DEVELOPMENT OF RADIOLIGANDS FOR IMAGING PHOSPHODIESTERASE-4 IN THE HUMAN BRAIN USING POSITRON EMISSION TOMOGRAPHY

by

Celia Maria Lourenco

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy in the Graduate Department of Pharmacology University of Toronto

© Copyright by Celia Maria Lourenco (2000)
The author has granted a non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of this thesis in microform, paper or electronic formats.

L'auteur a accordé une licence non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de cette thèse sous la forme de microfiche/film, de reproduction sur papier ou sur format électronique.

The author retains ownership of the copyright in this thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without the author's permission.

L’auteur conserve la propriété du droit d'auteur qui protège cette thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

0-612-53822-2
Phosphodiesterase-4 (PDE4) is one of the main enzymes responsible for metabolizing cyclic
3',5'-adenosine monophosphate (cAMP) following stimulation of at least β-adrenergic, A2-
adenosine, and H1-histamine receptors in the brain. PDE4 expression is regulated in concert
with cAMP-mediated signaling in the short-term via phosphorylation (within 15 min) and in the
long term via de novo protein synthesis (within hours to days). Disturbances in cAMP-mediated
signaling may occur in depression, and the activation of components in this pathway, including
PDE4, is a potential mechanism of action of antidepressants. We developed a radioligand with
the future aim of imaging PDE4 in the brain of living depressed patients, using positron
emission tomography (PET). The selective PDE4 inhibitor R-rolipram was successfully labeled
with carbon-11 and displayed the best in vivo pharmacological profile in rats as compared to the
racemic form or to another PDE4 inhibitor, [11C]Ro 20-1724. R-[11C]Rolipram showed higher
brain uptake in PDE4-rich areas (e.g., frontal cortex) over the brain stem (poor in PDE4), and
selectivity for PDE4 over PDE1, another abundant PDE in the brain. R-[11C]Rolipram yielded a
favorable metabolism profile, with no radioactive metabolites crossing the blood-brain barrier,
and was considered safe for injection in humans. Acute and chronic challenges with drugs
expected to increase PDE4 regulation in neurotransmitter systems linked to PDE4, including the
noradrenaline reuptake inhibitor desipramine (DMI) and the serotonin reuptake inhibitor
fluoxetine, augmented R-[11C]Rolipram binding in rat brain. DMI and fluoxetine increased R-
[11C]Rolipram specific binding by 10-41% across brain regions. In vitro validation with R/S-
[3H]Rolipram supported these findings, since acute and chronic DMI, and acute fluoxetine,
significantly increased the Bmax in rat frontal cortex by 27-54%. In contrast, activation of
dopamine-D\(_1\) receptors (likely linked to PDE1) by SKF 81297 had no significant effect on \(R\)\([-^{11}\text{C}]\)rolipram brain retention, showing that the radioligand discriminates among different systems in detecting the regulation of PDE4. \(R\)\([-^{11}\text{C}]\)Rolipram is unlike \([^{18}\text{F}]\)fluorodeoxyglucose or \([^{15}\text{O}]\)water, which monitor variations in neuronal activity regardless of the systems involved. PET imaging with \(R\)\([-^{11}\text{C}]\)rolipram in normal human volunteers showed a high signal in gray matter areas of the brain. These studies demonstrated that \(R\)\([-^{11}\text{C}]\)rolipram has potential for imaging PDE4 in the living human brain using PET.
ACKNOWLEDGEMENTS

Several people to whom I wish to express my sincerest gratitude have contributed to the work in this thesis. Beginning with my supervisor, Jean, whose endless support, patience, and guidance have allowed me to complete the most intellectually challenging achievement of my life, this thesis. Followed by my supervisory committee members, Dr. Jerry Warsh, Dr. Peter Li, and Dr. Sylvain Houle, as well as members of the PET Centre, including Dr. Alan Wilson and Dr. Shitij Kapur, whose advice was instrumental to this work. Several other members of the PET Centre, as well as countless numbers of graduate, undergraduate, and summer students also contributed significantly to this research. Steve, Jiny, and Armando are commended for their efficiency in synthesizing the radioligands. Doug and Kevin provided the best help one can get in the in vivo studies of this research. Corey, Erin, Eric, Rob, Vito, Miran, Chris, Sam, Nicole, Lily, Jespal, and Cesar also helped with the in vivo studies, and Carmen was my savior for the in vitro work. Members of Dr. Warsh's lab, including Kin Po and Marty Green, must also be acknowledged for their patience and guidance in the in vitro experiments. I also thank Dr. Niznik for his suggestions and kindness in letting me use the Skatron.

I must also thank two extremely important people in my life, my mother Maria and my mother-in-law Lidia, without whom I could not finish this work. I thank them for their endless support in babysitting my son and for encouraging me to finish the arduous task of writing this thesis.

I dedicate this thesis to the two most important people in my life, my husband Pedro and my son Matthew. Pedro, you have been my number one supporter throughout the last five years and without your patience, encouragement, and faith in me, I would not have been able to achieve this degree. Matthew, without you in my life there would be no sunshine. I love you both with all my heart.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>ii</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>iv</td>
</tr>
<tr>
<td>TABLE OF CONTENTS</td>
<td>v</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xi</td>
</tr>
<tr>
<td>LIST OF SCHEMES</td>
<td>xiv</td>
</tr>
<tr>
<td>ABBREVIATIONS</td>
<td>xv</td>
</tr>
<tr>
<td>CHAPTER 1.0: INTRODUCTION</td>
<td></td>
</tr>
<tr>
<td>1.1 General introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.2 Characteristics of PDE4</td>
<td>2</td>
</tr>
<tr>
<td>1.2.1 General PDE4 function and family subtypes</td>
<td>4</td>
</tr>
<tr>
<td>1.2.2 PDE4 distribution</td>
<td>6</td>
</tr>
<tr>
<td>1.2.3 PDE4 structure and regulation</td>
<td>7</td>
</tr>
<tr>
<td>1.2.3.1 How PDE4 structure affects regulation</td>
<td>7</td>
</tr>
<tr>
<td>1.2.3.2 Short-term regulation</td>
<td>9</td>
</tr>
<tr>
<td>1.2.3.3 Long-term regulation</td>
<td>10</td>
</tr>
<tr>
<td>1.2.3.4 How do the PDE4 subtypes interact to control cAMP levels?</td>
<td>11</td>
</tr>
<tr>
<td>1.2.4 Rolipram binding sites of PDE4 and other PDE4 inhibitors</td>
<td>12</td>
</tr>
<tr>
<td>1.3 Relevance to neuropsychiatric disorders</td>
<td>15</td>
</tr>
<tr>
<td>1.3.1 Current hypothesis in depression</td>
<td>15</td>
</tr>
<tr>
<td>1.3.1.1 Classic hypothesis: disruption in monoaminergic systems</td>
<td>15</td>
</tr>
<tr>
<td>1.3.1.2 The effects of stress</td>
<td>16</td>
</tr>
<tr>
<td>1.3.1.3 Mechanism of action of antidepressants</td>
<td>18</td>
</tr>
<tr>
<td>1.3.2 PDE4 link with Major Depressive Disorder</td>
<td>21</td>
</tr>
<tr>
<td>1.4 Positron emission tomography and second messenger systems</td>
<td>23</td>
</tr>
<tr>
<td>1.4.1 Positron emission tomography</td>
<td>23</td>
</tr>
<tr>
<td>1.4.2 PET imaging in depression</td>
<td>26</td>
</tr>
</tbody>
</table>
CHAPTER 2.0: MATERIALS AND METHODS


2.1.1 General

2.1.2 Chemistry

2.1.3 Radiochemistry

2.2 Animals

2.3 Biodistribution of the radioligands in rats

2.4 Competition studies

2.4.1 Materials

2.4.2 Blocking with PDE4-selective inhibitors

2.4.3 Competition with other drugs

2.4.4 Statistics

2.5 Metabolite analysis of R-[11C]rolipram

2.5.1 Solid phase extraction and thin layer chromatography analysis of metabolites in rat plasma

2.5.2 TLC analysis of metabolites in rat brain

2.6 Dosimetry and whole body biodistribution studies of R-[11C]rolipram

2.6.1 Measurement of whole body distribution of activity

2.6.2 Dosimetry calculations

2.7 Regulation of R-[11C]rolipram binding in rat brain following diverse in vivo drug treatments

2.7.1 Materials

2.7.2 Acute treatments

2.7.3 Chronic treatments

2.7.4 Determination of in vivo R-[11C]rolipram specific binding following
selected acute and chronic drug challenges

2.7.5 Plasma levels of $R$-$[^{14}C]$rolipram following selected acute treatments

2.7.6 Validation of selected in vivo results by measurement of $K_i$ and $B_{max}$ with $R/S$-$[^{3}H]$rolipram

2.7.7 Statistics

2.8 PET imaging of $R$-$[^{11}C]$rolipram in normal human volunteers

2.8.1 PET imaging

2.8.2 Image analysis

2.8.3 Analysis of metabolites in human venous and arterial plasma

CHAPTER 3.0: RESULTS

3.1 Synthesis and purification of precursors

3.2 Radiochemistry

3.3 Biodistribution studies in rats

3.3.1 Time course of $[^{14}C]$Ro 20-1724 and $R/S$-$[^{14}C]$rolipram

3.3.2 Time course of $R$-$[^{14}C]$rolipram and $S$-$[^{14}C]$rolipram

3.4 Competition studies

3.4.1 Competition of $[^{14}C]$Ro 20-1724, $R/S$-, and $R$-$[^{14}C]$rolipram with PDE4-selective inhibitors

3.4.2 Dose-dependent effect of $R$- and $S$-rolipram on $R$-$[^{14}C]$rolipram uptake

3.4.3 Competition of $R/S$- and $R$-$[^{11}C]$rolipram with a PDE1 inhibitor and a selective noradrenergic reuptake blocker

3.4.4 Competition of $S$-$[^{11}C]$rolipram uptake by PDE4 inhibitors and other drugs

3.5 Metabolite analysis of $R$-$[^{14}C]$rolipram in rat plasma and brain

3.6 Dosimetry and whole body biodistribution studies of $R$-$[^{14}C]$rolipram

3.7 Regulation of the in vivo binding of $R$-$[^{14}C]$rolipram in rat brain following diverse acute and chronic drug treatments

3.7.1 Effect of acute treatments

3.7.1.1 Dose- and time-dependent effect of the AC activator forskolin

3.7.1.2 Dose- and time-dependent effect of the noradrenergic agent DMI

3.7.1.3 Time-dependent effect of the $\alpha_2$-adrenergic antagonist yohimbine

3.7.1.4 Effect of other noradrenergic drugs: clonidine, tranylcyromine,
and propranolol

3.7.1.5 Modulations of the serotonergic system
3.7.1.6 Effect of A2-adenosine, H2-histamine, and D1-dopamine receptors
3.7.1.7 Inhibition of PKA-mediated phosphorylation
3.7.1.8 Inhibition of protein synthesis by cycloheximide

3.7.2 Determination of in vivo R-[14C]rolipram specific binding following selected acute drug treatments
3.7.3 Effect of selected acute drug treatments on R-[14C]rolipram levels in rat plasma
3.7.4 Effect of chronic treatments
3.7.4.1 Modulation of β-adrenergic receptors
3.7.4.2 Effect of chronic antidepressant administration
3.7.4.3 Determination of in vivo R-[14C]rolipram specific binding following chronic DMI and fluoxetine
3.7.4.4 Effect of vesicle depletion
3.7.4.5 Effect of lesioning noradrenergic neurons with DSP-4

3.7.5 In vitro validation with R/S-[3H]rolipram
3.7.5.1 Effect of acute DMI and fluoxetine
3.7.5.2 Effect of chronic DMI and fluoxetine

3.8 PET imaging of R-[14C]rolipram in normal human volunteers
3.8.1 Regional distribution of R-[14C]rolipram in human brain
3.8.2 R-[14C]Rolipram in human arterial and venous plasma: metabolite analysis

CHAPTER 4.0: DISCUSSION

4.1 Radiosynthesis of the radioligands
4.2 R-[14C]Rolipram in vivo binding and selectivity for PDE4
4.3 S-[14C]Rolipram in vivo binding in rat brain
4.4 R-[14C]Rolipram displayed favorable metabolism and dosimetry characteristics
4.5 Modulation of R-[14C]rolipram binding following different treatments affecting cAMP-mediated signaling in the brain: sensitivity to PDE4 regulation
4.5.1 Effect of acute forskolin treatment
4.5.2 Effect of acute and chronic modulation of α2- and β-adrenergic receptors
4.5.2.1 α₂-Adrenergic modulators
4.5.2.2 β-Adrenergic modulation
4.5.3 Effect of drugs acting acutely and chronically on serotonergic receptors
4.5.3.1 5-HT₁A receptors
4.5.3.2 5-HT₂₅ receptors
4.5.4 Effect of agents acting on adenosine, histamine, and dopamine neurotransmitter receptors
4.5.4.1 Adenosine and histamine neurotransmission
4.5.4.2 Dopamine D₁ receptors
4.5.5 Effect of in vivo PKA and protein synthesis inhibition
4.5.6 Effect of monoamine vesicle depletion and noradrenergic lesioning
4.5.6.1 Chronic reserpine
4.5.6.2 Effect of DSP-4
4.5.7 Effect of acute and chronic antidepressant administration
4.5.7.1 Acute monoamine oxidase inhibition
4.5.7.2 Chronic monoamine oxidase inhibition
4.5.7.3 Effect of acute DMI treatment
4.5.7.4 Effect of acute fluoxetine challenge
4.5.7.5 Chronic DMI and fluoxetine
4.5.8 DMI results correlate with antidepressant treatment model
4.5.9 What about the effects of acute and chronic fluoxetine on cAMP-mediated signaling as a model of its mechanism of action?
4.5.10 How does R-[¹⁴C]rolipram binding in vivo compare with measurements using in vitro techniques?

4.6 Human PET imaging

CHAPTER 5.0: CONCLUSION

5.1 Conclusion
5.2 Future direction
5.3 Statement of research significant

REFERENCES
<table>
<thead>
<tr>
<th>LIST OF TABLES</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>I The phosphodiesterase superfamily</td>
<td>3</td>
</tr>
<tr>
<td>II Human volunteer age, gender, and study completed</td>
<td>51</td>
</tr>
<tr>
<td>III Ratio of the distribution of ( R-[\text{\textsuperscript{11}}\text{C}])rolipram to the other radioligands</td>
<td>66</td>
</tr>
<tr>
<td>IV Percentage of plasma radiolabeled metabolites and unchanged ( R-[\text{\textsuperscript{11}}\text{C}])rolipram in rats</td>
<td>80</td>
</tr>
<tr>
<td>V Whole body biodistribution of ( R-[\text{\textsuperscript{11}}\text{C}])rolipram in rats</td>
<td>82</td>
</tr>
<tr>
<td>VI Cumulative absorbed dose estimates for administration of ( R-[\text{\textsuperscript{11}}\text{C}])rolipram to humans</td>
<td>84</td>
</tr>
<tr>
<td>VII Percentage change in specific binding of ( R-[\text{\textsuperscript{11}}\text{C}])rolipram in rat brain regions following different drug treatments</td>
<td>100</td>
</tr>
<tr>
<td>VIII Percentage change in specific binding of ( R-[\text{\textsuperscript{11}}\text{C}])rolipram in the lung and heart following different drug treatments in rats</td>
<td>102</td>
</tr>
<tr>
<td>IX Percentage change in specific binding of ( R-[\text{\textsuperscript{11}}\text{C}])rolipram in the lung and heart following chronic treatment with DMI and fluoxetine</td>
<td>111</td>
</tr>
<tr>
<td>X In vitro binding affinity values of ( R/S-[\text{\textsuperscript{3}}\text{H}])rolipram in supernatant and pellet following acute fluoxetine or DMI treatment in vivo</td>
<td>116</td>
</tr>
<tr>
<td>XI ( R/S-[\text{\textsuperscript{3}}\text{H}])Rolipram ( B_{\text{max}} ) following acute treatment with DMI and fluoxetine as compared to controls</td>
<td>116</td>
</tr>
<tr>
<td>XII In vitro binding affinity values of ( R/S-[\text{\textsuperscript{3}}\text{H}])rolipram in supernatant and pellet following chronic fluoxetine or DMI treatment in vivo</td>
<td>117</td>
</tr>
<tr>
<td>XIII ( R/S-[\text{\textsuperscript{3}}\text{H}])Rolipram ( B_{\text{max}} ) following chronic treatment with DMI and fluoxetine as compared to controls</td>
<td>117</td>
</tr>
<tr>
<td>XIV Percent change in ( R-[\text{\textsuperscript{11}}\text{C}])rolipram retention in rat frontal cortex following different acute drug treatments as compared to control</td>
<td>162</td>
</tr>
<tr>
<td>XV Percent change in ( R-[\text{\textsuperscript{11}}\text{C}])rolipram retention in rat frontal cortex following different chronic drug treatments as compared to controls</td>
<td>163</td>
</tr>
</tbody>
</table>
**LIST OF FIGURES**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Schematic of PDE4 function in neurotransmitter systems of the brain</td>
<td>5</td>
</tr>
<tr>
<td>2.</td>
<td>Mechanisms of PDE4 regulation</td>
<td>8</td>
</tr>
<tr>
<td>3.</td>
<td>Model of the mechanism of action of antidepressants</td>
<td>19</td>
</tr>
<tr>
<td>4.</td>
<td>The decay of the $^{11}$C nucleus</td>
<td>24</td>
</tr>
<tr>
<td>5.</td>
<td>Ring of scintillation detectors in a PET scanner</td>
<td>25</td>
</tr>
<tr>
<td>6.</td>
<td>Chemical structures of Ro 20-1724 and rolipram</td>
<td>29</td>
</tr>
<tr>
<td>7.</td>
<td>HPLC chromatogram of $[^{11}]$C]Ro 20-1724</td>
<td>57</td>
</tr>
<tr>
<td>8.</td>
<td>HPLC chromatogram of $[^{11}]$C]rolipram</td>
<td>58</td>
</tr>
<tr>
<td>9.</td>
<td>Time course of $[^{11}]$C]Ro 20-1724 and $R/S-[^{11}]$C]rolipram in rat brain regions</td>
<td>60</td>
</tr>
<tr>
<td>10.</td>
<td>Time course of $[^{11}]$C]Ro 20-1724 and $R/S-[^{11}]$C]rolipram in selected rat organs</td>
<td>61</td>
</tr>
<tr>
<td>11.</td>
<td>Time course of $R-[^{11}]$C]rolipram in rat brain and periphery</td>
<td>63</td>
</tr>
<tr>
<td>12.</td>
<td>Comparison of regional brain $R-[^{11}]$C]rolipram uptake with $R/S-[^{3}]$H]rolipram binding in rats</td>
<td>64</td>
</tr>
<tr>
<td>13.</td>
<td>Time course of $S-[^{11}]$C]rolipram in rat brain and periphery</td>
<td>65</td>
</tr>
<tr>
<td>15.</td>
<td>Competition of rolipram and Ro 20-1724 with $[^{11}]$C]Ro 20-1724 and $R/S-[^{11}]$C]rolipram uptake in rat periphery</td>
<td>69</td>
</tr>
<tr>
<td>16.</td>
<td>Competition of rolipram and Ro 20-1724 with $R-[^{11}]$C]rolipram uptake in rat brain regions</td>
<td>70</td>
</tr>
<tr>
<td>17.</td>
<td>Competition of different doses of $R$- and $S$-rolipram with $R-[^{11}]$C]rolipram uptake in rat brain regions</td>
<td>72</td>
</tr>
<tr>
<td>18.</td>
<td>Dose-response curve for $R-[^{11}]$C]rolipram uptake in rat frontal cortex following injection with increasing doses of $R$- or $S$-rolipram</td>
<td>73</td>
</tr>
<tr>
<td>19.</td>
<td>Competition of different doses of $R$- and $S$-rolipram $R-[^{11}]$C]rolipram uptake in selected rat peripheral organs</td>
<td>74</td>
</tr>
<tr>
<td>20.</td>
<td>Competition of $R/S-[^{11}]$C]rolipram with vinpocetine and DMI in rat brain and periphery</td>
<td>75</td>
</tr>
<tr>
<td>21.</td>
<td>Competition of $R-[^{11}]$C]rolipram with vinpocetine and DMI in rat brain and periphery</td>
<td>76</td>
</tr>
</tbody>
</table>
22. Competition of Ro 20-1724, R-, and S-rolipram with S-[14C]rolipram uptake in rat brain and periphery

23. Competition of S-[14C]rolipram with vinpocetine and DMI in rat brain and periphery

24. TLC chromatograms of R-[14C]rolipram metabolism in rat plasma and brain

25. Dose- and time-dependent effect of acute forskolin on R-[14C]rolipram rat regional brain distribution


27. Time-dependent effect of acute DMI on R-[14C]rolipram rat regional brain distribution

28. Time-dependent effect of acute yohimbine on R-[14C]rolipram rat regional brain distribution

29. Effect of acute clonidine and tranylcypromine on R-[14C]rolipram rat regional brain distribution

30. Effect of acute propranolol and clenbuterol on R-[14C]rolipram rat regional brain distribution

31. Effect of acute fluoxetine, 8-OH-DPAT, WAY 100635, DOI, and ritanserin on R-[14C]rolipram rat regional brain distribution

32. Effect of acute CGS 21680, thioperamide, and SKF 81297 on R-[14C]rolipram rat regional brain distribution

33. Effect of the PKA inhibitor HA 1004 on rat regional brain distribution of R-[14C]rolipram following challenge with yohimbine, DMI, or forskolin

34. Effect of the protein synthesis inhibitor cycloheximide on rat regional brain distribution of R-[14C]rolipram following challenge with CGS 21680

35. Effect of several acute drug treatments on the liver uptake of R-[14C]rolipram

36. Effect of several acute drug treatments on the blood levels of radioactivity

37. Effect of acute fluoxetine, DMI, or forskolin on the blood levels of R-[14C]rolipram

38. Effect of chronic treatment with propranolol on rat regional brain distribution of R-[14C]rolipram

39. Effect of chronic treatment with DMI, tranylcypromine, fluoxetine, or 8-OH-DPAT rat regional brain distribution of R-[14C]rolipram
40. Percentage change in $R-[^{11}C]$rolipram specific binding following chronic administration of DMI or fluoxetine

41. Radioactivity levels in liver and blood of rats treated chronically with DMI or fluoxetine, 45 min following $R-[^{11}C]$rolipram injection

42. Effect of chronic reserpine treatment on $R-[^{11}C]$rolipram rat regional brain distribution

43. Effect of lesioning noradrenergic neurons with DSP-4 on $R-[^{11}C]$rolipram distribution in rat brain regions

44. $R-[^{11}C]$Rolipram PET images from one of the control subjects

45. Decay-corrected time-activity curves of $R-[^{11}C]$rolipram in selected human brain regions

46. Time course of radioactivity levels in human arterial and venous plasma following $R-[^{11}C]$rolipram injection

47. Percentage of unmetabolized $R-[^{11}C]$rolipram in venous and arterial plasma as a function of time post-injection

48. Model of the effect of acute and chronic treatment with noradrenergic reuptake inhibitors on the concentration of cAMP in the postsynaptic neuron

49. Model of the effect of acute and chronic treatment with serotonergic reuptake inhibitors on the concentration of cAMP in the postsynaptic neuron
# LIST OF SCHEMES

<table>
<thead>
<tr>
<th>Scheme</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scheme 1.</td>
<td>Demethylation of Ro 20-1724 and radiosynthesis of $[^{11}\text{C}]$Ro 20-1724</td>
<td>55</td>
</tr>
<tr>
<td>Scheme 2.</td>
<td>Demethylation of $R/S$-rolipram and radiosynthesis of $R/S$-, $R$-, and $S$-$[^{11}\text{C}]$rolipram</td>
<td>56</td>
</tr>
</tbody>
</table>
ABBREVIATIONS

%ID/g percent of injected dose per gram of tissue
%IDBW/g percent of injected dose per gram of tissue normalized for body weight

$[^{11}]$CCH$_3$ carbon-11 methyl iodide
$[^{18}]$F FDG F-18 fluorodeoxyglucose

$^{11}$C carbon-11
$^{13}$N nitrogen-13
$^{18}$O oxygen-15

$^{18}$F fluorine-18

$^{32}$P phosphate-32

5-HT serotonin
6-OH-DA 6-hydroxy-dopamine

AC adenylyl cyclase

BaO barium oxide

BBB blood-brain barrier

BDNF brain-derived neurotrophic factor

$B_{\text{max}}$ maximal binding density

Buffer A 50 mM Tris-Cl, 5 mM MgCl$_2$, 0.1 mM dithiothreitol, and protease inhibitor cocktail

cAMP cyclic adenosine 3',5'-monophosphate

CDCl$_3$ deuterated chloroform

CRE cAMP-response element

CREB cAMP-response element binding protein

CTX cortex

DMF dimethylformamide

DMI desipramine

DNA deoxyribonucleic acid

ECS electroconvulsive shock

ED$_{50}$ dose required for 50% of maximal effect

EtOAc ethyl acetate

GC guanylyl cyclase

$G_i$ G-inhibitory protein

$G_s$ G-stimulatory protein

HPLC high performance liquid chromatography
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HRMS</td>
<td>high resolution mass spectra</td>
</tr>
<tr>
<td>IC&lt;sub&gt;50&lt;/sub&gt;</td>
<td>concentration at which 50% inhibition occurs</td>
</tr>
<tr>
<td>K&lt;sub&gt;d&lt;/sub&gt;</td>
<td>dissociation constant</td>
</tr>
<tr>
<td>K&lt;sub&gt;i&lt;/sub&gt;</td>
<td>inhibition constant</td>
</tr>
<tr>
<td>K&lt;sub&gt;m&lt;/sub&gt;</td>
<td>Michaelis-Menten constant</td>
</tr>
<tr>
<td>LRMS</td>
<td>low resolution mass spectra</td>
</tr>
<tr>
<td>MAO</td>
<td>monoamine oxidase</td>
</tr>
<tr>
<td>MeOH</td>
<td>methanol</td>
</tr>
<tr>
<td>MRI</td>
<td>magnetic resonance imaging</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NA</td>
<td>noradrenaline</td>
</tr>
<tr>
<td>NGF1-A</td>
<td>nerve growth factor transcription factor</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NT-3</td>
<td>neurotrophin-3</td>
</tr>
<tr>
<td>PDE</td>
<td>phosphodiesterase</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>PKA</td>
<td>cAMP-dependent protein kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase-C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase-C</td>
</tr>
<tr>
<td>R&lt;sub&gt;f&lt;/sub&gt;</td>
<td>relative front</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>R&lt;sub&gt;t&lt;/sub&gt;</td>
<td>retention time</td>
</tr>
<tr>
<td>Ser</td>
<td>serine</td>
</tr>
<tr>
<td>SH3</td>
<td>Src homology-3</td>
</tr>
<tr>
<td>SPE</td>
<td>solid phase extraction</td>
</tr>
<tr>
<td>SSRI</td>
<td>serotonin-selective reuptake inhibitor</td>
</tr>
<tr>
<td>TBAOH</td>
<td>tetrabutyl-ammonium hydroxide</td>
</tr>
<tr>
<td>THF</td>
<td>Tetrahydrofuran</td>
</tr>
<tr>
<td>TLC</td>
<td>thin layer chromatography</td>
</tr>
<tr>
<td>UCR</td>
<td>upstream conserved region</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>V&lt;sub&gt;max&lt;/sub&gt;</td>
<td>maximal velocity</td>
</tr>
<tr>
<td>β&lt;sup&gt;+&lt;/sup&gt;</td>
<td>positron</td>
</tr>
</tbody>
</table>
CHAPTER 1.0: INTRODUCTION

1.1 General Introduction

Positron Emission Tomography (PET) is a non-invasive imaging technology that uses radioactive substances injected in the blood stream for studying the functions of organs, including the brain, in the living person. For example, brain glucose metabolic activity can be imaged with $[^{18}F]FDG$ (Szelies et al., 1994), blood flow with $[^{15}O]water$ (Quarles et al., 1993), and the density of cell-surface receptors with radiolabeled agonists or antagonists, such as the agonist $R-[^{11}C]SKF 82957$ (DaSilva et al., 1998) and the antagonist $[^{11}C]raclopride$ (Antonini et al., 1993; Kapur et al., 1998) for dopamine $D_1$ and $D_2$ receptors, respectively. This thesis concerns with the development of PET radioligands for imaging for the first time the intracellular enzyme phosphodiesterase type-4 (PDE4) in the living human brain.

PDE4 is one of the main phosphodiesterase enzyme families that metabolizes the second messenger cyclic adenosine 3',5'-monophosphate (cAMP) in the brain, thereby contributing to intracellular signaling. Alterations in cAMP-mediated signaling have been demonstrated to regulate PDE4 activity within 15 min via phosphorylation (Sette and Conti, 1996), and PDE4 density following prolonged (hours to days) increases in cAMP levels (Manning et al., 1996; Takahashi et al., 1999; Torphy et al., 1995). Selective PDE4 inhibitors have been shown to display efficacy in clinical trials of depression (Fleischhacker et al., 1992; Zeller et al., 1984), as well as in animal models of this neuropsychiatric disorder (Schmiechen et al., 1990; Wachtel, 1983; Wachtel and Schneider, 1986). Recent cellular and molecular studies have suggested that the cAMP-signaling system, including PDE4, plays a role in the mechanism of action of antidepressants (Duman et al., 1997; Nibuya et al., 1996; Takahashi et al., 1999; Wachtel, 1990).
PDE4 was reported to be abundantly expressed in the brain (Engels et al., 1995a; Kaulen et al., 1989; Schneider et al., 1986), suggesting that a meaningful imaging signal could be obtained in brain PET studies using a selective PDE4 radioligand. The objective of this thesis was to label a series of PDE4-selective inhibitors with carbon-11 (\(^{11}\)C), and characterize their \textit{in vivo} potential in rats and humans for imaging PDE4 in the human brain using PET. Future research stemming from this thesis could unravel the function of PDE4 in antidepressant efficacy and in the etiology of neuropsychiatric disorders \textit{in vivo}.

1.2 Characteristics of PDE4

Adenylyl cyclases (AC) and guanylyl cyclases (GC) produce the second messengers cAMP and guanosine 3',5'-monophosphate (cGMP), respectively. A superfamily of PDEs regulates the break down of the second messengers, thus acting in concert with the cyclases in controlling intracellular signaling. The PDE superfamily is divided into at least eleven families, according to their genetic sequence, preferred substrate (cAMP, cGMP, or both cyclic nucleotides), and different selective inhibitors (Table I) (Beavo, 1995; Conti and Jin, 1999; Fisher et al., 1998a; Fisher et al., 1998b; Soderling et al., 1999; Fawcett et al., 2000). The existence of these different families of PDEs and of diverse forms of AC and GC, suggests that the precise tailoring of cAMP and cGMP concentrations is important for normal cell function. The cAMP-specific PDE4 enzymes are of particular interest because they have been demonstrated to be involved in the regulation of several processes, including learning and memory (Bolger et al., 1993; Qiu et al., 1991), cell survival (Hulley et al., 1995; Zhu et al., 1998), and inflammation (Torphy et al., 1992a). Additionally, alterations in PDE4 may play a role in depression (Duman et al., 1997; Wachtel, 1983; 1990; Wachtel and Schneider, 1986).
<table>
<thead>
<tr>
<th>Nomenclature</th>
<th>Gene Family (preferred substrate)</th>
<th>No. of Gene Products (splice variants)</th>
<th>Distribution</th>
<th>Selective Inhibitors</th>
</tr>
</thead>
<tbody>
<tr>
<td>PDE1</td>
<td>Ca²⁺/calmodulin Dependent (cGMP&gt;cAMP)</td>
<td>3 (&gt; 9)</td>
<td>Brain</td>
<td>Vinpocetine</td>
</tr>
<tr>
<td>PDE2</td>
<td>cGMP-stimulated (cGMP = cAMP)</td>
<td>1 (2)</td>
<td>Brain, adrenals</td>
<td>MEP-1, EHNA</td>
</tr>
<tr>
<td>PDE3</td>
<td>cGMP-inhibited (cGMP = cAMP)</td>
<td>2 (&gt; 2)</td>
<td>Heart, liver, smooth muscle, brain</td>
<td>SKF 94120</td>
</tr>
<tr>
<td>PDE4</td>
<td>cAMP-specific (cAMP only)</td>
<td>4 (&gt; 15)</td>
<td>Brain, immune system, lung, heart, testis</td>
<td>Rolipram, denbufylline, Ro 20-1724</td>
</tr>
<tr>
<td>PDE5</td>
<td>cGMP-specific (cGMP only)</td>
<td>2 (2)</td>
<td>Kidney, smooth muscle</td>
<td>Zaprinast</td>
</tr>
<tr>
<td>PDE6</td>
<td>Photoreceptor (cGMP only)</td>
<td>3 (2)</td>
<td>Retina</td>
<td>Zaprinast</td>
</tr>
<tr>
<td>PDE7</td>
<td>cAMP-specific, rolipram-insensitive (cAMP only)</td>
<td>1 (2)</td>
<td>Skeletal muscle</td>
<td>?</td>
</tr>
<tr>
<td>PDE8</td>
<td>cAMP-specific, IBMX-insensitive (cAMP only)</td>
<td>2 (&gt; 2)</td>
<td>Gonads, intestines</td>
<td>Dipyridamole</td>
</tr>
<tr>
<td>PDE9</td>
<td>cGMP-specific (cGMP only)</td>
<td>1 (?)</td>
<td>Spleen, small intestine, brain</td>
<td>SCH 51866</td>
</tr>
<tr>
<td>PDE10</td>
<td>Dual substrate, cAMP/cGMP-specific (cAMP = cGMP)</td>
<td>1 (?)</td>
<td>Brain, testis</td>
<td>?</td>
</tr>
<tr>
<td>PDE11</td>
<td>Dual substrate, cAMP/cGMP-specific (cAMP = cGMP)</td>
<td>1 (3)</td>
<td>Skeletal muscle, prostate, kidney, liver</td>
<td>Zaprinast, dipyridamole</td>
</tr>
</tbody>
</table>

*Data obtained from Conti, 1995; Beavo, 1995; Soderling et al., 1999; Fisher et al., 1998; Fisher et al., 1998; and Fawcett et al., 2000.
1.2.1 General PDE4 function and family subtypes

PDE4 is one of the main phosphodiesterase enzyme families responsible for selectively terminating the action of cAMP formed following the stimulation of β-adrenergic, A2-adenosine, H2-histamine, and possibly serotonin (5-HT)1a, 5-HT1c, and 5-HT, receptor-linked AC in the brain (Fig. 1) (Chang et al., 1997; Donaldson et al., 1988; Duman et al., 1997; Holden et al., 1987; Stanley et al., 1989; Whalin et al., 1989; Ye et al., 1997; Ye et al., 2000). Following activation of these postsynaptic receptors, the α subunit of the guanosine (G)-binding stimulatory protein (Gαs) stimulates AC to convert adenosine triphosphate (ATP) into cAMP, which activates cAMP-dependent kinases or exchange factors, leading to the regulation of a myriad of processes, including ion channel conductance (Cooper et al., 1995; Jurevicius and Fischmeister, 1996), protein phosphorylation (Govoni et al., 1988), gene transcription (Asanuma et al., 1996; Nibuya et al., 1996), and cell differentiation (Cook and McCormick, 1993). The presynaptic α2-adrenergic and H2-histamine receptors regulate the turnover and release of noradrenaline (NA) and histamine, respectively, by inhibiting the synthesis of cAMP through inhibitory G-proteins (Gi) (Bucher et al., 1990; Fredholm and Lindgren, 1988; Itoh, 1991; Oishi et al., 1989). A proportion of PDE4 activity was shown to be present on presynaptic noradrenergic neurons (Scuvée-Moreau et al., 1987; Wachtel and Schneider, 1986) and PDE4 function may be linked at least with the presynaptic α2-adrenergic receptor, since inhibiting PDE4 was reported to increase NA release (Kehr et al., 1985; Markstein et al., 1984; Schoffelmeer et al., 1985). Overall, the noradrenergic system appears to be a main neurotransmitter system where PDE4 acts by regulating noradrenergic responses both pre- and postsynaptically (Wachtel and Schneider, 1986; Whalin et al., 1989; Yamashita et al., 1997; Ye et al., 1997; Ye and O'Donnell, 1996).

Four genes encoding PDE4 subtypes have been characterized to date and are designated PDE4A, B, C, and D (Engels et al., 1994; Obernolte et al., 1993; Houslay et al., 1998). All four human PDE4 enzyme subfamilies exhibit similar kinetics of cAMP hydrolysis with a Vmax range
Fig. 1 Schematic of PDE4 function in neurotransmitter systems of the brain. The $\alpha_2$-adrenergic and possibly $H_3$-histamine autoreceptors are presynaptic receptors associated with PDE4. These receptors regulate the synthesis and release of noradrenaline and histamine, respectively, via inhibition of AC by the inhibitory G-protein $G_i$. Thus, when $\alpha_2$-adrenergic or $H_3$-histamine receptors are activated, cAMP synthesis is diminished and PDE4 expression is hypothesized to be decreased presynaptically. In contrast, the postsynaptic $\beta$-adrenergic, $A_2$-adenosine, $H_3$-histamine, and possibly 5-HT$_6$, 5-HT$_\alpha$, and 5-HT$_\beta$, receptors increase cAMP synthesis and likely PDE4 expression, via stimulation of AC by the stimulatory G-protein, $G_s$. The presynaptic transporter and the enzyme monoamine oxidase are also important components of the noradrenergic and serotonergic neurotransmitter systems that may function in concert with PDE4. Common antidepressants act by inhibiting noradrenaline or serotonin reuptake at the presynaptic transporter or by inhibiting monoamine oxidase activity, which is hypothesized to lead to an increase in PDE4 expression.
from 0.3 μmol/min/mg of protein to 11 μmol/min/mg of protein and a low $K_m$, varying from 1 μM to 8 μM (Hughes et al., 1997). The family members are distributed in different brain regions and cellular domains and are differentially regulated.

1.2.2 PDE4 distribution

The family of PDE4 enzymes is widely distributed throughout the body, being present in all major organs (Engels et al., 1994; Engels et al., 1995b; Swinnen et al., 1989). Tracheal and bronchial smooth muscles contain high PDE4 concentrations (de Boer et al., 1992; Schudt et al., 1991), but heart muscle appears to contain higher levels of PDE3 than PDE4 (Picq et al., 1995). Renal and reproductive tissues also reportedly express the PDE4 enzymes (Mehats et al., 2000; Obemolte et al., 1997) as do various types of cells in the immune system, however, red blood cells are devoid of PDE4 activity (Beavo, 1995; Tenor and Schudt, 1996). In the rat, the highest levels of these enzymes are expressed in brain as compared with other organs (Schneider et al., 1986). Within the brain, PDE4 activity is more abundant in neurons than glia (Tohda et al., 1994). PDE4 enzymes are localized in both particulate and soluble cellular fractions from many different tissues and cell lines (Schneider et al., 1986; Tenor and Schudt, 1996). The relative proportions between cell membrane and cytosol fractions of brain regions depends on the PDE4 subtypes expressed (Iona et al., 1998; Lobban et al., 1994).

The distribution of PDE4A, B, C, and D mRNA has been investigated in rat and human brain (Bolger et al., 1994; Engels et al., 1995a; Engels et al., 1994). Although present in human brain (Engels et al., 1994), PDE4C expression is low or absent in rat brain (Engels et al., 1995a). PDE4A is abundant in the rat olfactory system, cortex, hippocampus, and cerebellum. PDE4B is more highly expressed in the rat hippocampus and cerebellum, followed by lower levels in the cortex, olfactory system, and basal ganglia. PDE4D expression is more predominant in the rat hippocampus and cerebellum, followed by lower expression in the olfactory system and midbrain. PDE4A mRNA is abundantly expressed in the rat brain, especially in cortical regions,
being approximately five times as abundant as PDE4B and D mRNA (Engels et al., 1995a). This distribution of PDE4 mRNA subtypes in the rat is similar to the distribution of PDE4 proteins in the mouse brain (Cherry and Davis, 1999). PDE4A and B activities were shown to comprise the major fraction of PDE4 activity in rat brain membranes, whereas the cytosolic PDE4 activity was represented mostly by the PDE4D subfamily (McPhee et al., 1995). However, binding of the PDE4 inhibitor R/S-[3H]rolipram in rat cortex was found to be approximately equally distributed between cytosolic and membrane preparations (Schneider et al., 1986), suggesting that the overall distribution of PDE4 enzymes is similar between the two cellular fractions.

1.2.3 PDE4 structure and regulation

1.2.3.1 How PDE4 structure affects regulation

The regulation of the PDE4 family is very complex and may occur following both short- (within 15 min) and long-term (hours to days) changes in cAMP concentrations. Short-term mechanisms involve phosphorylation of the PDE4 protein, whereas, long-term mechanisms are characterized by altered PDE4 gene transcription (Fig. 2). The mode of regulation of the different PDE4 subtypes depends on their amino acid structure. Mammalian PDE4s exhibit a common structural pattern: all contain a conserved catalytic domain of ~270 amino acids close to the carboxy terminus, with >70-80% amino acid identity (Conti et al., 1995b; Jin et al., 1992; Manganiello et al., 1995). The amino terminus is very divergent, with over 15 splice variants having been identified and longer variants containing two conserved regions, upstream conserved region (UCR) 1 and 2 (Beard et al., 2000; Houslay and Milligan, 1997; Hughes et al., 1996; Houslay et al., 1998). Short splice variants contain only UCR2 or neither of the UCRs. A serine residue (Ser-54) in UCR1 of rat PDE4D3 was reported to be a target for cAMP-dependent protein kinase (PKA)-mediated phosphorylation (Conti et al., 1995b; Sette and Conti, 1996; Sette et al., 1994c). Additionally, PDE4D3 and the long variants PDE4A5 and B1, were shown to be activated by phosphatidic acid, while the short forms PDE4A1, B2, D1, and D2 were not
Fig. 2 Illustration of the two main mechanisms of PDE4 regulation: (1) short-term phosphorylation of some PDE4 subtypes by cAMP-dependent protein kinase (PKA) occurring within approximately 15 min of stimulation of cAMP synthesis, leading to an increase in PDE4 activity and (2) increase in de novo PDE4 protein synthesis as a result of long-term (>2 hours) incubation of cells with agents augmenting cAMP levels. The PDE4D3 subtype has been shown to be expressed in quiescent cells, and become rapidly activated via phosphorylation (Sette et al., 1994a,b). Chronic elevations in cAMP levels are thought to stimulate the PKA-mediated phosphorylation of cAMP-response element binding protein (CREB), which binds to cAMP-response-element (CRE), leading to an increase in PDE4 gene transcription.
Other regions within the amino terminus are involved in membrane targeting of rat PDE4A by binding to SH3 domains of membrane-bound tyrosine kinases, bringing PDE4A in close proximity to enzymes and effectors (O'Connell et al., 1996; Pooley et al., 1997; Shakur et al., 1993; Shakur et al., 1995). Interestingly, removal of parts of the amino terminal domain of PDE4A (Hughes et al., 1996; Jin et al., 1992; Shakur et al., 1993) or PDE4D1 (Kovala et al., 1997) was demonstrated to generate a catalytically more active enzyme, suggesting that some PDE4 subtypes contain an intramolecular inhibitory domain present at the amino terminus (Kovala et al., 1997). The carboxyl terminus may also be an important point of regulation, since the human PDE4B2 (Lenhard et al., 1996) and PDE4D3 (Hoffmann et al., 1999) were found to be potential targets for mitogen-activated protein kinase at carboxyl end serine residues, inhibiting enzyme activity in at least PDE4D3 (Hoffmann et al., 1999). A recent publication by Xu and colleagues (2000) reported the 3-dimensional structure of the catalytic domain of PDE4B2B using X-ray crystallography. This finding should help in advancing our understanding of the PDE4 family of enzymes.

Differences in the promoter regions of the PDE4 genes may also contribute to the regulation of the expression of these enzymes. Intronic promoters in the rat PDE4B and D genes are thought to control the expression of these genes in the testicular Sertoli cell, while upstream promoters are likely responsible for the expression of these genes in the brain (Monaco et al., 1994).

1.2.3.2 Short-term regulation

The short-term (within 15 min) regulation of PDE4 activity is mediated by phosphorylation of PDE4 proteins, mainly of the PDE4D subtype (Conti et al., 1995a; Houslay et al., 1998). The PDE4D gene encodes at least three mRNA variants that differ in the 5' region (Sette et al., 1994a). The shorter splice variants PDE4D1 and PDE4D2 are regulated by cAMP at the level of transcription or mRNA stability (see long-term regulation below) (Sette et al., 1994a; Swinnen et
PDE4D3 mRNA, on the other hand, is a longer variant containing UCR1 and is constitutively expressed in thyroid cells in the absence of hormones (Sette et al., 1994a; Sette et al., 1994b). PKA-mediated phosphorylation was shown to enhance significantly the activity of PDE4D3 within ~15 min following the stimulation of rat thyroid cells by thyroid stimulating hormone (Oki et al., 2000; Sette et al., 1994a), or stimulation of U937 cells by prostaglandin E2 (Alvarez et al., 1995). The same activation coincided with 32P incorporation into Ser-54 of the enzyme (Sette and Conti, 1996) as demonstrated by incubating partially purified PDE4 from FRTL-5 cells with the catalytic subunit of PKA (Sette et al., 1994b). Rapid increases in PDE4 activity have also been observed following incubation with the AC activator forskolin in astroglial (Madelian and La Vigne, 1996) and vascular smooth muscle cells (Ekholm et al., 1997). PDE4D3 is expressed in rat brain (Sette et al., 1994b) and elevations in cAMP may also increase its activity in the brain. Activation of PDE4D3 through phosphorylation was reported to increase the potency of the selective PDE4 inhibitor and putative antidepressant rolipram by ~8 fold (Hoffmann et al., 1998), showing that the rapid regulation of PDE4D3 may have therapeutic implications.

1.2.3.3 Long-term regulation

Members of all four PDE4 subfamilies have been shown to undergo long-term regulation in different cell lines in vitro as well as in rat brain in vivo. Recent studies of the promoter region of PDE4 genes have revealed that at least the human and rat PDE4B genes contain potential cAMP-responsive enhancer (CRE) elements that could mediate increased transcription of these genes in response to activation of the cAMP pathway (Monaco et al., 1994; Huston et al., 1997). Indeed, several in vitro and in vivo experiments support that the cAMP-signaling pathway regulates the long-term expression of PDE4 subtypes. For instance, increases in intracellular cAMP levels were found to augment the mRNA and protein contents of PDE4A and B following prolonged (2-4 hours) stimulation of U937 cells by agents that increase cAMP levels, such as
rolipram or the β2-adrenergic selective agonist salbutamol (Manning et al., 1996; Torphy et al., 1992c; Torphy et al., 1995). Expression of PDE4A, B, and D was augmented in Mono Mac 6 cells after treatment with the cAMP analogue dibutyryl-cAMP or other cAMP-elevating agents (Verghese et al., 1995). Prolonged treatment of PC12 cells with the adenosine A2-selective agonist CGS 21680, which enhances cAMP, led to a dose-dependent increase in PDE4 activity, that was abolished by inhibitors of mRNA and protein synthesis (Chang et al., 1997). Challenge of human Jurkat T-cells with forskolin induced PDE4D1 and D2 expression within three hours (Erdogan and Houslay, 1997). Interestingly, the PDE4A4 splice variant was downregulated by this treatment (Erdogan and Houslay, 1997), demonstrating that PDE4 variants may also be downregulated by certain cAMP-augmenting treatments. Chronic in vivo treatment with antidepressants, including desipramine (DMI), fluoxetine, sertraline, and tranylcypromine, was shown to significantly increase the expression of PDE4A and B mRNA and protein in rat frontal cortex (Takahashi et al., 1999; Ye et al., 1997) and hippocampus (Ye et al., 2000). Conversely, continuous infusion of the β-adrenergic antagonist propranolol for 14 days or lesioning of noradrenergic neurons using 6-hydroxy-dopamine (6-OH-DA), significantly decreased at least PDE4A expression in rat cortex (Ye et al., 1997; Ye and O'Donnell, 1996), showing that PDE4 subtypes are also downregulated following certain treatments in vivo. According to these in vitro and in vivo reports, the long-term increase in PDE4 expression is mediated by the cAMP-signaling pathway: the transcriptional activator cAMP-response element binding (CREB) protein becomes activated upon PKA-mediated phosphorylation, allowing CREB to bind to and activate CRE present on PDE4 genes, leading to an increase in PDE4 gene transcription (see Fig. 2) (Habener, 1990; Monaco et al., 1994; Huston et al., 1997).

1.2.3.4 How do the PDE4 subtypes interact to control cAMP levels?

Looking at the evidence obtained with the PDE4D family in different cell lines may help explain how the different PDE4 enzymes and variants interact to control cAMP levels. As
mentioned above, the long PDE4D variant, PDE4D3, is constitutively expressed in thyroid cells cultured in the absence of hormone. Stimulation of these cells with thyroid stimulating hormone produces a rapid activation of PDE4D3 via phosphorylation (Conti et al., 1995a; Oki et al., 2000; Sette et al., 1994a; Sette et al., 1994b). However, prolonged stimulation of these cells with hormone induces the de novo synthesis of the short forms PDE4D1 and D2 (Sette et al., 1994b). Therefore, in the short-term, cAMP concentrations are likely rapidly regulated by phosphorylation and activation of the long form of PDE4 enzymes already present within the cell. With prolonged increases in cAMP levels, shorter forms of PDE4 enzymes are assembled by de novo protein synthesis to regulate long-term changes in cAMP-mediated signaling.

1.2.4 Rolipram binding sites to PDE4 and other PDE4 inhibitors

Rolipram is a selective PDE4 inhibitor that is well documented in the literature. This compound has been shown to bind to PDE4 in rat brain homogenates with high affinity ($K_a \sim 1-2$ nM) (Borisy et al., 1993; Schneider et al., 1986), while only inhibiting PDE4 activity at much higher concentrations ($K_i > 0.25$ μM) (Amegadzie et al., 1995; Wilson et al., 1994). Previous studies have revealed that rolipram is a competitive inhibitor of PDE4 suggesting that it binds only to the catalytic site (Livi et al., 1990; Nemoz et al., 1989). In contrast, other reports have demonstrated complex rolipram inhibition kinetics, implying that multiple binding forms of PDE4 subtypes exist (Amegadzie et al., 1995; McLaughlin et al., 1993; Torphy et al., 1992b). These discrepant findings have led to the suggestion that there is a high-affinity rolipram-binding site in addition to the catalytic site on PDE4. Attempts to identify the rolipram binding site(s) in recombinant human PDE4A (Hughes et al., 1997; Jacobitz et al., 1996) or PDE4B (Rocque et al., 1997) by truncating the amino and/or carboxy terminus, or in PDE4C by expressing different splice variants of the amino terminus (Owens et al., 1997), have indicated that [3H]rolipram binds to a single site that exhibits both high- and low-affinity conformations. The high-affinity binding state was shown to require amino acid residues at both the amino terminus and catalytic domain,
whereas the low affinity binding state only required residues in the catalytic domain (Hughes et al., 1997; Rocque et al., 1997). Evidence showing that the amino terminus is involved in forming the high-affinity binding conformation include findings that solubilization of PDE4 enzymes from guinea-pig eosinophils increases the sensitivity to inhibition by rolipram by over 10 fold (Souness et al., 1992; Souness and Scott, 1993), likely due to the release of the membrane-bound amino terminus (Lobban et al., 1994; Shakur et al., 1995). Additionally, certain amino terminal splice variants of rat PDE4A (McPhee et al., 1995) and human PDE4D (Bolger et al., 1997) that were shown to be confined to the cytosol displayed a similar 10 fold higher sensitivity to inhibition by rolipram as compared to membrane-bound variants of the enzyme subtypes. However, freeing the amino terminus from the membrane may not be critical in the formation of the high-affinity conformation of PDE4 since certain particulate human PDE4B variants were reported to occur with ~10 fold higher sensitivity to rolipram than cytosolic forms (Huston et al., 1997). In fact, phosphorylation may be more important in the regulation of the high-affinity conformational state of PDE4 proteins than the subcellular distribution. Depending on whether PDE4 proteins are soluble or membrane-bound, regions of the enzymes may be phosphorylated by different protein kinases leading to an alteration in the kinetics of the enzymes and of rolipram inhibition. For instance, association of PDE4A variants with Src family tyrosyl kinases has been shown to mimick the enhanced rolipram inhibition seen for particulate as compared with cytosolic PDE4A (McPhee et al., 1999), and increases in PDE4 activity have been found to be correlated with augmented Src tyrosine kinase activity (Taylor et al., 1997). On the other hand, PKA phosphorylation of cytosolic PDE4D3 at the amino terminus (Ser-54) has been reported to increase the potency of rolipram by ~8 fold (Hoffmann et al., 1998).

Schneider and colleagues (1986) showed that high-affinity binding of \[^{1}H\]rolipram is Mg\(^{2+}\)-dependent, and recent studies (Xu et al., 2000; Laliberte et al., 2000) have confirmed that the formation of the high-affinity conformation is dependent on the presence of Mg\(^{2+}\) using in
**vitro** studies. Phosphorylation has been suggested to alter the PDE4 molecule and rolipram binding by enhancing the binding of Mg\(^{2+}\) to PDE4, and in fact, the sole presence of excess amounts of Mg\(^{2+}\) may facilitate the switch to the high-affinity conformation (Xu et al., 2000; Laliberte et al., 2000). Other studies have also implied that phosphorylation of PDE4D3 disrupts the interaction between a putative inhibitory domain and the catalytic site, thus allowing the formation of the high-affinity \[^{3}H\]rolipram-binding conformation of PDE4D3 (Beard et al., 2000; Lim et al., 1999). More studies in the near future will surely clarify the mechanisms involved in the formation of the high-affinity conformation of the PDE4 enzymes and which variants exhibit this phenomenon. Interestingly, the effective therapeutic concentrations of rolipram *in vivo* have been shown to be very similar to the high-affinity \(K_a\) and not to the apparent \(K_i\) of enzyme inhibition (Bolger et al., 1993; Schmiechen et al., 1990). Therefore, the high-affinity conformation may be the pharmacologically significant form of the enzymes in the brain. All PDE4 subtypes were demonstrated to display high-affinity \[^{3}H\]rolipram binding (Amegadzie et al., 1995; Hughes et al., 1997; McLaughlin et al., 1993; Pillai et al., 1993), showing that this inhibitor labeled with a positron emitter may have potential for imaging all the subtypes of PDE4 *in vivo* in PET studies.

Other high-affinity selective inhibitors of PDE4 include Ro 20-1724 (IC\(_{50}\) ~30-80 nM), denbufylline (IC\(_{50}\) ~6 nM), and RP 73401 (IC\(_{50}\) ~1 nM) (Borisy et al., 1993; Hughes et al., 1997; Kaulen et al., 1989). Several xanthine derivatives such as 3-isobutyl-1-methylxanthine (IC\(_{50}\) ~2-50 \(\mu\)M), caffeine (IC\(_{50}\) >30 \(\mu\)M), and theophylline (IC\(_{50}\) ~50-300 \(\mu\)M), are non-selective inhibitors of PDE4, inhibiting all PDEs (Beavo, 1995). None of the known PDE4 inhibitors, including Ro 20-1724 and rolipram, are substrates of the enzymes (Omburo et al., 1997).
1.3 Relevance to Major Depression

Increasing evidence of disruptions in the cAMP-signaling system, including PDE4, in major depression has led to an increased interest in studying the role of this system in this neuropsychiatric disorder (Duman, 1998; Wachtel, 1990; Shelton et al., 1996). The successful development of a PET radioligand for imaging PDE4 could allow for the study of this enzyme family in vivo in the normal brain and in brain disorders.

1.3.1 Current Hypotheses in Depression

1.3.1.1 Classic hypotheses: disruptions in the monoaminergic systems

The classic hypotheses in the etiology of depression point towards deficiencies in catecholamines, particularly noradrenaline (Schildkraut, 1965), or in 5-HT neurotransmission (Lapin and Oxenkrug, 1969) in the brain. Indeed, several studies have provided evidence that supports the implication of the noradrenergic and serotonergic systems in depression. For example, increased β-adrenergic and 5-HT$_2$, but not 5-HT$_1$, receptor binding was reported in the frontal cortex of suicide victims with a concomitant decrease in the presynaptic 5-HT reuptake transporter (Mann et al., 1986). Similar increases in 5-HT$_2$ and trends towards an increase in 5-HT$_1$ receptors were observed in post-mortem elderly patients of affective disorder (McKeith et al., 1987). The density and affinity of the high-affinity state of α$_2$-adrenergic receptors (adrenoceptors) were also reported to be increased in the brain of depressed suicides (Meana et al., 1992). In contrast, α$_1$-adrenoceptor density was shown to be reduced in suicide brain (Gross-Isseroff et al., 1990). Other studies published a decrease in β-adrenoceptor sensitivity to the agonist isoproterenol, as observed in lymphocytes (Mann et al., 1985) or leukocytes (Pandey et al., 1979) from drug-free depressed patients. Significant reductions in receptor-coupled G$_i$ (and G$_j$) protein levels and function were shown to occur in mononuclear leukocytes from depressed patients (Avissar et al., 1996; Avissar et al., 1997), which could contribute to the reduced
sensitivity of β-adrenoceptors. In fact, synthesis of cAMP following β-adrenoceptor stimulation was reported to be decreased in lymphocytes (Ebstein et al., 1988; Extein et al., 1979), correlating with depression severity and β-adrenoceptor downregulation (Mazzola-Pomietto et al., 1994). In disagreement with these findings, other studies have published no evidence of changes in α_2, β_1, and β_2-adrenoceptors in the prefrontal cortex from subjects with major depression (Klimek et al., 1999). Serotonergic binding sites, such as the 5-HT_1 and 5-HT_2 receptors, and the presynaptic reuptake site were also reported to remain unaltered in suicide victims (Owen et al., 1986). Additionally, no significant changes were demonstrated in G_α and G_α levels in leukocytes from depressed subjects (Young et al., 1994) and in polymorphonuclear lymphocyte cAMP responses to histamine H_2, prostaglandin-E, or β-adrenoceptors (Kanof et al., 1989). Because of these inconsistent reports, research has been redirected towards the study of components beyond the cell surface receptors and their immediate effectors (Duman, 1998; Schultz and Schmidt, 1986; Wachtel, 1990; Wachtel and Schneider, 1986; Popoli et al., 2000).

Recent important findings have been obtained by investigating the molecular and cellular adaptations following antidepressant administration in the brain, leading to the proposition of a novel hypothesis of depression: that “stress-induced vulnerability and the therapeutic action of antidepressants occur via intracellular mechanisms that decrease or increase, respectively, neurotrophic factors necessary for the survival and function of particular neurons” (Duman et al., 1997). The changes in the expression of neurotrophic factors by various classes of antidepressants may be the common ground in the mechanism of action of these drugs.

1.3.1.2 The effects of stress

Several studies support the molecular and cellular theory of depression proposed by Duman and colleagues (1997). Stress and other environmental insults may damage specific populations of neurons and, thereby, contribute to the pathophysiology of depression in vulnerable individuals (Duman et al., 1997; Post, 1997; Smith et al., 1995c; Smith et al., 1995d).
Specifically, stress and the release of glucocorticoids have been found to damage neurons in the brain, including the induction of neuronal atrophy and toxicity in brain regions, especially the hippocampus (McEwen, 1999; Rossby et al., 1995; Sapolsky, 1996; Smith et al., 1995d; Wong et al., 1996). Accordingly, depressed subjects have displayed decreases in cortical thickness, neuronal sizes, and neuronal and glial densities in cortical regions, and stress may contribute to this brain pathology (Rajkowska et al., 1999). Stress acts by altering the expression of neurotrophins controlled by the cAMP signaling pathway (Smith et al., 1995c; Tao et al., 1998). Single and repeated immobilization stress was reported to markedly reduce brain-derived neurotrophic factor (BDNF) mRNA levels, and repeated stress increased neurotrophin-3 (NT-3) mRNA expression in the dentate gyrus and hippocampus of rats (Smith et al., 1995d). BDNF was also shown to be increased in rat brain regions following stress such as in the hypothalamus and pituitary (Smith et al., 1995b). BDNF function may be activated by several neurotransmitter systems showing abnormalities in depression and suicide, including the 5-HT system. For example, studies revealed that BDNF markedly stimulates the sprouting of both intact and lesioned 5-HT axons in vivo in rats, leading to a hyperinnervation at the BDNF infusion site in the fronto-parietal cortex and dorsal hippocampus (Mamounas et al., 2000). Endogenous deficiencies in BDNF were shown to produce aggressiveness coupled with a dysfunction in several 5-HT receptors in mouse cortex (Lyons et al., 1999), while chronic BDNF administration was reported to upregulate tryptophan hydroxylase in serotonergic nuclei and 5-HT levels in rat brain (Siuciak et al., 1998). Interestingly, a significant decrease in temporal cortex levels of a transcription factor that activates BDNF expression, namely CREB (Tao et al., 1998), was found in depressed patients not on antidepressants, while treated patients demonstrated no difference from the control group (Dowlatshahi et al., 1999; Dowlatshahi et al., 1998). While not excluding the involvement of NA and 5-HT receptors or of other components in the cAMP second messenger system, such as G, and PKA, in the etiology of depression, these reports suggest that
there is a disturbance in the function of CREB and BDNF in depression, possibly produced by stress. Alternatively, the changes observed in CREB and BDNF expression may occur as a result of vulnerability secondary to disruptions in other neuronal functions, such as the synthesis and release of monoamine neurotransmitters and subsequent function of their receptors and second messenger systems in the brain.

1.3.1.3 Mechanism of action of antidepressants

Several studies have pointed towards an implication of neurotrophins in the mechanism of action of antidepressants. Infusion of BDNF itself into rat brain was shown to exhibit antidepressant efficacy in animal tests of antidepressant activity (Siuciak et al., 1997). Some observations in the treatment of depression are that acute antidepressant administration causes an increase in 5-HT and NA levels by inhibiting the presynaptic reuptake or breakdown of these monoamines, while prolonged antidepressant treatment downregulates certain 5-HT and NA receptors (e.g., β-adrenergic and 5-HT₁₆ receptors), and activates components of the cAMP signal transduction pathway (Albert et al., 1996; Attar-Levy et al., 1999; Blier et al., 1990; Karoum et al., 1984; Manji et al., 1991). For example, chronic treatment with different classes of antidepressants, including the tricyclic antidepressants, 5-HT-selective reuptake inhibitors (SSRI), and monoamine oxidase (MAO) inhibitors, as well as electroconvulsive shock (ECS), was shown to augment the cAMP-mediated regulation of CREB and BDNF expression (Fig. 3) (Duman, 1998). An increase in BDNF was reported to be necessary but not sufficient for the full effect of antidepressants, including the induction of mossy fiber sprouting in the hippocampus in vivo in rat brain (Vaidya et al., 1999), suggesting that there are other proteins contributing to the chronic effect of antidepressants. This is not surprising since antidepressant administration increases cAMP-mediated signaling at all levels of the signal transduction pathway. Specifically, chronic antidepressant treatment, including ECS, was found to augment the coupling between Gα and AC in rat cerebral cortex (Ozawa and Rasenick, 1991) and promoted
Antidepressant Treatments

Inhibit 5-HT and NA Reuptake or Breakdown

5-HT or NA

5-HT or NA

βAR
SHT₂,β,δ

SHT₂,α₁AR

Gs Adenylyl Cyclase

Gx

PDE4 Inhibitor

↑ cAMP

↑ PKA

↑ CREB

↑ BDNF

Nucleus

Ca²⁺-Dependent Kinases

Trophic Actions: Increased Function, Synaptic Remodeling

Fig. 3 Model of the mechanism of antidepressant treatment on postsynaptic intracellular signaling in the NA and 5-HT neurotransmitter systems. Antidepressant treatment increases the synaptic level of NA and/or 5-HT, which activate postsynaptic receptors linked to the cAMP signaling system. Prolonged stimulation of postsynaptic receptors leads to their downregulation, however, the intracellular cAMP signaling system has been recently found to become upregulated by chronic antidepressant treatment. For example, augmentations in the activity and expression of PKA, CREB, and BDNF have been reported to occur and these changes may promote remodeling of synapses. The amelioration of depressive symptoms may then result from the increased neuronal plasticity (adapted from Duman, 1997).
the translocation of PKA from the cytosol to the nucleus (Nestler et al., 1989). An increase in PKA and Ca\(^{2+}\)/calmodulin-dependent kinase function was reported to occur in the brain following chronic antidepressants (Popoli et al., 2000). PKA activation was shown to increase phosphorylation of CREB and CRE-mediated gene transcription in the rat cortex, hippocampus, amygdala, and hypothalamus (Frechilla et al., 1998; Thome et al., 2000). The increase in CREB activity following chronic antidepressant administration may lead to an upregulation in the transcription of several genes, including PDE4 (Takahashi et al., 1999) and BDNF (Tao et al., 1998). In fact, chronic antidepressants completely reversed the downregulation of BDNF mRNA in the rat hippocampus (Nibuya et al., 1995) in response to chronic restraint stress. Additionally, the mRNA levels of NGF1-A, a transcription factor that activates the expression of the neurotrophin nerve growth factor, were increased with chronic antidepressants (Morinobu et al., 1997). In contrast, stress-induced levels of the immediate early gene transcription factor c-fos mRNA were downregulated by antidepressants (Morinobu et al., 1995), suggesting that a decrease in c-fos expression may also be a common postreceptor action of antidepressants. Overall, these findings support the notion that the common mechanism of action of chronic antidepressants is via their effect on cAMP-mediated increases in gene transcription and neurotrophin expression, leading to an increase in neuronal plasticity, even though levels of some 5-HT and NA receptors may be partially downregulated.

Although the improvement in depressive symptoms is known to occur only following chronic antidepressant administration, researchers have reported evidence of significant acute effects on the expression of some of the genes described above. Acute ECS and tranylcypromine were reported to produce a significant increase in c-fos and NGF1-A mRNA and immunoreactivity in rat frontal cortex that was maximal within 1-4 hours following treatment relative to controls (Morinobu et al., 1997). Other transcription factors were also increased in rat frontal cortex including c-jun, junB, and fosB mRNA (Morinobu et al., 1997). Inhibition of 5-HT and NA
reuptake was also demonstrated to induce the expression of c-fos in the amygdala (Morelli et al., 1999). Interestingly, the acute effect of ECS and tranylcypromine was shown to be mediated in part by the β-adrenergic, α₁-adrenergic, 5-HT₂A, and 5-HT₃C receptors, since antagonists at these receptors significantly inhibited the increase in c-fos expression (Morinobu et al., 1997). Single administration of paroxetine, a SSRI, was revealed to significantly induce the phosphorylation of CREB in rat frontal cortex and hippocampus (Morinobu et al., 2000) and acute ECS was shown to increase BDNF mRNA levels in rat frontal cortex (Nibuya et al., 1995). Therefore, these findings suggest that the effect of antidepressants may begin immediately following administration in vivo, even though the alleviation of depressive symptoms only occurs after some time. The delayed symptomatic effect may be due to the time required for changes in neuronal plasticity to occur following the activation of a myriad of proteins, including AC, kinases, transcription factors, and neurotrophins in the cAMP signal transduction pathway.

1.3.2 PDE4 link with Major Depressive Disorder

As one of the main enzyme families that specifically break down cAMP in the brain, PDE4 plays a potentially important role in the amplification of the signal from cAMP to the upregulation of gene transcription by CREB (see Fig. 3). The first reports suggesting a role for PDE4 in depression were the ones demonstrating that selective PDE4 inhibitors have efficacy in animal tests of depression, such as reserpine-induced hypothermia and hypokinesia in mice, potentiation of yohimbine lethality, and the olfactory bulbectomized rat model (Schultz and Schmidt, 1986; Wachtel, 1983; Wachtel and Schneider, 1986; Mizokawa et al., 1988). The inhibitor rolipram showed potency in the animal tests, and subsequent clinical trials indicated that it has some efficacy at alleviating symptoms (Fleischhacker et al., 1992; Hebenstreit et al., 1989; Horowski and Sastre-Y-Hernandez, 1985; Zeller et al., 1984), but produces side effects such as nausea, vomiting, and weight gain (Overstreet et al., 1989; Robichaud et al., 1999). The mechanism of action of rolipram at alleviating depressive symptoms is thought to be through
inhibition of PDE4, leading to an increase in cAMP levels (Wachtel et al., 1990; Nemoz et al., 1985). Interestingly, rolipram has been reported to interact with the central noradrenergic system as commonly observed with other antidepressants, such as the tricyclics. For example, chronic injection of rolipram in rats was reported to cause a significant downregulation of central α₂- and β-adrenoceptors (Przegalinski et al., 1985; Schultz and Schmidt, 1986; Ye and O'Donnell, 1996), and to significantly reduce drinking response to the β-adrenoceptor agonist isoprenaline with a similar potency as the tricyclic antidepressants amitriptyline and imipramine (Przegalinski et al., 1988). Rolipram treatment has also been reported to be as effective as DMI in clinical trials of depression (Bobon et al., 1988) and in preventing muricide behavior, a test of antidepressant activity (Mizokawa et al., 1988). Although the effects of rolipram have been shown to be mediated in part by the noradrenergic system, they may not depend on the presence of monoamines, likely due to the ability of rolipram to increase cAMP levels by inhibiting PDE4 beyond postsynaptic β-adrenoceptors (Przegalinski et al., 1985; Wachtel and Schneider, 1986).

Importantly, recent reports have shown that rolipram may also alter neurotrophin gene expression through the cAMP signal transduction system, in agreement with the molecular and cellular theory of depression (Duman et al., 1997). For example, coadministration of rolipram with DMI was demonstrated to enhance the expression of BDNF in rat hippocampus (Fujimaki et al., 2000). The effect of rolipram on BDNF expression is likely mediated by the cAMP signaling system since incubation of U937 monocytes with rolipram increased CREB phosphorylation (MacKenzie and Houslay, 2000). Moreover, acute rolipram treatment was found to significantly increase CREB binding to CRE two hours after administration in vivo in rats (Asanuma et al., 1996). Rolipram has also been shown to induce c-fos-like immunoreactivity in rat brain (Dragunow and Faull, 1989), another recently observed effect of antidepressants (Morinobu et al., 1997). Interestingly, chronic antidepressant treatment using DMI, fluoxetine, sertraline, or tranylcypromine, led to a significant increase in the expression of
PDE4A and B mRNA and protein in rat frontal cortex (Takahashi et al., 1999) and hippocampus (Ye et al., 2000). In contrast, the non-antidepressant psychotropic drugs cocaine and haloperidol had no effect (Takahashi et al., 1999). The similar molecular, cellular, and behavioral effects observed between rolipram and other common antidepressants, and the upregulation of PDE4 subtypes following antidepressant treatments, suggests that the PDE4 family of enzymes play a role in the mechanism of action of antidepressants.

1.4 Positron Emission Tomography and Second Messenger Systems

1.4.1 Positron emission tomography

PET is a medical imaging technology that has the ability to produce images of selective body functions and can often distinguish between normal and abnormal physiology in cases where no major anatomical changes have occurred (for a review, see Sedvall, et. al, 1988, Maziere and Maziere 1990, or Frey, 1989). In PET, a radiotracer is usually injected, but can also be swallowed or inhaled. When the atoms of the tracer decay, photons are emitted. The distribution of the tracer in the patient is measured by recording this photon emission using the PET scanner. By tracing the path of the radioactive substance, normal and abnormal biological functions of tissues can be determined.

PET tracers incorporate a positron-emitting isotope, such as $^{15}$O, $^{11}$C, $^{13}$N and $^{18}$F. These isotopes decay rapidly (within minutes to hours), and the positrons released collide with their antiparticles, electrons, resulting in annihilation of the two particles and the release of two antiparallel, high intensity photons. The photons travel through biological tissues with minimal absorption (Fig. 4). An external system using a ring of scintillation detectors, placed around the head of a subject, is used to record the point of annihilation (Fig. 5). Antiparallel annihilation photons are recorded by coincidence coupling of two scintillation detectors placed at 180° to
Fig. 4 The $^{11}$C nucleus (half-life 20.4 min) decays releasing a positron ($\beta^+$) which travels through tissues until it encounters its antiparticle, an electron ($e^-$). The $\beta^+$ annihilates with the $e^-$ releasing two photons of 511 Kev at 180° to each other. The photons are detected by the PET camera (adapted from Sedvall et al., 1988).
Fig. 5 The subject lies down on a table with the head positioned at the centre of a ring of crystals. The radioligand labeled with $^{11}$C is injected intravenously. High intensity photons detected in coincidence by two opposite crystals are registered as coming from a single annihilation event occurring in the brain. After several registrations over a period of 90 min, a committed computer system reconstructs an image of the brain showing the regional distribution of activity (adapted from Sedvall et al., 1988).
each other. Over the course of a PET scan, many coincidence lines are drawn and areas of overlapping lines appear as more highly concentrated areas of radioactivity. This information is processed and used to reconstruct an image in a similar fashion to computer assisted tomography.

PET scanners have become increasingly sophisticated over the past two decades, and current models have the ability to measure the distribution of radioactivity within the whole living human brain with a resolution in the order of 3-5 mm³. As a result, PET may be useful in a wide range of clinical psychopharmacology areas and provide important information on the pharmacodynamics and receptor occupancy of new drugs in addition to the study of cerebral metabolism and blood flow in humans. A new potential area for imaging that is now emerging in the PET field due to the availability of radioligands is the cAMP signaling system as highlighted elsewhere (Fujita and Innis, 2000) and described in this thesis.

1.4.2 PET Imaging in depression

Reductions in regional cerebral glucose metabolism have been reported with the widely employed radiotracer [¹⁸F]FDG in depression (Baxter et al., 1989; Drevets et al., 1997; Mayberg et al., 1997). However, high efforts have been placed on the development of radioligands for imaging specific receptors or enzymes reported to be implicated in neuropsychiatric disorders in studies in vitro. The 5-HT₁A antagonist [¹¹C]WAY 100635 (Drevets et al., 1999) and the 5-HT₂ receptor antagonist [¹⁸F]setoperone (Blin et al., 1988; Meyer et al., 1999) are two examples of PET radioligands developed for assessing the densities of 5-HT₁A and 5-HT₂ receptors, respectively, in depression. The apparent density of 5-HT₁A receptors was found to be abnormally decreased, whereas no changes were discovered with 5-HT₂ receptors in depressed patients (Blin et al., 1988; Drevets et al., 1999). No radioligands have yet been successfully developed for imaging second messenger signal transduction systems in depression.
1.4.3 Imaging second messenger systems

Recent interest in the study of second messenger systems in the brain has led to the synthesis of novel PET radiotracers for imaging the AC and the protein kinase C (PKC) second messenger systems. The AC activator forskolin was labeled with $^{11}$C for imaging AC by Sasaki and colleagues (Sasaki et al., 1995; Sasaki et al., 1991). However, no further studies have been published using $[^{11}]$C-forskolin, possibly due to a complex radiosynthesis and low radiochemical yields. Tracers that were synthesized for imaging PKC include $[^{18}]$F- and $[^{11}]$C-1,2-diacylglycerol (Hammadi et al., 1994; Takahashi et al., 1994; 2000) and [7β-methoxy $^{11}$C]methoxy-staurosporine (Sasaki et al., 1996). As with $[^{11}]$C-forskolin no further data has been published on these radiotracers. In addition to the development of the PDE4 inhibitor R-$[^{11}]$C-rolipram described in this thesis, another group in Japan has recently synthesized and tested in vivo in mice the PDE4 inhibitor $[^{18}]$FKF 19316 (Hatano et al., 1999) for imaging PDE4. With the successful development of radioligands for imaging PDE4, a new window will open for PET: that of visualizing an important component of the cAMP signaling system.

1.5 Research Rationale, Hypotheses, and Strategy

1.5.1 Research rationale and ultimate objective

The rationale for this research is based on the evidence of disruptions in cAMP-mediated signaling and possible implication of PDE4 in the etiology and treatment of neuropsychiatric disorders, including depression. A PET radioligand has not yet been successfully developed for studying the function of PDE4 in the living human brain. Such a tool would be critical to determine the role of PDE4 in disease state, as the neurochemistry in the living condition may be very different from that in post-mortem tissues or in cellular and animal models of brain disease. PDE4 is expressed abundantly in the rat (Kaulen et al., 1989) and human brain (Engels et al.,
suggesting that meaningful measurements would be obtained with a selective PDE4 radioligand in human PET studies. The ultimate objective of this thesis was to develop a selective radioligand for imaging PDE4 in the living human brain using PET. Future work stemming from this research could unravel the role of PDE4 in the living human brain in neuropsychiatric disorders before and after treatment.

1.5.2 Research goals

The development of a new PET radioligand usually involves a series of experiments to characterize its potential for PET imaging. First, due to the short physical half-life of PET tracers (e.g., half-life of $^{11}$C is 20.4 min), it is essential that the radioligand be easily and rapidly (within 45 min) synthesized for prompt injection into animals or humans. Second, for brain imaging, it is necessary that the radioligand penetrate the blood-brain barrier (BBB) and provide a high signal-to-noise contrast. The brain uptake or signal of the radioligand is determined in an animal model such as the rat. Third, the specificity and selectivity of binding are determined in animals to ensure that the tracer binds selectively to the site of interest. Fourth, metabolism studies are performed in animals to characterize the metabolic profile of the radioligand in plasma and determine if radiolabeled metabolites enter the brain, since the presence of radiolabeled metabolites in the brain increases the difficulty in pharmacokinetic modeling of the regional brain distribution of radioactivity. Fifth, the human radiation absorbed dose estimates are calculated using rat biodistribution data to evaluate the safety of injecting the radioligand in humans. Sixth, additional in vivo animal studies are executed for a radioligand that binds to a site that is highly regulated (such as PDE4) in order to assess if the radioligand can detect changes in the regulation of the site. If all of the in vivo characterization studies in animals yield favorable results, then the final major step in evaluating a new PET radioligand is to perform imaging studies in normal human volunteers to determine the regional brain uptake and signal, and the metabolism profile of the radiotracer in plasma.
Several high-affinity and selective PDE4 inhibitors have been described over the last 20 years (Beavo, 1995), such as the prototypical Ro 20-1724 (IC$_{50}$ ~23-190 nM) and rolipram (IC$_{50}$ ~2-7 nM) (Barnette et al., 1995b; Borisy et al., 1993; Koe et al., 1990; Schmiechen et al., 1990; Schneider et al., 1986). The para methoxy in the chemical structure of these inhibitors (Fig. 6) is favorable for labeling with $^{13}$C, since it may be feasible to demethylate the methoxy using common demethylating reagents and then remethylate the resulting precursor using [$^{13}$C]methyl iodide ([C]CH$_3$I) with established radiochemical techniques (DaSilva et al., 1993b). The first thesis research goal was to label R/S-Ro 20-1724 and R/S-rolipram with $^{13}$C, and perform pharmacological characterization studies in vivo in rats, to assess their potential for imaging PDE4 with PET. The radioligand producing the best characteristics would then be further evaluated in vivo, first in rats, then in humans. PET radioligands are used in tracer doses, therefore only the high-affinity [$^3$H]rolipram-binding conformational state of PDE4 is expected to be labeled in vivo in imaging studies. In vitro binding assays were thus also performed in this project to determine the $B_{\text{max}}$ and the high-affinity $K_d$ in order to validate the results obtained in vivo following selected acute and chronic drug treatments hypothesized to regulate PDE4 expression.

![Chemical structures of Ro 20-1724 and rolipram](image)

**Fig. 6** Chemical structure of the PDE4-selective inhibitors Ro 20-1724 and rolipram (*denotes the chiral centre).
1.5.3 Hypotheses

The main hypotheses tested were the following:

1) $R/S$-Ro 20-1724 and/or $R/S$-rolipram can be successfully demethylated and labeled with $^{11}$C using $[^{11}\text{C}]\text{CH}_2\text{I}$ in high yields, purity, and specific activity;

2) $R/S-[^{11}\text{C}]\text{Ro }20-1724$ and/or $R/S-[^{11}\text{C}]\text{rolipram}$ display a high signal and selectivity for PDE4 in rat brain regions.

The more active enantiomer of the radioligand showing the most potential in the preliminary studies would then be evaluated:

3) The more active enantiomer shows an acceptable metabolism and dosimetry profile;

4) Binding of the more active enantiomer in vivo in rat brain demonstrates sensitivity to the regulation of PDE4 following challenges with drugs increasing or decreasing cAMP-mediated signaling, and finally

5) The more active enantiomer displays a high regional brain uptake and a favorable metabolism profile in human PET imaging studies.

1.5.4 Aims

The following main experiments were planned out in order to test the hypotheses:

1) $R/S$-Ro 20-1724 and $R/S$-rolipram will be demethylated and the desmethyl precursors will then be labeled with $^{11}$C.

2) Time course, biodistribution, and competition studies will be performed with $R/S-[^{11}\text{C}]\text{Ro }20-1724$ and $R/S-[^{11}\text{C}]\text{rolipram}$ in rats to determine their regional brain uptake, distribution, and selectivity for binding to PDE4 in vivo. Chiral high performance liquid chromatography (HPLC) will be used to separate the $R$-desmethyl from the $S$-desmethyl derivative of the racemate. The $^{11}$C-labeled pure enantiomer will be synthesized and evaluated in vivo in rats.
The less active \textsuperscript{13}C-labeled enantiomer will also be synthesized to determine its potential for measuring non-specific binding.

3) Metabolism and dosimetry studies will be executed in rats only with the most promising radioligand.

4) \textit{In vivo} studies will be carried out in rats using acute and chronic drug treatments that alter cAMP-mediated signaling in order to determine the ability of the selected tracer to detect changes in the regulation of PDE4 enzymes.

5) Initial human PET imaging will be performed to determine the regional brain distribution and its metabolism profile.

PDE4 activity and cAMP levels were not measured in this work, and therefore no comparison will be made with our results. Completion of the aims listed above is expected to lead to the development of the first PET radioligand for imaging PDE4 \textit{in vivo} in the living human brain using PET. Following the construction of a pharmacokinetic model, measurements of PDE4 with PET in the future may provide new knowledge on the etiology of depression and on the mechanism of action and appropriate dosage of antidepressants.
CHAPTER 2.0: MATERIALS AND METHODS

2.1 Synthesis of the Phosphodiesterase-4 Inhibitors R/S-[\(^{13}\)C]Ro 20-1724, R/S-, R-, and S-[\(^{13}\)C]Rolipram

2.1.1 General

Racemic Ro 20-1724 and rolipram were purchased from Sigma-Research Biochemicals (Oakville, Canada). R-Rolipram and S-rolipram were a generous gift from Berlex-Canada Inc. (Lachine, Canada). Tetrahydrofuran (THF) and dimethylformamide (DMF) were obtained from Aldrich (Oakville, Canada), and were freshly distilled under nitrogen from LiAlH\(_4\) (THF), or under reduced pressure from BaO and stored over 4 Å molecular sieves (DMF). All other chemicals were obtained from commercial sources and used without further purification. All dealkylating reactions were monitored by analytical HPLC using an Alltech Econosil C\(_{18}\) column (250 x 4.6 mm, 10 μ) eluted with CH\(_3\)CN/ammonium formate (0.1 M) 45/55 at a flow rate of 1 ml/min. Products were detected using ultraviolet (254 nm) and scintillation radioactivity detectors. Thin layer chromatography (TLC) analysis was performed on plastic-backed silica gel TLC plates (IB-F, J.T. Baker), eluted with dichloromethane/triethylamine/methanol (94/5/1, v/v/v). The TLC plates were analyzed by an automatic TLC-linear analyzer (Tracemaster-20, Berthold) for radioactivity, and by ultraviolet (λ = 254 nm). Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Unity 500 spectrometer at 500 MHz for \(^1\)H-NMR or 127.5 MHz for \(^{13}\)C-NMR spectra in deuterated chloroform (CDCl\(_3\)), using tetramethylsilane as an internal standard. Electron impact (70 eV) low-resolution (MS) and high-resolution mass spectra (HRMS) were obtained on a Micromass 70-250S mass spectrometer. MS data are expressed in m/z (intensity relative to base peak = 100).
2.1.2 Chemistry

DESMETHYL-Ro 20-1724. Iodotrimethylsilane (385 μl, 2.69 mmol) was slowly added under nitrogen through a silicon septum in a 5 ml reaction vial to a solution of Ro 20-1724 (150 mg, 0.54 mmol) in anhydrous CHCl₃ (1.4 ml) at room temperature. The reaction mixture was stirred for 64 h at room temperature. The solution was then successively treated with methanol (100 μl), and aqueous saturated solutions of sodium bisulfite (300 μl) and sodium bicarbonate (200 μl). The mixture was stirred until it became colorless, then it was diluted with methylene chloride (20 ml), stirred in the presence of Celite (10 min), and filtered. The resulting solution was filtered a second time through Celite. Following evaporation, the resultant product was recrystallized from ethyl acetate (EtOAc) to give desmethyl-Ro 20-1724 as a white powder (127 mg, 90%): mp 112-114 °C; ¹H-NMR (CDCl₃): δ 0.98 (t, J = 7.4 Hz, 3H, CH₃), 1.50 (sext., 2H, J = 7.5 Hz, CH₂CH₃), 1.78 (quint., 2H, J = 7.1 Hz, OCH₂CH₂), 2.67 (dd, J = 13.6 and 7.0 Hz, 1H, ArCH₃) and 2.76 (dd, J = 13.4 and 6.1 Hz, 1H, ArCH₃), 3.19 (dd, J = 8.9 and 6.3 Hz, 1H, CH₂N) and 3.44 (vrt. t, J = 8.9 Hz, 1H, CH₂N), 3.96 (dt, J = 15.1 and 6.6 Hz, 1H, CHN), 4.00 (t, J = 6.6 Hz, 1H, CH₂OAr), 6.63 (d, J = 7.8 Hz, 1H, 5-H or 6-H), 6.73 (d, J = 8.1 Hz, 1H, 6-H or 5-H), 6.77 (s, 1H, 2-H); ¹³C-NMR (CDCl₃): δ 14.1 (CH₃), 20.2 (CH₂CH₃), 32.5 (CH₂CH₂O), 42.1, 46.7, 55.6 (CHN), 70.0 (CH₂OAr), 115.4 (Ar), 116.4 (Ar), 122.8 (6-C), 130.0 (1-C), 146.7 (ArO), 148.3 (ArO), 166.3 (CO); MS (m/z, %): 264 (M⁺, 24%), 180 (M⁺-C₃H₄N₂O, 100%), 124 (M⁺-C₃H₁₂N₂O, 59%), 85 (C₃H₅N₂O⁺, 66%); HRMS calculated for C₁₄H₂₀N₂O₃ (M⁺) 264.1474, found 264.1476.

R/S-DESMETHYL-ROLIPRAM. To a solution of R/S-rolipram (300 mg, 1.09 mmol) in anhydrous CHCl₃ (3.5 ml) at room temperature, iodotrimethylsilane (520 μl, 3.60 mmol) was slowly added under nitrogen through a silicon septum in a 5 ml reaction vial. The reaction mixture was stirred for 64 h at room temperature, and then quenched with water (200 μl),
stirred, and transferred into an Erlenmeyer flask using EtOAc (75 ml). Aqueous saturated solutions of sodium bisulfite (300 µl) and sodium bicarbonate (200 µl) were added to the stirred mixture until it became colorless. The resulting solution was washed with brine and extracted (EtOAc) three times. The combined organic extracts were dried over Na₂SO₄, filtered, and evaporated. The resulting resin was purified by column chromatography (silica gel, gradient EtOAc/hexanes/triethylamine 39/60/1-49/50/1 (v/v/v)). The fractions containing R/S-desmethyl-rolipram (as analyzed by analytical HPLC) were combined and further purified by column chromatography as above to give the desired precursor as a resin (71 mg, 25%): 'H-NMR (CDCl₃): δ 1.61-1.71 (m, 2H, CH₂CH₂CHO), 1.75-1.84 (m, 2H, CH₂CH₂CHO), 1.84-1.98 (m, 4H, 2 x CH₂CH₂CHO), 2.46 (dd, J = 17.0 and 8.9 Hz, 1H, CH₂CO) and 2.71 (dd, J = 17.0 and 8.9 Hz, 1H, CH₂CO), 3.37 (dd, J = 9.3 and 7.6 Hz, 1H, CH₂N) and 3.75 (vbr. t, J = 8.5 Hz, 1H, CH₂N), 3.62 (quint., J = 8.4 Hz, 1H, CHAr), 4.82 (m, 1H, CHOAr), 5.64 (br s, 1H, NH), 6.12 (br s, 1 H, ArOH), 6.72 (m, 2H, 2-H and 5-H or 6-H), 6.87 (d, J = 8.5 Hz, 1H, 6-H or 5-H);

R- AND S-DESMETHYL-ROLIPRAM. Dealkylation of racemic rolipram (200 mg, 0.73 mmol and 3.3 equivalents of iodosiltrimethylsilane) was repeated twice as described above, followed by column chromatography purification of R/S-desmethyl-rolipram (as above) obtained from all attempted dealkylating reactions. Enantiomeric separation of the combined fractions containing R/S-desmethyl-rolipram was performed by successive chiral semi-preparative HPLC (Chirex S-Leu & R-NEA column, 250 x 10 mm, Phenomenex, CA, U.S.A., hexane/ethanol 95/5 (v/v), 10 ml/min, S-desmethyl-rolipram Rₜ = 15.0 min, R-desmethyl-rolipram Rₜ = 17.5 min). The fractions enriched in R- or S-desmethyl-rolipram were combined
and further purified (hexane/ethanol 97/3 (v/v), 10 ml/min, S-desmethyl-rolipram $R_1 = 33.5$ min, R-desmethyl-rolipram $R_1 = 35.0$ min) until pure $R$- or S-desmethyl-rolipram was obtained, both as white powders. The enantiomeric purity of the isolated products was determined by chiral analytical HPLC with a Chirex S-Leu & R-NEA column (250 x 4.6 mm, Phenomenex) eluted with hexane/ethanol 95/5 (v/v) at a flow rate of 2 ml/min (S-desmethyl-rolipram $R_1 = 18.5$ min, and R-desmethyl-rolipram $R_1 = 20.5$ min).

**R-Desmethyl-rolipram**: mp 124.0-124.5 °C; $^1$H-NMR (CDCl$_3$): $\delta$ 1.60-1.70 (m, 2H, CH$_2$CHO), 1.74-1.83 (m, 2H, CH$_3$CHO), 1.83-1.97 (m, 4H, 2 x CH$_2$CHO), 2.45 (dd, $J = 16.8$ and 9.0 Hz, 1H, CH$_2$CO) and 2.70 (dd, $J = 17.0$ and 8.9 Hz, 1H, CH$_2$CO), 3.36 (dd, $J = 9.5$ and 7.6 Hz, 1H, CH$_2$N) and 3.74 (vitr. t, $J = 8.9$ Hz, 1H, CH$_2$N), 3.60 (quint., $J = 8.4$ Hz, 1H, CHAr), 4.81 (m, 1H, CHOAr), 5.79 (s, 1H, NH), 6.70 (m, 1H, 5-H or 6-H), 6.71 (s, 1H, 2-H), 6.73 (br s, 1H, ArOH), 6.86 (m, 1H, 6-H or 5-H); $^{13}$C-NMR (CDCl$_3$): $\delta$ 23.9 (CH$_2$CHO), 32.9 (CH$_3$CHO), 38.2, 40.1, 49.9 (CH$_2$CO), 80.7 (CHOAr), 111.4 (Ar), 114.5 (Ar), 119.1 (Ar), 133.8 (1-C), 145.1 (ArO), 145.4 (ArO), 177.8 (CO); MS (m/z, %): 261 (M$^+$, 8%), 193 (M$^+-$C$_6$H$_4$, 66%), 136 (M$^+-$C$_7$H$_{12}$NO, 100%); HRMS calculated for C$_{15}$H$_{19}$NO$_2$ (M$^+$) 261.1365, found 261.1366.

**S-Desmethyl-rolipram**: mp 125.0-126.0 °C; $^1$H-NMR (CDCl$_3$): $\delta$ 1.58-1.68 (m, 2H, CH$_2$CHO), 1.73-1.82 (m, 2H, CH$_2$CHO), 1.82-1.96 (m, 4H, 2 x CH$_2$CHO), 2.45 (dd, $J = 17.0$ and 8.9 Hz, 1H, CH$_2$CO) and 2.69 (dd, $J = 16.9$ and 9.0 Hz, 1H, CH$_2$CO), 3.36 (vitr. t, $J = 8.5$ Hz, 1H, CH$_2$N) and 3.73 (vitr. t, $J = 9.0$ Hz, 1H, CH$_2$N), 3.58 (quint., $J = 8.4$ Hz, 1H, CHAr), 4.80 (m, 1H, CHOAr), 6.0 (br s, 1H, NH), 6.69 (d, $J = 8.3$ Hz, 1H, 5-H or 6-H), 6.71 (s, 1H, 2-H), 6.84 (d, $J = 8.1$ Hz, 1H, 6-H or 5-H), 7.12 (br s, 1H, ArOH); $^{13}$C-NMR (CDCl$_3$): $\delta$ 23.9 (CH$_2$CHO), 32.8 (CH$_3$CHO), 38.4, 40.1, 50.0 (CH$_2$CO), 80.6 (CHOAr), 111.6 (Ar), 114.6 (Ar), 119.1 (Ar), 133.8 (1-C), 145.1 (ArO), 145.4 (ArO), 178.2 (CO); MS (m/z, %): 261
(M⁺, 7%), 193 (M⁺-C₅H₅, 64%), 136 (M⁺-C₆H₁₂NO, 100%); HRMS calculated for C₁₃H₁₆NO₃ (M⁺) 261.1365, found 261.1365.

2.1.3 Radiochemistry

[¹³C]Ro 20-1724. [¹³C]CH₃I, produced from [¹³C]CO₂ as described previously (DaSilva et al., 1996), was swept at 10 ml/min with N₂ and trapped in a 1 ml reaction vial containing desmethyl-Ro 20-1724 (1 mg) and tetrabutylammonium hydroxide (TBAOH, MeOH 1 M solution, 1.1 equivalent) in DMF (185 µL) at -15 to -40°C. When radioactivity accumulation in the reaction vial was maximal (at approx. 3 min), the solution was heated at 85°C for 5 min. After cooling, the mixture was quenched with the HPLC solvent (0.5 ml), and then [¹³C]Ro 20-1724 was purified by semi-preparative HPLC (Ecosil C₁₈, 250 x 10 mm, 10 µ, CH₃CN/ammonium formate (0.1 M) 40/60 (v/v), 6 ml/min, Rₜ = 8.5 min). After evaporation of the solvent, the residue was dissolved in saline (10 ml), passed through a 0.22 µm filter into a sterile vial containing aqueous sodium bicarbonate (1 ml, 8.4%). The purity and specific activity of the final formulation of [¹³C]Ro 20-1724 (pH 6.5-8) was established by analytical HPLC (Ecosil C₁₈, 250 x 4.6 mm, 10 µ, CH₃CN/ammonium formate (0.1 M) 40/60 (v/v), 3 ml/min, Rₜ = 3.7 min). Identity of the radioactive product as [¹³C]Ro 20-1724 was determined by co-elution of authentic standard using analytical HPLC.

R/S-[¹³C]ROLIPRAM. R/S-[¹³C]Rolipram was prepared from R/S-desmethyl-rolipram and [¹³C]CH₃I, purified, and analyzed using similar conditions as described above for [¹³C]Ro 20-1724 (semi-preparative HPLC: 7 ml/min, Rₜ = 8 min; analytical HPLC: 4 ml/min, Rₜ = 3 min). Identity of R/S-[¹³C]rolipram was determined by coinjection of authentic R/S-rolipram.

R-[¹³C]ROLIPRAM. R-[¹³C]Rolipram was synthesized, purified, and analyzed using the same procedure as for R/S-[¹³C]rolipram, except that it was prepared from R-desmethyl-rolipram and [¹³C]CH₃I. Identity of R-[¹³C]rolipram was determined by coinjection of authentic R-
rolipram. In addition, radioactive TLC analysis of \( R-[^{11}C]\)rolipram showed one peak with the same \( R_t \) as cospotted authentic \( R/S \)-rolipram (\( R_t \sim 0.5 \), UV). For human studies, formulations were tested for sterility (microbiology laboratory, Toronto General Hospital) and pyrogens (Endosafe kit, Charles River Laboratories, Inc.).

\( S-[^{11}C]\)ROLIPRAM. \( S-[^{11}C]\)Rolipram was prepared from \( S \)-desmethyl-rolipram using the same procedure and HPLC conditions as for \( R/S-[^{11}C]\)rolipram. Identity of \( S-[^{11}C]\)rolipram was determined by coinjection of authentic \( S \)-rolipram.

2.2 Animals

The \textit{in vivo} pharmacological evaluation of the radioligands was performed in male Sprague-Dawley rats (Charles River, Montreal, Canada), and females were also tested in the dosimetry studies. All experiments were conducted in accordance with the recommendations of the Canadian Council on Animal Care and with approval from the Animal Care Committee at the Centre for Addiction and Mental Health. The animals were maintained on a 12-hour light/dark cycle, and housed in groups of two, with food and water \textit{ad libitum}. Acute experiments and dosimetry studies were carried out with rats weighing 200–275g. Chronically treated animals weighed 275–350g on the experiment day, except for those treated with reserpine, which displayed a weight loss (weighing \( \sim 150-180g \) at the end of the experiment). Rat weights different from these ranges are indicated below. For intravenous injections \textit{via} a tail vein, the rats were immobilized in a rodent restrainer (Harvard Apparatus, Canada) and the tail immersed in a warm water bath (\( \sim 45 \) °C) for vasodilation. The dose of \( R/S-[^{11}C]\)Ro 20-1724, \( R/S-, R-, \) or \( S-[^{11}C]\)rolipram administered \textit{via} tail vein was 13.3–66.6 MBq (0.36–1.8 mCi) in \( \sim 0.3 \) ml of the formulations, or as otherwise indicated. For each experiment, all animals received approximately the same mass dose (0.1–0.6 \( \mu \)g/mCi) of the tested radioligand.
2.3 Biodistribution of the Radioligands in Rats

Animals were administered \(^{[1]}\text{C}\)Ro 20-1724, \(R/S\)-, \(R\)-, or \(S\)-\(^{[1]}\text{C}\)rolipram intravenously as described above. Rats were killed by decapitation at 5, 15, 30, 45, 60 or 90 min (\(n = 4\) per time point) after radiotracer injection, except for \(^{[1]}\text{C}\)Ro 20-1724 where the 90 min point was omitted due to a poor signal at earlier time points. A blood sample (~1.5 ml) was collected from the trunk into a heparinized test tube and the brain quickly removed from each rat. The brain was dissected into the following regions: olfactory bulbs, olfactory tubercles, hypothalamus, frontal cortex, cerebellum, hippocampus, striatum, rest of cortex, thalamus, and brain stem. Peripheral organs including the whole heart and a sample of the lung and liver, were also removed. All tissues were washed in saline, blotted, and counted (decay-corrected) in a gamma-counter (Canberra Packard, Cobra II) along with aliquots of the injected solution as standards, and then weighed. Radioactivity remaining in the tails and syringes was counted in a dose-calibrator (Capintec CRC-712 M) and taken into account in the calculation of the injected dose. Radioactivity levels in the different brain and peripheral regions were expressed as percent of injected dose per gram of tissue (%ID/g).

2.4 Competition Studies

2.4.1 Materials

The selective PDE4 inhibitors \(R/S\)-Ro 20-1724, \(R/S\)-, \(R\)-, and \(S\)-rolipram were dissolved in warm (~50 °C) ethanol/1,2-propanediol/0.9% saline 5/20/75 (v/v/v) (Kant et al., 1980). The selective PDE1 inhibitor vinpocetine hydrochloride (purchased from Tocris, MO) was dissolved in warm 0.9% saline and the NA reuptake inhibitor desipramine (DMI) hydrochloride (Sigma-RBI, MA) was dissolved in 0.9% saline at room temperature (pH >4.5).
2.4.2 Blocking with PDE4-selective inhibitors

The pharmacological binding specificity of \( R/S-[^{11}\text{C}]\text{rolipram} \) or \( [^{11}\text{C}]\text{Ro 20-1724} \) uptake was evaluated using a coinjection of unlabeled rolipram (10 mg/Kg) or \( \text{Ro 20-1724} \) (10 mg/Kg with \( [^{11}\text{C}]\text{Ro 20-1724} \) and 30 mg/Kg with \( R/S-[^{11}\text{C}]\text{rolipram} \) via tail vein as described above. Blocking of \( R-[^{11}\text{C}]\text{rolipram} \) binding to PDE4 was assessed by coinjection of unlabeled \( \text{Ro 20-1724} \) (30 mg/Kg), \( R/S-\text{rolipram} \) (10 mg/Kg), \( \text{R-rolipram} \) (0.01–1 mg/Kg), or \( S-\text{rolipram} \) (0.5–30 mg/Kg), with the radiotracer via a tail vein. Competition studies with \( S-[^{11}\text{C}]\text{rolipram} \) were performed using a coinjection of \( \text{Ro 20-1724} \) (30 mg/Kg), \( R-\text{rolipram} \) (0.5 mg/Kg), or \( S-\text{rolipram} \) (3 mg/Kg) with the radioligand, also via a tail vein. For each radioligand formulation, two different test drugs (\( n = 5 \) rats per drug) and a control group of 4 rats (treated with vehicle), or four different drug treatments (\( n = 4 \) each) were tested. Rats were killed at 30 min following \( [^{11}\text{C}]\text{Ro 20-1724} \) or 45 min after \( R/S-, R-, \) and \( S-[^{11}\text{C}]\text{rolipram} \) administration. The peripheral tissue and regional brain distribution of radioactivity were then determined as described for the time course (section 2.3).

2.4.3 Competition with other drugs

Competition with other drugs was performed with the more potent \( R/S- \) and \( R-[^{11}\text{C}]\text{rolipram} \) to rule out the potential binding of these tracers to other specific sites other than PDE4. The less active \( S-[^{11}\text{C}]\text{rolipram} \) was also tested. Rats were administered the PDE1 inhibitor vinpocetine (10 mg/Kg, i.p., 15 min prior to radioligand injection) or the NA transporter blocker DMI (10 mg/Kg, i.p., 30 min prior for \( R/S- \) and \( R-[^{11}\text{C}]\text{rolipram} \), and 4.5 h prior for \( S-[^{11}\text{C}]\text{rolipram} \)) followed by the intravenous injection of the radioligand. Each radioligand formulation was used with two test drugs (\( n = 5 \) rats per drug) and a control group of 4 rats (treated with vehicle). The animals were killed at 45 min following radiotracer injection and the regional brain and
peripheral tissue levels of radioactivity were determined as described for the time course (section 2.3).

2.4.4 Statistics

Statistical analysis was carried out using one-way ANOVA followed by Bonferroni's post-hoc comparisons tests. Since no significant difference was found in the regional brain retention of radioactivity among the different control groups in the competition studies with R/S-, R-, or S-\[^{11}\text{C}\]rolipram, the data from all the controls were pooled for each radioligand (\( n = 16 \) for R/S- or R-\[^{11}\text{C}\]rolipram and \( n = 8 \) for S-\[^{11}\text{C}\]rolipram) and used in the statistical calculations. Statistics with a value of \( p < 0.05 \) were considered significant.

2.5 Metabolite Analysis of \( R-[^{11}\text{C}]\)Rolipram

2.5.1 Solid phase extraction and TLC analysis of metabolites in rat plasma

\( R-[^{11}\text{C}]\)Rolipram metabolism was examined in the plasma of two male rats (~275 g), 30 min following the injection of ~148 MBq (4 mCi; ~0.6 ml of formulation) of the radioligand via a tail vein. Upon decapitation, blood from the trunk (~ 7 ml) was collected into a heparinized tube and assayed immediately for metabolites. The brain was removed and analyzed for metabolites as described below (section 2.5.2). The blood was centrifuged (1,000 X g, 5 min) and a sample (1 ml) of the resulting plasma was mixed with 10% acetic acid in water (3 ml), containing \( R/S\)-rolipram (22.5 \( \mu \)g) as an internal standard. The solution was then passed through a pre-activated C-18 Sep-Pak Plus (Waters Co.) for solid phase extraction (SPE). Hydrophilic polar metabolites were further eluted with 10% acetic acid (3 X 4 ml), while hydrophobic metabolites and the parent compound were eluted with 95% ethanol (4 ml). All eluted fractions, the C-18 Sep-Pak, and samples (1 ml) of the whole blood and plasma were
counted for radioactivity (automated gamma-counter). The organic fraction was evaporated to dryness under vacuum, re-suspended in methanol, and analyzed by TLC. A control experiment was performed with rat blood mixed with ~13 MBq (350 µCi) of authentic R-[¹¹C]rolipram and incubated for 30 min at room temperature, to determine recovery capabilities and validate the procedure.

Plasma protein binding of R-[¹¹C]rolipram was assessed via centrifugation utilizing the Centrifree (Amicon) kit for separation of free from protein bound microsolute (MW cutoff: 30,000). Plasma from control rat containing authentic R-[¹¹C]rolipram was centrifuged (1,000 X g, 30 min) at room temperature, and the resulting filtrate (protein-free fraction) was counted in a gamma-counter.

2.5.2  *TLC analysis of metabolites in rat brain*

The brain from the rats was removed upon decapitation and kept over ice in a test-tube containing 10 ml ethanol (80% in water). Brains were homogenized (Polytron) in ice-cold ethanol (80% in water, 10 ml) containing R/S-rolipram (40 µg), and then centrifuged (82,000 X g, 15 min). Approximately 17 MBq (450 µCi) of authentic R-[¹¹C]rolipram was added to a control brain prior to homogenization. The supernatant was evaporated to dryness, resuspended in acetonitrile (100 µl), and then analyzed by TLC as described above.

2.6  *Dosimetry and Whole Body Biodistribution Studies of R-[¹¹C]Rolipram*

2.6.1  *Measurement of whole body distribution of activity*

Biodistribution studies were performed for the more potent R-[¹¹C]rolipram only. Two male and female rats were used for each data point. Male rats were anesthetized with diethyl ether and the penis ligated. Approximately 44 MBq (1.2 mCi) of the radioligand was injected into a
tail vein and the animals were then sacrificed by decapitation at 5, 15, 30, or 60 min after injection. A blood sample (~1.5 ml) was obtained from the trunk. Urine was collected from the male rats by exposing the bladder and using a syringe. The following whole tissues were then dissected out: pituitary, eyes, ascending, transverse, and descending large intestine, small intestine, testicles, ovaries, heart, lung, spleen, adrenals, stomach, bone, muscle, fat, bladder, blood, brain, urine, liver, kidneys, and carcass. Gastro-intestinal contents were also collected. Tissues were washed in saline, blotted, and counted for $^{11}$C activity in a gamma-counter (decay-corrected) along with aliquots of the injected solution as standards, and then weighed. Radioactivity remaining in the tails, syringes, and carcass was counted in a dose-calibrator and taken into account in the calculation of the injected dose. Data were calculated as % injected dose/tissue (%ID/tissue) and %ID/g of tissue.

2.6.2 Dosimetry calculations

The expected human dosimetry for $R$-$[^{11}]$rolipram was calculated using the rat whole body distribution data, following the MIRD formalism (Loevinger et al., 1988). The rat %ID/g values in the selected source organs, including the brain, lungs, kidneys, spleen, and liver, were modified to reflect human values based on the different proportions of organ to total body mass in rat and human. The contents of the small and large intestine were added together and measured. Because of the short physical half-life of $^{11}$C compared to the mean rate of transport through the human small intestine (Eve, 1966), the total radioactivity in the gastro-intestinal tract was assigned to the small intestine in the calculation. Other organs were examined to rule out unexpected high uptake by those organs but the data were not used in the final dosimetry calculations. Residence times were obtained by integration under the organ time vs. activity curves, with the effective half-life of $R$-$[^{11}]$rolipram assumed to be equal to the physical half-life of $^{11}$C for times exceeding the last data point (60 min). Residence times were entered into
the MIRDOSE 3.1 program (Stabin, 1996) to obtain the absorbed doses to selected target organs per unit of administered activity.

2.7 Regulation of R-[\textsuperscript{11}C]Rolipram Binding in Rat Brain Following Diverse \textit{In vivo} Drug Treatments

2.7.1 Materials

The regulation of R-[\textsuperscript{11}C]rolipram \textit{in vivo} binding to PDE4 was assessed using treatments of the following drugs dissolved in 0.9% sterile saline or as otherwise indicated below (pH >4.5): DMI hydrochloride (RBI, MA), forskolin dihydrochloride (Calbiochem, CA), S-(-)-propranolol hydrochloride (Sigma, MO), clenbuterol hydrochloride (Sigma, MO), CGS 21680 hydrochloride (RBI, MA) dissolved in 5/95 (v/v) ethanol/0.9% saline, thioperamide maleate (RBI, MA), yohimbine hydrochloride (RBI, MA) dissolved in 5/20/75 ethanol/1,2-propanediol/0.9% saline (v/v/v), clonidine hydrochloride (RBI, MA), SKF 81297 hydrobromide (a kind gift from SmithKline and Beecham, PA) dissolved in 5/20/75 ethanol/1,2-propanediol/0.9% saline (v/v/v), fluoxetine hydrochloride (RBI, MA) dissolved in 5/95 ethanol/0.9% saline (v/v), tranylcypromine hydrochloride (RBI, MA), (±)-8-hydroxy-2-(di-n-aminopropyl)tetralin (8-OH-DPAT) hydrobromide (RBI, MA), WAY 100635 dihydrochloride (produced in-house by Dr. Alan Wilson), (±)-2,5-dimethoxy-4-iodophenyl-2-aminopropane (DOI) hydrochloride (RBI, MA), ritanserin hydrochloride (RBI, MA) dissolved in 5/20/75 ethanol/1,2-propanediol/0.9% saline (v/v/v), cycloheximide (Tocris, MO) dissolved in 5/20/75 ethanol/1,2-propanediol/0.9% saline (v/v/v), HA 1004 hydrochloride (RBI, MA), N-2-chloroethyl-N-ethyl-2-bromobenzylamine (DSP-4) hydrochloride (Sigma, MO or RBI, MA), reserpine (RBI, MA) dissolved in 0.6% glacial acetic acid in sterile water, and zimelidine dihydrochloride (RBI, MA).
Materials for determining the \( K \) and \( B_{\text{max}} \) of \( R/S-[^3]H \)rolipram binding were obtained from commercial sources (e.g., Fisher, Sigma, Beckman, RBI, Skatron). \( R/S-[^3]H-\text{methyl} \)Rolipram (250 \( \mu \)Ci, 85 Ci/mmol) was purchased from Amersham Pharmaceuticals (USA). Homogenization buffer (buffer A) consisted of 50 mM Tris-HCl, 5 mM MgCl\(_2\), 0.1 mM dithiothreitol, and protease inhibitor cocktail (Boehringer Ingelheim), at pH \( \sim 7.5 \). The reaction buffer contained 50 mM of Tris-HCl, 5 mM of MgCl\(_2\), 50 \( \mu \)M of 5'-AMP, and 0.05% bovine serum albumin, at pH \( \sim 7.5 \).

2.7.2 Acute treatments

To assess the ability of \( R-[^{13}]C \)rolipram to detect rapid changes in the regulation of PDE4 in the brain, rats were treated acutely with different drugs affecting neurotransmitter systems at different sites.

AC ACTIVATION The time-dependent effect of the AC activator forskolin (6.5 mg/Kg, i.v., coinjection or 15 min prior to kill, or 15 mg/Kg, i.v., 3 h prior to radioligand injection, \( n = 5 \) per group) was executed to assess the effect of increasing cAMP levels directly.

NORADRENERGIC SYSTEM Challenges to modulate the noradrenergic system included: 1) time- (10 mg/Kg, i.p., 30 min, 1.25, 4.5, or 9 h prior to radioligand injection, \( n = 5 \) per group except 4.5 h prior time point where \( n = 9 \)) and dose-dependent (5 or 10 mg/Kg, i.p., 30 min prior; or 10 or 25 mg/Kg, i.p., 4.5 h prior, \( n = 5 \) per group except 4.5 h prior time point where \( n = 9 \)) treatments with DMI; 2) time-dependent administration of the \( \alpha_2 \)-adrenergic antagonist yohimbine (10 mg/Kg, i.p., 15 min, 1.25, or 3 h prior, \( n = 5 \) per group); 3) dose-dependent effect of the \( \alpha_2 \)-adrenergic agonist clonidine (0.1 or 1 mg/Kg, i.p., 3 h prior, \( n = 4 \) or 9, respectively); 4) treatment with the \( \beta \)-adrenergic antagonist propranolol (20 mg/Kg, i.v., 5 min prior, \( n = 5 \); or i.p., 3 h prior, \( n = 4 \)); and 5) treatment with the selective \( \beta_2 \)-adrenoceptor agonist clenbuterol (10 mg/Kg, i.p., 3 h prior).
SEROTONERGIC SYSTEM  The effect of the 5-HT system on \textit{R-[13}C\textit{]rolipram uptake in rat brain was determined using the following treatments: 1) the 5-HT reuptake inhibitor fluoxetine (5 mg/Kg, i.p., 3 h prior, \( n = 9 \)); 2) the 5HT1A agonist 8-OH-DPAT (1 mg/Kg, i.p., 3 h prior, \( n = 5 \)); 3) the 5HT1A antagonist WAY 100635 (3 mg/Kg, i.p., 3 h prior, \( n = 5 \)); 4) the 5HT2A/C agonist DOI (5 mg/Kg, i.p., 3 h prior, \( n = 5 \)); and 5) the 5HT2A/C antagonist ritanserin (2.5 mg/Kg, s.c., 4 h prior, \( n = 5 \)).

MONOAMINE OXIDASE INHIBITION  The effect of inhibiting monoamine metabolism was studied using the MAO inhibitor tranylcypromine (10 mg/Kg, i.p., 3 h prior, \( n = 5 \)).

ADENOSINE, HISTAMINE, AND DOPAMINE RECEPTORS  To study the regulation of \textit{R-[13}C\textit{]rolipram binding to PDE4 in the adenosine-A2, histamine-H3, and dopamine-D1 systems, respectively, treatments were performed with: 1) the adenosine-A2a agonist CGS 21680 (2 mg/Kg, i.p., 3 h prior, \( n = 9 \)); 2) the histamine H3-antagonist thioperamide (10 mg/Kg, i.p., 4 h prior, \( n = 9 \)); and 3) the dopamine-D1 agonist SKF 81297 using two time points (10 mg/Kg, i.p., 5 min or 3 h prior, \( n = 9 \) and \( n = 10 \), respectively).

PKA AND PROTEIN SYNTHESIS INHIBITION  Acute pretreatment with the PKA inhibitor HA 1004 or the protein synthesis inhibitor cycloheximide was performed to inhibit PKA-mediated phosphorylation and \textit{de novo} protein synthesis of PDE4, respectively. HA 1004 (20 mg/Kg) was administered 45 min prior to DMI (10 mg/Kg, i.p., 4.5 h prior to radiotracer injection, \( n = 5 \)), forskolin (6.5 mg/Kg, i.v., coinjection, \( n = 5 \)), or yohimbine (10 mg/Kg, i.p., 15 min prior, \( n = 5 \)). Cycloheximide (20 mg/Kg) was injected 30 min prior to CGS 21680 (2 mg/Kg, i.p., 3 h prior to radiotracer injection, \( n = 5 \)).

For each formulation of \textit{R-[13}C\textit{]rolipram, two drug treatments (\( n = 5 \)) and a control group (\( n = 4 \)) were tested. The rats were then injected intravenously with the radioligand and sacrificed 45 min later. The concentration of radioactivity was measured in several brain regions and expressed as \%\textit{ID/g} of tissue as described before, then multiplied by rat body weight.
(\%IDBW/g) to normalize for differences in body weight, in order to make comparisons between different drug treatments. Final data were calculated as the percentage change from the control group, which consisted of the pooled acute controls.

2.7.3 Chronic treatments

Different chronic studies hypothesized to increase or decrease the expression of PDE4 enzymes in the brain were performed to determine if R-[\textsuperscript{14}C]rolipram can detect changes in the density of PDE4 enzymes.

PDE4 UPREGULATION Treatments performed to increase PDE4 expression included the antidepressants DMI (10 mg/Kg, i.p., twice daily, 14 days, \(n = 14\)), fluoxetine (5 mg/Kg, i.p., once daily, 14 days, \(n = 15\)), and tranylcypromine (7 mg/Kg, i.p., once daily, 7 days, followed by 10 mg/Kg, i.p., once daily, 7 days, \(n = 15\)), the 5HT\textsubscript{1A} agonist 8-OH-DPAT (1 mg/Kg, s.c., 14 days, \(n = 7\)), and the \(\beta\textsubscript{2}\)-agonist clenbuterol (10 mg/Kg, i.p., 14 days, \(n = 7\)).

PDE4 DOWNREGULATION Treatments expected to decrease PDE4 expression included the \(\beta\)-antagonist propranolol (20 mg/Kg, i.p., 14 days, \(n = 14\)), the vesicle depleting agent reserpine (1 mg/kg, s.c., 2 days, followed by 0.5 mg/Kg, s.c., 10 days, 24 h washout, \(n = 3\); or 1 mg/Kg, s.c., 5 days, 2 or 24 h washout, \(n = 10\) or 9, respectively), and the noradrenergic lesioning agent DSP-4 (50 mg/Kg, i.p.; 1 injection, with or without zimelidine, 5 mg/Kg, 30 min prior, 14 day washout; or 50 mg/Kg, i.p., 2 injections, 7 days apart, no zimelidine; or 50 mg/Kg, i.p., 1 injection, no zimelidine, 7 day washout, \(n = 7\) for each group except for the rats receiving two DSP-4 injections where \(n = 5\)). Groups of 7 rats and 7 controls (injected with vehicle) were tested with each radioligand formulation. R-[\textsuperscript{14}C]Rolipram was injected 24 hours following the last treatment or as otherwise indicated in brackets above, and animals were sacrificed 45 min later. Brain regions were dissected out and assayed for radioactivity accumulation expressed as
%IDBW/g as described above. Final data were calculated as the percentage change from the control group, which consisted of the pooled chronic controls.

2.7.4 Determination of in vivo R-[11C]rolipram specific binding following selected acute and chronic drug challenges

Selected acute and chronic drug treatments that produced a significant change in regional brain radioactivity uptake were further studied to determine the amount of specific binding by the radioligand. Acute treatments were repeated as above with yohimbine (10 mg/Kg, i.p., 3 h prior to radioligand injection), clonidine (1 mg/Kg, i.p., 3 h prior), tranylcypromine (10 mg/Kg, i.p., 3 h prior), CGS 21680 (2 mg/Kg, i.p., 3 h prior), forskolin (6.5 mg/Kg, i.v., coinjection), DMI (10 mg/Kg, i.p., 4.5 h prior), fluoxetine (5 mg/Kg, i.p., 3 h prior), and thioperamide (10 mg/Kg, i.p., 4 h prior). Chronic treatments were carried out with the antidepressants DMI (10 mg/Kg, i.p., twice daily, 14 days) and fluoxetine (5 mg/Kg, i.p., once daily, 14 days) as above. The acute (n = 4 per treatment) or chronically (n = 7 per treatment) treated animals were injected intravenously with R-[11C]rolipram and a blocking dose of R-rolipram (1 mg/Kg) to saturate the PDE4 sites and thus allow the measurement of non-specific binding of the radioligand, and the rats were then sacrificed 45 min later. The regional brain accumulation of radioactivity was calculated as %IDBW/g as described above. The specific binding in brain regions for either control or drug treated groups was calculated by subtracting the non-specific binding (radioactivity levels in rats coinjected with unlabeled R-rolipram) from the total binding (R-[11C]rolipram administration alone). The percentage change in specific binding elicited by the drug treatment as compared to controls was then calculated using the following formula:

\[
\text{%Change in Specific binding} = \left( \frac{\text{Specific binding in drug group} - \text{Specific binding in control group}}{\text{Specific binding in control group}} \right) \times 100
\]
2.7.5 **Plasma levels of R-[\textsuperscript{11}C]rolipram following selected acute treatments**

To assess the effect of selected treatments described in section 2.7.4 on radioligand plasma levels and metabolism, the percentage of plasma unmetabolized R-[\textsuperscript{11}C]rolipram was measured at 45 min following radioligand injection in rats treated acutely with DMI, fluoxetine, and forskolin as in section 2.7.4, using two rats (275–350 g) per tested drug. Two control rats were administered saline. The animals were injected with 155–192 MBq (4.2–5.2 mCi) of R-[\textsuperscript{11}C]rolipram, sacrificed 45 min later, and whole blood (~7 ml) was collected from the trunk into heparinized test tubes. A control experiment was performed with rat blood mixed with 19.8 MBq (534 µCi) of authentic R-[\textsuperscript{11}C]rolipram and incubated for 45 min in a warm water bath at 36 °C, to confirm the recovery capabilities and validate the procedure. The percentage of radiolabeled metabolites and unchanged R-[\textsuperscript{11}C]rolipram was then measured using SPE and TLC as described above for the metabolism studies (section 2.5.1).

2.7.6 **Validation of selected in vivo results by measurement of $K_d$ and $B_{\text{max}}$ with R/S-[\textsuperscript{3}H]rolipram**

The $K_d$ and $B_{\text{max}}$ of R/S-[\textsuperscript{3}H]rolipram binding to rat frontal cortex was determined following the acute and chronic treatment with DMI and fluoxetine as described in section 2.7.4. above. On the kill day, rats ($n = 8$ per drug or vehicle treatment) were immobilized in a rodent restrainer and injected with saline, instead of radioligand, via a tail vein as described in the in vivo pharmacological binding studies to mimic the stress conditions in those experiments. The animals were decapitated 45 min later and their brain removed immediately. The frontal cortex was rapidly dissected on ice, weighed, frozen in liquid N\textsubscript{2}, and stored at ~80°C until assayed. The tissue was homogenized in 10 parts (v/w) buffer A by a Polytron (Brinkman) homogenizer. Separation of supernatant and particulate (crude membrane) fractions was achieved by 30 min 35,500 X g centrifugation at 4 °C, with one washing step of the membrane fraction (pellet) by resuspension in buffer A with the polytron, and recovered by centrifugation as before. Protein
concentration was measured employing a microplate reader in a modification of the protein-dye method of Bradford (Bradford, 1976). Incubations were performed in glass test-tubes in a total volume of 0.1 ml of reaction buffer with 0.2-15 nM \( R/S-[^{3}H] \) rolipram and 10 \( \mu l \) of supernatant or pellet corresponding to 0.2-0.6 mg of protein/ml (added last), at 23 °C for 1 h. Non-specific binding was defined by the presence of 1 \( \mu M \) \( R/S \)-rolipram in the incubation medium. Reactions were stopped and membrane-bound tracer was separated from free by rapid filtration and washing of the glass fiber filter mats using ice-cold buffer A in a Skatron Cell Harvester. Filters were presoaked with 0.3% polyethylenimine in buffer A for at least two hours and allowed to dry before use. Holes were punched out for each individual test tube and the filters were shaken for ~1 h with 5 ml of scintillation cocktail (Sigma-Fluor) before determination of radioactivity by liquid scintillation spectrometry (Beckman LS5000CE counter). Specific binding was determined by subtracting the non-specific from the total. Data was analyzed by non-linear regression using the GraphPad Prism (version 3.02) program to obtain the \( B_{max} \) and \( K_d \).

2.7.7 Statistics

Statistical analysis of the in vivo modulation studies was carried out using MANOVA followed by Bonferroni's post-hoc comparisons tests. Since no significant difference was found in the regional brain retention of radioactivity among the different control groups in the acute or chronic studies, the data from all the controls were pooled (\( n = 44 \) for the acute and \( n = 62 \) for the chronic studies) and used in the statistical calculations. Analysis of the in vitro results was performed using the Student's T-test or One-Way ANOVA with Bonferroni's comparisons test. Statistics with a value of \( p < 0.05 \) were considered significant. Some studies demonstrated \( p \) values slightly above 0.05 and repeating them with more rats could render the results significant.
2.8 PET Imaging of $R-[^{11}C]$Rolipram in Normal Human Volunteers

2.8.1 PET imaging

Human PET imaging studies were conducted using $R-[^{11}C]$rolipram, following approval by the University of Toronto Human Subjects Review Committee. A total of 12 normal human volunteers, 4 females and 8 males age range 20–51 years old, participated in PET studies with either venous or arterial blood sampling after giving written informed consent (Table II). Subjects were screened for history of significant systemic diseases, psychiatric illness (modified SCID), head injury, alcohol or substance abuse, and current use of medication. Additional exclusion criterion included pregnant women and subjects under 18 years of age. A 10 min transmission scan was obtained with a rotating $^{68}$Ge pin source prior to the PET scan to correct for attenuation of skull tissues and bones. Approximately 370 MBq (~10 mCi in 10 ml) of $R-[^{11}C]$rolipram was injected intravenously, using a PHD2000 Harvard Apparatus syringe pump at 30 ml/min (average specific activity was 1375 ± 433 Ci/mmol, which corresponds to ~2 μg of $R$-rolipram). Sequential images of the brain (one image per min for the first 15 min, then 15 X 5 min frames, for a total of 30 frames) were acquired over a 90 min period following radioligand injection, using a GEMS PC2048-15B brain PET scanner (15 slices, resolution 4.5 X 4.5 X 4.5 mm FWHM in air, with 6.5 mm inter-slice separation). Following acquisition, the images were corrected for attenuation and reconstructed by filtered back-projection (Hann filter, 5 mm) (Houle et al., 1997).

2.8.2 Image analysis

In the preliminary analysis of the PET images, regions of interest (ROIs) were drawn onto the PET scans and decay-corrected time-activity curves were constructed for the pre-frontal cortex, thalamus, striatum, and cerebellum. Each subject also underwent a magnetic resonance
Table II  Human volunteer age, gender, and study completed.

<table>
<thead>
<tr>
<th>AGE</th>
<th>GENDER</th>
<th>STUDY</th>
</tr>
</thead>
<tbody>
<tr>
<td>23</td>
<td>F</td>
<td>Venous</td>
</tr>
<tr>
<td>23</td>
<td>F</td>
<td>Venous</td>
</tr>
<tr>
<td>31</td>
<td>F</td>
<td>Venous</td>
</tr>
<tr>
<td>33</td>
<td>M</td>
<td>Venous</td>
</tr>
<tr>
<td>36</td>
<td>M</td>
<td>Venous</td>
</tr>
<tr>
<td>42</td>
<td>M</td>
<td>Venous</td>
</tr>
<tr>
<td>20</td>
<td>M</td>
<td>Arterial</td>
</tr>
<tr>
<td>22</td>
<td>M</td>
<td>Arterial</td>
</tr>
<tr>
<td>24</td>
<td>F</td>
<td>Arterial</td>
</tr>
<tr>
<td>29</td>
<td>M</td>
<td>Arterial</td>
</tr>
<tr>
<td>49</td>
<td>M</td>
<td>Arterial</td>
</tr>
<tr>
<td>51</td>
<td>M</td>
<td>Arterial</td>
</tr>
</tbody>
</table>
imaging (MRI) scan (GEMS Signa 1.5 tesla scanner spin-echo sequence T_{2}-weighed image) for the anatomical positioning of the ROIs in future R-[^{13}C]rolipram PET image analysis using pharmacokinetic modeling.

2.8.3 Analysis of metabolites in human venous and arterial plasma

Arterial sampling PET studies were performed in order to determine the kinetic parameters for the derivation of a kinetic model describing the binding of R-[^{13}C]rolipram in the brain. The input function for the kinetic model is the free arterial plasma radioligand concentration, corrected for labeled metabolites, as a function of time postinjection. Subjects (1 female and 5 males, age range 20–51) underwent one PET study with arterial blood sampling over the course of 90 min, followed by a MRI scan, as described above. Blood samples (~7 ml) were obtained through an arterial line inserted in the radial artery by an anesthesiologist. Local anesthesia was used in inserting the catheter to minimize subject discomfort.

Venous sampling PET studies were performed in order to characterize the metabolic profile of the radioligand and determine the possibility of using venous, as opposed to arterial, plasma for pharmacokinetic modeling. Subjects (3 females and 3 males, age range 23–42) underwent one PET study with venous blood sampling over the course of 90 min, followed by a MRI scan, as described above. Blood samples (~7 ml) were obtained from a vein in the arm.

The arterial and venous plasma were obtained by centrifugation (1,000 X g, 5 min) of arterial and venous whole blood samples (~7 ml), respectively, collected from the subjects at –5, 2 (venous only), 5, 10, 15, 20, 30, 45, 60, and 90 min after radioligand injection. Analysis of radiolabeled metabolites was performed using the same procedure as the one employed for rat plasma (section 2.5.1). A control experiment was carried out with the blood sample collected 5 min prior to injection of the radioligand. The blood was mixed with ~18.5 MBq (500 μCi) of
authentic \textit{R-}\textsuperscript{[\textsuperscript{14}\textit{C}]rolipram} and incubated for 90 min at room temperature, to assess the recovery capabilities and validate the procedure.
CHAPTER 3.0: RESULTS

3.1 Synthesis and Purification of Precursors

Selective dealkylation of Ro 20-1724 was carried out with iodos(trimethyl)silane (Scheme 1), followed by decomposition of the silane-complex and EtOAc recrystallization to give desmethyl-Ro 20-1724 in high yields (>90%). In contrast, dealkylation of R/S-rolipram with iodos(trimethyl)silane (Scheme 2) was not selective and yielded the desmethyl, descyclopentyl, and catechol derivatives. The putative desmethyl and catechol derivatives were analyzed by $^1$H-NMR and showed the absence of the methyl group in the former, and both the methyl and cyclopentyl groups in the latter (data not shown). Successive silica gel column chromatography purifications were necessary to separate the phenolic derivatives from unreacted racemic rolipram. Both R- and S-desmethyl-rolipram were isolated by successive chiral semi-preparative HPLC (Scheme 2) in pure form (>99% ee), as established by chiral analytical HPLC.

3.2 Radiochemistry

$[^{13}]$C]Ro 20-1724, and R-, R/S-, and S-$[^{13}]$C]rolipram were prepared by O-$[^{13}]$C]methylation of the corresponding phenolic precursors with $[^{13}]$C]CH$_3$I in the presence of TBAOH (Scheme 1 and 2) in high radiochemical yields (45-75%, decay-corrected, based on $[^{13}]$C]CH$_3$I), purity (>99%), and specific activity (18.5-92.5 GBq/µmol or >500-2,500 mCi/µmol, at end-of-synthesis), in a synthesis time of 30 min (including quality control assays). Final product formulations were sterile and pyrogen-free. Chemical and radiochemical purity as well as specific activity of each radiotracer were determined by analytical HPLC (see Fig. 7 and 8 for representative HPLC
Scheme 1 Demethylation of Ro 20-1724 and radiosynthesis scheme of $[^{11}\text{C}]\text{Ro }20-1724$. 
Scheme 2 Demethylation of \( R/S \)-rolipram, enantiomeric separation of \( R \)- and \( S \)-desmethyl-rolipram and radiosynthesis scheme of \( R/S^- \), \( R^- \), and \( S^-[^{11}C] \)rolipram.
Fig. 7 HPLC chromatograms of a representative $[^{11}]$C Ro 20-1724 radiosynthesis run. Reaction products were separated using semi-preparative HPLC and the peak corresponding to Ro 20-1724 UV (panel a) and radioactivity (panel c) was collected into a sterile vial. A sample (100 µl) of the final formulation containing $[^{11}]$C Ro 20-1724 was analyzed for quality control by analytical HPLC, showing UV peaks (panel b) at the solvent front and one corresponding to authentic Ro 20-1724, and one radioactivity peak (panel d) with the same retention time as Ro 20-1724.
Fig. 8 HPLC chromatograms of a representative R-[\textsuperscript{11}C]rolipram radiosynthesis run. Reaction products were separated using semi-preparative HPLC and the peak corresponding to rolipram UV (panel a) and radioactivity (panel c) was collected into a sterile vial. A sample (100 \mu l) of the final formulation containing R-[\textsuperscript{11}C]rolipram was analyzed for quality control by analytical HPLC, showing UV peaks (panel b) at the solvent front and one corresponding to authentic R-rolipram, and one radioactivity peak (panel d) with the same retention time as R-rolipram.
chromatograms of [11C]Ro 20-1724 and R-[11C]rolipram), which demonstrated a single radioactive peak corresponding to the desired radioligand. Similar results were obtained for R/S- and S-[11C]rolipram. Attempts to produce R- and S-[11C]rolipram using less precursor (0.3 mg) and 66% less base were successful and yielded the desired radioligands in similar yields, purity, and specific activities in comparison to reactions using 1 mg of precursors.

3.3 Biodistribution Studies in Rats

3.3.1 Time course of [11C]Ro 20-1724 and R/S-[11C]rolipram

Gradual washout of radioactivity vs. time was observed in all brain areas with [11C]Ro 20-1724 (Fig. 9A) and R/S-[11C]rolipram (Fig. 9B). [11C]Ro 20-1724 exhibited low regional brain uptake and 26-50% lower retention of radioactivity in brain regions as compared to blood (Fig. 9A). A higher regional brain retention (>3 fold) was observed with R/S-[11C]rolipram as compared to [11C]Ro 20-1724, even though Ro 20-1724 is slightly more lipophilic than rolipram (calculated log P values: 1.83 and 1.36, respectively, using Clog P program, BioByte Corp.). R/S-[11C]Rolipram revealed a higher uptake of radioactivity in areas previously reported to have a higher density of PDE4 enzymes, such as the frontal cortex (% ID/g = 0.57 ± 0.12), and lower in the brain stem (lower in PDE4 expression; % ID/g = 0.26 ± 0.06), 45 min postinjection. In comparison to [11C]Ro 20-1724, R/S-[11C]rolipram showed a higher radioactivity accumulation in the brain, heart, and lung, and lower retention in the liver (Fig. 10).

3.3.2 Time course of R-[11C]rolipram and S-[11C]rolipram

The more active enantiomer R-[11C]rolipram was synthesized and tested in vivo in rats in order to increase the signal obtained in the brain with R/S-[11C]rolipram. Following i.v. injection of R-[11C]rolipram, regional brain radioactivity uptake was rapid, peaking within 5 min
Fig. 9 Time course of A) $[^{11}\text{C}]$Ro 20-1724 and B) $R/S-[^{11}\text{C}]$rolipram in rat brain regions. Radioligands were injected via tail vein and the animals sacrificed at different time points. Data are mean % injected dose/g of tissue (wet weight) ± SD (CTX: cortex; n = 4 per time point).
Fig. 10 Time course of A) $[^{11}]C$Ro 20-1724 and B) $R/S-[^{11}]C$rolipram in selected organs. Radioligands were injected via tail vein and the animals sacrificed at different time points. Data are mean %injected dose/g of tissue (wet weight) ± SD (CTX: cortex; n = 4 per time point).
As with the racemic form, the frontal cortex and brain stem demonstrated the highest and lowest concentration of radioactivity, respectively, throughout the time window studied (e.g., %ID/g = 0.83 ± 0.13 in the frontal cortex and 0.37 ± 0.04 in the brain stem, at 45 min). The distribution of \( R-[^{11}C] \)rolipram in rat brain regions agreed with the \textit{in vitro} binding of \( R/S-[^{3}H] \)rolipram as published by Schneider and colleagues (1986), with low values present in the hypothalamus and brain stem and high values in cortical and olfactory regions (Fig. 12). Of all the organs sampled, the brain retained the highest quantities of radioactivity, followed by the liver, lung, and then the heart (Fig. 11B). Activity levels were lower in blood as compared to the other tissues sampled (Fig. 11B). \( S-[^{11}C] \)Rolipram was tested in order to determine if it can be used to measure \textit{non-specific binding} of \( R-[^{11}C] \)rolipram in human PET studies. \( S-[^{11}C] \)Rolipram regional brain retention decreased rapidly to levels below those measured in blood, demonstrating a very low regional brain selectivity (Fig. 13A). \( S-[^{11}C] \)Rolipram brain uptake was lower than liver, lung, and heart (Fig. 13B). Table III describes the ratio of the mean regional brain uptake of \( R-[^{11}C] \)rolipram to the other radiotracers investigated at 45 min postinjection. The regional brain retention of \( R-[^{11}C] \)rolipram was 2.5–4.6 and 1.5–1.6 fold higher than \( [^{11}C] \)Ro 20-1724 and \( R/S-[^{11}C] \)rolipram, respectively. In comparison to \( S-[^{11}C] \)rolipram, the retention of \( R-[^{11}C] \)rolipram in brain regions was 5-10 fold higher (Table III).

### 3.4 Competition Studies

#### 3.4.1 Competition of \( [^{11}C] \)Ro 20-1724, \( R/S, \) and \( R-[^{11}C] \)rolipram with PDE4-selective inhibitors

The regional brain uptake of the radioligands at early time points (up to 10 min) reflects mostly blood flow rather than specific binding to PDE4. However, \( ^{11}C \) has a short physical half-life (20.4 min), such that later time points (after 60 min) incur a higher error due to noise from lower radioactivity levels. Thus, to obtain meaningful results in the competition studies, a time
Fig. 11 Time course of \( R-[^{11}C] \)rolipram in A) rat brain regions and B) selected organs. Radioligand was injected via tail vein and the animals sacrificed at different time points. Data are mean %injected dose/g of tissue (wet weight) ± SD (CTX: cortex; \( n = 4 \) per time point).
Fig. 12 Comparison of regional brain $R$-[$^{11}$C]Rolipram uptake with $R/S$-[$^3$H]Rolipram binding in rats. $R$-[$^{11}$C]Rolipram distribution data are the bank of controls ($n = 44$) at 45 minutes postinjection, normalized for body weight. $R/S$-[$^3$H]Rolipram maximal binding values are from Schneider et al. (1986).
Fig. 13 Time course of $S-^{[11}C$rolipram in A) rat brain regions and B) selected organs. Radioligand was injected via tail vein and the animals sacrificed at different time points. Data are mean %injected dose/g of tissue (wet weight) ± SD (CTX: cortex; n = 4 per time point).
Table III  Ratio of the distribution of \( R-[\text{^{13}C}]\)rolipram to \( [\text{^{13}C}]\)Ro 20-1724, \( R/S-\), and \( S-[\text{^{13}C}]\)rolipram in rat brain regions.

<table>
<thead>
<tr>
<th>REGION</th>
<th>Ratio Over [\text{^{13}C}]Ro 20-1724$^a$</th>
<th>Ratio Over ( R/S-[\text{^{13}C}])Rolipram$^a$</th>
<th>Ratio Over ( S-[\text{^{13}C}])Rolipram$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal Cortex</td>
<td>4.6</td>
<td>1.6</td>
<td>10</td>
</tr>
<tr>
<td>Rest of Cortex</td>
<td>4.6</td>
<td>1.6</td>
<td>10</td>
</tr>
<tr>
<td>Olfactory Bulb</td>
<td>4.6</td>
<td>1.6</td>
<td>9</td>
</tr>
<tr>
<td>Striatum</td>
<td>4.3</td>
<td>1.5</td>
<td>9</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>3.8</td>
<td>1.5</td>
<td>8</td>
</tr>
<tr>
<td>Olfactory Tubercle</td>
<td>4.0</td>
<td>1.6</td>
<td>8</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>3.9</td>
<td>1.6</td>
<td>8</td>
</tr>
<tr>
<td>Thalamus</td>
<td>3.9</td>
<td>1.6</td>
<td>8</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>3.3</td>
<td>1.5</td>
<td>6</td>
</tr>
<tr>
<td>Brain Stem</td>
<td>2.5</td>
<td>1.6</td>
<td>5</td>
</tr>
<tr>
<td>Blood</td>
<td>0.8</td>
<td>1.1</td>
<td>2</td>
</tr>
</tbody>
</table>

$^a$ Ratio was calculated as: mean %ID/g of tissue (wet weight) of \( R-[\text{^{13}C}]\)rolipram $\div$ mean %ID/g of tissue (wet weight) of \( [\text{^{13}C}]\)Ro 20-1724, \( R/S-\) or \( S-[\text{^{13}C}]\)rolipram. Values of \( [\text{^{13}C}]\)Ro 20-1724 are at 30 min postinjection, and \( R-,\ R/S-,\) and \( S-[\text{^{13}C}]\)rolipram at 45 min postinjection.
point was chosen that showed a high regional brain uptake of radioactivity and tissue contrast following injection of the radioligands. Specifically, the 30 min or 45 min time point following injection of $[^{11}C]Ro 20-1724$ (Fig. 9A) or $R/S-$ (Fig. 9B) and $R-[^{11}C]rolipram$ (Fig. 11A), respectively, displayed a better tissue contrast and was chosen for the competition studies. Co-administration of $R/S$-rolipram at a high dose as used previously (10 mg/Kg, i.v.) (Kehr et al., 1985; Schmiechen et al., 1990) significantly reduced $[^{11}C]Ro 20-1724$ binding in brain regions, whereas the same dose of unlabeled Ro 20-1724 (10 mg/Kg, i.v.) had only a small effect (Fig. 14A). $R/S-[^{11}C]Rolipram$ uptake was significantly blocked in all brain regions following the administration of unlabeled $R/S$-rolipram (10 mg/Kg, i.v.) or a higher dose of Ro 20-1724 (30 mg/Kg, i.v.) as published previously (Kant et al., 1980) (Fig. 14B). The reduction in radioactivity was highest in the frontal cortex (85%) and lowest in the brain stem (61%). The uptake of $R/S-[^{11}C]rolipram$ was also reduced in organs known to express high-affinity $R/S-[^{3}H]rolipram$ binding sites other than the brain, such as the heart (significant) and lung, while $[^{11}C]Ro 20-1724$ retention was unaffected (Fig. 15) (Schneider et al., 1986). Due to its poor brain penetration, signal, and tissue contrasts, $[^{11}C]Ro 20-1724$ was not developed further.

Similar to $R/S-[^{11}C]rolipram$, coinjection with $R/S$-rolipram or Ro 20-1724 reduced $R-[^{11}C]rolipram$ retention to non-specific levels across brain regions (Fig. 16A). The binding reduction was also highest in the PDE4-rich frontal cortex (92%) and lowest in the brain stem (78%). Both $R/S$-rolipram and Ro 20-1724 significantly decreased tracer levels in the heart and the liver demonstrated a significant increase in activity levels with $R/S$-rolipram cotreatment (Fig. 16B).

### 3.4.2 Dose-dependent effect of R- and S-rolipram on $R-[^{11}C]rolipram$ uptake

$R-[^{11}C]Rolipram$ binding was further characterized using increasing doses of $R$- or $S$-rolipram in order to determine the effective dose required to block $R-[^{11}C]rolipram$ specific
Fig. 14  Rat regional brain distribution of A) $[^{11}C]$Ro 20-1724 and B) $R/S-[^{11}C]$rolipram 30 and 45 min, respectively, following i.v. co-injection with the competitors rolipram or Ro 20-1724. Data are mean %injected dose/g of tissue (wet weight) ± SD (*p<0.05, One-Way ANOVA with Bonferroni's comparisons test; control: n = 4 for $[^{11}C]$Ro 20-1724 and n = 16 for $R/S-[^{11}C]$rolipram, treated groups: n = 5; CTX: cortex).
Fig. 15  Uptake of A) $[^{11}\text{C}]{\text{Ro}}~20-1724$ and B) $R/S-[^{11}\text{C}]{\text{Rolipram}}$ in selected organs 30 and 45 min, respectively, following i.v. co-injection with the competitors rolipram or Ro 20-1724 in rats. Data are mean %injected dose/g of tissue (wet weight) ± SD (*p<0.05, One-Way ANOVA with Bonferroni's comparisons test; control: $n = 4$ for $[^{11}\text{C}]{\text{Ro}}~20-1724$ and $n = 16$ for $R/S-[^{11}\text{C}]{\text{Rolipram}}$, treated groups: $n = 5$; CTX: cortex).
Fig. 16 Distribution of R-[\textsuperscript{11}C]\textsuperscript{-}rolipram in rat A) regional brain and B) periphery 45 min following i.v. co-injection with the competitors rolipram or Ro 20-1724. Data are mean %injected dose/g of tissue (wet weight) ± SD (*p<0.05, One-Way ANOVA with Bonferroni's comparisons test; control: n = 16, rolipram and Ro 20-1724: n = 5; CTX: cortex).
uptake. R- and S-rolipram dose-dependently reduced R-[\textsuperscript{14}C]rolipram regional brain uptake (Fig. 17). The dose-response curves for R- and S-rolipram were similar across brain regions (Fig. 18 displays the frontal cortex as an example). The ED\textsubscript{50} of R- and S-rolipram was approximately 0.037 and 0.5 mg/Kg, respectively (Fig. 18), indicating that R-rolipram is \( \sim 13.5 \) fold more potent than S-rolipram at blocking R-[\textsuperscript{14}C]rolipram binding in vivo in all brain regions tested. Interestingly, saturating doses of S-rolipram (>3 mg/Kg) did not block radioligand uptake to the same level as Ro 20-1724, R/S-, or R-rolipram. For example, R-rolipram reduced frontal cortex radioactivity concentration to 10% (Fig. 18A), whereas, S-rolipram reduced it to 19% (Fig. 18B). Radioactivity accumulation was also dose-dependently reduced in peripheral organs, with doses of R- and S-rolipram that reduced radioactivity retention to non-specific levels in the brain also significantly decreasing R-[\textsuperscript{14}C]rolipram uptake to non-specific levels in the PDE4-rich lung and heart (Fig. 19A and B). Specifically, the effect of R-rolipram was significant with all doses in the heart and with 0.2 or 0.5 mg/kg in the lung (Fig. 19A). Coinjection with S-rolipram significantly blocked radioactivity uptake in the heart and lung only at lower doses (Fig. 19B), while the liver and blood demonstrated significant increases in activity levels with higher doses (Fig. 19B).

3.4.3 Competition of R/S- and R-[\textsuperscript{14}C]rolipram with a PDE1 inhibitor and a selective noradrenergic reuptake blocker

Pretreatment with the selective PDE1 inhibitor vinpocetine (Sauer et al., 1988) did not reduce R/S- (Fig. 20) or R-[\textsuperscript{14}C]rolipram (Fig. 21) distribution in any brain region or organ, supporting binding selectivity of the radiotracers for PDE4. Competition was also performed using the noradrenergic reuptake inhibitor DMI (Johnson et al., 1980), since previous reports have indicated that PDE4 plays a role in the presynaptic turnover of NA (Kehr et al., 1985; Wachtel and Schneider, 1986). However, DMI also did not reduce the regional brain or
Fig. 17  Rat regional brain distribution of $R$-$[^{11}\text{C}]$rolipram 45 min following i.v. coinjection with different doses of A) $R$-rolipram or B) $S$-rolipram. Data are mean %injected dose/g of tissue ± SD (*p<0.05, One-Way ANOVA with Bonferroni's comparisons test; control: $n = 16$, treated groups: $n = 4$, except for $S$-rolipram, 10 mg/Kg, where $n = 5$; CTX: cortex).
Fig. 18  Percentage of \( R-[\text{\textsuperscript{11}}\text{C}] \)rolipram inhibition in rat frontal cortex 45 min following coinjection with increasing doses of A) \( R \)-rolipram or B) \( S \)-rolipram. The E\( D_{50} \) for \( R \)- and \( S \)-rolipram was 0.037 mg/Kg and 0.5 mg/kg, respectively.
Fig. 19 Distribution of $R$-$[^{11}C]$rolipram in selected organs 45 min following i.v. coinjection with different doses of A) $R$-rolipram or B) $S$-rolipram. Data are mean %injected dose/g of tissue ± SD ($^*p<0.05$, One-Way ANOVA with Bonferroni’s comparisons test; control: $n = 16$, treated groups: $n = 4$, except for $S$-rolipram, 10 mg/Kg, where $n = 5$; CTX: cortex).
Fig. 20 Distribution of radioactivity in rat A) regional brain and B) periphery 45 min following R/S-[\textsuperscript{11}C]rolipram administration in rats treated with vinpocetine (10 mg/Kg, i.p., 15 min prior to radioligand injection) or DMI (10 mg/Kg, i.p., 30 min prior). Data are mean %injected dose/g of tissue (wet weight) ± SD (control: $n = 16$, DMI: $n = 5$; CTX: cortex).
Fig. 21 Distribution of radioactivity in rat A) regional brain and B) periphery 45 min following administration of \( R-[11\text{C}]\) rolipram in rats treated with vinpocetine (10 mg/Kg, i.p., 15 min prior to radioligand injection) or DMI (10 mg/Kg, i.p., 30 min prior). Data are mean % injected dose/g of tissue (wet weight) ± SD (control: \( n = 16 \), vinpocetine: \( n = 10 \), other treatments: \( n = 5 \); CTX: cortex).
peripheral uptake of R/S- (Fig. 20) of R-[¹⁴C]rolipram (Fig. 21), implying that the radioligands do not bind to the NA transporter.

3.4.4 Competition of S-[¹¹C]rolipram uptake by PDE4 inhibitors and other drugs

S-[¹¹C]Rolipram binding in brain regions was significantly inhibited following coinjection with Ro 20-1724, R-, or S-rolipram (Fig. 22A). R-Rolipram reduced S-[¹¹C]rolipram binding by 50-61%, whereas, S-rolipram reduced its binding by 37-53% across brain regions. The brain was the only organ showing significant decreases in radioactivity retention following coinjection with the PDE4-selective competitors while the lung, heart, and liver demonstrated no significant change (Fig. 22B). Pretreatment with vinpocetine or DMI had no effect on regional brain distribution of radioactivity (Fig. 23A). The heart and liver demonstrated significant increases in radioactivity accumulation following DMI administration (Fig. 23B).

3.5 Metabolite Analysis of R-[¹¹C]Rolipram in Rat Plasma and Brain

Solid phase extraction of rat plasma 30 min postinjection of R-[¹¹C]rolipram, demonstrated that ~53% of the total radioactivity eluted as hydrophilic metabolites in the aqueous fraction and the remaining radioactivity as hydrophobic eluant in the ethanol fraction. Analysis of radioactivity in the organic fraction using TLC revealed the presence of ~26% hydrophobic metabolites and ~20% unchanged R-[¹¹C]rolipram out of total radioactivity in plasma (Table IV, Fig. 24A). The radioactive peak corresponding to R-[¹¹C]rolipram coeluted with unlabeled R/S-rolipram (R, ~ 0.5, UV) and demonstrated the same R, as that obtained in the control experiment using authentic R-[¹¹C]rolipram. Extraction efficiency of radiotracer from brain was 90% (10% remained in the pellet fraction). TLC analysis of the homogenized brain extracts from both the injected and control rats indicated only the presence of unchanged R-[¹¹C]rolipram since a single
Fig. 22 Distribution of $S$-[$^{11}$C]rolipram in rat A) regional brain and B) periphery 45 min following co-injection with the competitors $R$-rolipram, $S$-rolipram, or Ro 20-1724. Data are mean %injected dose/g of tissue (wet weight) ± SD (*p<0.05, One-Way ANOVA with Bonferroni's comparisons test; control: $n = 8$ treated groups: $n = 4$; CTX: cortex).
Fig. 23 Distribution of radioactivity in A) regional brain and B) periphery 45 min following injection with S-[11C]rolipram in rats treated with vinpocetine (10 mg/Kg, i.p., 15 min prior to radioligand injection) or DMI (10 mg/Kg, i.p., 4.5 h prior). Data are mean %injected dose/g of tissue (wet weight) ± SD (*p<0.05, One-Way ANOVA with Bonferroni's comparisons test; control: n = 8, treated groups: n = 4; CTX: cortex).
Fig. 24 TLC chromatograms of rat #1: A) ethanol fraction after SPE plasma extraction; B) brain extract, 30 min post-injection of R-[¹¹C]rolipram; and C) brain extract, control mixed with authentic R-[¹¹C]rolipram.

Table IV Percent distribution of plasma radiolabeled metabolites and unchanged R-[¹¹C]rolipram in the water and organic fractions following SPE and TLC, 30 min postinjection in rats*.

<table>
<thead>
<tr>
<th></th>
<th>Hydrophilic Metabolites</th>
<th>Hydrophobic Metabolites</th>
<th>Unclassified R-[¹¹C]Rolipram</th>
<th>C-18 Sep-Pak</th>
<th>Percent Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat #1</td>
<td>56</td>
<td>29</td>
<td>20</td>
<td>0.1</td>
<td>105</td>
</tr>
<tr>
<td>Rat #2</td>
<td>50</td>
<td>23</td>
<td>19</td>
<td>0.1</td>
<td>97</td>
</tr>
<tr>
<td>Control (n = 1)</td>
<td>0.1</td>
<td>0</td>
<td>114</td>
<td>0</td>
<td>114</td>
</tr>
</tbody>
</table>

*Calculated as percentage of activity in plasma sample (1 ml).

Aqueous fraction from C-18 Sep-Pak.

Determined by TLC analysis of ethanol fraction.

The sum of percent activity of the metabolites, R-[¹¹C]rolipram, and C-18 Sep-Pak.
radioactive peak was detected (Fig. 24B) with the same R, as control (Fig. 24C). The peak in Figure 24B was slightly skewed, probably because of the large volume of solution (100 µl) to be analyzed, which required several spottings in order to be loaded onto the TLC plate. That is, some of the spottings may have been off-centered, producing a wide spot at baseline, leading to a large elution area and a slightly asymmetrical peak. Also, the whole process of extraction, spotting, elution, and acquisition of radioactivity, is lengthy, and the number of counts remaining in tissues can be substantially low due to the rapid physical decay of C-11 (20.4 min), leading to a reduction in resolution. In contrast, due to high radioactivity concentration, the control required only a single spotting, producing a very small elution area and a high resolution, leading to the sharp peak observed in Figure 24C. The distribution of radioactivity in control rat blood following centrifugation was approximately 50% in cells and 50% in plasma, indicating that there is a substantial amount of R-[11C]rolipram accumulated in blood cells. The plasma protein binding fraction measured in the control rat was found to be ~64%.

3.6 Dosimetry and Whole Body Biodistribution Studies of R-[11C]Rolipram

The decay-corrected accumulation of radioactivity in whole tissues (%ID/organ or tissue) is depicted in Table V. Most tissues demonstrated a rapid uptake of R-[11C]rolipram, with levels gradually decreasing 5 min following radiotracer injection, with the exception of the gastrointestinal (GI) contents and the urine, which exhibited an increase in radioactivity accumulation with time. Approximately 15% and 11% of the injected dose was measured in the urine and GI contents at 60 min, respectively, showing that both the kidneys and the hepatobiliary system were responsible for eliminating R-[11C]rolipram from the rat body. Approximately 60% of the total radioactivity excreted by 60 min occurred via the kidneys and the remaining via the hepatobiliary route. A mean of 91% of the total injected dose was recovered in the organs and
<table>
<thead>
<tr>
<th>Organ or Tissue</th>
<th>5</th>
<th>15</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pituitary</td>
<td>0.01 ± 0.00</td>
<td>0.01 ± 0.00</td>
<td>0.00 ± 0.00</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Eyes</td>
<td>0.07 ± 0.02</td>
<td>0.05 ± 0.01</td>
<td>0.05 ± 0.01</td>
<td>0.04 ± 0.01</td>
</tr>
<tr>
<td>Ascending/transverse LI*</td>
<td>0.72 ± 0.09</td>
<td>0.56 ± 0.15</td>
<td>0.54 ± 0.05</td>
<td>0.58 ± 0.25</td>
</tr>
<tr>
<td>Descending LI*</td>
<td>0.28 ± 0.11</td>
<td>0.26 ± 0.12</td>
<td>0.16 ± 0.04</td>
<td>0.14 ± 0.04</td>
</tr>
<tr>
<td>Small intestine</td>
<td>3.32 ± 0.54</td>
<td>3.76 ± 1.81</td>
<td>3.01 ± 0.26</td>
<td>2.78 ± 0.38</td>
</tr>
<tr>
<td>Testicles</td>
<td>0.62 ± 0.01</td>
<td>0.58 ± 0.01</td>
<td>0.42 ± 0.01</td>
<td>0.32 ± 0.00</td>
</tr>
<tr>
<td>Ovaries</td>
<td>0.06 ± 0.02</td>
<td>0.06 ± 0.01</td>
<td>0.05 ± 0.02</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>Heart</td>
<td>0.63 ± 0.11</td>
<td>0.50 ± 0.07</td>
<td>0.38 ± 0.06</td>
<td>0.20 ± 0.02</td>
</tr>
<tr>
<td>Lung</td>
<td>0.86 ± 0.10</td>
<td>0.71 ± 0.03</td>
<td>0.54 ± 0.05</td>
<td>0.37 ± 0.04</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.19 ± 0.03</td>
<td>0.24 ± 0.06</td>
<td>0.16 ± 0.04</td>
<td>0.10 ± 0.02</td>
</tr>
<tr>
<td>Adrenals</td>
<td>0.06 ± 0.01</td>
<td>0.04 ± 0.01</td>
<td>0.02 ± 0.01</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.65 ± 0.1</td>
<td>0.54 ± 0.04</td>
<td>0.46 ± 0.05</td>
<td>0.32 ± 0.08</td>
</tr>
<tr>
<td>Bone</td>
<td>0.29 ± 0.04</td>
<td>0.28 ± 0.04</td>
<td>0.24 ± 0.05</td>
<td>0.17 ± 0.05</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.26 ± 0.09</td>
<td>0.33 ± 0.08</td>
<td>0.27 ± 0.21</td>
<td>0.21 ± 0.12</td>
</tr>
<tr>
<td>Fat</td>
<td>0.17 ± 0.12</td>
<td>0.08 ± 0.05</td>
<td>0.18 ± 0.27</td>
<td>0.03 ± 0.03</td>
</tr>
<tr>
<td>Bladder</td>
<td>0.04 ± 0.02</td>
<td>0.08 ± 0.03</td>
<td>0.13 ± 0.08</td>
<td>0.14 ± 0.05</td>
</tr>
<tr>
<td>Blood</td>
<td>0.39 ± 0.06</td>
<td>0.46 ± 0.08</td>
<td>0.30 ± 0.04</td>
<td>0.20 ± 0.04</td>
</tr>
<tr>
<td>Brain</td>
<td>1.77 ± 0.16</td>
<td>1.72 ± 0.27</td>
<td>1.26 ± 0.13</td>
<td>0.84 ± 0.04</td>
</tr>
<tr>
<td>Urine</td>
<td>0.20 ± 0.07</td>
<td>0.25 ± 0.35</td>
<td>6.56 ± 3.15</td>
<td>15.06 ± 2.25</td>
</tr>
<tr>
<td>GI contents*</td>
<td>1.69 ± 0.43</td>
<td>4.24 ± 1.66</td>
<td>6.97 ± 1.91</td>
<td>11.41 ± 1.01</td>
</tr>
<tr>
<td>Liver</td>
<td>9.92 ± 0.53</td>
<td>7.30 ± 0.38</td>
<td>5.62 ± 1.27</td>
<td>3.99 ± 0.80</td>
</tr>
<tr>
<td>Kidneys</td>
<td>2.04 ± 0.26</td>
<td>2.30 ± 0.20</td>
<td>1.72 ± 0.26</td>
<td>1.22 ± 0.24</td>
</tr>
<tr>
<td>Carcass</td>
<td>69.45 ± 6.19</td>
<td>69.13 ± 2.93</td>
<td>58.08 ± 8.78</td>
<td>46.42 ± 10.26</td>
</tr>
</tbody>
</table>

*Data are mean %injected dose/organ or tissue ± SD.
*LI = Large intestine; GI = Gastro-intestinal.
carcass at the time points studied. Calculation of cumulative absorbed doses for administration of $R$-$[^{11}]$C]rolipram to humans revealed that the urinary bladder wall had the highest absorbed dose, being ~12 fold higher than in other critical organs such as the gonads (Table VI).

3.7 Regulation of the In Vivo Binding of $R$-$[^{11}]$C]Rolipram in Rat Brain Following Diverse Acute and Chronic Drug Treatments

The preliminary studies above showed that $R$-$[^{11}]$C]rolipram demonstrated a high signal and binding selectivity to PDE4, as well as good metabolism and dosimetry profiles, thus having potential for imaging PDE4 in the human brain using PET. An important aspect that remained to be explored in the development of $R$-$[^{11}]$C]rolipram is its ability to detect changes in the regulation of PDE4. Studies were performed to assess whether $R$-$[^{11}]$C]rolipram binding in rat brain reflected putative changes in the regulation of PDE4 following acute and chronic treatments with drugs altering cAMP-mediated signaling. As with the competition studies above, animals were injected via a tail vein with the radioligand, sacrificed 45 min later, and radioactivity accumulation in different brain regions and organs was assessed immediately as described in the materials and methods.

3.7.1 Effect of acute treatments

3.7.1.1 Dose- and time-dependent effect of the AC activator forskolin

Acute challenges with the AC activator forskolin (Simonds, 1999) were performed using dose levels as published previously in behavioral studies (Wachtel and Löschmann, 1986; Wachtel et al., 1987). The 6.5 mg/Kg dose coinjected with the radiotracer yielded a significant increase of 29–41% in radioactivity levels across brain regions, 45 min following radioligand injection (Fig. 25). The olfactory tubercles showed the highest change whereas the hippocampus the lowest. In contrast, a higher dose of 15 mg/Kg administered 3 hours prior to the radioligand or a treatment performed after the radioligand injection (6.5 mg/Kg, 15 min
Table VI Cumulative absorbed dose estimates for administration of $R-[^{125}\text{I}]$rolipram to humans.

<table>
<thead>
<tr>
<th>Organ</th>
<th>mGy/MBq</th>
<th>rad/mCi</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole Body</td>
<td>0.0014</td>
<td>0.0052</td>
</tr>
<tr>
<td>Red marrow</td>
<td>0.0012</td>
<td>0.0043</td>
</tr>
<tr>
<td>Ovaries</td>
<td>0.0012</td>
<td>0.0043</td>
</tr>
<tr>
<td>Testes</td>
<td>0.0012</td>
<td>0.0043</td>
</tr>
<tr>
<td>Small Intestine</td>
<td>0.0076</td>
<td>0.0282</td>
</tr>
<tr>
<td>Brain</td>
<td>0.0027</td>
<td>0.01</td>
</tr>
<tr>
<td>Heart Wall</td>
<td>0.0012</td>
<td>0.0043</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.0035</td>
<td>0.013</td>
</tr>
<tr>
<td>Liver</td>
<td>0.0053</td>
<td>0.0197</td>
</tr>
<tr>
<td>Lung</td>
<td>0.0019</td>
<td>0.0069</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.0011</td>
<td>0.0042</td>
</tr>
<tr>
<td>Urinary Bladder Wall</td>
<td>0.0142</td>
<td>0.0526</td>
</tr>
</tbody>
</table>
Fig. 25 Dose- and time-dependent percentage change in \( R-[\text{\(^{11}\)C}]\)rolipram regional brain distribution over controls following acute treatment with forskolin in rats. Animals were injected via tail vein with the radioligand, and sacrificed 45 min later. Data are mean of %change in radioactivity uptake over control group ± SD, normalized for body weight (*p<0.05, MANOVA with Bonferroni's comparisons test; control: \( n = 44 \), treated groups: \( n = 5 \); CTX: cortex).
prior to decapitation) produced no significant change in \( R-[\text{C}] \)rolipram regional brain distribution (Fig. 25). Importantly, blood radioactivity levels were significantly increased over regional brain values and may have affected the uptake of the radioligand in the brain.

### 3.7.1.2 Dose- and time-dependent effect of the noradrenergic agent DMI

The noradrenergic reuptake inhibitor DMI (Johnson et al., 1980) produced a dose-dependent change in \( R-[\text{C}] \)rolipram uptake across rat brain regions, 45 min following radioligand injection (Fig. 26). The 10 mg/Kg dose of DMI (4.5 hours prior to radioligand injection) had the optimal effect and significantly increased radioactivity levels in brain regions by 22-36% as compared to controls. The hippocampus displayed the lowest change and the hypothalamus the highest. In contrast, the higher and lower doses of 25 and 5 mg/kg, respectively, showed no significant effect. Blood radioactivity levels were not significantly altered by any of the DMI treatments (Fig. 26). Figure 27 depicts the radioactivity uptake in the frontal cortex as a function of time following treatment with the most effective dose of DMI, 10 mg/Kg. Retention peaked at \(~26\%\) over controls, was highest with the 4.5 hours pretreatment, and subsided by 9 hours. Other brain regions displayed a similar time-dependent effect of DMI as the frontal cortex.

### 3.7.1.3 Time-dependent effect of the \( \alpha_2 \)-adrenergic antagonist yohimbine

Rats were treated with the \( \alpha_2 \)-adrenergic antagonist yohimbine (Giralt and Garcia-Sevilla, 1989) at 0.25, 1.25, or 3 hours prior to radiotracer injection, resulting in an increase in radioactivity levels across brain regions at all time points (Fig. 28). The yohimbine challenge at 15 min prior to radioligand injection was most effective, demonstrating a significant increase in radioactivity retention of 25–47%. The thalamus yielded the highest uptake and the hippocampus the lowest. Yohimbine appeared to act rapidly at altering \( R-[\text{C}] \)rolipram regional brain binding \textit{in vivo}, since longer pretreatments were less potent. Blood radioactivity levels
Fig. 26 Dose- and time-dependent percentage change in $R$-$[^{11}C]$rolipram regional brain distribution over controls following acute treatment with desipramine (DMI) in rats. Animals were injected via tail vein with the radioligand and sacrificed 45 min later. Data are mean % change in radioactivity uptake over control group ± SD, normalized for body weight (*$p<0.05$, MANOVA with Bonferroni's comparisons test; control: $n=44$, treated groups: $n=5$, except for 10 mg/Kg, 4.5 h prior where $n=9$; CTX: cortex).
Fig. 27 Time-dependent effect of DMI (10 mg/Kg, i.p.) on \( R-[^{11}\text{C}]\)rolipram uptake in the frontal cortex as compared to controls. Rats were injected via tail vein with the radioligand and sacrificed 45 min later. Data are mean percentage change in radioactivity levels over controls ± SD (control: \( n = 44 \); treated groups: \( n = 5 \) per time point except at 4.5 h where \( n = 9 \)).
Fig. 28 Time-dependent percentage change in $R-[^{11}C]rolipram$ regional brain distribution over controls following acute treatment with yohimbine (10 mg/Kg, i.p.) in rats. Animals were injected via tail vein with the radioligand and sacrificed 45 min later. Data are mean %change in radioactivity uptake over control group ± SD, normalized for body weight (*p<0.05, MANOVA with Bonferroni's comparisons test; control: $n = 44$, treated groups: $n = 5$, CTX: cortex).
were also significantly higher with the 0.25 min prior treatment as compared to control and could have contributed to the effects observed in the brain.

3.7.1.4 Effect of other noradrenergic drugs: clonidine, tranylcypromine, propranolol, and clenbuterol

Acute administration of the α2-adrenergic agonist clonidine (Giralt and Garcia-Sevilla, 1989) at 1 mg/Kg dose significantly increased $R$-$[^{11}\text{C}]$rolipram retention across brain regions by 17-40%, as well as radioactivity levels in the blood 45 min following radioligand injection (Fig. 29). The brain stem demonstrated the highest uptake and the hippocampus the lowest. In contrast, the lower dose of 0.1 mg/Kg had no significant effect on $R$-$[^{11}\text{C}]$rolipram distribution across brain regions or blood radioactivity levels 45 min following radioligand injection (Fig. 29). The MAO inhibitor tranylcypromine (Giralt and Garcia-Sevilla, 1989) significantly augmented radioactivity accumulation by 17-29% across brain regions, with no significant change in blood levels (Fig. 29). The most pronounced change occurred in the frontal cortex and striatum and the least in the olfactory tubercles. The increased radioactivity levels detected in blood following clonidine or tranylcypromine may also have influenced the brain uptake of $R$-$[^{11}\text{C}]$rolipram following these drug treatments. In contrast, the postsynaptic β-adrenergic antagonist propranolol (Prichard and Tomlinson, 1986) showed no significant effect on $R$-$[^{11}\text{C}]$rolipram binding in brain regions, or on blood radioactivity levels at either a shorter or a longer time point (Fig. 30). The β2-adrenoceptor-selective agonist clenbuterol (O’Donnell and Frazer, 1985), however, significantly increased radioactivity retention in most brain regions (Fig. 30). The greatest effect was observed in the hypothalamus with a 32% augmentation in radioactivity levels, and the smallest in the hippocampus (only a 15% increase). Blood radioactivity levels were not significantly altered following clenbuterol treatment.
Fig. 29 Percentage change in \( R-[{}^{11}\text{C}] \) rolipram rat regional brain distribution over controls following treatment with clonidine (0.1 or 1 mg/Kg, i.p., 3 h prior), and tranylcypromine (10 mg/Kg, i.p., 3 h prior). Animals were injected via tail vein with the radioligand and sacrificed 45 min later. Data are mean % change in radioactivity uptake over control group ± SD, normalized for body weight (\(^*p<0.05\), MANOVA with Bonferroni's comparisons test; control: \( n = 44 \), clonidine (1 mg/Kg): \( n = 9 \), clonidine (0.1 mg/Kg): \( n = 4 \), tranylcypromine: \( n = 5 \); CTX: cortex).
Fig. 30 Percentage change in $R-[^{11}C]rolipram$ rat regional brain distribution over controls following treatment with propranolol (20 mg/Kg, i.v., 5 min prior or i.p., 3 h prior), and clenbuterol (10 mg/Kg, i.p., 3 h prior). Animals were injected via tail vein with the radioligand and sacrificed 45 min later. Data are mean % change in radioactivity uptake over control group ± SD, normalized for body weight (*p<0.05, MANOVA with Bonferroni's comparisons test; control: $n = 44$, treated groups: $n = 4$, except for propranolol 5 min prior, where $n = 5$; CTX: cortex).
3.7.1.5 Modulations of the serotonergic system

Effect of acute administration with various serotonergic agents is shown in Figure 31. Administration of the 5-HT reuptake inhibitor fluoxetine (Malagie et al., 1995) yielded a significant increase of 15-27% in radioligand absorption across brain regions 45 min following radioligand injection. Similar to DMI, treatment with fluoxetine produced the smallest change in the hippocampus and the greatest in the hypothalamus. In contrast, injection with the 5HT_{1A} agonist 8-OH-DPAT or the antagonist WAY 100635 (Casanovas et al., 1999) had no significant effect on R-[^{11}C]rolipram distribution in brain regions. The 5HT_{2A/C} agonist DOI (Marona-Lewicka and Nichols, 1997) also produced no significant change in radiation retention, demonstrating only a slight decrease (~10%) across brain regions. Similarly, the 5HT_{2A/C} antagonist ritanserin (Leysen et al., 1985) produced no significant change. Blood radioactivity levels showed no significant changes following any of the 5-HT agents.

3.7.1.6 Effect of adenosine-A_{2}, histamine-H_{1}, and dopamine-D_{1} receptors

To further explore the regulation of R-[^{11}C]rolipram uptake and binding to PDE4 in vivo, neurotransmitter systems other than the noradrenergic and serotonergic were investigated, including the adenosine, histamine, and dopamine systems (Fig. 32). Although stimulation of adenosine-A_{2A} receptors by the agonist CGS 21680 (Phillis, 1990) yielded no significant change in activity levels across brain regions 45 min following radioligand injection, the olfactory bulbs displayed an increase of 21% as compared to controls, which could reach significance if more animals are used. Increasing synaptic histamine turnover by inhibiting the histamine-H_{1} autoreceptor with thioperamide (Oishi et al., 1989; Yanai et al., 1994) produced an increase in R-[^{11}C]rolipram that was significant in the frontal cortex, thalamus, cerebellum and brain stem. The thalamus and frontal cortex demonstrated the highest uptake of 27%, closely followed by cerebellum (26%) and brain stem (25%), and the olfactory bulbs the lowest (7%) over controls. Blood radioactivity levels were not significantly altered with CGS 21680 or thioperamide. The
Fig. 31 Percentage change in $R-[^{11}\text{C}]$rolipram rat regional brain distribution over controls following treatment with fluoxetine (5 mg/Kg, i.p., 3 h prior), 8-OH-DPAT (1 mg/Kg, i.p., 3 h prior), WAY 100635 (3 mg/Kg, i.p., 3 h prior), DOI (5 mg/Kg, i.p., 3 h prior), and ritanserin (2.5 mg/Kg, s.c., 4 h prior). Rats were injected via tail vein with the radioligand and sacrificed 45 min later. Data are mean % change in radioactivity uptake over control group ± SD, normalized for body weight (*p<0.05, MANOVA with Bonferroni’s comparisons test; control: $n = 44$, fluoxetine: $n = 9$, other treated groups: $n = 5$; CTX: cortex).
treated groups: n = 9, except for SKF 81297 3 h prior, where n = 10; CTX: cortex.

body weight (p<0.05, MANOVA with Bonferroni's comparisons test: control: n = 44,
are mean %change in radiotracer uptake over control group ± SD, normalized for
were injected via tail vein with the radioligand and sacrificed 45 min later. Data
were | unless & p. 4 h prior), and SKF 81297 (10 mg/kg, i.p. 5 min or 3 h prior) in rats. Animals
were treated with CGS 21680 (2 mg/kg, 1.5 h prior) or piperazine (10 mg/kg).

FIG. 22
Percentage change in %11C[J]olipram uptake over controls following acute

Blood
Brain
Hypothalamus
Cerebellum
Thalamus
Hippocampus
Striatum
Olfactory Bulb
Rest of CTX
Frontal CTX

% Change over control group

SKF 81297 (3 h prior)
SKF 81297 (5 min prior)
Thiorperamide
CGS 21680
dopamine-D$_1$ receptor agonist SKF 81297 (Arnt et al., 1988) was employed as a negative control, since the dopamine system has been shown to employ mostly PDE1 and not PDE4 (Polli and Kincaid, 1994). SKF 81297 produced no significant effect on regional brain $R$-$[^{11}\text{C}]$rolipram distribution at either 5 min or 3 hours prior to radioligand injection.

3.7.1.7 Inhibition of PKA-mediated phosphorylation

The selective PKA inhibitor HA 1004 (De Sarro et al., 1988; Shea et al., 1995) was used in this study to determine if the mechanism of action of yohimbine, DMI, and forskolin treatments at increasing $R$-$[^{11}\text{C}]$rolipram regional brain retention in vivo as described above, was mediated by PKA. HA 1004 treatment alone 1 hour prior to radioligand injection produced no significant change in activity levels across brain regions (Fig. 33). HA 1004 injection 45 min prior to yohimbine unexpectedly yielded a significant potentiation of the radioactivity levels by $>2$ fold across brain regions and blood as compared to yohimbine treatment alone (Fig. 33A). When HA 1004 was administered prior to DMI, activity levels were not significantly affected except for a significant potentiation in the thalamus and a decrease in blood as compared to DMI treatment alone (Fig. 33B). HA 1004 injection prior to forskolin demonstrated no significant change in the distribution of $R$-$[^{11}\text{C}]$rolipram across brain areas (Fig. 33C). However, the frontal cortex yielded a 25% reduction, which may have reached significance if more rats had been used in the study. Interestingly, blood levels were significantly decreased with the HA 1004/forskolin combination as compared to forskolin injection alone, and may imply that PDE4 enzymes are expressed in blood cells.

3.7.1.8 Inhibition of protein synthesis by cycloheximide

The protein synthesis inhibitor cycloheximide was used to probe for the possibility that the increases in $R$-$[^{11}\text{C}]$rolipram uptake following acute treatments occur due to new PDE4 protein synthesis. Treatment with cycloheximide was administered prior to CGS 21680 challenge as reported previously in cell cultures (Chang et al., 1997). Cycloheximide alone significantly
Fig. 33  Effect of pre-treatment with the PKA inhibitor HA 1004 (20 mg/Kg, i.p., 45 min prior to yohimbine, DMI, or forskolin) on R-[11C]rolipram regional brain retention following challenge with A) yohimbine (10 mg/Kg, i.p., 15 min prior, n = 5), B) DMI (10 mg/Kg, i.p., 4.5 h prior, n = 5) or C) forskolin (6.5 mg/Kg, i.v., coinjection, n = 5) in rats. Animals were injected via tail vein with the radioligand and sacrificed 45 min later. Data are mean % change in radioactivity uptake over control group ± SD, normalized for body weight (# p<0.05 as compared to drug treatment in the absence of HA 1004, One-Way ANOVA; control: n = 44, treated groups in absence of HA 1004: n = 5, except for DMI where n = 9; CTX: cortex).
augmented radioligand retention across brain regions as compared to no treatment (Fig. 34). When administered prior to CGS 21680, no inhibition of R-[\textsuperscript{11}C]rolipram retention in brain regions was observed, instead a synergism was detected that was significant in blood but not in any brain region as compared to CGS 21680 treatment alone. The fact that blood levels were significantly increased with the CGS 21680/cycloheximide treatment may have also produced a non-specific increase in the regional brain uptake of R-[\textsuperscript{11}C]rolipram.

3.7.2 Determination of in vivo R-[\textsuperscript{11}C]rolipram specific binding following selected acute drug treatments

Selected acute modulations that demonstrated an increase in rat regional brain distribution of R-[\textsuperscript{11}C]rolipram were repeated in the presence of a saturating dose (1 mg/Kg) of unlabeled R-rolipram in order to block PDE4 and thus measure the non-specific binding of the radioligand. Radioactivity uptake following administration of the drug modulator in the absence of R-rolipram was taken as total binding and used in the calculation of specific binding as described in section 2.7.4. DMI, yohimbine, clonidine, and tranylcypromine treatments yielded an increase of 18-41% in the percentage change in specific binding over control values across rat brain regions (Table VII). Fluoxetine displayed a different profile, producing pronounced increases up to ~26% in some, and decreases up to ~16% in other brain areas (e.g., frontal cortex and brain stem, respectively) (Table VII). Similarly, thioperamide also yielded an increase in radioactivity concentrations in the frontal cortex (20%) and decreases in other brain regions, including the brain stem (-14%). On the other hand, forskolin injection elevated the percentage change in specific binding by 14-26% in most brain areas, with the exception of no change in the brain stem. The effect of CGS 21680 on R-[\textsuperscript{11}C]rolipram regional brain uptake was also heterogeneous, showing a notable specific binding increase of 26% in the olfactory bulbs, followed by modest increases in the cerebellum (14%) and brain stem (13%), and virtually no change in other regions such as the hippocampus (Table VII).
Fig. 34 Effect of pre-treatment with the protein synthesis inhibitor cycloheximide (20 mg/Kg, i.p. 30 min prior to CGS 21680) on $R-[^{11}C]$rolipram regional brain retention following challenge with CGS 21680 (2 mg/Kg, i.p., 3 h prior) in rats. Animals were injected via tail vein with the radioligand and sacrificed 45 min later. Data are mean % change in radioactivity uptake over control group ± SD, normalized for body weight (# $p<0.05$ as compared to CGS 21680 treatment in the absence of cycloheximide, One-Way ANOVA; control: $n = 44$, treated groups: $n = 5$; CTX: cortex).
### Table VII  Percentage change in specific binding* of \(R-[^{12}]C\)rolipram in rat brain regions following different drug treatments.

<table>
<thead>
<tr>
<th>DRUG TREATMENT$^a$</th>
<th>FSK</th>
<th>DMI</th>
<th>YOH</th>
<th>CLON</th>
<th>FLUOX</th>
<th>TRAN</th>
<th>THIO</th>
<th>CGS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Frontal CTX</td>
<td>24.6</td>
<td>28.3</td>
<td>27.6</td>
<td>24.2</td>
<td>26.1</td>
<td>32.0</td>
<td>19.8</td>
<td>3.6</td>
</tr>
<tr>
<td>Rest of CTX</td>
<td>18.4</td>
<td>23.7</td>
<td>24.0</td>
<td>18.8</td>
<td>15.5</td>
<td>20.0</td>
<td>7.2</td>
<td>9.6</td>
</tr>
<tr>
<td>Olfactory Bulb</td>
<td>23.5</td>
<td>32.4</td>
<td>35.5</td>
<td>36.1</td>
<td>22.6</td>
<td>30.4</td>
<td>-6.3</td>
<td>26.4</td>
</tr>
<tr>
<td>Olfactory Tubercle</td>
<td>25.8</td>
<td>35.3</td>
<td>26.3</td>
<td>29.6</td>
<td>19.5</td>
<td>19.0</td>
<td>5.5</td>
<td>4.7</td>
</tr>
<tr>
<td>Striatum</td>
<td>21.4</td>
<td>28.8</td>
<td>30.2</td>
<td>30.5</td>
<td>21.6</td>
<td>31.3</td>
<td>-5.4</td>
<td>7.6</td>
</tr>
<tr>
<td>Hippocampus</td>
<td>18.0</td>
<td>26.0</td>
<td>25.0</td>
<td>18.4</td>
<td>18.4</td>
<td>19.0</td>
<td>-5.4</td>
<td>-0.9</td>
</tr>
<tr>
<td>Thalamus</td>
<td>14.0</td>
<td>23.7</td>
<td>26.8</td>
<td>26.7</td>
<td>-11.8</td>
<td>23.6</td>
<td>2.9</td>
<td>12.0</td>
</tr>
<tr>
<td>Cerebellum</td>
<td>17.1</td>
<td>30.1</td>
<td>26.4</td>
<td>25.0</td>
<td>13.8</td>
<td>22.5</td>
<td>10.0</td>
<td>13.9</td>
</tr>
<tr>
<td>Hypothalamus</td>
<td>15.8</td>
<td>41.0</td>
<td>26.3</td>
<td>40.9</td>
<td>13.2</td>
<td>25.1</td>
<td>-11.4</td>
<td>10.5</td>
</tr>
<tr>
<td>Brain Stem</td>
<td>3.2</td>
<td>26.4</td>
<td>25.9</td>
<td>32.8</td>
<td>-16.2</td>
<td>17.9</td>
<td>-14.1</td>
<td>12.6</td>
</tr>
</tbody>
</table>

*Specific binding was calculated by subtracting the %IDBW/g in the presence of the R-rolipram blocking dose (1 mg/Kg, i.v., coinjected with the radioligand) or non-specific binding, from the %IDBW/g in the absence of R-rolipram or total binding.

$^a$To determine total binding, rats were administered: forskolin (FSK: 6.5 mg/Kg, coinjected with radioligand, i.v., \(n = 5\)); DMI (10 mg/Kg, 4.5 h prior to radioligand injection, \(n = 9\)); yohimbine (YOH: 10 mg/Kg, i.p., 15 min prior, \(n = 5\)); clonidine (CLON: 1 mg/Kg, i.p., 3 h prior, \(n = 5\)); fluoxetine (FLUOX: 5 mg/Kg, i.p., 3 h prior, \(n = 9\)); tranylcypromine (TRAN: 10 mg/Kg, i.p., 3 h prior, \(n = 5\)); thioperamide (THIO: 10 mg/Kg, i.p., 4 h prior, \(n = 9\)); or CGS 21680 (CGS: 2 mg/Kg, i.p., 3 h prior, \(n = 9\)), injected with R-[\(^{12}\)C]rolipram and sacrificed 45 min later. To determine non-specific binding, animals were treated again with the drug challenges as above (\(n = 4\) per drug treatment, except fluoxetine where \(n = 8\)), and then coinjected with a blocking dose of R-rolipram (1 mg/Kg, i.v.), and sacrificed 45 min later.
Peripheral regions rich in PDE4 enzymes also demonstrated changes in the specific binding of \( R-[^{11}C] \)rolipram, following some of the drug treatments (Table VIII). Forskolin was the only one that produced substantial decreases in both the lung and heart, while thioperamide reduced radioactivity levels in the lung only. Agents that increase NA release, such as DMI and yohimbine, demonstrated a large increase in \( R-[^{11}C] \)rolipram specific binding in both the heart and lung. On the other hand, clonidine, which decreases NA levels, showed no effect in either organ. Increases in endogenous 5-HT concentrations by fluoxetine, or in monoamines by tranylcypromine, yielded an augmentation in radioactivity accumulation in the lung only, while the adenosine-A\(_{2a}\) agonist CGS 21680 increased activity levels in both heart and lung, as compared to controls. \( R-[^{11}C] \)Rolipram specific binding was not detected in the liver, since coinjection with unlabeled \( R \)-rolipram did not significantly reduce radioactivity accumulation following any of the drug challenges in this organ (Fig. 35). In fact, \( R-[^{11}C] \)rolipram accumulation in the liver was significantly elevated with the \( R \)-rolipram coinjection in forskolin, clonidine, and thioperamide treated rats (Fig. 35), suggesting that these drug treatments may affect radioligand metabolism. Similarly, blood radioactivity levels were not substantially altered following most drug treatments in the presence of \( R \)-rolipram as compared to the absence of the competitor, with the exception of forskolin and thioperamide, where a significant increase was obtained (Fig. 36), which may have augmented the non-specific binding of \( R-[^{11}C] \)rolipram across brain regions.

3.7.3 Effect of selected acute drug treatments on \( R-[^{11}C] \)rolipram levels in rat plasma

Radioactivity levels in the blood were increased following several drug treatments as compared to controls. Therefore, acute in vivo treatments with forskolin, DMI, and fluoxetine were repeated as before in order to study their effect on the plasma level of \( R-[^{11}C] \)rolipram as compared to saline. Administration of DMI yielded no significant alteration in percentage
Table VIII  Percentage change in *specific binding* of \( R-[^{14}C] \)rolipram in the lung and heart following different drug treatments in rats.

<table>
<thead>
<tr>
<th>DRUG TREATMENT*</th>
<th>FSK</th>
<th>DMI</th>
<th>YOH</th>
<th>CLON</th>
<th>FLUOX</th>
<th>TRAN</th>
<th>THIO</th>
<th>CGS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>-71</td>
<td>75</td>
<td>92</td>
<td>-4</td>
<td>28</td>
<td>32</td>
<td>-38.7</td>
<td>85</td>
</tr>
<tr>
<td>Heart</td>
<td>-173</td>
<td>65</td>
<td>80</td>
<td>-1</td>
<td>4</td>
<td>-4</td>
<td>5.8</td>
<td>28</td>
</tr>
</tbody>
</table>

*Specific binding* was calculated by subtracting the %IDBW/g in the presence of the \( R \)-rolipram blocking dose (1 mg/Kg, i.v., coinjected with the radioligand) or *non-specific binding*, from the %IDBW/g in the absence of \( R \)-rolipram or *total binding*.

*b* To determine *total binding*, rats were administered: forskolin (FSK: 6.5 mg/Kg, coinjected with radioligand, i.v., \( n = 5 \)); DMI (10 mg/Kg, 4.5 h prior to radioligand injection, \( n = 9 \)); yohimbine (YOH: 10 mg/Kg, i.p., 3 h prior, \( n = 5 \)); clonidine (CLON: 1 mg/Kg, i.p., 3 h prior, \( n = 5 \)); fluoxetine (FLUOX: 5 mg/Kg, i.p., 3 h prior, \( n = 9 \)); tranylcypromine (TRAN: 10 mg/Kg, i.p., 3 h prior, \( n = 5 \)); thiopramide (THIO: 10 mg/Kg, i.p., 4 h prior, \( n = 9 \)); or CGS 21680 (CGS: 2 mg/Kg, i.p., 3 h prior, \( n = 9 \)), injected with \( R-[^{14}C] \)rolipram and sacrificed 45 min later. To determine *non-specific binding*, animals were treated again with the drug challenges as above (\( n = 4 \) per drug treatment, except fluoxetine where \( n = 8 \)), and then coinjected with a blocking dose of \( R \)-rolipram (1 mg/Kg, i.v.), and sacrificed 45 min later.
Fig. 35 Uptake of $R-[^{11}C]$rolipram in rat liver following challenge with DMI (10 mg/Kg, i.p., 4.5 h prior radioligand injection, $n = 9$), fluoxetine (5 mg/Kg, i.p., 3 h prior, $n = 9$), forskolin (6.5 mg/Kg, i.v., co-injected, $n = 5$), yohimbine (10 mg/Kg, i.p., 15 min prior, $n = 5$), clonidine (1 mg/Kg, i.p., 3 h prior, $n = 5$), tranylcypromine (10 mg/Kg, i.p., 3 h prior, $n = 5$), CGS 21680 (2 mg/Kg, i.p., 3 h prior, $n = 9$), or thioperamide (10 mg/Kg, i.p., 4 h prior, $n = 9$). $R$-Rolipram (1 mg/Kg, i.v., $n = 4$ per treatment, except for fluoxetine where $n = 8$) was co-injected with the radioligand in order to determine non-specific binding following the drug challenges. Data are mean $\pm$ SD, expressed as percentage of injected dose/g of wet tissue normalized for rat body weight ($#p<0.05$ as compared to drug treatment in the absence of $R$-rolipram blocking dose, One-Way ANOVA).
Fig. 36 Uptake of $R$-$[^{11}\text{C}]$rolipram in rat blood following challenge with DMI (10 mg/Kg, i.p., 4.5 h prior radioligand injection, $n = 9$), fluoxetine (5 mg/Kg, i.p., 3 h prior, $n = 9$), forskolin (6.5 mg/Kg, i.v., co-injected, $n = 5$), yohimbine (10 mg/Kg, i.p., 15 min prior, $n = 5$), clonidine (1 mg/Kg, i.p., 3 h prior, $n = 5$), tranylcypromine (10 mg/Kg, i.p., 3 h prior, $n = 5$), CGS 21680 (2 mg/Kg, i.p., 3 h prior, $n = 9$), or thioperamide (10 mg/Kg, i.p., 4 h prior, $n = 9$). $R$-Rolipram (1 mg/Kg, i.v., $n = 4$ per treatment, except for fluoxetine where $n = 8$) was coinjected with the radioligand in order to determine non-specific binding following the drug challenges. Data are mean ± SD, expressed as percentage of injected dose/g of wet tissue normalized for rat body weight (#p<0.05 as compared to drug treatment in the absence of $R$-rolipram blocking dose, One-Way ANOVA).
unchanged $R$-$[^{11}\text{C}]$rolipram levels in rat plasma, 45 min following radioligand injection as compared to saline (Fig. 37). Although not significant due to a small number of rats used in the study, fluoxetine showed $\sim20\%$ decrease and forskolin $\sim25\%$ increase in percentage unchanged $R$-$[^{11}\text{C}]$rolipram in rat plasma as compared to saline, which may signify that these drug treatments alter the metabolism of the radioligand (Fig. 37).

3.7.4 Effect of chronic treatments

3.7.4.1 Modulation of $\beta$-adrenoceptors

Chronic treatments with propranolol and the $\beta_2$-selective agonist clenbuterol (O'Donnell and Frazer, 1985) were performed in rats to decrease and increase the regulation of PDE4, respectively (Fig. 38). Twenty fours hours after the last propranolol injection, $R$-$[^{11}\text{C}]$rolipram uptake was unexpectedly significantly elevated by $10$–$17\%$ in 6 out of the 10 brain areas dissected as compared to controls. The elevation was lowest in the hippocampus and highest in the hypothalamus and frontal cortex. In contrast, no significant change was observed following chronic clenbuterol administration and blood levels were not significantly changed as compared to controls with either drug treatment.

3.7.4.2 Effect of chronic antidepressant administration

Chronic treatment with antidepressants was carried out to upregulate PDE4 density in rat brain (Fig. 39). DMI significantly increased $R$-$[^{11}\text{C}]$rolipram retention across all rat brain regions as compared to controls. The lowest increase occurred in the striatum and hypothalamus at $17\%$ and the highest in the olfactory bulbs at $24\%$. Chronic fluoxetine increased $R$-$[^{11}\text{C}]$rolipram regional brain uptake significantly in the frontal cortex (14%) and cerebellum (12%). In contrast, the MAO inhibitor tranylcypromine produced no significant change in radioactivity levels, showing rather a trend towards a decrease that was most pronounced in the thalamus ($\sim14\%)$. Repeated administration of 8-OH-DPAT showed no
Fig. 37 Percentage unchanged $R-\text{[}^{11}\text{C}]$rolipram in plasma 45 min following radioligand injection in rats treated with DMI (10 mg/Kg, i.p., 4.5 h prior to radioligand administration), fluoxetine (5 mg/kg, i.p., 3 h prior), and forskolin (6.5 mg/Kg, i.v., co-injected). Control animals were treated with saline 3 h prior or co-injected. Data are mean %unchanged $R-\text{[}^{11}\text{C}]$rolipram in rat plasma of 2 rats per treatment.
Fig. 38  Percentage change in R-[¹¹C]rolipram uptake following chronic treatment with propranolol (20 mg/Kg, i.p., once daily, 14 days) or clenbuterol (10 mg/Kg, i.p., once daily, 14 days) as compared to controls. Animals were injected with radioligand and sacrificed 45 min later. Data are mean of % change in radioactivity uptake over control group ± SD, normalized for body weight (*p<0.05, MANOVA with Bonferroni's comparisons test; control: n = 62, propranolol: n = 14, clenbuterol: n = 7; CTX: cortex).
Fig. 39 Percentage change in R-[¹¹C]rolipram uptake following chronic treatment with DMI (10 mg/Kg, i.p., twice daily, 14 days), tranylcypromine (10 mg/Kg, i.p., once daily, 14 days), fluoxetine (5 mg/Kg, i.p., once daily, 14 days), or 8-OH-DPAT (1 mg/Kg, s.c., once daily, 14 days) as compared to controls. Rats were injected via tail vein with radioligand and sacrificed 45 min later. Data are mean of % change in radioactivity uptake over control group ± SD (*p<0.05, MANOVA with Bonferroni's comparisons test; control: n = 62, DMI: n = 14, tranylcypromine and fluoxetine: n = 15, 8-OH-DPAT: n = 7; CTX: cortex).
significant effect on $R$-$[^{14}C]$rolipram regional brain distribution. Blood levels were not significantly affected by any of these antidepressant administrations.

3.7.4.3 Determination of in vivo $R$-$[^{14}C]$rolipram specific binding following chronic DMI and fluoxetine

Chronic treatment with DMI and fluoxetine substantially increased $R$-$[^{14}C]$rolipram specific binding by 16–26% and 10-18%, respectively, across brain regions (Fig. 40). The smallest changes occurred in the brain stem and the highest in the olfactory regions for both chronic treatments. The PDE4-rich lung and heart also demonstrated an increase in specific binding levels, being more pronounced following chronic DMI (Table IX). No specific binding was detected in the liver, since radioactivity levels were significantly higher in the presence of $R$-rolipram than in the absence of the competitor (Fig. 41). Blood levels were not significantly altered with any of the chronic treatments alone or with the blocking dose of $R$-rolipram (Fig. 41).

3.7.4.4 Effect of vesicle depletion

Monoamine-containing vesicles from rat brain were depleted with reserpine with the goal of decreasing the regulation of PDE4 at central noradrenergic and serotoninergic receptors. Twenty four, but not two, hours after the last reserpine injection, regional brain $R$-$[^{14}C]$rolipram retention was unexpectedly significantly increased in several brain areas as compared to controls (Fig. 42). The greatest change occurred in the olfactory bulbs at 22% and the lowest in the hippocampus at 10%. A longer treatment with 1 mg/Kg, s.c., for 2 days followed by 0.5 mg/Kg, s.c., for 10 days produced a high mortality with only 3/12 rats surviving. The remaining 3 rats demonstrated no significant change in regional brain radioactivity uptake, although levels did show a reduction that could have reached significance if more rats had survived (Fig. 42). Blood levels were not significantly altered in any of the treatment protocols.
Fig. 40 Percentage change in specific binding of $R^{[11]C}$rolipram across rat brain regions as compared to controls following chronic treatment with DMI (10 mg/Kg, i.p., twice daily, 14 days) or fluoxetine (5 mg/Kg, i.p., once daily, 14 days). Animals were injected with the radiotracer and sacrificed 45 min later. Data are %change in specific binding. Specific binding was calculated by subtracting the mean non-specific binding (antidepressant treatment in the presence of $R$-rolipram blocking dose of 1 mg/Kg, $n = 7$ per group) from the mean total binding (antidepressant treatment in the absence of $R$-rolipram, $n = 7$ for DMI and $n = 15$ for fluoxetine; control bank $n = 62$).
Table IX  Percentage change in *specific binding* of R-[\(^{11}\)C]rolipram in the lung and heart following chronic treatment with the antidepressants DMI and fluoxetine.

**DRUG TREATMENT**

<table>
<thead>
<tr>
<th>Region</th>
<th>Chronic DMI</th>
<th>Chronic Fluoxetine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lung</td>
<td>13</td>
<td>11</td>
</tr>
<tr>
<td>Heart</td>
<td>24</td>
<td>7</td>
</tr>
</tbody>
</table>

*Specific binding* was calculated by subtracting the %IDBW/g in the presence of the R-rolipram blocking dose (1 mg/Kg, i.v., coinjected with the radioligand) or *non-specific binding*, from the %IDBW/g in the absence of R-rolipram or *total binding*.

To determine *total binding*, rats were administered DMI (10 mg/Kg, i.p., twice daily, 14 days; \(n = 7\)) or fluoxetine (5 mg/Kg, i.p., once daily, 14 days; \(n = 15\)), allowed a 24 h washout, and injected with R-[\(^{11}\)C]rolipram via tail vein. To determine *non-specific binding*, chronic DMI and fluoxetine treatments were repeated as above (\(n = 7\) per group), but R-[\(^{11}\)C]rolipram was coinjected with a blocking dose of R-rolipram (1 mg/Kg) administered *via* tail vein, and the animals were killed 45 min later.
Fig. 41  Radioactivity levels in the liver and blood 45 min following \( R-[{\text{11C}}] \) rolipram injection in rats chronically treated with DMI (10 mg/kg, i.p., twice daily, 14 days; \( n = 7 \)) or fluoxetine (5 mg/Kg, i.p., once daily, 14 days; \( n = 15 \)). To determine non-specific binding, treatments were allowed a 24 hour washout and a blocking dose of \( R \)-rolipram (1 mg/Kg; \( n = 7 \) per group) was co-administered with the radioligand in order to determine non-specific binding. Data are mean \( \pm \) SD, expressed as percentage of injected dose/g of tissue normalized for body weight (#p<0.05 as compared to chronic drug treatment alone, One-Way ANOVA).
Fig. 42 Percentage change in \( R-[^{11}\text{C}] \)rolipram uptake following chronic treatment with reserpine (1 mg/Kg, s.c., once daily, 5 days, 2h or 24 h washout; or 1 mg/Kg, s.c., for 2 days, followed by 0.5 mg/Kg, s.c., 10 days, 24 h washout) as compared to controls. Rats were injected via tail vein with the radioligand and sacrificed 45 min later. Data are mean of %change in radioactivity uptake over control group ± SD (*p<0.05, MANOVA with Bonferroni's comparisons test; control: \( n = 62 \), reserpine 5 days 24 h washout: \( n = 9 \), 2 h washout: \( n = 10 \), 12 day: \( n = 3 \); CTX: cortex).
3.7.4.5 Effect of lesioning noradrenergic neurons with DSP-4

Similar to reserpine, DSP-4 was employed to reduce the regulation of PDE4 in the noradrenergic system of the brain. In this study, R-[1^C]rolipram regional brain retention was not significantly changed following different DSP-4 treatment paradigms, although, a trend towards a reduction in activity levels was observed with the 14 day treatments as compared to controls (Fig. 43).

3.7.5 In vitro validation with R/S-[3H]rolipram

In vitro experiments were performed to validate the in vivo results obtained with the DMI and fluoxetine acute and chronic challenges as described above. The K_d and B_max of R/S-[3H]rolipram were determined in binding assays using frontal cortex homogenates from rats treated acutely or chronically with DMI, fluoxetine, or saline vehicle. Since PDE4 is expressed in both supernatant and pellet, both cellular fractions were evaluated.

3.7.5.1 Effect of acute DMI and fluoxetine

The K_d values for R/S-[3H]rolipram binding in the rat frontal cortex were similar to values published in the literature (Schneider et al., 1986). No difference in K_d was obtained following acute treatment with DMI or fluoxetine as compared to controls (Table X). The B_max for R/S-[3H]rolipram binding in the frontal cortex was significantly increased by 47 and 54% in the supernatant following DMI and fluoxetine treatment, respectively (Table XI). In contrast, no significant change was detected in the pellet with either treatment.

3.7.5.2 Effect of chronic DMI and fluoxetine

The K_d values of R/S-[3H]rolipram binding in either the control or treated groups were within the range as previously published in the literature (Table XII) (Schneider et al., 1986). There was no significant effect on K_d following either chronic DMI or fluoxetine treatment as compared to controls (Table XII). Chronic fluoxetine had no significant effect on R/S-
Fig. 43 Percentage change in $R-[^{11}\text{C}]$rolipram uptake following treatment with DSP4 (50 mg/Kg, i.p.). One group received zimelidine, i.p., 30 min prior to DSP4 to protect 5-HT neurons. Rats were injected via tail vein with the radioligand and sacrificed 45 min later. Data are mean of %change in radioactivity uptake over control group ± SD (*p<0.05, MANOVA with Bonferroni's comparisons test; control: n = 62, treated groups: n = 7 except for the group injected twice with DSP4 where n = 5; CTX: cortex).
Table X  *In vitro* binding affinity$^\text{§}$ values of $R/S$-$[^3\text{H}]$rolipram in supernatant and pellet following acute fluoxetine or DMI treatment *in vivo*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Supernatant</th>
<th>Pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.2 ± 1.2</td>
<td>3.1 ± 1.2</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>3.0 ± 1.7</td>
<td>3.1 ± 0.9</td>
</tr>
<tr>
<td>DMI</td>
<td>2.4 ± 1.0</td>
<td>2.5 ± 1.7</td>
</tr>
</tbody>
</table>

$^\text{§}$Binding affinity is expressed as the dissociation constant, $K_d$, in nM.

*Control values consist of $n = 8$: $n = 4$ treated with fluoxetine vehicle and $n = 4$ with DMI vehicle. No significant difference in $K_d$ was detected between the two control groups, thus they were grouped together and used in the statistical calculations. Treated groups: $n = 8$.

Table XI  $R/S$-$[^3\text{H}]$Rolipram $B_{\text{max}}$ following acute treatment with DMI and fluoxetine as compared to controls.$^\text{§}$

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Supernatant</th>
<th>Pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$B_{\text{max}}$</td>
<td>%Change</td>
</tr>
<tr>
<td>Control</td>
<td>80 ± 19</td>
<td>N/A</td>
</tr>
<tr>
<td>DMI</td>
<td>118 ± 27</td>
<td>47*</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>123 ± 20</td>
<td>54*</td>
</tr>
</tbody>
</table>

$^\text{§}$B$_{\text{max}}$ values are in fmol/mg of protein in rat frontal cortex.

*p<0.05 as compared to controls; One-Way ANOVA with Bonferroni's comparisons test.

*Control values consist of $n = 8$: $n = 4$ treated with fluoxetine vehicle and $n = 4$ with DMI vehicle. No significant difference in $B_{\text{max}}$ was detected between the two control groups, thus they were grouped together and used in the statistical calculations.

N/A = non-applicable.
Table XII *In vitro* binding affinity values of *R/S-*[^3]H*rolipram in supernatant and pellet following chronic fluoxetine or DMI treatment *in vivo*.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Supernatant</th>
<th>Pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control DMI</td>
<td>0.9 ± 0.1</td>
<td>1.1 ± 0.4</td>
</tr>
<tr>
<td>DMI</td>
<td>1.1 ± 0.5</td>
<td>1.3 ± 0.4</td>
</tr>
<tr>
<td>Control Fluoxetine</td>
<td>1.5 ± 0.5</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>1.7 ± 0.3</td>
<td>1.6 ± 0.4</td>
</tr>
</tbody>
</table>

[^3]Binding affinity is expressed as the dissociation constant, $K_d$, in nM.

['Controls: $n = 5$ for each drug treatment vehicle, treated groups: $n = 8$.

Table XIII *R/S-*[^3]H*rolipram $B_{max}$ following chronic treatment with DMI and fluoxetine as compared to controls[^5].

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Supernatant</th>
<th>Pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$B_{max}$</td>
<td>%Change</td>
</tr>
<tr>
<td>Control DMI</td>
<td>66 ± 6</td>
<td>N/A</td>
</tr>
<tr>
<td>DMI</td>
<td>83 ± 14</td>
<td>27*</td>
</tr>
<tr>
<td>Control Fluoxetine</td>
<td>87 ± 14</td>
<td>N/A</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>82 ± 17</td>
<td>-5</td>
</tr>
</tbody>
</table>

[^5]$B_{max}$ values are in fmol/mg of protein in rat frontal cortex.

*p*<0.05 as compared to controls, Student's t-test; Controls: $n = 5$ for each drug treatment vehicle, treated groups: $n = 8$.

N/A = non-applicable.
$[^3]H$rolipram binding in either supernatant or pellet (Table XIII). However, the $B_{max}$ in the supernatant from DMI-treated rats was significantly increased by 27% as compared to controls, while no significant change was detected in the pellet (Table XIII).

3.8 PET Imaging of $R-[^{11}C]$Rolipram in Normal Human Volunteers

3.8.1 Regional distribution of $R-[^{11}C]$rolipram in human brain

High radioactivity accumulation was observed throughout the brain (Fig. 44), consistent with the wide distribution of PDE4 enzymes and the favorable properties of $R-[^{11}C]$rolipram as a PDE4 radioligand. Regional brain uptake was rapid initially and peaked at 10 min, followed by a slow washout (Fig. 45). High $R-[^{11}C]$rolipram levels were observed in the thalamus, which then converged to similar levels of binding as in the cortical and striatal areas at 40 min (Fig. 45). The peak uptake in the thalamus corresponded to 5.1±1.1 %ID/L. Lower binding was observed in the cerebellum.

3.8.2 $R-[^{11}C]$Rolipram in human arterial and venous plasma: metabolite analysis

Human plasma radioactivity levels were high throughout the scanning period, reaching a maximum at 5 min postinjection (Fig. 46), then gradually declining vs. time, with high protein binding (~97%). Although the dose-corrected total radioactivity was similar between venous and arterial plasma, a difference was obtained in the percentage unchanged $R-[^{11}C]$rolipram in arterial vs. venous plasma. Arterial plasma revealed that the percentage unchanged $R-[^{11}C]$rolipram concentration decreased gradually to 48% ± 5% at 90 min postinjection, with the remaining radioactivity corresponding to polar metabolites unlikely to cross the BBB (Fig. 47). In comparison, venous plasma displayed a slightly higher level of unchanged $R-[^{11}C]$rolipram, representing 65% ± 7% of the radioactivity in plasma at 90 min (Fig. 47).
Fig. 44 $R$-$[^{11}\text{C}]$Rolipram PET images (left – transverse; right – sagittal) from one of the control subjects, corresponding to the summed 0-90 min scan.

Fig. 45 Decay-corrected time-activity curves in the cerebellum, striatum, thalamus and prefrontal cortex of one subject following intravenous injection of $R$-$[^{11}\text{C}]$rolipram.
Fig. 46 Time course of radioactivity levels in human arterial and venous plasma following $R-[{}^1\text{C}]$rolipram injection. (Arterial: $n = 7$; venous: $n = 5$).

Fig. 47 Percentage of unmetabolized $R-[{}^1\text{C}]$rolipram in venous and arterial plasma as a function of time post-injection. (Arterial $n = 7$; venous $n = 5$).
CHAPTER 4.0: DISCUSSION

PDE4 is an essential component of the cAMP-signal transduction system and plays an important role in signaling and cell function by controlling cAMP levels formed following the stimulation of G-protein-coupled receptors in different neurotransmitter systems of the brain (Cherry and Davis, 1999; Yamashita et al., 1997; Ye et al., 1997). The ultimate objective of this project is to measure PDE4 levels in the living human brain using PET. The goal of this thesis was to synthesize the PDE4-selective inhibitors R/S-[14C]Ro 20-1724 and R/S-[14C]rolipram and characterize their in vivo pharmacological binding profile. The more active enantiomer of the radioligand showing the best profile would then be evaluated further to establish its ability to detect variations in PDE4 regulation and determine its potential for imaging PDE4 in the living human brain using PET, whereas, the less active enantiomer would be tested for its potential to measure the non-specific binding of the more active radioligand.

4.1 Radiosynthesis of the Radioligands

Previous reports have shown that R-rolipram (IC50 ~ 2-5 nM), R/S-rolipram (IC50 ~ 5-7 nM), S-rolipram (IC50 ~ 42-95 nM), and Ro 20-1724 (IC50 ~ 39-190 nM) bind selectively to the PDE4 high-affinity site (Barnette et al., 1995a; Borisy et al., 1993; Muller et al., 1996; Schneider et al., 1986; Torphy et al., 1992b) and produce behavioral effects (Egawa et al., 1997; Schmiechen et al., 1990). These PDE4 inhibitors contain a methoxy in the para position (see Fig. 6), which may be demethylated and then remethylated with [14C]CH3I, thus introducing the positron-emitting 14C isotope into the molecule, using PET radiochemistry approaches. Using 5 equivalents of iodotrimethylsilane (Olah et al., 1991; Vickery et al., 1979), Ro 20-1724 was
selectively dealkylated at the methoxy position in high yields (>90%). In contrast, dealkylation with BBr₃ (1.1 or 3.3 equivalents, for 0.5–1 h) or BI₃ (1 equivalent, 5 min) in CH₂Cl₂ (Lansinger and Ronald, 1979; McOmie et al., 1968) resulted in the demethylated derivative in poor yields (<40%), with more by-products and a higher proportion of unreacted starting material. The reaction of R/S-rolipram with iodoxotrimethylsilane was not selective, producing the desmethyl, descyclopentyl, and catechol derivatives in significant amounts. The highest yield of desmethyl-rolipram was obtained using 3.3 equivalents of iodoxotrimethylsilane. Under these conditions, most of the starting material was dealkylated, which is important due to the difficulty of separating the mono-hydroxy derivatives from rolipram by column chromatography. Reaction of racemic rolipram with BBr₃ (1.1 equivalent, 1 h reaction time) or BI₃ (1 equivalent, 5 min) in CH₂Cl₂ primarily produced the descyclopentyl and catechol derivatives, and a minimal amount of desmethyl-rolipram, along with a high quantity of unreacted rolipram. Due to its lower reactivity, higher selectivity for sterically hindered methoxy groups was previously reported in similar O-dealkylation reactions using iodoxotrimethylsilane as compared to reactions with BBr₃ (Vickery et al., 1979). Contrary to R/S-desmethyl-Ro 20-1724, R/S-desmethyl-rolipram could not be purified by recrystallization. Enantiomeric separation of R- and S-desmethyl-rolipram from racemic desmethyl-rolipram was achieved readily by successive chiral semi-preparative HPLC.

Radiosynthesis of the four PDE4 inhibitors labeled with ¹³C was accomplished successfully by O-[¹³C]methylation of the corresponding phenolic precursors with [¹³C]CH₃I, using a similar approach as has been previously used in the radiosynthesis of [¹³C]tetrabenazine (DaSilva et al., 1993a). As expected, similar results were obtained in the [¹³C]methylation reactions and all HPLC preparations of R-, R/S-, and S-[¹³C]rolipram. Optimization of the radiochemical reactions was attempted by reducing the heating period, changing the reaction temperature, or increasing the [¹³C]CH₃I bubbling flow. The best conditions as described in chapter 2 produced
$R/S-[{}^{13}]C$Ro 20-1724, $R$-, $R/S$-, and $S-[{}^{13}]C$rolipram in high radiochemical yields and purity as shown by HPLC. No isomerization was expected in these reactions with $R$- and $S$-desmethylrolipram since functional groups on the chemical structure of these compounds are distant from the chiral centre, where racemization could occur. In tracer doses, these radiotracers are expected to bind selectively to the high-affinity conformational binding state over the low-affinity state of PDE4. Therefore, binding of $R-[{}^{13}]C$rolipram in the brain in vivo in PET studies is expected to reflect the distribution of the high affinity binding conformation of the enzymes.

4.2 $R-[{}^{13}]C$Rolipram in Vivo Binding and Selectivity for PDE4

The rat regional brain uptake of $R-[{}^{13}]C$rolipram was high, with a rank order of binding that concurred with the distribution of $R/S-[{}^{3}]H$rolipram published previously (Kaulen et al., 1989; Schneider et al., 1986). Higher levels of radioactivity were observed in cortical and olfactory regions and lower levels in the hypothalamus and brain stem. The regional brain accumulation of $R-[{}^{13}]C$rolipram was 50% higher than $R/S-[{}^{13}]C$rolipram at 45 minutes postinjection, likely due to the 10-30 fold higher potency of $R$-rolipram as compared to the $S$-enantiomer at binding to PDE4 (Owens et al., 1997; Schneider et al., 1986). Brain levels of $[{}^{13}]C$Ro 20-1724 were lower than blood, with very low regional brain tissue contrasts. In fact, $R-[{}^{13}]C$rolipram revealed a 2.5–4.6 fold greater regional brain activity retention than $[{}^{13}]C$Ro 20-1724, even though Ro 20-1724 ($\log P = 1.83$) is slightly more lipophilic than rolipram ($\log P = 1.36$). This result is consistent with the 5-10 fold higher potency of $R$-rolipram at eliciting behavioral effects (Kehne et al., 1991; Kehne et al., 1987) and with its higher binding affinity ($IC_{50} \sim 1-7$ nM) for the high-affinity conformation of PDE4 as compared to Ro 20-1724 ($IC_{50} \sim 23-190$ nM) (Borisy et al., 1993; Kaulen et al., 1989; Schneider et al., 1986; Torphy et al., 1992b).
Competition studies revealed that coinjection of PDE4-selective inhibitors with the radioligands blocked R/S- and R-[\textsuperscript{3}H]rolipram regional brain uptake more effectively and to a greater extent than [\textsuperscript{3}H]Ro 20-1724, likely due to the lower brain penetration and affinity of [\textsuperscript{3}H]Ro 20-1724. A higher dose of Ro 20-1724 (30 mg/Kg) (Kant et al., 1980) was used to compete with R/S- and R-[\textsuperscript{3}H]rolipram binding to PDE4, since 10 mg/Kg was not sufficient to reduce [\textsuperscript{3}H]Ro 20-1724 binding to the levels reached with 10 mg/Kg of rolipram. Therefore, due to its poor in vivo binding characteristics, [\textsuperscript{3}H]Ro 20-1724 was not evaluated further.

Coinjection of R/S-rolipram and Ro 20-1724 significantly reduced the binding of R/S- and R-[\textsuperscript{3}H]rolipram to non-specific levels in brain regions, implying that these radiotracers bind specifically to PDE4 in rat brain. Both R/S- and R-[\textsuperscript{3}H]rolipram uptake was significantly reduced by Ro 20-1724 or rolipram treatment in the heart, but not in the lung or liver. This is in accordance with previous studies reporting that the heart contains detectable levels of the high-affinity [\textsuperscript{3}H]rolipram binding site (Schneider et al., 1986).

The brain also expresses high levels of PDE1, which hydrolyzes both cAMP and cGMP (Beavo, 1995; Polli and Kincaid, 1994). The amino acid sequence of all mammalian PDE enzymes is 25-40% conserved (Manganiello et al., 1995), making PDE1 an additional potential binding site for R/S- and R-[\textsuperscript{3}H]rolipram in the brain. However, pretreatment with a high dose (10 mg/Kg) of the brain penetrating PDE1 selective inhibitor vinpocetine (Sauer et al., 1988; Yamashita et al., 1997; Gulyas et al., 1999) did not alter R- or R/S-[\textsuperscript{3}H]rolipram uptake, indicating that the radioligands do not bind specifically to PDE1. This result is in accordance with the well-established selectivity of rolipram for PDE4 (Conti et al., 1995b; Yamashita et al., 1997). Furthermore, Schneider and colleagues (1986) tested rolipram affinity to other potential binding sites and found that it does not bind specifically to \(\alpha\)- and \(\beta\)-adrenergic, dopamine, 5-HT, diazepam, adenosine, \(\gamma\)-aminobutyric acid, or opiate receptors in rat brain membranes. Previous studies have shown that rolipram acts presynaptically to increase the synthesis and
release of NA (Kehr et al., 1985). \( R/S- \) and \( R-[^{13}C]\)rolipram binding selectivity was thus also evaluated using a potential competitor, DMI, which binds to the presynaptic NA transporter site. However, high doses of DMI did not reduce radioactivity concentrations in any brain area, suggesting that \( R/S- \) and \( R-[^{13}C]\)rolipram do not bind to the NA transporter. These preliminary studies show that \( R/S- \) and \( R-[^{13}C]\)rolipram satisfied important properties required for a PET radioligand by displaying high regional brain signal and tissue contrast, and exhibiting in vivo binding selectivity for PDE4 over PDE1 and the NA transporter in the brain.

Since \( R-[^{13}C]\)rolipram demonstrated a better pharmacological profile than \( [^{13}C]Ro \) 20-1724 and a ~50% higher regional brain uptake than \( R/S-[^{13}C]\)rolipram, only the \( R\)-enantiorner radioligand was developed further. The in vivo binding of \( R-[^{13}C]\)rolipram was further characterized using coinjections of increasing doses of unlabeled \( R\)-rolipram in order to obtain a dose-response curve and determine the ED\(_{50}\) for blocking \( R-[^{13}C]\)rolipram uptake in brain areas. Coinjections of \( S\)-rolipram were also performed in order to assess its in vivo potency relative to the more active enantiomer. As expected, \( R\)- and \( S\)-rolipram produced a dose-dependent reduction in \( R-[^{13}C]\)rolipram in all brain areas. \( R\)-Rolipram was ~13.5 fold more potent than \( S\)-rolipram since the ED\(_{50}\) for \( R\)-rolipram was 0.037 mg/Kg while the ED\(_{50}\) for \( S\)-rolipram was 0.5 mg/Kg for all brain areas tested. Interestingly, saturating doses of \( S\)-rolipram did not lower \( R-[^{13}C]\)rolipram accumulation to the same level as saturating doses of \( R\)-rolipram, \( R/S\)-rolipram, or Ro 20-1724. A possible explanation for this result is that the \( S\)-enantiomer does not bind to the same saturable non-specific binding sites of \( R-[^{13}C]\)rolipram as \( R\)-rolipram and Ro 20-1724. It is well established that \( R/S\)-rolipram binds selectively to the PDE4 family of enzymes (Schneider et al., 1986; Yamashita et al., 1997), and that \( R\)-rolipram inhibits similarly all four PDE4 subtypes with a high-affinity (Hughes et al., 1997; Wang et al., 1997). Most studies have focussed on \( R/S- \) or \( R\)-rolipram binding to the PDE4 enzymes, and it is not clear whether \( S\)-
rolipram binds similarly to all of the PDE4 variants. Studies have shown that the two enantiomers can have very different potencies in behavioral paradigms in vivo. For example, R-rolipram was shown to be up to 2000 fold more potent than S-rolipram at improving deficits in learning and memory in rats (Egawa et al., 1997). In contrast, other reports revealed that R-rolipram has only a 15 fold higher potency than S-rolipram at inducing head twitches in rats (Schmiechen et al., 1990). These different results could be explained by a different binding profile of both enantiomers to both specific and non-specific binding sites.

4.3 S-[^1]C]Rolipram in Vivo Binding in Rat Brain

In light of the fact that S-rolipram is less potent than R-rolipram, the potential for using S-[^1]C]rolipram to measure the non-specific binding of R-[^1]C]rolipram in PET studies was evaluated. In comparison to R-[^1]C]rolipram, the regional brain uptake of the S-enantiomer radioligand was 5-10 fold lower at 45 min postinjection. The lowest retention of S-[^1]C]rolipram was observed in the brain as compared to the peripheral organs sampled, likely due to its lower affinity for PDE4. Competition studies demonstrated that the minimum dose of R-rolipram (0.5 mg/Kg) and S-rolipram (3 mg/Kg) effective at reducing brain R-[^1]C]rolipram retention to non-specific levels also decreased accumulation of S-[^1]C]rolipram by 37-61%, whereas the PDE1 inhibitor vinpocetine had no effect. Although S-[^1]C]rolipram exhibited a weaker signal than R-[^1]C]rolipram, the less active enantiomer radioligand showed a substantial binding to PDE4, since 35-61% of its brain uptake was blocked by PDE4-selective competitors. Therefore, using S-[^1]C]rolipram for measuring non-specific binding, after subtraction from the R-[^1]C]rolipram scan, would result in a loss of the specific binding component in the PDE4 signal. Furthermore, as described above, S-[^1]C]rolipram may bind differently to non-specific and/or specific sites than
$R-[^{13}C]$rolipram, which indicates that $S-[^{13}C]$rolipram is unsuitable for measuring the non-specific binding of $R-[^{13}C]$rolipram.

4.4 $R-[^{13}C]$Rolipram Displayed Favorable Metabolism and Dosimetry Characteristics

In whole body distribution studies of $R-[^{13}C]$rolipram, ~60% of the total radioactivity excreted by 60 min occurred via the kidneys and the remaining via the hepatobiliary route. Cumulative absorbed dose estimates for administration of $R-[^{13}C]$rolipram to humans were low with the urinary bladder wall being the limiting organ. A typical 10 mCi dose of $R-[^{13}C]$rolipram per PET scan is safe for injection in humans since the absorbed dose estimate in the urinary bladder wall remains ~10 fold lower than the maximum allowed for this organ per PET study according to Food and Drug Administration (USA) guidelines.

Rolipram was previously reported to undergo ether cleavage at the methoxy and cyclopentyloxy groups, and hydroxylation in the cyclopentyl and pyrrolidine rings (see Fig. 6 for the chemical structure), followed by the formation of aromatic and aliphatic sulphates, yielding at least eight different metabolites unlikely to cross the BBB (Krause et al., 1993). Analysis of plasma radioactivity at 30 min postinjection in the rat revealed extensive metabolism, with ~20% unchanged $R-[^{13}C]$rolipram and the majority of the radioactivity corresponding to polar metabolites. Analysis of rat brain extracts demonstrated a single radioactive peak with the same $R$, as authentic $R-[^{13}C]$rolipram, suggesting that radioactivity in the brain corresponded to unchanged radioligand and that no radioactive metabolites crossed the BBB. These results are in agreement with previous studies reporting that $[^{3}H]$rolipram is metabolized into several compounds (Krause et al., 1993), while only unchanged $R/S-[^{3}H]$rolipram is found in rat brain homogenates (Krause and Kühne, 1988). Additionally, the
pharmacokinetics and biotransformation of rolipram were found to be similar between rat, monkey, and human (Krause and Kühne, 1988; Krause et al., 1993), with no differences between the two enantiomers (Krause et al., 1990). Therefore, \( R-[^{11}C] \) rolipram appears to have the acceptable metabolic characteristics for imaging PDE4 enzymes using PET.

No reference was found describing which cytochrome P450 (CYP450) enzymes metabolize rolipram. Given that rolipram is extensively biotransformed into hydroxylated/dealkylated products, it should be kept in mind that future administration of \( R-[^{11}C] \) rolipram to humans could be subject to altered metabolism if drugs that induce and/or inhibit the CYP450 enzymes that metabolize the radioligand are coadministered. For instance, the MAO inhibitor tranylcypromine has been found to be a potent inhibitor of CYP2C19 (Baker et al., 1999) and could significantly inhibit radioligand metabolism if CYP2C19 is a main enzyme metabolizing \( R-[^{11}C] \) rolipram. Administration of barbiturates, for example, is known to potently induce CYP3A4 and could thus increase the metabolism of coadministered drugs that are metabolized by this enzyme (Glue and Clement, 1999). Additionally, different subjects could display different \( R-[^{11}C] \) rolipram metabolism due to polymorphisms in the expression of certain CYP450 enzymes, including CYP2C19, CYP2D6, and CYP2C9 (Coutts and Urichuk, 1999). Altered radioligand metabolism could lead to different levels of unchanged \( R-[^{11}C] \) rolipram in human plasma, affecting both the \textit{specific} and \textit{non-specific} regional brain uptake of \( R-[^{11}C] \) rolipram, which may have implications in PET imaging and pharmacokinetic modeling. Other factors such as age, gender, race, and the environment may also have a substantial effect on the activity of the CYP450 enzyme system (Glue and Clement, 1999; Coutts and Urichuk, 1999), and should be taken into consideration in interpreting results of PET studies using radioligands, such as \( R-[^{11}C] \) rolipram, that are extensively metabolized within the imaging time window (~90 min).
4.5 Modulation of \( R-[^{13}\text{C}] \text{Rolipram Binding Following Different Treatments Affecting} \)
\( \text{cAMP-Mediated Signaling in the Brain: Sensitivity to PDE4 Regulation} \)

Exploratory experiments were performed using different drug challenges expected to alter
\( \text{cAMP-mediated signaling directly or indirectly in order to assess the ability of} \ R-[^{13}\text{C}] \text{rolipram} \)
to detect changes in the regulation of PDE4. Different acute and chronic drug treatments were
executed using doses as published in the literature and time periods in accordance with the rapid
\((-15 \text{ min})\) or prolonged (within hours to days) regulation of PDE4.

4.5.1 Effect of acute forskolin treatment

Forskolin enhances \( \text{cAMP concentrations in the brain } in \ \text{vivo} \) (Sano et al., 1984) by binding
to AC (Simonds, 1999), and was selected as an agent that increases \( \text{cAMP-mediated signaling more directly at the intracellular level. Compared to controls, forskolin coinjection with the} \)
radioligand produced a significant increase of 29-41% in \( R-[^{13}\text{C}] \text{rolipram uptake across all brain regions in vivo.} \)
The effect of forskolin was rapid since a lower dose coinjected with \( R-[^{13}\text{C}] \text{rolipram was more effective than a higher dose administered 3 hours prior to the} \)
radioligand. This result is in agreement with the rapid effect of forskolin at increasing \( \text{cAMP levels in vitro in cell cultures (Madelian and La Vigne, 1996) and in vivo in mouse brain (Sano} \)
et al., 1984). For instance, forskolin has been shown to increase PDE4 activity by over 100%
within 15 minutes of treatment, independent of \( \text{de novo protein synthesis in astroglial (Madelian} \)
and La Vigne, 1996) and vascular smooth muscle cells (Ekholm et al., 1997). This rapid effect
is rather dependent on PKA-mediated phosphorylation (Ekholm et al., 1997), and is likely a
result of at least increased activity of PDE4D3 (Hoffman et al., 1998). However, the
physiological effects of forskolin, including hypotension and cardiac inotropy (Seamon and
Daly, 1986), as well as its central effects, may influence the pharmacokinetics of \( R-[^{13}\text{C}] \text{rolipram in vivo.} \)
Because forskolin activates most AC enzymes present in the brain
(Simonds, 1999), forskolin increases cAMP synthesis in many brain areas and affects several neuronal processes, which may differentially regulate PDE4. For example, forskolin increases the release of NA (Markstein et al., 1984), acetylcholine (Allgaier et al., 1990), and dopamine (Katz et al., 1983) in the brain, and alters potassium channel conductance (Hoshi et al., 1988). Moreover, some of these effects may be mediated independently of AC (Allgaier et al., 1990; Hoshi et al., 1988). In our study, blood radioactivity levels were significantly increased (by >83%) following forskolin injection, raising the question of whether plasma levels of unmetabolized R-[\(^{14}\)C]rolipram were increased as compared to controls. Indeed, metabolism studies showed that the concentration of unchanged R-[\(^{14}\)C]rolipram in rat plasma at 45 minutes postinjection was ~25% higher with forskolin treatment. This increase may have contributed to a higher brain absorption of the radioligand, since non-specific binding was also elevated by >2 fold across brain regions as compared to controls. Thus, despite the high increase in regional brain radioactivity uptake (up to 41%), R-[\(^{14}\)C]rolipram specific binding was calculated to be increased by only ~25% in the frontal cortex and olfactory regions, and to a lesser extent in other brain areas. No specific binding was detected in peripheral organs likely due to a high non-specific binding in those tissues as well as the potential presence of radiolabeled metabolites, overshadowing the signal. An experiment to determine if the increase in blood radioactivity levels was independent of AC activation was attempted using the inactive forskolin analogue, 1,9-dideoxyforskolin (RBI), however, it was unsuccessful due to the lack of solubility of the compound in water and unknown BBB permeability of the emulsion form.

Despite the heterogeneous actions of forskolin, its ability to increase cAMP levels appears to be important for PDE4 regulation since forskolin and rolipram were reported to synergistically increase cAMP levels in the brain and improve behavior in animal tests of antidepressant activity (Wachtel and Löschmann, 1986; Wachtel et al., 1987). Interestingly, both acute and chronic administration of NKH477, a novel and potent water-soluble forskolin
derivative, significantly augmented the expression of BDNF and trkB mRNA in rat frontal cortex and hippocampus, in accordance with increases in cAMP levels (Morinobu et al., 1999). Thus, activation of AC by forskolin may induce the rapid expression of downstream components of the cAMP-signaling pathway previously suggested to play a potentially important role in the treatment of depression. Moreover, we detected higher R-[^11]C]rolipram specific binding following forskolin treatment in regions reported to have high PDE4D mRNA expression, such as the olfactory system (Engels et al., 1995a). Therefore, it is plausible that the increase in specific binding produced by forskolin is partly due to an augmentation in the regulation of PDE4 subtypes, such as PDE4D, in addition to the blood flow and changes in R-[^11]C]rolipram metabolism effects of the drug.

4.5.2 Effect of acute and chronic modulation of \( \alpha_2 \) - and \( \beta \)-adrenoceptors

Previous studies have identified the noradrenergic system as one of the main neurotransmitter systems in the brain expressing and regulating PDE4 activity (Przegalinski et al., 1985; Schultz and Schmidt, 1986; Yamashita et al., 1997; Ye et al., 1997; Ye and O' Donnell, 1996). Noradrenergic pathways project from the locus coeruleus and lateral tegmentum in the pons to brain regions such as the thalamus, hippocampus, amygdala, septum, frontal cortex, occipital cortex, and cerebellum (Lanca, 1998; Mongeau et al., 1997). PDE4 activity is reported to be present both pre- and postsynaptically in the noradrenergic system, metabolizing cAMP at both sites (Scuvièe-Moreau et al., 1987; Wachtel and Schneider, 1986). To determine the ability of R-[^11]C]rolipram to detect the regulation of PDE4 in this system, treatments were performed with several \( \alpha_2 \) - and \( \beta \)-adrenergic agents.

4.5.2.1 \( \alpha_2 \)-Adrenergic modulators

The \( \alpha_2 \)-adrenergic autoreceptor is negatively coupled to AC, decreasing the synthesis of cAMP. Antagonism of \( \alpha_2 \)-adrenoceptors by yohimbine leads to inhibition of the negative
feedback in the turnover of synaptic NA, rapidly increasing (within 15 min) central sympathetic neuron discharge (McCall et al., 1983) and the concentration of NA at the synapse by >90% (Pacak et al., 1992; Szemeredi et al., 1991). Increased synaptic endogenous NA levels were reported to stimulate postsynaptic β-adrenoceptors leading to an increase in cAMP levels and PDE4 regulation (Ye et al., 1997). Additionally, behavioral models for testing the potential antidepressant action of rolipram demonstrated an in vivo link between PDE4 and yohimbine action, since acute treatment with rolipram in mice potentiated yohimbine lethality (Wachtel, 1983). In vivo yohimbine administration in our study produced a time-dependent increase in \( ^{13}C \)-rolipram regional brain retention. Shorter pretreatment times (e.g., 15 min prior to radioligand injection) were more effective, yielding rapid radioactivity accumulations of 25–47% significantly over control values, which is in accordance with the rapid effect of yohimbine at increasing NA levels in the synapse in vivo (Pacak et al., 1992; Szemeredi et al., 1991). \( ^{13}C \)-Rolipram specific binding was 25–36% higher than controls, with no increase in non-specific binding, suggesting that the effect of yohimbine on radioligand uptake was due to a specific increase in PDE4 regulation. High specific binding elevations of 92% and 80% were also detected in the lung and heart, respectively. Yohimbine has also been reported to display pharmacological effects, such as increases in peripheral blood pressure (McCall et al., 1983), which may have enhanced the delivery of \( ^{13}C \)-rolipram to the brain. Indeed, blood radioactivity levels were significantly increased by ~29% over controls. However, the proportion of this increase that corresponds to authentic \( ^{13}C \)-rolipram is not known, and it could be that the augmented blood radioactivity levels are represented mostly by metabolites, as is the case with the fluoxetine treatment in this thesis. Studies are necessary to test this possibility if further validation using yohimbine is intended. Nonetheless, the increase in \( ^{13}C \)-rolipram specific binding in some brain regions was slightly higher (by ~7%) than the
elevation in blood radioactivity levels, suggesting that part of the effect of yohimbine may be due to an increase in the regulation of PDE4 as detected by \( R-[^{11}\text{C}] \)rolipram binding.

Clonidine has opposite effects to yohimbine, decreasing the synthesis and release of NA by stimulating presynaptic \( \alpha_2 \)-adrenoceptors. Thus, clonidine inhibits the firing of central NA-containing neurons (Svensson et al., 1975) and reduces peripheral blood pressure (Garty et al., 1990). Acute or chronic treatment with rolipram in rats was shown to reduce the effect of clonidine on food intake, demonstrating a behavioral link between PDE4 and clonidine action likely mediated by the noradrenergic system (Przegalinski and Jurkowska, 1987). \textit{In vivo} clonidine treatment in rats was hypothesized to decrease \( R-[^{11}\text{C}] \)rolipram regional brain retention due to a reduction in NA levels (Itoh et al., 1990) and thus decreased stimulation of postsynaptic \( \beta \)-adrenoceptors. A lower dose of clonidine (0.1 mg/Kg), expected to be more selective for \( \alpha_2 \)-adrenoceptors, revealed no significant effect on \( R-[^{11}\text{C}] \)rolipram regional brain uptake, suggesting that activation of \( \alpha_2 \)-adrenoceptors did not significantly affect PDE4 regulation \textit{in vivo} in rats. Interestingly, different results were obtained with a high dose of clonidine (1 mg/Kg) (Svensson et al., 1975; Szmigielski et al., 1986), a dose at which a significant augmentation in regional brain \( R-[^{11}\text{C}] \)rolipram uptake of 17-40\% was obtained in this study, with a similar enhancement in \textit{specific binding} of 18-41\% over controls. Peripheral organs, such as the lung and heart, demonstrated no measurable change in \textit{specific binding}. Although clonidine is known to decrease blood pressure (Philippu et al., 1973), radioactivity levels in blood were marginally significantly increased by \(-16\%\) with clonidine as compared to controls. The exact reason for the increase in blood radioactivity levels is unknown, but could have been produced by inhibition of radioligand metabolism or displacement from tissue depots. Clonidine has been shown to have a number of effects that are difficult to explain in the brain, especially at higher doses (1 mg/Kg). For instance, as opposed to 0.1 mg/Kg, the higher dose of clonidine (1 mg/Kg) used in our study could lose its selectivity for the presynaptic \( \alpha_2 \)-
adrenoceptors, stimulating also postsynaptic α-adrenoceptors, and affecting 5-HT neurotransmission by inhibiting a great majority of 5-HT neurons in the dorsal raphe nucleus (Svensson et al., 1975, Szmigielski et al., 1986). Clonidine has also been shown to bind with high-affinity to non-adrenoceptor sites, which have been termed imidazoline binding sites or imidazoline-preferring receptors (Olmos et al., 1994; Zhu et al., 1997). It is not known whether imidazoline receptors regulate PDE4, however, 0.6-2.4 mg/Kg doses of clonidine have been shown to produce a paradoxical increase (by 56%) in NA levels in rat brain, an effect that is possibly mediated by the imidazoline receptors (Meana et al., 1997). In conclusion, the exact effect of the 1 mg/Kg dose of clonidine in our study is not known, but it is possible that it increased R-[^14]C]rolipram specific binding by NA stimulation or through other neurotransmitter systems directly or indirectly linked to PDE4.

4.5.2.2 β-Adrenergic modulation

*In vitro* stimulation of β2-adrenoceptors in cell cultures (Manning et al., 1996; Torphy et al., 1992c) and *in vivo* modulation of central β-adrenoceptors (Ye et al., 1997; Ye et al., 2000; Ye and O'Donnell, 1996) have been shown to alter the expression of PDE4 subtypes. Clenbuterol is a centrally acting selective agonist at β2-adrenoceptors, increasing cAMP synthesis through the activation of AC (O'Donnell and Frazer, 1985). Administration of clenbuterol was reported to reduce the ability of isoproterenol to increase levels of cAMP in slices of rat cerebral cortex, an effect that was significant as early as one day following administration, and highest after 8 days of chronic clenbuterol treatment (O'Donnell and Frazer, 1985). This effect of clenbuterol was shown to be due to a desensitization in the second messenger pathway, and not due to a downregulation of β2-adrenoceptors (O'Donnell and Frazer, 1985). One mode via which β2-adrenoceptors become desensitized is through increased PDE4 expression (Giembycz, 1996; Seybold et al., 1998; Torphy et al., 1995). It was then hypothesized that acute and chronic clenbuterol injection could upregulate PDE4 activity, leading to an increase in R-[^14]C]rolipram
binding in the brain. Indeed, the uptake of the radioligand was significantly increased by 15-32% in several brain regions following acute clenbuterol administration, suggesting that this drug treatment upregulated the expression of PDE4. In contrast, no significant effect on regional brain R-[11C]rolipram uptake was observed following chronic clenbuterol. The mechanism of action behind the effect of acute clenbuterol treatment on R-[11C]rolipram retention is not known. β2-Adrenoceptor density is lower in the brain as compared to the periphery (Molinoff, 1984). By comparison, β1-adrenoceptors are more highly expressed in the cortex, and are possibly the main receptors regulating PDE4 in the noradrenergic system (Molinoff, 1984; Ye and O'Donnell, 1996). Moreover, brain developmental studies have shown that PDE4 activity is more closely associated with β1-adrenoceptors (Zhang et al., 1999). Alternatively, although speculative at this point, it is possible that the acute effect of clenbuterol involves increased expression of PDE4, whereas, the effect of chronic clenbuterol in the brain activates other pathways not involving PDE4. In conclusion, although acute treatment with clenbuterol increased radioligand uptake in this study, chronic stimulation of these receptors did not appear to significantly alter PDE4 binding in the rat brain as measured by R-[11C]rolipram uptake. More studies are necessary to analyze the effect of clenbuterol treatment on PDE4 regulation.

Chronic infusion with the non-selective β-adrenergic antagonist propranolol has been shown to upregulate β-adrenoceptors and downregulate PDE4A expression in rats (Ye et al., 1997; Ye and O'Donnell, 1996). Chronic, but not acute, treatment using intermittent injections of propranolol in our study contradicted these reports since it produced an elevation in R-[11C]rolipram retention in rat brain, that was significant in some brain areas, including the frontal cortex. As a β-blocker, propranolol has physiological effects such as decreased heart rate and hypotension (Prichard and Tomlinson, 1986). These effects, however, would probably decrease the brain uptake of the radioligand since propranolol was reported to decrease slightly
regional cerebral blood flow (Olesen et al., 1978), and thus do not explain the results.
Propranolol also antagonizes other brain receptors such as the 5-HT₁ subtypes (Boulenguez et al., 1992; Hide et al., 1989), and could affect cAMP synthesis by blocking the inhibitory effect of some of the 5-HT₁ receptor subtypes on AC (Uphouse, 1997). Alternatively, the increase in R-[¹³C]rolipram binding could have resulted from a rebound effect: β-adrenoceptors are upregulated by chronic propranolol (Ye and O'Donnell, 1996; Ye et al., 1997) and after a 24-hour washout of the drug, the receptors would become exposed to endogenous NA leading to an upregulation in PDE4 expression. Ye and colleagues (1996, 1997) did not report such a rebound effect on PDE4 expression. This may be because of two main differences between their drug treatment protocol and ours. First, they performed a constant infusion of propranol at a higher dose (30 mg/Kg) than in our study (20 mg/Kg), whereas our study involved intermittent injections (once a day) of the drug. It is possible that with the intermittent administration of propranolol, some β-adrenoceptors were activated by endogenous NA during the washout period occurring between the daily injections. Second, and importantly, Ye and colleagues (1996, 1997) assayed the rat brains 14 days after the start of the infusion without any washout period. Without a washout period, β-adrenoceptors remained blocked until the animals were sacrificed, likely not allowing for any sensitized β-adrenoceptors to be stimulated by endogenous NA, due to the presence of the antagonist.

4.5.3 Effect of drugs acting acutely and chronically on serotonergic receptors

At least 7 different mammalian 5-HT receptor families have been described to date, with several subtypes within each family (Uphouse, 1997). The 5-HT₆, 5-HT₁₅, and 5-HT₁ receptors increase AC activity and cAMP synthesis (Baker et al., 1998; Dumuis et al., 1991; Markstein et al., 1999; Ruat et al., 1993a; Ruat et al., 1993b; Torres et al., 1995), and are downregulated by chronic antidepressant treatment (Bijak, 1997; Bijak et al., 1997; Mullins et al., 1999; Sleight et al., 1995; Uphouse, 1997). The high-affinity 5-HT₁₅ autoreceptors are present in the soma and
dendrites of 5-HT neurons, and inhibit AC. Their activation was shown to decrease firing of 5-HT neurons and decrease release of 5-HT from synaptic terminals (Baumann and Rothman, 1998; Kreiss and Lucki, 1997). In contrast, 5-HT$_2$ receptors mediate neuroexcitation through a different second messenger system, the effector phospholipase C (PLC) and the phosphoinositide pathway. One of the second messengers produced upon activation of PLC is inositol triphosphate, which stimulates the release of calcium from intracellular stores, leading to the activation of calcium dependent kinases and possibly also calcium dependent AC, contributing to cross talk between different second messenger systems (Uphouse, 1997). PDE4 enzymes may be linked to some 5-HT receptors since chronic treatment with the selective 5-HT reuptake inhibitor fluoxetine was shown to increase the expression of PDE4A and B in rat brain (Takahashi et al., 1999; Ye et al., 2000), independent of β-adrenoceptor activation (Ye et al., 2000). To study the *in vivo* modulation of PDE4 enzymes by the serotonergic system, treatments were performed with centrally active 5-HT receptor agonists and antagonists.

4.5.3.1 5-HT$_{1A}$ receptors

The 5-HT$_{1A}$ agonist 8-OH-DPAT binds to and activates the dendritic 5HT$_{1A}$ autoreceptors (Albert et al., 1996), leading to a decrease in the synthesis and release of 5-HT and an inhibition in 5-HT cell firing (Baumann and Rothman, 1998). Acute 8-OH-DPAT administration decreased 5-HT release and baseline 5-HT content by 60-80% within 40 minutes, remaining low for at least 3 hours (Kreiss and Lucki, 1997). In our studies, however, pretreatment with 8-OH-DPAT 3 hours prior to $R$-[H]$\text{C}$]rolipram injection in rats produced no significant change in radioligand binding across brain regions, suggesting that the reduction in 5-HT levels elicited by 8-OH-DPAT does not affect PDE4 regulation. Prolonged administration of 5-HT$_{1A}$ agonists, including 8-OH-DPAT, has been shown to lead to the desensitization of the 5-HT$_{1A}$ autoreceptor, thus attenuating the negative feedback on the turnover of 5-HT in different brain regions (Albert et al., 1996; Kreiss and Lucki, 1997). The desensitization in 5-HT$_{1A}$ receptor
function may lead to an increase in 5-HT input at different 5-HT receptors, including those that activate cAMP production, thus potentially increasing PDE4 regulation. However, repeated injections of 8-OH-DPAT yielded no significant change in $R$-$[^{11}\text{C}]$rolipram distribution across brain regions. 8-OH-DPAT has been reported to also activate 5-HT$_1$ receptors leading to an increase in cAMP (Lovenberg et al., 1993; Uphouse, 1997), and this effect could oppose the actions of the 5-HT$_{1A}$ receptor following chronic treatment with the agonist. Similar to acute treatment with 8-OH-DPAT, a single injection of the 5-HT$_{1A}$ antagonist WAY 100635 had no effect on $R$-$[^{11}\text{C}]$rolipram brain retention.

4.5.3.2 5-HT$_{2A/C}$ receptors

The 5-HT$_{2A/C}$ agonist DOI and the antagonist ritanserin also yielded no significant effect on radioligand accumulation in rat brain following acute challenges, showing only slight decreases across brain regions, down by 13% in the hippocampus, with no changes in blood radioactivity levels. Interestingly, a similar treatment of DOI (2 mg/Kg, i.p., 3 hours prior) as used in our study (3 mg/Kg, i.p., 3 hours prior) downregulated and upregulated the levels of BDNF mRNA in rat hippocampus and neocortex, respectively, whereas ritanserin blocked this effect (Vaidya et al., 1997), showing that this drug may differentially affect the expression of neurotrophins in different brain regions. It may be worth performing chronic treatments with DOI in future experiments in order to determine the function of 5-HT$_{2A/C}$ receptors in regulating cAMP-mediated signaling and PDE4 in vivo. Taken together, these results suggest that drugs acting acutely or chronically on the 5-HT$_{1A}$ and 5-HT$_{2A/C}$ receptors do not significantly influence PDE4 regulation or $R$-$[^{11}\text{C}]$rolipram binding in rat brain in vivo.

4.5.4 Effect of agents acting on adenosine, histamine, and dopamine neurotransmitter receptors

As mentioned above, the noradrenergic system has been reported to be the main neurotransmitter system implicated in the function and regulation of PDE4 in the brain. The
selectivity of PDE4 for this neurotransmitter system may partly explain the clinical efficacy of rolipram in improving symptoms of depression (Fleischhacker et al., 1992; Laux et al., 1988; Zeller et al., 1984). However, studies have demonstrated that PDE4 is also expressed in other neurotransmitter systems, such as histamine and adenosine (Donaldson et al., 1988; Whalin et al., 1989), but probably not in the dopamine system (Polli and Kincaid, 1994). Therefore, in order to explore the ability of other neurotransmitter systems to regulate \( R-[^{13}\text{C}] \text{rolipram} \) binding to PDE4, drugs acting on adenosine, histamine, and dopamine receptors were tested.

4.5.4.1 Adenosine and histamine neurotransmission

The adenosine-A\(_2\) and the histamine-H\(_2\) receptors stimulate cAMP production by activating AC via \( G_{\alpha} \) (Agullo et al., 1990; Boknik et al., 1997; Correia-de-Sá and Ribeiro, 1994). Stimulation of adenosine-A\(_2\) receptors also increases 5-HT release (Barraco et al., 1996), and may thus regulate PDE4 indirectly via 5-HT receptors. Exposure of PC12 cell cultures to the A\(_2\) agonist CGS 21680 was shown to upregulate PDE4 activity by 100% within 4 hours in a PKA-dependent fashion (Chang et al., 1997). Adenosine-A\(_2\) receptors are reported to be abundantly expressed in brain regions showing high expression of PDE4 enzymes, such as the olfactory tubercle (Engels et al., 1995a; Jarvis and Williams, 1989). It was then hypothesized that acute treatment with CGS 21680 in vivo 3 hours prior to radioligand injection, would increase the regulation of PDE4. CGS 21680 produced an increase (non-significant) in radiotracer uptake of 21% in the olfactory bulbs, with modest increases in some of the other brain areas sampled, including the thalamus. These results were replicated in a second study (total \( n = 9 \) rats). The changes in radioligand regional brain uptake corresponded to very similar changes in specific binding, with an elevation of 26% in the olfactory bulb, and other regions demonstrating very little or no change. In addition to its effect on adenosine-A\(_2\) receptors and 5-HT release, CGS 21680 increases the release of neurotransmitters such as acetylcholine (Sebastiao and Ribeiro, 1996), which could indirectly interfere with the effect of CGS 21680 on
the binding of \( R-[^{13}C] \)rolipram to PDE4 in brain regions. A 85% and 28% elevation in \( R-[^{13}C] \)rolipram \textit{specific binding} was measured in the rat lung and heart, respectively, which is consistent with a higher expression of adenosine-\( A_{2a} \) receptors in these two organs in comparison to the mouse brain (Marian et al., 1999). CGS 21680 has been shown to display cardiovascular effects, increasing heart rate in rats, but lowering blood pressure and total peripheral resistance (Nekooeian and Tabrizchi, 1996). While no significant change in radioactivity levels was measured in blood, a significant increase (24%) was observed in the liver, suggesting that brain \( R-[^{13}C] \)rolipram uptake could have been affected by metabolism.

Injection with the \( H_3 \)-histamine autoreceptor antagonist thioperamide has been shown to increase histamine release by two-fold (Fujimoto et al., 1991; Itoh, 1991; Oishi et al., 1989). Higher histamine levels activate postsynaptic \( H_3 \) receptor-linked AC to produce cAMP, and may thus increase the regulation of PDE4 enzymes (Agullo et al., 1990; Holden et al., 1987). In this study, thioperamide produced a significant increase in \( R-[^{13}C] \)rolipram \textit{in vivo} accumulation of 25-27% in the frontal cortex, thalamus, cerebellum, and brain stem as compared to controls. Blood radioactivity levels were not significantly augmented (increase of only 15%), however, measurement of \( R-[^{13}C] \)rolipram \textit{specific binding} yielded only an increase of 20% in the frontal cortex, with other regions displaying much lower values and even decreases as compared to control. This effect is similar to that of forskolin, as described above, since an increase in radioligand \textit{non-specific binding} as high as 3 fold was detected following thioperamide treatment. Histamine release has been shown to increase BBB permeability to small molecule tracers (Schilling and Wahl, 1994), but was reported to decrease permeability of an endothelial cell monolayer by stimulating cAMP through the \( H_2 \) receptor (Takeda et al., 1992). Inhibition of \( H_3 \) receptors was also revealed to increase NA release and augment vasopressor and cardiostimulatory responses (Malinowska et al., 1998). Therefore, the effects of this drug
treatment on pharmacokinetic parameters such as blood flow and BBB permeability could have increased the regional brain uptake of $R$-[\textsuperscript{11}C]rolipram non-specifically.

4.5.4.2 Dopamine-D\textsubscript{1} receptors

Activation of dopamine-D\textsubscript{1} receptors also augments cAMP levels via stimulation of AC in the brain. However, the dopamine system has been reported to be mainly linked to PDE1 (Polli and Kincaid, 1994), the other abundant PDE in the brain (Sutor et al., 1998), and not PDE4, and was thus targeted as a negative control. Different treatments with the dopamine-D\textsubscript{1} agonist SKF 81297 had no significant effect on $R$-[\textsuperscript{11}C]rolipram retention in rat brain regions in vivo, that was also observed in the blood. This result is in accordance with the finding that PDE1 seems to be the main enzyme family expressed in dopamine-rich brain regions (Polli and Kincaid, 1994), and is important in this study since it suggests that the in vivo binding of $R$-[\textsuperscript{11}C]rolipram in rat brain shows selectivity for neurotransmitter systems where PDE4 is expressed.

4.5.5 Effect of in vivo PKA and protein synthesis inhibition

Inhibition of protein synthesis or PKA-mediated phosphorylation has been a widely used approach to study the mechanism of action of different drug treatments on the regulation of PDE4 enzymes in cell cultures (Chang et al., 1997; Kochetkova et al., 1995; Manning et al., 1996; Torphy et al., 1995). At least one study reported the successful use of the general protein synthesis inhibitor cycloheximide in vivo in rats in order to study the effect of the $\beta$-adrenergic agonist isoproterenol on cAMP-PDE activity (Oleshansky and Neff, 1975). HA1004 is a water-soluble selective PKA inhibitor that crosses the BBB. HA 1004 and cycloheximide were injected in vivo in order to study the mechanisms of action of selected drug treatments on $R$-[\textsuperscript{11}C]rolipram distribution in rat brain. Administration of HA 1004 alone produced a trend towards a decrease of $\sim$10% in $R$-[\textsuperscript{11}C]rolipram retention across brain regions, suggesting that if more rats had been used, a significant effect of HA 1004 could have been detected. HA 1004 injected prior to yohimbine, one of the drugs hypothesized to upregulate PDE4 through
increased NA release, did not block the increase in $R$-[14C]rolipram binding in the brain. Instead, a significant potentiation was observed. The reasons for this effect are not known, but are possibly due to increased blood flow to the brain or other pharmacokinetic effects that arise as a result of the HA 1004/yohimbine combination treatment, since the blood levels of radioactivity were significantly increased as compared to yohimbine treatment alone. HA 1004 administration prior to DMI and forskolin produced no significant effect on $R$-[14C]rolipram regional brain binding. Interestingly, a slight decrease in radioactivity levels was observed in PDE4-rich brain regions with the HA 1004/forskolin combination treatment, and a significant reduction was observed in the blood with HA 1004 treatment prior to DMI and forskolin. These decreases in radioactivity in brain regions may have been due to inhibition of PKA by HA 1004. Several blood cell types reportedly express high levels of PDE4 activity, including neutrophils, eosinophils, lymphocytes, monocytes, and macrophages, but not erythrocytes (Tenor and Schudt, 1996; Wang et al., 1999). Although the fraction of PDE4-rich cells in the circulation is small, it is possible that HA 1004 inhibited PDE4 regulation to a certain extent in the blood cells leading to the decrease in radioactivity levels. However, HA 1004 also acts as a calcium antagonist (De Sarro et al., 1989; De Sarro et al., 1990). Furthermore, inhibition of PKA itself in vivo contributes to a myriad of effects, since these enzymes are widely distributed throughout the brain, functioning in several neurotransmitter systems linked to the cAMP second messenger system (Popoli et al., 2000). Therefore, although HA 1004 may have inhibited to a certain degree PKA linked to PDE4, it may also have antagonized PKA enzymes in other systems not associated with PDE4, making the effect of this treatment complex and the results difficult to interpret.

Coincubation of cell cultures with cycloheximide and the adenosine-$A_2\alpha$ agonist CGS 21680 was shown by Chang and colleagues (1997) to block the synthesis of novel PDE4 proteins. In the present study, in vivo treatment with cycloheximide alone yielded a significant increase in
$R-[^{14}C]$rolipram binding across brain regions. Treatment of cycloheximide prior to CGS 21680 had a synergistic effect on the binding of $R-[^{14}C]$rolipram in brain regions. The blood, especially, demonstrated a significant increase in radioactivity levels with the cycloheximide/CGS 21680 cotreatment as compared to controls that was also much higher than the regional brain concentrations of radioactivity, suggesting that $R-[^{14}C]$rolipram uptake in brain regions may have been increased non-specifically due to enhanced delivery. As with the PKA inhibitor HA 1004, cycloheximide produces several effects, especially because it inhibits protein synthesis without any selectivity for PDE4. The lack of selectivity of HA 1004 for PKA or of cycloheximide for inhibiting PDE4 protein synthesis adds to the complexity of effects, making it difficult to obtain a meaningful result with these drug treatments in vivo.

4.5.6 Effect of monoamine vesicle depletion and noradrenergic lesioning

4.5.6.1 Chronic reserpine

A typical behavioral model used to test the antidepressant activity of drugs is the reserpine-induced hypothermia and hypokinesia (Wachtel and Schneider, 1986). In fact, rolipram was shown to exhibit efficacy in reversing reserpine-induced hypothermia and hypokinesia, implying that rolipram has antidepressant activity (Wachtel and Schneider, 1986). Reserpine treatment has been shown to reduce NA and 5-HT levels in the brain and periphery (Ross, 1979; Wakade, 1980), an effect that has been explored in the treatment of hypertension (Griebenow et al., 1997). Some receptors such as the $\beta_1$- and $\alpha_{1b}$-adrenoceptors, become selectively upregulated in rat brain with reserpine administration (Grimm et al., 1992). The $\beta_1$-adrenoceptors are linked to PDE4 (Ye and O'Donnell, 1996), and reduced stimulation of these receptors brought on by chronic infusion with propranolol or treatment with 6-OH-DA lesioning of noradrenergic neurons was shown to downregulate PDE4A activity (Ye et al., 1997). Thus, it was hypothesized that chronic reserpine administration would decrease the expression of PDE4 subtypes, leading to a decrease in $R-[^{14}C]$rolipram regional brain uptake. However,
administration of reserpine for 5 days, followed with a 24 hour, but not a 2 hour, washout, led to an unexpected augmentation in R-[\(^{11}\)C]rolipram distribution across brain regions, being significant in most areas. A 3 day treatment with a dose of reserpine as used in our study (1 mg/Kg, s.c.), was previously shown to produce a significant increase in tyrosine hydroxylase activity, and thus NA synthesis, 3 days following the last injection (Lambas-Senas et al., 1986). Although uncertain, it is possible that in our study, the 24-hour washout period provided sufficient time for new monoamines to be released, even in small amounts, activating sensitized or upregulated postsynaptic receptors, leading to an increase in PDE4 expression. Moreover, other reports have found no change in the basal level of cAMP 18 h following at least a 2 day treatment with 5 mg/Kg of reserpine, even though β-adrenoceptors were supersensitive (Gillespie et al., 1980; Newman et al., 1985). This finding may imply that receptors, other than monoamine receptor subtypes, positively linked to AC, compensate for the monoamine depletion by sustaining the basal levels of cAMP. We also performed a 12-day administration of reserpine using a 1 mg/Kg dose in the first two days followed by 0.5 mg/Kg in the remaining 10 days and a 24 h washout period. This protocol yielded no significant change in radioligand uptake throughout the brain regions, however a slight decrease of 5–19% was observed that could have reached significance if more rats had survived. In spite of this, the decreased R-[\(^{11}\)C]rolipram binding in the 3 surviving rats may signify that only prolonged depletion of monoamines with reserpine treatment leads to a decrease in R-[\(^{11}\)C]rolipram binding to PDE4 \textit{in vivo} even after a 24 h washout period. Shorter treatments are very effective at reducing NA levels by >50% in different brain regions (Lambas-Senas et al., 1986; Wakade, 1980), but PDE4 density could remain unchanged as compared to controls.

4.5.6.2 Effect of DSP-4

DSP-4 is a strong alkylating agent that interacts with the noradrenergic uptake mechanism at the level of the neuronal membrane and either permanently inactivates this process or triggers
the degeneration of noradrenergic terminals (Jaim-Etcheverry and Zieher, 1980). The compound permanently impairs noradrenergic neurons in the brain, for periods lasting as long as 8 months after a single injection, and has been used as a noradrenergic neurotoxin in studying the behavioral effects of rolipram (Kehne et al., 1991). NA levels were reported to be rapidly reduced by >80% in cortical regions, even 32 days after a single 50 mg/Kg intraperitoneal injection of DSP-4 (Jaim-Etcheverry and Zieher, 1980; Schuerger and Balaban, 1995; Wolfman et al., 1994), leading to a significant upregulation of α₁, α₂, and β-adrenoceptors in the cerebral cortex (Wolfman et al., 1994). DSP-4 is somewhat selective for NA neurons, showing no effect on dopamine neurons and only a mild decrease in cortical and hippocampal metabolites of 5-HT (Ho et al., 1995; Wolfman et al., 1994). Nonetheless, preinjection with a 5-HT-selective reuptake inhibitor, such as zimelidine and fluoxetine, has been used to protect serotonergic nerve terminals and direct the effect of DSP-4 to noradrenergic neurons (Eison et al., 1988; Heal et al., 1986; Rossby et al., 1995). Lesioning of noradrenergic neurons using 6-OH-DA has been previously shown to significantly decrease the expression of PDE4A in rat cortex (Ye et al., 1997). It was thus hypothesized that treatment with DSP-4 would decrease the regulation of PDE4 enzymes due to a decrease in NA release, and reduction in cAMP synthesis by postsynaptic β-adrenoceptors. All of the DSP-4 treatments assessed 14 days following injection, with or without serotonergic nerve terminal protection with zimelidine, produced a small decrease in R-[^11C]rolipram regional brain retention. These results could signify that DSP-4 has the ability to slightly decrease the expression of PDE4 in the brain to a certain extent. However, blood radioactivity levels followed a similar trend as observed in the brain regions with the different DSP-4 treatments, implying that R-[^11C]rolipram blood levels may have been reduced, possibly leading to a decrease in radioligand brain uptake independent of PDE4 regulation.
The measurement of receptor downregulation following chronic drug treatments is very difficult to achieve in vivo in these types of experiments. This is because the regional brain uptake of $R-[^{11}C]$rolipram is dependent not only on pharmacodynamic changes but also on the pharmacokinetic effects of the in vivo drug challenges, which may affect radiotracer levels in blood. Also, in vivo pharmacological manipulations targeted towards the noradrenergic neurotransmitter system are not free from influencing other systems, which could for example, compensate for the reduction in the activity of the noradrenergic system in the normal living animal by increasing cAMP-mediated signaling and PDE4 function. Additionally, changes in radioligand uptake, even increases in binding, may not entirely reflect the absolute changes in the density of PDE4, since binding of $R-[^{11}C]$rolipram to PDE4 may not be in equilibrium at the time point tested (45 min). The use of pharmacokinetic modeling in future human PET studies could eliminate some of the limitations of the ex vivo approach used in the rat studies since an index of $R-[^{11}C]$rolipram specific binding would be calculated from a time-activity curve obtained over the course of 90 min, as opposed to the single time point tested in the rat.

4.5.7 Effect of acute and chronic antidepressant administration

4.5.7.1 Acute monoamine oxidase inhibition

Tranylcypromine enhances the synaptic levels of monoamines, primarily NA and 5-HT, by inhibiting MAO enzymes irreversibly in the presynaptic terminal (Blier et al., 1990; Sharp et al., 1997; Thase et al., 1995; Mallinger and Smith, 1991). Acute treatment with tranylcypromine is hypothesized to increase the stimulation of a variety of receptors due to the increased monoamine accumulation, leading to a net increase in the regulation of PDE4. In this study, a single tranylcypromine injection increased regional brain $R-[^{11}C]$rolipram specific binding by 18–32%. Tranylcypromine produces hypotension, which is reversed within hours (Thase et al., 1995), but can significantly affect the pharmacokinetics of drugs that are coadministered. However, in the present study, radioactivity levels were not significantly altered in the blood
and only slightly increased in the liver with tranylcypromine as compared to controls, suggesting that the brain uptake of the radioligand was not significantly affected by blood flow. The lung exhibited an elevation in specific binding of 32% above controls, but no change was measured in the heart. Acute tranylcypromine has been shown to rapidly produce (within 4 hours) a significant increase in the expression of genes that may play a role in the mechanism of action of antidepressants, such as c-fos and NGF1-A, in rat frontal cortex (Morinobu et al., 1997). The effect of acute tranylcypromine administration on PDE4 expression is not yet known. Given that the specific binding of R-[\textsuperscript{14}C]rolipram was increased in rat frontal cortex 3 hours following acute tranylcypromine administration in our study, it may be worth determining in future experiments whether PDE4 expression is also upregulated by acute tranylcypromine, thus possibly implicating PDE4 in its mechanism of action.

4.5.7.2 Chronic monoamine oxidase inhibition

Takahashi and colleagues (1999) demonstrated that chronic treatment with different antidepressants, including DMI, tranylcypromine, mianserin (\(\alpha_2\)-adrenergic agonist and weak NA reuptake inhibitor), fluoxetine, and sertraline (SSRI), increased PDE4A and B protein and mRNA expression, whereas chronic cocaine (dopamine transporter blocker) or haloperidol (dopamine D\textsubscript{2} receptor antagonist) had no significant effect. Thus, chronic antidepressant treatments were also performed here to investigate the ability of R-[\textsuperscript{14}C]rolipram binding to detect PDE4 modulations \textit{in vivo} in rat brain. Repeated tranylcypromine administration yielded unexpected decreases in radioligand retention across all brain regions, in contrast to Takahashi and colleagues (1999). It is possible that the hypotensive effects of tranylcypromine were pronounced with chronic injections, leading to a decrease in the brain delivery and uptake of R-[\textsuperscript{14}C]rolipram, since blood levels of radioactivity were also decreased following chronic tranylcypromine as compared to controls.
4.5.7.3 Effect of acute DMI treatment

The antidepressant DMI is a NA transporter blocker that has been shown to increase the synaptic levels of NA, thus increasing α- and β-adrenergic receptor stimulation, and PDE4 regulation (Johnson et al., 1980; Takahashi et al., 1999; Ye et al., 1997). The dose- and time-dependent acute treatments with DMI demonstrated a maximal effect on regional brain radioligand accumulation with the 10 mg/Kg dose at 4.5 hours prior to radioligand injection, that subsided with the 9 hours prior treatment. The peak effect corresponded to a 22–36% increase in radioactivity levels that was significant as compared to controls. The maximal accumulation of DMI in rat cortex was reported to occur at ~1 hour following intraperitoneal injection (Hrdina and Dubas, 1981), and this prolonged time course in the brain uptake of DMI likely contributed indirectly to the delayed peak accumulation of R-[14C]rolipram in brain regions in vivo. DMI treatment may have also affected the BBB penetration of the radioligand, since DMI was previously shown to increase cerebral permeability through an adrenergic-dependent mechanism in vivo in the rat (Preskorn et al., 1980). However, the effect of DMI on BBB permeability was reported to be rapid and transient, disappearing within 15 min (Preskorn et al., 1980), thus occurring much earlier than radioligand administration in our studies. Calculation of specific binding yielded an increase of 21-41% in radioligand uptake, with a minimal effect on non-specific binding and no change in the plasma level of unmetabolized R-[14C]rolipram as compared to controls, implying that DMI had no effect on the brain delivery of the radioligand. The increase in radioligand specific binding following DMI treatment is in agreement with the high expression and function of PDE4 enzymes in the noradrenergic system (Yamashita et al., 1997; Ye et al., 1997; Ye and O'Donnell, 1996). Therefore, R-[14C]rolipram appears to have detected an increase in PDE4 regulation, such as an augmentation in affinity and/or density of PDE4, with DMI treatment. These in vivo results were validated in the in vitro study using R/S-[3H]rolipram, which demonstrated a significant increase of 47% in Bmax.
(maximal binding) in the supernatant of rat frontal cortex, confirming the increase in the expression of PDE4 sites in this brain region. Measurement of $K_d$ showed no significant change in binding affinity as compared to controls, suggesting that the increase in $R-[^{11}C]\text{rolipram}$ regional brain binding is solely mediated by the augmentation in $B_{max}$. These results concur with previous findings that acute stimulation of $\beta$-adrenoceptors by isoproterenol in vivo in rats upregulates cAMP-specific PDE activity within 1 hour in the pineal gland, which is dependent on de novo protein synthesis (Oleshansky and Neff, 1975). Additionally, incubation of cell cultures with the $\beta_2$-adrenergic receptor agonist salbutamol also increased PDE4 protein levels within 2-4 hours (Manning et al., 1996; Torphy et al., 1992c), showing that PDE4 enzymes can be upregulated rapidly in response to increases in cAMP-mediated signaling. $R-[^{11}C]\text{Rolipram}$ distribution was also found to be increased in the heart (65%) and lung (75%), two organs rich in $\beta$-adrenoceptors and PDE4 expression (Nemoz et al., 1989; Schneider et al., 1986; Torphy et al., 1993). The present results suggest that $R-[^{11}C]\text{rolipram}$ detected an increase in PDE4 density elicited by acute DMI injection.

4.5.7.4 Effect of acute fluoxetine challenge

Fluoxetine inhibits the 5-HT transporter selectively, leading to an accumulation of endogenous 5-HT in the synapse and increasing the stimulation of postsynaptic $5-HT_1$, $5-HT_2$, and $5-HT_3$ receptors, which are positively linked to AC and activate cAMP synthesis (Baker et al., 1998; Dumuis et al., 1991; Markstein et al., 1999; Ruat et al., 1993a; Ruat et al., 1993b; Torres et al., 1995). The increased release of 5-HT in the synapse (Malagie et al., 1995) and stimulation of postsynaptic 5-HT receptors linked to AC may lead to the activation of PDE4 enzymes. Indeed, fluoxetine was the only 5-HT agent that significantly elevated (by 15-27%) $R-[^{11}C]\text{rolipram}$ distribution across all brain regions following acute treatment, while agonists and antagonists at $5-HT_1$, or $5-HT_2$ receptors had no significant effect on radioligand retention in the brain as described above. This finding suggests that changes in $R-[^{11}C]\text{rolipram}$
uptake after fluoxetine treatment are mediated by other 5-HT receptor subtypes. In periphery, only the lung showed an increase in specific binding of 22%. While radioactivity levels were higher in blood as compared to controls, the percentage of unchanged \( R-[^{11}\text{C}] \)rolipram was actually decreased in plasma with the fluoxetine treatment, suggesting that more radioactive metabolites were produced in the presence of the antidepressant. In consequence, the elevated regional brain radioligand retention does not appear to have been caused by increased plasma levels of \( R-[^{11}\text{C}] \)rolipram, but by enhanced specific binding.

Although radioactivity uptake or total binding was significantly increased across brain regions following acute fluoxetine, \( R-[^{11}\text{C}] \)rolipram specific binding was only augmented in certain brain areas, including the olfactory and cortical areas, striatum, and hippocampus, while other regions demonstrated a decrease, including the thalamus (-12%) and brain stem (-16%). In fact, acute fluoxetine was the only treatment showing a decrease in specific binding in some brain regions even though the total binding was increased. This heterogeneous effect could be possibly explained by a selective effect on the serotonergic system, since tranylcypromine, a drug that increases the synaptic levels of both NA and 5-HT (Sharp et al., 1997; Giralt and Garcia-Sevilla, 1989), produced no such result. Since agonists and antagonists at 5-HT\(_{1A}\) or 5-HT\(_{2A/2C}\) receptors had no significant effect on radioligand distribution in the brain, showing only slight decreases, it is proposed that other 5-HT receptors modulated the augmentation in \( R-[^{11}\text{C}] \)rolipram regional brain specific binding. Some likely candidates are the 5-HT\(_{4}\), 5-HT\(_{6}\), and 5-HT\(_{7}\) receptors, which increase cAMP synthesis as mentioned above (Baker et al., 1998; Dumuis et al., 1991; Markstein et al., 1999; Ruat et al., 1993a; Ruat et al., 1993b; Torres et al., 1995) and may potentially mediate the upregulation in PDE4 function following fluoxetine treatment. In support of this proposition, the increase in \( R-[^{11}\text{C}] \)rolipram specific binding occurred in brain areas previously reported to express high concentrations of the 5-HT\(_{4}\) receptor, such as the hippocampus and caudate (Meneses, 1999; Torres et al., 1995), the 5-HT\(_{6}\) receptor,
such as the prefrontal cortex, striatum, olfactory tubercle, and hippocampus (Meneses, 1999; Ruat et al., 1993a), and the 5-HT, receptor such as the hippocampus and cortex (Meneses, 1999; Ruat et al., 1993b). These brain areas also demonstrate moderate-to-high levels of mRNA for different PDE4 subtypes (Engels et al., 1995a). On the other hand, the brain regions yielding decreases in $R$-$[^{14}C]$rolipram specific binding, i.e., the thalamus and brain stem, have been reported to express high levels of 5-HT receptors that could decrease the regulation of PDE4.

For example, the 5-HT$_{1c}$ receptor, which inhibits AC, was shown to be expressed in the thalamus, whereas, the 5-HT$_{2}$ receptor was reported to occur in the brain stem (Wright et al., 1995). Since, the in vivo administration of the 5-HT$_{3A/C}$ agonist DOI had no significant effect on $R$-$[^{14}C]$rolipram uptake across brain regions, including the brainstem, it is possible that other 5-HT receptor subtypes played a role in the effect of fluoxetine on the levels of radioactivity in the brainstem. Therefore, it appears that the in vivo uptake of $R$-$[^{14}C]$rolipram is reflective of changes in the regulation of PDE4 after acute fluoxetine treatment.

The in vitro validation of the acute fluoxetine treatment on radioligand distribution supported the in vivo findings. Similar to DMI, $K_v$ values for $R/S$-$[^3H]$rolipram binding in rat frontal cortex following fluoxetine injection were not significantly different from controls. However, a significant increase of 54% in $B_{max}$ was observed in the supernatant, with no significant change in the pellet. In our experiments, fluoxetine was injected 3 hours prior to $R$-$[^{14}C]$rolipram, a time period that is sufficient to upregulate PDE4 protein expression in cell cultures and in vivo in rats as described above. Therefore, the increased specific binding of $R$-$[^{14}C]$rolipram following acute fluoxetine in vivo appears to have been due to an increase in the density of PDE4 proteins.

4.5.7.5 Chronic DMI and fluoxetine

Chronic DMI treatment significantly increased $R$-$[^{14}C]$rolipram uptake across all brain regions, whereas fluoxetine showed lower increases and was significant only in the frontal
cortex and cerebellum. The percentage increase in specific binding as compared to controls was 17–26\% and 10–18\% with DMI and fluoxetine, respectively. Interestingly, chronic fluoxetine did not produce a decrease in R-[\textsuperscript{11}C]rolipram specific binding in any brain region as compared to acute administration (described above). The effects of fluoxetine treatment on PDE4 regulation are indirect by increasing the synaptic levels of 5-HT, which can activate different subfamilies of pre- and postsynaptic 5-HT receptors. For instance, 5-HT receptors negatively linked to AC, such as 5-HT\textsubscript{1C}, and involved in the feedback inhibition of 5-HT release, such as 5-HT\textsubscript{1A}, have been reported to be desensitized following chronic fluoxetine (Le Poul et al., 1997; Invernizzi, et al., 1996; Berendsen, 1995), and could contribute to the difference in R-[\textsuperscript{11}C]rolipram specific binding following chronic as compared to acute fluoxetine. In vitro experiments using R/S-[\textsuperscript{3}H]rolipram in rat frontal cortex validated that the DMI treatment significantly increased the \( B_{\text{max}} \) in the supernatant by 27\%, with no change in \( K_d \). Thus, the in vivo increase in R-[\textsuperscript{11}C]rolipram regional brain binding following chronic DMI was likely due to an upregulation in the density of PDE4. In contrast, no significant change in \( B_{\text{max}} \) or \( K_d \) was obtained with the chronic fluoxetine administration, in either the supernatant or pellet, suggesting that this treatment had no significant effect on PDE4 expression in the frontal cortex in vitro. Previous reports (Ye et al., 2000) have demonstrated no change in PDE4A levels in rat cortex following chronic fluoxetine. However, Takahashi et al. (1999) demonstrated an increase in PDE4A and B expression in the frontal cortex, which agrees with the present in vivo findings, and with the high expression of PDE4A mRNA in cortical regions (Engels et al., 1995a), but not with our in vitro findings. Ye and colleagues (2000) did publish that chronic fluoxetine increased PDE4A expression in the hippocampus, a region abundant in PDE4A (Engels et al., 1995a) and in 5-HT\textsubscript{4}, 5-HT\textsubscript{6}, and 5-HT, receptors (Markstein et al., 1999; Meneses, 1999; Ruat et al., 1993a; Ruat et al., 1993b; Torres et al., 1995). Our in vivo results also demonstrated an increase in R-[\textsuperscript{11}C]rolipram specific binding in the hippocampus, but a
higher elevation was obtained in the frontal cortex and olfactory regions. Thus, while the \textit{in vivo} results following chronic fluoxetine agree with some previous studies (Takahashi et al., 1999), the \textit{in vitro} validation results and other previous reports (Ye et al., 2000) are inconsistent. Experimental error may have contributed to the lack of change in $B_{\text{max}}$ in the chronic fluoxetine-treated animals as compared to controls, since the fluoxetine-treated and the control animals were each split into two groups, which were assayed on two different days. Nonetheless, the results from chronic DMI and fluoxetine experiments demonstrated the same trend, with no difference in $B_{\text{max}}$ between chronic fluoxetine and vehicle treatments. The $B_{\text{max}}$ in the supernatant of the control group treated with fluoxetine vehicle (5% ethanol in saline) was, however, substantially higher than in the control treated with DMI vehicle (saline), which contradicts the \textit{in vivo} binding results (with no difference between control groups). Further investigation into the effect of chronic fluoxetine on PDE4 regulation \textit{in vivo} and \textit{in vitro} is warranted. For example, chronic fluoxetine administration together with treatment of its vehicle solution in controls could be repeated in a higher number of animals. Alternatively, other brain regions rich in 5-HT$_1$, 5-HT$_6$, and 5-HT$_7$ receptors, such as the hippocampus, could be tested to determine if the $B_{\text{max}}$ of $R/S$-$[^3]$H]binding correlates with the increase in $R$-$[^{11}]$C]rolipram binding \textit{in vivo}.

\textbf{4.5.8 DMI results correlate with antidepressant treatment model}

The \textit{in vivo} acute and chronic results obtained following DMI treatment concur with the model of antidepressant action proposed by Duman and colleagues (1997) (Fig. 48). According to the model, in the absence of antidepressant treatment, basal production of cAMP occurs due to normal noradrenergic activity acting at postsynaptic $\beta$-adrenoceptors, and due to some constitutive activity of the receptors and AC. Acute DMI treatment elicits a rapid increase in the chain of events from NA release – $\beta$-adrenoceptor activation - cAMP synthesis. Chronic
Fig. 48 Model of the effect of acute and chronic noradrenergic reuptake inhibitor (e.g., DMI) treatment on the concentration of cAMP and PDE4 expression in the postsynaptic neuron (NE = NA; adapted and modified from Duman et al., 1997).
administration, however, yields a long-term downregulation of β-adrenoceptors (O'Donnell and Frazer, 1985; Ye et al., 1997). But since NA release is still higher than in the basal or normal condition, postsynaptic cAMP production is higher relative to no treatment. Thus, in the long-term, postsynaptic receptor density is reduced and the intracellular signaling system is elevated yielding a net augmentation in signal transduction, including PDE4 activation (Duman et al., 1997; Takahashi et al., 1999; Ye et al., 2000). The increase in PDE4 activity may contribute to the attenuation in NA- and forskolin-stimulated cAMP production in rat brain reported following chronic ECS (Newman et al., 1986; Takahashi et al., 1999), and the delay in the improvement of depressive symptoms by antidepressants (Takahashi et al., 1999). Combination of PDE4 inhibitors with antidepressants to further increase cAMP levels chronically could be pursued as an alternative therapy in resistant cases, since a combination of the non-selective PDE inhibitor papaverine with previously unsuccessful antidepressant medications was shown to be effective at improving depressive symptoms in a case study (Malison et al., 1997).

Interestingly, the effect of DMI treatment on R-[11C]rolipram binding in rat brain concurs with the antidepressant treatment model since acute DMI augmented R-[11C]rolipram specific binding in vivo (21-41%) and R/S-[3H]rolipram B_max in the supernatant (47%) to a higher level than chronic DMI administration (only 17-26% increase in specific binding and 27% in B_max). These results suggest that R-[11C]rolipram regional brain uptake in vivo detects the differential changes in PDE4 expression inside neurons following the different DMI challenges, and that PDE4 density regulation occurs rapidly in adapting to the changes in cAMP levels. The effect of DMI downstream of cAMP levels has been shown to involve an elevation in the expression of neurotrophins important for neuronal plasticity, such as BDNF (Duman et al., 1997; Nibuya et al., 1995). Coadministration of rolipram with DMI enhanced the expression of BDNF in rat hippocampus (Fujimaki et al., 2000), further supporting the notion that PDE4 plays a role in the mechanism of action of DMI. The results following in vivo challenges with DMI strongly
suggest that \( R-[^{1}{C}] \text{rolipram} \) has potential for studying the effects on PDE4 of antidepressants acting through the noradrenergic system using PET. Future experiments to test this notion further, could include \textit{in vivo} acute and chronic treatment of rats with the selective NA reuptake inhibitor reboxetine followed by measurement of \( R-[^{1}{C}] \text{rolipram} \) regional brain uptake in parallel with assessment of NA levels and PDE4 protein expression (Western blotting) as compared to vehicle treated controls.

4.5.9 \textit{What about the effects of acute and chronic fluoxetine on cAMP-mediated signaling as a model of its mechanism of action?}

Studies have demonstrated that administration of fluoxetine influences the cAMP-second messenger system. For example, chronic, but not acute, administration of fluoxetine was reported to significantly increase CRE-mediated gene transcription, CREB mRNA, and CREB phosphorylation in several limbic brain regions thought to mediate the action of antidepressants, including the cerebral cortex, hippocampus, amygdala, and hypothalamus (Nibuya et al., 1996; Thome et al., 2000). Importantly, chronic fluoxetine has also been reported to increase the expression of PDE4A and B in the rat frontal cortex (Takahashi et al., 1999), and the expression of PDE4A in the hippocampus, independent of \( \beta \)-adrenoceptor stimulation (Ye et al., 2000).

The 5-HT\(_{1A}\) receptor, which acts through the PLC system, and the 5-HT\(_{1A}\) subtype, which inhibits AC, have been widely studied for their role in suicide and depression as well as in relation to the mechanism of action of antidepressant drugs (Pandey et al., 1995; Meyer et al., 1999; Berendsen, 1995). However, recent evidence suggests that chronic treatment with SSRIs downregulates other 5-HT receptors, including 5-HT\(_{2A}\) (Bijak, 1997; Bijak et al., 1997) and 5-HT\(_{1C}\) (Sleight et al., 1995), which are positively linked to AC. Since, the cAMP-signaling system has been recently implicated in the molecular and cellular theory of depression (Duman et al., 1997), 5-HT receptors directly linked to AC may be of high importance in the mechanism of action of SSRIs. Therefore, it is possible that the mechanism of action of fluoxetine at
improving depressive symptoms is through indirect stimulation of at least 5-HT₄, 5-HT₆, and 5-HT₃ receptors, increasing cAMP-mediated signaling.

As proposed for antidepressants acting at the noradrenergic reuptake site (Fig. 48), acute treatment with SSRIs may also produce a pronounced and transient increase in cAMP levels through activation of 5-HT₄, 5-HT₆, and/or 5-HT₃ receptors, whereas chronic administration could yield a smaller sustained augmentation in cAMP (Fig. 49). In accordance with this model, chronic antidepressant exposure has been reported to enhance 5-HT₃ receptor-mediated cAMP accumulation in rat frontocortical astrocytes (Shimizu et al., 1996). Importantly, our results with fluoxetine also concur with this proposed model. Specifically, the *in vivo specific binding* of R-[¹¹C]rolipram was increased with acute fluoxetine in brain regions rich in both Gα₃-coupled 5-HT receptors and PDE4 enzymes, such as the hippocampus with an 18% increase, while it was only augmented by 10% in the same region of chronically treated animals. Acute treatment with fluoxetine also significantly increased by 54% the Bₘₐₓ of R/S-[³H]rolipram binding in rat frontal cortex, however, no significant change in Bₘₐₓ was detected with chronic fluoxetine. Although more studies are required to ascertain the effect of acute and chronic fluoxetine administration on R-[¹¹C]rolipram binding to PDE4 *in vivo*, these preliminary results suggest that the radioligand may have potential for studying the effects of antidepressants acting through the serotonergic system using PET.

**4.5.10 How does R-[¹¹C]rolipram binding *in vivo* compare with measurements using *in vitro* techniques?**

In tracer doses, R-[¹¹C]rolipram and [³H]rolipram are expected to bind selectively to the high-affinity conformation of PDE4 enzymes, which has been shown to be the site in the brain correlated with rolipram efficacy in animal tests of depression (Schmiechen et al., 1990). Although PKA-mediated phosphorylation could be one of the mechanisms involved in the regulation of the switch between the low- and high-affinity conformation of PDE4 enzymes
Fig. 49 Model of the effect of acute and chronic selective serotonergic reuptake inhibitor (e.g., fluoxetine) treatment on the concentration of cAMP and PDE4 expression in the postsynaptic neuron (5-HT: serotonin; adapted and modified from Duman et al., 1997).
(Hoffman et al., 1997), more studies are required to understand this likely complex process. As a consequence, it is not completely understood how changes in cAMP levels, cAMP-mediated signaling, and PDE4 mRNA and/or protein expression following the different drug challenges described above, translate into changes in the expression of the high-affinity conformation as measured by $R$-[11C]rolipram or [$^3$H]rolipram binding. Northern and Western blot analysis of PDE4 mRNA and protein expression, respectively, may not necessarily reflect the changes in the expression of PDE4 high-affinity conformation. This is because, presumably, these *in vitro* techniques, especially Northern blotting, measure the total expression of PDE4 enzyme subtypes, irrespective of their conformational binding state. However, the high-affinity binding site may comprise only a small fraction (1-10%) of the total quantity of enzyme expressed (Hughes et al, 1997) and, although speculative, it is possible that certain drug treatments affect the number of enzymes in their high-affinity conformation, without affecting the total number of enzymes expressed in the brain, or vice versa. PET imaging with $R$-[11C]rolipram is concerned with measuring the density of the high-affinity conformation of PDE4 enzymes in brain regions. Thus, at this stage of the project, validation studies were only performed using tracer doses of [$^3$H]rolipram in *in vitro* binding assays, since these provide an alternative method to *in vivo* $R$-[11C]rolipram binding in assessing the effect of acute and chronic drug treatments on the expression of the high-affinity conformation of PDE4. Additionally, brain supernatant and pellet fractions were assayed separately in these experiments, since [$^3$H]rolipram high-affinity binding to PDE4 has been previously shown to occur in both cytosol and membrane in rat brain (Schneider et al., 1986). However, PET does not have the resolution to decipher the cytosolic versus membrane distribution of radioligands. Furthermore, given the wide distribution of PDE4 enzymes in different neurotransmitter systems, it may also be difficult to separately study the pre- and postsynaptic expression of the PDE4 high-affinity conformation. More detailed experiments could be performed in animals in order to compliment/validate the PET studies,
and could also be targeted at determining how drug treatments affect mRNA and/or protein expression of PDE4 enzymes in comparison to the high-affinity conformation of PDE4 as measured by \( R-[{^{11}}C]\)rolipram binding.

4.6 Human PET imaging

The \( R-[{^{11}}C]\)rolipram brain PET images demonstrated that the radioligand was rapidly absorbed by brain regions representing the gray matter. This is consistent with the wide distribution of PDE4 and the higher density and expression of these enzymes in gray over white matter (Kaulen et al., 1989; Tohda et al., 1994; Weiss and Costa, 1968). This finding may be important since antidepressant treatment has been reported to produce changes in neuronal plasticity via increases in the expression of neurotrophins, regulated by the cAMP signaling system and PDE4 (Nibuya et al., 1995; Nibuya et al., 1996; Siuciak et al., 1997; Smith et al., 1995a; Vaidya et al., 1999). The higher PDE4 expression in the neuronal compartment may signify that imaging PDE4 enzymes using \( R-[{^{11}}C]\)rolipram with PET would allow for the study of the effects of antidepressant treatments on neuronal activity, selectively.

The distribution of radioactivity in human brain was different from that obtained in the rat brain, possibly demonstrating a species difference in the concentration of the high-affinity conformational state of PDE4. PET scans of the human brain showed the highest uptake in the thalamus and striatum, followed by prefrontal cortex and then cerebellum with lower levels. The \textit{in vivo} rat studies yielded the highest uptake in the cortical regions, with thalamus, striatum, and cerebellum showing lower radioactivity levels. The high uptake of \( R-[{^{11}}C]\)rolipram in the striatum in humans could imply that receptors, other than the dopamine-D\(_{1}\), positively linked to AC and expressed in this brain region, such as some metabotropic glutamate receptor subtypes (Wang et al., 1995), are linked to PDE4. Further studies are required in order to test this suggestion.
Analysis of venous and arterial blood samples showed the presence of ~65% and ~48% unchanged \(^{11}\text{C}\)rolipram at 90 minutes, respectively. The cause of the difference between venous and arterial \(^{11}\text{C}\)rolipram levels is not clear. The total radioactivity level was the same between arterial and venous plasma, however the proportion of \(^{11}\text{C}\)rolipram and its \(^{11}\text{C}\)-labeled metabolites is different between the plasma and cell fractions for arterial blood as compared to venous. For instance, there may have been an enhanced non-specific binding of \(^{11}\text{C}\)rolipram to oxygenated hemoglobin, yielding a lower level of the unchanged radioligand in the arterial plasma fraction. Alternatively, radiolabeled metabolites may have been deposited in peripheral tissues after capillary transit, yielding a higher proportion of unmetabolized \(^{11}\text{C}\)rolipram in venous plasma. The presence of radiolabeled metabolites in plasma may not pose a problem for PET imaging since radiolabeled metabolites of \(^{3}\text{H}\)rolipram (Krause et al., 1993) and \(^{11}\text{C}\)rolipram (present work) were not shown to cross the BBB in rats. The metabolism of rolipram was reported to be very similar between rat and human, with the same types of metabolites found in urine (Krause and Kühne, 1993; Krause et al., 1993), thus it is likely that these metabolites will not cross the BBB in humans either. However, the extensive metabolism of \(^{11}\text{C}\)rolipram reduces the amount of unchanged radioligand in the brain for PET imaging. The use of arterial blood sampling poses a greater inconvenience due to reduced subject compliance and also to the necessity for recruiting an anesthesiologist for insertion of the radial catheter. We are currently working on testing different approaches for obtaining a reliable pharmacokinetic model for quantifying PDE4 levels with \(^{11}\text{C}\)rolipram and PET, if possible, without the invasive arterial catheter insertion.
Table XIV Percent change in $R$-$[^{11}]C$rolipram retention in rat frontal cortex following different acute drug treatments as compared to control.

<table>
<thead>
<tr>
<th>DRUG TREATMENT</th>
<th>% Change in Frontal Cortex $R$-$[^{11}]C$Rolipram Retention</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forskolin</td>
<td></td>
</tr>
<tr>
<td>6.5 mg/Kg, i.p., 15 min prior to kill</td>
<td>↑ 8%</td>
</tr>
<tr>
<td>6.5 mg/Kg, i.v., coinjected</td>
<td>↑ 35% *</td>
</tr>
<tr>
<td>15 mg/Kg, i.v., 3 hr prior</td>
<td>↓ 1%</td>
</tr>
<tr>
<td>DMI</td>
<td></td>
</tr>
<tr>
<td>10 mg/Kg, i.p., 30 min prior</td>
<td>↑ 6%</td>
</tr>
<tr>
<td>10 mg/Kg, i.p., 1.25 hr prior</td>
<td>↑ 5%</td>
</tr>
<tr>
<td>10 mg/Kg, i.p., 4.5 hr prior</td>
<td>↑ 26% *</td>
</tr>
<tr>
<td>10 mg/Kg, i.p., 9 hr prior</td>
<td>↑ 5%</td>
</tr>
<tr>
<td>Yohimbine</td>
<td></td>
</tr>
<tr>
<td>10 mg/Kg, i.p., 15 min prior</td>
<td>↑ 41% *</td>
</tr>
<tr>
<td>10 mg/Kg, i.p., 1.25 hr prior</td>
<td>↑ 17%</td>
</tr>
<tr>
<td>10 mg/Kg, i.p., 3 hr prior</td>
<td>↑ 20%</td>
</tr>
<tr>
<td>Clonidine</td>
<td></td>
</tr>
<tr>
<td>1 mg/Kg, i.p., 3 hr prior</td>
<td>↑ 24% *</td>
</tr>
<tr>
<td>0.1 mg/Kg, i.p., 3 hr prior</td>
<td>↓ 0.1%</td>
</tr>
<tr>
<td>Clenbuterol</td>
<td></td>
</tr>
<tr>
<td>10 mg/Kg, i.p., 3 hr prior</td>
<td>↑ 16%</td>
</tr>
<tr>
<td>Propranolol</td>
<td></td>
</tr>
<tr>
<td>20 mg/Kg, i.v., 5 min prior</td>
<td>↑ 5%</td>
</tr>
<tr>
<td>20 mg/Kg, i.p., 3 hr prior</td>
<td>↑ 4%</td>
</tr>
<tr>
<td>Tranlycypromine</td>
<td></td>
</tr>
<tr>
<td>10 mg/Kg, i.p., 3 hr prior</td>
<td>↑ 29% *</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td></td>
</tr>
<tr>
<td>5 mg/Kg, i.p., 3 hr prior</td>
<td>↑ 22% *</td>
</tr>
<tr>
<td>8-OH-DPAT</td>
<td></td>
</tr>
<tr>
<td>1 mg/Kg, i.p., 3 hr prior</td>
<td>↓ 1%</td>
</tr>
<tr>
<td>WAY 100635</td>
<td></td>
</tr>
<tr>
<td>3 mg/Kg, i.p., 3 hr prior</td>
<td>↑ 2%</td>
</tr>
<tr>
<td>DOI</td>
<td></td>
</tr>
<tr>
<td>5 mg/Kg, i.p., 3 hr prior</td>
<td>↓ 11%</td>
</tr>
<tr>
<td>Ritanserin</td>
<td></td>
</tr>
<tr>
<td>2.5 mg/Kg, s.c., 4 hr prior</td>
<td>↓ 4%</td>
</tr>
<tr>
<td>CGS21680</td>
<td></td>
</tr>
<tr>
<td>2 mg/Kg, i.p., 3 hr prior</td>
<td>↑ 0.1%</td>
</tr>
<tr>
<td>Thioperamide</td>
<td></td>
</tr>
<tr>
<td>10 mg/Kg, i.p., 4 hr prior</td>
<td>↑ 27% *</td>
</tr>
<tr>
<td>SKF81297</td>
<td></td>
</tr>
<tr>
<td>10 mg/Kg, i.p., 5 min prior</td>
<td>↓ 6%</td>
</tr>
<tr>
<td>10 mg/Kg, i.p., 3 hr prior</td>
<td>↓ 5%</td>
</tr>
</tbody>
</table>

*Drug treatments were performed either prior to $R$-$[^{11}]C$rolipram injection or coinjected with the radioligand as described in the table. Rats were sacrificed 45 min following radioligand injection, the frontal cortex and other brain regions removed, and assayed for radioactivity retention. The data are displayed as % change from radioactivity retention in the control bank (p < 0.05 as compared to control; MANOVA with Bonferroni’s compartmental tests). Only frontal cortex results are presented for sake of clarity.
Table XV Percent change in R-[\textsuperscript{11}C]rolipram retention in rat frontal cortex following different chronic drug treatments as compared to controls.

<table>
<thead>
<tr>
<th>DRUG TREATMENT(^a)</th>
<th>% Change in Frontal Cortex R-[\textsuperscript{11}C]Rolipram Retention</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propranolol</td>
<td>↑ 17%(^*)</td>
</tr>
<tr>
<td>20 mg/Kg, 14 days</td>
<td></td>
</tr>
<tr>
<td>Clenbuterol</td>
<td>↑ 4%</td>
</tr>
<tr>
<td>10 mg/Kg, 14 days</td>
<td></td>
</tr>
<tr>
<td>DMI</td>
<td>↑ 22%(^*)</td>
</tr>
<tr>
<td>10 mg/Kg, i.p., 14 days, twice daily</td>
<td></td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>↑ 19%(^*)</td>
</tr>
<tr>
<td>10 mg/Kg, i.p., 14 days, once daily</td>
<td></td>
</tr>
<tr>
<td>Tranylcypromine</td>
<td>↓ 11%</td>
</tr>
<tr>
<td>7 mg/Kg, i.p. 7 days; 10 mg/Kg, i.p., 7 days</td>
<td></td>
</tr>
<tr>
<td>8-OH-DPAT</td>
<td>0%</td>
</tr>
<tr>
<td>1 mg/Kg, s.c., 14 days, once daily</td>
<td></td>
</tr>
<tr>
<td>Reserpine</td>
<td>↑ 11%</td>
</tr>
<tr>
<td>1 mg/Kg, s.c., 5 days, 24 hr washout</td>
<td></td>
</tr>
<tr>
<td>1 mg/Kg, s.c., 5 days, 2 hr washout</td>
<td></td>
</tr>
<tr>
<td>DSP-4</td>
<td>↓ 10%</td>
</tr>
<tr>
<td>50 mg/Kg, i.p., 1 injection 14 days prior with zimelidine protection</td>
<td></td>
</tr>
<tr>
<td>50 mg/Kg, i.p., 1 injection 14 days prior, no zimelidine</td>
<td></td>
</tr>
<tr>
<td>50 mg/Kg, i.p., 2 injections 7 days apart</td>
<td></td>
</tr>
<tr>
<td>50 mg/Kg, i.p., 1 injection 7 days washout</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\)Animals were allowed a 24 hour washout or as otherwise indicated in the table and then injected with R-[\textsuperscript{11}C]rolipram via tail vein, and sacrificed 45 min later. The frontal cortex and other brain regions were removed and assayed for radioactivity retention. The data are displayed as % change from radioactivity retention in the control bank (\(^*\) \(p < 0.05\) as compared to control; MANOVA with Bonferroni's comparisons tests). Only frontal cortex results are presented for sake of clarity.
CHAPTER 5.0: CONCLUSION

5.1 Conclusion

$R$-[$^{11}$C]Rolipram demonstrated the most favorable characteristics for imaging PDE4 with PET in comparison to $R/S$-$[^{11}$C]rolipram and $[^{11}$C]Ro 20-1724. First, a rapid and reliable radiosynthesis is a required property for PET radioligands due to the short physical half-life of positron emitting isotopes, such as $^{11}$C (half-life = 20.4 min). $R$-[$^{11}$C]Rolipram was synthesized rapidly and reliably within 30 min, in high purity, yields and specific activities, meeting the first requirement. Second, for brain imaging, it is necessary that the radioligand cross the BBB and bind selectively to the protein of interest with high affinity, giving a high signal. $R$-[$^{11}$C]Rolipram demonstrated good brain permeability, appropriate regional distribution that concurred with known PDE4 concentrations, and binding selectivity for PDE4 enzymes in rat brain, producing a high signal that was >4 fold higher than the radioactivity levels measured in blood. Third, metabolite analysis in plasma and brain homogenates revealed that no radiolabeled metabolites crossed the rat BBB, implying that radiolabeled metabolites should not interfere with the pharmacokinetic modeling of $R$-[$^{11}$C]rolipram distribution in the brain. Fourth, dosimetry studies demonstrated that the radioligand is safe for injection into humans. Fifth, in vivo and in vitro regulation experiments in rats suggested that $R$-[$^{11}$C]rolipram detected changes in the expression of PDE4 following selected acute and chronic drug treatments that affect cAMP-mediated signaling in neurotransmitter systems where PDE4 is thought to function. Table XIV and XV summarize the %change in $R$-[$^{11}$C]rolipram retention in the frontal cortex of rats treated acutely and chronically with different agents, respectively, as compared to controls. Some treatments, including acute forskolin, thioperamide, and the high dose (1 mg/Kg) of clonidine, may have substantially affected the blood flow and/or metabolism of the
radioligand, thus altering the delivery of \( R-[^{11}\text{C}] \)rolipram to the brain and peripheral organs and increasing specific as well as non-specific binding. However, not all treatments affected the regional brain uptake or blood levels of \( R-[^{11}\text{C}] \)rolipram. In particular, drugs acting at 5-HT\(_{1A}\) and 5-HT\(_{2A/C}\) receptors, or dopamine-D\(_4\) receptors, or a low dose of clonidine (0.1 mg/Kg) stimulating presynaptic \( \alpha_2 \)-adrenoceptors, demonstrated no significant effect on \( R-[^{11}\text{C}] \)rolipram binding \textit{in vivo} in rat brain or the blood levels of the radioligand. The most significant increases in the \textit{in vivo} regional brain distribution of the radioligand were observed with drugs increasing the synaptic levels of NA, such as acute yohimbine and acute and chronic DMI, or by increasing the synaptic levels of 5-HT using acute and chronic fluoxetine. None of the drug treatments performed to downregulate PDE4 in the noradrenergic system, including reserpine and DSP-4, significantly decreased \( R-[^{11}\text{C}] \)rolipram binding \textit{in vivo}, possibly due to a compensation by other neurotransmitter systems also linked to the cAMP-signaling system directly or indirectly. Importantly, the \textit{in vivo} pharmacological modulation experiments demonstrated that \( R-[^{11}\text{C}] \)rolipram was able to discriminate between drug challenges acting at different neurotransmitter systems of the brain. Agents affecting the noradrenergic system, such as acute yohimbine and acute and chronic DMI, or treatments inhibiting 5-HT uptake (acute and chronic fluoxetine), were the most effective at enhancing the radioligand binding in rat brain. Increases in histamine release by thioperamide also significantly increased \( R-[^{11}\text{C}] \)rolipram accumulation in certain brain regions. In contrast, treatments targeted at other neurotransmitter systems not using PDE4 had no significant effect, such as stimulation of dopamine-D\(_4\) receptors by SKF 81297. Moreover, \( R-[^{11}\text{C}] \)rolipram was sensitive to differential modulations of different receptors within the noradrenergic and serotonergic systems, showing that the \textit{in vivo} binding of the radioligand can reflect receptor-specific effects. For example, stimulation of \( \alpha_2 \)-adrenoceptors by a low dose of clonidine had no significant effect, while a higher dose of clonidine and inhibition of these receptors by yohimbine significantly increased radioligand
uptake in the brain. Similarly, stimulation of 5-HT$_{1A}$ or 5-HT$_{2A/2C}$ receptors had no significant effect, while acute and chronic indirect stimulation of other 5-HT receptors, possibly 5-HT$_{6}$, 5-HT$_{7}$, and/or 5-HT$_{4}$, by fluoxetine significantly increased $R$-$[^{11}]$C rolipram accumulation in vivo in rat frontal cortex. Finally, PET imaging of $R$-$[^{11}]$C rolipram in normal human volunteers displayed a high regional brain distribution of radioactivity, concentrating in regions of gray matter, suggesting that imaging with $R$-$[^{11}]$C rolipram could be directed at studying PDE4 function in the neuronal compartment. The sensitivity of the in vivo binding of $R$-$[^{11}]$C rolipram to changes in PDE4 regulation in the noradrenergic and serotonergic systems is an essential characteristic for future human brain PET studies, especially before and after treatment in depression, since antidepressant administration has been shown to alter PDE4 expression in rat brain regions. Furthermore, the selectivity of the tracer for only certain neurotransmitter systems shows that $R$-$[^{11}]$C rolipram is akin to a receptor-binding radioligand like $[^{11}]$C raclopride, which images dopamine-D$_{2}$ receptors selectively, and not like $[^{18}]$FFDG or $[^{15}]$O water, which image glucose metabolism or blood flow, respectively, in all neurotransmitter systems across the brain. These results support further development of $R$-$[^{11}]$C rolipram for studying and imaging PDE4 in the human brain using PET.

5.2 Future Direction

Future work in the development of $R$-$[^{11}]$C rolipram will be aimed at constructing a pharmacokinetic model for measuring the distribution of the radioligand in living human brain with PET, since venous sampling is not sufficient for obtaining the radioligand binding index of apparent $B_{max}$. $S$-$[^{11}]$C Rolipram was not found to be ideal for measuring non-specific binding due to its significant potency in binding to PDE4, leading to a reduction of the specific signal, and due to differences in non-specific saturable binding as compared to $R$-$[^{11}]$C rolipram.
Another approach is to use a low binding region as the non-specific compartment. Since PDE4 expression was found to be low in white matter regions, these areas may possibly be used in determining non-specific binding. A bolus plus infusion protocol could be carried out in order to achieve radioligand binding equilibrium in the ROIs and in the white matter, thus allowing a more reliable measurement of apparent $B_{\text{max}}$ by subtracting the non-specific region from the ROIs (Houle et al., 1996). Alternatively, high- and low-specific activity PET scans could be performed on the same subject in order to obtain two points for measuring the $K_i$ and $B_{\text{max}}$ using non-linear regression analysis.

The sensitivity of $R-[^{11}\text{C}]$rolipram to detect changes in the expression of the PDE4 high-affinity conformation could be assessed further following drug treatments modulating several subtypes of 5-HT receptors (e.g., 5-HT$_{1A}$, 5-HT$_{2A}$, 5-HT$_{1D}$) or glutamate metabotropic receptors possibly using PDE4 to metabolize cAMP. The levels of the second messenger cAMP and PDE4 activity could also be measured in parallel to the different drug challenges, in order to study the correlation between changes in cAMP concentrations in brain regions with changes in $R-[^{11}\text{C}]$rolipram binding. Such studies could support the hypothesis that the alterations in $R-[^{11}\text{C}]$rolipram binding in vivo in rat brain are due to changes in cAMP levels and regulation of PDE4. Additionally, Northern and Western blot analyses could be carried out to determine the mRNA and protein expression, respectively, of PDE4 enzymes following selected treatments to compliment the results obtained with $R-[^{11}\text{C}]$rolipram in vivo. It must be kept in mind, however, that in vitro measurements in tissues do not always agree with in vivo findings. Since PET is an in vivo imaging technique, in vivo evaluation of $R-[^{11}\text{C}]$rolipram in animal PET studies, such as in primates, using pharmacokinetic modeling, would provide invaluable results for completing the development of $R-[^{11}\text{C}]$rolipram as a PET radioligand.
5.3 Statement of Research Significance

$R-[^{11}C]$Rolipram is the first radioligand that shows promise for imaging PDE4 in the living human brain using PET. So far, all the characteristics of $R-[^{11}C]rolipram$ meet those desired in a PET radioligand for brain imaging. Recent reports have pointed towards disruptions in cAMP-mediated signaling in the etiology and treatment of neuropsychiatric disorders, most notably depression. As an important component of the cAMP signaling system controlling the action of cAMP, PDE4 contributes to the functions of this second messenger system, and possibly also other signaling systems through cross-talk. $R-[^{11}C]Rolipram$ demonstrated low human absorbed dose estimates, no radiolabeled metabolites in rat brain, and a good signal in human brain. Additionally, the radioligand discriminated between pharmacological challenges acting at different neurotransmitter systems, demonstrating selectivity for discrete brain functions, as oppose to $[^{18}F]$FDG or $[^{15}O]$water, which image glucose metabolism and blood flow, respectively, non-selectively across the brain, regardless of the neurotransmitter system. With the development of a successful pharmacokinetic model, possibly making use of a reference region, $R-[^{11}C]rolipram$ should be comparable to other radioligands developed to image specific neuroreceptors, such as $[^{11}C]$raclopride (for dopamine-$D_2$) or $[^{11}C]$setoperone (for 5-HT$_2$). The major difference would be a wider distribution of $R-[^{11}C]rolipram$ specific binding throughout the brain as compared to the other radioligands. As a result of the present research, there is now the potential for studying the role of PDE4 in normal individuals as well as in neuropsychiatric disorders before and after treatments in the living human brain using PET.
REFERENCES


Arnt, J., K.P. Bøgesø, J. Hyttel and E. Meier, 1988, Relative dopamine D, and D, receptor affinity and efficacy determine whether dopamine agonists induce hyperactivity or oral stereotypy in rats, Pharmacol & Toxicol 62, 121.


Avissar, S., Y. Nechamkin, G. Roitman and G. Schreiber, 1997, Reduced G protein functions and immunoreactive levels in mononuclear leukocytes of patients with depression, Am J Psychiatry 154, 211.


Barnette, M.S., M. Grous, L.B. Cieslinski, M. Burman, S.B. Christensen and T.J. Torphy, 1995a, Inhibitors of phosphodiesterase IV (PDE IV) increase acid secretion in rabbit isolated gastric glands: correlation between function and interaction with a high-affinity rolipram binding site, J Pharmacol Exp Ther 273, 1396.

Barnette, M.S., C.D. Manning, L.B. Cieslinski, M. Burman, S.B. Christensen and T.J. Torphy, 1995b, The ability of phosphodiesterase IV inhibitors to suppress superoxide production in guinea pig eosinophils is correlated with inhibition of phosphodiesterase IV catalytic activity, J Pharmacol Exp Ther 273, 674.


Bijak, M., 1997, Imipramine-induced subsensitivity to the 5-HT4 receptor activation, a possible mediation via an alteration in the postreceptor transduction mechanism involving adenylyl cyclase, Pol J Pharmacol 49, 345.

Bijak, M., K. Tokarski and J. Maj, 1997, Repeated treatment with antidepressant drugs induces subsensitivity to the excitatory effect of 5-HT4 receptor activation in the rat hippocampus, Naunyn Schmiedebergs Arch Pharmacol 355, 14.


Casanovas, J.M., M.T. Vilaro, G. Mengod and F. Artigas, 1999, Differential regulation of somatodendritic serotonin 5-HT1A receptors by 2-week treatments with the selective agonists alnespirone (S-20499) and 8-hydroxy-2-(Di-n-propylamino)tetrailin: microdialysis and autoradiographic studies in rat brain, J Neurochem 72, 262.


Conti, M., S. Iona, M. Cuomo, J.V. Swinnen, J. Odeh and M.E. Svoboda, 1995a, Characterization of a hormone-inducible, high affinity adenosine 3'-5'-cyclic monophosphate phosphodiesterase from the rat sertoli cell, Biochem 34, 7979.


Conti, M., G. Nemoz, C. Sette and E. Vicini, 1995b, Recent progress in understanding the hormonal regulation of phosphodiesterases, Endocrine Rev 16, 370.


Correa-de-Sá, P. and J.A. Ribeiro, 1994, Evidence that the presynaptic A2a-adenosine receptor of the rat motor nerve endings is positively coupled to adenylate cyclase, Naunyn-Schmiedeberg's Arch Pharmacol 350, 514.


Duman, R.S., 1998, Novel therapeutic approaches beyond the serotonin receptor, Biol Psychiatry 44, 324.


Eison, A.S., F.D. Yocca and G. Gianutsos, 1988, Noradrenergic denervation alters serotonin2-mediated behavior but not serotonin2 receptor number in rats: modulatory role of beta adrenergic receptors, J Pharmacol Exp Ther 246, 571.

Ekholm, D., P. Belfrage, V. Manganiello and E. Degerman, 1997, Protein kinase A-dependent activation of PDE4 (cAMP-specific cyclic nucleotide phosphodiesterase) in cultured bovine vascular smooth muscle cells, Biochim Biophys Acta 1356, 64.


Erdogan, S. and M.D. Houslay, 1997, Challenge of human jurkat T-cells with the adenylate cyclase activator forskolin elicits major changes in cAMP phosphodiesterase (PDE)
expression by up-regulating PDE3 and inducing PDE4D1 and PDE4D2 splice variants as well as down-regulating a novel PDE4A splice variant, Biochem J 321, 165.


Fredholm, B.B. and E. Lindgren, 1988, Protein kinase C activation increases noradrenaline release from the rat hippocampus and modifies the inhibitory effect of α2-adrenoceptor and adenosine A1-receptor agonists, Naunyn-Schmiedeberg’s Arch Pharmacol 337, 477.


Fujimaki, K., S. Morinobu and R.S. Duman, 2000, Administration of a cAMP phosphodiesterase 4 inhibitor enhances antidepressant-induction of BDNF mRNA in rat hippocampus, Neuropsychopharmacol 22, 42.


Grimm, L.J., J.A. Blendy, K.J. Kellar and D.C. Perry, 1992, Chronic reserpine administration selectively up-regulates $\beta_1$- and alpha$_1$B-adrenergic receptors in rat brain: An autoradiographic study, Neurosci 47, 77.


Hulley, P., J. Hartikka and L. H, 1995, Cyclic AMP promotes the survival of dopaminergic neurons in vitro and protects them from the toxic affects of MPP+, J Neural Transmission 46, 217.


Jurevicius, J. and R. Fischmeister, 1996, cAMP compartmentation is responsible for a local activation of cardiac Ca²⁺ channels by b-adrenergic agonists, Proc Natl Acad Sci USA 93, 295.


Kehn, W., G. Debus and R. Neumeister, 1985, Effects of Rolipram, a Novel Antidepressant, on Monoamine Metabolism in Rat Brain, J Neural Transmission 63, 1.


Krause, W. and G. Kühne, 1988, Pharmacokinetics of rolipram in the rhesus and cynomolgus monkeys, the rat and the rabbit. Studies on species differences, Xenobiotica 18, 561.


Le Poul, E., N. Laaris, M. Hamon and L. Lanfumey, 1997, Fluoxetine-induced desensitization of somatodendritic 5-HT1A autoreceptors is independent of glucocorticoid(s), Synapse 27, 303.


Loevinger, B., T. Budinger and E. Watson, 1988, MIRD Primer for absorbed dose calculations (New York).


develop aggressiveness and hyperphagia in conjunction with brain serotonergic abnormalities, Proc Natl Acad Sci U S A 96, 15239.


Madelian, V. and E. La Vigne, 1996, Rapid Regulation of a Cyclic AMP-Specific Phosphodiesterase (PDE IV) by Forskolin and Isoproterenol in LRM55 Astroglial Cells, Biochem Pharmacol 51, 1739.


Olesen, J., K. Hougaard and M. Hertz, 1978, Isoproterenol and propranolol: ability to cross the blood-brain barrier and effects on cerebral circulation in man, Stroke 9, 344.


Ozawa, H. and M.M. Rasenick, 1991, Chronic electroconvulsive treatment augments coupling of the GTP-binding protein Gs to the catalytic moiety of adenylyl cyclase in a manner similar to that seen with chronic antidepressant drugs, J Neurochem 56, 330.


Post, R.M., 1997, Molecular biology of behavior, Arch Gen Psychiatry 54, 607.


Przegalinski, E., L. Baran, J. Siwanowicz, G. Nowak, L. Antkiewicz-Michaluk and J. Vetulani, 1985, Chronic Treatment with the Potential Antidepressant Drug Rolipram: the Effect on the Behavioural Responses to Adrenergic and Dopaminergic Receptor Agonists with Some Biochemical Correlates, J Neural Transmission 64, 211.


Przegalinski, E. and T. Jurkowska, 1987, Effect of repeated treatment with antidepressant drugs or electroconvulsive shock (ECS) on the increase in food intake induced by clonidine injected into the paraventricular nucleus, Arch Int Pharmacodyn 290, 257.


Sano, M., A. Seto-Ohshima and A. Mizutani, 1984, Forskolin supresses seizures induced by pentylenetetrazol in mice, Experientia 40, 1270.

Sapena, R., D. Morin, R. Zini and J.P. Tillement, 1994, Cyclic AMP and inositol phosphate accumulations in rat brain cortical slices following chronic citalopram or desipramine administration, Drugs Exptl Clin Res 20, 93.

Sapolsky, R.M., 1996, Stress, Glucocorticoids, and Damage to the Nervous System: The Current State of Confusion, Stress 1, 1.


Schoffelmeer, A.N.M., G. Wardeh and A.H. Mulder, 1985, Cyclic AMP facilitates the electrically evoked release of radiolabelled noradrenaline, dopamine and 5-hydroxytryptamine from rat brain slices, Naunyn-Schmiedeberg's Arch Pharmacol 330, 74.

Schudt, C., S. Winder, S. Forderkunz, A. Hatzelmann and V. Ullrich, 1991, Influence of selective phosphodiesterase inhibitors on human neutrophil functions and levels of cAMP and Ca\textsuperscript{i}, Naunyn-Schmiedeberg's Arch Pharmacol 344, 682.


Scuée-Moreau, J., I. Giesbers and A. Dresse, 1987, Effect of rolipram, a phosphodiesterase inhibitor and potential antidepressant, on the firing rate of central monoaminergic neurons in the rat, Arch Int Pharmacodyn 288, 43.


Sharp, T., S.E. Gartside and V. Umbers, 1997, Effects of co-administration of a monoamine oxidase inhibitor and a 5-HT1A receptor antagonist on 5-hydroxytryptamine cell firing and release, Eur J Pharmacol 320, 15.

Shea, T.B., E.P. Klinger and C.M. Cressman, 1995, Calcium influx recruits an additional class of kinases to hyperphosphorylate tau, Neuroreport 6, 1309.


Smith, M.A., S. Makino, R. Kvetnansky and R.M. Post, 1995d, Stress and glucocorticoids affect the expression of brain-derived neurotrophic factor and neurotrophin-3 mRNAs in the hippocampus, J Neurosci 15, 1768.


Takeda, T., Y. Yamashita, S. Shimazaki and Y. Mitsui, 1992, Histamine decreases the permeability of an endothelial cell monolayer by stimulating cyclic AMP production through the H2-receptor, J Cell Sci 101, 745.


Uphouse, L., 1997, Multiple serotonin receptors: too many, not enough, or just the right number?, Neurosci Biobehav Rev 21, 679.


Wachtel, H. and H.H. Schneider, 1986, Rolipram, a novel antidepressant drug, reverses the hypothermia and hypokinesia of monoamine-depleted mice by an action beyond postsynaptic monoamine receptors, Neuropharmacol 25, 1119.


Wang, J. and K.M. Johnson, 1995, Regulation of striatal cyclic-3',5'-adenosine monophosphate accumulation and GABA release by glutamate, J Pharmacol Exp Ther 275, 877.


Weiss, B. and E. Costa, 1968, Regional and subcellular distribution of adenyl cyclase and 3',5'-cyclic nucleotide phosphodiesterase in brain and pineal gland, Biochem Pharmacol 17, 2107.


