HEPOXILIN BINDING ACTIVITY IN THE RAT BRAIN

By

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

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ABSTRACT

Hepoxilin binding activity in the rat brain

Degree of Master of Science 1999
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Previous work from our laboratory has shown that hepoxilins have neuromodulatory actions in the rat brain and that specific hepoxilin binding activities exist in human neutrophils. In this study, we investigate the existence of hepoxilin-specific binding in rat brain homogenates using \(^{3}\)H-(8R)-HxA\(_3\)Me, and the responsible protein. Specific binding was most abundant in the hippocampus and thalamus and the binding was time- and substrate-dependent with high affinity \(K_d=43.1\pm6.7\) nM and \(B_{\text{max}}=42.3\pm7.3\) pmol/mg of protein). Competitive binding was best observed with unlabeled HxA\(_3\), HxB, and less with 11,12-EET. Binding studies of HxA\(_3\) with frozen sections and frozen homogenates using SDS-PAGE were inconclusive as a stronger signal was observed for nonspecific binding of the ligand. This unexpected observation may relate to the known instability of the hepoxilin binding protein in frozen tissues. Results with fresh homogenates of brain suggest the presence of hitherto uncharacterized HxA\(_3\) specific binding activity in rat brain.
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### LIST OF ABBREVIATIONS

- **BSA**: bovine serum albumin
- **cAMP**: cyclic adenosine monophosphate
- **DMSO**: dimethyl sulphoxide
- **DDH₂O**: double-distilled water
- **DHT**: dihydroxyeicosatrienoic acid
- **DTT**: DL-dithiothreitol
- **EET**: epoxyeicosatrienoic acid
- **11,12-EET**: 11,12-cis-epoxy-5Z,8Z,14Z-eicosatrienoic acid
- **EtOH**: ethanol
- **EGTA**: ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
- **EtOAc**: ethyl acetate
- **FABP**: fatty acid-binding protein
- **FA**: free acid
- **FMRF amide**: Phe-Met-Arg-Phe amide
- **G protein**: GTP-binding protein
- **G₁**: GTP-binding protein (inhibitory component)
- **G₁**: GTP-binding protein (stimulatory component)
- **GTP**: guanosine triphosphate
- **HxA₃**: hepoxilin A₃, 8(S/R)-hydroxy-11(S),12(S)-trans-epoxy-eicosa-5Z,9E,14Z-trienoic acid
- **HxA₃-C**: hepoxilin A₃-C, 8(S/R),12(S)-dihydroxy-11(R)-glutathionyl-eicosa-5Z,9E,14Z-trienoic acid
- **HxB₃**: hepoxilin B₃, 10(S/R)-hydroxy-11(S),12(S)-trans-epoxy-eicosa-5Z,8Z,14Z-trienoic acid
- **HxA₃-AZT**: 20-azido(tri-n-butyl)benzoate of 20-hydroxy-(8S)-HxA₃ methyl ester
- **(8S)-HxA₃Me-[¹²⁵]I-AZI**: 20-azidiodobenzoate of 20-hydroxy-(8S)-HxA₃ methyl ester
- **HETE**: hydroxyeicosatetraenoic acid
- **8(S/R)-HETE**: 8(S/R)-hydroxy-5Z,9E,11Z,14Z-eicosatetraenoic acid
- **12(S/R)-HETE**: 12(S/R)-hydroxy-5Z,8Z,10E,14Z-eicosatetraenoic acid
- **HPETE**: hydroperoxyeicosatetraenoic acid
- **InsP₃**: inositol 1,4,5-triphosphate
- **LTB₄**: leukotriene B₄, 5(S),12(S/R)-dihydroxy-6E,8Z,10E,14Z-eicosatetraenoic acid
- **HEPES**: N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]
- **MeOH**: methanol
- **Me**: methyl ester
PMSF  phenyl methyl sulfonyl fluoride
PG   prostaglandin
PGD₂  9α,15(S)-dihydroxy-11-oxo-prosta-5Z,13E-dien-1-oic acid
PGE₂  11α,15(S)-dihydroxy-9-oxo-prosta-5Z,13E-dien-1-oic acid
PGF₂α  9α,11α,15(S)-trihydroxy-prosta-5Z,13E-dien-1-oic acid
6-keto-PGF₁α  9α,11α,15(S)-trihydroxy-6-oxo-prost-13E-en-1-oic acid
NaCl  sodium chloride
NaH₂PO₄  sodium phosphate monobasic
Na₂HPO₄  sodium phosphate
NaI  sodium iodide
SDS  sodium dodecyl sulfate
SDS-PAGE  sodium dodecyl sulfate polyacrylamide gel electrophoresis
Na₂S₂O₃  sodium metabisulfite
SPB  sodium phosphate buffer
TCA  trichloroacetic acid
TxB₂  thromboxane B₂, 9α,11α,15(S)-trihydroxy-thromboxa-
      5Z,13E-dien-1-oic acid
Tris-HCl  tris(hydroxymethyl)aminomethane hydrochloride
TLC  thin layer chromatography
TrXA₃  trioxilin A₃, 8(S),11(S,R),12(S)-trihydroxyeicosa-
      5Z,9E,14Z-trienoic acid

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SECTION ONE: INTRODUCTION

1.1 EICOSANOIDs

The term 'eicosanoids' was introduced by Corey et al. (1980) and it refers to the unsaturated lipids constituted by a chain of twenty carbons. The term is applied to fatty acids as well as the oxygenated products derived from them through a variety of enzymatic systems namely, cyclooxygenase, lipoxygenases and cytochrome P450. Most of the oxygenated eicosanoids have diverse and potent pharmacological activities. They are formed by virtually every tissue in the body. The biological activities of eicosanoids vary between organs in a given species and may also vary from species to species. Their biological activities even differ during different stages of development. Diverse activities of eicosanoids include actions in blood clotting, inflammation, control of vascular tone, renal function, reproductive system, neural and neuroendocrine systems (Shimizu and Wolfe, 1990).

All the oxygenated eicosanoids are biosynthesized by mono- and dioxygenation of the three following precursors: homo-γ-linolenic acid, arachidonic acid and eicosapentaenoic acid. These three precursors are found esterified to phospholipids, cholesteryl esters and triglycerides. The main storage site for polyunsaturated fatty acids is phospholipids. However, cholesteryl esters often contain arachidonate (Deby, 1988).

1.1.1 Arachidonic Acid

Arachidonic acid (AA), the major eicosanoid precursor acid, is a polyunsaturated essential fatty acid with 20 carbons and 4 double bonds at positions 5, 8, 11 and 14 (designation 20:4 Δ5,8,11,14). It is derived from linoleic acid (18:2, ω-6) which is abundant in certain vegetable oils. The relative concentration of AA is highest in phosphatidylcholine (PC) and phosphatidylinositol (PI). The approximate relative concentrations of AA in cerebral cortex are 1.7 mg for PC, 3.8 mg for phosphatidylethanolamine (PE), 0.2 mg for phosphatidylserine (PS) and 1.06 mg for PI in one gram of dry weight of the brain. However, since the turnover rate of AA in PC and PE is relatively low, PI is the major supplier of free AA for eicosanoid biosynthesis. The
availability of AA in the cytosol of the cell determines the eicosanoid formation and is controlled by the balance of the release of AA by hydrolysis and its re-esterification into phospholipids by acyl transferases (Anggard, 1988; Piomelli, 1996).

1.1.2 Arachidonic Acid Cascade

External stimuli or cell-damaging insults trigger the release of arachidonic acid via phospholipase reaction. Part of the available arachidonic acid interacts with protein targets within the cell and part of it is utilized as the substrate for the rate-limiting oxidative reactions. The three main classes of oxygenase enzymes include cytochrome P450, cyclooxygenase and various lipoxygenases (Fig. 1). Cytochrome P450 catalyzes the monooxygenation of AA to form a family of epoxyeicosatrienoic acids (EETs) which in turn may be transformed to dihydroxyeicosatrienoic acids (DHTs) by epoxide hydrolase. Cytochrome P450 can also metabolize AA to a family of non-chiral hydroxyeicosatetraenoic acids (HETEs) such as 20- and 19-HETEs. Cyclooxygenase initiates the conversion of AA to PGG₂ by oxygenation and ring closure of arachidonate and then to PGH₂. PGH₂ is then metabolized to various prostaglandins (PGE₂, PGF₂α and PGD₂), prostacyclins and thromboxanes (Piomelli, 1996). Lipoxygenases catalyze the addition of a hydroperoxide group from molecular oxygen to a particular carbon atom in the arachidonic molecule to form a chiral (usually S) hydroperoxyeicosatetraenoic acid (HPETE). 5-lipoxgenase converts AA to 5-HPETE which then gets metabolized to 5-HETE and leukotrienes including LTA₄, LTB₄, LTC₄, LTD₄ and LTE₄ (Yamamoto, 1992). 12-lipoxgenase introduces molecular oxygen into the 12(S)-position of arachidonic acid to yield 12(S)-HPETE which then gives rise to hepxilins or is subsequently reduced by peroxidases to 12-HETE (Yoshimoto and Yamamoto, 1995). There are other types of lipoxygenases converting AA to various HPETEs, such as 8-, 9-, 11- and 15-HPETEs, which lead to various HETES and other metabolites. Additionally, AA can be oxidized nonenzymatically into HPETEs and HETEs although this reaction is not under stereochemical control resulting in racemic (S/R) products.
Fig. 1. The arachidonic acid cascade outlining the major metabolic pathways.
1.1.3 12-Lipoxygenase

12-lipoxygenase was first discovered in human (Hamberg and Samuelsson, 1974) and bovine (Nugteren, 1975) platelets. Two isoforms of 12-lipoxygenase, a 'platelet-type' and a 'leukocyte-type' were found based upon the biochemical, immunological and molecular biological criteria, such as their activities toward C18 and C20 fatty acids, immunoreactivity toward anti-platelet or anti-leukocyte enzyme antibody, sequence homology with 15-lipoxygenase and their occurrence (Yamamoto, 1992). 12-lipoxygenases have been found in various cells and tissues in various animal species. For example, platelet-type 12-lipoxygenase was found in human (Hamberg and Samuelsson, 1974), rat (Chang et al., 1982), bovine (Nugteren, 1975) and mouse (Chen et al., 1994) platelets. Leukocyte-type 12-lipoxygenase was found in rat leukocytes (Watanabe and Haeggström, 1993), rat pineal gland (Yoshimoto et al., 1984 and Hada et al., 1994), and rat pancreatic islets (Turk et al., 1984).

12-lipoxygenase is the principal lipoxygenase in the brain (Sautebin et al., 1978; Spagnuolo et al., 1979; Wolfe and Pappius, 1984; Shimizu et al., 1987). Although it is not clear whether the enzyme originates from neurons or glia, 12-lipoxygenase activity was found in a cultured neuroblastoma cell line (Adesuyi et al., 1985) and cultured astrocytes (Ishizaki and Murota, 1991). 12-lipoxygenase activity was also found in rat anterior pituitary (Pilote et al., 1982) and the metabolite generated from the 12-lipoxygenase pathway, 12-HPETE, was reported to stimulate melatonin biosynthesis in rat pineal (Sakai et al., 1988).

1.2 HEPOXILINS

1.2.1 Formation

The hepoxilin pathway was discovered over a decade ago (Pace-Asciak and Martin, 1984). The name of hepoxilins combines the structure, Hydroxy EPOXide and the first reported biological activity of the compound, release of Insulin. Hepoxilins are hydroxy epoxide derivatives of arachidonic acid formed through the intramolecular isomerization of 12(S)-HPETE, the initial product formed by 12-lipoxygenase (Fig. 2)
Fig. 2 The synthesis of hepoxilins.
(Pace-Asciak, 1984b; Pace-Asciak et al, 1983a). The 12(S)-HPETE isomerase, or hepoxilin synthase has not yet been characterized. It is unknown whether the lipoxygenase and hepoxilin synthase are coupled or separated in different cell compartments. Two hepoxilins, HxA, and HxB, which are trans-epoxides with a configuration of 11(S) and 12(S), are formed through the rearrangement of 12(S)-HPETE (Pace-Asciak, 1984a). HxA has a hydroxyl group at carbon 8 while HxB at carbon 10. A structure similar to HxB was first isolated when Jones et al. (1978) discovered the formation of a trihydroxy metabolite of arachidonic acid in human platelets. Then HxA was isolated from rat lung and characterized by Pace-Asciak et al. (1983b).

Reynaud et al. (1994b) demonstrated that 8(S) and 8(R)-epimers of HxA, were formed exclusively, with minor amount of HxB, in rat pineal gland from 12(S)-HPETE by a specific heat-labile protein, hepoxilin synthase. This enzyme appears to be a cytosolic protein and it does not require any cofactors to transform 12-HPETE to hepoxilins. Hepoxilin synthase is also present in rat brain as the formation of HxA, was detected (Pace-Asciak, 1988). Characterization of this enzyme is still in progress.

1.2.2 Metabolism

Unlike HxB, which is more chemically stable and resistant to both enzymatic and nonenzymatic hydrolysis, HxA, can be metabolized by three different pathways (Fig. 3). The first pathway leads to the formation of a trihydroxy metabolite termed trioxilin A3 (TrXA3) through the action of an epoxide hydrolase. This product is mostly inactive and a partially purified protein appears to be selective for HxA, as the substrate rather than leukotriene A4 or styrene oxide (Pace Asciak et al., 1986; Pace-Asciak and Lee, 1989). In acid media, HxA, is unstable chemically and is rapidly hydrolyzed into TrXA3. Methyl ester of HxA, is much more stable chemically. The second pathway results in the formation of a glutathione conjugate of HxA3, termed HxA3-C, through the action of glutathione S-transferase. It is detected when trichloropropene oxide is present to inhibit the more active hepoxilin epoxide hydrolase (Pace-Asciak and Lee, 1989). The third pathway was identified recently by Reynaud et al. (1997) in human neutrophils and it
Hepoxilin A, Trioxilin A, w-Oxidation a-Hydroxy-hepoxilin Hepoxilin A,-C

Fig. 3 The metabolism of hepoxilins
leads to the formation of a 20-hydroxy catabolite through the action of an apparently specific ω-hydroxylase.

1.2.3 Biological actions

Actions in the central nervous system

Incubation of homogenates of the rat cerebral cortex with arachidonic acid has been shown to lead to the formation of HxA₃, analyzed as its stable trihydroxy derivative, trioxilin A₃, by high resolution gas chromatography/electron impact mass spectrometry. It was estimated that the cerebral cortex generated 5.0 ± 0.2 ng/mg protein of HxA₃. The formation of HxA₃ was also detected in median eminence (11.7 ± 1.6 ng/mg protein), pituitary (12.3 ± 0.7 ng/mg protein) and pons (26.6 ± 0.2 ng/mg protein) (Pace-Asciak, 1988). Unlike the formation of HxA₃ in platelets, the formation of HxA₃ in the brain was not blocked by the dual cyclooxygenase and lipoxygenase blocker, BW 755C (Pace-Asciak, 1988). As HxA₃ is not found in circulation, the formation of HxA₃ in the brain may suggest biological actions of HxA₃ in the central nervous system (CNS).

In *Aplysia* sensory cells, 12-lipoxgenase metabolites were demonstrated to act as second messengers in neurotransmission (Piomelli *et al.*, 1987; Piomelli and Greengard, 1990). 12-HPETE applied to *Aplysia* sensory neurons mimicks the actions of the molluscan tetrapeptide, Phe-Met-Arg-Phe amide (FMRF-amide), which leads to the hyperpolarization of the membrane, a decrease in the duration of the action potential, an increase in potassium conductance and an inhibition of synaptic transmission. Subsequent studies identified the active metabolites to be HxA₃ (Piomelli *et al.*, 1989) and 12-keto-5,8,10,14-eicosatetraenoic acid (Piomelli *et al.*, 1988). HxA₃ was released by hippocampal slices and the release was greatly stimulated by exogenous AA, which could be blocked by BW 755C. HxA₃ was also proved to be active in rat hippocampal CA1 neurons. HxA₃ at micromolar concentrations caused hyperpolarization of membrane potential, and an increase in post spike train afterhyperpolarization (AHP) and the amplitude of the inhibitory postsynaptic potentials (IPSP) and these actions were mimicked by arachidonic acid (Carlen *et al.*, 1989). HxA₃ also inhibited the 4-aminopyridine-induced release of norepinephrine from tritiated norepinephrine-loaded
hippocampal slices (Pace-Asciak et al., 1990b). Later studies have shown that the threshold concentrations of hepoxilins to elicit the above actions lie in the 3-10 nM range (Carlen et al., 1994). HxA, -C was also found to hyperpolarize the membrane potential, enhance the amplitude and duration of AHP, and increase IPSP with a threshold of low nanomolar concentrations (Pace-Asciak et al., 1990a). The actions of hepoxilins in the CNS suggest that they are neuromodulatory compounds. Despite the fact that actions of hepoxilins in the CNS have been identified, the mechanism of these actions is still unknown. It is possible that hepoxilins elicit these actions through specific binding proteins.

1.3 EICOSANOID RECEPTORS

Biochemical, physiological and pharmacological studies have suggested the presence of various eicosanoid receptors in different tissues. The availability of tritiated eicosanoids of high specific activity has facilitated the identification of receptors for various eicosanoids. For example, receptors PGD, have been identified in platelets (Cooper and Ahern, 1979). Receptors for PGE have been found in a wide range of tissues including adipocytes (Gorman and Miller, 1973), liver (Okamura and Terayama, 1976) and pineal (Cardinali et al., 1979). The presence of PGF, receptors have been found in the liver (Okamura and Terayama, 1976), pineal (Cardinali et al., 1979) and skin (Lord et al., 1978) while PGI, receptors have been found in platelets (Siegl et al., 1979). The existence of 12-HETE binding proteins has been found in a human epidermal cell line (Gross et al., 1990), keratinocytes (Arenberger et al., 1993), and Langerhans cells (Arenberger et al., 1992). Binding parameters for various eicosanoid receptors in different tissues are summarized in Table I.

The structure of the above prostanoid (PGD, PGE, PGF, and PGI,) receptor proteins consists of seven hydrophobic transmembrane domains and thus they belong to a rhodopsin-type receptor superfamily. The third and seventh transmembrane domains and the second extracellular loop are the highly conserved regions. The arginine residue in the seventh transmembrane domain is conserved in all prostanoid receptors. This residue
<table>
<thead>
<tr>
<th>Type of receptors</th>
<th>Tissue distribution</th>
<th>$K_d$ (nM)</th>
<th>$B_{max}$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>PGD$_2$</td>
<td>Platelets</td>
<td>53</td>
<td>210 sites/platelet</td>
<td>Cooper and Ahern, 1979</td>
</tr>
<tr>
<td></td>
<td>Brain synaptic membranes</td>
<td>28</td>
<td>0.45 pmol/mg protein</td>
<td>Shimizu et al., 1982</td>
</tr>
<tr>
<td>PGE</td>
<td>Adipocytes</td>
<td>0.2</td>
<td>0.051 pmol/mg protein</td>
<td>Gorman and Miller, 1973</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>17.6</td>
<td>0.68 pmol/mg protein</td>
<td>Okamura and Terayama, 1977</td>
</tr>
<tr>
<td></td>
<td>Liver</td>
<td>13.6</td>
<td>3.66 pmol/mg protein</td>
<td>Okamura and Terayama, 1977</td>
</tr>
<tr>
<td></td>
<td>Pineal</td>
<td>1.2</td>
<td>1-2 fmol/mg protein</td>
<td>Cardinali et al., 1979</td>
</tr>
<tr>
<td></td>
<td>Brain membrane</td>
<td>4.2</td>
<td>17.4 fmol/mg protein</td>
<td>Malet et al., 1982</td>
</tr>
<tr>
<td>PGF$_{2\alpha}$</td>
<td>Liver</td>
<td>23.1</td>
<td>0.41 pmol/mg protein</td>
<td>Okamura and Terayama, 1977</td>
</tr>
<tr>
<td></td>
<td>Pineal</td>
<td>1.7</td>
<td>1-2 fmol/mg protein</td>
<td>Cardinali et al., 1979</td>
</tr>
<tr>
<td></td>
<td>Skin</td>
<td>1.0</td>
<td>0.28 pmol/mg protein</td>
<td>Lord et al., 1978</td>
</tr>
<tr>
<td>PGI$_1$</td>
<td>Platelets</td>
<td>12.1</td>
<td>93 sites/platelet</td>
<td>Siegl et al., 1979</td>
</tr>
<tr>
<td>12-HETE</td>
<td>Epidermal cell line</td>
<td>2.6</td>
<td>216,000 sites/cell</td>
<td>Gross et al., 1990</td>
</tr>
<tr>
<td></td>
<td>Keratinocytes</td>
<td>3.84</td>
<td>232,000 sites/cell</td>
<td>Arenberger et al., 1993</td>
</tr>
<tr>
<td></td>
<td>Langerhans cells</td>
<td>3.32</td>
<td>691,000 sites/cell</td>
<td>Arenberger et al., 1992</td>
</tr>
</tbody>
</table>
is proposed to be the binding site for the $\alpha$-carboxylic acid of prostanoid molecules. The overall homology of amino acid sequences among these receptors is approximately 30%. Until now, the molecular characterization of 12-HETE receptor has not been attained.

1.3.1 Eicosanoid receptors in the CNS

In the CNS, receptors for PGD$_2$ and PGE$_2$ have been identified (see below).

PGD$_2$ receptors (DP receptors)

PGD$_2$ has been shown to have various actions in the CNS, including sleep induction (Hayaishi et al., 1987; Ueno et al., 1983), hormone release (Kinoshita et al., 1982), analgesia (Horiguchi et al., 1986) and neurotransmitter release (Shimizu and Wolfe, 1990). Specific binding of PGD$_2$ has been observed in rat brain synaptic membranes ($K_d = 28 \pm 7$ nM and $B_{\text{max}} = 0.45$ pmol/mg protein) (Shimizu, 1982).

The actions of PGD$_2$ on platelet aggregation, and relaxation of smooth muscle is coupled to $G_\text{i}$ and adenylate cyclase which leads to an increase in intracellular cAMP. However, the molecular mechanisms of PGD$_2$ actions in the CNS are not known yet. A cDNA for mouse DP receptor has been isolated from the mouse genomic library. The receptor is a protein with of 357 amino acids with a molecular weight of 40,012 (Negishi et al., 1995).

PGE receptors (EP receptors)

In the CNS, PGE$_2$ has been shown to have actions on analgesia (Horiguchi et al., 1986; Poddubiuk, 1976), hormone release (Ojeda et al., 1977), anticonvulsion (Poddubiuk 1976; Wolfe, 1982) and neurotransmitter release (Shimizu and Wolfe, 1990). Specific binding of PGE$_2$ has been proven in rat brain membranes with $K_d$ and $B_{\text{max}}$ values of $4.2 \pm 0.9$ nM and $17.4 \pm 3.9$ fmol/mg protein, respectively (Malet et al., 1982).

Based on their responses to various agonists and antagonists, PGE receptors are subdivided pharmacologically into four subtypes, EP1, EP2, EP3 and EP4.

EP1 receptors are abundantly expressed in the kidneys. The action of EP1 involves calcium influx which is independent of phospholipase C activation, indicating
that the EP1 receptor may activate a novel type of calcium channels. EP1 receptor is coupled to a G protein but the identity of the G protein is not known yet. Human EP1 receptor consists of 402 amino acids and a molecular weight of 41,858 (Negishi et al., 1995).

EP2 and EP4 receptors have been detected in the lungs, and thymus and ileum respectively. They are both coupled to $G_\alpha$ and stimulate adenylate cyclase. In contrast to other PGE receptors, EP4 receptors possess long carboxyl-terminal tails (153 amino acid residues). The mouse EP4 receptor consists of 513 amino acid residues with a molecular weight of 56,157. EP2 receptor is much smaller than EP4 receptor and it consists of 358 amino acid residues with a molecular weight of 39,380 (Negishi et al., 1995).

EP3 receptors are widely distributed in the sensory ganglia and neurons in the brain to modulate various neuronal activities (Sugimoto et al., 1994). EP3 receptors are coupled to multiple signal transduction pathways, mainly by the inhibition of adenylate cyclase via $G_i$ and some of the actions are mediated by calcium mobilization. A cDNA for mouse EP3 receptor was cloned and it consists of 365 amino acid residues with a molecular weight of 40,007. Subsequently, the homologues of mouse EP3 receptor have been cloned for other species including human, rats, cows and rabbits (Negishi et al., 1995).

1.4 THE BRAIN

As the autoradiographic study and electrophoresis of HxA$_3$ binding in the frozen tissue preparations used in this study were inconclusive regarding the positive identification of a specific protein involved in hepoxilin binding, an extensive treatment of brain anatomy and physiology and interpretation of this with respect to hepoxilin binding and potential action is not warranted in this thesis. A general treatment, however, is provided.

The brain is an imperative organ regulating all physiological processes from cellular metabolism to the overall functioning of the entire body. It is protected with one
of the strongest structures of the body, the cranium. Homeostasis in the brain is maintained by a unique mechanism, the blood-brain barrier.

The blood-brain barrier is made up of solid walls of brain capillaries formed by endothelial cells that are joined by tight junctions. All brain capillaries are surrounded by the foot processes of astrocytes. The blood-brain barrier restricts the entrance of potentially harmful substances from the blood into the brain, and allows essential nutrients to enter the brain. The astrocytes store the metabolites and transfer them from capillaries to neurons. They also take up excess potassium and neurotransmitters from the extracellular fluid during intense neuronal activity. The structure of the blood-brain barrier only allows lipid-soluble substances to pass through. Some of the water-soluble substances, e.g. glucose, pass through the barrier by the transport of carrier proteins on the plasma membrane of the brain endothelial cells.

The brain has four major divisions: a) brainstem, which includes midbrain, pons and medulla oblongata; b) cerebellum; c) cerebrum and d) diencephalon which composes the thalamus, hypothalamus, epithalamus and ventral thalamus. Due to the complexity of the brain, only some of the structures are mentioned in this thesis as they apply to this study (for review, see Carola et al., 1992; Brodal, 1998).

1.4.1 Cerebellum

The cerebellum is the second largest part of the brain. Its surface consists of a thin, highly convoluted layer of gray matter, called the cerebellar cortex. White matter containing the afferent and efferent fibers is found underneath the cerebellar cortex. In the central parts of the cerebellum, masses of gray matter, called intracerebellar nuclei, are embedded in the white matter.

The cerebellum is a processing center which coordinates muscular movements by regulating the tension and tone of the muscles, timing and precision of movements; and monitoring the sensory inputs e.g. relative body position from those motor systems. Recent animal experiments suggest a role of the cerebellum during motor learning by changes in synaptic efficacy in the cerebellar cortex. Furthermore, recent studies have shown that the cerebellum can measure temporals with great accuracy and this timing
function can be used in non-motor tasks. For example, patients with cerebellar damage have impaired ability in tapping their fingers at a certain rhythm and also in discriminating different sound rhythms. Research activities are still ongoing about the functions of the cerebellum.

1.4.2 Diencephalon

Thalamus

The thalamus is composed of two masses of gray matter surrounded by a layer of white matter and it is located in the center of the cranial cavity. Anatomists divided the gray matter into several nuclei. The name of each nucleus is usually derived from its geographical location within the thalamus. In a general way, depending upon their fiber connections, most of the major thalamic nuclei can be classified as specific relay nuclei or as association nuclei. Specific relay nuclei receive ascending signals and project to well defined cortical region with specific function. Association nuclei do not receive direct fibers from ascending system but project to association areas of the cortex. Other thalamic nuclei have predominantly subcortical connection.

The thalamus is an intermediate relay structure and processing center for all sensory information, except smell, going to the cerebral cortex from the spinal cord, brainstem, cerebellum, basal ganglia and other sources. Four major areas of activities are involved in the thalamus: 1. sensory systems: sensory input travels along the fibers from the thalamic nuclei to the sensory areas of the cerebral cortex where the information is translated to appropriate sensation; 2. motor systems: some thalamic nuclei receive the neural input from the cerebellum and basal ganglia and then project and influence the motor cortex; 3. general neural background activity: thalamic nuclei monitor neurophysiological activities such as the sleep-wake cycles and the electrical brain waves which are expressed in the cerebral cortex; 4. Expression of the cerebral cortex: as a result of its connection with the limbic system, the thalamus also regulates the expression of emotions and behaviors.
**Hypothalamus**

The hypothalamus is a small region just below the thalamus. It is the highest integrating center for the autonomic nervous system. It modifies blood pressure, rate and force of heartbeat, peristalsis and glandular secretion in the digestive system, sweat glands and salivary glands secretion, and the control of the urinary bladder. The hypothalamus also regulates body temperature, water and electrolyte balance, food intake, sleep-wake pattern and sexual responses. It also controls the release of oxytocin and antidiuretic hormone and other hormones released from the pituitary gland.

**1.4.3 Cerebrum**

The cerebrum consists of two cerebral hemispheres, which are composed of a cortex (gray matter), white matter and basal ganglia. In human, the cerebral lobe is subdivided into five lobes: the frontal, parietal, temporal, occipital and the central lobes.

The white matter underneath the cortex contains three type of fibers: 1) association fibers which interconnect neurons in different parts of the cortex of the same hemisphere; 2) commissural fibers, such as the anterior commissure and the corpus callosum, which are axons projecting from the cortex of one hemisphere to the same cortical area of the opposite hemisphere; 3) projection fibers which include axons from the cortex to other structures in the brain, such as the thalamus, basal ganglia, brainstem, and to the spinal cord.

The basal ganglia is a core of gray matter which includes the caudate nucleus, putamen, globus pallidus, subthalamic nucleus and substantia nigra. The basal ganglia receives afferent information from the cerebral cortex, process and integrate these inputs, and then relay them to the thalamus which then projects to the motor, premotor and limbic cortical regions. Parkinson’s disease and Huntington’s disease result from the malfunction of the basal ganglia.

The neurons of the cerebral cortex receive impulses from lower parts of the CNS, majority of which are relayed through the thalamus. About two-thirds of the neurons are pyramidal cells in the cortex. The remaining of the cortical neurons contains a heterogeneous group of cells, collectively called nonpyramidal cells, and are most likely
the interneurons. Different parts of the cerebral cortex elicit different functions. For instance, the motor cortex is involved in the motor control of voluntary movements. The auditory cortex plays a crucial role in hearing, equilibrium and to a certain degree, emotion and memory. The primary visual cortex receives the images from the retina of the eye and then the information is further processed by the surrounding area. The olfactory cortex is involved with the perception of odors.

**Hippocampus**

Hippocampus is a C-shaped structure in the limbic system and it forms the hippocampal formation with the subiculum, dentate gyrus and the entorhinal cortex. Most of the neurons of the dentate gyrus are small granule cells and the hippocampal cell layer consists of large pyramidal cells with pyramidal cell dendrites and incoming axons below and above. Various types of interneurons, mainly GABAergic basket cells, are found to inhibit the pyramidal cells. The hippocampus can be divided into three distinct fields, namely CA1, CA2 and CA3. The granule cell axons contact the pyramidal cell dendrites and the pyramidal cells send their axons out of the hippocampus.

The internal neuronal pathway is rather complicated in the hippocampus. First, pyramidal cells in the entorhinal area send their axons, called perforant path, to the hippocampus, where many of them end in the dentate gyrus. Then the granule cells of the dentate gyrus send their axons, called mossy fibers, to the apical dendrites of the pyramidal cells of CA3. The CA3 pyramidal cells then send their axons to the apical dendrites of the CA1 pyramidal cells, which then are directed to the subiculum and back to the entorhinal area. All links in this pathway are excitatory using glutamate as the neurotransmitter. However, what kind of information processing takes place in the pathway in the hippocampus is not known.

Two aspects of connections of the hippocampal formation are crucial for its function: 1) connections with various cortical association areas; 2) direct and indirect connections with other limbic structures such as cingulate gyrus and the septal nuclei.

Hippocampal function has been proven to be involved in the memory of recent events (Milner, 1970; Squire 1987). A clinicopathological human case study of a patient
with bilateral removal of the hippocampal formation and the surrounding regions, including amygdala, suffered from anterograde amnesia (memory loss for the events happened after the brain damage) and impaired long-term memory (Scoville and Milner, 1957). This indicated that the damage of the hippocampal formation led to the occurrence of the amnesic syndrome. Later, a case study by Zola-Morgan and co-workers (1986) showed that the damage of the pyramidal cells in the CA1 region in the hippocampus was sufficient to produce a clinically significant anterograde amnesia.

Now, it seems established that other areas including the perirhinal cortex and the adjoining cortex of the hippocampal gyrus contribute to memory loss by their connections to the hippocampal formation, and to other parts of the brain, such as thalamus, amygdala, mammillary body and parts of the prefrontal cortex (Brodal, 1998).

The mechanism about the role of the hippocampus in enduring memories is still unknown. Hippocampus does not seem to be the storage site for memory because damage of this area does not cause a loss of distant or well-established memories. The mechanism of this interaction is one of the highly studied phenomena in current neuroscience (Amaral and Insausti, 1990).
1.5 RATIONALE AND OBJECTIVES

Previous work has shown that:

1. Hepoxilins have neuromodulatory effects in mammalian hippocampal neurons and *Aplysia* neurons;
2. Hepoxilins modulate neurotransmission in mammalian hippocampal neurons;
3. Hepoxilin-specific binding activity is present in human neutrophils.

The objective of my study was to first localize hepoxilin-specific binding activity in rat brain. I have adopted a classical method of determining specific binding sites by using radioactive ligand binding first in tissue homogenates and then through radioactive autoradiographic localization in frozen tissue sections.

My second objective was to characterize the hepoxilin binding using SDS-PAGE. A radioactive photoaffinity analog of hepoxilin was synthesized to label the specific binding proteins in tissue homogenates.

These studies may suggest the presence of a binding protein through which hepoxilins elicit their neuromodulatory actions. The presence of such a binding protein may suggest actions of hepoxilins in specific regions in the brain and these may imply important physiological and/or pathological actions of hepoxilins in the central nervous system.

HYPOTHESIS

A specific HxA, binding activity (protein?) is present in the CNS with regional specificity.
SECTION TWO: MATERIALS AND METHODS

2.1 BINDING OF [\textsuperscript{3}H]-\textit{(8R)}-HxA\textsubscript{3}-Me IN HOMOGENIZED RAT BRAIN TISSUE

General structures of rat brain are illustrated in Fig. 4 (Paxinos and Watson, 1997).

2.1.1 Materials

The solutes used to prepare Yamamoto synthetic cerebral spinal fluid (YSCSF) including NaCl, KCl, KH\textsubscript{2}PO\textsubscript{4}, MgSO\textsubscript{4}, CaCl\textsubscript{2}, glucose, NaHCO\textsubscript{3}, Tris-HCl, bovine serum albumin (BSA) and protease inhibitors including leupeptin, pepstatin A and PMSF were purchased from Sigma Chemical Co. (St. Louis, MO). Aprotinin was obtained from Boehringer Mannheim (Mannheim, Germany). Protein assay was obtained from Bio-Rad Laboratories (Hercules, CA). Diethyl ether was purchased from Caledon (Georgetown, ON). Ecolite scintillation fluid was purchased from ICN (St. Laurent, Quebec). Pure (8R)-HxA\textsubscript{3}Me and [\textsuperscript{3}H]-\textit{(8R)}-HxA\textsubscript{3}Me (specific radioactivity 169 Ci/mmol) were prepared by Dr. P. Demin in our laboratory by total chemical synthesis (Demin \textit{et al.}, 1994).

2.1.2 Brain tissue preparation

Adult male Wistar rats (180-250g, Charles River Breeding Laboratories, St. Constant, Quebec) were anesthetized with ether and decapitated with a guillotine. The brain was dissected and placed in freshly prepared ice-cold oxygenated (95%O\textsubscript{2}/5% CO\textsubscript{2}) Yamamoto synthetic cerebral spinal fluid (YSCSF) containing (in mM) NaCl 124.0, KCl 2.7, KH\textsubscript{2}PO\textsubscript{4} 1.2, MgSO\textsubscript{4} 4.1, CaCl\textsubscript{2} 2.0, glucose 10.0, and NaHCO\textsubscript{3} 26.0 at pH 7.4. Frontal cerebral cortex, rostral cerebral cortex, and the cerebellum were dissected out and equilibrated in oxygenated YSCSF at 30°C for 1-2 hrs. Coronal sections (300 \textmu m thick) of the brain were prepared using a vibratome (Campden Instruments, UK). Different structures from the brain slices, including the cerebral cortex, hippocampus, thalamus, and the forebrain with the hippocampus and thalamus were dissected, separated and allowed to equilibrate in oxygenated YSCSF at 30°C for 1-2 hrs. Each combined
Fig. 4 General structures of the rat brain
structure was homogenized with a glass-Teflon tissue grinder (Dorval Omni Mixer, Power 3.5 for 12 strokes, Newtown, Connecticut) in 1-10 ml of Buffer A (50 mM Tris-HCl 100 mM NaCl) containing 0.3 μM aprotinin, 1.0 μM leupeptin, 1.0 μM pepstatin A and 1 μM PMSF at pH 7.4. Protein concentration was determined by the technique of Bradford (1976) using Bio-Rad protein assay and BSA as protein standard. The freshly prepared homogenates were kept on ice and used for the binding experiments on the same day.

2.1.3 Incubation with [3H]-(8R)-HxA3Me

50,000 cpm of [3H]-(8R)-HxA3Me (final concentration: 269 pM) in the presence or absence of 1 μg of (8R)-HxA3Me (to determine non-specific binding; final concentration 2.8 μM) was dissolved in 2 μl of DMSO. Homogenized tissue (90 μg of protein) in 1 ml of Buffer A containing 0.3 μM aprotinin, 1.0 μM leupeptin, 1.0 μM pepstatin A and 1 μM PMSF was added to the tube to which the ligand had been added and incubated at 37°C for 30 min. The incubations were terminated by filtering the reaction mixture through pre-chilled Whatman GF/B glass microfibre filter (Whatman, Maidstone, England) and washing the filter with 3 ml of ice-cold Buffer A three times. The glass microfibre filters were then put into scintillation vials containing 8 ml of Ecolite scintillation fluid and the radioactivity in the vial was determined the following day in a Beckman liquid scintillation counter (Model LS 3800).

2.2 BINDING STUDIES IN HOMOGENIZED HIPPOCAMPUS AND THALAMUS

2.2.1 Materials

(10S)-HxB3,Me and (10R)-HxB3,Me were prepared by Dr. P. Demin and TrXA3,FA was prepared by Dr. D. Reynaud in our laboratory. 12(S/R)-HETE, PGE2, PGF2α, PGD2, TxB2, 6-keto-PGF1α, 11,12-EET, (8R)-HETE, (8S)-HETE and LTB4 were purchased from Cayman Chemicals (Ann Arbor, MI). Other chemicals used have been listed elsewhere.
2.2.2 Brain tissue preparation

The hippocampus and thalamus from adult male Wistar rats (180-250 g) was dissected and homogenized as described. The homogenates were used fresh for the binding experiments.

2.2.3 Incubation with \([^{3}\text{H}]-(8\text{R})-\text{HxA}_3\text{Me}\)

50,000 cpm of \([^{3}\text{H}]-(8\text{R})-\text{HxA}_3\text{Me}\) (final concentration: 269 pM) in the presence or absence of (8R)-HxA3Me or other unlabeled analogs specified was dissolved in 2 µl DMSO. Protein concentration of the homogenates was adjusted with Buffer A containing 0.3 µM aprotinin, 1.0 µM leupeptin, 1.0 µM pepstatin A and 1 µM PMSF. Reactions were started upon the addition of the homogenates into the tube containing the ligand. All binding experiments were carried out in 1 ml of Buffer A for 30 min at 37°C unless otherwise specified, and performed in triplicate. The reactions were then terminated by filtration and the radioactivity bound on the filters containing the tissues was determined as described.

2.2.4 Statistics

Binding parameters were determined from saturation isotherms by computer analysis using the BioSoft EBDA and LIGAND programs modified for an Apple Macintosh computer (Munson and Rodbard, 1980).

2.3 METABOLISM OF HxA, BY RAT BRAIN

2.3.1 Materials

Ethyl acetate (EtOAc), benzene, ethanol (EtOH) and acetic acid were obtained from Caledon (Georgetown, ON) and other chemicals have been listed elsewhere.
2.3.2 Brain tissue preparation

Homogenates of hippocampus and thalamus were prepared as described in Buffer A (pH 7.4) containing 0.3 μM aprotinin, 1.0 μM leupeptin, 1.0 μM pepstatin A and 1 μM PMSF. Coronal sections of rat brain were prepared using a vibratome as mentioned above.

2.3.3 Incubation with [3H]-(8R)-HxA3Me or [3H]-(8R)-HxA3FA

This part of the study includes the metabolism of HxA3 by homogenates of the hippocampus and thalamus and by intact brain sections. The fresh tissue homogenates (500 μg) were incubated with 200,000 cpm of [3H]-(8R)-HxA3Me (dissolved in 3 μl DMSO) in 4 ml of Buffer A (final concentration: 269 pM) containing 0.3 μM aprotinin, 1.0 μM leupeptin, 1.0 μM pepstatin A and 1 μM PMSF at 37°C for 30 min with gentle shaking. Freshly prepared intact coronal sections of the forebrain (200 μm thick), which contained the hippocampus and thalamus, and the cerebellum were then cut into pieces and incubated with 500,000 cpm of [3H]-(8R)-HxA3FA (dissolved in 5 μl DMSO) in 2 ml of Buffer A (final concentration: 1.3 nM) at pH 7.4 for 30 min. In both cases, the reaction was stopped by adding 4 ml of EtOAc.

2.3.4 Extraction of samples

The reaction mixture was then extracted three times with 4 ml of EtOAc. The EtOAc phases were combined and washed with 1 ml of double-distilled water (DDH2O) to remove the remaining salt from the buffer. The final extract was dried with nitrogen gas and redissolved in 0.5 ml of benzene. Sample containing about 100,000 cpm was then spotted onto a TLC plate (Silica Gel 60, Merck, Darmstadt, Germany) and developed with a solvent system of EtOAc/acetic acid, 99.5/0.5 (v/v) for 50 min at 23°C. Then, the TLC plate was dried and scanned for radioactivity on a TLC radiochromatogram scanner (Berthold, Germany). After, the TLC plate was scraped according to the radioactive zones detected by the scanner. 1 ml of EtOH/DDH2O (1/1, v/v) was added to the silica particles in a scintillation vial to elute the material from the silica gel. After 10 min, 8 ml of Ecolite scintillation fluid (ICN, St. Laurent, Quebec) was then added to the silica.
mixture and the radioactivity was counted in a Beckman liquid scintillation counter (Model LS 3800).

2.4 IODINATION OF HxA₃ PHOTOCHEMISTRY AZIDO ANALOG

2.4.1 Materials

The solutes, NaH₂PO₄, Na₂HPO₄, used for the sodium phosphate buffer (SPB) and Chloramine T were purchased from Sigma Chemical Co. (St. Louis, MO). Na₂S₂O₅ were obtained from Fisher Scientific (Nepean, ON). HxA₃-AZT was prepared by Dr. Peter Demin (Demin et al., 1997). Na¹²⁵I was purchased from Amersham Pharmacia Biotech (Baie d’Urfé, Quebec). Methanol (MeOH) and hexane were obtained from Caledon (Georgetown, ON).

2.4.2 Radio-iodination of HxA₃-AZT

The preparation of the photoaffinity analog was carried out under reduced light. HxA₃-AZT (1μg) was dissolved in 2 μl of MeOH and then 8 μl of 0.1M SPB. 0.6 μCi of Na¹²⁵I (6 μl, about 2000 Ci/mmol) and 0.6 μg Chloramine T in 4 μl of 0.1M SPB (16 mM NaH₂PO₄, 84 mM Na₂HPO₄, pH 7.5) were added. The reaction was carried on for 10 min. Then, 50 μl of 0.1M SPB, 100 μl EtOAc and 0.45 μg of Na₂S₂O₅ in 3 μl 0.1M SPB were added to terminate the reaction. The EtOAc phase containing (8S)-HxA₃Me-[¹²⁵I]-AZI was transferred to a siliconized glass tube. The EtOAc phase was then washed with 50 μl DDH₂O.

2.4.3 Purification of (8S)-HxA₃Me-[¹²⁵I]-AZI

The total extract containing (8S)-HxA₃Me-[¹²⁵I]-AZI in EtOAc was spotted onto a TLC plate (Silica Gel 60, Merck, Darmstadt, Germany) and developed using a solvent system of hexane/EtOAc 1/1 (v/v) for 40 min. The TLC plate was exposed to an X-ray film (Hyperfilm-MP, Amersham Pharmacia Biotech, Baie d’Urfé, Quebec) for 5 min. The band representing (8S)-HxA₃Me-[¹²⁵I]-AZI was cut out and extracted with 1 ml of
EtOAc three times; the EtOAc phase was washed with 1 ml of DDH2O. The final product was stored at -20°C until use.

2.5 AUTORADIOGRAPHY OF BRAIN SECTIONS

2.5.1 Materials
Kodak D-19 developer, indicator stopping bath and rapid fixer were purchased from a local photography store. (8S)-HxA3Me-[125I]-AZI was prepared as mentioned. Other chemicals used have been listed elsewhere.

2.5.2 Preparation of brain sections
Rat brains from male Wistar rats (180-250g) were dissected and quickly frozen over liquid nitrogen with OCT compound in a cryomold (Sigma, St. Louis, MO) and stored at -70°C. Brain sections of 20 µm thick were cut (Hospital for Sick Children, Pathology Department) in a temperature-controlled cryostat (Leitz, Model 1720, Germany) at -20°C. Sections were mounted on poly-L-lysine slides (Sigma Chemical Co., St. Louis), placed in a dessicator and dried under vacuum at -20°C overnight. The sections were then stored at -70°C until use.

2.5.3 Incubation with radioactive HxA3
Brain sections were thawed at room temperature for about 30 min and then preincubated in Buffer A (pH 7.4) for 1 hr. Four different strategies have been developed for incubation (see Table II for sequential approaches used). First, the immersion method was attempted (Fig. 5A). 4,000,000 cpm of (8S)-HxA3Me-[125I]-AZI (final concentration: 56 pM) in the presence or absence of 40 µg of (8R)-HxA3Me (final concentration: 5.6 µM), or 3,000,000 cpm of [3H]-(8R)-HxA3Me (final concentration: 1.3 nM) in the presence or absence of 60 µg of (8R)-HxA3Me (final concentration: 14 µM), was dissolved in 20 µl of DMSO and mixed in 12-20 ml of the Buffer A in a siliconized glass container. The brain sections were then immersed into the incubation mixture. The second approach was the Drop Method I (Fig. 5B). A circle surrounding
Table II: Approaches used for incubating frozen rat brain sections in autoradiography study

<table>
<thead>
<tr>
<th>Approach</th>
<th>Method Used</th>
<th>Ligand Used</th>
<th>Procedures</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Immersion</td>
<td>(8S)-HxA₃Me-[^125]I-AZI</td>
<td>Brain sections were immersed into Buffer A containing 56 pM of (8S)-HxA₃Me-[^125]I-AZI ± 5.6 μM of unlabeled (8R)-HxA₃Me</td>
</tr>
<tr>
<td>2</td>
<td>Immersion</td>
<td>[³H]-[(8R)-HxA₃Me</td>
<td>Brain sections were immersed into Buffer A containing 1.3 nM of [³H]-[(8R)-HxA₃Me ± 14 μM of unlabeled (8R)-HxA₃Me</td>
</tr>
<tr>
<td>3</td>
<td>Drop Method I</td>
<td>[³H]-[(8R)-HxA₃Me</td>
<td>1.3 nM of [³H]-[(8R)-HxA₃Me ± 14 μM (8R)-HxA₃Me in 1 μl EtOH was added into a drop of Buffer A (200 μl) covering the brain sections</td>
</tr>
<tr>
<td>4</td>
<td>Drop Method II</td>
<td>[³H]-[(8R)-HxA₃Me</td>
<td>1.3 nM of [³H]-[(8R)-HxA₃Me ± 14 μM (8R)-HxA₃Me in 200 μl Buffer A was added in the form of a drop onto the brain sections</td>
</tr>
<tr>
<td>5</td>
<td>Immersion</td>
<td>[³H]-[(8R)-HxA₃FA</td>
<td>Brain sections were immersed into Buffer A containing 1.3 nM of [³H]-[(8R)-HxA₃FA ± 14 μM of unlabeled (8R)-HxA₃Me</td>
</tr>
<tr>
<td>6</td>
<td>Drop Method I</td>
<td>[³H]-[(8R)-HxA₃FA</td>
<td>1.3 nM of [³H]-[(8R)-HxA₃FA ± 14 μM (8R)-HxA₃Me in 1 μl EtOH was added into a drop of Buffer A (200 μl) covering the brain sections</td>
</tr>
</tbody>
</table>
Preincubation

Brain sections on poly-L-lysine coated slides

Buffer A containing the radioligand

Incubate at 37°C for 30 min.

Fig. 5A Immersion method used for incubating frozen brain sections.
Radioligand was first dissolved in DMSO or EtOH and then in Buffer A. After preincubation, brain sections were then immersed into the solution and incubated at 37°C for 30 min.
After preincubation, a drop of Buffer A (200 µl) was added onto the brain sections surrounded by a pap pen-circle and then the radioligand dissolved in EtOH was added into the drop and mixed with a pipet.
the tissue section was drawn with the pap pen (Sigma, St. Louis, MO) to retain the drop of solution from spreading out. 200 µl of Buffer A was added to each tissue section in the form of a drop and 40,000 cpm of (8S)-HxA3Me-[125I]-AzI (final concentration: 56 pM) or 50,000 cpm of [3H]-(8R)-HxA3Me (final concentration: 1.3 nM) in the presence or absence of 1 µg of (8R)-HxA3Me (final concentration: 14 µM) in 1 µl of ethanol was then added into the drop. The third strategy using the immersion method was to dissolve $3 \times 10^6$ cpm of [3H]-(8R)-HxA3FA (final concentration: 1.3 nM) in the presence or absence of 60 µg of (8R)-HxA3FA (final concentration: 14 µM) in 100 µl of EtOH and then added to 12 ml of Buffer A at pH 7.4 and then the brain sections were immersed into the solution. The fourth approach utilized the Drop Method II (Fig. 5C) using 200 µl of Buffer A containing 50,000 cpm (final concentration: 1.3 nM) of [3H]-(8R)-HxA3FA in the presence or absence of 1 µg (final concentration: 14 µM) of (8R)-HxA3FA (dissolved in 2µl of ethanol (EtOH)) onto the tissue section. The drop was retained on the tissue sections using a circle drawn with the pap pen as described. Brain sections were then incubated for 30 min at 37°C. The slides were then immersed into ice-cold Buffer A, washed for 30 sec for four times and then dipped in ice-cold DDH₂O. The slides were dried with cold air at room temperature and exposed to an X-ray film (Hyperfilm-[3H], Amersham Pharmacia Biotech, Baie d'Urfé, Quebec) and stored at 4°C for about 2 weeks. After the exposure, the film was processed in Kodak D19 developer for 5 min at 4°C, then in a stopping bath for 1 min and fixed for 7 min.

2.5.4 Computer-assisted image-processing system

Computerized image analyses of the autoradiographic data were performed using the MCID System (Imaging Research, St. Catherine's, ON), carried out at the Clarke Institute of Psychiatry, courtesy of Dr. J. N. Nobrega.
Preincubation

Buffer A containing radioligand

Brain sections surrounded by pap pen-circle

Slide with brain sections

Incubate at 37°C for 30 min.

Fig. 5C Drop method II used for incubating frozen brain sections.
After preincubation, radioligand which was first dissolved in EtOH and then in Buffer A was added as a premixed drop onto the brain sections surrounded by pap pen-circle.
2.6 CHARACTERIZATION OF HxA<sub>3</sub>-BINDING ACTIVITY USING ELECTROPHORESIS

2.6.1 Materials

Trichloroacetic acid (TCA), Coomassie Blue, bromophenol blue, glycerol, SDS, DTT, Triton X-100 and ribonuclease were obtained from Sigma Chemical Co. (St. Louis, MO). Tris-HCl (electrophoresis grade) and reagents used for electrophoresis were purchased from Bio-Rad Laboratories (Hercules, CA). Acetic acid was obtained from Caledon (Georgetown, ON). Other chemicals have been listed elsewhere.

2.6.2 Brain tissue preparation

Homogenized rat hippocampus and thalamus were prepared and protein concentration was determined as described. The tissues were stored at -70°C until photolabeling.

2.6.3 Photolabeling of homogenized hippocampus and thalamus

Homogenates were thawed and 125 μg of protein in 1 ml of Buffer A (pH 7.4) with 1 mM EGTA, 0.3 μM aprotinin, 1.0 μM leupeptin, 1.0 μM pepstatin A and 1 μM PMSF, was incubated with 110 pM (8S)-HxA<sub>3</sub>Me-[<sup>125</sup>I]-AZI (400,000 cpm) in the presence/absence of excess of 5 μg of unlabeled HxA<sub>3</sub>Me (final concentration 14 μM) dissolved in 2 μl DMSO for 30 min at 37°C. The mixture was then exposed to an UV lamp for 5 sec for crosslinking (Yip 1988), courtesy of Dr. C.C. Yip, Banting Institute. The source of the UV lamp is 32 cm from the top of samples and the efficiency of photoincorporation is about 40%. Then the mixture was subjected to SDS-PAGE or differential centrifugation at 10,000 g for 15 min at 4°C and 100,000 g for 60 min at 4°C (Beckman Optima TL Ultracentrifuge) prior to SDS-PAGE.

2.6.4 SDS-PAGE

Proteins of the photolabeled crude homogenates, or those of the 100,000 g supernatant were precipitated with 6% TCA for 15 min. Then protein pellets from each fraction were resuspended in 1X Laemmli sample buffer containing 62.5 mM Tris-HCl
(pH 6.8), 2% SDS, 10% glycerol, 0.025% bromophenol blue, 15 mM DTT (Laemmli, 1970). Samples were then heated at 100°C for 5 min and electrophoresed on 10-20% polyacrylamide gel in a vertical slab gel unit (Model SE 400, Hoefer Scientific Instruments, San Francisco, CA) at 150V for 1 hr and then 65V overnight. Then the gel was stained with Coomassie blue (Coomassie blue 0.25%, MeOH 50%, acetic acid 10%) for 30 min, destained (MeOH 20%, acetic acid 10%) for about 3 hr. The gel was then washed with DDH2O three times for 15 min, soaked in the drying solution (Novex Experimental Technology, San Diego, CA) for 20 min, dried on cellophane (Novex Experimental Technology, San Diego, CA) overnight and then exposed to an X-ray film (Hyperfilm-MP, Amersham Pharmacia Biotech, Baie d’Urfé, Quebec) for about 3 days at -70°C.
SECTION THREE: RESULTS

3.1 BINDING OF $[^3H]-(8R)$-HxA$_3$Me IN HOMOGENIZED RAT BRAIN TISSUE

In order to get a quick idea of localization of hepxilin binding in the brain, various structures of rat brain were dissected and homogenized. Specific binding was determined from the difference between total binding (in the presence of $[^3H]-(8R)$-HxA$_3$Me) and nonspecific binding, represented by the binding in the presence of $[^3H]-(8R)$-HxA$_3$Me and excess of unlabeled (8R)-HxA$_3$Me. Fig. 6 shows the specific binding of $[^3H]-(8R)$-HxA$_3$Me in different areas of rat brain with a protein concentration of 90 µg/ml. The specific binding of $[^3H]-(8R)$-HxA$_3$Me was most abundant in the hippocampus and thalamus (111.3 ± 26.7 and 295.3 ± 15.9 fmol/mg protein, or 39.2 ± 9.4 % and 46.3 ± 2.5 % of total binding, respectively). Specific binding of 44.8 ± 19.5 fmol/mg protein (15.6 ± 6.8 % of total binding) was displayed in the forebrain from which the hippocampus and thalamus were removed. Specific binding of less than 41.3 fmol/mg protein (<14 % total binding) was found in the frontal cortex, rostral cortex, cerebral cortex and the cerebellum. The specific binding of $[^3H]-(8R)$-HxA$_3$Me in the hippocampus and thalamus is significantly different from other regions tested in the brain (Mann-Whitney test with p < 0.05).

3.2 BINDING STUDIES IN HOMOGENIZED HIPPOCAMPUS AND THALAMUS

In order to characterize the HxA$_3$ binding activity, binding studies were performed in crude homogenized mixture of the hippocampus and thalamus, the two structures with the most abundant specific binding. Specific binding in the crude homogenates increased with protein concentration in the incubation mixture and plateaued at about 200 µg of protein/ml (Fig. 7). Protein concentration of 125 µg/ml was chosen for further experiments.

Fig. 8 shows the effect of incubation temperature on the specific binding of $[^3H]-(8R)$-HxA$_3$Me with 125 µg/ml of protein. Specific binding, which is expressed as
Specific binding of $[3^H]-(8R)$-HxA$_3$Me in freshly homogenized rat brain structures.

Homogenized fresh rat brain structures were prepared as described. 90 μg of homogenates was incubated with 50,000 cpm (269 pM) of $[3^H]-(8R)$-HxA$_3$Me in the presence or absence of 1 μg (2.8 μM) of unlabeled (8R)-HxA$_3$Me in 1 ml of buffer for 30 min at 37°C. Radiolabeled tissue was separated from the free ligand by filtration. Total binding in fmol/mg protein for different regions was: frontal cortex, 265.1 ± 12.2; rostral cortex, 216.4 ± 26.1; cerebral cortex, 295.4 ± 90.9; cerebellum, 252.5 ± 11.0; forebrain, 287.0 ± 26.9; hippocampus, 284.0 ± 28.4; thalamus, 637.9 ± 23.2. Specific binding was calculated by subtracting the nonspecific binding (in the presence of excess (8R)-HxA$_3$Me) from the total binding (in the absence of excess (8R)-HxA$_3$Me). Data shown is specific binding for each region represented as % of total binding. Data represent the mean ± S.E.M. of 3 separate experiments in triplicate.
Fig. 7  Relationship of protein concentration of homogenized fresh rat hippocampus and thalamus to specific binding of $[^3\text{H}]$-(8R)-HxA$_3$Me.

Binding was carried out with fresh homogenates of rat hippocampus and thalamus at the protein concentration indicated during incubation with 50,000 cpm (269 pM) of $[^3\text{H}]$-(8R)-HxA$_3$Me in the presence or absence of 1 µg (2.8 µM) of unlabeled (8R)-HxA$_3$Me in 1 ml of buffer for 30 min at 37°C. Future experiments were carried out in the presence of 125 µg protein/ml. 1000 cpm of specific binding of $[^3\text{H}]$-(8R)-HxA$_3$Me equals to 5.4 fmol. Data represent the mean ± S.D. of triplicate.
Fig. 8 Effect of temperature on the binding of $[^3\text{H}]$-(8R)-HxA$_3$Me to fresh rat hippocampus and thalamus homogenates.

Homogenized fresh rat hippocampus and thalamus (125 µg of protein) were incubated with 50,000 cpm (269 pM) of $[^3\text{H}]$-(8R)-HxA$_3$Me in 1 ml of buffer for 30 min at the temperature indicated. Specific binding was obtained by subtracting the nonspecific binding (in the presence of 1 µg (2.8 µM) of unlabeled (8R)-HxA$_3$Me) from total binding (in the absence of unlabeled (8R)-HxA$_3$Me). Specific binding was then expressed as percentage of total binding. Total binding at 4 °C, 23°C and 37°C was 565.3 ± 44.2, 361.8 ± 10.9, and 441.1 ± 12.2 fmol/mg protein. Values represent the mean ± S.D. of triplicate.
percentage of total binding, was lowest at 23°C. A similar amount of specific binding was observed with incubation temperatures of 4°C and 37°C.

Fig. 9 shows the time course of the specific binding of [³H]-(8R)-HxA₃Me with a protein concentration of 125 µg/ml at 37°C. Specific binding increased with time and appeared to level off at 30 min. Binding of the radioligand then remained stable for at least 90 min. The binding was reversible and largely displaceable when 1 µg of unlabeled (8R)-HxA₃Me (2.8 µM) was added. Under these binding conditions, specific binding reached about 50% of total binding. The specific binding of [³H]-(8R)-HxA₃Me in crude homogenates of the thalamus and hippocampus was saturable with radioligand concentrations within the range of 7-286 nM (Fig. 10).

Binding parameters were determined from three separate saturation isotherms (performed in triplicate) and subsequent Scatchard analysis of specific binding. Due to the limited availability of [³H]-(8R)-HxA₃Me, we could not evaluate saturation by utilizing progressively increasing concentrations of the radiolabeled of HxA₃. Instead, we examined the reduction of [³H]-(8R)-HxA₃Me binding with increasing concentrations of unlabeled HxA₃. Fig. 10 shows a representative saturation isotherm and its Scatchard plot from one of the three experiments. The equilibrium dissociation constant (Kₐ), represented by the slope, and the number of binding sites at the saturation (Bₘₐₓ), represented by the x-intercept, were determined from the Scatchard analysis of the data from three separate experiments performed in triplicate determined by the LIGAND program. One population of binding sites with a Kₐ of 43.1 ± 6.7 (± S.E.M.) nM and a Bₘₐₓ of 42.3 ± 7.3 (± S.E.M.) pmol/mg of protein was found. The Hill coefficient was not significantly different from 1.0, indicating that only one population of binding sites is present and the binding of one molecule of the ligand will not affect the binding of the next molecule of the ligand.

To evaluate the specificity of [³H]-(8R)-HxA₃ Me binding, an experiment was performed in the absence or presence of various related unlabeled compounds ((8R)-HxA₃Me, (8S)-HxA₃Me, (10R)-HxB₃Me, (10S)-HxB₃Me, TrXA₃, PGE₂, PGF₁₂, PGD₂, TxB₂, 6-keto-PGF₁₂, 11,12-EET, 12(S/R)-HETE, (8R)-HETE, (8S)-HETE and LTB₄). Since previous experiments have shown that 500 ng of unlabeled (8R)-HxA₃Me (1.4 µM)
Fig. 9 Time course of $[^3H]-(8R)$-HxA$_3$Me binding to fresh homogenates of rat hippocampus and thalamus and its dissociation with unlabeled (8R)-HxA$_3$Me

This figure shows the time course of total binding (○) to homogenates of rat hippocampus and thalamus (125 μg protein) incubated with 50,000 cpm (269 pM) of $[^3H]-(8R)$-HxA$_3$Me in 1ml of buffer at 37°C, and its dissociation (●) after 60 min with 1 μg of unlabeled (8R)-HxA$_3$Me (2.8 μM) added in 1 μl of DMSO. Nonspecific binding (□) is shown in the lower curve in which 1 μg (2.8 μM) of unlabeled compound was mixed before incubation with the labeled compound. Incubation time was selected to be 30 min. for future experiments. 1000 cpm of bound radioactivity equals to 43 fmol/mg protein. Data represent the mean ± S.D. of triplicate. The experiments were replicated twice with similar results.
Fig. 10 Saturation isotherm from a typical experiment showing specific binding of $[^3H]-(8R)-HxA_3Me$ to fresh rat hippocampus and thalamus homogenates.

Homogenates of rat hippocampus and thalamus (125 µg protein) were incubated with 50,000 cpm (269 pM) of $[^3H]-(8R)-HxA_3Me$ with various concentrations of unlabeled (8R)-HxA$_3$Me (0 – 286 nM) in 1 ml of buffer for 30 min at 37 °C. Inset: Scatchard plot of the specific binding is illustrated with the best fit using EBDA/LIGAND computer program. The following constants can be derived from this plot: $K_d$ (slope) = 38 nM; $B_{max}$ (x-intercept) = 35.7 pmol/mg protein. Data averaged from the three separate experiments (each performed in triplicates) showed the following parameters: $K_d = 43.1 \pm 6.7$ (± S.E.M.) nM and a $B_{max}$ of 42.3 ± 7.3 (± S.E.M.) pmol/mg protein.
was almost as effective as 1 µg (2.8 μM) in competing with radioactive HxA₃, we decided to test all the unlabeled ligands at 500 ng/ml (1.4 - 1.6 μM). All drugs were added in 2 µl of DMSO at the same time as [³H]-(8R)-HxA₃ Me. As shown in Fig. 11, maximum competition against [³H]-(8R)-HxA₃ Me binding was observed with unlabeled (8R)-HxA₃ Me, although the (8S) diastereomer also competed with the labeled ligand almost to the same extent. Also, significant amount of competition was observed with (10R)-HxB₃ Me and (10S)-HxB₃ Me (89% and 97% of the specific binding observed with 0.5 µg of (8R)-HxA₃ Me (1.4 μM), respectively). 11,12-EET competed for the binding of the radioligand to a lesser extent (68% of the specific binding with 0.5 µg of (8R)-HxA₃ Me (1.4 μM)). A variety of PGs, HETEs, TxB₂, LTB₄ and TrXA₃ failed to compete for the binding of [³H]-(8R)-HxA₃ Me.

3.3 METABOLISM OF HxA₃ IN RAT BRAIN

In order to analyze whether metabolism of the radioligand took place during the binding experiments, the method of thin layer chromatography (TLC) was used. In freshly prepared homogenates of hippocampus and thalamus, the methyl ester of [³H]-(8R)-HxA₃ was converted within 30 min into the free acid form [³H]-(8R)-HxA₃ FA (75%) and partly into TrXA₃ FA (16%). Next, the metabolism of [³H]-(8R)-HxA₃ FA was determined in freshly prepared intact tissue sections. A TLC scan of the ethyl acetate extract of the incubation mixture is shown in Fig. 12. 77% of the total radioactivity remained as unmetabolized [³H]-(8R)-HxA₃ FA and 16% appeared as TrXA₃ FA. These experiments indicate that HxA₃ is essentially mostly available for binding to the intact tissue section or homogenates and that the results of the binding experiments are mostly due to intact HxA₃. As shown in Fig. 11, the metabolite, TrXA₃, in fact does not compete for HxA₃ binding.
Fig. 11 Specificity of binding of $[^3\text{H}]-$(8R)-HxA$_3$Me to homogenized fresh rat hippocampus and thalamus.

Homogenates of rat hippocampus and thalamus (125 µg protein) were incubated in 1ml of buffer for 30 min at 37°C with 50,000 cpm of $[^3\text{H}]-$$(8R)$-HxA$_3$Me (269 pM) in the absence (total binding) and presence of 1 µg of unlabeled (8R)-HxA$_3$Me (2.8 µM) (nonspecific binding) or in the presence of the indicated compounds (500 ng, concentration ranges from 1.4-1.6 µM). Data are expressed as percentage of the specific binding of $[^3\text{H}]-$$(8R)$-HxA$_3$Me observed by competition of the radioligand with the addition of 1 µg (2.8 µM) of (8R)-HxA$_3$Me. Values represent the mean ± S.D. of triplicates. The experiments were replicated twice with similar results.
Fig. 12 Metabolism of HxA₃ in fresh rat brain.
Thin layer radiochromatograms of: (A) standard of (8R)-HxA₃FA (100,000 cpm); (B) ethyl acetate extract (100,000 cpm) after incubation of [³H]-(8R)-HxA₃FA (500,000 cpm) with intact brain sections for 30 min at 37°C in 2 ml of Buffer A; (C) ethyl acetate extract (100,000 cpm) after incubation of [³H]-(8R)-HxA₃Me (200,000 cpm) with a homogenate of the hippocampus and thalamus for 30 min at 37°C in 4 ml of Buffer A; (D) standard of [³H]-(8R)-HxA₃Me (100,000 cpm).
3.4 PREPARATION OF (8S)-HxA₃Me-[¹²⁵I]-AZI

Fig. 13A shows an autoradiograph of the TLC plate after radio-iodination. (8S)-HxA₃Me-[¹²⁵I]-AZI, represented by P in the figure, is contaminated with other impurities in the crude reaction mixture (left panel). After purification of the ligand by preparative TLC i.e. scraping out the corresponding radioactive zone and extraction with EtOAc, the purity of the ligand reached >95% (Fig. 13B) and the yield was about 20%.

3.5 AUTORADIOGRAPHY OF BRAIN SECTIONS

Fig. 14 shows autoradiographs of the sagittal (A) and coronal (B) sections of the rat brain incubated with (8S)-HxA₃Me-[¹²⁵I]-AZI in the presence or absence of excess of unlabeled HxA₃Me utilizing the immersion method. Apparent HxA₃ specific binding activity was found in the thalamus in the first two experiments. However, the consistent trend of subsequent experiments (n>3) was that more binding of (8S)-HxA₃Me-[¹²⁵I]-AZI was obtained in the sections representing nonspecific binding (results not shown). This surprising finding warranted further study. Solutions in which (8S)-HxA₃Me-[¹²⁵I]-AZI was added in the presence of unlabeled HxA₃Me contained twice the radioactivity of solution in which only (8S)-HxA₃Me-[¹²⁵I]-AZI was added even though the same amount of (8S)-HxA₃Me-[¹²⁵I]-AZI was used in both cases. It was also observed that the resolution of the brain structures obtained from this radioligand was relatively low.

We therefore reverted to the use of the tritiated ligand, [¹H]-(8R)-HxA₃Me. We mixed [¹H]-(8R)-HxA₃Me with or without unlabeled (8R)-HxA₃Me and investigated its binding using the immersion method. Again, more binding of [¹H]-(8R)-HxA₃Me in the brain sections representing nonspecific binding (i.e. with unlabeled HxA₃ added) was observed (Fig. 15). The coronal section shown is an example from 18 coronal sections taken from different levels of the brain. This observation was accompanied by the unequal radioactivity in the incubation solution as mentioned above. Since these experiments point to a lack of solubility of the ligand, we decided to attempt to overcome this problem by investigating binding with a drop method in which the labeled substrate was added in 2 µl EtOH on top of the tissue. The result obtained from the Drop Method I
Fig. 13 Autoradiography of TLC plates after radio-iodination of the HxA₃ analog and after purification.

After radio-iodination of HxA₃-AZT (A); after purification of (8S)-HxA₃Me-[¹²⁵I]-AZI (B). The reaction mixture was spotted onto a TLC plate and developed in a solvent system of hexane/EtOAc 1/1 (v/v) for 40 min. The TLC plate was then exposed to an X-ray film for 5 min and the film was developed. S represents the origin where sample was spotted; P represents the product (8S)-HxA₃Me-[¹²⁵I]-AZI; and F represents the solvent front. Radioactive purity of the [¹²⁵I] product (P) was > 95%.
Fig. 14A  Binding of (8S)-HxA₃Me-[¹²⁵I]-AZI in brain sagittal frozen sections using the immersion method

The upper picture represents the total binding upon incubation with 4,000,000 cpm of (8S)-HxA₃Me-[¹²⁵I]-AZI in 20 ml of Buffer A (56 pM) using the immersion method and the lower one represents the nonspecific binding upon incubation with 400,000 cpm of (8S)-HxA₃Me-[¹²⁵I]-AZI (56 pM) and 40 µg of unlabeled HxA₃Me (5.6 µM) in 20 ml of Buffer A. T, thalamus.
Fig. 14B  Binding of (8S)-HxA₃,Me-[¹²⁵I]-AZI in brain frozen coronal sections using the immersion method

The upper picture represents the total binding upon incubation with 4,000,000 cpm of (8S)-HxA₃,Me-[¹²⁵I]-AZI in 20 ml of Buffer A (56 pM) using the immersion method and the lower one represents the nonspecific binding upon incubation with 400,000 cpm of (8S)-HxA₃,Me-[¹²⁵I]-AZI (56 pM) and 40 μg of unlabeled HxA₃,Me (5.6 μM) in 20 ml of Buffer A. T, thalamus.
Fig. 15 Binding of [3H]-(8R)-HxA₃Me in brain frozen coronal sections using the immersion method.

The upper picture represents the total binding upon incubation with 3,000,000 cpm of [3H]-(8R)-HxA₃Me in 12 ml of Buffer A (1.3 nM) using the immersion method. The lower picture represents the nonspecific binding upon incubation with 3,000,000 cpm of [3H]-(8R)-HxA₃Me (1.3 nM) and 60 µg of unlabeled HxA₃Me (14 µM) in 12 ml of Buffer A.
is shown in Fig. 16. Although labeling was more discrete and clear, significant uneven labeling of all the brain sections was observed with asymmetrical labeling between the left and the right sides of the brain. This must be an artifact due to labeling but unlikely to any differences in the binding activity between the left and the right sides of the brain. Brain sections labeled by Drop Method II were not helpful either (results not shown) since unequal radioactivities were also obtained, even though the incubation solutions were sonicated, warmed and constantly vortexed before the addition of the labeled solution onto the brain sections.

A last attempt involved dissolving the free carboxylic acid, [3H]-HxA3,FA as a salt, in the presence or absence of unlabeled (8R)-HxA3,FA in the buffer. Brain sections were incubated in the solution either by the immersion method or Drop Method II. Although the uneven amount of soluble radioactivity was corrected in solutions in the presence and absence of unlabeled hepxilin, as shown in Fig. 17, a stronger signal was still obtained from the brain sections representing nonspecific binding of the ligand with the immersion method. Similar results were achieved using Drop Method II (results not shown). Preincubation of the brain sections with 5 μg/ml of (8R)-HxA3,Me (14 μM) before incubation with [3H]-HxA3,Me in the presence or absence of (8R)-HxA3,Me gave similar results (not shown). These experiments were therefore inconclusive.

3.6 CHARACTERIZATION OF HxA3-BINDING ACTIVITY USING ELECTROPHORESIS

Coomassie blue staining and autoradiography of SDS-PAGE after photolabeling with (8S)-HxA3,Me-[125I]-AZI in the frozen homogenates of the hippocampus and thalamus are shown in Fig. 18. The Coomassie staining of the SDS-PAGE showed that equal amount of proteins were loaded in the pair of lanes representing the total and nonspecific binding. Autoradiography of SDS-PAGE of the crosslinking experiment with crude homogenates revealed higher binding of (8S)-HxA3,Me-[125I]-AZI in all protein bands in the lanes presenting nonspecific binding (in the presence of excess unlabeled HxA3,Me). Hence none of the protein bands demonstrated specific binding of (8S)-HxA3,Me-[125I]-AZI
**Fig. 16** Binding of $[^3H]-(8R)$-HxA$_3$Me in brain frozen coronal sections using the Drop Method I.

The upper picture represents the total binding upon incubation with 50,000 cpm of $[^3H]-(8R)$-HxA$_3$Me in 200 µl of Buffer A (1.3 nM) using Drop Method I. The lower picture represents the nonspecific binding upon incubation with 50,000 cpm of $[^3H]-(8R)$-HxA$_3$Me (1.3 nM) and 1 µg of unlabeled HxA$_3$Me (14 µM) in 200 µl of Buffer A.
Fig. 17 Binding of \([^3H]-(8R)-\text{HxA}_3\text{FA}\) in brain frozen coronal sections using the immersion method.

The upper picture represents the total binding upon incubation with 3,000,000 cpm of \([^3H]-(8R)-\text{HxA}_3\text{FA}\) in 12 ml of Buffer A (1.3 nM) using the immersion method. The lower picture represents the nonspecific binding upon incubation with 3,000,000 cpm of \([^3H]-(8R)-\text{HxA}_3\text{FA}\) (1.3 nM) and 60 μg of unlabeled HxA3FA (14 μM) in 12 ml of Buffer A.
Fig. 18 Photolabeling of frozen homogenates of rat hippocampus and thalamus with (8S)-HxA₃Me-[¹²⁵I]-AZI

Frozen homogenates of rat hippocampus and thalamus (125 µg of protein/ml) were thawed, then incubated with 400,000 cpm of (8S)-HxA₃Me-[¹²⁵I]-AZI (110 µM) ± 5 µg of unlabeled HxA₃Me (14 µM) for 30 min at 37°C. After 5 sec of UV crosslinking, samples were analyzed on a 10-20% gradient gel. Left panel represents the Coomassie blue staining of the gel; right panel represents the autoradiograph of the gel.
which could be competed by unlabeled HxA₂Me. Similar results were obtained with fractionations of frozen homogenates (10,000g, 100,000g and soluble proteins) after photolabeling (not shown).

Incubating (8S)-HxA₂Me-[^¹²⁵I]-AZI in the presence or absence of HxA₂Me with 0.05% Triton X-100 and/or 0.1% ribonulease did not help in lowering the amount of nonspecific binding in the experiment (results not shown).
SECTION FOUR: DISCUSSION

4.1 [3H]-(8R)-HxA₃Me BINDING IN HOMOGENIZED RAT BRAIN TISSUE

This part of the study revealed that hepxilin-specific binding is present in the central nervous system. The initial binding studies provided us with an approximate localization of hepxilin binding in the brain. 50,000 cpm (269 pM) of [3H]-(8R)-HxA₃Me was chosen as the concentration used to perform the study because of cost and practicality. HxA₃ binds specifically and selectively to the hippocampus and thalamus, but not to the cerebral cortex or the cerebellum. As a result, further studies investigating the nature of the HxA₃ specific binding were carried out in the hippocampus and thalamus. Pooling the tissues of the most active regions, hippocampus and thalamus, in the brain in further studies can increase the availability of proteins and the amount of binding activity.

4.2 BINDING OF [3H]-(8R)-HxA₃Me IN HOMOGENIZED HIPPOCAMPUS AND THALAMUS

In order to optimize the binding conditions, homogenates of the hippocampus and thalamus were incubated with HxA₃ at different temperatures. Incubation of the homogenized hippocampus and thalamus at different temperatures revealed that the binding was temperature-independent. Although the extent of specific binding at 4°C was the same as that at 37°C, an incubation temperature of 37°C was chosen to enhance the solubility and prevent precipitation, if any, of this lipidic ligand due to lower temperature.

The time course showed that specific binding in homogenates of the hippocampus and thalamus is time-dependent. Addition of unlabeled (8R)-HxA₃Me when binding had reached a plateau reversed the binding of [3H]-(8R)-HxA₃Me. Reversible binding of HxA₃ suggests a possible involvement of a receptor protein with HxA₃ binding activity. Subsequent saturation isotherms proved that the binding is dose-dependent and saturable. Scatchard analysis revealed a single population of binding sites with a Kₐ of 43.1 ± 6.7
nM and a $B_{\text{max}}$ of 42.3 ± 7.3 pmol/mg of protein. Both of these values are somewhat different than those previously reported in human neutrophils ($K_d$ 79.3±9.1 nM and $B_{\text{max}}$ of 14.7 ± 2.4 pmol/mg of protein) (Reynaud et al., 1996). This implies that the binding sites has a higher affinity and is more abundant in the CNS than in human neutrophils.

Based on the above observations, we can conclude that HxA$_3$-specific binding activity is present in the hippocampus and thalamus as the criteria for specific binding activity have been fulfilled:

1. Reversibility: binding of HxA$_3$ was reversed by the addition of unlabeled HxA$_3$ (Fig. 9);
2. Saturability: HxA$_3$ binding is saturable by adding increasing amount of ligand (Fig. 10);
3. Dose-dependence: level of HxA$_3$ binding increased with the dose of ligand added and saturated at about 0.1 μM (Fig. 10);
4. Time-dependence: HxA$_3$ binding activity increased with time and leveled off at 30 min (Fig. 9).

Interestingly, HxA$_3$ binding parameters in the study are similar to those reported for the intracellular receptor of the second messenger, inositol 1,4,5-triphosphate (InsP$_3$), in rat brain, i.e. $K_d$ 40 nM and $B_{\text{max}}$ 20 pmol/mg of protein (Worley et al., 1987). Recent studies by Nigam et al. (1990) demonstrated that hepxilins activate phospholipase D and not phospholipase C, since InsP$_3$ release is not increased. Whether HxA$_3$ binds to the InsP$_3$ receptors or a similar protein is not known at this time but appears important for future study.

In the competition experiments, both epimers, 8R and 8S, of HxA$_3$ appear to be equally active in competing with the binding of $[^3\text{H}]$-(8R)-HxA$_3$Me. The non-stereoselectivity of the hydroxyl group at carbon 8 suggests that the hydroxyl group is not a functional group needed for specific HxA$_3$ binding. Although the exact spatial requirement of the functional group required for binding is not known yet, the binding of $[^3\text{H}]$-(8R)-HxA$_3$Me in homogenates of the hippocampus and thalamus appears to be specific to the epoxide moiety at C-11 and C-12 position, as evidenced by the ability of both epimers of HxA$_3$, HxB$_3$ and to a lesser extent 11,12-EET, to compete for HxA$_3$, 
binding. Other related compounds, which are C-20 compounds with various degrees of unsaturation derived from similar pathways, such as the PGs, HETEs, LTB₄, TxB₂ and the hepoxilin metabolite TrxA₂, which lack the epoxide moiety, all failed to compete with HxA₃ binding (see Fig. 19 for the structures of these compounds). The specificity of HxA₃ binding in rat brain appears to be similar to that previously observed in human neutrophils (Reynaud et al., 1996). The binding activity in human neutrophils is considerably HxA₃-specific. However, HxB₁ and 11,12-EET were not included in the competition studies in human neutrophils. Unpublished results in our laboratory have shown that only one particular epimer of 11,12-EET is active in releasing intracellular calcium in human neutrophils in a similar fashion as HxA₃. Future experiments with different epimers will be necessary for the identification of the functional group required for HxA₃ binding. Despite the fact that HxB₁ is effective in competing with the binding of HxA₃, unpublished results in our laboratory have shown that although HxB₁, as opposed to HxA₃ (Dho et al., 1990), is not active in releasing intracellular calcium in human neutrophils, it appears to potently inhibit the calcium releasing actions of HxA₃, confirming that HxB₁ binds to the hepoxilin binding activity.

In order to define the binding activity observed related to a potential receptor, saturability, reversibility, specificity, consistency in time course and dose-dependent functional response elicited/inhibited by the ligand are essential criteria (Hechter, 1978). The HxA₃ binding in the thalamus and hippocampus is dose-dependent, time-dependent, reversible and saturable. All these criteria have been satisfied in our study with the exception of ‘function’. No functional study with this binding activity has been performed yet. Over the past decade, extensive reports regarding the physiological role of HxA₃ in the hippocampus have been published (Carlen et al., 1989; Pace-Asciak et al., 1990a, Pace-Asciak et al., 1990b, Carlen et al., 1994), suggesting that HxA₃ binding may be important to function. In the human neutrophils, HxA₃ binding has been shown to be coupled to calcium release (Reynaud et al., 1995a). The biological action of HxA₃ in the thalamus needs further investigation.

20-carbon unsaturated fatty acids, particularly AA, are the major constituents of the excitable membranes of the CNS (Okuda et al., 1994). As mentioned earlier, the
Fig. 19 Chemical structures of compounds tested as putative competitors of hepoxilin binding in brain.
metabolism of AA leads to the production of a series of biologically active lipids, including prostaglandins, leukotrienes, thromboxanes and hepxilins. Many of these compounds have diverse and potent biological actions in the CNS. For instance, PGD$_2$ acts as a sleep inducer (Hayaishi et al., 1987; Ueno et al., 1983). It modifies pain sensation biphasically (Horiguchi et al., 1986), modifies olfaction (Watanabe et al., 1986), produces hypothermia (Ueno et al., 1982), and inhibits the release of luteinizing hormone-releasing hormone (Kinoshita et al., 1982). On the other hand, PGE$_2$ induces wakefulness (Hayaishi, 1988), modifies pain sensation (Horiguchi et al., 1986; Poddubiuk, 1976), produces hyperthermia (Milton, 1976), stimulates the release of luteinizing hormone-releasing hormone (Ojeda et al., 1977), produces anticonvulsive (Poddubiuk 1976; Wolfe, 1982) and antidiuretic effects (Wolfe and Coceani, 1979) and regulates food-intake (Wolfe and Coceani, 1979). PGF$_{2\alpha}$ also has an antidiuretic effect (Ishikawa et al., 1981) and it inhibits oxytocin release (Prilusky and Deis, 1976). Moreover, these three PGs have been shown to modulate neurotransmitter release (Shimizu and Wolfe, 1990). Eicosanoid receptors, for instance, PGD$_2$ (Malet et al., 1982) and PGE$_2$ (Shimizu et al., 1982) receptors, have been found in the CNS. Therefore, the findings of the present experiments may suggest a hepxilin-specific binding protein in the hippocampus and thalamus through which HxA$_3$ may elicit its actions in these areas. As HxA$_3$ has been proven to have neuromodulatory actions in the hippocampus, it is possible that it may elicit similar actions in the thalamus.

Only one dose of the unlabeled compound/analogs was tested in the competition experiments. It is necessary to compete the binding of [H]-HxA$_3$Me with different doses of the unlabeled ligands so that the IC$_{50}$s can be determined. This would be very informative for future studies in terms of characterization of the binding sites.

One shortcoming of our present data is that the binding studies have only been performed in fresh crude homogenates of brain tissue. Our results cannot conclude in which cell types the HxA$_3$ binding activity is located. Preparation of a single cell-type, or use of specific cell cultures would be an excellent way to investigate whether the binding activity is located in neurons, glia and/or other cell types.
Previous work has shown that HxA₃ binding protein appears to be intracellular in human neutrophils, since only the methyl ester form of HxA₃ binds specifically to intact cells (Reynaud et al., 1995b). It is not unusual to have intracellular eicosanoid receptors as studies of Rao and co-workers have indicated the presence of intracellular PGE and PGF₂α receptors in bovine corpora lutea. Their results showed that PGE and PGF₂α receptors were located not only on the plasma membrane but also the membranes of intracellular organelles such as nuclei (Rao and Mitra, 1979), lysosomes (Mitra and Rao, 1978a), rough endoplasmic reticulum, and cis and trans Golgi elements (Mitra and Rao, 1978b) in bovine corpora lutea. HxA₃ has been shown to act as a second messenger in neurotransmission in *Aplysia* sensory cells (Piomelli et al., 1987; Piomelli et al., 1989; Piomelli and Greengard, 1990) and in mammalian hippocampal CA1 neurons (Carlen et al., 1989; Carlen et al., 1994 Pace-Asciak et al., 1990a; Pace-Asciak et al., 1990b). The concept of lipoxygenase metabolites as intracellular messengers were confirmed later using the patch-clamping method in atrial cells of the guinea pig and rat (Kim and Clapham, 1989; Kim et al., 1989; Kurachi et al., 1989a,b). Arachidonic acid and LTC₄ have been shown to mimic the action of phenylephrine on the cardiac potassium channels and the action can be blocked by nordihydroguaiaretic acid (NDGA, a lipoxygenase blocker) or AA-861 (a specific 5-lipoxygenase inhibitor). Our data suggest that HxA₃ binds to an intracellular binding protein and then functions as an intracellular messenger (CNS modulation, norepinephrine release and changes in intracellular calcium level). This finding contrasts with the classical concept of eicosanoid actions as autocoids.

An obvious extension of our studies relates to the post-HxA₃ binding events e.g. second messenger system, G protein coupling and phosphorylation etc. Receptors for prostaglandins are known to be G protein-coupled receptors. In most cases, binding of the ligand to the receptors stimulates or inhibits adenylate cyclase activity (Takechi et al., 1996). For example, prostacyclin receptor subtype 1 (IP₁) is mainly coupled to Gₛ, which stimulates the production of cAMP with ligand binding (Namba et al., 1994). PGE₂ receptor has been shown to be G protein coupled in rat hypothalamus (Wisner et al., 1989) and in rat brain membrane (Yumoto et al., 1986). Also, peptidoleukotriene (LTC₄, LTD₄, and LTE₄) receptors (Shimizu and Wolfe, 1990; Crooke et al., 1989) and LTB₄
receptors (Goldman and Goetzl, 1984) appear to exhibit their biological activities through G protein-phosphoinositide turnover. Preliminary results from our laboratory have revealed that the hepoxilin binding in human neutrophils appears to be G protein coupled. Incorporation of GTP-γS into intact human neutrophils inhibited the release of intracellular calcium and the binding of tritiated hepoxilin (Pace-Asciak et al., 1999). To investigate whether G proteins are coupled to the binding activity in the hippocampus and thalamus, measurements of cAMP need to be carried out. In human neutrophils, the binding leads to a stimulation of cAMP (unpublished) while HxA₃ leads to a decrease in cAMP production in rat pineal (Reynaud et al., 1994a). Similar studies as well as investigation of the effect of GTP-γS on the binding of [³H]-(8R)-HxA₃,Me in homogenates of the hippocampus and thalamus need to be performed.

The mechanism of actions of HxA₃ in brain is still unclear. Hepoxilins have been shown to activate the release of diacylglycerol (DAG) and AA in human neutrophils (Nigam et al., 1990). Later studies have also shown that HxA₃ can increase intracellular calcium concentration in human neutrophils (Dho et al., 1990). It can be speculated that the actions of HxA₃ in the brain may also be mediated by these pathways.

4.3 METABOLISM OF HxA₃ IN RAT BRAIN

Previous studies have shown that HxA₃ can be metabolized into TrxA₃ in rat lung (Pace-Asciak et al., 1983a; Pace-Asciak et al., 1983b). This part of the study was set out to analyze if HxA₃ is metabolized in rat brain to evaluate the relationship of its structure to the degree of binding. This was carried out in two sets of experiments i.e. with intact coronal sections and with homogenates. After incubating coronal sections of the rat brain with [³H]-(8R)-HxA₃,FA, the majority of the radioactivity remained as unmetabolized [³H]-(8R)-HxA₃,FA. Only a small proportion appeared as the metabolite TrXₐ₃. Similarly, after incubating the homogenates of hippocampus and thalamus with [³H]-(8R)-HxA₃,Me, the majority of the radioactivity was transformed to and stayed as [³H]-(8R)-HxA₃,FA. Hence active esterases hydrolyzed the methyl ester group of HxA₃ to the free acid. The methyl ester derivative of HxA₃ was used in most of the studies because of
its stability and lipophilicity, which enables the compound to enter the cell. Only a small proportion of the radioactivity was metabolized to TrXA₃ in these studies. Although TrXA₃ is present in the incubation mixture, the specific binding observed in the series of experiments reported here cannot be due to TrXA₃ because TrXA₃, as revealed in the competition experiment, does not compete for the binding of [³H]-(8R)-HxA₃Me.

4.4 PREPARATION OF (8S)-HxA₃Me-[¹²⁵I]-AZI

To investigate the protein nature of hepoxilin binding in human neutrophils, our laboratory developed a method of synthesizing a hepoxilin analog containing both azido- and trialkyltin functionalities distant from the active binding functional groups (Fig. 20) (Demin et al., 1997). The azido group serves for the photoaffinity labeling of the binding protein to form a covalent-bound hepoxilin wherein radioactive ¹²⁵I is introduced through the trialkyltin group. As this ligand is labeled with ¹²⁵I, its crosslinking to the binding protein would permit rapid autoradiographic study of the protein in tissue sections as short exposure times are required for its detection by X-ray film. Moreover, this photoaffinity label can be used for subsequent characterization of the binding protein at the molecular level by SDS-PAGE. The use and general approach of creation of such photoaffinity label in the eicosanoid series has been reported (Abramovitz et al., 1993).

Preliminary results in our laboratory have shown that this photoaffinity label antagonizes the actions of HxA₃ in increasing intracellular calcium levels in human neutrophils (not shown). This confirms binding of this label to the Hx binding sites and validates the use of this label in further investigation of the hepoxilin-specific binding activity.

However, binding studies in tissue homogenates using this photoaffinity label were not successful since this ligand bound to the glass filters used for filtration in an inconsistent way. Centrifugation techniques are also not attainable as this ligand binds avidly to plastic tubes.
Fig. 20  Chemical structure of (8S)-HxA₃Me-[¹²⁵I]-AZI
4.5 AUTORADIOGRAPHY OF RAT BRAIN SECTIONS

The method of autoradiography using an *in vitro* labeling technique has been used for decades to localize the receptors of various neurotransmitters and drugs (Quirion *et al.*, 1981; Atweh and Kuhar, 1977; Young and Kuhar, 1980a; Young and Kuhar, 1980b; Palacios *et al.*, 1981a; Palacios *et al.*, 1981b; Palacios *et al.*, 1981c). The use of this technique has the advantage of localizing receptors in the microscopic structures in the brain. Recently, due to the discovery of the actions of eicosanoids in the CNS, several studies have been performed to localize specific eicosanoid binding proteins in the brain using the autoradiography technique. For instance, high specific binding of PGD₂ was observed in the hypothalamus, anterior thalamic nuclei, cerebellar and cerebral cortices and the limbic system (preoptic area, septum, olfactory bulb, hippocampus (dentate gyrus), and amygdala (Yamashita *et al.*, 1983). High PGE₂ binding was found in the preoptic area, hypothalamus, amygdala and subiculum of the hippocampus in the monkey brain (Watanabe *et al.*, 1989). Prostacyclin receptors labeled by [³H]isocarbacyclin were observed in the thalamus, lateral septal nucleus, hippocampus, cerebral cortex, striatum and dorsal cochlear nucleus (Takechi *et al.*, 1996). Unfortunately, the autoradiographic localization of HxA₃ binding protein is not conclusive as carried out in this study due to the following problems.

Different technical difficulties were encountered. Various strategies were attempted to overcome the problems in this work. Labeling of brain sections using (8S)-HxA₃Me-[¹²⁵I]-AZI was first used because of its availability in our laboratory and because this [¹²⁵I]-labeled ligand requires only short exposure times on X-ray film. We employed two methods of labeling intact brain sections i.e. the generally used immersion technique where the thin brain sections on microscope slides are immersed in glass jars containing the labeled compound; and the drop technique where the tissue section is covered in a 'drop' of buffer and the labeled compound is added to this drop. The latter is of use when a limited amount of radioactive ligand is available although both methods suffer greatly in our hands from the lack of or uneven solubility of the lipidic ligand used. We found this to be a serious setback providing inconsistencies in results. In the former method, inconsistencies related to the fact that when unlabeled ligand was added to the label, more
radiolabel was found in solution and hence the nonspecific binding was found to be greater than the specific binding. In the latter method, the addition of the ligand to the tissue section covered with buffer lead to uneven mixing resulting in areas of the tissue receiving more label than others.

Brain sections were immersed in a buffer containing (8S)-HxA₃Me-[¹²⁵I]-AZI in the presence or absence of unlabeled HxA₃Me and incubated under reduced light. Apparent specific binding in the thalamus was found in the first two experiments. However, other experiments showed a stronger signal in the brain sections representing the nonspecific binding of (8S)-HxA₃Me-[¹²⁵I]-AZI. We found that when we dissolved the same amount of the radioligand in the presence or absence of excess of unlabeled ligand in DMSO (final concentration 1%) and then in Buffer A (pH 7.4) in two separate glass siliconized containers in the absence of tissue, double amount of radioactivity was found in the container with (8S)-HxA₃Me-[¹²⁵I]-AZI containing an excess of unlabeled HxA₃Me. The possible explanation of this phenomenon was due to the binding of the ligand to the glassware, even though the glassware was siliconized to prevent binding of the ligand. When excess of unlabeled HxA₃Me was present, it bound to the surface of the glass container and left most of the (8S)-HxA₃Me-[¹²⁵I]-AZI free in the buffer. However, when (8S)-HxA₃Me-[¹²⁵I]-AZI was present alone, most of the ligand bound to the glassware. As a result, the radioactivity detected was a lot lower than expected. Therefore, incubating the brain sections in these solutions gave a stronger signal in the nonspecific binding. Another problem observed with this part of the study was the low resolution of the brain structures when labeled with (8S)-HxA₃Me-[¹²⁵I]-AZI. Therefore, we attempted to use [³H]-(8R)-HxA₃Me to label the brain sections, as tritium-label usually gives better resolution on film.

However, when [³H]-(8R)-HxA₃Me with or without unlabeled (8R)-HxA₃Me was dissolved in DMSO and then in Buffer A, double amount of radioactivity was still found in the solution with excess of (8R)-HxA₃Me present. The explanation for this problem was possibly due to the binding of the ligand to the glassware as mentioned above. Subsequent immersion of the brain sections into the solutions gave a stronger signal for nonspecific binding. Another problem was encountered with this radioligand. Aliquots
taken from both incubation solutions revealed an unstable count of radioactivity. The possible explanation of this problem was the insolubility of the ligand in the buffer. Insolubility of the ligand creates uneven micelles in the buffer and it contributes to the unstable radioactive count in the solution. Two approaches were attempted to solve these two problems. 1) Addition of 0.0001-0.01% of Triton X-100 in an attempt to improve the solubility of the ligand; and/or 2) addition of 0.001-0.1% BSA as a carrier for the ligand. However, both approaches failed to solve the problem of higher binding found in the samples with excess unlabeled HxA₃.

We therefore developed Drop Method I to try to prevent the binding of the ligand to the glassware. In this method, by adding the ligand directly to the tissue on the slides, we can be sure that the expected amount of radioactivity was added. Hence a drop of Buffer A covering the whole brain section was first added and then 1-2 µl of an ethanol solution of [³H]-(8R)-Hx₃Me with or without unlabeled Hx₃Me was added directly over the tissue into the drop of the buffer. The ligand was mixed in the buffer by pipetting the solution up and down several times. The tissue sections were then incubated at 37°C in a moist chamber with gentle shaking. However, we observed uneven labeling of the tissue by the ligand. This may possibly be due to inadequate mixing of the ligand with the buffer or precipitation of the ligand.

Another approach (Drop Method II) was developed in which the ligand was first mixed with the buffer prior to its addition to the tissue sections. Sonication, continuous vortexing and gentle heating (37°C) of the buffer containing the ligand were attempted to stabilize the solubility of the ligand. Nevertheless, unequal and unstable levels of radioactivity of [³H]-(8R)-Hx₃Me in the presence and absence of (8R)-Hx₃Me were still observed.

The final attempt was to use the free acid, [³H]-(8R)-Hx₃FA in salt form, instead of the methyl ester, [³H]-(8R)-Hx₃Me, with the brain sections using the immersion method. [³H]-(8R)-Hx₃FA is more hydrophilic than [³H]-(8R)-Hx₃Me. [³H]-(8R)-Hx₃FA can be easily dissolved in the buffer at neutral pH as it would be present in the ionized form. As seen from the metabolism experiment, [³H]-(8R)-Hx₃Me was transformed to [³H]-(8R)-Hx₃FA when it enters the cell. [³H]-(8R)-Hx₃FA can also
enter the cell when it is dissolved in EtOH but not in DMSO (Reynaud et al., 1999). As a result, the use of \[^3H\]-\((8R)\)-HxA\(\text{Me}\) or \[^3H\]-\((8R)\)-HxA\(\text{FA}\) should not make any difference to the binding itself but it does affect the degree of and stability of its solution in buffer. Solutions containing \[^3H\]-\((8R)\)-HxA\(\text{FA}\) in the presence or absence of \((8R)\)-HxA\(\text{FA}\) revealed the expected level of radioactivity and this was stable over time. Incubation of the brain sections with these ligands using the immersion method or the drop method still revealed a stronger signal in the nonspecific binding. We now can be certain that this observation is not due to problems of solubility or binding of the ligand to the container since the radioactivity of aliquots taken from both solutions were similar and remained so before and after the incubation. Interestingly, for some unknown reason, the resolution of the brain structures obtained from \[^3H\]-\((8R)\)-HxA\(\text{FA}\) was not as good as that from \[^3H\]-\((8R)\)-HxA\(\text{Me}\).

In conclusion, although the technical difficulties in this series of experiments appeared mostly resolved, autoradiographic difference to provide clear evidence of hepoxilin binding were largely unsuccessful for reasons that remain unknown. It is possible that HxA\(\text{A}\) binding protein does not exist, or brain tissue has such an avid binding of fatty acid-like substances that it is impossible to carry out such studies unless specific cell types are used in isolation.

Unpublished results with human neutrophils have revealed that the binding protein is located in the membrane fraction when sonicates of fresh cells were photolabeled by the photoaffinity analog of HxA\(\text{A}\). However, photolabeling of sonicates of previously frozen cells revealed that the binding protein was released into the cytosolic fraction. Since in the autoradiographic study described herein, labeling was done in previously frozen brain sections, the binding protein, if exists, may have been released into the cytoplasm resulting in the observed differences in binding. Co-factors or the G protein associated with the binding protein may have dissociated from the binding complex during tissue freezing making the binding protein non-functional. One way to verify this possibility is to perform a binding experiment with unfrozen tissue sections. This may be technically difficult. Another alternative would be to fix the brain sections before freezing and then perform the binding studies. In that case, the release of the
binding complex to the cytosol can be prevented as cellular compartments in the sections are fixed prior to freezing of the sections.

It can be speculated that HxA₃ binding activity is modified by a cytosolic protein, the fatty acid-binding protein (FABP). The alteration of the properties of PGE₂ receptor by FABP in rat epididymal adipocytes has been reported (Cohen-Luria et al., 1993). The modulation of PGE binding by cytosolic protein may be due to either the direct interaction of the protein with the internal domain of the receptor, or indirect interaction with GTP-binding protein. Since FABP has been demonstrated in the brain (Galarza De Bo et al., 1992; Bass et al., 1984), whether HxA₃ binding activity gets modified by these cytosolic proteins after the binding protein gets released into the cytosol requires investigation. Also, it is not known whether any other product e.g. the metabolite of HxA₃, TrXA₃, modifies HxA₃ binding as such, even though TrXA₃ does not bind to the HxA₃ binding sites.

Consistent stronger signal in the nonspecific binding experiments may imply that HxA₃ in micromolar concentrations is able to re-activate inactive binding activity. This idea is supported by the evidence of homologous regulation of prostaglandin receptors. PG receptors can be up-regulated or down-regulated by the PGs themselves (Lefkowitz et al., 1977; Rice et al., 1980). Preincubation of the brain sections with 5 μg/ml of HxA₃ did not seem to make any difference to the subsequent binding of labeled HxA₃, although this may be due to an inappropriate dosage used. Future studies with different amounts of hepxilin are necessary to explore this direction.

4.6 ELECTROPHORESIS OF PUTATIVE HxA₃-BINDING PROTEIN

Analysis of the putative HxA₃-binding protein by SDS-PAGE was not successful in this study because higher nonspecific binding of (8S)-HxA₃Me-[¹²⁵I]-AZI was found with the frozen crude homogenates and also in all the subcellular fractions derived from the labeled homogenates. Possible explanation of this phenomenon may be due to non-existence of the binding protein, or if the binding protein exist, the inactivation or modification of the binding protein through freezing as mentioned in the last section.
Future experiments should involve the crosslinking of ligand with freshly prepared homogenates. In this case, any effect of freezing and thawing, if any, on HxA₃ binding can be determined.

The problems noticed in these experiments are observed only in brain tissue. Several other experiments using leukocytes, platelets and other cell types did not result in inconsistencies in binding. Specific bands on SDS-PAGE were observed (unpublished results). Hence use of specific neuronal cell types instead of crude tissue may be significant in localization of the binding protein on SDS gels.

4.7 FUTURE CONSIDERATIONS

Much of the work planned for the immediate future has been alluded to elsewhere. These include:

1. Competition of HxA₃ against InsP₁ binding;
2. Competition experiment using specific epimers of 11,12-EET and determining the IC₅₀'s of HxB₁ and 11,12-EET against HxA₃ binding;
3. Functional study on the action of HxA₃ in the thalamus;
4. Investigation of the effect of GTP-γS on HxA₃ binding and the determination of cAMP and intracellular calcium levels due to HxA₃ addition in hippocampus and thalamus;
5. Identification of the cell type in which HxA₃ binding activity is located in order that the actions of hepoxilin can be understood;
6. Binding of HxA₃ on fresh tissue sections or fixed sections prior to freezing ought to be attempted to investigate whether all the difficulties we experienced are due to some intrinsic properties of HxA₃ binding which is altered during tissue freezing;
7. Binding experiment using freshly prepared homogenates of the brain after microdissection of the microscopic structures;
8. Photolabeling of freshly prepared homogenates of hippocampus and thalamus as an alternative to characterize the binding protein using SDS-PAGE.
SUMMARY

The following novel findings have been made in this study:

1. We have demonstrated in this study that specific binding activity for HxA3 is present in rat hippocampus and thalamus. [3H]-(8R)-HxA3Me was used as radioligand to determine specific hepoxilin binding in different homogenized structures in rat brain;

2. [3H]- (8R)-HxA3Me binds to homogenates of the hippocampus and thalamus in a saturable, reversible, time-, and dose-dependent manner. And all these observations satisfied the criteria, saturability, reversibility, time- and substrate-dependence, for specific binding;

3. Competitive binding was best observed with HxA3 and HxB3 and to a lesser extent with 11,12-EET, and it suggested that the epoxide moiety at C-11 and C-12 are essential for HxA3 specific binding;

4. Scatchard analysis of the data revealed a single population of binding sites with $K_d$ and $B_{max}$ of $43.1 \pm 6.7 \text{ nM}$ and $42.3 \pm 7.3 \text{ pmol/mg of protein}$.

In binding studies using slide-mounted tissue sections, two radiolabeled ligands, (8S)-HxA3Me-[125I]-AZI or [3H]- (8R)-HxA3Me were used to investigate specific binding sites. However, this part of the study was not conclusive under all the conditions attempted as higher binding was found in the sections representing the nonspecific binding of the ligand. The reasons for this have not been defined.

The photoaffinity label (8S)-HxA3Me-[125I]-AZI was used to attempt to characterize the HxA3 binding protein in the frozen homogenates of the hippocampus and thalamus by SDS-PAGE. This part of the work was also inconclusive as a stronger signal was also obtained from the nonspecific binding of the ligand.

As HxA3 has been shown to elicit neuromodulatory actions in CA1 neurons of the hippocampus, the HxA3 specific binding activity demonstrated herein suggests the existence of a binding protein through which HxA3 elicits its actions. However, the
existence of HxA₃ binding protein was not observed in this study due to the failure of identifying HxA₃ binding protein in frozen rat brain sections and in frozen homogenates through use of SDS-PAGE. This may suggest that the HxA₃ binding protein does not exist in the hippocampus and thalamus, or binding of the lipidic ligand to FABP, or modification of the binding sites due to freezing make the attempts that we have developed unsuccessful. Autoradiographic study and characterization of HxA₃ binding activity was done in frozen tissues only in this study. As HxA₃ binding protein has been demonstrated in other cell types, e.g. human neutrophils, the existence of specific HxA₃ binding protein in the CNS warrants further experimentation.

In human neutrophils, HxA₃ binding activity is G protein-coupled. It is not known at this point if this phenomenon can be extended to other cell types, or is restricted to human neutrophils. Further studies of the HxA₃ binding activity is important for the understanding of the hepoxilin system in the normal state and its possible role in diseases of the CNS.
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