EXPLAINING THE PATTERNS OF DISTRIBUTION OF EXOGENOUS RADIOLABELED ANANDAMIDE AND RELATED COMPOUNDS IN THE MOUSE BRAIN

Thesis Presented by

Kun Hu

To

The Bouvé Graduate School of Health Sciences
in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Pharmaceutical Sciences with Specialization in Pharmacology

NORTHEASTERN UNIVERSITY
BOSTON, MASSACHUSETTS

August 15th, 2016
Northeastern University
Bouvé College of Health Sciences

Dissertation Approval

Dissertation title: Explaining the Patterns of Distribution of Exogenous Radiolabeled Anandamide and Related Compounds in the Mouse Brain

Author: Kun Hu

Program: Doctor of Philosophy in Pharmaceutical Sciences
Approval for dissertation requirements for the Doctor of Philosophy in Pharmaceutical Science

Dissertation Committee (Chair):

Signature: ____________________________ Date__________

Printed Name: _____Samuel John Gatley__________

Other committee members:

Signature: ____________________________ Date__________

Printed Name: _____Dmitry Blinder__________

Signature: ____________________________ Date__________

Printed Name: _____Barbara Waszczak__________

Signature: ____________________________ Date__________

Printed Name: _____Ralph Loring__________

Signature: ____________________________ Date__________

Printed Name: _____David Janero__________

Associate Dean of Graduate Education, Bouvé College of Health Sciences:

Signature: ____________________________ Date__________

Printed Name: _____Jeanine Mount__________
Table of Contents

ABSTRACT .......................................................................................................................... v

ACKNOWLEDGEMENTS ..................................................................................................... vii

LIST OF ABBREVIATIONS ................................................................................................. viii

LIST OF TABLES ................................................................................................................ x

LIST OF FIGURES ............................................................................................................... xi

I. INTRODUCTION .............................................................................................................. 1
   A. statement of the problem .............................................................................................. 1
   B. The endocannabinoids and related compounds ......................................................... 4
   C. Synthesis, metabolism and signaling of the endocannabinoids ................................ 5
   D. Manipulating the endocannabinoid system ............................................................... 7
   E. Expression and regulation of FAAH and MAGL ......................................................... 9
   F. Human trials of FAAH inhibitors ............................................................................. 11
   G. Imaging the metabolism of the endocannabinoids ................................................... 12
   H. Hypothesis and specific aims ................................................................................... 16

II. Materials and methods .................................................................................................... 19
   A. Animals ....................................................................................................................... 19
   B. Radioactive compounds ............................................................................................. 19
   C. Drugs and biochemicals ............................................................................................. 19
   D. Equipment and instruments ....................................................................................... 19
   E. Autoradiography and regions of interest analysis ..................................................... 20
   F. Microdissection .......................................................................................................... 21
   G. Radio-TLC analysis .................................................................................................... 23
   H. Radio-HPLC analysis .................................................................................................. 26
   I. statistics ....................................................................................................................... 27

III. Results ............................................................................................................................. 28
   A. Aim 1: To evaluate the use of [14C-arach.]anandamide and [14C]arachidonic acid (AA) for imaging FAAH activity and PLA2 activity in the mouse brain ................................................ 28
   B. Aim 2: To evaluate the use of an anandamide analogue - [14C]myristoylthanolamine (MEA) - for imaging FAAH activity ......................................................................................... 36
   C. Aim 3: To synthesize NAEs labeled at the ethanolamine moiety and evaluate the use of [14C-EA]anandamide and [14C-EA]MEA for imaging FAAH activity ................................................. 45
   D. Aim 4: To evaluate the use of [3H]2-AG and [14C]2-AG for imaging MAGL activity .... 65
E. RadioTLC and autoradiography of brain from mice injected with N-(16-[18F]-fluorohexadecanoyl)ethanolamine ([18F]FHEA) ................................................................. 68

F. New hypothesis .................................................................................................................. 70

G. The patterns produced with [14C]iodoantipyrine and [14C]benzyl alcohol (rCBF tracers) are similar to those produced with anandamide and related compounds .................................................. 73

H. Arecoline increases the uptake of various tracers in the brain through activation of central muscarinic receptors .................................................................................................................. 76

IV. DISCUSSION ......................................................................................................................... 84

A. Radiotracers for ex vivo autoradiography and nuclear medical imaging ...................... 84

B. The factors that determine brain uptake and distribution of labeled anandamide and related compounds (How patterns are formed and what they mean) ...................................................... 91

C. How to interpret changes in brain uptake and distribution of radiotracers ................. 101

D. Limitations of our study ........................................................................................................ 110

V. SUMMARY AND CONCLUSIONS ..................................................................................... 111

VI. REFERENCES ...................................................................................................................... 115
Abstract:

N-Arachidonoylethanolamine, also called anandamide, is a lipid signaling molecule that acts on cannabinoid and vanilloid receptors. In the brain endocannabinoid system it acts as a retrograde neuromodulator, being produced post-synaptically and activating presynaptic cannabinoid CB1 receptors that control release of classical neurotransmitters. It is involved in the regulation of numerous physiological functions such as pain sensation, inflammation, mood, appetite and memory. Anandamide is synthesized on demand in the brain and is hydrolyzed after its action mainly by the enzyme fatty acid amide hydrolase (FAAH). Inhibitors of FAAH are candidate drugs against pain and inflammation. We aimed to develop a non-invasive method to map FAAH activity in the brain in order to facilitate evaluation of such drugs. However, several lines of investigation indicated that the pattern of radiolabel deposition after administration of $[^{14}\text{C}]$anandamide did not reflect FAAH activity.

Autoradiography of mouse brain after administration of $[^{3}\text{H}]$-arachidonoylanandamide shows a heterogeneous distribution pattern which was considered to reflect regional FAAH activity (Glaser et al. 2006). However, using carbon-14 instead of tritium we found that similar patterns were produced after injection of either labeled arachidonic acid or $[^{14}\text{C}]$-arachidonoylanandamide, suggesting that regional levels of FAAH activities do not control the disposition of radioactivity. To examine regional FAAH activity without the interference of other enzymatic activities related to the metabolism of the fatty acids, we undertook the radiosynthesis of anandamide labeled in the ethanolamine moiety instead of the acyl moiety, $[^{14}\text{C}]$-ethanolamineanandamide, and used this material, and also $[^{14}\text{C}]$ethanolamine in radiotracer experiments. Autoradiographic experiments with $[^{14}\text{C}]$-ethanolamineanandamide showed heterogeneous patterns of incorporation of radiolabel in the brain, which were distinct from the more homogenous distribution of radioactivity produced with $[^{14}\text{C}]$ethanolamine. Radio TLC and HPLC analyses showed that $[^{14}\text{C}]$ethanolamine was released from anandamide and then mainly
incorporated into phosphatidylethanolamine (PE). We then tested the effects of the FAAH inhibitor URB597 on the regional uptake of radiolabel from anandamide labeled at either the acyl or the ethanolamine moiety. Even though URB597 (3mg/kg) blocked the production of labeled phospholipids; it did not prevent the formation of a heterogeneous pattern at both 15 minute and 100 minute time points. Our results indicate that un-metabolized anandamide is distributed heterogeneously to different brain parts before it is hydrolyzed and that this initial distribution, rather than FAAH activity is the major factor controlling the brain distribution pattern of radiolabel from anandamide.

Since we found that similar patterns are formed with $^{14}$C-labeled fatty acids and their ethanolamides, we hypothesized that regional cerebral blood flow or delivery of the molecules plays a major role in formation of the patterns. In support of this notion, two blood flow tracers, iodoantipyrine and benzyl alcohol, showed initial distribution patterns similar to those obtained using arachidonic acid and anandamide. Further support for this view was obtained in studies using arecoline. This muscarinic agonist was shown to increase the incorporation of labeled arachidonic acid in the rat brain, an effect associated to increased PLA$_2$ activity (DeGeorge et al. 1991). However, we found that arecoline also increased brain uptake of radiolabel from $[^{14}$C-ethanolamine]anandamide, $[^{125}$I]RTI-55, $[^{14}$C]benzyl alcohol and $[^{125}$I]iomazenil.

The simplest explanation is that these changes are due to a global increase in cerebral blood flow induced by arecoline. Our results indicate that the heterogeneous disposition of radiolabel from arachidonic acid and anandamide largely depends on regional cerebral blood flow, or in other words the different rates of delivery of the molecules to different parts of the brain. This supports our conclusions from finding similar deposition patterns for labeled arachidonic acid and either isotopomers of $[^{14}$C]anandamide and also our radiochromatographic results in mice pretreated with FAAH inhibitors.
Acknowledgements

First of all, I would like to thank my advisor Dr. Gatley for the continuous support and guidance in my Ph.D study. He patiently trained me all the techniques and guided me through analyzing results and thinking up possible mechanisms, and most importantly, he inspired and encouraged me to design my own experiments to answer questions and solve problems. Without him, this project might have ended at the first aim. Because of the support from him, my six-year work has been very exciting and rewarding, and I feel myself grown into a scientist with a confident, independent mind.

I would also like to thank my committee members: Dr. Waszczak, Dr. Janero, Dr. Loring and Dr. Blinder. They have spent a lot of time in my proposal and progress report meetings, and even more time and efforts in reading drafts and helping me editing this thesis. Their insightful comments and suggestions have made this thesis as good as possible.

I am grateful to all my colleagues in Dr. Gatley’s lab, especially Dr. Duclos and Shilpa Sonti. Dr. Duclos spent a lot of time teaching me techniques in radiochemical synthesis and analysis, and was always there when I need help. Shilpa, a hardworking and cheerful friend in the lab, offered to help me in many of my experiments. Yu Miao, Xiaotian Jiang, Richa Pradhan, Xue Shui and many other students in the lab also helped me in my project. It felt wonderful working as a team.

Last but not least, I would like to thank my family and friends. My father, a remarkable paleontologist, nurtured my interest in science since I was little. My mother helped me find Northeastern and have spent a lot of money and energy to support me. My husband and best friend Weiwei was always there to listen, to encourage and to celebrate every small successful step in my research. Lei Guan, a colleague of Weiwei and Murui Han, my dear friend from Dr. Kim’s lab have offered valuable help in those busy weekends before deadlines.

This research was partly supported by the Office of Science (BER), U.S. Department of Energy.
List of Abbreviations

%IA/g: percentage of injected activity per gram

2-AG: 2-arachidonyl glycerol

2DG: 2-deoxy-D-glucose

\[^{18}\text{F}]2\text{-FDG}: \[^{18}\text{F}]2\text{-deoxy-2-fluoro-D-glucose}\]

\(\Delta9\text{-THC}\): (−)trans-delta-9-tetrahydrocannabinol

\[^{18}\text{F}]16\text{-fluoro-L-DOPA}: \text{L-3,4-Dihydroxy-6-}[^{18}\text{F}]\text{fluorophenylalanine}\]

AA: arachidonic acid

AA-CoA: arachidonoyl-coenzyme A

Abh4: α/β-hydrolase 4

ABHD6, ABHD12: αβ-hydrolase domain containing protein - 6 and - 12

Acyl-CoA: acyl-coenzyme A

AEA: anandamide

\[^{14}\text{C-arach.}]\text{anandamide}, \[^{3}\text{H-arach.}]\text{anandamide}: \text{anandamide labeled at the arachidonoyl moiety}\]

\[^{14}\text{C-EA}]\text{anandamide}: \text{anandamide labeled at the ethanolamine moiety}\]

\[^{11}\text{C}]\text{CURB}: \text{[}^{11}\text{C-carbonyl\}6-hydroxy-[1,10-biphenyl]-3-yl cyclohexylcarbamate}\]

BBB: blood brain barrier

CB1 receptor: cannabinoid receptor 1

CB2 receptor: cannabinoid receptor 2

CDTA: calcium-dependent transacylase

clogP: calculated logarithm of the partition coefficient

COX-2: cyclooxygenase-2

cpm: counts per minute

DAG: 1, 2-diacylglycerol

DAGL: diacylglycerol lipase

DMAP: 4-Dimethylaminopyridine

DHA: docosahexaenoic acid

DHEA: docosahexaenoylethanolamide
DLU/mm²: digital light units per mm²
DMSO: dimethyl sulfoxide
ECS: endocannabinoid system
EA: ethanolamine
EDCI: 1-ethyl-3-(3-dimethylaminopropyl)
EPEA: eicosapentaenoylethanolamide
FAAH: fatty acid amide hydrolase
FABPs: fatty acid binding proteins
[¹⁸F]FHEA: N-(16-[¹⁸F]-fluorohexadecanoyl)ethanolamine
GLUT1, GLUT3: Glucose transporter 1 and 3
HPLC: high performance liquid chromatography
Iomazenil: ethyl 7-iodanyl-5-methyl-6-oxo-4H-imidazo[1,5-a][1,4]benzodiazepine-3-carboxylate
i.v.: intravenous
i.p.: intraperitoneal
JZL184:4-nitrophenyl-4-[bis(1,3-benzodioxol-5-yl)(hydroxy)methyl]piperidine-1-carboxylate
lyso-PLD, lyso-PLC: lysophospholipase-D, lysophospholipase-C
MA: myristic acid
MAGs: monoacylglycerols
MAGL: monoacylglycerol lipase
MEA: myristoylethanolamine
MRI: magnetic resonance imaging
NAAA: N-acylethanolamine hydrolyzing acid amidase
NAEs: N-acylethanolamines
NAPE: N-acyl phosphatidylethanolamine
NAPE-PLD: N-acyl phosphatidylethanolamine-selective phospholipase D
PC: phosphatidylcholine
PE: phosphatidylethanolamine
PET: positron emission tomography
PI: Phosphatidylinositol
PLA₁: phospholipase A1
PLA₂: phospholipase A2
PLC: phospholipase C
PPAR-α: peroxisome proliferator-activated receptor alpha
rCBF: regional cerebral blood flow
Rf: retention factor
RTI-55: Methyl (1R,2S,3S)-3-(4-iodophenyl)-8-methyl-8-azabicyclo[3.2.1]octane-2-carboxylate;
s.c.: subcutaneous
SPECT: single photon emission computed tomography
TLC: thin-layer chromatography
URB597: [3-(3-Carbamoylphenyl)phenyl] N-cyclohexylcarbamate
List of Tables

Table 1. Quantification of the accumulation of radioactivity in various brain regions with OptiQuant.

Table 2. Effects of arecoline on whole brain radioactivity concentrations.

Table 3. Metabolically trapped radiotracers for brain studies.

Table 4. Radionuclides used for ex vivo and in vivo studies and mentioned in the dissertation.

Table 5. Ex vivo and in vivo radionuclide imaging modalities.

Table 6. Comparison of physical properties of radiolabels used in this study.
List of Figures

Figure 1. Trapping Mechanism for imaging FAAH activity using $[^3H]$anandamide.

Figure 2. Structures of some endocannabinoids and related lipids.

Figure 3. Patterns of mRNA expression and protein levels for FAAH and MAGL.

Figure 4. Example sections with regions of interest circled out for quantification of signal intensity using the OptiQuant software.

Figure 5. The extraction method for brain samples.

Figure 6. Example autoradiographs from mice injected with $[^{14}C]$AA and $[^{14}C]$-arach.[anandamide.

Figure 7. Comparison of regional deposition of radiolabel in the brain following administration of $[^{14}C]$AA and $[^{14}C]$-arach.[anandamide.

Figure 8. Comparison of relative signal intensity of different brain regions quantified from autoradiographs of mice injected with $[^{14}C]$AA and $[^{14}C]$-arachi.[anandamide.

Figure 9. Autoradiography with $[^3H]$MEA and $[^3H]$MA.

Figure 10. Autoradiography of mice brain sectioned at 2, 5, 10 or 80 minutes after administration of $[^{14}C]$MA.

Figure 11. Signal intensity from autoradiographs produced with $[^{14}C]$MA at different time points.

Figure 12. TLC analysis of samples from mice injected with $[^{14}C]$AA, $[^{14}C]$MEA or $[^{14}C]$MA.

Figure 13. TLC results of trial synthesis of $[^{14}C]$-EA.me.

Figure 14. Autoradiography with anandamide or MEA labeled at the ethanolamine moiety.

Figure 15. Brain autoradiography with $[^3H]$-arach.[anandamide showed reduced total activity and regional differences in mice treated with FAAH inhibitors and in FAAH knockout mice.

Figure 16. Brain autoradiography of mice pretreated with control or URB597 and injected with $[^{14}C]$-arach.[anandamide or $[^{14}C]$-EA.anandamide.

Figure 17. The effects of URB597 pre-treatment on regional deposition and total amount of brain radioactivity in mice injected with $[^{14}C]$-EA.anandamide.

Figure 18. Two-dimensional TLC analysis for brain and blood samples from mice pretreated with control or URB597 and injected with $[^{14}C]$-EA.anandamide.

Figure 19. $[^{14}C]$Ethanolamine is released from $[^{14}C]$-EA.anandamide and is incorporated into PE.

Figure 20. Detection of labeled ethanolamine by radio-HPLC in brain and blood aqueous extracts from mice pretreated with URB597 and injected with $[^{14}C]$-EA.ethanolamine.

Figure 21. One-dimensional TLC of blood and brain samples from mice treated with URB597 or vehicle showing the effect of URB597 on breakdown of $[^{14}C]$-EA.anandamide.
Figure 22. Pretreatment with JZL184 had little effect on regional or total accumulation of radiolabel from $[^3H]2$-AG.

Figure 23. Autoradiography and metabolite analysis with [$^{18}$F]FHEA.

Figure 24. Schematic representation of the formation of the autoradiographic patterns from labeled anandamide, AA and ethanolamine.

Figure 25. The patterns produced with [$^{14}$C]jodoantipyrine and [$^{14}$C]benzyl alcohol (rCBF tracers) are similar to those produced with anandamide and AA.

Figure 26. Arecoline increased incorporation of [$^{14}$C]AA.

Figure 27. Arecoline increased brain uptake of [$^{14}$C]AA, [$^{125}$I]RTI-55 and [$^{14}$C:EA]anandamide through activation of central muscarinic receptors.

Figure 28. Arecoline increased brain uptake of [$^{14}$C]benzyl alcohol and [$^{125}$I]iomazenil through activation of central muscarinic receptors.

Figure 29. The idea that AA metabolism involving PLA$_2$ activity (instead of anandamide metabolism involving FAAH activity) determines the pattern formed with [$^{14}$C-arach.]anandamide.

Figure 30. Illustrations explaining why the patterns are not determined by FAAH or PLA$_2$.

Figure 31. The three-stage model of how patterns are formed with radiotracers.

Figure 32. All possible factors in the three-stage model need to be considered in development of the two typical types of radiotracers.
I. Introduction

A. Statement of the Problem

The endocannabinoid system (ECS) is an important regulatory system in the brain as well as in peripheral tissues. It is involved in numerous physiological processes such as pain perception, inflammation, neuroprotection, memory, appetite and mood. Elements of the ECS, including the CB1 and CB2 receptors, endogenous ligands (endocannabinoids), and enzymes that are responsible for synthesis and degradation of endocannabinoids have been studied intensively, yet many aspects of how ECS works, such as ligand transport and retrograde signaling, are still incompletely understood, partly due to the unique characteristics such as lipophilicity of these ligands. Nevertheless, fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL), the most important metabolic enzymes for anandamide and 2-AG respectively, have been considered promising targets for development of medications for pain and inflammation, since their inhibition increases tissue concentrations of the endocannabinoids, and decreases synaptic activity in pathways involved in these processes.

Our project aimed to develop a non-invasive method to map in vivo FAAH activity in the brain in order to facilitate evaluation of FAAH inhibitors as candidate pain medications. Such an imaging method would, for example, facilitate quantification of the relationships between drug doses and dose regimens, and the degree of brain regional inhibition of FAAH. Since our own studies were initiated, several nuclear medicine radiotracers for FAAH such as $[^{11}C$-carbonyl]6-hydroxy-[1,10-biphenyl]-3-yl cyclohexylcarbamate ($[^{11}C$]CURB) have been developed(Wilson et al. 2011). These radiotracers bind irreversibly to FAAH and successfully used to image the FAAH protein levels in the human brain. However, a method for producing images of FAAH activity levels, which might be more important for monitoring the state of the endocannabinoid system and the effect of FAAH inhibitors has not yet been developed. In 2006, Glaser and her colleagues
suggested an *ex vivo* method to solve this problem: autoradiography of mouse brain after administration of \([^3\text{H}}\text{arach.}]\)anandamide show a heterogeneous distribution pattern which was considered to reflect regional FAAH activity (Glaser, et al. 2006). The concept of metabolic trapping mechanism (figure 1) underlies their approach: if the radiotracer is metabolized by the enzyme of interest and then the immediate metabolites which carry the radiolabel are trapped locally, the pattern of disposition of the radiolabel will give information about local metabolic flux through this enzyme. The best established radiotracers that utilize this principle are \([^{18}\text{F}}\text{2-deoxy-2-fluoroglucose}] (\([^{18}\text{F}}\text{2-FDG}) (Delbeke 1999) and L-3,4-Dihydroxy-6-\([^{18}\text{F}}\text{fluorophenylalanine}] (\([^{18}\text{F}}\text{16-fluoro-L-DOPA}) (Garnett et al. 1984) where the enzymes probed in positron tomographic studies are hexokinase and DOPA decarboxylase, respectively. Glaser’s (2006) method was partly based on previous studies by Rapoport and his colleagues using \([^3\text{H}}\text{arachidonic acid}. They argued that the local extent of deposition of radioactivity from labeled arachidonic acid reflected local activities of PL\(_\text{A}_2\) (DeGeorge et al. 1991, Rapoport et al. 1997). We started our studies by repeating autoradiography studies with radiolabeled anandamide but using carbon-14 instead of tritium for better quality images, with the hope to verify and further evaluate the usefulness of the method, and to better understand the metabolism of complex lipids in the brain.
Figure 1: Trapping Mechanism for imaging FAAH activity using $[^3]H$anandamide (Glaser et al. 2006). $[^3]H$AA is released from intravenously administered $[^3]H$anandamide by the action of FAAH and eventually incorporated in membranous phospholipids. By imaging at a time following the majority of tritium incorporation into membranes, but before appreciable tritium release from membranes by PLA$_2$, it is possible to temporally isolate $[^3]H$anandamide metabolite incorporation from downstream events in the brain. Thick arrows indicate prompt reactions and thin arrows indicate gradual reactions.*, tritium; FAAH, fatty acid amide hydrolase; AA, arachidonic acid, AA-CoA, arachidonoyl-coenzyme A; PI & PC, phosphatidylinositol and phosphatidylcholine; PLA$_2$, phospholipase A$_2$. 
B. The endocannabinoids and related compounds

The major psychoactive compound found in *Cannabis sativa*, (-)trans-delta-9-tetrahydrocannabinol (Δ9-THC), is an agonist for the G protein coupled cannabinoid receptors CB1 and CB2. The CB2 receptor is predominantly expressed in the immune system, whereas the CB1 receptor is expressed abundantly in the brain, modulating numerous physiological processes including learning, pain perception, appetite and neuroprotection. N-arachidonoylethanolamine (anandamide) and 2-arachidonoylglycerol (2-AG) are two major endogenous ligands that act on cannabinoid receptors, and are thus defined as endocannabinoids. Anandamide belongs to a class of lipids termed N-acylethanolamines (NAEs). Although anandamide binds to the brain cannabinoid receptor with high affinity (Ki = 52 nM) (Devane et al. 1992), it is present in the brain only at very low concentrations, on the order of pmol/g tissue (Sugiura et al. 2002). The second ligand 2-AG belongs to another class of lipids termed monoacylglycerols (MAGs), is expressed in the brain at much higher concentrations than anandamide, on the order of nmol/g tissue, although it has a relatively lower affinity (Ki =15 uM) to the brain cannabinoid receptor (Sugiura et al. 2006). Unlike anandamide, 2-AG acts as a full agonist at both CB1 and CB2 receptors (Sugiura et al. 2000) and is considered to be the major endocannabinoid in the brain. Other lipids that are structurally and pharmacologically similar to anandamide or 2-AG, such as noladinether (Hanus et al. 2001), virodhamine (Porter et al. 2002), N-arachidonyl dopamine (NADA) (Huang et al. 2002), and some other NAEs including the n-3 fatty acid ethanolamides docosahexaenoylethanolamine(DHEA) and eicosapentaenoylethanolamine (EPEA) (Brown et al. 2013, Cascio 2013), are also considered to be endocannabinoids. Two cannabinoid receptor-inactive NAEs, palmitoylethanolamine and oleoylethanolamide can activate PPAR-α receptors and have anti-inflammatory effects (Esposito et al. 2014) and appetite-suppressing effects (Rodriguez de Fonseca 2004) respectively. They are considered endocannabinoid-like compounds and are marketed as
dietary supplements. Structures of some endocannabinoids and related compounds are shown in Figure 2.

![Structures of some endocannabinoids and related lipids](image)

**Figure 2: Structures of some endocannabinoids and related lipids.** 2-AG, 2-arachidonoylglycerol; NADA, N-arachidonyldopamine. Myristic acid (not an endocannabinoid) was used in this project to compare its brain uptake and deposition pattern to that of arachidonic acid.

C. Synthesis, metabolism and signaling of the endocannabinoids

Unlike classical neurotransmitters, the lipophilic endocannabinoids are not stored in vesicles – rather, they are thought to be synthesized on demand at the postsynaptic site in response to an increased level of intracellular calcium (Di Marzo and Deutsch 1998) or activation of Gq/11-coupled receptors (Maejima et al. 2001, Kim et al. 2002, Kano 2014). There is also evidence showing that anandamide can be accumulated in lipid droplets (also known as adiposomes) inside the cells (Oddi et al. 2008), and 2-AG could be constitutively synthesized and stored until mobilized upon stimulation (Min et al. 2010).

The primary synthetic pathway of anandamide starts from the action of the calcium-dependent transacylase (CDTA) which transfers an arachidonoyl chain from phospholipid precursors to
phosphatidylethanolamine (PE) to generate N-acyl phosphatidylethanolamine (NAPE), which is then hydrolyzed by N-acyl phosphatidylethanolamine-selective phospholipase D (NAPE-PLD) to anandamide and phosphatidic acid (Di Marzo et al. 1994, Ahn et al. 2008). At least three alternative synthetic pathways for anandamide were identified. Anandamide can also be formed from NAPE by the sequential actions of PLA₂ and lysophospholipase-D (lyso-PLD) (Sun et al. 2004). NAPE was also shown to be hydrolyzed by phospholipase C (PLC) to phosphaanandamide, which is subsequently dephosphorylated by phosphatases (Liu et al. 2006). Another alternative pathway involves the enzyme α/β-hydrolase 4 (Abh4) which selectively hydrolyzes NAPE and lyso-NAPE to generate glycerophosphoarachidonoyl ethanolamide, which is then hydrolyzed to anandamide by a phosphodiesterase (Simon and Cravatt 2006).

For 2-AG, the primary synthetic pathway is the hydrolysis of an arachidonoyl containing phospholipid (phosphatidyl inositol bis-phosphate) (Jung et al. 2007) by PLC followed by the hydrolysis of the generated 1, 2-diacylglycerol (DAG) by diacylglycerol lipase (DAGL) (Bisogno et al. 2003, Ahn et al. 2008). Alternative synthetic pathways for 2-AG includes the cleavage of the phosphatidylinositol precursor by phospholipase A1 (PLA₁), followed by hydrolysis of the generated lyso-phosphatidylinositol by a lyso-PLC; however, the physiological importance of this pathway remains unclear (Cascio and Marini 2015, Lu and Mackie 2016).

Freshly synthesized endocannabinoids, particularly 2-AG, have to travel across the synaptic cleft to activate cannabinoid receptors on presynaptic neurons – a process termed retrograde signaling (Kano et al. 2009). It is still unclear how these lipophilic endocannabinoids travel across the aqueous extracellular space. Upon activation, the CB1 receptor couples to Gi/o proteins, which lead to inhibition of adenyl cyclase activity, regulation of ion channels and decreased neurotransmitter release from presynaptic neurons (Turu and Hunyady 2010). There is also evidence showing CB1 receptors can couple to Gs proteins in certain circumstances.
The functions of 2-AG and anandamide are different in modulation of synaptic plasticity. 2-AG can induce either transient or persistent suppression of neurotransmitter release, whereas anandamide can contribute to long term depression through both retrograde signaling and activation of transient receptor potential vanilloid receptor type 1 (Ohno-Shosaku and Kano 2014).

The levels of endocannabinoids in the brain are temporally and regionally controlled by the activities of local enzymes that are involved in their biosynthesis and degradation. Anandamide is hydrolyzed to arachidonic acid (AA) and ethanolamine, primarily by fatty acid amide hydrolase (FAAH) located in postsynaptic neurons (Cravatt et al. 1996, Ahn et al. 2008), while 2-AG is hydrolyzed to AA and glycerol, primarily by monoacylglycerol lipase (MAGL) in presynaptic neurons (Sugiura et al. 2006, Ahn et al. 2008). Degradation of anandamide or 2-AG is not restricted to one specific enzyme: 2-AG can also be metabolized by FAAH, αβ-hydrolase domain containing protein - 6 (ABHD6) and - 12(ABHD12) (Blankman et al. 2007); and both 2-AG and anandamide can be metabolized by cyclooxygenase-2 (COX-2), lipoxygenases, and cytochrome P450 (Piscitelli and Di Marzo 2012). Anandamide and related compounds such as palmitoylethanolamine are also substrates for N-acylethanolamine hydrolyzing acid amidase (NAAA) (Ueda et al. 2010, Alhouayek et al. 2015). Arachidonic acid produced by enzymatic hydrolysis of endocannabinoids is incorporated into the phospholipid pool, which provides precursors for the synthesis of new endocannabinoid ligands.

D. Manipulating the endocannabinoid system

Cannabinoid receptors, endocannabinoid messengers, and the enzymes that synthesize and hydrolyze the endocannabinoids, together constitute the endocannabinoid system: the functions of this system are tightly linked to those of other neurotransmitter systems and may vary across different brain regions. For example, activation of the CB1 receptor by Δ⁹-THC in the
hippocampus impairs long term memory (Puighermanal et al. 2009) whereas in the hypothalamic nuclei, activation of the same receptor increases appetite (Kirkham 2005); on the other hand, the antiemetic (Sharkey et al. 2014) and analgesic (Chiou et al. 2013) effects of cannabinoids are mediated by activation of cannabinoid receptors located in the brain stem. Levels of 2-AG are increased after closed head injury, and administration of 2-AG reduces post-traumatic brain edema and improves recovery (Panikashvili et al. 2001), suggesting that endocannabinoids have a neuroprotective function. 2-AG has also been shown to prevent beta amyloid-induced neurodegeneration and apoptosis in cultured hippocampal neurons (Chen et al. 2011), indicating that increasing endocannabinoid signaling could be a strategy in the treatment of neurodegenerative diseases.

Endocannabinoid signaling in the brain can be enhanced in several ways: 1) direct activation of the CB1 receptor by administration of CB1 receptor agonists, which could be endocannabinoids, plant-derived cannabinoids, or synthetic CB1 receptor agonists; 2) increasing levels of endocannabinoids via inhibition of metabolic enzymes, particularly FAAH and MAGL; 3) increasing the expression or sensitivity of the CB1 receptor in specific brain regions; 4) enhancing downstream signaling pathways by targeting specific elements in the signaling cascades. Practically, the first two approaches, which involve druggable targets – the CB1 receptor and the metabolic enzymes – are easier to implement. With regard to direct activation of the CB1 receptor with use of receptor agonists, Δ⁹-THC and other plant derived cannabinoids have antiemetic, analgesic, and appetite stimulating effects, and are known to be beneficial in treating wasting disease such as cancer and AIDS (Bar-Sela et al. 2014, Abrams and Guzman 2015); however, the psychoactive side effects and abuse liability associated with these compounds have led to concerns about their clinical use (MacDonald and Pappas 2016). Direct use of endocannabinoids has only transient effects, because of their rapid clearance by metabolic enzymes (Woodhams et al. 2015). The second strategy (inhibition of metabolic
enzymes) has advantages because signaling is initiated by endogenous ligands rather than synthetic agonists, and thus can be maintained for a long time, allowing therapeutic effects to be realized. Also, a FAAH (or MAGL) inhibitor would only increase activation of cannabinoid receptors where endocannabinoids are being generated whereas direct agonists activate all cannabinoid receptors. It is possible that the inhibitor-approach would avoid some of the undesirable psychoactive effects of Δ⁸-THC. However, much remains to be learned about the expression and regulation of the metabolic enzymes in different brain regions.

E. Expression and regulation of FAAH and MAGL

FAAH and MAGL both belong to the large family of serine hydrolase enzymes. The distribution of FAAH is heterogeneous among different brain regions: in the rat brain, in situ hybridization experiments showed that the expression of FAAH mRNA is relatively higher in the hippocampus and cortex, and lower in the hypothalamus, brain stem and pituitary (Thomas et al. 1997). Similar mRNA expression pattern was observed in mice (El Rawas et al. 2011) (Figure 3). However, different species may have different patterns of FAAH expression: for example, Egertova et al. (2004) used in situ hybridization as well as immunocytochemistry assays, to reveal that FAAH is highly expressed in the ventricular epithelium of the mouse brain, and not in the epithelial cells of the choroid plexus, whereas in the rat brain, the enzyme is expressed in the choroid plexus but not in the ventricular epithelium. In cerebellum and periaqueductal gray matter of the mouse brain, FAAH activity displays a diurnal variation – high at noon and low at midnight - but without significant changes in the protein levels of FAAH (Glaser and Kaczocha 2009). These regional and temporal differences need to be considered when developing therapeutic strategies that target FAAH, because functions of anandamide signaling may vary in different brain regions, and that the effectiveness of FAAH inhibition depends on the degree of FAAH activity at the targeted region. Several selective FAAH inhibitors, such as URB597 and
PF-3845, have been developed. Together with FAAH knockout mice, these are valuable tools for the study of anandamide and FAAH.

The expression of MAGL is also heterogeneous in the mouse brain, as shown with the use of \textit{in situ} hybridization; prefrontal cortex, nucleus accumbens shell and dorsal hippocampus express highest levels of MAGL mRNA (El Rawas et al. 2011). For several years, due to the lack of a selective inhibitor for MAGL, research on this enzyme lagged behind that on FAAH; however, the development of JZL184 as the first highly selective inhibitor of MAGL (it enhances 2-AG levels eight-fold without affecting anandamide in the mouse brain) (Pan et al. 2009) helped to unravel the function of MAGL. Unlike FAAH inhibition, chronic MAGL blockade desensitizes the CB1 receptor and causes functional antagonism of the endocannabinoid system (Schlosburg et al. 2010); consistent with this finding, MAGL knockout mice show partial desensitization of the CB1 receptor and enhanced learning in behavioral tests (Pan et al. 2011). These results suggest that 2-AG rather than anandamide plays the major (housekeeping) role in the endocannabinoid system, and excess 2-AG levels engage a negative feedback mechanism that downregulates the endocannabinoid tone.

The known patterns of mRNA expression and protein levels for FAAH and MAGL obtained with \textit{in situ} hybridization and ex vivo autoradiography are shown in Figure 3. The aim for this project was to develop a method for ex vivo imaging of FAAH \textbf{activity} levels, which could provide complementary information for monitoring the state of the endocannabinoid system and contribute to evaluation of the effect of FAAH inhibitors.
Figure 3. Patterns of mRNA expression and protein levels for FAAH and MAGL. A) In situ hybridization of FAAH mRNA in sagittal and coronal sections of adult rat brain. NC, neocortex; Hip, hippocampus; Tha, thalamus; Cb, cerebellum; CPU, caudate-putamen; Pn, pontine nuclei; AO, anterior olfactory nuclei; Hyp, hypothalamus; Amy, amygdala; Pir, piriform cortex (Thomas et al. 1997). B) Ex vivo autoradiography of $[^{18}F]$3-(4,5-dihydrooxazol-2-yl)phenyl (5-fluoropentyl)carbamate (a FAAH protein probe) in rat brain (Sadovski et al. 2013). C) In situ hybridization of FAAH mRNA and MAGL mRNA in mouse brain (El Rawas et al. 2011)

F. Human trials of FAAH inhibitors

Since our studies began, several clinical trials have been conducted with FAAH inhibitors. The clinicaltrials.gov website has 10 entries that mention FAAH. Three reports of the Pfizer compound PF-04457845 (Johnson et al. 2011) in human subjects have appeared: a phase I study (Li et al. 2012), a PET imaging study (Boileau et al. 2015) and a placebo-controlled trial of efficacy in pain resulting from osteoarthritis of the knee (Huggins et al. 2012). Unfortunately, the efficacy trial did not meet its primary endpoint. More recently, there has been an unfortunate event during a clinical trial in France, in which one subject died and four others were hospitalized during a phase I study of the FAAH inhibitor BIA 10-2474 (Begaud et al. 2016).
The lack of efficacy of PF-04457845 in human subjects, despite the efficacy in animal models (Ahn et al. 2011) and the French disaster have cast a pall on this field. The toxicity associated with BIA 10-2474 involved brain stem lesions dissimilar to any previously observed pharmaceutical toxicity and occurred after several consecutive days of treatment. It is most likely to be an off-target toxicity (Begaud et al. 2016). To our knowledge selective inhibitors of monoacylglycerol lipase such as JZL 184, which appears to alleviate neuropathic pain based on animal experiments (Ignatowska-Jankowska et al. 2015) have not been tested in human subjects; no studies are listed in clinicaltrials.gov.

G. Imaging the metabolism of the endocannabinoids

Our knowledge of the spatial localization and organization of the macromolecular components of the endocannabinoid system has come from the use of classical approaches involving microdissection and in vitro imaging modalities such as in situ hybridization, immunocytochemistry and receptor-binding autoradiography. Functional information has come from studies involving techniques such as electrophysiology, microdialysis, regional microinjection and animal behavior, synaptosomal preparations and measurements of regional endocannabinoid concentrations using liquid chromatography/mass spectroscopy.

Cannabinoid CB1 receptor and FAAH enzyme mapping at a spatial resolution of 5-10mm in the human brain have become possible because of the development of suitable radiotracers for positron emission tomography (PET) (Burns et al. 2007, Wilson et al. 2011). Functional studies of effects of cannabimimetic drugs have also been conducted in human using PET radiotracers able to quantify regional cerebral blood flow (e.g. [15O]water (Ter-Pogossian and Herscovitch 1985)) or glucose utilization [18F]2-FDG, and to detect changes in synaptic dopamine concentrations ([11C]raclopride (Volkow et al. 1994)). Anatomical and functional human brain studies of the cannabinoid system have also been performed using magnetic resonance
imaging (MRI) techniques. PET and MRI are minimally invasive and do not expose human research subjects to unreasonable risks; they also, obviously, provide information on the species of major interest in drug discovery and development. Additionally, these imaging modalities may have promise in medical diagnosis and in the monitoring or optimization therapies in individual patients suffering from conditions that target components of the cannabinoid system. An example may be Huntington’s Disease, where a large loss of cannabinoid CB1 receptors is seen in the substantia nigra (Glass et al. 1993). This is because Huntington’s Disease involves degeneration of striatonigral GABAergic neurons which express CB1 receptors on their nerve terminals in the substantia nigra.

While PET is primarily regarded as a human imaging modality, its minimally invasive nature also makes it useful for studies with valuable, large animals such as non-human primates, while instrumentation has been miniaturized to provide “microPET” devices offering 1 mm resolution in rodent species. Much higher spatial resolution can be obtained in ex vivo autoradiographic experiments, where following administration of radiotracer animals are euthanized and radioactivity distribution assessed in brain sections. While an ex vivo experiment with an individual animal is necessarily terminal, an advantage over PET is that short lived positon emitting radionuclides are not required. Readily available tritium, carbon-14 and iodine-125 labeled compounds can often be used. Also, animals that are conscious during the period of radiotracer distribution can be used; complications due to effects of anesthesia on brain functions are avoided. Ex vivo autoradiography in rodents is commonly employed during development and validation of PET radiotracers where the eventual aim is to conduct human studies. Imaging is combined with microdissection experiments, which generally require smaller amounts of radioactivity, and with radioanalytical experiments to determine concentrations of radioactive metabolites in the brain.
Our starting point was the work of Glaser et al. (2006) who, seeking to image FAAH activity, evaluated regional brain deposition of tritium after administration of $[^{3}\text{H}]$-arachidonic acid to mice. That study was based on several papers from an intramural laboratory at the NIH that had employed $[^{14}\text{C}]$arachidonic acid as a radiotracer in rats. It was shown that free arachidonic acid in the brain is rapidly incorporated into phospholipids, which essentially act as a “sink”. The central idea in the Glaser (2006) paper was that the appearance of free $[^{3}\text{H}]$arachidonic acid in the brain after administration of anandamide would necessitate operation of FAAH, and that the extent of formation of “fixed” labeled phospholipids would therefore reflect the activity of FAAH in particular brain areas, provided that the release of $[^{3}\text{H}]$arachidonic acid from anandamide was rate determining.

Even though the regional intensity of $[^{3}\text{H}]$AA incorporation following $[^{3}\text{H}]$anandamide administration might not be linearly correlated with the actual activity of FAAH, an ex vivo autoradiography method would be a convenient and valuable tool to assess changes of enzyme activity in the presence of potential FAAH inhibitors. However, autoradiographs of $[^{3}\text{H}]$AA accumulation obtained with use of $[^{3}\text{H}]$anandamide are very noisy (Glaser et al. 2006), making it difficult to quantify regional deposition of radiolabel in the brain. We reasoned that the use of carbon-14 instead of tritium would produce images with higher resolution and lower noise, and provide more detailed information with regard to differential enzyme activities in small brain regions. We therefore planned to examine the incorporation of $[^{14}\text{C}]$AA after intravenous administration of $[^{14}\text{C}]$anandamide, with the goal of uncovering more details about regional differences (Aim 1). Because FAAH also hydrolyzes other NAEs such as $[^{14}\text{C}]$palmitoyl ethanolamine, we anticipated that NAEs with shorter acyl chain, such as $[^{14}\text{C}]$myristoyl ethanolamine (MEA), would produce results similar to those of $[^{14}\text{C}]$ anandamide (Aim 2).

Duclos et al (2011) in our laboratory had also recently developed a methodology for radiolabeling the endocannabinoid 2-AG; such labeling had been difficult to achieve because 2-
AG is unstable and easily rearranges to 1-AG. The availability of radiolabeled 2-AG, along with that of the selective MAGL inhibitor JZL184 greatly facilitated research on 2-AG and MAGL. Thus we determined to extend the work of Glaser et al. (2006) to image radiolabeled AA incorporation following administration of radiolabeled 2-AG, with the expectation of revealing regional differences of MAGL activity (Aim 4).

In the original ex vivo autoradiography study by Glaser et al (2006), the imaging of [3H] AA incorporation following intravenous administration of [3H] AA was a control experiment, which showed a generally less pronounced, vague, homogenous distribution of the radiolabel. Because of the low signal and high noise, it is hard, in retrospect, to tell from their images whether the distribution is truly homogenous or simply appears so because of poor signal-to-noise. Based on the “trapping mechanism” shown in Figure 1, the incorporation of directly injected [3H]AA is independent of FAAH activity, but is dependent on the activities of acyl-CoA synthetase, acyltransferase and PLA2. Following administration of [3H]AA, any regional differences of radiolabel incorporation would reflect the differential metabolism of AA, involving the activities of Acyl-CoA synthetase, acyltransferase, and PLA2. Glaser et al. argued that since the action of PLA2 is slow, the time point when the brain is taken out for imaging would affect the distribution pattern: brains imaged shortly after the administration of [3H]AA would reflect the activity of acyl-CoA synthetase or acyltransferase, whereas a longer time would result in a pattern that is more influenced by the activity of PLA2.

Rapoport and his colleagues have published the results of many autoradiography experiments with the use of radiolabeled AA in animals that are treated with a variety of drugs to measure the changes in PLA2 signaling (Rapoport 2001, Qu et al. 2003, Rapoport 2003, Basselin et al. 2005, Chang et al. 2009). We do not yet know whether, following intravenous administration of radiolabeled AA, the incorporation of radiolabel merely reflects the activation of PLA2 but not of other enzymes, or whether PLA2 activity is proportional to the intensity of the incorporated
radiolabel. Nevertheless, we reasoned that these autoradiography methods established by Rapoport and his colleagues could be used to evaluate the effects of different drugs on the regional incorporation of radiolabel following intravenous administration of $[^{14}\text{C}]$anandamide or $[^{14}\text{C}]2$-AG (Aim 5).

The Rapoport group’s images of radiolabel incorporation following intravenous administration of radiolabeled AA did show a clearly heterogeneous distribution pattern in the brain, which was not obvious in the report by Glaser et al. (2006). Differences in the use of isotopes, times of exposure, animal species and strain, and ways of administering the radiolabel may explain this discrepancy. (See DISCUSSION section for more detailed analysis of this issue.) However, the similarity in patterns of radiolabel incorporation between $[^{3}\text{H}]$anandamide and $[^{3}\text{H}]$AA raised the question of whether $[^{3}\text{H}]$anandamide was indeed acting as a precursor of $[^{3}\text{H}]$AA, and whether the pattern actually reflected AA metabolism rather than FAAH activity. To address this possibility, we conducted similar autoradiography experiments with anandamide labeled at the ethanolamine moiety (Aim 3). Because incorporation of the radiolabeled ethanolamine would be independent of AA metabolism, we could eliminate the contribution of enzymes activities involved in AA metabolism, and thus produce a pattern which would be more consistent with actual FAAH activity. Successful autoradiography experiments in animals could be translated into non-invasive PET imaging, using carbon-11 rather than carbon-14, which could be a useful tool to monitor the changes in the metabolism of endocannabinoids and the biochemistry of complex lipids in humans and other large animals.

**H. Hypothesis and specific aims**

The original hypothesis was that heterogeneous distribution pattern of radiolabel in the mouse brain following intravenous injection of radio-labeled endocannabinoids (anandamide or 2-AG) reflects regional differences in the activities of the enzymes which metabolize the endocannabinoids (FAAH or MAGL), and the original specific aims were:
1. To evaluate the use of $[^{14}\text{C}]$arachidonoyl]anandamide and $[^{14}\text{C}]$arachidonic acid (AA) for imaging FAAH activity and PLA$_2$ activity in the mouse brain.

2. To evaluate the use of an anandamide analogue - $[^{14}\text{C}]$myristoyl]myristoylethanolamine (MEA) - for imaging FAAH activity.

3. To synthesize N-acylethanolamines labeled at the ethanolamine moiety and evaluate the use of $[^{14}\text{C}]$ethanolamine]anandamide and $[^{14}\text{C}]$ethanolamine]MEA for imaging FAAH activity.

4. To evaluate the use of $[^{3}\text{H}]$2-AG and $[^{14}\text{C}]$2-AG for imaging MAGL activity.

5. To test the efficiency of previously developed radiotracers in revealing changes of the metabolism of endocannabinoids caused by FAAH inhibitor, MAGL inhibitor and ethanol.

The results obtained for the first four aims indicated that the activity of FAAH or MAGL cannot be imaged by radiolabeled anandamide or 2-AG no matter where the label is. Similarity among the autoradiographs produced with the lipophilic tracers suggested that regional cerebral blood flow(rCBF) is the dominant factor that controls the deposition of radiolabel from intravenously administered $[^{14}\text{C}]$anandamide and $[^{14}\text{C}]$AA. A new hypothesis was developed: Initial delivery (determined by rCBF and permeability), diffusion rate of the compound across brain regions and back to the blood (stickiness to brain tissue, determined by its physical properties such as size, structure and lipophilicity) and binding/metabolism of the compound and its metabolites together determine the formation of the pattern and how it is changed over time. To support the new hypothesis, two more aims were designed:

1. To image rCBF with classic and new blood flow tracers ($[^{14}\text{C}]$jodoantipyrine and $[^{14}\text{C}]$benzyl alcohol).
2. To test the effect of arecoline on the incorporation of labeled AA (to repeat Rapoport’s experiment) and also on brain uptake of other tracers of which the metabolism does not involve PLA₂ activity (to challenge Rapoport’s explanation that the effect of arecoline was due to activation of PLA₂ rather than increased rCBF).

The results from the new aims supported the new hypothesis.
II. Materials and methods

A. Animals

We used male Swiss Webster mice (Charles River Laboratories, Cambridge, MA) weighing 25 ~30 g for all in vivo studies. Mice were maintained at the animal facility of Division of Laboratory Animal Medicine (DLAM) on 12 hour alternating light and dark period, with access to food and water ad libitum. Mice were treated in compliance with NIH guidelines for the use of laboratory animals and according to a protocol approved by the Institutional Animal Care and Use Committee (IACUC).

B. Radioactive compounds

The following tracers were used in this study: \[^{14}\text{C}]\text{AA}, \[^{14}\text{C}-\text{arach.}]\text{anandamide}, \[^{14}\text{C}]\text{ethanolamine}, \[^{14}\text{C}]\text{MA}, \[^{14}\text{C}]\text{jodoantipyrine} \text{and } \[^{14}\text{C}]\text{benzyl alcohol were purchased from American Radiolabeled Chemicals, Inc; }[^{14}\text{C}]\text{MEA}, ^{14}\text{C-EA}anandamide \text{and } ^{3}\text{H}2\text{-AG were provided by Dr. Richard DDuclos, Jr in our lab; }[^{125}\text{I}]\text{RTI-55 and }[^{125}\text{I}]\text{iomazenil were prepared by Dr. Samuel J Gatley from precursors purchased from ABX and Aobius, respectively.}

C. Drugs and biochemicals

The following were purchased from Fisher Scientific or Sigma-Aldrich: AA, anandamide, MA, MEA, 2-AG, JZL184, URB597, 200 proof ethanol (USP/NF) arecoline, methyldropine and atropine; Solvable, Ultima Gold™ XR LSC cocktail, high flash-point liquid scintillation counter cocktail were obtained from Perkin Elmer.

D. Equipment and instruments

The following items were used in this study: NVSL Manual Advance Vibroslice, Vibrating Microtome (Vibratome) (World Precision Instruments), Silica gel TLC plates, Reversed-Phase
C18 HPLC columns (Shimadzu), LS6500 Multi-Purpose Scintillation Counter (Beckman Coulter), Cyclone® Plus Storage Phosphor Imager, Resolution Storage Phosphor Screen and the OptiQuant software (Perkin Elmer Las Inc).

E. Autoradiography and regions of interest analysis

Autoradiography experiments to visualize the regional deposition of radiolabel in the mouse brain involved the i.v. administration of different $^{14}$C labeled compounds. The injection solution was prepared immediately before the start of the experiment. For injecting two mice, 10 µCi of the radiolabeled compound (lipophilic) was dried under argon air follow and re-dissolved in 25 µl of ethanol, and then 375 µl of pre-mixed emulphor/0.9% sodium chloride (saline) (1:18) was added, making the final ratio of the injection mixture to be 1(ethanol):1(emulphor):18(saline). The mixture was vortexed until the solution became clear. Two samples of 5µl of the injection mixture were quantified for the amount of radioactivity by liquid scintillation counter for verification of the injected activity and calculation of injected activity per gram (IA%/g). About 100 µl of the injection solution (20%) was lost in the syringe; therefore each mouse received 4 µCi of a radiolabeled compound in 200 µl of vehicle via tail vein injection. After 15 or 100 minutes (30 seconds for $[^{14}\text{C}]$benzyl alcohol, $[^{14}\text{C}]$iodoantipyrine and(delivery/blood flow tracers)), the mouse was euthanized by cervical dislocation, the brain removed immediately and chilled in ice cold 0.9% sodium chloride solution (saline). The cerebellum and brain stem were separated using forceps. Half of the cerebellum was weighed and dissolved in tissue solubilizer for quantification of the amount of radioactivity. The other half was stored in a 5 ml glass vial at -80°C for later TLC experiments. The forebrain was sliced on a vibratome into 300 µm thick coronal sections, collected on glass slides, and dried on a slightly warm heating plate overnight at 30°C. The sections were apposed to a storage phosphor screen for 5-21 days, which were scanned with Cyclone Plus imager to obtain autoradiograph of the brain sections. Because the signal intensity of the image is positively correlated to exposure time, sections that needed to be
compared for amounts of radioactivity were exposed to the same screen at the same time to ensure equal exposure time.

Autoradiographic analysis of regions of interest was done using the software OptiQuant supplied with the Cyclone imager. Cortex, hippocampus, ventricle areas, thalamus and striatum were circled on brain sections (Figure 4). The signal intensity in digital light units per mm² (DLU/mm²) was calculated by OptiQuant for each selected area. The average DLU/mm² values in the regions of interested were calculated in Microsoft Excel. To represent and compare the patterns of the autoradiographs obtained with use of different radiotracers, the relative DLU/mm² value in each brain region was calculated by dividing the absolute DLU/mm² value by the absolute DLU/mm² value in hippocampus of the same brain. Hippocampus was chosen as a reference region because its DLU/mm² value was found to be the lowest among the regions of interest in our preliminary study. While the absolute DLU/mm² values were used to quantify the amount of radioactivity that can enter the brain, the relative DLU/mm² values were used to compare regional differences of accumulation of radioactivity.

![Figure 4](image)

**Figure 4.** Example sections with regions of interest circled out for quantification of signal intensity using the OptiQuant software.

### F. Microdissection

Microdissection is an alternative method for quantification of regional distribution of radiolabel in the mouse brain. Compared with autoradiography, microdissection offers poorer spatial resolution, so that separation of small regions like ventricular epithelium or quantification of sub-
regional differences is difficult to achieve. However, it requires less time and less radioactive material. Also, it is more quantitative, because of regionally different self-absorption of beta-particles in autoradiography, which is not a problem with liquid scintillation counting of properly prepared samples. Self-absorption is a minor problem for carbon-14, but is more significant for the lower energy emissions from tritium.

Microdissection experiments were done with use of $[^3\text{H}]2$-AG to examine the metabolism of 2-AG via looking at regional distribution of radiolabel in the mouse brain. Tritiated 2-AG was synthesized by Dr. Duclos and stored in toluene at -80°C. The solution for i.v. injection was prepared just before starting each experiment, to limit the rearrangement or degradation of 2-AG. Toluene was evaporated under a flow of argon for protection against oxygen, and then $[^3\text{H}]2$-AG was re-dissolved in emulphor/ethanol/saline (1:1:18) immediately before administration to mice. Two samples of 5µl of the injection mixture were tested with the scintillation counter for the amount of radioactivity in order to verify the injected activity and for later calculation of IA%/g values. Mice were administered 1 µCi of $[^3\text{H}]2$-AG in 200 µl of vehicle via tail vein. After 15 minutes, animals were euthanized by cervical dislocation and the brains were removed immediately, and microdissected on a filter paper wetted with saline. Hypothalamus, olfactory tubercle, frontal cortex, hippocampus, striatum, cerebellum, brain stem, thalamus and rest of the brain were separated, weighed in tared scintillation vials, and dissolved in 1-2 ml of Solvable tissue solubilizer. After tissue samples were completely dissolved, 5 ml of scintillation fluid was added to each vial. Blood and urine samples were collected with fine tip transfer pipettes when mice were euthanized, and weighed in tared scintillation vials. Blood samples were dissolved in tissue solubilizer (Solvable) and then bleached with 50ul aliquots of 30% hydrogen peroxide until the color became light yellow. Five milliliter of scintillation fluid was added to urine samples and bleached blood samples. The amounts of radioactivity in the samples were quantified using the liquid scintillation counter. The counts per minute (CPM) and
H# (an index of quenching determined automatically by the use of the instrument’s internal radioactive source) were recorded for each individual sample and used to calculate the percent of injected activity per gram (%IA/g) for each sample. The relative %IA/g values were calculated by dividing the absolute %IA/g value in each region of interest by the absolute %IA/g value in hippocampus of the same brain.

The microdissection technique was also used to quantify regional distribution of radiolabel from \([^{14}\text{C}]\text{AA}, [^{14}\text{C}-\text{EA}]\text{anandamide,}[^{14}\text{C}]\text{benzyl alcohol,}[^{125}\text{I}]\text{RTI-55 and }[^{125}\text{I}]\text{iomazenil with arecoline or control pretreatment. These experiments were conducted following essentially the same procedures as for the 2-AG experiment described above, except that time points were chosen according to the purpose of each experiment. For }[^{14}\text{C}]\text{AA and }[^{14}\text{C}-\text{EA}]\text{anandamide, we waited for 15 minutes after tracer injection to euthanize the mice. This time point was chosen for consistency with studies of labeled AA by DeGeorge et al. (1991) and of labeled anandamide by Glaser et al. (2006). In other experiments we used shorter times because we wanted to capture the initial pattern of delivery of the tracer that closely reflects regional blood flow. By doing microdissection instead of sectioning in saline, we avoided diffusion of the tracer to adjacent brain regions which happens immediately after the animals were euthanized and continues in the sections until they are completely dried. We noticed this diffusion while doing autoradiography with \([^{14}\text{C}]\text{benzyl alcohol and }[^{14}\text{C}]\text{jodoantipyrine, which are water-soluble small molecules that diffuse quickly to adjacent brain regions, resulting in compromised resolution of the initial pattern of delivery.}

\textbf{G. Radio-TLC analysis}

TLC experiments were conducted to determine the chemical forms of radiolabel in the brain and blood following administration of different \(^{14}\text{C}\) labeled compounds, in order to study the metabolism of the radio tracers. Extraction of lipophilic metabolites of the radiotracer was done
as follows (figure 5): the 5 ml glass vial containing the half of the cerebellum saved from the autoradiography experiment was placed on ice. One milliliter of chloroform/methanol (2:1) was added and then the tissue was minced in the vial using a hand-held motorized homogenizer. Five hundred microliter of 40% urea and 500ul of 5% sulfuric acid were added to the vial to denature proteins, and the mixture was homogenized again until no particles of tissue were visible. The homogenate was split into two 1.5 ml centrifuge tubes, and centrifuged at 0°C, 18000 rpm for 5 minutes. After centrifugation three layers were formed: the chloroform fraction (lower layer), the methanol/water fraction (upper layer) and the protein deposit (middle sheet). A 200 ul pipette was used to remove a sample of the chloroform layer (larger tip sizes cause loss of chloroform by dripping). The pipette button was press down before touching the upper layer and was kept pressed while the tip was in the layers; the protein sheet was pushed away gently with the pipette tip to reach the organic phase; the pipette button was further pressed to blow a bubble from the tip to force out the small amount of liquid from the upper layer before aspiration; the pipette button was slowly released to aspirate the liquid from the chloroform layer and then the pipette was quickly removed from the vial and liquid from the chloroform layer was transferred to another 2ml tube. This procedure was repeated until most of the phase was transferred (with some left below the protein sheet). The upper layer was then poured to another clean 2ml tube and saved for later quantification and analysis of aqueous metabolites of the radio tracer. To increase the extraction yield for lipophilic metabolites trapped in and under the protein sheet, 200 ul of CHCl₃/MeOH 2:1 was added to the original tube containing the protein sheet. The tube was vortexed and centrifuged again under the same conditions. The method of separation was repeated and the separated extracts were combined with the extract from the 1st separation. When the chloroform extracts were contaminated with protein particles or aqueous droplets, the extracts were centrifuged again and re-separated. Blood samples were treated similarly to brain samples.
Figure 5. The extraction method for brain samples. The forebrain was immediately sliced using a vibratome and the cerebellum were cut in halves, weighed and kept in -80°C before analysis. Half of the cerebellum was dissolved in tissue solubilizer and then counted for total amount of radioactivity. The other half was extracted using the modified Folch method: The tissue was homogenized in 1ml of chloroform/methanol (2:1) and then 500ul of 40% urea and 500ul of 5% sulfuric acid were added to denature proteins, and the mixture was homogenized again until no particles of tissue were visible. The homogenate was split into two 1.5 ml centrifuge tubes, and centrifuged at 0°C, 18000 rpm for 5 minutes. After centrifugation the chloroform fraction (lower layer) was used for TLC analysis, the methanol/water fraction (upper layer) was counted or used for HPLC analysis and the protein deposit (middle sheet) was dissolved in tissue solubilizer and counted for radioactivity.
The chloroform fraction was dried under a stream of argon and re-dissolved in 50 μl of chloroform/methanol (2:1). The total amount of radioactivity in the chloroform extract was estimated by counting 5 ul of the extract. To visualize the metabolites efficiently with only a few days of exposure, a fraction of the extract containing at least 500 counts per minute (cpm) of radioactivity had to be spotted on a silica gel TLC plate, and to avoid smears from overloading or damaging the plate, the minimum amount of extract which had more than 500 cpm was spotted. The extracts from different samples were spotted along with 14C-labeled standards of the injected compound and its possible metabolites. An appropriate solvent system composed of chloroform and methanol, with addition of 1% of ammonium hydroxide or 1% acetic acid, was determined based on the polarity of the injected compound and its metabolites. After developing with the chosen solvent system in an airtight jar, the TLC plates were air-dried, then apposed to a phosphor storage screen for 1-5 day. The screen were scanned with the Cyclone Plus imager to obtain TLC images. The DLU ratios among different metabolites, which represent their relative amounts in the blood and cerebellum samples, were quantified using the instrument's OptiQuant software.

H. Radio-HPLC analysis

Samples of aqueous fractions of Folch extractions were analyzed by HPLC for the presence of [14C]ethanolamine. A Whatman Partisil SCX column (250 x 4.6mm) was used. The mobile phase was 50 mM potassium phosphate buffer, pH 3.5, and the flow-rate was 1 ml/min. Successive 2mL fractions of the eluate were assayed for C-14 using liquid scintillation counting. Authentic [14C]ethanolamine appeared in fractions 6 and 7.
I. Statistics

In quantifying relative signal intensity values of brain regions in autoradiographic experiments and in experiments evaluating the effects of pharmacological pretreatments (URB597, Arecoline) on brain deposition of radioactivity, p-values for comparisons of individual brain regions were calculated using student’s t-test in Microsoft Excel. Bar charts were expressed as mean ± standard deviation.
II. Results

A. Aim 1: To evaluate the use of \([^{14}C\text{-arach.}]\text{anandamide}\) and \([^{14}C\text{-arachidonic acid (AA)}]\) for imaging FAAH activity and PLA\(_2\) activity in the mouse brain

Glaser et al. (2006) showed, using a Biospace “beta-imager” to measure deposition of tritium in brain slices, that the mouse brain uptake of radiolabel from \([^{3}H\text{-arach.}]\text{anandamide}\) is heterogeneous, and they postulated that the heterogeneous pattern reflects regional differences in FAAH activity, representing incorporation of \([^{3}H]\text{arachidonic acid}\) released from labeled anandamide by FAAH into phospholipids. However, when Glaser et al. (2006) administered \([^{3}H]\text{arachidonic acid}\), to mice, they saw almost homogeneous deposition of radioactivity, which contradicted studies in the literature conducted using rats; Rapoport and co-workers found that a heterogeneous pattern developed in the brain using labeled AA, and that the patterns could be altered by a variety of drugs. Rapoport and his colleagues suggested that the heterogeneous pattern reflects PLA\(_2\) activity, which can be altered pharmacologically (Rapoport 2001, Qu et al. 2003, Rapoport 2003, Basselin et al. 2005, Chang et al. 2009). We started our project following Glaser’s method, but followed Rapoport’s group in using carbon-14 labeled materials, since this isotope is better suited to autoradiographic studies using phosphor-imaging devices.

We have compared the regional deposition of radiolabel in the brain following i.v. administration of \([^{14}C]\text{AAor}^{[14}C\text{-arach.}]\text{anandamide}\). Mice were given 4 \(\mu\)Ci of \([^{14}C]\text{AA}\) or \([^{14}C\text{-arach.}]\text{anandamide}\) via tail vein injection. After 10 or 100 minutes, animals were euthanized by cervical dislocation and the brains were taken out immediately and chilled in ice-cold 0.9% sodium chloride solution. The forebrain was sliced on a vibratome at the thickness of 300 \(\mu\)m. After the brain sections were dried, they were apposed to a storage phosphor screen, which was then scanned with the Cyclone Plus imager to obtain autoradiograph of the brain sections. The experiments were repeated four times for each time point and for each radiotracer. Representative autoradiographs obtained following administration of 4 \(\mu\)Ci of \([^{14}C\text{ AA}\) or \([^{14}C\text{-arach.}]\text{anandamide}\) are shown in Figure 6. The color intensity of the images for each brain was
individually adjusted with the software OptiQuant to show clear regional differences in accumulation of radioactivity. Autoradiographs for brains sectioned 100 minutes after injection are shown in both grayscale and color, with the different levels of signal intensity reflecting the different amounts of radioactivity.

Each radiotracer produced a heterogeneous distribution of radioactivity in the mouse brain. The use of $^{14}$C instead of $^3$H produced higher quality autoradiographs with lower noise and higher effective resolution. We could see detailed patterns of regional deposition of radiolabel in the brain following administration of $[^{14}$C]AA, which was not observed by Glaser et al. using $[^3$H]AA (Figure 7A). However, the pattern of the autoradiographs produced using $[^{14}$C]AA was very similar to those produced with use of $[^{14}$C-arach.]anandamide, both showing higher accumulation of radioactivity in cortex, thalamus and ventricular epithelium (figure 7B).

To quantify and compare the differences of accumulation of radioactivity in different brain regions, sections containing cortex, hippocampus, ventricles, thalamus and striatum were selected and the regions of interest were circled out on each section (Figure 7C). Using OptiQuant, the signal intensity in DLU/mm$^2$ was calculated for each region of interest. We compared the absolute values of signal intensity for different brain regions from $[^{14}$C]AA and $[^{14}$C-arach.]anandamide injected mice that were sacrificed at 10 or 100 minutes after tracer injection. Consistent with the observation of Glaser et al., the accumulation of radioactivity (as reflected in DLU/mm$^2$) produced with use of $[^{14}$C-arach.]anandamide is 3 to 5 fold higher than that produced with use of $[^{14}$C]AA (Table 1). This phenomenon shows that AA, which is a fatty acid with negative charge at pH 7.4 and therefore of higher polarity than the neutral compound anandamide, does not penetrate the blood brain barrier as well as anandamide.
Figure 6. Example autoradiographs from mice injected with $[^{14}C]$AA and $[^{14}C]$-arach.anandamide. Grey scale images show the same brain sections as colored images for mice sacrificed at 100 minutes after tracer injection. Patterns were similar between mice injected with $[^{14}C]$AA and $[^{14}C]$-arach.anandamide.
Figure 7. Comparison of regional deposition of radiolabel in the brain following administration of $[^{14}\text{C}]$AA and $[^{12}\text{C}$-arach.$]$.|anandamide. A) Compared with $[^{3}\text{H}]$AA and $[^{3}$H-arach.$]$.anandamide (Glaser et al, 2006), $[^{14}\text{C}]$AA and $[^{14}$C-arach.$]$.anandamide produced autoradiographs with higher resolution and lower noise. B) Similar patterns between autoradiographs produced with $[^{14}\text{C}]$AA and $[^{12}$C-arach.$]$.anandamide. 1-cortex, 2-striatum, 3-ventricular epithelium, 4-thalamus, 5-hippocampus. C) Example sections with circles drawn around regions of interest used for quantification of signal intensity using the OptiQuant software.
<table>
<thead>
<tr>
<th>Brain regions</th>
<th>time after injection</th>
<th>[^{14}\text{C}]\text{AA})</th>
<th>[^{14}\text{C}\text{-arach.}]\text{anandamide})</th>
<th>Signal intensity ratios [^{14}\text{C}\text{-arach.}]\text{anandamide} / [^{14}\text{C}]\text{AA})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortex</td>
<td>10min</td>
<td>1.97</td>
<td>9.45</td>
<td>4.79</td>
</tr>
<tr>
<td></td>
<td>100min</td>
<td>2.26</td>
<td>11.0</td>
<td>4.87</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>10min</td>
<td>1.33</td>
<td>5.84</td>
<td>4.38</td>
</tr>
<tr>
<td></td>
<td>100min</td>
<td>1.52</td>
<td>7.06</td>
<td>4.65</td>
</tr>
<tr>
<td>Ventricular epithelium</td>
<td>10min</td>
<td>3.74</td>
<td>14.1</td>
<td>3.76</td>
</tr>
<tr>
<td></td>
<td>100min</td>
<td>5.84</td>
<td>19.1</td>
<td>3.27</td>
</tr>
<tr>
<td>Thalamus</td>
<td>10min</td>
<td>1.89</td>
<td>8.25</td>
<td>4.36</td>
</tr>
<tr>
<td></td>
<td>100min</td>
<td>2.11</td>
<td>9.92</td>
<td>4.69</td>
</tr>
<tr>
<td>Striatum</td>
<td>10min</td>
<td>1.47</td>
<td>6.66</td>
<td>4.53</td>
</tr>
<tr>
<td></td>
<td>100min</td>
<td>1.76</td>
<td>8.26</td>
<td>4.70</td>
</tr>
</tbody>
</table>

Table 1. Quantification of the accumulation of radioactivity in various brain regions with OptiQuant. Values were averages from all the slices that contained the region of interest in each brain. The slices from the four mice injected with \[^{14}\text{C}]\text{AA}\) or \[^{14}\text{C}\text{-arach.}]\text{anandamide}\) and euthanized 10 or 100 minutes later were apposed to the same phosphor imaging plate for 36 days (n = 1).
The relative value of signal intensity in each brain region was calculated by dividing the absolute DLU/mm\(^2\) value by the absolute DLU/mm\(^2\) value in hippocampus of the same brain. Hippocampus was chosen as a reference region because its DLU/mm\(^2\) value appears to be the lowest among the regions of interest (Table 1). Despite overall lower accumulation of radioactivity in the brain following administration of \(^{14}\text{C}]\text{AA}, the relative values of signal intensity, which imply the relative amounts of radioactivity in various regions, are similar between \(^{14}\text{C}]\text{AA} and \(^{14}\text{C}-\text{arach.} \text{anandamide}\) (Figure 8), confirming the similar patterns produced with \(^{14}\text{C}]\text{AA} and \(^{14}\text{C}-\text{arach.} \text{anandamide}\) (Figure 7B). Compared to other regions, the ventricle areas appeared to show larger differences between 10 and 100 minutes time points and between \(^{14}\text{C}]\text{AA and } ^{14}\text{C}-\text{arach.} \text{anandamide}\). However, the differences were not significant (n=4, p>0.05). The ventricle areas were much smaller and the signal intensity values were over twice as much as that of the hippocampus, therefore there might be larger errors and variations in quantification of these areas. Overall, our autoradiography experiments with carbon-14 showed the advantage of using carbon-14 instead of tritium in autoradiographic studies, and also revealed similar patterns of distribution of radioactivity in the brain following administration of \(^{14}\text{C}]\text{AA and } ^{14}\text{C}-\text{arach.} \text{anandamide}.\)
Figure 8. Comparison of relative signal intensity of different brain regions quantified from autoradiographs of mice injected with $[^{14}\text{C}]$AA and $[^{14}\text{C}]$-arachidonananda. The patterns of radiolabel disposition were not significantly different between 10 and 100 minutes and between $[^{14}\text{C}]$AA and $[^{14}\text{C}]$-arachidonananda. (n=4, student’s t-test, p>0.05) CX, cortex; HP, hippocampus; VT, ventricular epithelium; TH, thalamus; ST, striatum.
While our results obtained with [\(^{14}\)C-arach.]anandamide are consistent with those of Glaser et al., who used \(^{3}\)H]anandamide, the similar patterns produced with [\(^{14}\)C]AA make the control experiments with \(^{3}\)H]AA by Glaser et al. (2006) questionable (see DISCUSSION). The faint, homogeneous, appearance of the autoradiographs produced with \(^{3}\)H]AA did not necessarily indicate a homogeneous distribution of radioactivity, rather, it could be the result of too much noise, which masked the actual heterogeneous distribution of radioactivity. The conclusion by Glaser et al. (2006), that the patterns of distribution of radioactivity reflect the regional differences of FAAH activity, is also questionable, because FAAH is not involved in incorporation of AA into the membrane phospholipids. The regional differences of incorporation of radiolabel following administration of [\(^{14}\)C]AA should reflect the regional differences of AA metabolism, involving acyl-CoA synthetase, acyltransferase, and PLA\(_2\) (Figure 1). Based on the observation that [\(^{14}\)C-arach.]anandamide enters the brain much more easily than [\(^{14}\)C]AA (Table 1), and is then hydrolyzed into [\(^{14}\)C]AA by FAAH in the brain, we speculated that anandamide possibly acts as a precursor of AA, which facilitates the delivery of radiolabel across the blood brain barrier, and therefore the regional differences of incorporation of radiolabel following administration of [\(^{14}\)C-arach.]anandamide may reflect the regional differences in AA metabolism rather than in FAAH activity.
B. Aim 2: To evaluate the use of an anandamide analogue - $[^{14}\text{C}]$myristoylethanolamine (MEA) - for imaging FAAH activity

B.1. Ex vivo autoradiography using $[^{3}\text{H}]$myristic acid (MA), $[^{3}\text{H}]$MEA and $[^{14}\text{C}]$MA

Having found that $[^{14}\text{C}]$AA and $[^{14}\text{C}]$-arach. anandamide produced similar radiolabel deposition patterns in the brain; we sought to determine whether this was also true for other fatty acids and their NAEs. One of our choices was myristic acid (MA, tetradecanoic acid) which is generally regarded as the simplest “long chain” fatty acid (alkanoic acids with shorter chain lengths are generally termed “medium chain” or “short chain”. Also, myristoylethanolamine (MEA) is an important lipid signaling messenger in plants, and was of interest in another project in our laboratory.

Dr. Duclos in our lab synthesized $[^{3}\text{H}]$MEA from $[^{3}\text{H}]$MA and non-radioactive ethanolamine. Autoradiography experiments described previously were repeated with use of $[^{3}\text{H}]$MEA and $[^{3}\text{H}]$MA. Mice were injected with 30 µCi of $[^{3}\text{H}]$MEA or $[^{3}\text{H}]$MA, and their brains were sectioned 15 minutes or 90 minutes later. Brain sections were exposed to an uncoated (tritium-sensitive) screen (instead of a more robust screen with a protective coating used for carbon-14) that cannot be penetrated by the weak beta-rays from tritium. The autoradiographs produced with $[^{3}\text{H}]$MA were very noisy which precluded clear identification of specific patterns, while $[^{3}\text{H}]$MEA produced only hints of heterogeneity in distribution of radioactivity(Figure 9). Our experience with tritiated myristic acid and its N-ethanolamine lead us to the conclusion that the combination of tritium labeled tracers and phosphor imaging it is not useful for autoradiography under circumstances where brain accumulation of radiolabel is low and administration of large activities of radioactive material is not practicable.
Figure 9. Autoradiography with [3H]MEA and [3H]MA. The autoradiographs produced with [3H]MEA showed heterogeneous distribution of radiolabel, while the autoradiographs produced with [3H]MA was too noisy that no pattern could be detected.

To achieve better quality images, we repeated the autoradiography experiments with use of [14C]MA. Mice were injected with 20 µCi of [14C]MA and their brains were sectioned 2, 5, 10 or 80 minutes later. We chose multiple time points because we noticed a drop of total amount of radioactivity at the later time point in the previous experiment with [3H]MA. This was not observed in autoradiography experiments with [14C]AA or [14C]-arachidonic acid. Heterogeneous distribution of radioactivity in the brain was observed with use of [14C]MA (Figure 10), and the pattern of distribution appeared to be similar to those produced with [14C]AA (Figure 6): Highest signal intensity was present in ventricular epithelium. Thalamus and cortex showed relatively higher signal intensity than hippocampus. Absolute and relative signal intensities of various brain regions were quantified as previously described.

The overall amount of radioactivity accumulated in the brain following [14C]MA administration was greatly reduced (about 50%) in the tissue sectioned 80 minutes after injection – a
phenomenon not observed with use of $[^{14}\text{C}]$AA or $[^{14}\text{C}]$arachid.anandamide (Figure 11). Freed et al. (1994) found that treatment of rats with an inhibitor of beta-oxidation increased incorporation of $[^{14}\text{C}]$palmitic acid but not of $[^{14}\text{C}]$arachidonic acid into brain lipids, indicating that arachidonic acid (20:4) undergoes beta-oxidation to a lesser extent than palmitic acid (16:0) and therefore very likely to a lesser extent than the similar myristic acid (14:0). Studies with purified cell organelles also indicate that arachidonic acid is oxidized more slowly than saturated or mono-unsaturated long chain fatty acids by both peroxisomes (Hiltunen et al. 1986) and by mitochondria (Lea et al. 2000). The radioactive carbon atoms from $[^{14}\text{C}]$myristic acid eventually become $[^{14}\text{C}]$CO$_2$, which is lost during exhalation. During operation of the beta-oxidation and tricarboxylate cycles, label is incorporated into many intermediary metabolites, which is probably a cause of the greater level of background in the autoradiographs made using $[^{14}\text{C}]$MA (Figure 10). In addition, saturated long chain fatty acids like MA are incorporated into phospholipids at the sn-1 position, which may undergo turnover at a higher rate that the sn-2 position where AA is incorporated (Shindou et al. 2009). The $^{14}\text{C}$ label in MA may thus be lost from membrane phospholipids more quickly than AA. Therefore, to use labeled MA and related compounds with their label on the saturated acyl chain as radiotracers, one needs to limit the waiting time after tracer injection to less than 10 minutes, in order to avoid the impact of labeled intermediary metabolites on the clarity of autoradiographs.
Figure 10. Autoradiography of mouse brain sectioned at 2, 5, 10 or 80 minutes after administration of $[^{14}C]$MA. Intensities of images were adjusted to the same level to show similar patterns.
Figure 11. Signal intensity from autoradiographs produced with $[^{14}\text{C}]\text{MA}$ at different time points. Regional distribution of radioactivity is similar between $[^{14}\text{C}]\text{AA}$ and $[^{14}\text{C}]\text{MA}$ at 5~10 minutes. The amount of activity produced with $[^{14}\text{C}]\text{MA}$ decreased about 50% at 80 minutes due to faster beta oxidation of MA, forming the noisy, blurred autoradiographs. Signal from AA is maintained because beta oxidation is difficult for polyunsaturated fatty acids. ($n=1$ for $[^{14}\text{C}]\text{MA}$) CX, cortex; HP, hippocampus; VT, ventricular epithelium; TH, thalamus; ST, striatum.

To determine the chemical forms of radioactivity in the brain following administration of the radiolabel, half of the cerebellum from the brain used for autoradiography was saved for TLC experiments. The cerebellum and blood samples were extracted following the methods described in II.E. Samples were homogenized in 1 ml of aqueous solution made up of 500μl of 40% urea and 500 μl of 5% sulfuric acid and extracted with 2 ml of chloroform/methanol (2:1). After centrifugation and separation, the organic fraction was concentrated to a volume of 50 μl, and 5 μl of the chloroform fraction was spotted on a silica gel TLC plate, along with standards of $[^3]$HMA, $[^3]$HMEA or $[^{14}]$CMA. The solvent system used to develop the TLC plate was chloroform/methanol/ammonium hydroxide (90:10:1, v/v). Because the free fatty acid MA is ionized in the solvent mixture containing ammonia, and it migrates more slowly than MEA on the TLC plate. (That is, ammonium hydroxide was added to distinguish MA from MEA.) After running with the solvent system in an airtight jar, the TLC plates were air-dried, then apposed to a tritium sensitive screen (for $[^3]$HMA and $[^3]$HMEA) or a regular (protectively coated) screen (for $[^{14}]$CMA). The screens were scanned with the Cyclone Plus imager and TLC spots were quantified using OptiQuant.

In cerebellum samples taken at both 15 minutes and 90 minutes after injection of $[^3]$HMEA, compounds of similar retention factor (Rf) values to those of $[^3]$HMA were detected (Figure 11A), indicating the probable existence of $[^3]$HMA as a metabolite of $[^3]$HMEA in the brain. No lipophilic compounds were detected by TLC in cerebellum samples from mice given $[^3]$HMA – a result that is in agreement with autoradiograph produced with use of $[^3]$HMA (Figure 9). Because $[^3]$HMA does not penetrate the blood brain barrier as well as $[^3]$HMEA, and undergoes β
oxidation rapidly, we cannot rule out the possibility that the amounts of [\textsuperscript{3}H]MA and its metabolites in the brain were too low to be detected by the tritium-sensitive screen. Tritiated phospholipids (possible metabolites of [\textsuperscript{3}H]MA), which would stay near the origin of the TLC plate due to their higher polarity, were not detected in all samples. Other metabolites of [\textsuperscript{3}H]MEA were found in blood and cerebellum samples (pink circles in Figure 12A). On the basis of their Rf values, these metabolites were tentatively assigned as diacylglycerol, triacylglycerol and ethyl myristate.

In blood and cerebellum samples from mice given [\textsuperscript{14}C]MA, four radioactive spots were detected on silica gel TLC for each sample when chloroform/methanol/ammonia (90:10:1, v/v) was used as mobile phase (Figure 12B). Two ran near the solvent front; the other two had Rf values in the region of 10-15%. The higher of these two had the same Rf value as authentic [\textsuperscript{14}C]MA. This was further examined by developing the TLC plate in Figure 9B again, using the more polar solvent system - chloroform/methanol (1:1, v/v). The second development was stopped before the solvent front reached the two compounds that had high Rf values in chloroform/methanol/ammonia (60:30:1, v/v). After the second run, the two compounds with low RF values were separated very well and it was apparent that only the blood sample contained [\textsuperscript{14}C]MA (Figure 12C). The cerebellum samples taken at either 10 min or 80 min after injection of [\textsuperscript{14}C]MA contained an unidentified compound that has slightly lower Rf value than that of [\textsuperscript{14}C]MA in either solvent system. Using chloroform/methanol/ammonia (60:30:1, v/v) a radioactive spot of similar lower Rf value to that seen in Figure 9B appears for cerebellum samples taken at either 15 min or 90 min after injection of [\textsuperscript{3}H]MEA (blue circles of lane 3 and 4 in Figure 9A). Based on polarities of possible radioactive lipid metabolites of [\textsuperscript{14}C]MA, we tentatively assign the spot of lower Rf than [\textsuperscript{14}C]MA as phospholipid(s) that incorporate [\textsuperscript{14}C]MA,
and the two spots of high Rf values as diacylglycerol and triacylglycerol. Similar assignments of lipid metabolites, on the basis of authentic samples on non-radioactive lipids, were made in our studies with $[^{18}\text{F}]$FHEA (Pandey et al. 2014). The faint spot seen in the lane of standard $[^{14}\text{C}]$MA could be ethyl myristate, formed from reaction of standard $[^{14}\text{C}]$MA with its solvent ethanol as the spot dries on the silica gel TLC plate. The TLC experiments with labeled MA and MEA helped us design similar radioanalytic procedures with samples from mice injected with $[^{14}\text{C}]$AA and $[^{14}\text{C-}\text{arach.}]$anandamide.
Figure 12. TLC analysis of samples from mice injected with $[^3]$HMA, $[^3]$HMEA or $[^{14}]$CMA. A) Lipid extractions of samples from mice injected with 30uCi of $[^3]$HMEA (lane 1-4) or $[^3]$HMA (lane 5,6) were analyzed by TLC using chloroform/methanol/ammonium hydroxide (90:10:1, v/v). No metabolites were detected for samples from mice received $[^3]$HMA, spots of similar Rf to standard $[^3]$HMA along with unidentified spots were detected in samples from mice received $[^3]$HMEA; B) lipid extractions of samples from mice injected with 20uCi of $[^{14}]$CMA were analyzed by TLC using the same condition as in A), the present of $[^{14}]$CMA was not clear because of poor separation of the lower spots; C) The TLC plate in B) was developed again in chloroform/methanol (1:1, v/v) and it was clear that $[^{14}]$CMA was present in the blood but not in the brain samples.
C. Aim 3: To synthesize NAEs labeled at the ethanolamine moiety and evaluate the use of \([^{14}\text{C}}\text{-EA}]\text{anandamide and }[^{14}\text{C}}\text{-EA}]\text{MEA for imaging FAAH activity}

In previous results we showed that when administered intravenously, \([^{14}\text{C}}\text{-arach.}]\text{anandamide is hydrolyzed by FAAH into }[^{14}\text{C}]\text{AA and ethanolamine in the brain, and then the arachidonoyl moiety labeled by }^{14}\text{C} \text{ is rapidly incorporated into phospholipids in a heterogeneous pattern, similar to that produced by i.v. }^-\text{administered }[^{14}\text{C}]\text{AA. The hypothesis that hydrolysis by FAAH is the rate limiting step that determines the heterogeneous pattern produced by }[^{14}\text{C}}\text{-arach.}]\text{anandamide is challenged by our results, because a similar pattern produced by }[^{14}\text{C}]\text{AA does not involve FAAH activity. Labeling the ethanolamine moiety instead of the AA moiety in anandamide provided us with a different way of looking at regional differences of anandamide metabolism, and possibly a better approach to image FAAH activity. One of the possibilities that we had envisaged was that in regions with high activity of FAAH, a greater release of the small water-soluble molecule }[^{14}\text{C}]\text{ethanolamine that could diffuse from brain to blood, we would see decreased levels of radioactivity. This would therefore be the opposite to our originally anticipated results using ethanolamides labeled in the acyl group, where greater FAAH activity was hypothesized to lead to increased regional brain radioactivity. Therefore, we decided to undertake an autoradiography study with }[^{14}\text{C}}\text{-EA}]\text{anandamide and other NAEs labeled at the ethanolamine moiety, and preparatory to these experiments we needed to obtain fatty acid ethanolamides labeled in the ethanolamine moiety.}

C. 1. Synthesis of \([^{14}\text{C}}\text{-EA}]\text{MEA}

Except for \([^{14}\text{C}}\text{-EA}]\text{anandamide, no fatty acid ethanolamide labeled at the ethanolamine moiety is commercially available; also, the price of }[^{14}\text{C}}\text{-EA}]\text{anandamide was prohibitively expensive compared with that of }[^{14}\text{C}]\text{anandamide labeled at the AA moiety. We therefore optimized a method for synthesis of }[^{14}\text{C}}\text{-EA}]\text{anandamide and other fatty acid amides labeled at the ethanolamine moiety, based on the method that Rick Duclos in our lab had developed for}
synthesis of \[^{3}H\]MEA from \[^{3}H\]MA and cold ethanolamine. The synthesis of a fatty acid ethanolamine is completed in a one-step reaction of two reactants: the fatty acid and the ethanolamine. Ideally, one part of the fatty acid would react with one part of ethanolamine; however in radiochemical synthesis, the unlabeled reactant is usually used in excess, to assure maximum use of the radiolabeled reactant. Therefore, the method for synthesis of \[^{14}C\text{-EA}]\text{MEA}\ was slightly changed from the method used for synthesis of \[^{3}H\]MEA: the unlabeled reactant MA was used in excess to use up as much \[^{14}C\]ethanolamine as possible in the reaction.

For the first trial synthesis of \[^{14}C\text{-EA}]\text{MEA}, one mg of MA was mixed with 0.1 μCi of \[^{14}C\]ethanolamine (1 μl x 0.1 μCi/μl) in a 1 ml reaction vial. The solvent ethanol was removed evaporatively in a stream of argon. Dichloromethane (0.5 ml), the solvent to be used for the reaction was added to the mixture and evaporated under argon to remove trace amounts of ethanol. A stir bar was added to the reaction vessel, followed by 0.5 ml of dichloromethane containing 1 mg of 1-ethyl-3-(3-dimethylaminopropyl) (EDCI) as a carboxyl activating agent and 0.05 mg of 4-Dimethylaminopyridine (DMAP) as a catalyst. The reaction mixture was stirred at room temperature for two days, and terminated by addition of 0.4 ml of 0.1M HCl. The reaction progress was monitored by radioTLC and the results were quantified with OptiQuam. Based on previous experience with synthesis of \[^{3}H\]MEA, we know the RF value of the expected product \[^{14}C\text{-EA}]\text{MEA}\ is about 0.6 when using chloroform/methanol (85:15) or chloroform/methanol/acetic acid (85:15:1) as the solvent system. At 2 hours after the reaction started, 79% of the radioactivity was in the expected product \[^{14}C\text{-EA}]\text{MEA}\ (Figure 13A, green circle) and less than 3% was in unreacted \[^{14}C\]ethanolamine. About 20% of the radioactivity came from a major byproduct with high Rf value, possibly N-, O- diacyl ethanolamine (Figure 13A, red circle). After 2 days of reaction, the amount of \[^{14}C\text{-EA}]\text{MEA}\ was greatly reduced to about 3%; in fact, 96% of the radioactivity was in the byproduct (putatively N-, O- diacyl
ethanolamine). When the reaction was repeated and the reaction mixture analyzed at 1 hour, 87% of the radioactivity was already in the byproduct N-, O- diacyl ethanolamine (Figure 13B).

Figure 13. TLC results of trial synthesis of [14C-EA]MEA. A) and B) Two trials of synthesis with use of DMAP as a catalyst resulted in uncontrolled production of N-, O- diacyl ethanolamine (near the top). C) Without DMAP, the amount of byproduct was kept low even with prolonged reaction time. Solvent system: chloroform/methanol (85:15) with or without 1% of acetic acid. Lane 1: [14C]ethanolamine; lane 2: reaction mixture. Green circles: [14C-EA]MEA; red circles: major byproduct.
The reason for uncontrolled formation of N-, O- diacyl ethanolamine can be accounted for by the action of DMAP. In the third DMAP was not added to the reaction and monitored the progress at 10, 20, 30, 50, 110 minutes and at 18 hours after the reaction started. TLC results showed that without DMAP, [14C-EA]MEA was successfully synthesized, with a yield of 78.3% at 110 minutes, and that a prolonged reaction time did not increase the amount of byproduct (Figure 13C). The condition of the third trial was used in scaled up reactions with 50 μCi of [14C]ethanolamine; however, the final yield after purification in this case was only about 20%. The loss of product during purification was negligible. Possible reasons for the low final yield are: a) excess MA in the scaled up reaction making the reaction mixture too concentrated and too acidic; or b) [14C]ethanolamine being stuck to the wall and not completely dissolved for reaction. In subsequent studies in our laboratory, the yield was improved by adding DMSO to the dichloromethane as a co-solvent.


The distribution pattern of radioactivity in the brain following administration of [14C-EA]anandamide was similar to that produced with use of [14C-arach.] anandamide (Figure 6, 8 and 14A). Furthermore, [14C-EA]MEA also produced similar results, with slightly smaller relative DLU/mm^2 values for ventricular epithelium, which was still highest among all brain regions. In contrast, the control experiment with use of [14C]ethanolamine produced an almost homogeneous distribution of radioactivity in the brain (regional relative DLU/mm^2 values were almost the same for all regions), except for the ventricular epithelium areas which had significantly higher relative DLU/mm^2 at 13.6 and 9.19 for two different time points (Figure 14C); these were the highest values obtained out of all autoradiography experiments. So far, ventricular epithelium was shown to contain the highest amount of radioactivity with every 14C labeled tracer tested. Besides higher enzyme activity or faster incorporation of radiotracer into
phospholipids at this region, the underlying mechanisms of the phenomenon may include particular structural and functional characteristics of the ventricles. The heterogeneous distribution of radioactivity in the brain following administration of [\(^{14}\)C-EA]anandamide and [\(^{14}\)C-EA]MEA should reflect some regional differences of metabolism of these NAEs, because \([^{14}\text{C}]\text{ethanolamine as a control radiotracer produced a very different result. The situation was unlike that pertaining to } [^{14}\text{C-}
\text{arach.}]\text{anandamide where a similar regional pattern was obtained to that when } [^{14}\text{C}]\text{arachidonic acid was used. Compared to NAEs labeled at the fatty acid moiety, NAEs labeled at the ethanolamine moiety are better tracers to use for studying regional differences of metabolism of NAEs, including regional differences of FAAH activity.}
Figure 14. Autoradiography with anandamide or MEA labeled at the ethanolamine moiety. Heterogeneous patterns were observed with $[^{14}C]$anandamide and $[^{14}C]$MEA. Accumulation of $[^{14}C]$ethanolamine is almost homogeneous except for the ventricular areas. (n=1) CX, cortex; HP, hippocampus; VT, ventricular epithelium; TH, thalamus; ST, striatum.
C.3. Effects of the FAAH inhibitor URB597 on ex vivo autoradiograms after injection of $[^{14}\text{C}}\text{-arach.}]\text{anandamide or }[^{14}\text{C}}\text{-EA}]\text{anandamide}$

To test our hypothesis that $[^{14}\text{C}}\text{-EA}]\text{anandamide would be better than }[^{14}\text{C}}\text{-arach.}]\text{anandamide}$ for imaging FAAH activity, we repeated the autoradiography experiments with both tracers in mice pretreated with the FAAH inhibitor URB597 or vehicle alone. We expected that URB597 treatment would block the formation of the patterns in autoradiographs produced with both $[^{14}\text{C}}\text{-EA}]\text{anandamide and }[^{14}\text{C}}\text{-arach.}]\text{anandamide}$. However, the pattern produced with $[^{14}\text{C}}\text{-EA}]\text{anandamide, but not }[^{14}\text{C}}\text{-arach.}]\text{anandamide, should closely reflect regional FAAH activity}$ since $[^{14}\text{C}}\text{ethanolamine, when freed by FAAH from }[^{14}\text{C}}\text{-EA}]\text{anandamide, would not produce further pattern except perhaps in the ventricles which were prominent in autoradiographs obtained after injection of }[^{14}\text{C}}\text{ethanolamine. We expected to see reduced pattern for URB597 treated mice injected with }[^{14}\text{C}}\text{-arach.}]\text{anandamide as well because inhibition of the release of }[^{14}\text{C}}\text{AA by FAAH would prevent the incorporation of }[^{14}\text{C}}\text{AA in brain phospholipids and the formation of a pattern reflecting PLA}_2\text{ activity. Indeed, Glaser et al. (2006) reported reduced total activity and regional differences in brain autoradiography with }[^{3}\text{H}}\text{-arach.}]\text{anandamide after mice were treated with FAAH inhibitors methyl arachidonyl fluorophosphonate (MAFP, 1 mg/kg i.p.) and CAY10435 (i.v. 1mg/kg, co-administered), as well as from FAAH knockout mice (Figure 15) (Glaser et al. 2006). However, both MAFP and CAY10435 are much less selective for FAAH than URB597. MAFP was also shown to be an irreversible inhibitor of cytosolic PLA}_2\text{ and calcium-independent PLA}_2\text{ (Balsinde and Dennis 1996, Lio et al. 1996).}$
We divided the mice into four groups (n=3): control15, control100, URB15 and URB100. Mice were given freshly prepared URB597 (3mg/kg) or vehicle (75% DMSO in saline) through intraperitoneal administration one hour before tracer injection. The dose, timing and administration route were chosen on the basis of studies in the literature (Fegley et al. 2005, Clapper et al. 2006, Piomelli et al. 2006). An experiment where we measured FAAH activity in brain homogenate after administration of URB597 to mice confirmed >90% inhibition of FAAH using this protocol (data not shown). Mice were euthanized 15 or 100 minutes after tracer injection. Brains were sectioned immediately for autoradiography as described before. The autoradiographs from the four groups were strikingly similar (Figure 16): URB597 pretreatment did not reduce the regional differences in disposition of the radiolabel from either [14C-EA]anandamide or [14C-arach.]anandamide.
Brain autoradiography of mice pretreated with control or URB597 and injected with [{\textsuperscript{14}}C-arach.]anandamide or [{\textsuperscript{14}}C-EA]anandamide. Mice were given URB597 (3mg/kg) or vehicle (75% DMSO in saline) through intraperitoneal administration one hour before tracer injection and euthanized 15 or 100 minutes later. Brain autoradiography showed that inhibition of the breakdown of anandamide by URB597 failed to block the heterogeneous distribution pattern of radiolabel from anandamide labeled at either the ethanolamine or the arachidonoyl moiety. (n = 3, representative images were adjusted to the same level of signal intensity to show similar patterns)

We quantified the autoradiographs from mice injected with [{\textsuperscript{14}}C-EA]anandamide and confirmed that URB597 did not prevent the formation of the pattern we saw in control animals (Figure 17A). At 15 minutes, URB597 treatment had even increased the relative signal intensity in cortex. However, in URB597 treated mice sacrificed at 100 minutes after tracer injection, the relative signal intensity (DLU/mm\textsuperscript{2}) for cortex, thalamus and striatum normalized to DLU/mm\textsuperscript{2} of hippocampus were significantly lower compared to control. Looking at the autoradiographs for URB100 (Figure 16), we can tell that the pattern was still heterogeneous, but had changed slightly -- the relative signal intensity of the hippocampus had increased. This may result from multiple metabolism pathways (such as through COX2 (Glaser and Kaczocha 2010)) besides
the action of FAAH on anandamide and its metabolites, drawing the label towards hippocampus at a later time point. Other than changing relative DLU/mm$^2$ in the hippocampus, we found that URB597 treatment was able to affect the absolute DLU/mm$^2$ in the whole brain (Figure 17B, C): For control groups, brain regions from mice euthanized at 100 minutes had 20%-40% more total activity than those from mice euthanized at 15 minutes (Figure 17B); but in URB treated groups, the trend inverted and mice euthanized at 15 minutes had about 40% more activity than the 100 minutes group. We apposed the sections of the four groups on the same phosphor imaging plate and found that the absolute signal from URB15 was the highest among the four groups (Figure 17C). Glaser et al. (2006) previously showed lower brain uptake in FAAH KO mice and in mice treated with MAFP and CAY10435. From her results it seemed that labeled anandamide is removed from the brain when the metabolism by FAAH is blocked. From our results, the reason URB597 treatment increased overall brain uptake of the tracer may be that URB597 inhibited peripheral breakdown of anandamide, and as a result more labeled anandamide could enter the brain. The differences of absolute signal intensity among the four treatment groups (normalized to control15) were almost identical in cortex, striatum and thalamus, again implying maintained “pattern” (Figure 17C). The slightly higher signal in hippocampus happened at URB100 (not significant compared to other regions, $p > 0.05$), which agrees with the autoradiographs in Figure 16 and quantified relative signal differences in Figure 17A.
Figure 17. The effects of URB597 pre-treatment on regional deposition and total amount of brain radioactivity in mice injected with [14C-EA]anandamide. A) Pre-treatment of URB597 failed to block the formation of the heterogeneous pattern of radiolabel deposition at 15 minutes after injection of [14C-EA]anandamide; however, regional differences were reduced at 100 minutes. B) Control treated group showed increased brain total activity at 100 minutes compared to that at 15 minutes, whereas URB597 treated group showed decreased brain total activity. C) URB597 treated mice euthanized at 15 minutes showed the highest total brain activity among all treatment groups; the percentage absolute signal changes of different brain regions (compared to control 15) were similar under the same treatment condition. Control15, control100, URB15 and URB100 indicates control/URB597 treated group euthanized at 15 minutes/100 minutes after tracer injection. CX, cortex; ST, striatum; HP, hippocampus; TH, thalamus. (*), p<0.05, student’s t-test.
Our results were not what we expected based on the reports by Glaser et al. (2006) and by Rapoport’s group. Although we had already concluded that FAAH activity does not determine the pattern seen in brain sections after administration of $[^{14}\text{C}]$-arach.anandamide, we still expected reduced regional differences after URB597 treatment, because inhibition of the breakdown of $[^{14}\text{C}]$-arach.anandamide by URB597 would definitely limit the availability of free labeled AA in the brain to be incorporated into phospholipids and form a pattern reflecting AA metabolism involving PL$\text{A}_2$ activity. For $[^{14}\text{C}]$-EA\text{anandamide}, we had shown that the heterogeneous distribution of radioactivity in the brain following administration of $[^{14}\text{C}]$-EA\text{anandamide} was quite different from the almost homogeneous pattern formed with the control tracer $[^{14}\text{C}]$ethanolamine; and the only difference between directly injecting $[^{14}\text{C}]$ethanolamine and indirectly delivering $[^{14}\text{C}]$ethanolamine through $[^{14}\text{C}]$-EA\text{anandamide} seemed to be the action of FAAH that hydrolyze $[^{14}\text{C}]$-EA\text{anandamide} to release free $[^{14}\text{C}]$ethanolamine. That was the reason we hypothesized that regional differences in the rate of the action of FAAH (regional differences in FAAH activity) may determine the formation of the heterogeneous pattern with $[^{14}\text{C}]$-EA\text{anandamide}.

Assuming that pretreatment with URB597 did inhibit FAAH (proved later using radioTLC analysis of brain tissue), then our results with both $[^{14}\text{C}]$-EA\text{anandamide} and $[^{14}\text{C}]$-arach.anandamide indicated that the radioactivity distribution pattern is formed before the action of FAAH, while labeled anandamide is still in its intact form. Our finding that URB597 could not block the heterogeneous pattern produced with $[^{14}\text{C}]$-arach.anandamide also challenged the notion that autoradiography with $[^{14}\text{C}]$AA is reflecting PL$\text{A}_2$ activity, since a very similar pattern is formed by $[^{14}\text{C}]$-arach.anandamide without available free $[^{14}\text{C}]$AA in the brain and thus without $[^{14}\text{C}]$AA metabolism involving PL$\text{A}_2$. If the pattern formed by $[^{14}\text{C}]$AA were due to PL$\text{A}_2$, we would detect an increased regional differences in the autoradiograph produced with $[^{14}\text{C}]$-arach.anandamide compared to $[^{14}\text{C}]$AA because intact $[^{14}\text{C}]$-arach.anandamide had
already formed a similar pattern and the following metabolism of released $[^{14}C]AA$ should enhance it. However, we observed no significant differences in the patterns produced with $[^{14}C$-arach.]anandamide and $[^{14}C]AA$ (Figure 8). It is therefore possible that similarly to i.v. administered $[^{14}C$-arach.]anandamide, injected $[^{14}C]AA$ itself forms a pattern before its incorporation into phospholipids and release by PLA$_2$, and that further metabolism involving PLA$_2$ is homogeneous across the brain so that the initial pattern formed by $[^{14}C]AA$ is maintained.

Autoradiograms made after administration of $[^{14}C]$ethanolamine itself on the one hand, and of $[^{14}C]$ethanolamine incorporated into $[^{14}C]$anandamide on the other, were very different. $[^{14}C]$ethanolamine is small, polar and very water soluble and may quickly diffuse across brain regions, accounting for its more homogeneous distribution pattern, while $[^{14}C$-EA]anandamide has a very high calculated octanol/water partition coefficient (clogP $\geq 7$) and is expected to dissolve with a high local volume of distribution in lipid-rich regions. However, it is likely that the bulk property of lipophilicity per se (defined by clogP value) is not the complete determinant of affinity of anandamide for brain lipids. Delta-8-THC has a very similar clogP value to anandamide (about 7) but unlike anandamide it clears from the mouse brain fairly rapidly (whole brain concentration at 60 min less than 25% of that at 5 min) (Charalambous et al. 1991). Interactions between anandamide and nanometer scale triglyceride-phospholipid-protein structures in brain membranes may determine the unique patterns produced with NAEs and their fatty acids.

Anandamide, either labeled at the ethanolamine moiety or the arachidonoyl moiety, is not a good probe for imaging FAAH activity in the brain, because first of all, the heterogeneous distribution of label is formed without the action of FAAH and thus does not represent the pattern of FAAH activity as previously suggested; secondly, the pattern is not greatly affected by FAAH inhibition and the slight change at a later time point is likely due to other complex
pathways dominating the metabolism of the label containing compounds (anandamide and its many metabolites) when FAAH is inhibited; and thirdly, the change of brain total activity in FAAH inhibited animal is likely due to peripheral inhibition of FAAH leading to more available labeled anandamide entering the brain. **In conclusion, a direct and sensitive measurement of brain regional or total FAAH activity cannot be achieved with labeled anandamide.**

**The original aim of this dissertation work could therefore not be achieved.**

C. 4. TLC and HPLC analysis

We performed TLC analysis for blood and brain samples from the four treatment groups in the experiment described in C.3. We initially performed 2-D TLC to capture a profile of metabolites from anandamide isotopomers. We confirmed that URB597 treatment did reduce the extent of the breakdown of anandamide labeled at either the arachidonic acid moiety or the ethanolamine moiety. We also visualized the formation of metabolites at 15 and 100 minutes post injection in brain and blood samples. (Figure 18A, B). Additionally, we compared the metabolite profile in brain after administration of $[^{14}\text{C}]$ethanolamine with that of $[^{14}\text{C-EA}]$anandamide. Labeled ethanolamine, whether directly injected or released from $[^{14}\text{C-EA}]$anandamide, was incorporated into $[^{14}\text{C}]$phosphatidylethanolamine (PE) identified using a non-radioactive standard of PE. The fraction of total brain $^{14}\text{C}$ accounted for as $[^{14}\text{C}]$PE was higher at 100 min than at 15 min. (Figure 19). Pretreatment of mice with URB597 reduced the loss of $[^{14}\text{C-EA}]$anandamide and also reduced the formation of $[^{14}\text{C}]$PE (formed after release of $[^{14}\text{C}]$ethanolamine from $[^{14}\text{C-EA}]$anandamide). HPLC analysis of blood and brain aqueous fractions from Folch extractions confirmed the presence of labeled ethanolamine in animals treated with URB597, consistent with the formation of $[^{14}\text{C}]$PE albeit to a lesser extent than in control animals (Figure 20). The increased level of $[^{14}\text{C-EA}]$anandamide and decreased levels of $[^{14}\text{C}]$ethanolamine and $[^{14}\text{C}]$PE in mice pretreated with URB597 was more marked at 15 min, but was evident at both time-points. These experiments indicated that while inhibition of FAAH was
not complete it was at least 80% at the 15 min time-point yet had no effect on autoradiographic images (next section). We did not test higher doses of URB597 because of difficulties in formulating this inhibitor in an acceptable vehicle. Furthermore, even if higher percentage (>80%) of FAAH inhibition could lead to an impact on the autoradiographic images, the strategy would still not be useful because we aimed to develop a sensitive measurement of subtle changes in FAAH activity. Similar TLC data indicating no difference in whole brain uptake of radiotracer between control and URB597 treated animals were obtained in our studies with [\(^{18}\text{F}\)]FHEA (Pandey et al. 2014).
Figure 18. Two-dimensional TLC analysis for brain and blood samples from mice pretreated with control or URB597 and injected with [14C-EA]anandamide. A) Brain samples (cerebellum) and B) blood samples from mice treated with control or URB597 and sacrificed at 15 or 100 minutes after injection of [14C-EA]anandamide showed that URB597 pre-treatment reduced the metabolism of anandamide and the formation of labeled phospholipids. Solvent system: first-dimension: chloroform/methanol/ammonium hydroxide = 65:35:5; second-dimension: chloroform/acetone/methanol/acetic acid/water = 30:40:10:10:5.
Figure 19. [$^{14}$C]Ethanolamine is released from [14C-EA]anandamide and is incorporated into PE. A) Two-dimensional TLC analysis showed radiolabel from injected [$^{14}$C]ethanolamine or [14C-EA]anandamide was incorporated into PE; URB597 pretreatment reduced the production of labeled PE. Solvent system: first-dimension: chloroform/methanol/ammonium hydroxide = 65:35:5; second-dimension: chloroform/acetone/methanol/acetic acid/water = 30:40:10:10:5.
Figure 20. Detection of labeled ethanolamine by radio-HPLC in brain and blood aqueous extracts from mice pretreated with URB597 and injected with [14C-EA]ethanolamine. Retention time for ethanolamine was 12-14 minutes (fraction# 6-7). An unknown aqueous metabolite (retention time ~ 4 minutes) was found in greater quantity than ethanolamine. Column used: Whatman Partisil SCX column(250x4.6mm); mobile phase: 50mM potassiumphosphate buffer, pH 3.5; flow-rate: 1ml/min.
To simplify the procedure and the quantification of the breakdown of anandamide, we modified the solvent system and used chloroform/methanol/ammonium hydroxide (60:30:1) in the one-dimensional TLC. We were able to separate anandamide from the phospholipids which contain the label from metabolized anandamide (Figure 21A). Quantification of the signal intensity of anandamide and its metabolites on the TLC plate showed that URB597 did indeed inhibit FAAH as there was significantly more un-metabolized anandamide in brains from mice treated with URB597 than in control brains (Figure 21A and B): For the URB15 group, about 90% of the radiolabel was in the form of un-metabolized anandamide at 15 minutes after tracer injection, and we observed a heterogeneous pattern similar to the control in the autoradiography experiment described in C.3 (Figure 16). **Our TLC and autoradiography results together indicate that i.v. administered anandamide itself distributes heterogeneously in the brain and that the pattern of distribution is not controlled by its metabolism.**
Figure 21. One-dimensional TLC of blood and brain samples from mice treated with URB597 or vehicle showing the effect of URB597 on breakdown of [14C-EA]anandamide. A) Representative images of one-dimensional TLC showed that URB597 pretreatment reduced metabolism of anandamide. B) Quantification of radio-TLC showed that at 15 minutes after tracer injection, 90% of radiolabel in the brain was un-metabolized anandamide in URB597 treated animals, indicating that the heterogeneous pattern observed in autoradiography was formed by mostly un-metabolized anandamide and thus was not determined by metabolism of anandamide. Solvent system: chloroform/methanol/ammonium hydroxide =60:30:1 (n=3, student's t-test; (*), p < 0.05; n.s., not significant)
D. Aim 4: To evaluate the use of $[^3]H$2-AG and $[^{14}]C$2-AG for imaging MAGL activity

We have shown that the brain distribution of radiolabel from i.v. injected labeled anandamide is not controlled by the metabolism of anandamide, and thus labeled anandamide cannot be used as a probe for imaging FAAH activity. It is likely that the distribution of anandamide is largely determined by its physical properties as a lipid. We suspected that labeled 2-AG might behave similarly and the distribution pattern of radiolabel from 2-AG also might not be controlled by its metabolism (by monoacylglycerol lipase(MAGL)--rather than by FAAH). Autoradiography with labeled 2-AG had never been performed before since labeled 2-AG was not available. Dr. Duclos was the first to introduce a rapid method of synthesizing labeled 2-AG (Duclos et al. 2011). We moved on to reveal the brain disposition of radiolabel from 2-AG and to test whether blocking its metabolism using the new MAGL inhibitor JZL184 could affect the total amount or regional differences of radioactivity in the brain.

Although $^3$H labeled compounds do not produce autoradiographs that are as good as those produced with $^{14}$C labeled compounds, these compounds can nevertheless be used for quantification of regional distribution of radiolabel using microdissection, which requires less radioactivity and is faster than autoradiography. Tritiated 2-AG was synthesized by Dr. Duclos and stored in toluene at -80°C. The solution for i.v. injection was prepared freshly before each experiment. Toluene was evaporated in a small tube under a flow of argon and vehicle for injection into animals (emulphor/ethanol/saline; 1:1:18) was added to redissolve $[^3]H$ 2-AG.

Mice were pretreated with JZL184 (16mg/kg) or vehicle (20%DMSO in PEG:TWEEN 4:1) (Long et al. 2009) via i.p. injection 30 minutes before giving 1 μCi of $[^3]H$ 2-AG via tail vein injection, and were euthanized by cervical dislocation after 15 minutes. The brains were taken out immediately and microdissection was performed as described in methods (II.F). The amount of radioactivity in hypothalamus, olfactory tubercle, frontal cortex, hippocampus, striatum,
cerebellum, brain stem, thalamus and rest of the brain were quantified in percentage injected activity per gram (%IA/g) values. A higher %IA/g value indicates higher concentration of the radiolabel in the dissected region. Our results with both young and old mice groups showed that inhibition of MAGL by JZL184 was not able to significantly alter the total amount of radioactivity in the brain, nor was it able to block the regional differences of the accumulation of radiolabel in the brain (Figure 22). The result that brain deposition of 2-AG was not controlled by JZL184 was not surprising and was in consistent with the result from anandamide and URB597. It further suggested that a direct and sensitive measurement of the activity of FAAH or MAGL could not be achieved by imaging with their radiolabeled substrates -- anandamide or 2-AG.
Figure 22. Pretreatment with JZL184 had little effect on regional or total accumulation of radiolabel from [³H] 2-AG. A) Old and B) young mice were pretreated with JZL184 (16mg/kg, i.p.) 30 minutes before tracer injection and their brains were microdissected 15 minutes after tracer injection. Some brain regions showed significant differences (student's t-test, n=5, p<0.05), however the changes were not consistent in different age groups and could be random. HY, hypothalamus; OT, olfactory tubercle; HP, hippocampus; ST, striatum; CB, cerebellum; BS, brain stem; CX, cortex; TH, thalamus; RB, rest of brain; BL, blood; UR, urine.
E. RadioTLC and autoradiography of brain from mice injected with N-(16-[18F]-fluorohexadecanoyl)ethanolamine ([18F]FHEA)

Based on the trapping mechanism for imaging FAAH activity we were evaluating using radiolabeled ethanolamides of arachidonic acid and myristic acid, we collaborated with PET researcher Dr TR DeGrado at the Brigham and Women’s Hospital (later at the Mayo Clinic in Rochester, MN) to evaluate [18F]16-fluorohexadecanoylethanolamine (FHEA). These studies have been published (Pandey et al. 2014). FHEA (which can alternatively called 16-fluoropalmitoylethanolamine) has 16 carbons in its acyl group with one of the H-atoms on the terminal methyl group replaced with an atom of fluorine-18, a positron emitter with a half-life of 110 min (Figure 23.A). It is thus structurally more similar to MEA than to AEA. We expected to see similar characteristics between [18F]FHEA and [14C]MEA when used as imaging probes, such as heterogeneous distribution across the brain, subject to hydrolysis by FAAH, and fast-fading resolution due to fast beta-oxidation.

[18F]FHEA brain uptake and metabolism was tested in the presence and absence of the FAAH inhibitor URB597 (1mg/kg, i.p.) in Swiss Webster mice. Microdissection experiments showed that pretreatment with FAAH inhibitor did not affect the total or regional uptake of the radiotracer in the brain, similar to our results with [14C-EA]anandamide and [14C-arach.]anandamide. We also did the autoradiography experiment as described before. Mice were given 20uCi of [18F]FHEA and brain were sectioned 5 minutes later. We observed heterogeneous distribution pattern similar to the result from [14C]MEA, as we expected (Figure 23.A). TLC analysis of the brains from control or URB597 pretreated mice injected with [18F]FHEA showed that FAAH inhibition did decrease the hydrolysis of [18F]FHEA and the incorporation of 18F into phospholipids (Figure 23.B), indicating that FHEA is a substrate for FAAH. However, brain uptake and distribution of radiolabel from FHEA is not sensitive to the metabolism state of FHEA. The result from FHEA is in agreement with results from other NAEs we tested, including anandamide and MEA. In conclusion, we found that metabolic trapping of NAEs does not
control the heterogeneous pattern formed from labeled NAEs. **Our results with the fluorine-18 labeled fatty acid ethanolamide confirmed that this approach to developing a PET radiotracer for FAAH is probably not fruitful.**

![Figure 23](image.png)

**Figure 23. Autoradiography and metabolite analysis with [18F]FHEA.** A) Structure of [18F]FHEA and example brain autoradiograph from mice injected with [18F]FHEA. B) Metabolite analysis of cerebellum by r-TLC measurement in a polar solvent system (chloroform/methanol/ammonium hydroxide, 60:30:1) after 5 min post-injection of [18F]FHEA to both control and URB 597 treated Swiss Webster mice (n = 3, (*)p<0.05, one tail Student’s t-test). (LPE, lysophosphatidylethanolamine; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DG, diglyceride; UNK, unknown; FA, fatty acid; TG, triglyceride; CE, cholesterol ester.) (Pandey et al. 2014)
F. New hypothesis

At this point in our studies, the autoradiography results with [\(^{14}\text{C}\)]AA, [\(^{14}\text{C}\)-arachidonic acid]anandamide and [\(^{14}\text{C}\)-EA]anandamide had disproved our original hypothesis that the heterogeneous pattern of radiolabel uptake from anandamide is determined by and is reflective of FAAH activity. Also, the fact that FAAH inhibition had little effect on the disposition pattern of radiolabel from anandamide excludes the possibility that metabolism of anandamide through other enzymes contributes significantly to the pattern. Furthermore, our data also questioned the role of PLA\(_2\) activity on the pattern produced with labeled AA.

It appears that intact anandamide (and also arachidonic acid) distributes heterogeneously in the brain shortly after crossing the blood brain barrier and that the distribution pattern stays approximately stable for a while (at least 100 minutes). In other words, the most important factor that determines the heterogeneous distribution of these tracers is likely to be dominated by the initial delivery of the compound to each brain region, which is determined by (1) the relative blood flow to each area and (2) by the relative permeability of the blood brain barrier in each area to the tracers. These two factors of course control the initial distribution of any radiotracer, In the present case, three additional factors come into play: (3) there is little additional transfer of tracer from blood to brain after the initial "bolus" has passed through the brain vasculature; (4) the radiolabeled fatty acids and fatty acylethanolamines diffuse extremely slowly back into the blood from the brain due to their high solubility in brain lipids, and (5) their radioactive metabolites also leave the brain very slowly. Based on the observation that all the autoradiographs we obtained from fatty acids and NAEs were very similar, and that the autoradiographs produced with labeled ethanolamine, which is a water soluble small molecule, were almost homogeneous in appearance, we developed a new hypothesis that initial delivery (determined by rCBF and permeability), diffusion rate of the compound and its metabolites across brain regions and back into the blood (stickiness to brain tissue,
determined by its physical properties such as size, structure and lipophilicity) and binding/metabolism of the compound and its metabolites together determine the formation of the pattern and how it is changed over time. Permeability of the blood brain barrier to a particular tracer also affects the initial delivery and overall brain uptake of the tracer, but it will not contribute much to the “pattern” because there should be little regional differences in permeability. All of our previous data can be well explained by this new hypothesis: For anandamide labeled at the arachidonoyl moiety (Figure 24.A), the pattern is formed at initial delivery due to differences in rCBF rates, and because it is “sticky” to membranes (Figure 24.E), the labeled molecules diffuse very slowly from where they have been delivered, and get hydrolyzed by FAAH and move on to the incorporation/release cycle of AA. The process starting from the action of FAAH (including the following step involving PLA₂) has to be almost homogeneous to make the pattern stable. Now we reasoned that PLA₂ doesn’t contribute to the pattern, we can speculate that for labeled AA (Figure 24.B), the pattern is also determined at initial delivery by rCBF, and similarly, because AA is “sticky” and metabolized homogenously later on, the pattern stays. For labeled ethanolamine (Figure 24.C, E), the effect of rCBF was quickly erased before it can be visualized due to fast diffusion of ethanolamine across brain regions and back to the blood, thus a almost homogenous pattern was obtained. For anandamide labeled at the ethanolamine moiety (Figure 24.D), the pattern reflecting rCBF is also maintained because the action of FAAH and the following metabolism of ethanolamine is almost homogenous.
Figure 24. Schematic representation of the formation of the autoradiographic patterns from labeled anandamide, AA and ethanolamine. The autoradiographic patterns produced with [14C-arach.]anandamide (A), [14C]AA (B) or [14C-EA]anandamide (D) are determined by initial delivery immediately after the tracer molecules cross the blood brain barrier and before their metabolism, and further action of FAAH and/or PLA2 had little impact on the patterns. C) The pattern of the initial delivery of ethanolamine was not preserved because ethanolamine and its metabolites can diffuse quickly across brain regions and back to the blood, and the process of incorporation of ethanolamine into PE is almost homogeneous in the brain. E) Anandamide tends to "sink" into membranes due to its lipid structure and therefore diffuse very slowly, whereas ethanolamine can diffuse freely across the membranes because it is small and water soluble. BBB, blood brain barrier; AEA, anandamide; AA, arachidonic acid; PI, phosphatidylinositol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; EA, ethanolamine; p-EA, phosphorylcholine; ATP, adenosine triphosphate; ADP, adenosine diphosphate; PPI, diphosphate; CTP, cytidine triphosphate; CDP, cytidine diphosphate; CMP, cytidine monophosphate; DAG, diacylglycerol; "pattern", a heterogeneous pattern is determined at this point; "pattern", this metabolic step makes little contribution to a heterogeneous pattern.
G. The patterns produced with $[^{14}\text{C}]{\text{iodoantipyrine}}$ and $[^{14}\text{C}]{\text{benzyl alcohol}}$ (rCBF tracers) are similar to those produced with anandamide and related compounds.

To test our new hypothesis, we need to identify the pattern of rCBF so that we can compare it with the patterns obtained with labeled anandamide and related compounds. The time point for euthanasia (the time when the blood flow stops) is very important and it directly impacts the fidelity of the obtained rCBF image. If the animal is sacrificed too early, the amount of radioactivity in the brain may be too low and the regional differences may still be accumulating; if the animal is sacrificed too late, the effects of diffusion and binding/metabolism may start to manifest in the image, but more importantly compounds that cross the blood brain barrier easily (“freely diffusible” tracers) also move back into the blood very quickly, as the tracer-rich blood during first-pass through the brain is replaced by blood with a much lower concentration of tracer than the brain. There is no single universally perfect time-point for euthanasia, and for any given tracer the time at which the regional tracer distribution is the best approximation to regional perfusion depends on its kinetics in the blood and on its volume of distribution in brain regions. For many radiotracers the distribution in the first minute probably gives a good approximation to flow. We decided to evaluate several radioactive compounds whose early regional brain distributions have been reported by others to largely reflect blood flow.

The classic rCBF probe is $[^{14}\text{C}]{\text{iodoantipyrine}}$ (Flora et al. 1962). However, the resolution of the rCBF image produced with $[^{14}\text{C}]{\text{iodoantipyrine}}$ is limited by its relatively fast diffusion across brain regions (Greenberg et al. 1999). We thought that $[^{14}\text{C}]{\text{benzyl alcohol}}$ could be another tracer to image rCBF since it was reported by Cornforth et al. (1982) to be well extracted after intracarotid injection. We proposed that if we are fast enough in taking out and then sectioning or dissecting the brain shortly after injection of the rCBF probes, we would be able to obtain a distribution pattern that is very close to the actual pattern of rCBF.
Autoradiography experiment with $[^{14}\text{C}]$iodoantipyrine and $[^{14}\text{C}]$benzyl alcohol were performed following previously describe procedures except that mice were euthanized at 30 seconds after tracer injection to capture the pattern of the initial delivery which is largely determined by rCBF. Each mouse received 4 µCi of the tracer dissolved in ethanol/emulphor/saline (1:1:18). As expected, the images produced with $[^{14}\text{C}]$iodoantipyrine and $[^{14}\text{C}]$benzyl alcohol are similar, which show higher amount of radioactivity at thalamus, the ventricle areas, and cortex (Figure 25). Compared to the images obtained with labeled AA and anandamide, the rCBF images have relatively less amount of radioactivity in the cortex, however the overall patterns look similar, indicating that rCBF plays an important role in formation of the pattern produced with labeled AA or anandamide, and that further metabolism/binding of AA or anandamide possibly drives the label towards the cortex.

![Chemical structures](image1)

Figure 25. The patterns produced with $[^{14}\text{C}]$iodoantipyrine and $[^{14}\text{C}]$benzyl alcohol (rCBF tracers) are similar to those produced with anandamide and AA. Each mouse received 4 µCi of the tracer dissolved in ethanol/emulphor/saline (1:1:18). For $[^{14}\text{C}]$iodoantipyrine and $[^{14}\text{C}]$benzyl alcohol, mice were euthanized at 30 seconds instead of 15 minutes to capture the pattern of the initial delivery.
The autoradiography experiments with $^{14}$Ciodoantipyrine and $^{14}$Cbenzyl alcohol revealed that there are limitations for both tracers. Although $^{14}$Ciodoantipyrine has been used for decades as a rCBF tracer, the resolution of the images produced with $^{14}$Ciodoantipyrine is lower than that produced with $^{14}$Cbenzyl alcohol. We speculated that $^{14}$Ciodoantipyrine continues to diffuse across brain regions during and after sectioning until the sections are dried. Although $^{14}$Cbenzyl alcohol seemed to diffuse more slowly and did produce sharper images, the preparation of the injection solution, which includes evaporating ethanol from the stock solution of $^{14}$Cbenzyl alcohol is difficult and wasteful because benzyl alcohol is volatile and tends to evaporate along with the ethanol. During and after drying of the brain sections, $^{14}$Cbenzyl alcohol continues to evaporate from the tissue so that exposure should be done immediately after the sections are dried. The air above the drying sections was vented to the lab hood system. A second exposure four weeks later resulted in images with much less overall signal. Evaporation of $^{14}$Cbenzyl alcohol could also affect the fidelity of the rCBF images because the rates of evaporation might be different for sections of different thickness or for brain regions with different cellular structures.

To resolve the problems mentioned above, we decided to quantify rCBF using the microdissection technique. With this method, less amount of radioactivity is required (1µCi instead of 4 µCi per animal) and quantification of regional differences is much faster and easier. Furthermore, the time during which diffusion can happen is limited to less than 5 minutes (versus 30 minutes of sectioning and hours of drying) which is the time required to take out and microdissect the brain. Evaporation is also halted right after the separated brain regions are placed in individual capped vials. We decided to use this technique in our following experiments.
H. Arecoline increases the uptake of various tracers in the brain through activation of central muscarinic receptors

Our hypothesis that rCBF contributes greatly to the pattern formed with labeled AA and anandamide does not agree with the idea of Rapoport (1996) that PLA$_2$ activity controls the pattern. Actually, Rapoport and his colleagues had excluded the contribution of rCBF to the incorporation of fatty acids into the brain based on several studies: they showed that the incorporation of [³H]palmitate is not affected by cholinergic stimulation which increases rCBF (DeGeorge et al. 1991); and that the incorporation of [³H]palmitate and [¹¹C]AA is not affected by hypercapnia which would have increased rCBF. (Yamazaki et al. 1994, Chang et al. 1997).

We argue that rCBF is an important factor that determines the first step -- the initial delivery of the tracers to the brain, and the uptake of tracers will definitely be affected by changes of rCBF if only rCBF and no other factor in the following stages (diffusion and metabolism/binding) is changed. However, this is rarely the case that only one factor is changed by any medication or procedure. The observed changes in the pattern and calculated incorporation coefficient result from the combination of all changes -- which could cancel each other out -- in this complex process. PLA$_2$ activity is only one of the factors that could be changed. Therefore we doubt the feasibility of quantifying or monitoring PLA$_2$ activity based on the pattern of AA incorporation in the brain.

H. 1. Arecoline increased incorporation of [¹⁴C]AA

Arecoline is a muscarinic cholinergic agonist shown to increase the incorporation of labeled AA in rat brain, and the underlying mechanism was suggested to be activation of PLA$_2$ which is coupled to activation of central muscarinic receptors (DeGeorge et al. 1991, Jones et al. 1996). However, other workers have documented increases in rCBF after administration of arecoline (Maiese et al. 1994). We considered it difficult to rule out the contribution of increased rCBF on
increased incorporation of AA, and we planned to test the effect of arecoline on brain uptake of labeled AA and other compounds, including blood flow tracers. Our reasoning was that if the effects of arecoline on brain uptake of labeled AA was similar to that of other tracers which are not related to PLA₂, then it was likely that the increased brain uptake of AA was also largely due to increased rCBF by arecoline.

We firstly tested the effect of arecoline on brain uptake of labeled AA in mice. We followed the methods from an arecoline experiment in rats (DeGeorge et al. 1991) except that we administered the tracer via tail vein injection to mice instead of 5-minute intravenous infusion. Mice received methylyatropine (5mg/kg) subcutaneously to block peripheral effects of arecoline. After 17 minutes, control (saline) or arecoline (15mg/kg) were administered via intraperitoneal injection, and one minute later 4uCi of [14C]AA were injected into the tail vein. After 15 minutes, mice were sacrificed and the brains were sectioned for autoradiography as previously described. Our result is consistent with the findings in rats: mice received arecoline showed increased brain uptake of [14C]AA compared to the control group, however the pattern of uptake was similar between arecoline and control groups, indicating a global effect of arecoline on all brain regions (Figure 26). We also noticed about 30 seconds after administration of arecoline, mice started to tremor vigorously -- a typical behavioral response to stimulation of M2 muscarinic receptor in the brain (Bymaster et al. 2003).
Figure 26. Arecoline increased incorporation of $[^{14}C]AA$. A) Brains from mice treated with arecoline showed higher uptake of $[^{14}C]AA$ compared to mice injected with saline (sections were exposed on the same phosphor imaging plate and images were captured at the same scale of signal intensity). B) The scale of signal intensity was adjusted in OptiQuant for saline treatment group to show regional differences in $[^{14}C]AA$ uptake. The patterns are similar between arecoline and saline groups, but arecoline treated group showed higher signal intensity.

H. 2. Arecoline increased brain uptake of various tracers similarly

We have confirmed the effect of arecoline on brain uptake of $[^{14}C]AA$, next we used the microdissection technique to quantify the changes and to compare the effects among $[^{14}C]AA$ and other tracers that are unrelated to PLA$_2$ activity. The tracers we tested were $[^{14}C]$-EA anandamide, $[^{14}C]$benzyl alcohol, $[^{125}I]$RTI-55 and $[^{125}I]$iomazenil. Previously we have found that the heterogeneous pattern of brain uptake of $[^{14}C]$-EA anandamide was formed before metabolism of anandamide and is similar to the pattern obtained with $[^{14}C]AA$. Because the label is on the ethanolamine moiety, which is released from anandamide immediately by FAAH in the brain, the distribution and incorporation of the label is independent of metabolism of the arachidonoyl moiety which involves PLA$_2$. For $[^{14}C]$benzyl alcohol, we have shown that it can be used as a blood flow tracer to directly measure the changes in rCBF.$[^{125}I]$RTI-55 is a probe for dopamine transporters and its initial distribution pattern is largely determined by rCBF. Similarly, $[^{125}I]$iomazenil is a probe for GABA$_A$ receptors and its early distribution was shown to correlate
with rCBF (Suzuki et al. 2012). If arecoline increases brain uptake of these unrelated tracers similarly, it is likely that a direct increase in delivery of the tracers as a result of increased rCBF accounts for the effect.

We formulated a mixture of dual-tracer for intravenous injection. Each mouse received 1uCi of [$^{14}$C-AA and 0.1uCi of [$^{125}$I]RTI-55 in 200 ul of ethanol/emulphor/saline (1:1:18, v/v). The same doses (DeGeorge et al. 1991) and procedure described above was used: mice were pretreated with methylatropine (5mg/kg, s.c.) 17 minutes before receiving either saline or arecoline (15mg/kg, i.p.) and received the tracers via tail vein one minute later. After another minute, mice were sacrificed and the brains were immediately microdissected into small regions and weighed in gamma counter tubes. Cortex, hippocampus, striatum, thalamus, cerebellum, brain stem and rest of the brain along with blood sample were collected. Amounts of [$^{125}$I]RTI-55 were immediately obtained from gamma counter and [$^{14}$C-AA readings were counted after all tissues were dissolved in Solvable tissue solubilizer, and then assayed in the liquid scintillation counter using UltimaGold cocktail. We found that at one minute after injection of the tracers, arecoline treated mice showed significant and similar increases of uptake of the two tracers compared to control (Figure 27A, Table 2). We used one minute instead of 15 minutes this time because we wanted to capture the early distribution of tracers that reflects mostly rCBF. Next, we repeated the experiment co-administrating 1uCi of [$^{14}$C-EA]anandamide and 0.1uCi of [$^{125}$I]RTI-55. This time we euthanized the animals at 15 minutes because we would like to see how the distribution of [$^{125}$I]RTI-55 changes in 15 minutes due to its affinity to dopamine transporters. We did notice that at 15 minutes the amount of activity at striatum had doubled compared to 1 minute, indicating that the influence of binding had started to manifest in the pattern of distribution. Again, arecoline treatment boosted the uptake of both tracers in all brain regions (Figure 27B, Table 2). We then used atropine instead of methylatropine to block both peripheral and central muscarinic receptors before injection saline or arecoline, and then co-administered [$^{14}$C-
EA]nanandamide and $[^{125}I]$RTI-55 again. Treatment with atropine blocked the increased uptake of $[^{125}I]$RTI-55 and $[^{14}C]$EA]nanandamide uptake (Figure 27C, table 2), indicating that the increased uptake of both tracers were due to activation of central muscarinic receptors by arecoline.

![Figure 27](image)

**Figure 27.** Arecoline increased brain uptake of $[^{14}C]$AA, $[^{125}I]$RTI-55 and $[^{14}C]$EA]nanandamide through activation of central muscarinic receptors. A) Mice were pretreated with methylatropine (s.c.) 17 minutes before injection of arecoline or control (i.p.), and were given tracers via tail vein injection 1 minute later and sacrificed after another minute  B) Mice were treated as in A but euthanized 15 minute after injection of tracers C) Mice were given atropine instead of methylatropine and treated as in B. %IA/g, percentage of injected activity per gram; ST, striatum; TH, thalamus; CB, cerebellum; BS, brain stem; HP, hippocampus; CX, cortex; RB, rest of the brain; BL, blood. (n=5, statistics shown in table 2)
We continued our arecoline experiments with $^{14}$Cbenzyl alcohol and $^{125}$Iiomazenil to further confirm that the effect of arecoline is universal to all tracers, to support our hypothesis that the increased brain uptake of tracers by arecoline is largely due to increased delivery via increased rCBF. We tried not to use either methylatropine or atropine at first to see whether the effect of arecoline would be different if peripheral muscarinic receptors were not blocked. Similar to previously described, each mouse received a mixture of 1uCi of $^{14}$Cbenzyl alcohol and 0.1uCi of $^{125}$Iiomazenil 1 minute after administration of arecoline or control, and was sacrificed 15 seconds later to obtain the distribution of tracers in the brain representing the initial delivery. 15-second was the shortest time we could manage and we hoped to capture the distribution pattern that is almost entirely determined by rCBF. We found that without methylatropine pretreatment, arecoline failed to increase brain uptake of either $^{14}$Cbenzyl alcohol or $^{125}$Iiomazenil. Pretreatment of atropine made the amounts of activity in all brain regions even lower for both tracers (Figure 28A). We also noticed that mice received no methylatropine treatment have less tremor than those who received methylatropine in previous experiments. We repeated the experiment with methylatropine or atropine pretreatment and did see the expected effect of arecoline in mice received methylatropine: the differences between arecoline and control reach significance in some brain regions for $^{14}$Cbenzyl alcohol and $^{125}$Iiomazenil and in the whole brain for $^{125}$Iiomazenil. Atropine significantly blocked the effect of arecoline (Figure 28B).
Figure 28. Arecoline increased brain uptake of $[^{14}\text{C}]{\text{benzyl alcohol}}$ and $[^{125}\text{I}]{\text{iomazenil}}$ through activation of central muscarinic receptors. A) Without methylatropine pretreatment, arecoline failed to alter brain uptake of both tracers and atropine significantly lowered brain uptake of both tracers B) with pretreatment of methylatropine, the effect of arecoline was significant in highlighted brain regions and in whole brain for $[^{125}\text{I}]{\text{iomazenil}}$. (n=5, statistics shown in table 2)
In summary (Table 2), our data indicate that effect of arecoline on increased brain uptake of radiotracers is via activation of central muscarinic receptors, and peripheral muscarinic stimulation by arecoline seems to cancel out this central effect. A possible mechanism of this phenomenon might include peripheral vasodilatation which could increase the delivery of the tracer to peripheral tissues and reduce the blood flow and the delivery of the tracers to the brain. When activating only central muscarinic receptors, arecoline leads to increased brain delivery of AA, anandamide, benzyl alcohol, RTI-55 and iomazenil, indicating that the effect is likely due to a global increase in rCBF instead of altered metabolism of certain tracers.

<table>
<thead>
<tr>
<th>Radiotracer</th>
<th>Pretreatment</th>
<th>Euth. Time (sec)</th>
<th>Arecoline % inc</th>
<th>p-value</th>
<th>Expt #</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{[14C]}\text{EA})anandamide</td>
<td>methylatropine</td>
<td>900</td>
<td>24%</td>
<td>0.07</td>
<td>a</td>
</tr>
<tr>
<td>(^{[14C]}\text{EA})anandamide</td>
<td>atropine</td>
<td>900</td>
<td>11%</td>
<td>0.1</td>
<td>b</td>
</tr>
<tr>
<td>(^{[14C]}\text{arachidonic acid})</td>
<td>methylatropine</td>
<td>60</td>
<td>34%</td>
<td>0.02</td>
<td>c</td>
</tr>
<tr>
<td>(^{[125I]}\text{RTI})</td>
<td>methylatropine</td>
<td>900</td>
<td>30%</td>
<td>0.02</td>
<td>a</td>
</tr>
<tr>
<td>(^{[125I]}\text{RTI})</td>
<td>atropine</td>
<td>900</td>
<td>0%</td>
<td>0.4</td>
<td>b</td>
</tr>
<tr>
<td>(^{[125I]}\text{RTI})</td>
<td>methylatropine</td>
<td>60</td>
<td>30%</td>
<td>0.04</td>
<td>c</td>
</tr>
<tr>
<td>(^{[14C]}\text{benzyl alcohol})</td>
<td>methylatropine</td>
<td>15</td>
<td>28%</td>
<td>0.03</td>
<td>d</td>
</tr>
<tr>
<td>(^{[14C]}\text{benzyl alcohol})</td>
<td>atropine</td>
<td>15</td>
<td>-1%</td>
<td>0.6</td>
<td>d</td>
</tr>
<tr>
<td>(^{[125I]}\text{iomazenil})</td>
<td>methylatropine</td>
<td>15</td>
<td>34%</td>
<td>0.01</td>
<td>d</td>
</tr>
<tr>
<td>(^{[125I]}\text{iomazenil})</td>
<td>atropine</td>
<td>15</td>
<td>-8%</td>
<td>0.9</td>
<td>d</td>
</tr>
</tbody>
</table>

**Table 2. Effects of arecoline on whole brain radioactivity concentrations.** Mice (male, 25-30g) were administered radiotracers intravenously in a volume of 0.2mL ethanol/emulphor/saline (1:1:18; v/v). They were pretreated subcutaneously with methylatropine or atropine 18 min before radiotracers and arecoline or saline was given intraperitoneally 1 min before radiotracers. Experiments: (a) \(^{[14C]}\text{EA}\)anandamide plus RTI-55; (b) \(^{[14C]}\text{EA}\)anandamide plus RTI-55; (c) arachidonic acid plus RTI-55; (d) benzyl alcohol plus iomazenil. (n=5, student’s t-test)
IV. Discussion

A. Radiotracers for ex vivo autoradiography and nuclear medical imaging

 Autoradiographic methods have been used to study physiology and biochemistry in the living brains of experimental animals for over 50 years. The general methodology involves intravenous administration of radioactive compound followed by euthanasia at a time when the distribution of radioactivity is a good reflection of the process under study. Extensive experimentation is required to validate choices of tracer and methodology. General brain function has been studied using tracers of blood flow and of glucose metabolism. The “agent-of-choice” for blood flow measurement is generally considered to be iodoantipyrine (Ohno et al. 1979). This compound is an example of a “freely diffusible indicator” (Lassen et al. 1967) that crosses the blood brain barrier easily and is not trapped by any biochemical mechanism, but has a high volume of distribution in brain tissue. At short times after administration, before much of the tracer has undergone back-diffusion into the blood, its local distribution is a good indicator of local rates of blood flow. Iodoantipyrine can be labeled with either $^{14}$C or $^{125}$I for studies of regional cerebral blood flow (rCBF). The standard radiotracer for autoradiographic studies of cerebral glucose metabolism is $[^{14}\text{C}]2$-deoxy-D-glucose (2DG) (Kennedy et al. 1975, Sokoloff 1979). This analog of D-glucose crosses the blood brain barrier and enters brain cells (using GLUT1 and GLUT3 transporters, respectively) with similar efficiency to glucose itself, and also readily undergoes the first step of glycolysis—formation of the glucose-6-phosphate. The 6-phosphate of 2-deoxyglucose, however, is not a substrate for the second step of glycolysis—in which glucose-6-phosphate is isomerized to fructose-6-phosphate. This isomerization requires the presence of an O-atom on carbon-2, which of course is lacking in 2-deoxyglucose. Since 2-deoxyglucose-6-phosphate is dephosphorylated only very slowly in non-gluconeogenic tissues, its concentration accumulates in brain regions following intravenous injection in proportion to the rates of regional glucose utilization, when adjustments are made for residual...
unphosphorylated 2DG in tissues, for the concentration of glucose in plasma, and for the relative efficiency of phosphorylation of 2DG and glucose (Sokoloff 1979). Typically, animals are euthanized 45 minutes after administration of $[^{14}\text{C}]2\text{DG}$. The development of the 2DG autoradiographic method coincided with the development of the nuclear imaging modality, positron emission tomography (PET) and the discovery of $[^{18}\text{F}]2\text{-deoxy-2-fluoro-D-glucose}$ (2FDG or FDG) which exhibits similar biochemical behavior to 2DG, and which has made possible studies of local rates of glucose metabolism in brains of human subjects (Reivich et al. 1979). The term “metabolic trapping”, which is now applied generally to radiotracers whose tissue kinetics are slowed by phosphorylation or other biochemical process(es), appears to have been coined in relation to FDG (Gallagher et al. 1978). Other examples of metabolically trapped PET tracers include: $[^{18}\text{F}]6\text{-fluoro-L-DOPA}$ (Garnett et al. 1984), where the trapped species is vesicularized $[^{18}\text{F}]6\text{-fluorodopamine}$; $[^{18}\text{F}]3\text{-deoxy-3'-fluoro-thymidine}$, which is trapped as phosphorylated species (Shields et al. 1998); and $[^{11}\text{C}]\text{N-methylpiperidin-4-yl acetate}$ and other positron emitting acetylcholine analogs where the labeled moiety is retained in tissue after cleavage of the acetate by acetylcholinesterase (Kikuchi et al. 2005).

Nuclear medicine radiopharmaceuticals that are distinct from the metabolically trapped tracers are those that are retained in tissue by tight binding to macromolecules (hormone or neurotransmitter receptors, transporters or enzymes). There are very many examples of such radiotracers, which are often termed “radioligands”, that can be used to image binding sites in the brains of humans and non human primates, among the first to be discovered being $[^{11}\text{C}]\text{raclopride}$ for imaging the dopamine D2 receptor (Ehrin et al. 1985), and $[^{11}\text{C}]\text{nomifensine}$ for imaging the dopamine reuptake transporter (Aquilonius et al. 1987). A tracer that binds very tightly to its target will typically continue to accumulate in target-rich brain regions for several minutes not begin to decrease for an hour or longer, whereas the concentration of radionuclide
in receptor poor regions will reach peak concentrations within a few minutes and its tissue concentration decline thereafter.

A tracer that was trapped with 100% efficiency by its target process or binding site would not be sensitive to changes in the density of its binding site, at least for densities over some threshold level. Its accumulation in tissue would then indicate flow and blood brain barrier permeability only. The optimum affinity of an in vivo/ex vivo radioligand for its target is not necessarily a radiolabeled form of the drug or research compound that binds with the highest known affinity to the target, because blood flow would be a major determinant of its accumulation.

The classic example of a metabolically trapped tracer where cerebral blood flow strongly affects extraction is $[^{15}\text{N}]\text{ammonia}$ (Phelps et al. 1981). Among other methodologies, Phelps et al. (1981) examined brain kinetics and metabolism of $[^{15}\text{N}]\text{ammonia}$ under conditions where cerebral blood flow was altered with hypercapnia; the results showed that changes in extraction are due to changes in both capillary transit time and capillary recruitment. The predominant enzyme that fixes $^{15}\text{N}$ in the brain is glutamine synthetase, which is ubiquitous, and the residence time of free $[^{15}\text{N}]\text{ammonia}$ in the brain is estimated to be less than three seconds (Cooper et al. 1979). Notwithstanding its very short half-life, $^{13}\text{N}$, which is the longest-lived radioisotope of nitrogen, has been used in important biochemical and physiological experiments (Cooper 2011).

An extensive recent review of PET imaging is available (Gunn et al. 2015). Another nuclear medicine imaging modality that should be mentioned is SPECT (single photon emission computed tomography), which is less sensitive and less quantitative than PET (Goffin and van Laere 2016). However, it has the advantage of not requiring positron emitting radiotracers, so that commonly available clinical radiopharmaceuticals can be used. Many compounds labeled with $^{123}\text{I}$ (half-life = 13 h) including RTI-55 and iomazenil, which we used in $^{125}\text{I}$-labeled forms,
can be used in SPECT brain imaging experiments in humans and animals though $^{123}$I radioligands are generally synthesized in-house from commercially obtained $[^{123}]$iodide.

It is important to appreciate that no ex vivo or nuclear medicine imaging agent yields a tissue radioactivity distribution that is a perfect representation of the physiological or biochemical process or concentration of binding site that is under study in all tissues, under all physiological or pathological conditions and at a single time-point. Probably, estimates of local cerebral metabolic rates for glucose in normal brain using 2DG or FDG come closest (Dienel et al. 1991), and studies of the brain using these tracers are widely used. A MEDLINE search using the terms “brain” and “2-deoxyglucose” or “fluorodeoxyglucose” reveals over 8,000 and over 4,000 papers, respectively. FDG/PET is most widely used for non-quantitative or semi-quantitative clinical imaging to evaluate disease progression and the extent of metastasis in patients with cancer.

In ex vivo autoradiographic experiments, each experimental animal gives data for a single time-point. The in vivo imaging modalities of PET and SPECT, however, in principle permit the whole time-course of radioactivity in brain regions to be measured in individual animals (including humans). Such dynamic as opposed to static imaging removes the reliance on radiotracer distribution at a single time-point and allows the application of tracer kinetic analysis to the whole time-course of radioactivity. This facilitates estimation of tracer metabolic rates or radioligand binding potentials (Mintun et al. 1984, Sawada et al. 1990). Tracer kinetic modeling is an important aspect of PET research, but will not be discussed in detail here. In brief, one approach to kinetic analysis of PET scan data using either radioligands or metabolically trapped tracers is that of compartmental modeling. The simplest 3-compartment model involves tracer in blood, “free” tracer in tissue and trapped or bound tracer in tissue (Huang et al. 1981, Logan et al. 1987, Votaw et al. 1993). The other general approach is to use one of the graphical, model-independent methods. The Patlak method is used with quasi-irreversibly bound or
trapped tracers, including FDG (Patlak et al. 1983). The Logan method is used with “reversible” tracers where tissue radioactivity is declining by the end of the study, such as $[^{11}C]$raclopride (Logan et al. 1990).

Whatever the analysis method, quantitative PET and SPECT permit repeat studies of, for example, drug treatments, so that a single animal can act as its own control, and longitudinal studies of disease progression or chronic drug effects in individual subjects become possible. On the other hand, ex vivo autoradiography offers much better spatial resolution, and does not require access to expensive tomographs, or to tracers labeled with short lived radionuclides. Furthermore, it is easy to combine rodent autoradiographic studies with radioanalytical studies of the chemical form of radioisotopes in tissues, and with pharmacological challenges. For these reasons, ex vivo autoradiographic and tissue microdissection studies using $^{14}$C, $^3$H and $^{125}$I labeled tracers as well as positron emitters are often used in radiopharmaceutical discovery, development and validation studies.
<table>
<thead>
<tr>
<th>Radiotracer</th>
<th>Initially trapped species</th>
<th>Process studied</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Deoxy-D-glucose</td>
<td>Hexose-6-phosphate</td>
<td>Glucose metabolic rate</td>
<td>1</td>
</tr>
<tr>
<td>2-Deoxy-2-fluoro-D-glucose</td>
<td>Hexose-6-phosphate</td>
<td>Glucose metabolic rate</td>
<td>2</td>
</tr>
<tr>
<td>Ammonia</td>
<td>Glutamine</td>
<td>Perfusion</td>
<td>3</td>
</tr>
<tr>
<td>L-6-Fluoro-DOPA</td>
<td>Fluorodopamine</td>
<td>Dopamine synthesis</td>
<td>4</td>
</tr>
<tr>
<td>3'-Deoxy-3'-fluorothymidine (FLT)</td>
<td>FLT-5-phosphate</td>
<td>DNA synthesis</td>
<td>5</td>
</tr>
<tr>
<td>Leucine</td>
<td>Leucyl-tRNA</td>
<td>Protein synthesis</td>
<td>6</td>
</tr>
<tr>
<td>[11C]N-Methyl-piperidin-4-yl acetate</td>
<td>1-Methyl-4-piperidinol</td>
<td>Acetylcholinesterase activity</td>
<td>7</td>
</tr>
<tr>
<td>Arachidonic acid</td>
<td>Acyl-coenzyme A</td>
<td>Phospholipase A2 activity</td>
<td>8</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>Nuclide</th>
<th>Half-life</th>
<th>Decay mode; particle emission</th>
<th>Photons</th>
<th>Applications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tritium</td>
<td>12.3 y</td>
<td>β⁺; 18.6 keV</td>
<td>None</td>
<td>General laboratory use</td>
</tr>
<tr>
<td>Carbon-11</td>
<td>20 min</td>
<td>β⁺⁺; 960 keV</td>
<td>511 keV</td>
<td>PET</td>
</tr>
<tr>
<td>Carbon-14</td>
<td>5730 y</td>
<td>β⁺; 156 keV</td>
<td>None</td>
<td>General laboratory use</td>
</tr>
<tr>
<td>Nitrogen-13</td>
<td>10 min</td>
<td>β⁺; 1200 keV</td>
<td>511 keV</td>
<td>PET</td>
</tr>
<tr>
<td>Fluorine-18</td>
<td>110 min</td>
<td>β⁺⁺; 630 keV</td>
<td>511 keV</td>
<td>PET</td>
</tr>
<tr>
<td>Iodine-123</td>
<td>13 h</td>
<td>EC; 127 keV</td>
<td>159 keV</td>
<td>SPECT</td>
</tr>
<tr>
<td>Iodine-125</td>
<td>60 d</td>
<td>EC; 35 keV</td>
<td>35 keV</td>
<td>General laboratory use</td>
</tr>
</tbody>
</table>

Table 4. Radionuclides used for ex vivo and in vivo studies and mentioned in the dissertation. EC = electron capture; β⁺ = beta particle (negatron); β⁺⁺ = beta particle (positron); SPECT = single photon emission computed tomography; PET = positron emission tomography.
<table>
<thead>
<tr>
<th>Modality</th>
<th>Approx. resolution</th>
<th>Ex/in vivo</th>
<th>Nuclides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Film autoradiography</td>
<td>10 micron</td>
<td>Ex vivo</td>
<td>$^3$H, $^{14}$C, $^{125}$I</td>
</tr>
<tr>
<td>Emulsion autoradiography</td>
<td>1 micron</td>
<td>Ex vivo</td>
<td>$^3$H</td>
</tr>
<tr>
<td>Phosphor imaging</td>
<td>100 micron</td>
<td>Ex vivo</td>
<td>$^3$H, $^{14}$C, $^{125}$I, $^{18}$F</td>
</tr>
<tr>
<td>Beta-imaging</td>
<td>100 micron</td>
<td>Ex vivo</td>
<td>$^3$H, $^{14}$C, $^{125}$I</td>
</tr>
<tr>
<td>PET</td>
<td>millimeter</td>
<td>In vivo</td>
<td>$^{11}$C, $^{13}$N, $^{18}$F</td>
</tr>
<tr>
<td>SPECT</td>
<td>millimeter</td>
<td>In vivo</td>
<td>$^{123}$I</td>
</tr>
</tbody>
</table>

Table 5. Ex vivo and in vivo radionuclide imaging modalities.

Film/emulsion: activation of silver halide grains to subsequent development (oxidation to silver grains)

Phosphor imaging: activation of europium-doped barium fluorobromide grains deposited on a flexible support. Subsequent irradiation with a long wavelength laser stimulates emission of lower wavelength light which can be scanned with a photomultiplier tube to form an image of the original radioactivity. Compared with photographic films, phosphor imaging plates are more sensitive and give a linear response over a large range, but have lower potential spatial resolution.

Beta-imaging: beta particles emitted from the surface of tissue sections held in a vacuum chamber are accelerated and focused on a detector using electric fields.

PET: a pair of annihilation photons (511 keV) is emitted back-to-back when a positron interacts with an atomic electron, so that when two detectors on opposite sides of the subject register counts in the same instant, it is known that an annihilation event occurred on the line joining the detectors. A computer reconstructs an image of radioactivity distribution from millions of such coincidence events.

SPECT: a nuclear medicine "scintillation camera" capable of generating a 2-dimensional image is rotated around the subject gathering information from many angles. A computer reconstructs a 3-D representation of the radioactivity distribution.
B. The factors that determine brain uptake and distribution of labeled anandamide and related compounds (How patterns are formed and what they mean)

We started this project aiming to develop a radiotracer for imaging regional values of FAAH activity in the brain, which could provide complementary information to brain regional concentrations of the FAAH protein achieved by radiolabeled high affinity or irreversible inhibitors of FAAH. We considered $[^{14}\text{C}]$-arach.anandamide, a labeled FAAH substrate, to be a good candidate for imaging FAAH activity using ex vivo autoradiography in rodents. Since $[^{14}\text{C}]$AA released from $[^{14}\text{C}]$-arach.anandamide is rapidly incorporated into brain phospholipids and its release from phospholipids is relatively slow, we hypothesized that it was possible to map FAAH activity in the brain assuming that release of labeled AA from $[^{14}\text{C}]$-arach.anandamide by FAAH is rate limiting in the process of radiolabel incorporation.

Previous experiments with $[^{3}\text{H}]$-arach.anandamide and $[^{3}\text{H}]$AA produced heterogeneous and homogeneous autoradiography respectively, and supported our notion that the heterogeneous incorporation of radiolabel in the brain is due to and reflect regional differences in FAAH activity (Glaser et al. 2006). However, we obtained almost identical patterns with $[^{14}\text{C}]$-arach.anandamide and $[^{14}\text{C}]$AA, indicating that FAAH activity is not a major determinant of label disposition. Further experiments with tritiated ($[^{3}\text{H}]$MA and $[^{3}\text{H}]$MEA) and carbon-14 ($[^{14}\text{C}]$MA) biochemicals confirmed that labeled fatty acids distribute heterogeneously in the brain and the patterns are similar to those for their NAEs. However, the noise associated with the tritium label can mask the signal. When the signal is relatively low (fatty acids are charged and do not cross the blood brain barrier as well as NAEs), the regional differences cannot be seen due to high noise. In other words, the patterns produced with labeled fatty acids using practicable amount of tritium are obscured because of the low sensitivity of this nuclide. At this point our original hypothesis was rejected and we had to pause and rethink the factors that determine brain
uptake and distribution of the radiotracers we were using, and whether imaging FAAH activity using a labeled substrate was possible.

Based on the fact that similar autoradiographic patterns were produced with $[^{14}\text{C}]$anandamide and $[^{14}\text{C}]$AA, we realized that FAAH is not the rate-limiting enzyme in the process of radiolabel incorporation and that the pattern from $[^{14}\text{C}]$-arach.anandamide may actually reflect regional differences in metabolism of AA in the brain (Figure 29). It is likely that anandamide simply serves as a precursor for efficient delivery of labeled AA into the brain. Actually, labeled AA was shown to produce heterogeneous autoradiographic pattern in rats and has been suggested to be useful in imaging PLA$_2$ activity in the brain (DeGeorge et al. 1991). Decreased uptake and/or altered pattern with FAAH inhibitor treatment observed by Glaser et al does not contradict the new notion because blockage of anandamide metabolism prevents the formation of labeled AA and thus prevented the formation of the pattern which was considered to represent metabolism of AA.
Figure 29. The idea that AA metabolism involving PLA₂ activity (instead of anandamide metabolism involving FAAH activity) determines the pattern formed with [¹⁴C-arach.]anandamide. This idea was suggested based on the observation that autoradiographs produced with [¹⁴C-arach.]anandamide and [¹⁴C]AA were similar, and the notion by Rapoport et al. that the heterogeneous pattern produced with [¹⁴C]AA is determined by the rate of incorporation and release of AA which is controlled by PLA₂ activity. This idea was not supported by autoradiographs produced in mice pretreated with the FAAH inhibitor URB597 and was rejected later on.
After concluding that anandamide labeled at the acyl part of the amide cannot be used to image FAAH activity because the corresponding acid itself forms a heterogeneous pattern, we were motivated to synthesize anandamide and other NAEs labeled at the ethanolamine moiety. \[^{14}\text{C-EA}]\text{anandamide and }[^{14}\text{C-EA}]\text{MEA did produced heterogeneous patterns that were very different from those produced with the control tracer }[^{14}\text{C}]\text{ethanolamine, which were almost homogeneous except for the ventricle areas. Our first thought was that }[^{14}\text{C-EA}]\text{anandamide can be used to image FAAH activity because labeled ethanolamine released from }[^{14}\text{C}]\text{ethanolamine distributes almost evenly in the brain so it makes little contribution to the formation of the pattern, therefore the heterogeneous pattern formed by }[^{14}\text{C-EA}]\text{anandamide is likely due to FAAH activity. We did not realize then that FAAH activity was not the only difference between injecting }[^{14}\text{C-EA}]\text{anandamide and }[^{14}\text{C}]\text{ethanolamine. However we did start to have doubts on the similarity of the patterns produced with all the fatty acids and NAEs we tested, we could not explain why the pattern from }[^{14}\text{C-EA}]\text{anandamide can reflect FAAH activity while a very similar pattern from }[^{14}\text{C-arach.}]\text{anandamide can reflect PLA}_{2} \text{ activity instead. We reasoned that there might be a universal mechanism responsible for the unique disposition of all the lipids substrates we used (likely due to their physical properties).}

With these doubts we continued to test the usefulness of \[^{14}\text{C-EA}]\text{anandamide as a probe for FAAH activity. To our surprise, FAAH inhibition by URB597 had little effect on the distribution pattern of radiolabel from either }[^{14}\text{C-EA}]\text{anandamide or }[^{14}\text{C-arach.}]\text{anandamide, indicating that the pattern is already formed before the action of FAAH when anandamide is just delivered to the brain, and further metabolism of anandamide by FAAH (or metabolism of labeled AA involving PLA}_{2} \text{ for }[^{14}\text{C-arach.}]\text{anandamide) contributes very little to the pattern (Figure 29B). Realizing that un-metabolized anandamide itself can form a heterogeneous pattern we started to believe that AA itself can behave similarly and form the similar pattern before it is incorporated into phospholipids (Figure 30B). Based on our results, the patterns seen with}
labeled AA and [\(^{14}\text{C}-\text{arach.}\)]anandamide are not likely to reflect \(\text{PLA}_2\) activity, since a similar pattern is obtained with \([^{14}\text{C-EA}]\)anandamide where no \(\text{PLA}_2\) is involved in incorporation of the label, and also obtained with un-metabolized \([^{14}\text{C-arach.}]\)anandamide where no labeled AA is released for incorporation and later release by \(\text{PLA}_2\) (figure 30A). At this point we could explain the similarity between the pattern from \([^{14}\text{C-EA}]\)anandamide and that from \([^{14}\text{C-arach.}]\)anandamide: they both largely reflect the initial distribution of anandamide upon delivery, but not the activity of either FAAH or \(\text{PLA}_2\).
Figure 30. Illustrations explaining why the patterns are not determined by FAAH or PLA₂. A) The similarity among patterns formed with [¹⁴C]AA, [¹⁴C-arach.]anandamide and un-metabolized [¹⁴C-arach.]anandamide (when FAAH is inhibited) indicated that metabolism through FAAH and PLA₂ has little contribution to the pattern, otherwise the pattern from [¹⁴C-arach.]anandamide without FAAH inhibition would be different (enhanced) from un-metabolized [¹⁴C-arach.]anandamide (when FAAH is inhibited). B) Our results suggested that AA also distributes heterogeneously upon delivery and further metabolism has little impact on the pattern that is already formed.
Although our radiochromatographic data show that the fatty acids and NAEs we studied do undergo metabolism in the brain, we have excluded the contribution of metabolism to the formation of the heterogeneous autoradiographic patterns. Now we have to determine the actual factors that determine the distribution of these labeled lipids immediately after they entered the brain. Our data strongly suggested that certain physical properties of these lipids make them concentrate in certain brain areas but not others, unlike the water soluble ethanolamine that distributes almost evenly in the brain. We first thought that lipophilicity might be one of the factors and the pattern could reflect a map of lipid/membrane richness in the brain. However, not all lipophilic compounds show the same pattern: Delta-8THC has a very similar clogP value to anandamide (about 7) but unlike anandamide it clears from the mouse brain fairly rapidly (whole brain concentration at 60 min less than 25% of that at 5 min) (Charalambous et al. 1991).

We revisited the steps taken for the intravenously injected tracer molecules to distribute in a certain way throughout the brain: first of all the tracer molecules have to cross the blood brain barrier to enter the brain. Molecules can cross the blood brain barrier in several ways. Passage of very small compounds depends on the tightness of junctions between endothelial cells of brain capillaries, which could be altered under certain conditions. Polar compounds which are naturally occurring, such as glucose or amino acids, cross endothelial cells using specific carriers. Most drugs active in the brain have Lipinski compliant or rule-of-five properties (Lipinski 2016) including moderate lipophilicity, and diffuse across the endothelial cell membranes but have sufficient water solubility to cross aqueous spaces including the endothelial cell cytoplasm. Fatty acid binding proteins (FABPs) are believed to be involved in intracellular trafficking (Smathers and Petersen 2011), and certain molecules can be pumped from the endothelial cell cytoplasm into the capillary blood by p-glycoprotein transporters, thus preventing access to the brain parenchyma (Subramanian et al. 2016). The situation can thus
be complex, but our working assumption is that N-acylethanolamides move from the blood to the brain by simple diffusion. The extent of transfer is relatively small, however, because of the high lipophilicity of the ethanolamides. Also, binding to blood proteins such as albumin or alpha-1 acid glycoprotein may limit the fraction of anandamide in the plasma that is free to diffuse. We assume that differences in net permeability of different tracers (such as AA and anandamide) will lead to differences in the total extraction of radioactivity by the brain, but will not contribute to the distribution pattern because there are no large regional differences in permeability.

Besides permeability, the other factor affecting delivery of the radiotracer from the blood to the brain is the amount of blood that passes certain brain regions in a given time, or rCBF. It is known that rCBF is heterogeneous across the brain, and obviously, the initial delivery of any radiotracer to the brain is heterogeneous based on rCBF (Kety 1994). After their initial delivery, the physical properties of the tracers determine whether the pattern of rCBF is preserved or quickly erased: the fatty acids and NAEs tend to dissolve readily in brain lipids and diffuse very slowly from where they are delivered. We speculate that this was the reason that we obtained similar heterogeneous patterns with all the lipids we examined. Furthermore, the tracer molecules will be metabolized to various compounds that carry the label and the actions of metabolism could also affect distribution of the label in various ways, depending on the physical properties of the radiolabel before and after the action. If the tracer or its metabolites have high affinity towards certain proteins, the label will slowly concentrate in the regions with higher amount of the specific proteins.

Taking all the factors together, we proposed a three-stage model to explain the different distribution patterns we obtained from labeled fatty acids, NAEs and ethanolamine (Figure 31). The model predicts that the autoradiographic pattern of a radiotracer is constantly changing and is determined by a combination of factors including initial delivery (determined by rCBF), diffusion rate of the compound across brain regions (stickiness to brain tissue, determined by its
physical properties such as size, structure and lipophilicity) and binding/metabolism of the compound. This model can explain all the data we obtained (Figure 24), and it can also be used to predict and explain the autoradiographic pattern produced with any labeled compound based on the information about its physical properties. A summary of the radioactive compounds used in this study and their physical properties are listed in Table 6.

<table>
<thead>
<tr>
<th>Tracer</th>
<th>MW</th>
<th>PSA</th>
<th>HBD</th>
<th>HBA</th>
<th>clogP</th>
<th>clogD</th>
<th>FRB</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachidonic acid</td>
<td>304</td>
<td>37</td>
<td>1</td>
<td>2</td>
<td>6.9</td>
<td>3.9</td>
<td>14</td>
</tr>
<tr>
<td>Anandamide</td>
<td>348</td>
<td>49</td>
<td>2</td>
<td>3</td>
<td>5.7</td>
<td>5.7</td>
<td>16</td>
</tr>
<tr>
<td>RTI-55</td>
<td>385</td>
<td>30</td>
<td>0</td>
<td>3</td>
<td>3.4</td>
<td>1.1</td>
<td>3</td>
</tr>
<tr>
<td>Iomazenil</td>
<td>411</td>
<td>64</td>
<td>0</td>
<td>6</td>
<td>1.7</td>
<td>1.9</td>
<td>3</td>
</tr>
<tr>
<td>Benzyl alcohol</td>
<td>108</td>
<td>20</td>
<td>1</td>
<td>1</td>
<td>1.0</td>
<td>1.2</td>
<td>1</td>
</tr>
<tr>
<td>Myristic acid</td>
<td>228</td>
<td>37</td>
<td>1</td>
<td>2</td>
<td>6.1</td>
<td>3.6</td>
<td>12</td>
</tr>
<tr>
<td>Myristoylethanolamine</td>
<td>272</td>
<td>49</td>
<td>2</td>
<td>3</td>
<td>5.0</td>
<td>5.0</td>
<td>14</td>
</tr>
<tr>
<td>Ethanolamine</td>
<td>61</td>
<td>46</td>
<td>3</td>
<td>2</td>
<td>-1.3</td>
<td>-3.4</td>
<td>1</td>
</tr>
<tr>
<td>Iodoantipyrine</td>
<td>314</td>
<td>24</td>
<td>0</td>
<td>3</td>
<td>1.1</td>
<td>1.5</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 6. Comparison of physical properties of radiolabels used in this study. MW, molecular weight (size); PSA, polar surface area; HBD, hydrogen bond donor; HBA, hydrogen bond acceptor; clogP, calculated logP (lipophilicity); clogD, calculated logD(at pH 7); FRB, free rotation bonds.
Figure 31. The three-stage model of how patterns are formed with radiotracers. Stage 1 is initial delivery of the tracer molecules from the blood to the brain, the pattern of initial delivery is determined by regional cerebral blood flow and it is heterogeneous. Stage 2 is diffusion—initially delivered tracer molecules will diffuse across brain regions and back to the blood, and the rate of diffusion is determined by their physical properties. For example, ethanolamine is a small water soluble molecule so it diffuses very quickly and the pattern of initial delivery is erased; in contrast, anandamide and AA can dissolve in membrane phospholipids and diffuse very slowly, as a result the pattern of initial delivery is maintained. Stage 3 is further changes of the pattern caused by metabolism and/or affinity/binding of the tracer and its metabolites to certain targets in the brain. Areas with higher metabolic rates will drive more tracers there through concentration gradients. The three stages overlap and the dominant factors that control the pattern depend on the properties of the tracers and time of imaging.
C. How to interpret changes in brain uptake and distribution of radiotracers

We now know that the autoradiographic pattern produced by a radiotracer is formed under the influence of a number of factors and is changing with time. At a certain time point, it is possible that one factor dominates and controls the total amount and/or distribution of radiolabel from a tracer. By picking the right time point, we could use the tracer to image the baseline level and regional differences of the particular factor and changes of this factor caused by a disease, a drug or a procedure. For example, to image the levels of dopamine transporters with labeled RTI-55 in humans, one needs to wait 14-24 hours after injection of the tracer for the striatal uptake to reach maximum level for stable measurement of dopamine transporters (Seibyl et al. 1998). In contrast, to image rCBF with [\(^{14}\)C]idoantipyrine in mice, one needs to remove the brains within one minute and freeze the brain immediately to prevent postmortem diffusion of the tracer (Jay et al. 1988, Greenberg et al. 1999). The usefulness of any tracer for imaging a target factor (such as level of a protein, enzyme activity or rCBF), depends on whether it is possible to pick a period when only the target factor controls most of the uptake or distribution pattern of the radiolabel. If there is no such period and a combination of factors including the target factor contribute to the uptake or distribution pattern at all times, it is difficult to quantify changes of the target factor based on changes in the obtained image, unless one can isolate and subtract the contribution of all the other interfering factors. This general issue has been extensively studied by researchers interested in quantitative positron emission tomographic (PET) imaging of receptor-binding and metabolically trapped radiotracers (Mori et al. 1990, Logan et al. 2011).

Autoradiography with labeled AA, DHA and palmitic acid (PA) has been studied since 1989 by Rapoport and his colleagues (DeGeorge et al. 1989, Rapoport et al. 1997, Rapoport 2001). They found that intravenously infused labeled fatty acids are incorporated into different brain phospholipids (sn-2 position of PI and PC for AA, sn-2 position of PE and PC for DHA and sn-1
position of PC and PE for PA) and the label distributes heterogeneously in the brain (Rapoport et al. 1997). Based on the observation that the incorporation of PA was not affected by cholinergic stimulation by arecoline or by acute changes in brain functional activity, while the incorporation of AA and DHA was influenced, they suggested that the changes in incorporation were due to changes in PLA$_2$ activity, with higher levels of incorporation indicating higher PLA$_2$ activity, because at equilibrium, the amount of incorporation at sn-2 is proportional to the amount of release from sn-2 by the action of PLA$_2$. Even though they accepted that increased rCBF is coupled to cholinergic stimulation and increased brain functional activity in these experiments, they have excluded the contribution of increased rCBF to the increased incorporation of AA and DHA, based on the evidence that the incorporation of PA is not affected by the changes in rCBF induced by cholinergic stimulation (DeGeorge et al. 1991), hypercapnia (Yamazaki et al. 1994) and unilateral brain damage (Yamazaki et al. 1994).

We now surmise that there are flaws in the argument that the changes seen in brain incorporation of AA and DHA are solely due to changes in PLA$_2$ activity. First of all, the evidence that disproves the contribution of changed rCBF is weak. $[^3]$HPA was the only tracer tested by the Rapoport group whose incorporation was not affected by changes in rCBF. Incorporation of PA into phospholipids differs from that of AA in that it occurs at sn-1 rather than at sn-2, the site of action of PLA$_2$. The degree of incorporation of fatty acids into phospholipids was different at baseline for $[^3]$H]PA (51.7%±0.6%), $[^14]$C]AA (72.9%±0.8%) and $[^14]$C]DHA (64.7%±0.9%) (Rapoport et al. 1997). It is possible that when rCBF is raised, this increases delivery to the same extend for all fatty acids, but that PA, which, unlike AA and DHA, is subject to mitochondrial beta-oxidation, also suffers greater loss due to metabolism. This would explain why at 15min after tracer injection (the only time point tested) there was no net increase in incorporation of PA. The brain may have greater flexibility for storing AA and DHA (essential fatty acids, relying on diet) for later use as precursors and therefore increased incorporation can
be stimulated by certain drugs. The brain may also have the mechanism to stabilize the storage for PA (a saturated, non-essential fatty acid). A group of factors including carrier proteins, transferases, phospholipases, beta oxidation and de novo lipogenesis may work together to buffer the increase or decrease in PA incorporation. Indeed, there is evidence showing that the PA content in the brain is regulated very differently from those of the essential polyunsaturated fatty acids. For instance, labeled PA in diet does not enter the brain of the developing rat and all brain PA is from de novo lipogenesis (Marbois et al. 1992). In contrast, n-3 and n-6 polyunsaturated fatty acids are sourced from the diet and have to enter the brain via highly specific mechanisms (Edmond et al. 1998, Edmond 2001). For these reasons, it is inappropriate to conclude that increased rCBF is unlikely to account for increases in plasma-derived [14C]AAand [14C]DHA incorporation based on the observation that increased rCBF is not coupled to increased [3H]PA incorporation.

Actually, the changes in incorporation of [14C]AA and [14C]DHA induced by drugs or procedures tested by Rapoport and his colleagues can all be more simply explained by our three-stage model with rCBF playing the most essential role at 15 minutes for these labeled fatty acids. The changes of rCBF and the changes of the incorporation coefficient are positively correlated and cannot be separated: arecoline increases rCBF and increases incorporation (DeGeorge et al. 1991); pentobarbital decreases rCBF and decreases incorporation (Yamazaki et al. 1994), brain functional activity (glucose metabolism) correlates with rCBF and also correlates with the incorporation coefficient (Rapoport et al. 1997). It might be coincidence that rCBF happens to correlate with PLA₂ activity in these experiments, but it is not convincing to assume that PLA₂ is the only or most important factor that controls brain uptake and distribution of [14C]AA and [14C]DHA. As discussed before, the total amount and distribution of radiolabel in the brain is determined by a combination of factors including rCBF, diffusion rate and metabolism/binding of the label and its metabolites. The impact of a particular factor depends on physical properties of
the tracer as well as the time point, and only when the target factor controls most of the uptake or distribution pattern can one accurately measure the target factor. For imaging with labeled fatty acids at 15 minutes, the impact of rCBF clearly dominates (evidenced by similar patterns produced with labeled fatty acids, NAEs and blood flow tracers), and it is considerably more difficult to measure changes due to contributions of another factor. An observed increase in incorporation coefficient induced by a drug or procedure is the summed up effects of all the three stages, and if rCBF is increased without changes of other factors, it is obvious that the increase in rCBF accounts for the increase in incorporation. However, for the drugs and procedures tested by Rapoport and this colleagues, the only known factor that is changed by the drug is rCBF, with changes in diffusion rate and metabolism/binding possible, but largely unknown. Diffusion rate could be altered if the structure of membrane is altered or if altered metabolism produced metabolites with different diffusion rates. The possible changes in factors associated with metabolism/binding could be even more complicated, and PLA₂ is only one of the factors that could be changed. It is also possible that PLA₂ activity is decreased but not increased by the tested drug or procedure, but the increase in rCBF has a larger effect and the overall observed effect is increased incorporation. It is possible to quantify and compare a drug induced change of rCBF and change of incorporation coefficient of labeled AA or DHA, and the difference between the two provides the information that other factors besides rCBF might also be altered by the drug, but it is difficult to pick one from the many other factors and predict if it is altered in any way.

A review paper by Rapoport’s group in which they presented a mathematical model of their studies with [³H]palmitic acid, [¹⁴C]arachidonic acid and [¹⁴C]docosahexadecanoic acid argued that net fatty acid incorporation into brain is independent of rCBF (Robinson et al. 1992). However, they acknowledged that their argument was based on several assumptions, one of which was that there is no recruitment of capillaries associated with increases in flow. Capillary
recruitment would increase brain blood volume, and they show in their review that increased brain capillary blood volume would lead to increased fatty acid uptake. In reality, rCBF is often increased due to vasodilatation and increased brain blood volume. For example, the mechanism that arecoline increases rCBF involves cholinergic dilation of cerebral blood vessels via activation of central muscarinic receptors (Uchida et al. 2000, Patil and Stearns 2002), especially M5 receptors since the effect is abolished in M5 knockout mice (Yamada et al. 2001). It is also possible that arecoline increases the delivery of radiotracer from blood to the brain via altering the structure of the blood brain barrier, making it more permeable to the tracers. If that is the case we would get smaller increases for radiotracers that are already very permeable. However, we observed similar increases for all types of radiotracers, with %IA/g ranging from 0.5 to more than 10, indicating that changes in blood brain barrier are unlikely to account for increased brain uptake. In conclusion, the observed increase in brain uptake of radiotracers after arecoline treatment is largely due to increased rCBF through dilation of cerebral blood vessels caused by activation of central muscarinic receptors.

It is not clear why the results of the present study with [¹⁴C-arachidonoyl]anandamide are not in agreement with those obtained earlier using tritiated anandamide and arachidonic acid (Glaser et al. 2006). There were a number of technical differences: 1) we used Swiss-Webster mice rather than C57BL/6 mice; 2) we used ¹⁴C rather than ³H, and we imaged using a phosphor-imager rather than a beta-imager; 3) we cut sections of unfixed brain using a vibratome, whereas Glaser et al. used a rapid fixation technique followed by microtome sectioning; 4) we used tracers of higher specific radioactivity; 5) we used pretreatment with FAAH inhibitor rather than (intravenous) co-injection with tracer; 6) we did not use FAAH knockout mice in our experiments. Taking these six points in order, some possible explanations for discrepant results may involve:
1) **Mouse strain.** A recent study (Seeger and Murphy 2016) demonstrated differences in tissue accumulation of fatty acids and their partitioning into lipid classes between the Swiss-Webster and C57BL/6 strains. In constant infusion experiments using carbon-14 labeled palmitic, arachidonic and docosahexaenoic acids, the area under the plasma concentration-time curve for arachidonic acid was significantly lower for C57BL/6 mice. The curves for palmitic and docosahexaenoic acid, however, were not significantly different. Additionally, incorporation of arachidonic acid but not the other fatty acids into diacylglycerols was significantly lower in C57BL/6 mice. More differences between the two mouse strains were noted for liver and especially heart, than for brain, but it is clear that conclusions drawn from one strain of mice do not necessarily apply to another.

2) **Tritium vs carbon-14.** Tritium is lost during oxidation of C-atoms bearing tritium, as $^3$H-atoms abstracted by enzymatic cofactors then appear as $^3$H$_2$O water. Thus regions found to have relatively lower radioactivity after administration of tritium radiotracers may be regions where tritium has been lost in the absence of loss of the carbon skeleton. The tritium atoms on commercially available [${}^3$H]arachidonic acid are located at the four double bonds. Some fraction of this label must be lost from [${}^3$H]arachidonic acid in vivo, since an important function of arachidonic acid is to serve as the precursor of prostaglandins and related signaling molecules via cyclooxygenase-catalyzed oxidations and rearrangements (Schneider et al. 2000, McGinley and van der Donk 2003). Additionally, anandamide is a substrate of cyclooxygenases, forming eicosanoids that retain the ethanolamide moiety (Fowler et al. 1997, Hermanson et al. 2014). Thus it is possible that brain regional differences in metabolism of [${}^3$H]anandamide, other than hydrolysis of the amide bond by FAAH could contribute to regional heterogeneity in autoradiograms.

3) **Preparation of sections.** In the study of Glaser et al. (2006), brains were transferred into 2% paraformaldehyde plus 2%glutaraldehyde in phosphate buffer (PB) on ice for 1 h. The brains
were then washed three times with iced PB, and cryoprotected overnight at 4°C in 30%sucrose in PB. Serial cryosections (40 microns) were made and promptly fixed for 5 min in 2% OsO₄ in PB. The slides were rinsed in distilled water and desiccated for at least 3 days.” It seems possible that the fixation process may have removed unmetabolized anandamide from the surface of the sections, but left in place labeled arachidonic acid (derived from anandamide) incorporated into phospholipids. The beta particles from tritium are very weak, and their range in tissue is <1 micron, so loss of unfixed anandamide from the top layer of cells would be sufficient to alter the appearance of autoradiograms. Also, it seems possible that physical changes in the surface associated with the fixation process might alter the efficiency with which the weak beta particles leave the section, possibly on a regionally selective basis.

4) **Administration of serine hydrolase inhibitors.** We pretreated animals by intraperitoneal injection of the selective FAAH inhibitor, URB597, 3h before administration of radiotracer. Thus immediate effects of administration of the FAAH-blocking drug on initial extraction of radiotracer by the brain should have been minimal. In contrast, Glaser et al. (2006), working before the wide availability of specific inhibitors of FAAH, administered the relatively non-specific inhibitors CAY10435 and MAFP as mixtures with the radiotracer (plus non-radioactive anandamide—see point 5, below). They may have reduced extraction of the tracer during passage through the brain vasculature, rather than reducing the degree of hydrolysis of anandamide by FAAH, by acute pharmacological effects.

5) **Specific radioactivity of labeled anandamide.** We, unlike Glaser et al. (2006), did not dilute the radioactive anandamide with non-radioactive (“carrier”) anandamide. Our decision on this point was motivated by the desire to avoid possible pharmacodynamics effects of carrier anandamide, in other words, to preserve the tracer principle (Hevesy 1923). In this we followed the usual practice in nuclear medicine, of using “no carrier added” radioactive probes, since ultimately one would want to evaluate FAAH activity in humans, and the practice avoids the
possibility of physiological or toxicological actions, as well as fulfilling a requirement for tracer kinetic modeling. A 4 microcurie injection of \[^{14}\text{C}]\text{anandamide}\) of specific activity 50 mCi/mmol corresponds to a concentration of 400 nM in the injection bolus, while injection of \[^{3}\text{H}]\text{anandamide}\) with a loading dose of 10 mg/Kg body weight in a 25g mouse (i.e. 250 microgram) corresponds to a concentration of 5,000 nM in the injection bolus. Thus Glaser et al. (2006) administered about 12 times the amount of anandamide as in the present study. Glaser and Kazochka (2010) used a loading dose of 1 mg/Kg, so the total dose of anandamide was very similar to ours.

Since we measured a brain uptake of \[^{14}\text{C}]\text{anandamide}\) of about 2% injected radioactivity per gram of brain, one can estimate a brain concentration in our work of about 1.6 nmol/g (about 20 nmol/g for Glaser et al. 2006). Most measurements of the average concentration of anandamide in the brains of rodents are in the range 0.1-1 nmol/g (Fegley et al. 2005, Richardson et al. 2007, Muccioli and Stella 2008, Chen et al. 2009), so that neither our autoradiographic studies nor those of Glaser et al. (2006) corresponded to true tracer conditions. A possible explanation for the discrepancy between our results and those of Glaser et al. (2006) is the existence of a binding protein that is saturated under the conditions of Glaser et al. (2006), so that most of the labeled anandamide is available for hydrolysis, but that binds essentially all the labeled anandamide under our conditions, so that there was no “free” anandamide. If so, the concentration (Bmax) of this hypothetical binding site would have to be well above the 1.6 nmol/g calculated for our brain uptake of \[^{14}\text{C}]\text{anandamide}\), and the dissociation constant (Km) well below this value (or 1600 nM). Fatty acid binding proteins would appear to be a possibility, but do not seem to meet these criteria for Bmax and Km (Matsumata et al. 2016). We consider it more likely that the “stickiness” of \[^{14}\text{C}]\text{anandamide}\) reflects its dissolution in the abundant structurally similar lipid pools of the brain, and its very poor solubility in aqueous compartments.
6) FAAH knockout mice. An important piece of evidence in support of the conclusion of Glaser et al. (2006) that regional accumulation of [$^3$H]anandamide indicated relative FAAH activity was that autoradiograms from animals in which the FAAH gene was deleted showed less regional heterogeneity. A possible explanation for these observations is related to point #2, above. Cyclooxygenase activity is upregulated in animals lacking FAAH (Glaser and Kaczocha 2010) and so the loss of tritium by oxidation of the arachidonic acid moiety might be responsible for reduced autoradiographic contrast.
D. Limitations of our study

The use of mice rather than rats in radiotracer validation studies is convenient for several reasons including the lower cost of purchasing and maintaining animals, the smaller amounts of radiotracers and drugs required, and the greater availability of genetically modified animals. However, a disadvantage of mice for radiotracer studies is that their smaller size makes it difficult to obtain samples of arterial blood during the radiotracer uptake period, and so to obtain quantitative measures of passage of tracers from blood to brain. Also, the volume of vehicle used in intravenous injections to mice is typically greater, relative to the total blood volume of the animal, than used in experiments with rats.
V. Summary and conclusions

Our initial concept for an in vivo radiotracer for FAAH was that labeled AA released from anandamide by FAAH would be rapidly incorporated into phospholipids, allowing the brain-regional disposition of label to indicate regional values of FAAH activity. An ex vivo radiotracer based on this approach might provide complementary information to that provided by radiolabeled high affinity or irreversible inhibitors of FAAH, which indicate brain regional concentrations of the FAAH protein. Although previous results using $[^3]$H-anandamide appeared to be consistent with our notion, the present data using $[^{14}]$C-anandamide do not support our expectation. One piece of evidence that FAAH activity is not a major determinant of label disposition is that the pattern seen with $[^{14}]$C-anandamide is almost identical to that seen with AA. Another is that the pattern seen with $[^{14}]$C-EA-anandamide, which lacks a labeled AA moiety for incorporation into phospholipids, is identical or very similar to that seen with $[^{14}]$C-arach. Anandamide. A third is that the selective FAAH inhibitor URB597 cannot block the pattern produced with anandamide labeled at either part, even though it can successfully block anandamide metabolism. Therefore, brain regional or total FAAH activity cannot be imaged using labeled anandamide no matter where the label is.

The finding of the similar distribution patterns for fatty acids and NAEs, and the contrasting homogeneous pattern for ethanolamine suggests that physical properties of the radiolabels determine how the patterns are formed. We examined previous results and developed a three-stage model scrutinizing the elements that can affect where the label go from the blood to the brain (Figure 31 and 32), and suggested that the constantly changing pattern is under the control of a combination of factors including rCBF, permeability, diffusion rate and metabolism/binding. For AA or anandamide, the initial pattern reflecting regional differences in delivery (rCBF) is long-lasting because the lipophilic compounds dissolve in brain lipids, with very little back-diffusion to the blood. Further experiments using blood flow tracers and
arecoline, a cholinergic stimulant that increases rCBF, confirmed our idea that early patterns of lipophilic radiotracers reflect delivery and can be affected by drugs that alter rCBF. In conclusion, rCBF is the most important determinant of the heterogeneous autoradiographic patterns produced with labeled anandamide and AA.

More broadly, this work has shown that in order to image a target factor (such as level of a protein, enzyme activity or rCBF), one needs to develop a tracer whose distribution is stable at a period when only the target factor controls most of the uptake or distribution pattern. From this study we can conclude that radiolabeled anandamide is unsuitable as an in vivo tracer for FAAH, because there is no such period when activity of FAAH controls the pattern whereas the impact of rCBF is strong and long-lasting. On the other hand, our results suggest that it may be possible to develop better lipid-based rCBF tracers, considering that currently available blood flow tracers have relatively high diffusion rates and require immediate freezing and cryosectioning techniques.
Figure 32. All possible factors in the three-stage model need to be considered in development of the two typical types of radiotracers. The “target” factor for protein-binding tracers and metabolically trapped tracers is at stage 3 of the model. The effects of rCBF (stage 1) and diffusion rate (stage 2) on the distribution of the candidate radiotracers can be predicted based on their physical properties. The accuracy of the tracer in measurement of the “target” factor depends on whether it is possible to choose a period when the “target” factor in stage 3 plays the predominant role in controlling the distribution of the radiolabel.

Besides blood flow tracers, there are typically two types of radiotracers that can be reliable measures of target “activity” or binding: protein-binding tracers and metabolically trapped tracers (Figure 31). For development of radiotracers that simply bind to the target (usually labeled irreversible inhibitors such as $[^{11}\text{C}]\text{CURB}$), one needs to keep in mind that early distribution is still affected by rCBF and diffusion rate, and needs to choose a period of time when the effect of rCBF is past while the effect of affinity is dominating. Development of radiotracers that are metabolically trapped is harder to achieve because the tracer must be metabolized by the target enzyme, and immediately trapped at the location of action, which does not always happen. A good example is $[^{18}\text{F}]\text{2-FDG}$ which has fast diffusion rate to erase the pattern of rCBF quickly, and does not have high affinity to any target, nor does it have complicated metabolism.
pathways. Although anandamide and AA are metabolically trapped, their slow diffusion rates make them trapped at where they are delivered, representing a map of rCBF rather than metabolic enzyme activity, and the complexity of their metabolism pathways makes it even harder to image one particular enzyme.

In conclusion, for future development of protein-binding and metabolically trapped radiotracers, all the possible factors discussed above must be considered in order to determine if it is possible to choose a period when the target factor plays the predominant role in controlling the distribution of the radiolabel.
VI. References


