>
<td>MRI – ASL and steady-state susceptibility contrast</td>
<td>PS2APP</td>
<td>2009</td>
<td>(60)</td>
</tr>
</tbody>
</table>
1.7 Structure and organization of this thesis

The body of this thesis comprises three manuscripts; Chapters 2 and 4 are reproduced from the original peer-reviewed publication with only minor edits.

Chapter 2 describes the development of a technique to measure CBV in the mouse brain using micro-CT. Key features of the described method are vascular perfusion under controlled pressure, registration of the micro-CT images to an MRI anatomical brain atlas and re-scaling of micro-CT intensities to CBV units with selectable exclusion of major vessels. As an application of the methodology, two hypotheses are tested, namely, 1- CBV differs over anatomical brain regions in the mouse; and 2- high energy demanding primary sensory regions of the cortex have locally elevated CBV.

Chapter 3 describes a study in the TgCRND8 model of AD in which regional patterns of brain volume and small-vessel cerebral blood volume (sv-CBV) are measured with MRI and micro-CT, respectively. These metrics are compared with amyloid plaque load as detected by immunohistochemistry.

Chapter 4 describes robust mapping of CBF over 3D brain regions using am-CASL. To provide physiological data for CBF modeling, the carotid artery blood velocity distribution is characterized using pulsed-wave Doppler ultrasound. These blood velocity measurements are utilized in simulations that optimize inversion efficiency for parameters meeting MRI gradient duty cycle constraints. A rapid slice positioning algorithm is developed and evaluated to provide accurate positioning of the labeling plane. To account for enhancement of T1 due to magnetization transfer, a binary spin bath model of MT is utilized to provide a more accurate estimate of CBF. Finally, a study of CBF is conducted on ten mice providing values of inversion efficiency and regional variation in CBF over 12 brain regions.

Chapter 5 provides a summary of the results of this thesis and a discussion of further technical considerations for the perfusion-related metrics developed and tested. The material covered in this chapter extends the discussion beyond that in the body of this thesis and considers future directions.
Chapter 2
Measurement of Cerebral Blood Volume in Mouse Brain Regions using Micro-computed Tomography

2.1 Foreword

The work in this chapter was previously published as:


2.2 Introduction

Micro-computed tomography (micro-CT) can provide detailed 3D images of the mouse vascular architecture(61). Recent applications of micro-CT to the mouse cerebral circulation include the systematic classification of major vessels(62), the detection of atherosclerotic lesions around the circle of Willis(63), and the co-registration of capillary-level views of the circulation with the macroscopic vasculature(64). The use of this technique in the mouse is motivated by a desire to better understand mouse models of human diseases.

This study describes the application of micro-CT to measure cerebral blood volume (CBV) for characterizing total vascularity in 3D regions of the mouse brain. CBV is defined as the total volume of blood in a given unit volume of brain(5). Measurement of CBV in local regions of the mouse brain is of particular interest for delineating the phenotypes of models of neurodegenerative diseases that alter cerebral vasculature, such as Alzheimer's disease(48).

Before the advent of suitable 3D imaging technologies, the CBV of the whole mouse brain was measured by detecting intravascular radionuclides(36). More recently, regional values of CBV were obtained using magnetic resonance imaging (MRI)(38); however, resolution was limited to 0.1 mm × 0.1 mm × 0.6 mm due to the time constraints of in vivo MRI scanning. Another
technique that allows a higher resolution for CBV mapping is multi-photon laser scanning microscopy (37); however, available optics and depth of light penetration limit this technique to the superficial 0.6 mm of cortex over small fields of view. Micro-CT measurement of CBV provides both high-resolution and whole brain coverage for characterizing 3D regions.

In this study, we present a methodology by which CBV can be measured as the percentage of a volume of tissue occupied by a perfused radio-opaque silicon rubber that remains intravascular. We utilize the principle that for a voxel filled with two components, tissue and radio-opaque contrast agent, the micro-CT image intensity is a weighted average of the attenuation coefficients of each component (65). Thus, the micro-CT image intensity is linearly related to the proportion of a voxel's volume that is occupied by radio-opaque contrast agent.

The procedure outlined in this study involves several innovative refinements to standard micro-CT specimen preparation and analysis. First, to permit reproducible measurement of CBV, radio-opaque vascular casts were prepared under controlled pressure. Second, to permit regional comparisons, micro-CT images were registered to an MRI anatomical brain atlas. Third, to better reflect the contribution of local microvessels to CBV, major vessels were excluded from the analysis.

We also address the hypotheses that differences in CBV exist over anatomical brain regions and that highly active primary sensory cortical areas have a particularly rich vascularization to meet their high metabolic demands (66; 67). Specifically, we examine the possibility that primary sensory cortex has a relatively high CBV in the non-stimulated condition, reflecting more dense patterns of vascularization.

2.3 Materials and Methods

The steps to measure CBV in regions of the mouse brain were: (i)- the cerebral vasculature was filled with Microfil (Flow Tech, Inc., Carver, MA, USA), a radio-opaque silicone rubber containing particulate lead chromate and lead sulfate and known for minimal shrinkage (41); (ii)- micro-CT images were acquired; (iii)- micro-CT images were re-scaled to CBV units; (iv)- CBV images were co-registered to an MRI anatomical brain atlas; (v)- CBV was measured over brain
regions; (vi)- small vessel CBV (sv-CBV) was calculated by major vessel masking; (vii)-
regional CBV values were analyzed. Each step is detailed in the following sections.

2.3.1 Filling the cerebral vasculature with Microfil

Nine female C57BL/6 mice (Charles River Laboratories, Wilmington, MA, USA), weighing 17–
25 g, were anesthetized with an intraperitoneal injection (IP) of 100 μg ketamine per gram of
body weight (Pfizer, Kirkland, QC, Canada), 20 μg of xylazine per gram of body weight (Bayer
Inc., Toronto, Canada) and 3 μg of the vasodilator, acepromazine maleate(68), per gram of body
weight (Vetoquinol, Lavaltrie, QC, Canada), then given an IP injection of heparin (200 U)
(Organon Canada Ltd., Toronto, Canada). The inferior vena cava and descending aorta were
ligated. A 24-gauge IV catheter (Becton Dickinson Infusion Therapy System Inc., UT, USA)
was inserted into the left ventricle, sealed in place using the adhesive Loctite 404 (McMaster-
Carr, GA, USA) and connected to a pressure- controlled pump (Model PS/200, Living Systems
Instrumentation, VT, USA). All incidental cuts were sealed. A slit in the right atrium provided
outflow.

To minimize variation in CBV due to vessel inflation, the pressure at which Microfil
polymerizes should be uniform. Warm heparinized (5 U/mL) phosphate buffered saline (Wisent
Inc., St-Bruno, QC, Canada) was perfused at 50 mm Hg for 5 min at a filling rate of 2 mL/min,
followed by Microfil at room temperature at 150 mm Hg for 10 min at a filling rate of 0.25
mL/min. The filling rate was determined based on the rate of volume change in a graduated
cylinder containing the infusate. The pump was stopped, the slit in the right atrium sealed and the
pump restarted at 30 mm Hg, approximating normal capillary pressure(69; 70). At this uniform
pressure, the Microfil polymerized over 90 min at room temperature. Microfil has previously
been shown to remain intravascular(40); we quantified the completeness of the perfusions in a
section that follows.

2.3.2 Acquiring micro-CT images

In preparation for micro-CT scans, dissected skulls (devoid of external soft tissue and lower jaw)
were fixed for 12 h at 4 ± 1 °C in 10% buffered formalin phosphate (Fisher Scientific Company,
Ottawa, Canada). To avoid partial volume and beam hardening artifacts, the skulls were
decalcified in 5% formic acid (Fisher Scientific Company, Ottawa, Canada) at 50 °C for 24 h and
then mounted in 1% agar (Sigma-Aldrich Co., St. Louis, MO, USA). Each micro-CT image was acquired over 2 h in which 720 views were obtained through 360° rotation with a GE eXplore Locus SP Specimen Scanner using peak voltage 80 kVp, current 80 μA and field of view at object of 20.48 mm. The measured line spread function had a full-width-at-half-maximum of 24 μm and the modulation transfer function at 10% was 34 line pairs/mm, based on previous work(71). The micro-CT images were reconstructed with isotropic cubic voxels of size 20 μm.

Fig. 2.1 Illustration of a representative inverted maximum intensity projection of a micro-CT image in three views, (A) sagittal, (B) horizontal and (C) coronal. For illustrative purposes, the images have been cropped using an MRI derived mask to show only CT data within the brain. Marked with arrows are the middle cerebral artery (MCA), the anterior cerebral artery (ACA) and the superior sagittal sinus (SSS)
2.3.3 Measuring completeness of the perfusion

A potential source of error is incomplete perfusion of the brain with Microfil, leading to underestimated CBV. Thus, the proportion of vessels filled with Microfil was measured. Specifically, five of the perfused specimens were paraffin embedded and sixteen 5 μm coronal sections were cut: four samples (spaced 10 μm apart) were cut at four locations (defined by frontal cortex, striatum, hippocampus and superior colliculus). To calculate the percentage of vessels filled with Microfil, we stained the sections with hematoxylin and under 20× magnification, scored vessels in randomly selected fields according to whether Microfil was seen in the lumen. The data were categorized by specimen (out of 5) and brain location (out of 4). To test whether the completeness of Microfil perfusion significantly differed between the locations examined, an analysis of variance (ANOVA) was performed on a linear mixed effect model of the data, with one fixed effect (location) and one random effect (specimen) using the statistical program R, available at http://www.r-project.org/.

2.3.4 Re-scaling the micro-CT images to CBV units

The CBV of each tissue voxel is the ratio of the concentration of Microfil in that tissue voxel to the concentration of Microfil in the vasculature. This was computed for each micro-CT image, by using simple volume averaging of the components of the voxel, which were assumed to be Microfil and tissue. The tissue's radio-opacity is similar to 1% agar and is much lower than that of Microfil.

To study the relative radio-opacities of the components of each voxel, an additional C57BL/6 mouse was perfused just with phosphate buffered saline (PBS), without Microfil, prior to scanning. This specimen was imaged in 1% agar together with an external slab of Microfil and the average radio-opacity in all components was compared. We also tested uniformity in the CT intensity through the depth of the image, by measuring the percentage variation in the line profile through the tissue.

Each micro-CT image was re-scaled into CBV units by the following equation:

\[
I_{\text{CBV}} = 100\% \frac{(I_{\text{ORIGINAL}} - I_{\text{AGAR}})}{(I_{\text{MICROFIL}} - I_{\text{AGAR}})},
\]  

[2.1]
where $I_{\text{ORIGINAL}}$ denotes the x-ray attenuation coefficient of the tissue voxels, $I_{\text{AGAR}}$ denotes the average x-ray attenuation coefficient of voxels in the 1% agar and $I_{\text{MICROFIL}}$ denotes the average x-ray attenuation coefficient of voxels completely filled with Microfil. Below, the terms “image intensity” and “x-ray attenuation coefficient” are used interchangeably since the CT images were processed in arbitrary units.

The background intensity, $I_{\text{AGAR}}$, was computed as the average intensity of approximately 10,000 voxels in the 1% agar. We applied a custom written program to automatically trace vessel centerlines and determine vessel diameters. This automated vessel tracking program traces the centerlines of tubular objects by maintaining equal distance from the lumen wall as determined by the image intensity gradient along rays perpendicular to the vessel centerline(72). To normalize the intensity of each individual image, $I_{\text{MICROFIL}}$ was computed as the average intensity of approximately 20,000 voxels at centerline positions of vessel segments with diameter between 0.1 and 0.2 mm. Use of these vessel segments ensured that the voxels were completely filled with Microfil.

**2.3.5 Co-registering micro-CT images to an MRI anatomical brain atlas**

To delineate CBV measurement regions, we used an MRI anatomical brain atlas that was registered to the micro-CT images. The MRI anatomical brain atlas, which has 62 labeled 3D regions(73), was prepared by averaging 20 female and 20 male C57BL/6 brain MRI images of 32 μm isotropic resolution(74). The MRI anatomical brain atlas was accessed at http://www.mouseimaging.ca/research/C57Bl6j_mouse_atlas.html.

Each micro-CT image was registered to the MRI anatomical brain atlas using vascular landmarks as a guide. As landmarks, we selected four easily identifiable bifurcations on major vessels in the mid-sagittal plane, which had low variability in position, namely: the branch point of the vertebral arteries and the basilar artery; the branch point of the medial orbitofrontal artery from the azygos of the anterior cerebral artery; the branch point of the rostral rhinal veins from the superior sagittal sinus; and the branch point of the transverse sinuses from the superior sagittal sinus. The position of each landmark in the MRI anatomical brain atlas was computed as the mean value of the coordinates identified on a subset of eight of its individual MRI images. The computer program Register, used to facilitate landmark identification, is distributed by the
Montreal Neurological Institute (http://www.bic.mni.mcgill.ca/software/). The same four landmarks were also identified on each of the micro-CT images by applying our automated vessel tracking program. The registration of micro-CT images to the MRI anatomical brain atlas was performed using a procrustes matching algorithm without scaling(75) applied to each landmark position in the micro-CT images and the corresponding landmarks in the MRI anatomical brain atlas.

**Fig. 2.2** The mid-sagittal section of a micro-CT image (A) and the corresponding section of the MRI anatomical brain atlas (B) with suitably chosen vascular landmarks (yellow) leads to accurate registration (C). Also shown is the corresponding major vessel mask (light gray) superimposed on the MRI anatomical brain atlas (D)
2.3.6 Measuring CBV over brain regions

Our goal was to reduce variability in CBV caused by registration error. Of the 62 regions in the MRI anatomical brain atlas, we selected all 13 regions that were larger than 5 mm$^3$. Selection of this threshold was based on a registration simulation that relied on the measured variability in the position of the vascular landmarks. This simulation involved generating five transformed copies of a given micro-CT image by adding random offsets derived from observed positional variation in vascular landmarks; for the transformed images the coefficient of variation (COV) was calculated region by region and an arbitrary cutoff at 10% COV led us to retain regions larger than 5 mm$^3$.

Misregistration error can significantly bias CBV when there is a great difference between the values of neighboring regions. To reduce this bias, we incrementally eroded the boundary of each region on the MRI anatomical brain atlas using a six neighbor binary morphological operator(76). We selected 0.15 mm as the depth of erosion because the CBV values of gray matter regions that border white matter regions stabilized after this amount of erosion.

The following computational method was applied to obtain CBV values. The average CBV and standard error were calculated for each of the selected 13 brain regions. To map CBV over the cerebral cortex, we solved Laplace's equation to generate field-lines from the inner to the outer cortical surface. This method has previously been applied to map human cortical thickness(77) and has recently been adapted to determine cortical thickness in the mouse brain(78). Next, CBV was averaged along each field-line and the value was projected to the mid-cortical surface; this was done through the entire thickness of the cortex, without eroding the boundary. To determine CBV through the depth of the cortex, the field-lines were divided into ten equal parts, which collectively defined ten layers.

2.3.7 Determining sv-CBV by major vessel masking

To emphasize the contribution of local microvessels to CBV, major vessels were excluded using a selectable diameter threshold that was arbitrarily chosen to be 100 μm. We formed a major vessel mask by our automated vessel tracking program to identify vessels in the micro-CT images greater than 100 μm in diameter. The metric, small-vessel CBV (sv-CBV), was defined as the total volume of blood, for vessels with diameter under 100 μm, as a percentage of the
region’s volume and was calculated analogously to CBV after deletion of data from the micro-CT images that was associated with the major vessel mask; sv-CBV was measured over the 13 brain regions and the cerebral cortex.

**Fig. 2.3** CBV maps over the surface of the cerebral cortex, averaged over nine mice: (A-C) show total CBV maps for the dorsal, right lateral and left lateral surfaces, respectively; (D-F) show the sv-CBV maps; (G and H) show the right and left surfaces marked with manually delineated visual, auditory and somatosensory areas (in blue, yellow, and red respectively). For each sensory modality a core region (dark shading) is indicated, which includes visual areas V1 and V2 and auditory areas A1 and A2. The large somatosensory area (pink) denotes primary somatosensory areas including the extensive barrel cortex region.
2.3.8 Analyzing regional CBV values

Using R, a statistical analysis was performed on the CBV data, which were categorized by specimen (N = 9), brain region (N = 13 and the type of metric (total CBV or sv-CBV). An ANOVA was performed on a linear mixed effect model of the data, which had two fixed effects (brain region and type of metric) and one random effect (specimen). The percentage difference between total CBV and sv-CBV was also determined for each micro-CT image. To determine if sv-CBV significantly differed between brain hemispheres, ten of thirteen regions were segmented into portions in each hemisphere and the mean sv-CBV was graphically compared. An ANOVA was performed with R, using a mixed effect linear model where hemisphere was the fixed factor and specimen was the random factor.

To compare sv-CBV over regions of the cortex, the primary sensory areas were delineated on right and left cortical maps based on published maps for the C57BL/6 mouse, prepared by staining cytochrome oxidase(79), cytochrome oxidase and myelin(80) and acetylcholinesterase(81).

All described animal procedures were approved by the Hospital for Sick Children Animal Care Committee, subject to the Canadian Council on Animal Care.

2.4 Results

A representative example of one of the Micro-CT images is shown in three maximum intensity projection views in Fig.2.1. All major arteries branching from the circle of Willis and major returning veins and sinuses were identifiable, such as the middle cerebral artery (MCA), the anterior cerebral artery (ACA) and the superior sagittal sinus (SSS) (marked with arrows in Fig.2.1). Inspection of the images provided no evidence that Microfil had leaked outside of vessels.

Histological analysis revealed that over the group of mice the average ± standard error of mean (SEM) percentage of vessels filled with Microfil was 93 ± 3%, which may have been an underestimate due to minor displacement of Microfil fragments during slide preparation. The
completeness of Microfil perfusion did not significantly differ between the locations examined (F = 0.72, p = 0.5).

The average radio-opacities of Microfil and 1% agar were 3700 ± 100 Hounsfield units and 0 ± 100 Hounsfield units, respectively. In support of the assumption that CT intensity varies negligibly through the depth of images, we found that the line profile through the unperfused brain tissue, described in section 2.3.4, had a maximum variation of 5 Hounsfield units.

A representative example of vascular-landmark registration of a micro-CT image to the anatomical brain atlas is shown in Fig.2.2 (A,B,C). The mean value (N = 9) for the standard deviation of the registered coordinates of the four vascular landmarks was 0.15 mm. A representative example of the overlay of a major vessel mask on the MRI anatomical brain atlas is shown in Fig.2.2 (D). In regions where multiple vessels converged, the diameters of the vessels tended to be overestimated by our automated vessel tracking program.

Cortical maps of total CBV, averaged across nine mice, are shown in Fig.2.3 (A,B,C) and maps of sv-CBV in Fig.2.3 (D,E,F). The posterior cortex had an elevated sv-CBV compared to the average cortical sv-CBV of 4.6±0.4. The primary sensory areas that are delineated(79–81) in Fig.2.3 (G,H) indicate a higher value of CBV in the primary visual and auditory areas relative to the average value of the cortex. However, there does not appear to be a higher than average value of the CBV in the primary somatosensory areas.

Table 2.1 summarizes CBV data sets for both total and sv-CBV. To illustrate the shapes, relative sizes and locations of the 13 brain regions, three sagittal slices have been labeled with each region in Fig.2.4.
**Table 2.1:** Total CBV and sv-CBV values for thirteen brain regions and the total brain reported as mean ± SEM (n=9).

<table>
<thead>
<tr>
<th>Region</th>
<th>Total CBV (%)</th>
<th>sv-CBV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amygdala</td>
<td>4.3±0.4</td>
<td>4.1±0.4</td>
</tr>
<tr>
<td>Arbor vitae</td>
<td>4.0±0.4</td>
<td>3.9±0.3</td>
</tr>
<tr>
<td>Cerebral cortex</td>
<td>7.9±0.7</td>
<td>4.6±0.4</td>
</tr>
<tr>
<td>Cerebellar cortex</td>
<td>6.6±0.5</td>
<td>4.9±0.5</td>
</tr>
<tr>
<td>Corpus callosum</td>
<td>3.1±0.5</td>
<td>2.9±0.4</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>3.7±0.4</td>
<td>3.7±0.4</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>4.3±0.5</td>
<td>4.3±0.5</td>
</tr>
<tr>
<td>Medulla</td>
<td>7.9±0.5</td>
<td>5.7±0.3</td>
</tr>
<tr>
<td>Midbrain</td>
<td>5.6±0.6</td>
<td>4.7±0.5</td>
</tr>
<tr>
<td>Olfactory bulbs</td>
<td>6.9±0.4</td>
<td>5.9±0.5</td>
</tr>
<tr>
<td>Pons</td>
<td>4.4±0.5</td>
<td>4.3±0.5</td>
</tr>
<tr>
<td>Striatum</td>
<td>2.8±0.3</td>
<td>2.8±0.3</td>
</tr>
<tr>
<td>Thalamus</td>
<td>3.7±0.5</td>
<td>3.6±0.5</td>
</tr>
<tr>
<td>Total brain</td>
<td>5.8±0.4</td>
<td>4.4±0.3</td>
</tr>
</tbody>
</table>
Significant differences were found between regions (F = 35.6, p < 0.0001) and by type of metric, namely, total CBV or sv-CBV (F = 28.8, p < 0.0001). The largest differences between total CBV and sv-CBV were found in the cerebral cortex, the cerebellar cortex, the medulla oblongata, the olfactory bulbs and the midbrain, respectively. As shown in Fig. 2.5, sv-CBV did not significantly differ between hemispheres (F-value = 3.4, p-value = 0.07).
As plotted in Fig. 2.6, the variation in CBV through the depth of the cortex showed no fine structure but did show a monotonic increase from inside to outside for both sv-CBV and for total CBV. The curve for sv-CBV was flatter than the curve for total CBV. The difference between the two curves was most pronounced at the outer surface of the cortex.

Fig. 2.5 Comparison of mean sv-CBV ± SEM for regions in left versus right hemisphere. For reference the line of identity has been plotted.
2.5 Discussion

This study demonstrates the use of micro-CT to generate CBV maps of the mouse, an application that is of particular interest for characterizing cerebral vascular disease phenotypes. Several innovative refinements to standard micro-CT specimen preparation and analysis procedures were developed to meet this objective. To minimize variation due to vessel inflation, we perfused the vascular network at a specified pressure by controlling inflow and outflow of the contrast agent. To permit regional comparisons, micro-CT images were registered to an MRI anatomical brain atlas. By re-scaling the intensities to CBV units and masking out major vessels, the sv-CBV values better reflected the local contributions of microvessels; this protocol also permits flexible selection of major vessel masks. We provided normative values of CBV in thirteen brain regions, and cortical maps to illustrate normative variations in the brain.

The total brain CBV reported here can be compared with measurements obtained in vivo. A reported value of in vivo CBV measured using intravascular radionuclides is 35–40 μL of blood per gram of brain tissue(36). By assuming a brain density of 1 g/mL, this method gives a total CBV of 3.5% to 4% in our scale. These values, which do not suppress the contributions of major
vessels, are in reasonable agreement with our sv-CBV measurement of 4.4 ± 0.3%, but lower than the total CBV of 5.8 ± 0.4%. A possible explanation for this difference is that by preparing the radio-opaque vascular casts at a pressure of 30 mm Hg, the venous vessels, which contain most of the blood in the brain, were distended past their normal physiological point. Most of this effect would have been accounted for by masking of the major sinuses and veins. However, the remaining slight difference is likely owing to the large amount of blood in the venules, which may also be distended in the pressure control setup. Nevertheless, the CBV values measured in this study are in reasonable agreement with the in vivo CBV values, as obtained using intravascular radionuclides.

To obtain reproducible values of CBV, we stress that the radio-opaque vascular casts must be prepared at a well-defined pressure. As the main contribution to the CBV comes from highly compliant venules and veins, failure to control this factor would likely lead to a large variation in results. We set the pressure close to an assumed average capillary pressure of about 30 mm Hg, which has not been directly measured in the mouse brain, but which provides a commonly accepted estimate for capillaries in the systemic circulation (69; 70). In this case, we can expect the sv-CBV values to be similar to the in vivo value. The close correspondence of sv-CBV between bilaterally symmetric regions (see Fig.2.5) supports the notion that the image registration was accurate. Another factor affecting reproducibility is the completeness of major vessel masking. The purpose of applying our automated vessel tracking program was to ensure greater accuracy of the major vessel masking. Since vessels with diameter greater than 100 μm represent approximately 1% of total brain volume, moderate overestimate of the mask can be expected to have a negligible effect on sv-CBV. Masking of finer vessels in the brain could also be performed, in future, with micro-CT scanners of higher resolution and signal-to-noise ratio.

As brain atlases become more refined, one likely interest will be to measure sv-CBV in increasingly fine regions. In such cases, registration will pose a fundamental limitation on the size of the region over which sv-CBV can be accurately mapped. The registration method used in this study was based on vascular landmarks that have 0.15 mm root mean squared variability in position. In Fig.2.3, a comparison is made between total CBV and sv-CBV. By masking the major vessels, the sv-CBV was shown to be more spatially uniform over structurally similar regions of the cortex. The ability to mask vessels also poses a limitation on the size of the region that can be studied. In small regions, a major vessel may constitute a large fraction of the
measurement volume; this artifact could outweigh microvascular variation and lead to false interpretation. For example, a 100 μm vessel that passes through a measurement volume of 1 mm$^3$ will increase an average CBV of 4% to about 5%, leading to a 25% overestimate. Since the masking is a function of resolution and SNR, improvements in micro-CT image quality will translate to greater control of the size of the region that can be mapped.

One of our scientific questions was whether primary sensory areas of cortex have a higher local sv-CBV compared to other cortical regions. This question was based on the hypothesis that sensory input areas such as visual, auditory and somatosensory cortices are highly active, with metabolic demands that might be met with a privileged blood oxygen supply. It is well known from hemodynamic based functional imaging techniques such as optical imaging of intrinsic signals(46) that under sensory stimulation, there is a significant CBV change in primary cortex compared to less active association cortex(45; 82). Although the changes described in these studies are transient responses to stimulation, it is possible that primary sensory cortex has a more permanent vascular specialization(66). Comparison of the cortical map of sv-CBV in Fig.2.3 with maps showing locations of the major sensory cortical areas, suggests that sv-CBV is elevated in both visual and auditory areas, though not convincingly in the primary somatosensory area. This likely suggests that there is not a specialized blood supply for the entire somatosensory area. One reason for this may be that the hypothesized increased blood oxygen supply to highly active brain areas may be most readily detectable at the capillary network level(66) whereas this definition of sv-CBV includes feeding arterioles and drainage venules that contain a significant proportion of the blood.

The sv-CBV increased from the inner to the outer boundary of the cerebral cortex, which was consistent with the depth variation of cortical microvessel density observed with confocal microscopy in rats for vessels with diameter under 50 μm(83) and in humans both with and without major vessel masking above 10 μm in diameter(84). Unlike those studies of microvessel density, however, the sv-CBV, plotted in Fig.2.6, did not sharply decline at the outer boundary, but appeared to level off toward the outer 20% of the depth. This pattern of variation supports a model of vascular organization where capillary-rich beds and their associated feeding arterioles and drainage venules are concentrated near the outer boundary of the cortex.
Mapping sv-CBV over an anatomical brain atlas could lead to better understanding of vascular degeneration in dementia models, as well as the angiogenesis following treatments. Our method of sv-CBV mapping improves on past work as it can be used to determine CBV phenotypes in small 3D regions of the mouse brain. With continued development of better mouse brain atlases and genetic models, we anticipate that this method to measure sv-CBV will lead to a better characterization of vascularity in mouse models of cerebral vascular disease.
Chapter 3
High Resolution MRI and Micro-CT Imaging show Microvascular and Neuroanatomical Changes in a Mouse Model of Alzheimer’s Disease

3.1 Foreword

At the time of completion of this thesis, the work in this chapter was not published in a peer-reviewed journal.

3.2 Introduction

Research on Alzheimer’s Disease (AD) etiology has been dominated by the amyloid cascade hypothesis, which holds that deposition of extracellular Aβ in the brain is causative in the biochemical progression to AD(85). In this hypothesis, an imbalance between Aβ production and clearance initiates a multi-step cascade leading to the histopathological hallmarks of AD including neurodegeneration and synaptic failure with associated failure of neurotransmitter pathways. A major line of support for the amyloid cascade hypothesis is that transgenic mice, expressing a mutant form of the human amyloid precursor protein (APP) gene, develop amyloid plaque burden and cognitive symptoms such as spatial learning deficits(86). One of the major challenges to this hypothesis has been that the breakdown of vulnerable neuronal systems, especially those involved in memory, have often appeared to be disassociated from amyloid deposition, while it has been argued that the clinical impairments are better explained by the regional pattern of neurofibrillary tangles(87). The amyloid cascade hypothesis has been supported, however, by controlled studies on APP mouse models that first develop diffuse and then fibrillar Aβ plaques, which appear to precede any neurological damage. In fact, Aβ42 immunization in an APP model known as TgCRND8 has been shown to reverse behavioural and cognitive abnormalities and is coincident with approximately half as many dense-core plaques(88).
Several studies support the notion that microvascular changes, which include decreases in vascular density, atrophic and coiling vessels, and glomerular loop formations, are associated with the progression of AD(48; 89). There is a known association between amyloid plaques and damage to the architecture of blood vessels in mouse models of AD(90). Microvascular abnormalities may not directly result in perfusion changes, but may hinder the clearance of amyloid plaques(56). Although there are several mechanisms for clearing Aβ, including degradation of amyloid by microglia or by proteases and drainage of amyloid via the periarterial interstitial fluid, it has been found that direct transport of Aβ across the blood-brain-barrier (BBB) is normally one of the most efficient(91; 92). This direct vascular clearance route can become compromised due to BBB damage or when the BBB is overwhelmed by excessive production of Aβ. Despite the growing evidence for vascular involvement in AD, however, limited work has been done to characterize regional changes in microvasculature.

Regional changes in the microvasculature can be characterized by 3D imaging techniques that measure cerebral blood volume (CBV). This has been developed in mice using magnetic resonance imaging (MRI) and at higher resolutions with micro-computed tomography (micro-CT), a technique that can produce detailed 3D images of the cerebral vasculature(93). In vivo measurement of CBV, using MRI in transgenic AD-afflicted mice, has revealed altered CBV in the cerebral cortex, the hippocampus and the thalamus(58). Changes in brain morphology that are characteristic of AD have also been observed in different transgenic mouse models of AD(94; 95). Relative to histological methods, these neuroimaging approaches provide a macroscopic view of regional variation, which can often be a prelude to more detailed investigations.

A promising model of AD is the double APP695 mutant, with Swedish and Indiana mutations, known as TgCRND8 that expresses a high ratio of Aβ42 to Aβ40 and presents with an aggressive amyloid pathology(96). These mice have low survival rates and their dense-core plaques, widespread by 16-35 weeks, have been associated with dystrophic neurites(97; 98), synaptic degeneration and inflammatory response with activated microglia and astrocytes appearing concurrently with plaques(99). As with other APP mouse models(100), TgCRND8 mice do not show the extent of neuronal loss observed in human AD(99), although selective loss has been reported for cholinergic neurons in the vicinity of plaques at seven month of age(101) and for neurons in the hippocampus at five months of age(102), a major site for plaque
accumulation in TgCRND8 mice (99). This neuronal degeneration may be associated with brain atrophy, though swelling occurs in 28% of axons in the vicinity of plaques of five month old TgCRND8 mice (98) and infiltration of enlarged microglial cells and hypertrophic astrocytes accompanies amyloid deposition (101); both of these latter factors may contribute to brain hypertrophy. Vascular abnormalities in the TgCRND8 model include blood brain barrier damage with increased tortuosity, reduced number of microvascular branches (103) and widespread cerebral amyloid angiopathy (104). Decreased microvessel density in the cerebral cortex of six month old TgCRND8 mice was observed on 2D immunofluorescent images labeled for platelet endothelial cell adhesion molecule-1 (PECAM-1) (103). Despite the abundance of microscopic evidence for vascular and morphological changes in the TgCRND8 brain, to date, no study has provided a macroscopic view of these changes over the entire brain.

The present study characterizes and compares, in five month old TgCRND8 mice, the following three metrics: (i)- regional brain volume measured by MRI; (ii)- small-vessel CBV (sv-CBV), measured by micro-CT, where sv-CBV is like CBV in that it measures the percentage of a region’s volume occupied by blood vessels, however, only for small-vessels with diameter less than 100 μm; and, (iii)- plaque load, measured by immunohistochemistry. Plaque load is the percentage of each region’s area seen on histological sections that is covered by amyloid plaques. These three metrics were evaluated over anatomical regions of the brain delineated with high-resolution MRI. Brain volume and sv-CBV were measured over 23 brain regions and plaque load assessed in 15 regions.

3.3 Materials and Methods

3.3.1 Animals

The TgCRND8 colony was maintained in a 129SvEv background and heterozygote experimental animals were derived from crosses of TgCRND8 males with C57BL/6 females. PCR-based genotyping was performed both at the age of weaning and at sacrifice, namely, 3 weeks and 23±2 weeks. A total of 20 TgCRND8 mice (16 males and 4 females) and 27 controls (13 males and 14 females), underwent the following procedures, approved by the Hospital for Sick Children Animal Care Committee, subject to the Canadian Council for Animal Care.
3.3.2 Specimen Preparation

The cerebral vasculature of each mouse was perfused under controlled pressure with a radio-opaque silicone rubber, Microfil (Flow Tech, Inc., Carver, MA, USA), using the previously described protocol (93). To prepare for MR imaging, the dissected skulls were sequentially soaked in the following solutions: 12 hours in 4% paraformaldehyde (providing chemical fixation), 5 days in phosphate buffered solution (PBS) and 0.01% sodium azide at 15°C and 7 days in 2 mM Prohance (Bracco Diagnostics Inc., Princeton, NJ) diluted in PBS at 15°C (providing MRI contrast enhancement).

3.3.3 MR imaging

MR imaging was performed using a protocol customized for mouse applications (105). Specimens were imaged using a 7.0-T MRI scanner (Varian Inc., Palo Alto, CA, USA) while immersed in a proton-free susceptibility-matching fluid (Fluorinert FC-77, 3M Corp., St. Paul, MN). The parameters used in the scans were optimized for grey/white matter contrast: T2 -weighted, 3D fast spin-echo (ETL=6), with TR/TE= 2000/15 ms, NEX=2, field-of-view 25 x 14 x 14 mm and matrix size = 450 x 250 x 250, providing images with 56 µm isotropic voxels. The total imaging time was 11 hours and 35 minutes.

3.3.4 Micro-CT imaging

To avoid partial volume and beam hardening artifacts in Micro-CT images, specimens were decalcified by soaking for 2 days in 8% formic acid (Fisher Scientific Company, Ottawa, ON, Canada) at 15°C following a two day soak in PBS. Micro-CT images were then acquired as previously described (93).

3.3.5 Aβ Immunohistochemistry

Aβ was stained on 4 µm paraffin embedded coronal brain sections, four sections per brain (at level of olfactory, striatum, hippocampus and cerebellum), with 4G8 anti-Aβ monoclonal antibody (1:1000, Signet 9220-02), followed by overnight incubation at 4 °C and development using Avidin/Biotinylated enzyme Complex kit (Vector Labs, PK-6100) with visualization using diaminobenzidine. Counterstaining was performed with hematoxalin and scanning was at 20X (Hamamatsu Nanozoomer 2.0 RS scanner).
3.3.6 Computation of brain volume, sv-CBV and plaque load

All MR images were non-linearly registered to a neuroanatomical atlas with 62 segmented regions, as previously described(73; 105). This procedure allowed each of the 62 regions defined in the atlas to be outlined on the given MR image. Each micro-CT image underwent rigid-body registration to the corresponding mouse brain’s MR image by manually selecting twenty landmarks at vessel bifurcations in each image; the root-mean-square error of this registration, expressed as mean±standard deviation for the group of 47 image pairs, was 12.5±4.8 μm. This landmark registration was facilitated by contrast between the Microfil and surrounding tissue in the MRI images. sv-CBV was computed by utilizing the principle that for a voxel filled with two components, tissue and radio-opaque contrast agent, the micro-CT image intensity is a weighted average of the attenuation coefficients of each component(93). To emphasize the contribution of local microvessels to CBV, major vessels with diameter over 100 μm were excluded using vessel tracking(72), as previously described(93).

Plaque load was calculated over each region of the brain using the following procedure: (i)-regions of the images, described in section 3.3.5, were manually outlined with guidance from the corresponding MRI image with a co-registered labeled regions mapped; (ii)- a bayesian classifier(106) with non-informative prior was manually trained based on image pixel colour with 900 training points, which included ambiguous cases, to distinguish pixels corresponding to amyloid from those corresponding to Microfil and the remaining background using the computer program Classify, distributed by the Montreal Neurological Institute. Fifteen regions were manually outlined on the images of the histological slides and the percentage area classified as amyloid plaque was computed for each region.

3.3.7 Statistical analysis

For each mouse brain, volume and sv-CBV were computed in the 23 largest anatomical regions (see Table 3.1) of the brain atlas with the smallest region included being the dentate gyrus. To test whether there was significant regional variation in brain volume, an ANOVA was performed on a linear mixed effect model of the data, with two fixed effects (region and genotype) and one random effect (specimen) using the statistical program R, available at http://www.r-project.org/. Regional variation in sv-CBV was tested using the same procedure. Specific regional differences were determined by conducting t-tests for individual regions and a false discovery rate, q , was
computed corresponding to each p-value for the combined set of 23 volume measurements and 23 sv-CBV measurements based on the false discovery rate method(107). Percentage increases in volume, illustrated in Fig. 3.1, were plotted after blurring the data with a kernel of 0.5 mm full width at half maximum. To generate cortical surface plots shown in Fig. 3.2, sv-CBV was averaged over field lines perpendicular to the cerebral cortex(77; 78), using the previously described procedure(93).

To test for bilateral symmetry of regional brain volume, nineteen of the twenty three brain regions were segmented into portions in each hemisphere and an ANOVA was performed using a mixed effect linear model with region, genotype and hemisphere as fixed factors and specimen as a random factor. An analogous ANOVA was applied to test for significant hemispheric variation in sv-CBV.

An ANOVA was performed to test for variation of whole brain volume or average brain sv-CBV with sex, in each case with a linear model with genotype and sex as fixed factors. An analogous test for age related effects was also conducted.

To test for regional variation of plaque load, an ANOVA was performed on a linear mixed effect model, with region as the fixed effect and specimen as the random effect. To test whether percentage change in brain volume was significantly dependent on plaque load, an ANOVA was performed on a linear mixed effect model of the data, with two fixed effects (region and plaque load) and one random effect (specimen). Significant variation in sv-CBV with plaque load was tested using an analogous ANOVA.

The sensitivity of the test for a relationship between sv-CBV and plaque load or percentage change in brain volume and plaque load was estimated using power analysis. For each response variable, statistical power was estimated based on a Monte Carlo simulation as a function of the linear coefficient corresponding to plaque load. This coefficient in the linear-mixed effect model with two fixed effects (region and plaque load) was systematically varied and ANOVA was performed to test for significance (p-value < 0.05) with power estimated for 500 trials per coefficient.

All computed parameters were expressed as mean ± standard-error-of-the-mean (SEM) unless stated otherwise.
3.4 Results and Discussion

3.4.1 Global and local brain atrophy

TgCRND8 mice (n=20) had a total brain volume of 474±4 mm$^3$ which was significantly (p=0.0007) smaller than control mice (n=27) at 493±4 mm$^3$. This is not a surprising finding as global brain atrophy is prevalent in human AD patients(108) and has also been reported in some APP mouse models(109). Based on a linear mixed effect model, regional brain volume varied according to genotype ($F_{1,45} = 14.2$, p=0.0005) with an interaction between region and genotype ($F_{22,990} = 7.2$, p<0.0001). As depicted in Fig. 3.1, caudal regions of the brain had more significant volume decreases than rostral regions. Specifically, TgCRND8 mice had significantly decreased regional volume relative to controls in the arbor vitae of the cerebellum (7.2 %, q=0.0001), cerebellar cortex (8.4 %, q=0.0007), superior colliculus (4.3 %, q=0.003), corpus callosum (7.9 %, q=0.0004), medulla oblongata (7.2 %, q=0.0001), midbrain (6.1 %, q=0.0002), pons (6.9 %, q<0.0001) and thalamus (4.5 %, q=0.003), whereas the regional volume in the amygdala (Fig. 3.1) increased relative to controls by 5.9 % (q=0.002).

This pattern of regional brain volume change contrasts quite sharply with the characteristic pattern observed in human AD, where the hippocampus (including entorhinal cortex), neocortex and amygdala are usually the main sites of severe atrophy while the cerebellum is typically one of the least affected regions(110). As suggested by the reduced volume in the corpus callosum and arbor vitae, the significant atrophy in the brain stem regions may also be associated with its high content of white matter, which was not separable from the grey matter of the brain stem due to insufficient imaging contrast. The regional volume changes in TgCRND8 were also dissimilar to past studies on other APP mouse models(94; 109), although the tendency of caudal regions to have greater atrophy has been previously reported(94) in PSAPP mice. One possible explanation for these differences in brain volume change is that specific promoter and background strain differences between APP models may strongly influence the starting location or the progression of the neurodegeneration. Interestingly, despite previously reported neuronal loss in the hippocampi of TgCRND8 mice(102), no significant hippocampal atrophy was detected. No significant left versus right differences in brain volume were observed in either TgCRND8 or control mice. As previously reported(74), regional brain volume did depend on sex (p=0.001), but no significant relationship between genotype and sex was found.
Fig. 3.1 Regional differences in brain volume between TgCRND8 and control mice illustrated over five coronal slices, where the left column depicts average MRI for all 47 non-linearly registered brain specimens, the center column depicts the segmented regions and the right column plots the percentage volume increase of TgCRND8 relative to controls. These maps were masked based on a 5% false discovery rate threshold. Acronym codes for the legend are: AMG=amygdala, ARB=arbor vitae, BAS=basal forebrain, CBL=cerebellum, ENT=entorhinal cortex, FRO=frontal cortex, PTC=parieto-temporal cortex, ICO=inferior colliculus, SCO=superior colliculus, CRP=corpus callosum, DEN=dentate gyrus, HIP=hippocampus, HYP=hypothalamus, MED=medulla oblongata, MID=midbrain, NCA=nucleus accumbens, OLB=olfactory bulbs, OLT=olfactory tubercle, PRG=periaquiductal grey, PNS=pons, STR=striatum, THA=thalamus. Note that the occipital cortex was not represented in these five slices.
<table>
<thead>
<tr>
<th>Brain region</th>
<th>Brain volume&lt;sup&gt;a&lt;/sup&gt; (mm&lt;sup&gt;3&lt;/sup&gt;)</th>
<th>sv-CBV&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>Brain region</th>
<th>Brain volume&lt;sup&gt;a&lt;/sup&gt; (mm&lt;sup&gt;3&lt;/sup&gt;)</th>
<th>sv-CBV&lt;sup&gt;a&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brain region</td>
<td>control</td>
<td>TgCRND8</td>
<td>control</td>
<td>TgCRND8</td>
<td>q-value&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Amygdala</td>
<td>14.8±0.1</td>
<td>15.7±0.2</td>
<td>0.002</td>
<td>4.4±0.1</td>
<td>3.7±0.2</td>
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<td>Arbor Vitae</td>
<td>13.0±0.1</td>
<td>12.1±0.1</td>
<td>0.0001</td>
<td>2.7±0.2</td>
<td>2.7±0.2</td>
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<tr>
<td>Basal Forebrain</td>
<td>5.61±0.07</td>
<td>5.6±0.1</td>
<td>0.75</td>
<td>4.0±0.2</td>
<td>3.5±0.2</td>
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<tr>
<td>Cerebellar cortex</td>
<td>58.0±0.9</td>
<td>53.1±0.7</td>
<td>0.0007</td>
<td>4.9±0.3</td>
<td>4.5±0.4</td>
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<tr>
<td>Entorhinal cortex</td>
<td>9.71±0.09</td>
<td>9.7±0.1</td>
<td>0.95</td>
<td>7.5±0.2</td>
<td>6.2±0.3</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>36.1±0.5</td>
<td>35.6±0.7</td>
<td>0.68</td>
<td>4.0±0.2</td>
<td>3.8±0.3</td>
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<tr>
<td>Occipital cortex</td>
<td>6.15±0.09</td>
<td>5.94±0.09</td>
<td>0.24</td>
<td>6.0±0.3</td>
<td>5.0±0.5</td>
</tr>
<tr>
<td>Parieto-temporal cortex</td>
<td>78.6±0.7</td>
<td>76.4±0.9</td>
<td>0.16</td>
<td>4.0±0.2</td>
<td>3.3±0.4</td>
</tr>
<tr>
<td>Inferior colliculus</td>
<td>6.36±0.07</td>
<td>6.23±0.06</td>
<td>0.33</td>
<td>5.3±0.3</td>
<td>4.7±0.4</td>
</tr>
<tr>
<td>Superior colliculus</td>
<td>10.14±0.08</td>
<td>9.70±0.09</td>
<td>0.003</td>
<td>4.2±0.3</td>
<td>3.8±0.3</td>
</tr>
<tr>
<td>Corpus callosum</td>
<td>23.9±0.2</td>
<td>22.0±0.3</td>
<td>0.0004</td>
<td>1.9±0.2</td>
<td>1.8±0.2</td>
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<tr>
<td>Dentate gyrus</td>
<td>3.77±0.03</td>
<td>3.66±0.04</td>
<td>0.15</td>
<td>3.8±0.2</td>
<td>3.6±0.3</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>22.1±0.1</td>
<td>21.5±0.2</td>
<td>0.13</td>
<td>2.5±0.2</td>
<td>2.3±0.2</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>11.05±0.08</td>
<td>11.0±0.1</td>
<td>0.68</td>
<td>5.1±0.2</td>
<td>4.2±0.2</td>
</tr>
<tr>
<td>Medulla oblongata</td>
<td>27.2±0.3</td>
<td>25.3±0.3</td>
<td>0.0001</td>
<td>3.9±0.3</td>
<td>3.3±0.3</td>
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<tr>
<td>Midbrain</td>
<td>15.8±0.1</td>
<td>14.9±0.1</td>
<td>0.0002</td>
<td>3.5±0.2</td>
<td>3.1±0.2</td>
</tr>
<tr>
<td>Nucleus Acumbens</td>
<td>4.09±0.07</td>
<td>4.2±0.1</td>
<td>0.64</td>
<td>3.5±0.2</td>
<td>3.4±0.2</td>
</tr>
<tr>
<td>Region</td>
<td>Mean ± SEM</td>
<td>q-Value</td>
<td>p-Value</td>
<td>Mean ± SEM</td>
<td>q-Value</td>
</tr>
<tr>
<td>--------------------</td>
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<td>---------</td>
</tr>
<tr>
<td>Olfactory bulbs</td>
<td>27.5±0.7</td>
<td>0.12</td>
<td>5.1±0.2</td>
<td>4.7±0.3</td>
<td>0.53</td>
</tr>
<tr>
<td>Olfactory tubercle</td>
<td>3.90±0.06</td>
<td>0.16</td>
<td>7.2±0.3</td>
<td>6.7±0.4</td>
<td>0.47</td>
</tr>
<tr>
<td>Periaqueductal grey</td>
<td>4.54±0.04</td>
<td>0.43</td>
<td>2.7±0.2</td>
<td>2.5±0.2</td>
<td>0.68</td>
</tr>
<tr>
<td>Pons</td>
<td>18.8±0.2</td>
<td>0.0004</td>
<td>3.0±0.2</td>
<td>2.8±0.2</td>
<td>0.68</td>
</tr>
<tr>
<td>Striatum</td>
<td>27.4±0.3</td>
<td>0.87</td>
<td>2.4±0.2</td>
<td>2.3±0.2</td>
<td>0.75</td>
</tr>
<tr>
<td>Thalamus</td>
<td>18.4±0.2</td>
<td>0.003</td>
<td>2.5±0.2</td>
<td>2.4±0.2</td>
<td>0.75</td>
</tr>
<tr>
<td>Whole brain</td>
<td>493±4</td>
<td>0.0007</td>
<td>3.9±0.2</td>
<td>3.4±0.2</td>
<td>0.17</td>
</tr>
</tbody>
</table>

*aValues expressed as mean±SEM for n=20 for TgCRND8 and n=27 for controls

*bq-values calculated for a two-sample t-test with FDR correction, as described in section 3.3.7

3.4.2 sv-CBV decreased in several brain regions

Although average whole brain sv-CBV in TgCRND8 mice was not significantly different from control mice (11.6 % decrease, p=0.2), TgCRND8 mice had significantly decreased sv-CBV relative to controls in the amygdala (16.7 %, q=0.05), entorhinal cortex (16.6 %, q=0.02) and the hypothalamus (18.2 %, q=0.02).

As shown in the cortical surface plots of sv-CBV in Fig. 3.2, reduced sv-CBV was observed spanning the entorhinal and neighbouring temporal cortex. No significant change in sv-CBV was observed in the overall parieto-temporal cortex as delineated by the MRI atlas. A greater effect on sv-CBV for the entorhinal cortex than the hippocampus proper was also previously reported in an APP mouse model identified as J20(59).
As tissue degenerates, the volume of blood in the microvasculature might decrease in proportion to the tissue volume or, alternatively, the parenchyma might decrease independently of the vasculature. In the former case, sv-CBV would remain constant with respect to brain volume changes as observed for 8 of 23 regions tested whereas in the latter case, sv-CBV would change by an equal but opposite amount. Of the regions where sv-CBV underwent significant change, the hypothalamus and entorhinal cortex had no significant change in regional brain volume. These regional decreases may be associated with the reported reduction in microvascular

\[ \text{As tissu} \text{e degenerates, the volume of blood in the microvasculature might decrease in proportion to the tissue volume or, alternatively, the parenchyma might decrease independently of the vasculature. In the former case, sv-CBV would remain constant with respect to brain volume changes as observed for 8 of 23 regions tested whereas in the latter case, sv-CBV would change by an equal but opposite amount. Of the regions where sv-CBV underwent significant change, the hypothalamus and entorhinal cortex had no significant change in regional brain volume. These regional decreases may be associated with the reported reduction in microvascular} \]

\[ \text{Fig. 3.2 Variation of sv-CBV illustrated over right and left cortical surface maps. From top to bottom, rows represent mean sv-CBV for 27 control mice, mean sv-CBV for 20 TgCRND8 mice, difference between mean sv-CBV for control and mean sv-CBV for TgCRND8 mice, and segmentation of cortical surfaces into three regions, namely, the entorhinal cortex (ENT), the frontal cortex (FRO) and the parieto-temporal cortex (PTC).} \]
branches and density in cortical regions of five month old TgCRND8 mice using immunofluorescent images labeled for PECAM-1(103). On the other hand, in the amygdala, tissue volume changes did not fully account for changes in sv-CBV as the percentage decrease of sv-CBV was substantially greater than the percentage volume increase.

These changes in regional sv-CBV can be compared with previous CBV measurements in APP mice. In one study, Wu and coworkers reported statistically significant CBV reductions in the cerebral cortex, hippocampus and thalamus(58), whereas in another study Weidensteiner and coworkers reported significantly lower CBV in the occipital cortex and higher CBV in the hippocampus(60). While the explanation for these conflicting data is not entirely clear, at least some of these differences may be due to variations in mouse model selection or differences in experimental techniques used to measure CBV.

Although there was statistically significant variation of sv-CBV according to region \((F_{22,990} = 165, p<0.0001)\) with a significant interaction between region and genotype \((F_{22,990} = 2, p=0.001)\), neither left versus right nor sex differences in sv-CBV were found.

**3.4.3 No compelling evidence that the regional variation in sv-CBV and brain volume was associated with amyloid plaque load**

As illustrated in a representative coronal section of Fig. 3.3, plaque load varied significantly with brain region \((F_{14,160} = 12, p<0.0001)\) and, as shown in Table 3.2, the hippocampal and cortical areas, which are primary sites of plaque accumulation in human AD(47), had relatively high plaque load, as reported previously for TgCRND8 mice(99).

The percentage change in brain volume was not found to depend significantly on plaque load \((F_{1,145} = 1.2, p=0.3)\), though it was significantly related to the interaction between region and plaque load \((F_{14,145} = 2.7, p=0.002)\). Similarly, sv-CBV did not significantly depend on plaque load \((F_{1,145} = 0.003, p=0.96)\), though it was significantly related to the interaction between region and plaque load \((F_{14,145} = 2.4, p=0.005)\).

Power analysis for this experiment revealed that a relationship between sv-CBV and plaque load with a coefficient of at least 1.1 \((\% svCBV / \% \text{ plaque load})\) would be detected with a probability of type II error of less than 0.2. At this strength of relationship, an observed interregional
variation in plaque load ($\sigma = 0.25\%$) translates to a variation of sv-CBV that is 17.2% of the observed interregional sv-CBV variation ($\sigma = 1.6\%$). It follows from this analysis that this study was powered to detect an effect of plaque load that would be subtle compared to the observed interregional variations in sv-CBV. The failure to detect such an effect suggests that any dependence of sv-CBV on plaque load, if present, is small. Similarly, power analysis revealed that a relationship between percentage change in brain volume and plaque load with a coefficient of at least 6.5 (% change in brain volume / % plaque load) would be detected with a probability of type II error of less than 0.2. At this strength of relationship, an observed interregional variation in plaque load ($\sigma = 0.25\%$) translates to a 1.6 % change in brain volume, which is approximately half of the observed whole brain atrophy. Therefore, any dependence of percentage change in brain volume on plaque load, if present, is not large.

Fig. 3.3 Amyloid plaques were observed in TgCRND8 mice, identified as dark spots distributed over this representative coronal section which was immunohistochemically stained with the 4G8 anti-Aβ monoclonal antibody.
Table 3.2: Regional plaque load

<table>
<thead>
<tr>
<th>Brain region</th>
<th>Plaque load&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>n&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amygdala</td>
<td>0.10±0.02</td>
<td>13</td>
</tr>
<tr>
<td>Cerebellar cortex</td>
<td>0.017±0.005</td>
<td>19</td>
</tr>
<tr>
<td>Entorhinal cortex</td>
<td>0.18±0.05</td>
<td>7</td>
</tr>
<tr>
<td>Frontal cortex</td>
<td>0.36±0.07</td>
<td>14</td>
</tr>
<tr>
<td>Occipital cortex</td>
<td>0.10±0.04</td>
<td>4</td>
</tr>
<tr>
<td>Parieto-temporal cortex</td>
<td>0.32±0.06</td>
<td>20</td>
</tr>
<tr>
<td>Corpus callosum</td>
<td>0.25±0.06</td>
<td>20</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>0.42±0.09</td>
<td>19</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>0.016±0.006</td>
<td>12</td>
</tr>
<tr>
<td>Medulla oblongata</td>
<td>0.006±0.002</td>
<td>9</td>
</tr>
<tr>
<td>Midbrain</td>
<td>0.02±0.01</td>
<td>11</td>
</tr>
<tr>
<td>Olfactory bulbs</td>
<td>0.4±0.1</td>
<td>7</td>
</tr>
<tr>
<td>Pons</td>
<td>0.003±0.001</td>
<td>10</td>
</tr>
<tr>
<td>Striatum</td>
<td>0.025±0.005</td>
<td>16</td>
</tr>
<tr>
<td>Thalamus</td>
<td>0.07±0.02</td>
<td>13</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values expressed as mean±SEM

<sup>b</sup>Number of mice for which region was sampled
3.5 Conclusion

This study provides a macroscopic view of regional changes in microvasculature and brain morphology associated with genetic differences between TgCRND8 mice and controls. While evidence is provided of atrophy and reduced sv-CBV in several brain regions, there is no compelling evidence that these regional changes are associated with the observed amyloid plaque burden. Given the result of power analysis, the study design was likely to detect relationships of even moderate strength between sv-CBV and plaque load or percentage change in brain volume and plaque load. Therefore, the null findings are surprising since amyloid plaques, comprising aggregates of insoluble mature fibrils of Aβ, are the most prominent histopathological characteristic in TgCRND8 mice, which have been shown to exhibit both neuronal and microvascular pathology. Future studies exploring the relationship between the observed morphological changes and soluble forms of Aβ may provide further insights.
Chapter 4 Robust Method for 3D Arterial Spin Labeling in Mice

4.1 Foreword

The work in this chapter was previously published as:


4.2 Introduction

Arterial spin labeling (ASL) provides a means for quantifying cerebral blood flow (CBF) over mouse brain regions in a non-invasive manner. Mouse ASL permits longitudinal screening of cerebrovascular phenotypes during the mouse lifespan of 2-3 years, which facilitates the study of vascular factors in disease. Recent ASL applications in mice include models of stroke(111), cerebral malaria(112), sickle cell disease(113) and Alzheimer's disease(60). There is also considerable interest in quantifying CBF in mice for studying blood flow regulatory mechanisms(114) and for the development of disease-specific therapies. The use of ASL in mice is generally motivated by a desire to better understand mouse models of human disease.

Design of robust mouse ASL involves producing images with high enough resolution and signal-to-noise-ratio (SNR) to detect important changes in regional CBF, while meeting experimental constraints of scan time and sample size. The limits on available signal strength and gradient performance as well as physiological differences between the species prevent a simple scaling down of human imaging protocols for ASL from achieving satisfactory results.

Adapting amplitude-modulated continuous ASL (am-CASL) to brain size and carotid blood velocity of mice requires optimization of the labeling gradient and $B_1$ waveform within the given hardware constraints. Previous work using simulation has shown that, for some combinations of labeling parameters, inversion efficiency with am-CASL may be unstable with respect to small variations in blood velocity(115). It is therefore necessary to map the stable regime to bracket the anticipated blood velocity distribution for the mouse. All ASL pulse sequences are designed to
equalize MT signal loss in label and control images. The control experiment in am-CASL uses an RF pulse, which is sinusoidally modulated at frequency \( f_m \) to cause arterial spins to doubly invert as they flow through two closely spaced inversion planes. The power and center frequency of the RF pulse in this control experiment are matched to those used for single inversion, thereby equalizing MT effects on brain tissue. To achieve optimal inversion efficiency, the adiabatic inversion inequalities must be fulfilled, namely, \( 1/T_{2b} << Gv / B_1 << \gamma B_1 \), where \( v \) is the speed of the inflowing spins, \( G \) is the strength of the MRI gradient during labeling, \( B_1 \) is the amplitude of the radio-frequency field and \( T_{2b} \) is the transverse relaxation time of blood. It is also necessary to fulfill a condition unique to am-CASL that balances the requirement for stable inversion behavior with minimal relaxation (115), namely: \( Gv / B_1 f_m \approx 1 \). Even after satisfying these conditions, inversion efficiency in am-CASL remains highly sensitive to velocity (116), which requires careful slice positioning to reduce variability in CBF. With mice smaller than humans by a factor of fifteen in each linear dimension, it is not possible to obtain high quality localizers rapidly enough to enable accurate slice positioning by eye. Another relevant constraint is the small size of the mouse brain since, for commonly realizable gradient strengths, the 1-2 cm offset from the labeling plane intersecting the common carotid artery to the caudal or rostral limits of the mouse brain, corresponds to an off-resonance frequency range of 5-20 kHz. The associated large enhancement of \( T_1 \) relaxation due to MT (117), which has been characterized for typical CASL parameters at 7 T in rat (118), must be accounted for in CBF modeling equations to obtain accurate CBF maps (31). Moreover, the equilibrium magnetization of the tissue is reduced by MT. Thus, the constraints of mouse brain size and blood velocity necessitate judicious selection of am-CASL parameters.

This study describes an experimental approach to implementing mouse am-CASL in a robust manner. A rapid slice positioning algorithm is developed to improve the consistency of labeling plane placement and thereby inversion efficiency. Errors in positioning the inversion plane are evaluated. For the purpose of optimizing inversion efficiency parameters, blood velocity distributions in the mouse common carotid arteries are characterized using pulsed-wave Doppler ultrasound. These blood velocity data are used for simulations of inversion efficiency, while satisfying gradient duty cycle and SAR constraints. The sensitivity of inversion efficiency to velocity is determined. Following these developments, 3D am-CASL is implemented. The final
stage of this method is to calculate CBF based on an MT corrected model applicable to the high CBF values of mice.

4.3 Materials and Methods

All animal procedures described in this study were approved by the Hospital for Sick Children Animal Care Committee, subject to the Canadian Council for Animal Care.

4.3.1 Development of a rapid slice positioning algorithm

A slice positioning algorithm was developed which takes advantage of the conspicuity of vessel bifurcations in rapidly acquired MRI localizer images that provide inflow contrast due to short TR. By identifying the corresponding vessel bifurcations in a reference 3D vascular image, the prescribed inversion plane can be mapped by landmark registration to the correct position in the *in vivo* MRI coordinate system. In particular, four easily recognizable vessel landmarks with low positional variability can be identified in mid-sagittal brain slices (as shown in Fig. 4.1a).

An MRI anatomical brain atlas was used as a reference coordinate system, with 62 labeled 3D regions(73), prepared by averaging 20 female and 20 male C57BL/6 brain MRI images of 32 μm isotropic resolution(74). The MRI anatomical brain atlas can be accessed at http://www.mouseimaging.ca/technologies/C57Bl6j_mouse_atlas.html.

In this reference coordinate system, the coordinates of these four vessel landmarks were identified using nine post-mortem micro-CT images of the cerebral vasculature of C57BL/6 mice that had previously been co-registered to an MRI anatomical brain atlas for blood volume measurements(93). Mean and standard deviation of each vessel landmark were calculated for the nine brains.

In a post-mortem micro-CT cerebral vasculature specimen(93), serving as the reference 3D vascular image, the centerlines of the common carotid arteries were traced using custom-written software. Based on the image intensity gradient along rays perpendicular to the vessel centerline, this software maintains equal distance from the lumen wall(72). The plane perpendicular to the common carotid artery was selected as the inversion plane.
Fig. 4.1: Illustration of process of correcting for MT starting with an in vivo mid-sagittal localizer (a) with localized vascular landmarks (1-confluence of transverse and superior sagittal sinuses; 2-confluence of anterior cerebral artery and great cerebral vein of Galen; 3-confluence of rostral rhinal veins and superior sagittal sinus; 4-confluence of medial orbitofrontal artery and azygos of the anterior cerebral artery) and calculated position of inversion slice (slanted blue lines). For illustration purposes, the common carotid artery including its bifurcation is shown overlaid on the mid-sagittal plane (b) of the MR image of a fixed mouse (posture is not identical between a & b). Also shown is the calculated position and orientation of the labeling plane. The mid-sagittal slice through the map of computed distance from the inversion slice is shown in (c) and the calculated ratio of CBF (post-MT correction) to CBF (pre-MT correction) is shown in (d). Note the systematic variation of the MT effect across the brain, decreasing from the caudal to the rostral direction.
The anatomical brain atlas is oriented so that the +x direction points from medial to right lateral, the +y direction points from caudal to rostral and the +z direction points from ventral to dorsal. In this coordinate system, in millimeter units, the inversion plane is centered midway between the arteries at C=[0.0,-7.0,-11.3] with normal vector of N=[0.0,0.80,0.60], as shown in Fig.4.1b. Thus, from C and N, it follows that the equation of the inversion plane is 4y+3z+61.9=0. The slanted blue lines in Fig.4.1a represent the components of an am-CASL double inversion, which are equidistant and symmetric to this calculated inversion plane.

Given the in vivo locations of the landmarks (as described in section 4.3.4), the in vivo position of the inversion plane was calculated by rigid body vascular landmark registration using a Procrustes matching algorithm without scaling(75). This algorithm calculates the rigid body transformation that best matches each landmark position in the reference micro-CT image to the corresponding in vivo MRI landmarks. Using this algorithm and reference data, the placement of the labeling plane can be computed rapidly and largely automatically by the operator at the MRI console.

4.3.2 Analysis of errors in inversion plane positioning

A simulation was conducted to determine the extent to which uncertainty in vascular landmark placement led to errors in inversion plane position. Ten thousand coordinates centered on the true position of each vascular landmark were randomly generated (normal distribution with standard deviation of 1 mm, which is the thickness of the MRI localizer scans). The rigid body transformation that maps each simulated coordinate to the true position was computed by the Procrustes matching algorithm without scaling. The inversion plane was transformed accordingly. Mean positional and angular shifts of the inversion plane were determined.

4.3.3 Optimization of spin inversion efficiency parameters

The time evolution of inflowing blood magnetization was simulated in the mouse common carotid artery. The time-velocity profile of blood was measured in the common carotid, internal carotid and vertebral arteries of five C57BL/6 mice (body weight 20-25 g) using a VisualSonics Vevo 2100 ultrasound scanner equipped with a 40 MHz linear array transducer. 2D Doppler color flow imaging was used to guide each pulse wave Doppler recording in which the intercept
angle between the blood flow direction and the ultrasound beam was minimized to under 60° by optimizing transducer orientation. Beam angle was accounted for in the data analysis (119).

Inversion efficiency of am-CASL, namely, \( \frac{M_{\text{Control}} - M_{\text{Label}}}{2M_0} \), was calculated in a representative blood velocity distribution for the common carotid artery (spanning four cardiac cycles). The Bloch equations were solved (Runge-Kutta ODE solver, Matlab) for a range of velocities (1 to 40 cm/s) assuming a 3.1 s label pulse with \( T_{2b} = 0.25 \) s (120) and labeling gradient \( G=13 \) mT/m\(^{-1}\) (constrained by maximum gradient amplitude output at 50% duty cycle). \( T_1 \) effects were neglected from the simulation. The simulations were conducted with \( B_1 \) varying over the range 1 to 20 μT and modulation frequency, \( f_m \), varying from 50 to 1000 Hz. Inversion efficiency was calculated as an average over phase (0 to \( \pi \)) of the amplitude modulation at the time the spins cross the center of the labeling plane. For each combination of parameters, inversion efficiency was weighted by the blood velocity distribution to provide velocity-weighted inversion efficiency.

The bias of inversion efficiency to inaccurate inversion plane orientation was evaluated by calculating the reduction in velocity-weighted inversion efficiency corresponding to a scaling of the velocity distribution by \( \cos(\theta) \). In this case, \( \theta \) was the mean angular shift of the inversion plane resulting from errors in landmark identification (as described in section 4.3.2).

### 4.3.4 Implementation of am-CASL in mice

3D am-CASL was performed on ten female C57BL/6 mice (20-25 g) by the following protocol: Each mouse was scanned using a 7 T MRI scanner (Varian Inc., Palo Alto, CA, USA) with a horizontal 30 cm inner diameter bore gradient. The mice were anesthetized with 1.2 % isoflurane and maintained at core body temperature of 36.5±0.5°C, while being continuously monitored for respiratory rate, temperature and heart rate (121).

To identify the vascular landmarks needed for positioning the inversion slice, a mid-sagittal localizer image was acquired using a spoiled gradient-echo pulse sequence (TE = 4 ms, TR = 100 ms, 128 x 128 matrix, NEX=1, 90° Gaussian excitation pulse, FOV = 30 mm x 30 mm x 1 mm thick, total scan time = 13 sec). Each of the four vascular landmarks described in section 4.3.1 could be easily recognized on 1 mm thick slices.
Prior to each am-CASL scan, inversion efficiency was measured with inversion and imaging planes positioned on the common carotids and separated by 4 mm. The average magnetization over the common carotid cross-section was determined from the intra-vascular signal in three coronal spoiled gradient-echo images of the common carotids (TE = 5.75 ms, TR = 200 ms, NEX=4, 90° gaussian excitation pulse, matrix = 300 x 300, FOV = 30 mm x 30 mm x 1 mm thick), one following regular labeling ($B_1 = 9 \mu T$, hard pulse of 100 ms duration), the next following sinusoidally modulation of the labeling pulse ($f_m=400$ Hz, $B_1=(\sqrt{2})9 \mu T$, 100 ms duration) and the last by reflection of the labeling pulse ($B_1 = 9 \mu T$, hard pulse of 100 ms duration) about the imaging plane (to obtain equilibrium magnetization $M_0$). These measurements required a total of 12 minutes of scan time for each mouse. This protocol was adapted from an established method of measuring inversion efficiency\(^{(122)}\).

Flow-induced adiabatic inversion in the common carotid artery was applied using a pulse with labeling time $t_L = 3.1$ s, $B_1 = 9 \mu T$ with $G = 13$ mTm\(^{-1}\) and $f_m = 400$ Hz using the slice positioning algorithm described in section 4.3.1. Control and label cycles were interleaved and a post-label delay of 500 ms was applied to reduce transit time dispersion artifacts and to clear the intravascular signal\(^{(31)}\). The whole brain was imaged using a 3D fast spin echo pulse sequence ($T_E^{eff} = 4$ ms, TR = 6.6 s, ETL = 12, 90° hard excitation and 180° hard refocusing pulses, 60 x 96 x 80 matrix, FOV = 60 mm x 34 mm x 28 mm and NEX = 1) resulting in a total CASL scan time of 141 min for each of the ten mice.

For estimating arterial transit time for use in CBF modeling, am-CASL was applied to a second batch of five C57BL/6 mice at multiple post-label delays, namely, 50, 100, 200, 400, 800, and 1600 ms, while using a modified imaging matrix of 60 x 48 x 30 and FOV = 36 mm x 29 mm x 18 mm, resulting in a total scan time of 158 min for each mouse.

To provide reference tissue $T_1$ and proton density for CBF quantification, a third batch of five C57BL/6 mice were imaged using an inversion recovery pulse sequence with inversion times of 0.1, 0.3 and 2.0 s using a 2 ms hyperbolic secant inversion pulse and a 3D fast spin echo pulse sequence ($T_E^{eff} = 6$ ms, TR = 5s, matrix = 60 x 96 x 80 with FOV = 60 mm x 34 mm x 28 mm, 90° hard excitation pulse and 180° hard refocusing pulses, ETL = 12, NEX = 1) resulting in a total scan time of 160 min for each mouse. The $T_1$ and proton density maps for the five mice
were registered to each other and to the control images of the am-CASL experiment, through a rigid-body landmark-matching algorithm.

4.3.5 Implementation of am-CASL in mice

CBF was quantified using a single-compartment biophysical model that assumes capillaries to be completely permeable to water (i.e. permeability-surface area product = infinity), while accounting for both transit time and magnetization transfer enhanced $T_1$ relaxation,$^{31; 123}$ namely:

$$f = \frac{\lambda (S_{ctrl} - S_{lab})}{2\alpha S^0 K(\delta, w, T_{lb}, T_1, T_{1SAT})},$$  \hspace{1cm} [4.1]$$

where:

$$K(\delta, w, T_{lb}, T_1, T_{1SAT}) = \exp\left(-\frac{\delta}{T_{lb}}\right)\exp\left(-\frac{w}{T_1}\right)\left[T_{1SAT} \left(1 - \exp\left(-\frac{(t_L - \delta)}{T_{1SAT}}\right)\right) - T_1 \left(1 - \exp\left(-\frac{\delta}{T_1}\right)\right)\right],$$  \hspace{1cm} [4.2]$$

$$S^0 = \frac{1}{n} \sum_{i=1}^{n} \left(\frac{S_{ctrl}}{M^a_L(t_L + w)}\right)_i,$$  \hspace{1cm} [4.3]$$

$$M^a_L(t_L + w) = \left[1 - \exp\left(-\frac{w}{T_1}\right)\left(1 - M^a_L(t_L)\right)\right],$$  \hspace{1cm} [4.4]$$

$$M^a_L(t_L) = \frac{P}{r},$$  \hspace{1cm} [4.5]$$

$$R_{1SAT} = q - \sqrt{q^2 - r},$$  \hspace{1cm} [4.6]$$

and where:

$$p = R_0 R M^b_0 + R_{fb} R_a + R_b R_a + R_a R,$$

$$q = \left(R_a + R M^b_0 + R_{fb} + R + R_{fba}\right)/2,$$  \hspace{1cm} and

$$r = \left(R_a + R_{fba} + R M^b_0\right)\left(R_b + R_{fb} + R\right) - \left(R M^b_0\right).$$
Apart from inclusion of finite $t_L$, equation [4.1] is equivalent to CASL model presented by Alsop and Detre in 1996(31), where $f$ represents cerebral blood flow, $S_{\text{ctrl}}$ is the magnitude signal in the control image, $S_{\text{lab}}$ is the magnitude signal in the label image, $S^0$ is the equilibrium tissue signal which has been corrected for MT at each voxel (equation [4.3]), $\lambda$ is the brain/blood partition coefficient set to equal the measured regional proton density after normalization to an average of 0.9 mL/g(124), $\alpha$ is measured inversion efficiency, $\delta$ is the mean value of arterial transit time as determined by curve fit in a selected region of grey matter cortex, $w$ is the post-label delay, $T_{1b}$ is the longitudinal relaxation time of blood, set to 2.3 s as previously reported in rat at 7 T(125), while $T_{1\text{SAT}}$ and $T_1$ refer to the longitudinal relaxation time of brain tissue with and without enhancement due to MT, respectively.

$T_{1\text{SAT}}$ and $M_a$ were calculated (equations [4.4] – [4.6]) based on a binary spin bath model of MT(117; 126), using the reference $T_1$ maps and the known position and orientation of the labeling plane. As described in equation [4.3], the correction of $S^0$ for MT accounted for the post-label delay during which no off-resonance irradiation was applied. This MT model assumed Lorentzian and superLorentzian lineshapes when computing the saturation rates, $R_{\text{rfA}}$ and $R_{\text{rfB}}$, of the liquid pool A and semi-solid pool B, respectively. Based on previously established curve fits of this MT model in rat temporal cortex at 4.7 T(127), this calculation utilized an exchange rate, $R$, of 12.3 Hz, a semi-solid pool $T_2$ of 8.5 $\mu$s, a longitudinal relaxation rate of the semi-solid pool, $R_b$, of 2.9 Hz and a semi-solid pool size, $M_0^b$, of 0.11 which is defined relative to the normalized liquid pool size, namely, $M_0^a = 1$. The longitudinal relaxation rate of the liquid pool, $R_a$, was calculated as previously described(126) based on the average $T_1$ as measured by inversion recovery on 5 mice (see section 4.3.4). Liquid pool $T_2$ was assumed to be 38.7 ms, as previously published for young mouse cortex at 7T(128). $T_{1\text{SAT}}$ and $M_a$ were calculated by applying this MT model to the distance offset between the labeling plane of each mouse in which 3D am-CASL was performed, as shown in Fig.4.1c. To assess the impact of the MT correction, CBF was calculated both with and without the use of the MT correction and the ratio was plotted, as shown in Fig.4.1d.

Each CBF image was registered to the MRI anatomical brain atlas through a rigid-body landmark-matching algorithm. Mean CBF was computed in the twelve largest anatomical regions of the brain as shown in Fig.4.2 (a, b and c).
To test whether there was significant regional variation in CBF, an ANOVA was performed on a linear mixed effect model of the data, with one fixed effect (region) and one random effect (specimen) using the statistical program R, available at http://www.r-project.org/. Significant regional variation in T₁ was tested using an analogous ANOVA.

To determine if CBF significantly differed between brain hemispheres, nine of the twelve brain regions were segmented into portions in each hemisphere and the mean CBF was graphically compared as illustrated in Fig. 4.3. An ANOVA was performed using a mixed effect linear model with hemisphere and region as fixed factors and specimen as a random factor.

**Fig. 4.2:** Regions of interest in MRI atlas (top row) for coronal (a), sagittal (b) and transverse (c) images with corresponding mean CBF map (d,e,f, respectively). In this figure, CBF images were interpolated by a factor of two. Legend: AMG=Amygdala; CRB=Cerebellar Cortex; CER=Cerebral Cortex; COR=Corpus Callosum; HIP=Hippocampus; HYP=Hypothalamus; MED=Medulla Oblongata; MID=Midbrain; OLF=Olfactory Bulbs; PON=Pons; STR=Striatum; THA=Thalamus
A power analysis for a t-test was conducted (two-sided, two-sample, probability of type I error = 0.05, probability of type II error = 0.2) using the measured variation in hippocampal CBF as a representative region of interest.

All computed parameters were expressed as mean ± standard-error-of-the-mean (SEM) unless stated otherwise.

**Fig. 4.3:** Bilateral symmetry of CBF for nine brain regions (amygdala, cerebellar cortex, cerebral cortex, corpus callosum, hippocampus, hypothalamus, olfactory bulbs, striatum, thalamus) illustrated by plotting left versus right portion of CBF. Error bars represent ±SEM for n=10. For reference, the line of symmetry is indicated.
Fig. 4.4:  (a) Dependence of velocity-weighted inversion efficiency on $B_1$ for an am-CASL experiment. Simulation parameters were $G = 13 \text{ mTm}^{-1}$ and $f_m = 400 \text{ Hz}$. Velocity-weighted inversion efficiency reaches its peak of 0.87 at $B_1 = 9 \mu\text{T}$.  (b) Dependence of velocity-weighted inversion efficiency on $f_m$ for an am-CASL experiment. Simulation parameters were $G = 13 \text{ mTm}^{-1}$, $B_1 = 9 \mu\text{T}$.  (c) Dependence of longitudinal magnetization on blood velocity for control pulse (dashed curve) and labeling pulse (solid curve) in an am-CASL experiment with $G = 13 \text{ mTm}^{-1}$, $B_1 = 9 \mu\text{T}$ and $f_m = 400 \text{ Hz}$.  (d) Dependence of inversion efficiency (thick curve) on blood velocity. Blood velocity distribution is also shown (thin curve). Simulation parameters were $G = 13 \text{ mTm}^{-1}$, $B_1 = 9 \mu\text{T}$ and $f_m = 400 \text{ Hz}$. 
4.4 Results

The simulation of inversion efficiency in am-CASL provided an optimized parameter set corresponding to a gradient of 13 mTm\(^{-1}\). As illustrated in Fig. 4.4a, the velocity-weighted inversion efficiency peaked at \(B_1 = 9\ \mu\text{T}\) and reached a maximum value of 0.87. The effect of varying modulation frequency, illustrated in Fig. 4.4b, was to increase inversion efficiency until saturation after 300 Hz. Fig. 4.4c illustrates the dependence of longitudinal magnetization on velocity for the labeling and control experiment. Despite the oscillatory nature of the magnetization in the control experiment at high velocities and the steep drop at low velocities, there is a relatively flat region for both control and label magnetization in the range of 5 and 15 cm/s. Further, in Fig. 4.4d, which plots the dependence of inversion efficiency on velocity alongside a representative velocity distribution for the common carotid artery, it is apparent that the inversion efficiency is relatively constant for the velocity window that contains the bulk of the velocity distribution. This, of course, is non-coincidental as the optimized parameter-set from the simulation (\(B_1 = 9\ \mu\text{T}\), \(f_m = 400\text{Hz}\) and \(G = 13\ \text{mTm}^{-1}\)) were derived from the pulse-wave Doppler ultrasound measurements. Similar time averaged mean velocities were observed in all feeding arteries to the brain, with mean±standard-deviation (SD) of 12±1 cm/s, 12±2 cm/s, 14±3 cm/s, 11±2 cm/s, 10±2 cm/s, 11±1 cm/s for the left common carotid artery, right common carotid artery, left internal carotid artery, right internal carotid artery, left vertebral artery and right vertebral artery, respectively.

Analysis of errors in inversion plane positioning determined that, in the event of a large variability in landmark positioning in the range of 1 mm per landmark, the mean positional and angular shifts of the inversion plane were 2.4 mm and 18.6°, respectively. Misalignment of this magnitude would result in an inversion efficiency loss of 0.5 %. The selected vascular landmarks, based on micro-CT images, were found to have low inter-mouse variation (standard deviation of 0.19 mm).

Statistically significant differences in CBF (\(F_{11,99} = 19.28, p<0.0001\)) and \(T_1\) (\(F_{11,44}=176.31, p<0.0001\)) were observed over the twelve regions examined, with values listed in Table 4.1. \(T_1\) for the total brain was 1.53± 0.01 s. Based on the MT model, the group average \(T_{\text{SAT}}\) and \(M_z^* (t_e + w)\) were 0.57 s and 0.48, respectively. CBF for brain was found to be 219±6 ml/100g/min, based on the measured inversion efficiency of 0.67 ± 0.03 and the fitted arterial
transit time of 0.08 ± 0.02 s. In comparison, CBF calculated without MT correction was 217 ± 5 ml/100g/min. As summarized in Table 4.1 and Fig.4.1d, accounting for MT led to notable increases in CBF in caudal regions of the brain, while CBF in rostral regions decreased due to MT correction. Maps of mean CBF with the corresponding regions of interest are shown for coronal, sagittal and transverse views in Fig.4.2. CBF was found to be highly symmetric bilaterally and there was no significant hemispheric difference in CBF ($F_{1,153} = 0.12$, $p=0.73$). The bilateral symmetry of volumetric CBF measurements is displayed in Fig.4.3. Based on the power analysis displayed in Fig.4.5, CBF differences as low as 10% should be statistically significant for studies conducted with 12 animals per group.

![Power Analysis for Hippocampal CBF](image)

**Fig. 4.5:** Power analysis (two-sample, two-sided, probability of type I error = 0.05 and probability of type II error = 0.2) for difference in hippocampal CBF
**Table 4.1:** Regional values (mean±SEM) of $T_1$ (n=5) and CBF (n=10)

<table>
<thead>
<tr>
<th>Brain region</th>
<th>$T_1$ (s)</th>
<th>CBF (mL/100g/min) uncorrected for MT</th>
<th>CBF (mL/100g/min) corrected for MT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amygdala</td>
<td>1.65±0.03</td>
<td>176±7</td>
<td>183±7</td>
</tr>
<tr>
<td>Cerebellar cortex</td>
<td>1.50±0.02</td>
<td>187±12</td>
<td>209±13</td>
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<tr>
<td>Cerebral cortex</td>
<td>1.59±0.01</td>
<td>240±6</td>
<td>240±6</td>
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<tr>
<td>Corpus callosum</td>
<td>1.54±0.01</td>
<td>202±6</td>
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<tr>
<td>Hippocampus</td>
<td>1.55±0.01</td>
<td>273±7</td>
<td>277±7</td>
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<td>Hypothalamus</td>
<td>1.50±0.01</td>
<td>228±9</td>
<td>231±9</td>
</tr>
<tr>
<td>Medulla</td>
<td>1.33±0.01</td>
<td>175±13</td>
<td>203±15</td>
</tr>
<tr>
<td>Midbrain</td>
<td>1.33±0.01</td>
<td>189±10</td>
<td>189±10</td>
</tr>
<tr>
<td>Olfactory bulbs</td>
<td>1.61±0.01</td>
<td>258±7</td>
<td>247±6</td>
</tr>
<tr>
<td>Pons</td>
<td>1.38±0.02</td>
<td>190±11</td>
<td>201±12</td>
</tr>
<tr>
<td>Striatum</td>
<td>1.63±0.01</td>
<td>190±5</td>
<td>189±5</td>
</tr>
<tr>
<td>Thalamus</td>
<td>1.45±0.01</td>
<td>202±7</td>
<td>199±7</td>
</tr>
</tbody>
</table>
4.5 Discussion

Selecting a mouse ASL protocol involves making a series of choices. First, the protocol for spatially-selective labeling can either be Pulsed ASL (PASL), where blood water magnetization is labeled in a large slab proximal to the imaging slice, or Continuous ASL (CASL), where blood water magnetization is continuously labeled as it flows through a plane intersecting the carotid arteries. CASL was selected for this work due to its inherently higher signal strength(129).

Second, an image readout portion of the protocol must be selected. This study focused on a 3D approach to ASL. By imaging the entire brain, one can screen mutant mice for regional CBF phenotypes, while permitting regions of interest to be defined retrospectively. Relative to single slice imaging, whole brain imaging also facilitated automated registration to an MRI atlas and subsequent data mining. Furthermore, 3D imaging has the advantage that overlap of slice excitation profiles and saturation of arterial blood by proximal slice excitation can be avoided.

Third, a choice must be made between implementing CASL using a separate coil for labeling or using only the imaging coil(130). Single-coil CASL was implemented to avoid the need for additional hardware. This choice forces one to choose a strategy to control for magnetization transfer (MT). This study utilized the am-CASL labeling protocol(34), which places lower demands on the MRI gradient than pseudo-continuous ASL while maintaining high inversion efficiency(30). Moreover, relative to the simultaneous proximal and distal irradiation method, am-CASL provides more consistent control of MT effects over the entire brain(9).

A slice positioning algorithm was developed to position the labeling plane perpendicular to the common carotid arteries to ensure that the apparent velocity through the plane is high enough to avoid inefficiency associated with low velocity. In the mouse, this slice positioning algorithm is robust, as demonstrated by the simulated reduction of only 0.5 % in inversion efficiency associated with the hypothetical case of large variability in landmark selection. Furthermore, this method is easy to implement when labeling blood in the long and parallel common carotid arteries. By utilizing the inversion plane position recommended in this study, comparable inversion efficiency is expected for the vertebral and common carotid arteries, given their similar orientation and velocity distribution. Variation in neck posture between micro-CT and MRI scans was reduced by the use of an anatomically-moulded sled during MRI scanning and by careful positioning during fixation in the previous micro-CT study. Observed variation in the angulation of the common carotids among micro-CT specimens was approximately 10 °,
suggesting that any systematic effect of postural differences on inversion efficiency was small compared to other factors. Labeling of the common carotid arteries is preferable to the internal carotid arteries since MT enhancement of $T_1$ in the vicinity of the labeling plane will reduce the magnitude of the difference signal and, hence, reduce the precision of the CBF estimate.

Pulsed Doppler ultrasound measurements provide an efficient method to characterize common carotid blood velocity in mice. Furthermore, the calculated inversion parameters were robust with respect to blood velocity variation. This suggests that re-measurement of blood velocity should only be necessary in cases where very large changes in carotid velocity are expected (e.g. a stroke model) or where measured inversion efficiency is sub-optimal. Although the difference between measured and simulated inversion efficiency may be partly explained based on the known bias in favor of measuring slower moving spins(131), another potential factor is $T_{2b}$.

There is considerable variation in the literature on measured $T_{2b}$(120; 132–134), which is likely related to both physiological factors such as oxygenation and hematocrit as well as the timescale over which refocusing occurs. Short refocusing intervals lead to significantly larger $T_{2b}$ estimates(135). The relatively rapid transition that occurs during am-CASL inversion can be treated as a short refocusing period and justifies the use of longer $T_{2b}$ values when calculating inversion efficiency. For comparison, inversion efficiency for a shorter $T_{2b}$ of 70 ms was estimated at 0.64 as compared to 0.87 with a $T_{2b}$ of 250ms. A benefit of utilizing measured inversion efficiency in calculations of CBF is to reduce variability due to individual velocity differences. Although the mean velocity as measured by ultrasound was consistent across the group, it is still possible to find individual mice with atypical velocity distributions, which can ultimately impact inversion efficiency.

As depicted in Fig.4.2 (d,e,f), the described 141 minute CASL protocol provided high enough SNR to reveal blood flow contrast between neighbouring brain structures such as the cerebral cortex and surrounding white matter. Based on recorded heart rate, respiration and temperature, physiological parameters were stable throughout each CASL scan. This is consistent with a previous study which found that under similar anesthesia conditions, CBF was stable over 70 minutes(136). Where necessary it should be possible to reduce scan times by using a shorter labeling period, leading to lower SNR. The reduced SNR may, however, increase variability in the CBF data, which may in turn compromise statistical power. Additional SNR could be gained
by reducing post-label delay to significantly less than 500ms, which was an overly conservative selection given that the measured arterial transit time was only 80ms.

As shown in Table 4.1, the observed regional CBF values were larger in gray than white matter structures. Brain stem structures such as the medulla, pons and midbrain, which have a mixture of gray and white matter, tended to have only slightly lower CBF than major gray matter regions such as the cerebral cortex. A diverse range of CBF values in mice have been reported under similar experimental conditions. For example, three single-coil CASL studies on horizontally loaded mice maintained at 37 °C and anesthetized with average isoflurane concentration (mean±SD) of 1.3±0.6 %, reported whole brain CBF (mean±SD) of 208±12 ml/100g/min(137), 215±27 ml/100g/min(114), and 165±13 ml/100g/min(60). The whole brain MT corrected CBF (mean±SD) reported in this study of 219±18 ml/100g/min, while similar to other single-coil CASL studies, is higher than that reported by autoradiography(138; 139). While the explanation for this difference between autoradiography and CASL is presently unclear, a similar discrepancy has been previously reported for single-coil CASL studies applied to the rat brain(140). Differences in body temperature(136), anesthesia level(141) and subject orientation(114) may account for some of the reported differences in CBF.

The need to account for MT enhancement of T_1 could be avoided by using a much higher gradient (> 200 mTm^{-1}) or much lower B_1. The former option is, however, challenging due to hardware overheating limits, whereas the latter option may restrict the inversion efficiency to impractically low values. In this study, CBF was modeled to account for MT effects, which has not been routinely applied in mouse ASL studies. While local CBF values were affected by the MT correction, the relatively minor global effect of the correction may partly explain why this effect has been underreported in past mouse ASL studies. As shown in Fig.4.1d and tabulated in Table 4.1, the observed pattern of variation of the MT effect on CBF is dependent on position and orientation of the labeling plane. MT effects were accounted for in the CBF modeling to avoid having to measure this effect for each mouse. To improve accuracy of MT modeling, this study utilized T_1 and T_2 of the liquid pool based on mouse brain data at 7 T. Four parameters in the binary spin bath model were based on 4.7 T rat brain data, namely, exchange rate, semi-solid pool size, semi-solid pool T_1 and semi-solid pool T_2. The accuracy of this extrapolation of MT parameters to 50% higher field strength is supported by a study that compared these MT parameters over a wider field strength range in three concentrations of agar and reported little
variation(126). Furthermore, CBF as computed by equation [4.1] was found to be relatively insensitive to substantial variations in those MT parameters that were adopted from 4.7 T. It is, however, possible that direct measurement of $T_{1SAT}$ and the equilibrium tissue signal may further refine CBF modeling(118; 142).

Two-compartment CBF models have been proposed as an enhancement for human ASL. The single-compartment model applied in this study assumes complete permeability of the capillary wall to water exchange (i.e. PS=$\infty$). Applying the two-compartment model, namely, the fast flow solution proposed by Parkes and Tofts(23), to data in this study using a PS reported for rat cerebral cortex of 3.3 ml/g/min(143), a blood volume of 0.04(36) and a blood water content of 0.7(144), provided a whole brain CBF estimate of 221 mL/100g/min, which is within 1% of the value computed using the single-compartment model. In fact, the single and two-compartment models differed by less than 10% provided that PS was selected to be over 1.7 ml/g/min. This suggests that the single-compartment model is realistic for use in computing mouse brain CBF.

The development of robust am-CASL in mice was motivated by a desire to pursue large-scale applications that would greatly benefit from high-throughput protocols. As demonstrated by the power analysis, it is feasible to detect small differences in CBF when screening moderate numbers of mutant mice.
Chapter 5
Discussion and Conclusion

5.1 Summary

This thesis developed and tested techniques for measuring two perfusion-related metrics in mouse brain regions, namely, CBV and CBF using micro-CT and ASL, respectively. These techniques could lead to improved characterization of vascularity in models of neurological diseases.

Chapter 2 described the development of a technique to measure CBV in the mouse brain using micro-CT. Key features of the described method were vascular perfusion under controlled pressure, registration of the micro-CT images to an MRI anatomical brain atlas and re-scaling of micro-CT intensities to CBV units with selectable exclusion of major vessels. Histological validation of the vascular perfusion showed that the average percentage of vessels filled was 93±3%. Comparison of thirteen brain regions in nine mice revealed significant differences in CBV between regions (p<0.0001), while cortical maps showed that primary visual and auditory areas had higher CBV than primary somatosensory areas.

Chapter 3 described a study in the TgCRND8 model of AD in which regional patterns of brain volume and sv-CBV were measured with MRI and micro-CT, respectively. These metrics were compared with amyloid plaque load as detected by immunohistochemistry. Widespread brain atrophy was observed when comparing data collected on twenty TgCRND8 mice and twenty-seven controls, including significant overall brain volume loss (4.0%, p=0.0007), with greater atrophy in caudal brain regions. Additionally, significantly reduced svCBV was observed in the amygdala (17.4%, p=0.04), the entorhinal cortex (17.5%, p=0.01) and the hypothalamus (18.3%, p=0.01). There was no compelling evidence that regional variation in sv-CBV or brain volume was associated with amyloid plaque load.
Chapter 4 described a technique for robust mapping of CBF over 3D brain regions using am-CASL. To provide physiological data for CBF modeling, the carotid artery blood velocity distribution was characterized using pulsed-wave Doppler ultrasound. These blood velocity measurements were utilized in simulations that optimize inversion efficiency for parameters meeting MRI gradient duty cycle constraints. A rapid slice positioning algorithm was developed and evaluated to provide accurate positioning of the labeling plane. To account for enhancement of $T_1$ due to magnetization transfer, a binary spin bath model of MT was utilized to provide a more accurate estimate of CBF. A study conducted on ten mice provided highly reproducible inversion efficiency (0.67±0.03), statistically significant variation in CBF over 12 brain regions (p<0.0001) and a whole brain CBF of 219±6 ml/100g/min.

The sections below provide a discussion of further technical considerations for the perfusion-related metrics developed and tested. The material covered in this chapter extends the discussion beyond that in the body of this thesis and considers future directions.

5.2 Further technical considerations

Several lessons were learned during development of the perfusion protocol in chapter 2, described as follows: (i)- administration of the anticoagulant, heparin, improved consistency of perfusions, but was best limited at 10 IU/ g of mouse body weight to avoid excessive mouse bleeding; (ii)- a pilot study conducted in preparation for CBV measurements, testing the effect of a standard vasodilator, acepromazine maleate, on a small sample of mice, provided evidence of higher success rate for perfusions; (iii)- initial attempts to cannulate all four feeding arteries to the brain were abandoned due to difficulty in accessing the arteries without causing irrecoverable structural damage; attempts to cannulate the aorta at different locations were complicated either by excessive bleeding of neighboring vessels of the thymus gland or technical difficulties in efficient reproduction of the cannulation; (iv)- during clearance of the blood with warm heparinized phosphate buffered saline, at pressures significantly greater than 50 mmHg, the replacement of blood was not isovolumetric and there was evidence of edema in the form of swollen tissues; (v)- during Microfil infusion, pressures exceeding 180 mmHg were sometimes accompanied by burst vessels in the cerebral vasculature; on the other hand, infusion pressures
under 120 mmHg were found to reduce consistency of perfusions; and, (vi)- of the several methods for sealing off the venous outlet vessels, including cauterization, ligation and adhesion, the simple approach of using super glue adhesive was found to be the most reproducible.

Following these considerations, the procedure described in chapter 2, provided a high percentage of quality perfusions, yielding approximately 85% success rate for the perfusions described in this thesis. Major lines of evidence that support the accuracy of CBV determination using this protocol included the observations: (i)- that 93 ± 3% of vessels were found to be filled with Microfil as assessed histologically; this may have been underestimated due to minor displacement of Microfil fragments during slide preparations, or alternatively, may be related to a small percentage of unperfused capillaries; (ii)- that the completeness of Microfil perfusion, as assessed histologically, did not significantly differ between the locations examined; (iii)- that the respective ratio of CBV in cortical gray matter to white matter (corpus callosum) was 2.5:1, which is supported by a past study on the rat (35); and, (iv)- that the pattern of variation of sv-CBV through cortical depth was similar to the previously observed microvessel density variation in rats and humans. It is worth noting that points (ii) and (iii) also support that the lower values of CBV reported in certain regions were truly related to differences in vessel density and not just a filling artifact.

Other possibilities for obtaining validation of CBV measurements have been proposed in the literature. For example, in an experimental protocol, MRI bolus tracking measurements of relative CBV were correlated with CBV estimated using stereological principles applied to fractional vessel area measurements on frozen sections of Microfil-perfused rat brains (28). A prerequisite to obtaining sound measures of CBV with histological validation is that the contrast agent must remain stable within the vasculature. In my experience, frozen sectioning of Microfil-perfused mouse brain specimens did not consistently retain the Microfil segments within the vessel lumen to meet the standard needed for histological validation. The Microfil was, however, found to be retained in the vessels upon switching from frozen to paraffin embedding, although the two day paraffin embedding protocol applied in this thesis causes Microfil to shrink by 60-85%, as determined by comparison of micro-CT images of cured pieces of Microfil before and after applying the protocol. Thus, although the paraffin embedding protocol was useful for measuring the proportion of vessels filled with Microfil, the large amount of observed shrinkage of Microfil made it difficult to estimate CBV reliably from the histological images. While other
imaging options, such as ones described in chapter 1, remain feasible alternatives to using micro-CT mapping of CBV, each is not without its own technical challenges.

The co-registration of micro-CT and MRI images allowed the strengths of both modalities to synergize for calculation of CBV. Micro-CT images provided excellent contrast of vessel lumen, but relatively poor contrast of soft tissue boundaries. On the other hand, the T₂-weighted anatomical MRI images provided superior contrast for delineating soft tissue boundaries, but relatively limited vessel contrast. Thus, by using images from both modalities, it was possible to obtain the resulting measure of micro-CT defined volume of vasculature per each MRI defined volume of region. To accomplish this task, vascular landmark registration was developed in chapter 2 and subsequently further refined and applied in chapter 3. The main distinction in the registration procedure of the studies of chapter 2 and 3 was the increase in the number of landmarks utilized, from 4 to 20, respectively. The ability to accurately select more landmarks became possible with the acquisition of 3D high resolution MRI images of each Microfil-perfused specimen in the study of chapter 3. There was no evidence of inaccurate registration associated with observed chemical shift of cured Microfil (4.0±0.3 ppm), as viewed in the MRI images. A drawback of manually selecting more landmarks is the longer time investment needed. In my experience, the manual work involved in tagging 20 landmarks per pair of MRI and micro-CT images could be accomplished in half an hour per specimen after initially learning the routine.

Although the whole brain svCBV (mean±SEM) was similar for the wildtype mice in the studies described in chapters 2 and 3, namely, 4.4±0.3 % and 3.9±0.2 %, respectively, considerable regional differences in svCBV were observed, as illustrated in Fig.5.1. Based on a separate analysis, sex differences among mice in the studies could not account for these differences. The improved registration method of chapter 3 relative to chapter 2 may partly account for the reduced white-matter svCBV values by mitigating partial volume effects. Variability of vascular architecture has been previously reported for several mouse inbred strains(145), suggesting that genetic background may account for differences in cerebral vascular anatomy. It is possible that these differences extend to the microvasculature and are reflected in the svCBV differences in Fig.5.1.
Regional comparison of sv-CBV (mean±SEM) for wildtype mice from studies of chapter 2 (9 female C57BL/6 mice) versus chapter 3 (13 male and 14 female C57BL/6 x 129SvEv mice) revealed that these data sets were significantly correlated (r=0.76, p=0.003). For reference, a solid line of identity has been plotted.

Since this thesis has focused on 3D metrics, it may seem odd that the measurement of plaque load, in chapter 3, was performed using a 2D histological protocol. Immunohistochemistry was selected for this thesis for its proven ability to detect amyloid plaques. In my view, these plaque load measurements of chapter 3 were a preliminary step forward, and future studies may derive greater benefit by pursuing the development of a protocol for 3D imaging of plaques in mouse models. Several useful lessons were learned from the histological approach taken in this thesis, including, (i) that plaque detection was valuable as a decisive indicator of genotype, in cases where discrepancies existed between PCR genotype measurements made at weaning versus at sacrifice (specifically, errors were found in 10 out of 47 mice genotyped); (ii) that global shrinkage of brain tissue by 40-50% due to use of the paraffin embedding protocol was a potential source of error in plaque load calculations, since water content of plaques and surrounding tissue may differ; (iii) that the manual segmentation of 15 regions over 4 slices for
each of 20 TgCRND8 brain specimens was very laborious and an automated approach would be required to extend this to full brain coverage; (iv) that efforts to perform landmark-based affine registration of the histological images to the corresponding MRI slices for the same specimen were unfruitful due to boundary damage to several of the amyloid images; and (v) that when training the intensity-based plaque classifier, it was beneficial to create a separate category for Microfil, which had a relatively similar colour distribution to the diaminobenzidine indicator of amyloid; the clustering of training points into the three groups is illustrated in Fig. 5.2.

![Image of intensity-based clustering of amyloid, Microfil and background pixels](image_url)

**Fig. 5.2** Intensity-based clustering of amyloid, Microfil and background pixels (i.e. all other pixels besides those assigned to amyloid or Microfil), illustrated using the red-green plane (which best illustrates the clustering) from a red-green-blue image.

In chapter 4, CBF was calculated using compartmental modeling assumptions. Based on convergence of the results of single and two-compartment models, it was concluded that the single-compartment model of chapter 4 was realistic for use in computing mouse brain CBF. Revisiting equations [1.1] and [1.2], one may wonder why this general model could not be directly solved for CBF. In fact, if the arterial input function could be precisely defined at each voxel, then CBF and \( \lambda \) could be solved from the deconvolved impulse-response function as the
maximum value and area under the curve, respectively. In this case, no assumptions about intercompartment exchange mechanisms would be needed. Another benefit of developing deconvolution methods is the ability to extract greater amounts of physiological information, such as the regional variation of PS for water.

The deconvolution approach is, however, highly noise sensitive and requires knowledge of the complete shape of both the arterial input function and the tissue concentration-time curve(8; 9). Thus, the arterial and tissue concentration-time curves would have to be precisely acquired at high temporal resolution. CASL, which was selected for this thesis to provide maximal SNR for mouse imaging, only provides a single time point measurement without information on shape of the dynamic tracer concentration curves and is, therefore, not suited for this approach. In human ASL, recently proposed pulse sequences that rapidly acquire data at multiple time points, while providing a well defined bolus, may lead to feasible options for using deconvolution in mouse ASL studies(146).

It is worth noting that even if deconvolution methods were applied to ASL data, they would not provide CBV, since water is a diffusible tracer with a volume of distribution equal to its partition coefficient, which is an order of magnitude larger than CBV. Therefore, an intravascular tracer would be required to solve for both CBF and CBV.

Another key assumption in CBF modeling is that the labeled blood water arrives in each voxel by uniform plug flow. In reality, the shape of the arterial input will undergo dispersion as the flow profile is not exactly plug flow, but more like an intermediate mixture of parabolic and plug flow(14). It is unclear whether accounting for dispersion during the relatively short 80ms arterial transit period of mice will substantially improve measurement of CBF. To be useful to phenotyping applications, any such correction would have to be generalizable to account for dispersion in mouse models of vascular disease.

As discussed in chapter 4, side effects of MT on the ASL experiment could be diminished if MRI gradients of over 200 mTm$^{-1}$ were used for the entire labeling period. In this case, the 1-2 cm offset from the labeling plane to the imaging plane in mice would correspond to an off-resonance frequency range of 80-170 kHz and MT would have a negligible effect on the ASL experiment.
Due to duty cycle limitations in commonly available MR hardware, which restricts the amplitude of the ASL labeling gradient, this thesis accounted for MT effects using a binary spin bath model. MT had a substantial effect on both $T_1$ and equilibrium magnetization though, interestingly, it had a relatively minor effect on whole brain CBF.

### 5.3 Future directions

Investigations of abnormal microvasculature explored in this thesis were confined to a specific mouse model of AD, namely, TgCRND8. Using tomographic imaging techniques, the study described in Chapter 3 was the first demonstration of brain volume and sv-CBV changes in TgCRND8 mice. This study did not find compelling evidence that the observed regional changes were associated with deposition of insoluble Aβ. However, both soluble and insoluble forms of Aβ have been implicated in neurotoxicity and cognitive dysfunction in rodent models of AD(88; 147). Future studies could explore relationships between soluble forms of Aβ and the perfusion-related metrics described in this thesis. Furthermore, TgCRND8 mice substantially older than five months of age, having experienced greater cell death, may show different patterns of brain volume and sv-CBV change. These later stage patterns may more closely resemble changes observed in human AD and may be better explained by plaque load.

CBF could also be measured in TgCRND8 mice using ASL. In this case, subtler perfusion-related changes could be detected due to improved statistical power relative to the sv-CBV measurement method. Another advantage of applying ASL to this mouse model is the ability to study CBF in a longitudinal manner, which could be useful for characterizing the progression of the disease in this mouse model.

TgCRND8 is only one of dozens of AD models, none of which perfectly mimics human AD(86). These mouse models of AD have surprisingly divergent phenotypes depending on the manipulated combinations of transgene, promoter, genetic background and strain. The techniques developed in this thesis could well be applied to strains other than the TgCRND8 with a view to building a database of characterized phenotypes associated with the microvasculature. This will enable a better understanding of the genotype-to-phenotype relationships associated with microvascular abnormalities.
Bibliography


