Cholesterol 24S-Hydroxylase: Involvement in Brain Injury and Disease

A Dissertation submitted to the Faculty of the Graduate School of Arts and Sciences of Georgetown University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Neuroscience

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Washington, DC

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Here we investigated whether traumatic brain injury (TBI) altered the regulation of cholesterol 24S-hydroxylase (Cyp46), an enzyme that converts cholesterol to the more hydrophilic 24S-hydroxycholesterol. We examined changes in Cyp46 expression in rats following fluid percussion injury. Under normal conditions, most Cyp46 was present in neurons, with very little measurable in glia. However, Cyp46 levels were significantly increased in microglia after TBI. 24-hydroxycholesterol induces activation of genes through the liver X receptor (LXR), and here we show apolipoprotein E (ApoE) and ATP-binding cassette transporter (ABC) A1 were increased after TBI, indicating that increased LXR activity coincided with increased Cyp46 levels. We found that activation of primary rat microglia by LPS in vitro caused increased Cyp46 levels. These data suggest that increased microglial Cyp46 activity is part of a system for removal of damaged cell membranes post-injury, by conversion of cholesterol to 24-hydroxycholesterol and by activation of LXR-regulated gene transcription. We also took a systematic look at the effects of 24S-hydroxycholesterol on fatty acid and cholesterol synthesis enzymes, initially focusing on the lipid...
regulatory proteins SREBP-1 and -2. In three different cell lines, 24S-hydroxycholesterol decreased SREBP-2 and increased levels of LXR regulated SREBP-1. In SY5Y neuroblastomas cells, 24S-hydroxycholesterol decreased cholesterol synthesis enzyme mRNA levels but did not alter fatty synthesis enzyme mRNA levels. In contrast, BV2 microglia 24S-hydroxycholesterol significantly increased mRNA levels of fatty acid synthesis enzymes but had no significant effect on cholesterol synthesis enzymes. After TBI we found that, consistent with the \textit{in vitro} results, SREBP-1 mRNA levels were increased while SREBP-2 mRNA levels were decreased. Cholesterol synthesis enzymes were significantly decreased after TBI, which we attribute to 24S-hydroxycholesterol activity. However, mRNA levels of the rate limiting step in fatty acid synthesis, acetyl CoA carboxylase were also significantly decreased which could not be due to the effects of 24S-hydroxycholesterol. Thus, other negative feed back mechanisms regulating fatty acid synthesis must be activated after TBI.
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This dissertation is dedicated to my daughter,

Moira Isabel Íñigo-Cartagena.

For her, the sky is the limit.
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<tbody>
<tr>
<td>AA</td>
<td>arachidonic acid</td>
</tr>
<tr>
<td>ABC</td>
<td>ATP-binding cassette transporter protein</td>
</tr>
<tr>
<td>ABCA1</td>
<td>ATP-binding cassette transporter protein</td>
</tr>
<tr>
<td>ACAT</td>
<td>acyl coenzyme A: cholesterol acyltransferase</td>
</tr>
<tr>
<td>ACC</td>
<td>acetyl CoA carboxylase</td>
</tr>
<tr>
<td>AD</td>
<td>Alzheimer's disease</td>
</tr>
<tr>
<td>Apo</td>
<td>apolipoprotein</td>
</tr>
<tr>
<td>ApoE</td>
<td>apolipoprotein E</td>
</tr>
<tr>
<td>APP</td>
<td>amyloid precursor protein</td>
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<tr>
<td>Aβ</td>
<td>beta amyloid</td>
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<tr>
<td>CEPT</td>
<td>cholesterol ester transfer protein</td>
</tr>
<tr>
<td>cSREBP</td>
<td>cytosolic SREBP</td>
</tr>
<tr>
<td>CTF</td>
<td>C-terminal fragment</td>
</tr>
<tr>
<td>Cyp46</td>
<td>cholesterol 24S-hydroxylase</td>
</tr>
<tr>
<td>DHA</td>
<td>docosahexaenoic acid</td>
</tr>
<tr>
<td>DIV</td>
<td>days in vitro</td>
</tr>
<tr>
<td>EPA</td>
<td>eicosapentaenoic acid</td>
</tr>
<tr>
<td>ER</td>
<td>endoplasmic reticulum</td>
</tr>
<tr>
<td>FAS</td>
<td>fatty acid synthase</td>
</tr>
<tr>
<td>FPP</td>
<td>farnesyl-pyrophosphate</td>
</tr>
<tr>
<td>Acronym</td>
<td>Definition</td>
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<tr>
<td>---------</td>
<td>------------------------------------------------</td>
</tr>
<tr>
<td>HDL</td>
<td>high density lipoprotein</td>
</tr>
<tr>
<td>HMG CoA</td>
<td>3-hydroxy-3-methylglutaryl CoA</td>
</tr>
<tr>
<td>IDL</td>
<td>intermediate density lipoprotein</td>
</tr>
<tr>
<td>JNK</td>
<td>c-jun N-terminal kinase</td>
</tr>
<tr>
<td>LCAT</td>
<td>lectithin cholesterol acyltransferase</td>
</tr>
<tr>
<td>LDL</td>
<td>low density lipoprotein</td>
</tr>
<tr>
<td>LPL</td>
<td>lipoprotein lipase</td>
</tr>
<tr>
<td>LPS</td>
<td>lipopolysaccharide</td>
</tr>
<tr>
<td>LXR</td>
<td>liver X receptor</td>
</tr>
<tr>
<td>LXRE</td>
<td>liver X receptor element</td>
</tr>
<tr>
<td>MS</td>
<td>multiple sclerosis</td>
</tr>
<tr>
<td>nSREBP</td>
<td>nuclear SREBP</td>
</tr>
<tr>
<td>OPC</td>
<td>oligodendrocyte progenitor cell</td>
</tr>
<tr>
<td>PD</td>
<td>Parkinson's disease</td>
</tr>
<tr>
<td>PUFA</td>
<td>polyunsaturated fatty acid</td>
</tr>
<tr>
<td>RIPA</td>
<td>radioimmunoprecipitation</td>
</tr>
<tr>
<td>S1P</td>
<td>sterol-regulated protease 1</td>
</tr>
<tr>
<td>SCAP</td>
<td>SREBP cleavage activating protein</td>
</tr>
<tr>
<td>SR-BI</td>
<td>scavenger receptor BI</td>
</tr>
<tr>
<td>SREBP</td>
<td>sterol regulatory binding protein</td>
</tr>
<tr>
<td>TBI</td>
<td>traumatic brain injury</td>
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VLDL  very low density lipoprotein
CHAPTER I: INTRODUCTION

Cholesterol Function and Metabolism in the Periphery

Cholesterol Function

Cholesterol is a small hydrophobic molecule that serves several functions. Cholesterol is a precursor in production of hormones, including estrogen, testosterone, cortisol and many others (Simpson and Waterman, 1988; Berg et al., 2007). Cholesterol also serves important functions in the membranes of all cells. Membrane fluidity is influenced by the concentration of cholesterol in the membrane. Higher concentrations of cholesterol provide increased rigidity to the membrane, making the membrane less fluid, and promote the ordered packing of embedded proteins (Alberts, 1989; Gross et al., 2005; Ikonen, 2008). High concentrations of cholesterol also prevent permeability of the membrane to small water soluble molecules (Alberts, 1989; Gross et al., 2005; Ikonen, 2008). Cholesterol is also integral to the formation of lipid rafts where receptors and other anchored proteins are brought together, facilitating some types of cell signaling (Fielding and Fielding, 2004; Gross et al., 2005).

Sources of Cholesterol

Cholesterol in the periphery comes primarily from two sources: Liver synthesis and dietary consumption (Ikonen, 2008). The body can produce its own cholesterol, and thus it has been suggested that cholesterol is not a necessary component of the diet (Keys et
al., 1965; Hodges et al., 1967; Engelhardt et al., 1991). However, in dietary cholesterol deprivation studies, benefits were limited and the long term consequences remain unknown (Hodges et al., 1967; Engelhardt et al., 1991). Although there is high variability across subjects, on average 70% of total body cholesterol is synthesized de novo while 30% is derived from the diet (Ikonen, 2008). Although the liver is the primary site of cholesterol synthesis, other peripheral organs synthesize cholesterol at lower levels, where it can be used as a precursor in hormone production (Simpson and Waterman, 1988). Knockout mice lacking the critical enzyme HMG CoA reductase, and thus the ability to synthesize cholesterol, do not survive beyond the blastocyst stage, emphasizing the importance of cholesterol in development (Ohashi et al., 2003).

**Cholesterol Transport**

Cholesterol transport mechanisms allow cholesterol from both the liver and diet to be packaged and put into circulation so that it is accessible to tissues that need more cholesterol. Cholesterol synthesized in the liver is exported to the gall bladder through the membrane bound proteins, ATP-binding cassette (ABC) G5 or ABC G8 (Kosters et al., 2006; Ikonen, 2008). Cholesterol is stored in the gall bladder between meals but during food digestion it is released with bile salts into the lumen of the intestine where it mixes with dietary cholesterol (Ikonen, 2008). In the intestines, triglycerides, phospholipids, cholesterol and bile salts are combined to form a cluster called a micelles which travel through the intestines (Ikonen, 2008).
Once cholesterol from the liver and the diet is combined in the intestines, it can re-enter the circulation system. Cholesterol from micelles is reabsorbed by cells in the intestinal wall through the channel NPC1L1 (Ikonen, 2008). Although NPC1L1 absorbs cholesterol and plant sterols from the intestinal lumen, plant sterols are efficiently effluxed back into the lumen by ABC G5 or ABC G8 (Phan and Tso, 2001; Hazard and Patel, 2007; Ikonen, 2008).

Cells in the intestinal wall function to package cholesterol into lipoproteins and release them into circulation. After the intestine absorbs cholesterol, it converts the cholesterol into a cholesteryl ester via the cytoplasmic enzyme acyl coenzyme A: cholesterol acyltransferase (ACAT) (Gallo et al., 1984) and then exports it into the lymphatic system, producing a chylomicron (Vine et al., 1997; Hussain et al., 2001; Gibbons et al., 2004; Ikonen, 2008). Chylomicrons are the largest lipoproteins consisting primarily of a cholesteryl ester and triglyceride core with an outer shell of free cholesterol and phospholipids (Hussain et al., 2001; Superko, 2001; Gibbons et al., 2004; Ikonen, 2008). The chylomicron outer surface also contains several proteins called apolipoproteins (Apo), including ApoAI, ApoAIV, ApoB48, ApoCII, ApoCIII, and ApoE (Mahley and Innerarity, 1983; Hussain et al., 2001; Superko, 2001; Bretillon et al., 2007).

Triglycerides in the chylomicron core can be hydrolyzed by the enzyme lipoprotein lipase (LPL), leaving a smaller lipoprotein referred to as chylomicron remnant (Goldberg
and Merkel, 2001; Frayn, 2003; Redgrave, 2004; Ikonen, 2008), and producing free fatty acids which can be used as energy (Goldberg and Merkel, 2001; Frayn, 2003).

Chylomicrons are not the only lipoprotein produced by the intestine. The intestinal wall can also export cholesterol in high density lipoproteins (HDLs) which consist primarily of free cholesterol and phospholipids (Ikonen, 2008). Both chylomicrons and HDLs travel by way of the lymphatic system to the liver where membrane lipoprotein receptors bind apolipoproteins on the surface of the chylomicron or HDL and subsequently these lipoproteins are endocytosed (Goldstein et al., 1985; Vine et al., 1997; Redgrave, 2004; Ikonen, 2008).

The liver can release cholesterol and lipids for other organs by exporting lipoproteins into the blood supply. The liver combines cholesterol and cholesterol esters from chylomicrons with triglycerides and free cholesterol synthesized de novo to produce very low density lipoproteins (VLDLs) which are released into blood (Gibbons et al., 2004; Ikonen, 2008). VLDLs are the second largest lipoproteins consisting of a triglyceride and cholesteryl ester core and an outer shell containing free cholesterol, phospholipids and ApoB100, ApoCI, ApoCII ApoCIII, and ApoE (Mahley and Innerarity, 1983; Goldberg and Merkel, 2001; Hussain et al., 2001; Superko, 2001; Gibbons et al., 2004; Bretillon et al., 2007). The secretion of VLDL is in relation to the availability of triglycerides as well as ApoB100 synthesis (Julius, 2003). Triglycerides levels in VLDL can be reduced by
the activity of LPL, thereby converting VLDLs to intermediate density lipoproteins (IDLs) (Superko, 2001). The enzyme cholesteryl ester transfer protein (CETP) exchanges triglycerides found in IDLs for free cholesterol found in HDLs producing slightly smaller low density lipoproteins (LDLs) (Superko, 2001; Yamashita et al., 2001) which contain primarily ApoB100 (Mahley and Innerarity, 1983).

Similar to the intestine, liver also produces nascent HDLs by exporting free cholesterol, ApoAI, ApoAII, ApoCI, ApoCII and ApoCIII through the phospholipid and cholesterol efflux protein, ABCA1 (Mahley and Innerarity, 1983; Goldberg and Merkel, 2001; Superko, 2001; Smith et al., 2004). Only a small portion of plasma HDL contain ApoE (Mahley and Innerarity, 1983). The enzyme lecithin cholesterol acyltransferase (LCAT) is activated by ApoAI and converts free cholesterol in these nascent HDLs to cholesteryl ester, giving a mature HDL\textsubscript{2} (Superko, 2001). These small HDL lipoproteins circulate in the blood serum along with the larger ApoB-containing lipoproteins VLDL and LDL. Tissue in need of cholesterol, especially structures that do not produce high levels of cholesterol, will access this cholesterol supply by expressing receptors recognizing the apolipoproteins on the surface of lipoproteins (Ikonen, 2008).
**Global cholesterol regulation**

Global cholesterol regulation is complex and it is related not only to cholesterol levels but also to carbohydrate levels (Mutungi et al., 2007; Torres-Gonzalez et al., 2007). Generally, liver cholesterol synthesis rates are stable. For example, decreasing dietary cholesterol alone has little effect on liver cholesterol synthesis rates. However when dietary cholesterol levels are decreased in the presence of increases in carbohydrates or increases in certain kinds of protein, liver cholesterol synthesis is also increased (Hodges et al., 1967; Engelhardt et al., 1991; Superko, 2001; Mutungi et al., 2007; Torres-Gonzalez et al., 2007).

When serum cholesterol levels are high, which is more often the case with western diets, cholesterol is cleared from the body. This occurs through a process called *reverse cholesterol transport* (Ikonen, 2008). Larger HDLs that have absorbed cholesterol from the periphery are bound by the surface membrane liver scavenger receptor BI (SR-BI) which removes cholesterol esters from the HDL into the cell and exports it through hepatic ABCG5 and G8 back through bile to the intestines where it can be excreted as waste (Trigatti et al., 2004; Kosters et al., 2006; Hazard and Patel, 2007; Ikonen, 2008). After cholesterol extraction the remaining HDL is smaller and referred to as HDL$_2$. This lipoprotein is then bound by a second liver surface receptor HL, which hydrolyzes phospholipids and triglycerides to release free fatty acids, resulting in a smaller lipoprotein HDL$_3$ (Goldberg and Merkel, 2001). This HDL$_3$ can either return to the
serum circulation where it accepts more cholesterol exported from tissues through ABCA1 or it can travel to the kidney where it is cleared from the body (Peterson et al., 1984).

**Cholesterol Synthesis Pathway**

Cholesterol synthesis is a highly regulated process depending in great part on the levels of cholesterol in the cell. Cholesterol synthesis starts with the conversion of acetyl CoA and acetoacetyl CoA into 3-hydroxy-3-methylglutaryl CoA (HMG CoA) by the enzyme HMG CoA synthase (Berg et al., 2007). HMG CoA is an intermediate which is reduced by the endoplasmic reticulum membrane bound enzyme HMG CoA reductase to form mevalonate. This is the rate limiting step in cholesterol synthesis (Berg et al., 2007).

Mevalonate is then modified in three reactions by mevalonate kinase, phosphomevalonate kinase and mevalonate-5-pyrophosphate decarboxylase to form isopentenyl pyrophosphate (Berg et al., 2007). This completes the first of three main stages in cholesterol synthesis (Berg et al., 2007). In the second stage three molecules of isopentenyl pyrophosphate are joined by farnesyl-pyrophosphate (FPP) synthase to form FPP. Following this, two molecules of FPP are combined by squalene synthase, thus forming squalene (Berg et al., 2007). In the third stage, linear squalene is cyclized to form sterol rings by the enzymes squalene monooxygenase and squalene epoxidase. Three methyl groups and a double bond are removed from this structure (lanosterol) in
19 steps to form cholesterol (Berg et al., 2007). The cholesterol synthesis pathway has been summarized in Figure 1-1.
Figure 1-1. Cholesterol Synthesis Pathway.
Cellular Cholesterol Homeostasis

Under conditions of low cellular cholesterol, cholesterol synthesis and cholesterol import into the cell can be increased. This regulation depends on the sterol regulatory element-binding protein (SREBP), a membrane bound protein normally located to the endoplasmic reticulum (ER). When cholesterol levels are sufficient, SREBP is held at the ER by SREBP cleavage activating protein (SCAP) (Brown and Goldstein, 1997; Schoonjans et al., 2000). When cholesterol levels are low, SCAP moves with SREBP to the Golgi, where the full length (or “cytosolic”) SREBP (cSREBP) can be cleaved by sterol-regulated protease (S1P) to release a cytoplasmic fragment that can travel to the nucleus (Brown and Goldstein, 1997; Schoonjans et al., 2000). This nuclear SREBP (nSREBP) acts as a transcription factor (Figure 1-6B) and signals for cholesterol production by upregulating expression of the rate-limiting enzyme in cholesterol synthesis, HMG CoA reductase (Schoonjans et al., 2000). Other enzymes in the cholesterol synthesis pathway are also under nSREBP regulation, including HMG CoA synthase, FPP synthase and squalene synthase (Olivier et al., 2000) (Figure 1-1).

nSREBP also signals for increased cholesterol influx by upregulating expression of the low-density lipoprotein (LDL) receptor (Schoonjans et al., 2000). This receptor allows cells to endocytose cholesterol in lipoproteins from the extracellular space (Goldstein et al., 1985).

When cellular cholesterol levels are high, cholesterol can also be exported from the cell via ABCA1 (Lehmann et al., 1997; Janowski et al., 1999; Laffitte et al., 2001; Fukumoto
et al., 2002; Vaughan and Oram, 2003) In peripheral cells, this is the primary mechanism of decreasing cellular cholesterol levels. Extracellular cholesterol is bound by a lipid acceptor such as ApoAI or ApoE (Rothblat et al., 1999). Lipoproteins accept cholesterol when it is exported from one cell and can deliver cholesterol to other cells, where it can be incorporated into membranes (Barres and Smith, 2001; Schmitz and Kaminski, 2001; Ikonen, 2008).

Cholesterol can also be converted to oxysterols by a subclass of P450 enzymes (Rothblat et al., 1999). In the periphery, this mechanism plays a smaller role in lowering cellular cholesterol levels but is a primary mechanism of lowering cellular cholesterol levels in the brain (Schmitz and Kaminski, 2001; Xie et al., 2003; Ikonen, 2008). These oxysterols can function in three ways. First, they serve as an additional mechanism to clear cholesterol from the cell. Cholesterol is very hydrophobic and unable to leave membranes except by cholesterol efflux through ABCA1 with ApoE (Vaya and Schipper, 2007). Conversion of cholesterol to an oxysterol makes cholesterol less hydrophobic and thus more soluble (Vaya and Schipper, 2007).

Secondly, some oxysterols function as signaling molecules by activating liver X receptors (LXR) (Lehmann et al., 1997; Janowski et al., 1999; Laffitte et al., 2001; Fukumoto et al., 2002). LXRs are nuclear hormone receptors (Janowski et al., 1999).
LXRs function by forming heterodimers with retinoid X receptors (Janowski et al., 1999). This heterodimer is located in the cytosol, but with ligand binding undergoes a conformational change and travels to the nucleus where it binds gene promoters containing a LXR element (LXRE) (Janowski et al., 1999). Activation of LXRs induces the upregulation of several genes involved in cholesterol efflux (Lehmann et al., 1997; Janowski et al., 1999). Although not all oxysterols have strong LXR activity, examples include peripherally produced 22-hydroxycholesterol, and 27-hydroxycholesterol as well as 24S-hydroxycholesterol, which is produced in the brain (Janowski et al., 1999; Fu et al., 2001). Genes under LXR control include ABCA1 and ApoE, which, as discussed above, are both important in cholesterol efflux (Fukumoto et al., 2002; Liang et al., 2004).

Finally, the presence of oxysterols is hypothesized to inhibit cSREBP cleavage (Thewke et al., 1998; Adams et al., 2004; Du et al., 2004). Oxysterols activate a protein Insig, which in turn binds SCAP. This retains both SCAP and cSREBP in the ER, thus prohibiting cleavage by SP1 in the Golgi (Adams et al., 2004; Radhakrishnan et al., 2007). Mechanisms of cholesterol production, cholesterol efflux and cholesterol influx are all part of the homeostatic controls keeping total cellular cholesterol levels constant.
**Cholesterol Mechanisms in the Brain**

Although much is known about regulation of cholesterol in cells in the periphery, much less is known about its control in the central nervous system. Although the brain is only 2% of total body mass, it contains 25% of unesterified cholesterol (Vaya and Schipper, 2007). After development, lipoproteins do not cross the blood brain barrier from the periphery and thus there is no cholesterol transport into the brain (Jurevics and Morell, 1995; Turley et al., 1996). In fact, the brain makes its own cholesterol (Jurevics and Morell, 1995; Turley et al., 1998; Lund et al., 2003; Xie et al., 2003). Rates of cholesterol synthesis in the brain correspond to total cholesterol levels, such that regions with more cholesterol have higher synthesis rates while regions with lower total levels have lower cholesterol synthesis rates (Turley et al., 1998). Most cholesterol synthesis occurs in white matter regions including brain stem and cerebral white matter, consistent with cholesterol’s role as a significant component of myelin (Turley et al., 1998). However, even in grey matter regions such as frontal cortex, parietal cortex and cerebellar folia, cholesterol synthesis occurs and may function there to maintain cell membranes (Turley et al., 1998).

As in the periphery, cholesterol in the brain is found associated with lipoproteins. Lipoproteins in the brain are unique in several ways. The brain lacks larger lipoprotein (e.g. LDL, VLDL) and only has the smaller HDLs. These HDLs contain mainly ApoAI
and ApoE (Turley et al., 1996; Kay et al., 2003) Although ApoAI is found in brain it is not synthesized there (Elshourbagy et al., 1985). Instead, the primary apolipoprotein synthesized in the brain is ApoE (Boyles et al., 1985; Pitas et al., 1987). As in the periphery, cells that have excess cholesterol can export it through ABCA1 to an ApoE containing HDL. These observations have led to the hypothesis that in the CNS these proteins act to transport cholesterol from areas with excess cholesterol to areas in need of cholesterol (Pitas et al., 1987; Barres and Smith, 2001; Kay et al., 2003). This process may be important for both neuronal development and neuronal plasticity and regeneration (Barres and Smith, 2001).

The brain also has its own homeostatic mechanisms for maintaining steady-state levels of cholesterol. Cholesterol 24S-hydroxylase (CYP46) is a brain enriched enzyme responsible for converting cholesterol from the plasma membrane into 24S-hydroxycholesterol (Lund et al., 1999; Ramirez et al., 2008) (Figure 1-2). Because cholesterol is hydrophobic and unable to leave membranes (Vaya and Schipper, 2007), conversion of it to 24S-hydroxycholesterol makes cholesterol less hydrophobic and more soluble (Vaya and Schipper, 2007). Because of this increased solubility, 24S-hydroxycholesterol can cross the cell membrane as well as the blood brain barrier (Lutjohann et al., 2000).
Figure 1-2. Structure of cholesterol and three oxysterols.
The relative importance of cholesterol conversion to 24S-hydroxycholesterol versus cholesterol efflux to lipoproteins in brain cholesterol clearance has been studied using a Cyp46 knockout mice (Lund et al., 2003; Xie et al., 2003). In the absence of Cyp46 activity, total brain cholesterol levels were similar to wildtype mice (Xie et al., 2003). However cholesterol excretion from the CNS was decreased by 64% (Xie et al., 2003). In addition, cholesterol synthesis rates decreased by 41%, which may be a compensatory homeostatic mechanism induced in these mice to maintain stable total cholesterol levels in the brain (Xie et al., 2003). These data indicate that the main mechanism for clearing cholesterol from the brain is its conversion to 24S-hydroxycholesterol (Xie et al., 2003). In humans, CSF levels of 24S-hydroxycholesterol levels average about 6.7 µM while serum levels average about 0.25 µM (Papassotiropoulos et al., 2002). While the brain can clear cholesterol by export of the HDLs through the arachnoid granulations and into the venal blood flow (Pitas et al., 1987), conversion of cholesterol to 24S-hydroxycholesterol allows the brain to clear cholesterol directly across the blood brain barrier over a much greater surface area (Lutjohann et al., 2000; Papassotiropoulos et al., 2002).

24S-hydroxycholesterol is also an activator of the nuclear transcription factors LXRs and can induce the upregulation of genes involved in cholesterol efflux (Lehmann et al., 1997; Janowski et al., 1999). Upregulation of genes such as ApoE and ABCA1 can cause increased production of HDL. This HDL can support removal of cholesterol from the
brain by its removal via arachnoid granulations (Pitas et al., 1987; Fukumoto et al., 2002; Kay et al., 2003; Liang et al., 2004). LXR activation has also been shown to increase SREBP levels (Whitney et al., 2002; Hoe et al., 2007). SREBPs regulate not only cholesterol synthesis, as discussed above, but also fatty acid synthesis (Horton et al., 2002; Chen et al., 2004). Thus, 24S-hydroxycholesterol may have significant regulatory effects on both cholesterol homeostasis and fatty acid homeostasis in the brain.

**Fatty Acid Function and Metabolism**

**Fatty Acid Function**

Fatty acids have been shown to serve four functions in living organisms (Berg et al., 2007). First, they serve as fuel storage molecules (Frayn, 2003; Miles and Nelson, 2007). Combining fatty acids and glycerol to form triglycerides occurs primarily in the liver and adipose tissue (Berg et al., 2007; Miles and Nelson, 2007). During rest or low level exercise these triglycerides are the primary source of energy (Frayn, 2003). Second, fatty acids are used in the assembly of phospholipids and glycoproteins, important components of cell membranes (Berg et al., 2007). Like cholesterol, fatty acids influence membrane fluidity. Double bonds in unsaturated fatty acids increase the fluidity of membranes because they cover more surface area and decrease membrane density (Alberts, 1989). Third, fatty acids can function as trafficking signals which target proteins to membranes (Berg et al., 2007). Short fatty acids such as myristic acid (Figure 1-3A) traffic proteins to the membrane when added post-translationally (Sowadski et al.,
1996). And finally, fatty acids are components used in the synthesis of hormone-like molecules such as prostaglandins, leukotrienes, and thromboxanes (Bhathena, 2000; Chen and Bazan, 2005; Wymann and Schneiter, 2008) and other intracellular messengers (Chen and Bazan, 2005; Gross et al., 2005).
Figure 1-3. Structure of endogenously produced fatty acids. A) lauric, myristic, and palmitic acid, products of FAS activity. B) oleic and stearic acids as well as sphingolipids are all derivatives of palmitic acid. Structure lengths are indicated in number of carbons ranging from 12 carbons (C12) to 18 carbons (C18). Oleic acid contains a double bond at the 9th carbon (omega-9).
Sources of Fatty Acids

Similar to cholesterol, fatty acids can be synthesized by the body or obtained in the diet and absorbed by the intestinal wall. The intestine cannot absorb triglycerides so these are first broken down in the digestive process into free fatty acids and combined with cholesterol and bile acid in micelles (Phan and Tso, 2001). Some fatty acids such as linoleate cross the intestinal wall by passive diffusion while others are taken up by fatty acid transporter proteins (Phan and Tso, 2001). Once absorbed into the intestines fatty acids are reassembled into triglycerides and exported to chylomicrons for transport by way of the lymphatic system to the liver (Goldstein et al., 1985; Vine et al., 1997; Hussain et al., 2001; Phan and Tso, 2001). In subjects consuming high fat diets, chylomicrons are the major source of fatty acids in the body (Miles and Nelson, 2007). Dietary fatty acid composition influences triglyceride levels in chylomicrons, with oleic acid, a 18 carbon chain with a double bond at the omega-9 carbon (Figure 1-3B), leading to the greatest triglyceride levels, linoleic acid, a 18 carbon chain with double bonds at the omega-6 and omega-9 carbons (Figure 1-4A), leading to intermediate triglyceride levels and palmitic acid (Figure 1-3A), a 16 carbon chain without double bonds, leading to lower triglyceride levels (Williams et al., 2004). The body is capable of producing most fatty acids and high levels of dietary absorption can lead to unhealthy levels of triglycerides (Frayn, 2003; Jessup et al., 2004; Redgrave, 2004; Fritsche, 2006). However, oleic acid deprivation showed decreased levels of this important fatty acid in some organs, including liver, kidney and heart. This suggests that endogenous synthesis
of some fatty acids may not be sufficient and dietary sources are necessary (Bourre et al., 1997). Animals are incapable of adding double bonds to fatty acids beyond 16 carbons in length. Thus, polyunsaturated fatty acids (PUFAs) beyond 16 carbons in length must be obtained from the diet (Berg et al., 2007). They are therefore referred to as essential fatty acids. These include linoleate and linolenate, both 18 carbons in length with multiple double bonds (Figure 1-4A). All polyunsaturated fatty acids longer than these are either obtained through the diet or are synthesized by the body using dietary linoleate or linolenate as base molecules (Berg et al., 2007). This includes important fatty acids with double bonds at the 6th carbon (omega-6 or n-6) or the 3rd carbon (omega-3 or n-3).

These PUFAs are used in cell membrane synthesis as well as the productions of signaling molecules (Fritsche, 2006). The n-6 PUFA arachidonic acid (AA) is a precursor in the production of leukotrienes and other proinflammatory eicosanoids while the n-3 PUFAs eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) which lead to anti-inflammatory eicosanoid production (Fritsche, 2006) (Figure 1-4B).
A. Essential Fatty Acids

Linoleic acid (C18:omega-6)  Linolenic acid (C18:omega-3)

B. Derivatives of Essential Fatty Acids

Arachidonic acid (C20:omega-6)  Docosahexaenoic acid (C22:omega-3)  Eicosapentaenoic acid (C20:omega-3)

Figure 1-4. Polyunsaturated Fatty Acids. A) Structure of the essential fatty acids linoleic and linolenic acid. B) Important PUFA derivatives include arachidonic acid, docosahexaenoic acid and eicosapentaenoic acid. Structure lengths are indicated in number of carbons ranging from 18 carbons (C18) to 22 carbons (C22). PUFA illustrated contain double bonds at either the 6th carbon (omega-6) or 3rd carbon (omega-3) as well as at other carbons.
The liver, intestine, and adipose tissue produce fatty acids for use in triglyceride production (Farese et al., 2000). Triglycerides are exported by the liver to LDLs and transported in serum where they serve as available energy sources for various tissues (Farese et al., 2000). Tissues in need of energy can endocytose triglyceride containing lipoproteins and break them down into fatty acids and glycerol once again (Haemmerle et al., 2006). Although the liver is the primary source of triglycerides, fatty acids are produced at varying levels throughout the body to serve functions other than energy storage such as cell signal production or the synthesis of membrane components (Fritsche, 2006; Sutterwala et al., 2007; Hannun and Obeid, 2008).

**Fatty Acid Synthesis**

Fatty acid synthesis utilizes two enzymes, acetyl CoA carboxylase (ACC) and fatty acid synthase (FAS) (Berg et al., 2007). ACC activity is the rate limiting step in fatty acid synthesis and is also referred to as the committed step since it is irreversible (Berg et al., 2007). In this step, acetyl CoA is combined with CO$_2$ to produce malonyl CoA. FAS is a single chain molecule which combines several enzymatic reactions (Berg et al., 2007). FAS facilitates the addition of two carbons to the fatty acid chain using one molecule of malonyl CoA. The addition of each malonyl CoA involves four steps: condensation, reduction, dehydration and again reduction. These steps are repeated until the fatty acid is 16 carbons in length giving primary final product palmitate (palmitic acid) (Berg et al., 2007) (Figure 1-3A). The shorter products of lauric acid (12 carbons) and myristic acid
(14 carbons) are also produced in smaller quantities but are usually quickly extended by FAS to form palmitic acid (Riouxb, 2007). Fatty acid synthesis is summarized in Figure 1-5. Further modifications of the fatty acid, such as elongation or double bonds are carried out by other enzyme systems (Jakobsson et al., 2006). A critical downstream modification is de novo sphingolipid synthesis where palmitic acid is the substrate of the rate limiting enzyme serine palmitoyltransferase (Sutterwala et al., 2007; Hannun and Obeid, 2008; Jungersted et al., 2008). Sphingolipid derivatives include ceramides and sphingosines, which are pro-apoptotic as well as sphingosine-1-phosphate, which promotes cell survival and proliferation (Wymann and Schneiter, 2008).
Figure 1-5. Fatty Acid Synthesis Pathway.
**Fatty Acid Homeostasis**

Cellular fatty acid levels can be controlled by negative feedback controls on ACC levels or activity (Mabrouk et al., 1990; Brownsey et al., 2006; Berg et al., 2007). When ATP levels are low, fatty acid synthesis as energy storage is not needed (Brownsey et al., 2006; Berg et al., 2007). Low ATP levels lead to AMPK phosphorylation of ACC, causing its inhibition (Brownsey et al., 2006; Berg et al., 2007). When fatty acid synthesis is high, palmitoyl CoA, a derivative of the end product palmitic acid, causes the disassembly of ACC filaments and inhibits the transport of citrate, a facilitator of ACC filaments, from the mitochondria to the cytoplasm (Mabrouk et al., 1990; Brownsey et al., 2006; Berg et al., 2007).

Various hormones have been shown to have regulatory effects on fatty acid synthesis. Hormones that increase with fasting and exercise, such as glucagon and epinephrine have been shown to inhibit ACC activity by unknown mechanisms. Insulin has been shown to upregulate ACC activity by activating phosphatases and to upregulate ACC synthesis through increased SREBP activity. Fatty acid synthesis rates have been shown to be altered by low-fat diets (Hudgins et al., 1996; Brooks and Lampi, 1999). Within two weeks of decreased dietary fat levels, both ACC and FAS activities increase (Brooks and Lampi, 1999). There is also increased palmitic acid levels in VLDL triglyceride (Hudgins et al., 1996). While cholesterol synthesis is almost exclusively controlled by SREBP activity, fatty acid synthesis has additional regulatory mechanisms (Horton et al.,
2002). For example, dietary PUFAs have also been shown to decrease both ACC and FAS levels in the liver (Yahagi et al., 1999) and FAS has been shown to have low-level response to LXR activation in the absence of SREBP activation (Horton et al., 2002).

**Fatty Acids in the Brain**

As is the case with cholesterol mechanisms, the majority of research on fatty acid mechanisms has been done in the periphery. In the mouse brain ACC and FAS levels are maximal 5 days after birth when cell proliferation in high but fall to 20% of maximal levels by 20 days after birth (Garbay et al., 1997). Some studies have shown that brain ACC activity is invariant with changes in nutritional content in contrast to peripheral ACC which has altered activity with diet (Spencer et al., 1993) while other studies showed that with food deprivation brain ACC activity decreased (Karami et al., 2006). Little is known about regulation of fatty acid synthesis in the CNS.

Brain fatty acids serve some of the same functions as in the periphery but not all. For instance, fatty acids do not serve as a fuel source in the brain (Ruderman et al., 1974). The brain exclusively uses glucose as its fuel source, although under starvation condition the brain may use ketone bodies as fuel and these can be made from fatty acids (Ruderman et al., 1974). As in the periphery, fatty acid synthesis plays a role in maintaining membrane fluidity (Alberts, 1989) and in cell signaling and cell membrane structure (Sutterwala et al., 2007; Hannun and Obeid, 2008; Jungersted et al., 2008). The
derivation of sphingolipids from palmitic acid leads to sphingomyelin production, a critical component of CNS myelin (Baumann and Pham-Dinh, 2001; Wymann and Schneiter, 2008) as well as choline phosphoglyceride, a component of synaptosomes (Koeppen et al., 1973). Oleic acid affects several signaling mechanisms in the CNS including activating isoenzymes of protein kinase C, modulating interactions between benzodiazepines and the GABA receptor, and modulating the effects of toxins on neuroblastomas (Jourdon et al., 1989; Khan et al., 1993; Witt and Nielsen, 1994; Bourre et al., 1997).

As in the periphery, the brain can synthesize most necessary fatty acids. In fact, the brain may be more capable of meeting fatty acid demands by endogenous synthesis. Since studies of dietary oleic fatty acid deprivation showed that while peripheral organs were lacking oleic acid, oleic acid levels were constant in brain, myelin, and nerve endings (Bourre et al., 1997). However, dietary sources of essential fatty acids or their derivatives are critical for brain development. The essential fatty acid derivative DHA, derived from the n-3 PUFA linolenic acid, is particularly important due to its role in neuronal connectivity and photoreceptor membranes (Uauy et al., 2001).

**SREBP regulation of Cholesterol and Fatty Acid Synthesis**

There are two genes encoding SREBPs, *SREBP-1* and *SREBP-2*. In addition differential transcription of *SREBP-1* generates two isoforms, *SREBP-1a* and *SREBP-1c*. These
isoforms differ only in a portion of exon 1 such that \( SREBP-1c \) has a truncated exon 1 (Figure 1-6A) (Brown and Goldstein, 1997). Expression of SREBP-1a is constitutive (Raghow et al., 2008) while SREBP-1c is under LXR control (Whitney et al., 2002). Mechanisms controlling expression of SREBP-2 are not well defined but it is known to have positive feedback regulation due to a SRE in its promoter region (Sato et al., 1996). Cleavage of full length cytosolic SREBPs (cSREBPs), as described previously, leads to cleavage products, nuclear SREBPs (nSREBPs) (Figure 1-6B), that regulate genes containing SRE elements in their promoters (Brown and Goldstein, 1997; Schoonjans et al., 2000; Raghow et al., 2008). All three SREBPs bind efficiently to SRE elements in the promoter region of several enzymes in both the cholesterol synthesis (Figure 1-1) and fatty acid synthesis pathways (Figure 1-2). However, studies of SREBP transgenic knock-in mice showed that relative mRNA levels of fatty acid synthesis enzymes ACC and FAS were more strongly regulated by SREBP-1a than SREBP-2 while cholesterol synthesis enzymes HMG CoA synthase, HMG CoA reductase, and squalene synthase were more strongly regulated by SREBP-2 than SREBP-1a (Horton et al., 1998; Horton and Shimomura, 1999; Horton et al., 2002). However SREBP-2 still increased ACC mRNA levels by more than 5 fold and FAS levels while SREBP-1a increased HMG CoA synthase levels by more than 5 fold, HMG CoA reductase levels by more than 30 fold. SREBP-1c increased levels of enzymes from both pathways including HMG synthase, squalene synthase, ACC and FAS at much lower levels ranging between 1 and 5 fold in comparison to wildtype mice (Horton et al., 1998). Although this study has been used to
classify SREBP-2 as controlling cholesterol synthesis while SREBP-1 controls fatty acid synthesis, there are considerable crossover effects (Figure 1-7).
Figure 1-6. Sterol Regulatory Binding Proteins.
Figure 1-7. Hypothetical Effects of Oxysterols on SREBPs and downstream Cholesterol and Fatty Acid Synthesis Enzymes. Some oxysterols have been shown to inhibit SREBP-2 translocation to the nucleus by immunohistochemical staining. cSREBP-1c is under LXR regulation and several oxysterols have been shown to activate LXR. Although all the enzymes shown are under SREBP control SREBP-1 has a stronger effect on fatty acid synthesis enzymes while SREBP-2 has a stronger effect on cholesterol synthesis enzymes.
Cholesterol and fatty acids are important in brain function. As discussed above, both are important for membrane integrity (Alberts, 1989; Gross et al., 2005; Berg et al., 2007; Ikonen, 2008). They are important for regulating cell signaling at the membrane in lipid rafts as well as intracellular signaling (Alberts, 1989; Fielding and Fielding, 2004; Chen and Bazan, 2005; Gross et al., 2005; Berg et al., 2007; Ikonen, 2008). However their regulation in the brain is not well defined. These mechanisms may be particularly important when there is damage to the brain, either chronic or acute. Both cholesterol and fatty acids have been shown to have effects on the immune system. In the periphery, high cholesterol levels are known to trigger inflammatory mechanisms (Hansson et al., 2006) and cholesterol clearance mechanisms are thought to be anti-inflammatory (Ansell et al., 2007; Navab et al., 2007; Sanossian et al., 2007).

Traumatic brain injury is a model with clinical relevance where inflammation is a major contributor to cellular damage (Dhillon et al., 1994; Dhillon et al., 1999; Gasparovic et al., 2001; Kamada et al., 2003; Kay et al., 2003) and both fatty acid and cholesterol metabolism are known to be altered (Dhillon et al., 1994; Dhillon et al., 1999; Gasparovic et al., 2001; Kamada et al., 2003; Kay et al., 2003).
Traumatic Brain Injury

Traumatic brain injury (TBI) is the cause of more than 50,000 deaths annually in the United States (Langlois et al., 2004). Although many patients survive TBI, it is estimated that 5 million people in the US live with long term disabilities resulting from TBI (NINDS, 2002). Beyond the cellular damage caused by the primary injury seen within minutes to a few hours after impact, TBI also results in many secondary injury effects which occur within hours to days after the impact (Menon and Wheeler, 2005; Morganti-Kossmann et al., 2007). Primary effects include cellular loss from the impact itself as well as loss of ATP, a necessary energy source for many cellular mechanisms, and failure of ionic pumps (Menon and Wheeler, 2005). Secondary effects include swelling of the astrocytic foot processes, contributing to the breakdown of the blood brain barrier (Fitch et al., 1999; Menon and Wheeler, 2005; Morganti-Kossmann et al., 2007). This breakdown allows peripheral macrophages to infiltrate the injury site (Fitch and Silver, 1997). In addition, local astroglia and microglia proliferate and become active (Cernak et al., 2004; Di Giovanni et al., 2005). Both peripheral macrophages and resident microglia release proinflammatory cytokines and promote oxidative stress (Delgado and Ganea, 2003; Langley and Ratan, 2004; Menon and Wheeler, 2005; Morganti-Kossmann et al., 2007). In addition, glial neurotransmitter reuptake is reversed leading to increased extracellular levels of the excitatory amino acid glutamate (Faden and Stoica, 2007; Park et al., 2008). This leads to excitotoxic neuronal death from depolarization of AMPA and
NMDA receptors, leading to excessively high intracellular calcium levels (Faden and Stoica, 2007; Park et al., 2008).

Although cellular necrosis is the cause of primary neuronal loss after TBI, apoptotic mechanisms have also been shown to play a role in secondary neuronal loss in surrounding tissue starting days after the initial injury and lasting for weeks (Langley and Ratan, 2004; Menon and Wheeler, 2005; Faden and Stoica, 2007; Morganti-Kossmann et al., 2007; Park et al., 2008). Long-term effects of TBI may include epilepsy as well as disabilities in cognition, sensory processing, verbal and non-verbal communication, and mental health (NINDS, 2002). In addition TBI increases the risk later in life of neurodegenerative diseases such as Alzheimer’s disease (AD) (Plassman et al., 2000) and Parkinson’s disease (PD) (Goldman et al., 2006). Neurodegeneration beyond the initial impact may be due to the inflammatory processes following TBI (Hoane et al., 2007; Laskowitz et al., 2007; Pan et al., 2007; Zhang et al., 2008), since inflammation contributes to the chronic neurodegeneration seen in AD (Akiyama et al., 2000; Bogdanovic et al., 2001; Fassbender et al., 2001; McGeer and McGeer, 2001; Bonotis et al., 2008; Rojo et al., 2008) and PD (Bar-On et al., 2008; Kim et al., 2008; McGeer and McGeer, 2008; Reynolds et al., 2008).
TBI patients show an increase in cholesterol in the CSF (Kay et al., 2003), most likely from injury induced neuronal damage (Faden, 1996) and the resulting membrane debris (Fagan et al., 1998; Gasparovic et al., 2001; Kamada et al., 2003). Free fatty acids, including palmitic and oleic acids are also increased after TBI, which may also derive from membrane degradation (Dhillon et al., 1994; Dhillon et al., 1999). The increase in essential fatty acid derivatives such as AA can have negative consequences by leading to proinflammatory eicosanoid production while EPA and DHA may be beneficial by promoting to anti-inflammatory eicosanoid production (Fritsche, 2006). In addition, the incorporation of palmitic acid, the final product on acetyl CoA carboxylase and fatty acid synthase activity, has been linked positively to cellular recovery and axonal regeneration after brain injury (Tone et al., 1987; Robinson and Rapoport, 1989). Thus both cholesterol and fatty acid mechanisms may be important factors following TBI.

Overall Significance and Aims
Cyp46 conversion of cholesterol to 24S-hydroxycholesterol is a possible mechanism by which excess cholesterol following TBI could be cleared from the extracellular space. In addition to its LXR effects, 24S-hydroxycholesterol has potential effects on both cholesterol and fatty acid synthesis. The goals of this research are first, to determine whether Cyp46 expression is regulated in response to a moderate TBI and the potential effects on cholesterol efflux mechanisms, and second, to determine what
downstream effects 24S-hydroxycholesterol may have on cholesterol and fatty acid
synthesis and whether these mechanisms are altered after TBI.
CHAPTER II: CORTICAL INJURY INCREASES CHOLESTEROL 24S-HYDROXYLASE LEVELS IN THE RAT BRAIN.

ABSTRACT

In traumatic brain injury (TBI), cellular loss from initial impact as well as secondary neurodegeneration leads to increased cholesterol and lipid debris at the site of injury. Cholesterol accumulation in the periphery can trigger inflammatory mechanisms while cholesterol clearance may be anti-inflammatory. Here we investigated whether TBI altered the regulation of cholesterol 24S-hydroxylase (Cyp46), an enzyme that converts cholesterol to the more hydrophilic 24S-hydroxycholesterol. We examined by western blot and immunohistochemistry changes in Cyp46 expression following fluid percussion injury. Under normal conditions, most Cyp46 was present in neurons, with very little measurable in glia. Cyp46 levels were significantly increased at 7 days post-injury, and cell type specific analysis at 3 days post-injury showed a significant increase in levels of Cyp46 (84%) in microglia. Since 24-hydroxycholesterol induces activation of genes through the liver X receptor (LXR), we examined protein levels of ATP-binding cassette transporter A1 and apolipoprotein E, two LXR regulated cholesterol homeostasis proteins. Apolipoprotein E and ATP-binding cassette transporter A1 were increased at 7 days post-injury, indicating that increased LXR activity coincided with increased Cyp46 levels. We found that activation of primary rat microglia by LPS in vitro caused increased Cyp46 levels. These data suggest that increased microglial Cyp46 activity is part of a system for removal of damaged cell
membranes post-injury, by conversion of cholesterol to 24-hydroxycholesterol and by
activation of LXR-regulated gene transcription.

INTRODUCTION
Traumatic brain injury (TBI) is the cause of more than 50,000 deaths annually in the
United States (Langlois et al., 2004). Although many patients survive TBI, it is
estimated that 5 million people in the US live with long term disabilities resulting from
TBI (NINDS, 2002). Long-term effects may include epilepsy as well as disabilities in
cognition, sensory processing, verbal and non-verbal communication, and mental
health (NINDS, 2002). In addition TBI increases the risk later in life of
neurodegenerative diseases such as Alzheimer’s disease (AD) (Plasman et al., 2000)
and Parkinson’s disease (PD) (Goldman et al., 2006). Neurodegeneration beyond the
initial impact may be due to the inflammatory processes following TBI (Hoane et al.,
2007; Laskowitz et al., 2007; Pan et al., 2007; Zhang et al., 2008), since inflammation
contributes to the chronic neurodegeneration seen in AD (Akiyama et al., 2000;
Bogdanovic et al., 2001; Fassbender et al., 2001; McGeer and McGeer, 2001; Bonotis
et al., 2008; Rojo et al., 2008) and PD (Bar-On et al., 2008; Kim et al., 2008; McGeer
and McGeer, 2008; Reynolds et al., 2008).

In the periphery, high cholesterol levels are known to trigger inflammatory
mechanisms (Hansson et al., 2006) and cholesterol clearance mechanisms are thought
to be anti-inflammatory (Ansell et al., 2007; Navab et al., 2007; Sanossian et al., 2007).

In the central nervous system, cholesterol homeostasis is poorly understood. Although the brain is very cholesterol-rich, cholesterol is not imported from the periphery, but rather synthesized in situ (Jurevics and Morell, 1995; Turley et al., 1996; Turley et al., 1998; Lund et al., 2003; Xie et al., 2003). Cholesterol removal from the brain can occur through several mechanisms. Cholesterol 24S-hydroxylase (Cyp46) is an endoplasmic reticulum associated enzyme responsible for converting cholesterol into 24S-hydroxycholesterol (Lund et al., 1999; Ramirez et al., 2008), which is removed from the CNS to the periphery. 24S-hydroxycholesterol is also an activator of the nuclear transcription factor liver X receptor (LXR) (Lehmann et al., 1997). LXR activation induces the upregulation of genes involved in cholesterol efflux including the ATP-binding cassette transporter A1 (ABCA1) (Fukumoto et al., 2002) and apolipoprotein E (ApoE) (Whitney et al., 2002; Liang et al., 2004). These multiple roles of 24S-hydroxycholesterol emphasize the importance of Cyp46 in maintaining cholesterol homeostasis at both the cellular level and in the brain as a whole.

In both humans and rodents, Cyp46 mRNA is found primarily in the brain with trace amounts found in testis (Lund et al., 1999; Nishimura et al., 2003). Immunostaining of cortex and cerebellum has indicated that Cyp46 is mainly expressed in neurons (Lund et al., 1999; Ramirez et al., 2008). Expression of Cyp46 is first seen at birth and increases rapidly, then remains stable throughout adulthood in both human and rodent
(Lund et al., 1999; Ohyama et al., 2006). The expression of Cyp46 is not affected by a variety of hormones or oxysterols, including 24S-hydroxycholesterol, or by the inhibition of cholesterol synthesis via statins or knockout of the cholesterol synthesis enzyme delta 24-reductase (Ohyama et al., 2006). In contrast, Cyp46 promoter activity in vitro was induced by oxidative stress and a dexamethasone/interleukin-6 treatment (Ohyama et al., 2006).

TBI is an acute form of cellular injury that may necessitate a redistribution of cholesterol due to damage to neuronal membranes and the proliferation of glial cells (Cernak et al., 2004; Di Giovanni et al., 2005). Following TBI, patients show an increase in cholesterol in the CSF (Kay et al., 2003). This excess cholesterol is likely from injury induced neuronal damage and the resulting membrane debris (Faden, 1996; Gasparovic et al., 2001; Kamada et al., 2003). Cyp46 conversion of cholesterol to 24S-hydroxycholesterol is a possible mechanism by which this excess cholesterol could be cleared from the extracellular space. Both ABCA1 and ApoE, cholesterol homeostasis factors downstream of Cyp46 activity, have been shown to increase at the site of acute brain injury (Poirier et al., 1991; Page et al., 1998; Fukumoto et al., 2002). Here we examined Cyp46 expression in response to a moderate TBI model that has been well characterized and contains many of the characteristic of moderate TBI found in clinical settings (Natale et al., 2003; Cernak et al., 2004; Di Giovanni et al., 2005). We found
that Cyp46 expression was upregulated at the site of injury, primarily in activated microglia.

METHODS

Animals:

Twenty-four male Sprague-Dawley rats were used in these experiments. All protocols involving animals were approved by the Georgetown University Institutional Animal Use and Care Committee and were in compliance with the standards stated in the Committee on Care and the Use of Laboratory Animals of the Institution of Laboratory Resources DHEW pub. No. [NIH] 85-23 2985. The CYP46 knock-out mouse was generated by Dr. David Russell and has been previously described (Lund et al., 2003). A targeting vector was used to delete exon 1 of the Cyp46 gene. CYP46 knock-out and wild-type background (C57Bl/6J;129S6/SvEv) control whole brain tissue homogenized in radioimmunoprecipitation assay (RIPA) buffer was generously provided by Dr. Russell.

Rat lateral fluid percussion trauma model: This model has been previously described in detail (Natale et al., 2003). Briefly, rats were anesthetized with sodium pentobarbital and intubated. A 5 mm craniotomy was created between the lambda and bregma sutures over the left parietal cortex where a female luer-loc was cemented in place. An isotonic saline filled fluid percussion device with a 5 mm tube was attached
by means of a male luer-loc fitting. A brief 2.5 atmosphere pressure pulse was given when a pendulum struck a piston at the opposite end of the device. This procedure led to moderate brain injury of parietal cortex as previously defined (Faden et al., 2003). Sham animals received a similarly located craniotomy but no percussion injury. Either 3 days or 7 days after injury or sham surgery animals were anesthetized with sodium pentobarbital. Animals used for immunohistochemistry were sacrificed following perfusion with isotonic saline and then 4% paraformaldehyde (PFA). These brains were frozen and sectioned coronally at 16 µm. There were three animals in each group. Separate animals (3 days or 7 days post-surgery (sham) or post-injury) were sacrificed for western blot. These animals were perfused with saline. Fresh brains were removed and the cortex was dissected out. Parietal cortex on the side of injury (or the sham surgery) was isolated and homogenized in RIPA buffer. There were three animals per group dedicated to this western blot method. In total there were six animals in each group with three being dedicated for immunohistochemistry and three for western blot.

Cell Culture:

Primary rat neurons: Hippocampal neurons were cultured from embryonic day 18-19 Sprague-Dawley rats at 150 cells/ mm² as described previously (Pak et al., 2001). Cells were plated on poly-D-lysine (30 µg/ml) and laminin (2 µg/ml) coated coverslips. Cells were maintained in neurobasal media (Gibco) with B27 (Gibco), 0.5 mM glutamine, and 12.5 µM glutamate until 9 days in vitro (DIV 9) or DIV 16.
**Primary rat microglia:** Cortical and midbrain tissue was taken from P2 rats and dissociated by pipetting in Leibowitz media (Gibco) containing 0.1% bovine serum albumin, 1000 unit/ml penicillin and 1000 µg/ml streptomycin (1x pen/strep) (Gibco). Cells were poured through a 100 µm mesh, spun at 2000 rpm for 5 min, and resuspended in growth media (DMEM high glucose media (Gibco) containing 2mM L-glutamine (Gibco), 1mM sodium pyruvate (Gibco), 1x pen/strep (Gibco) and 10% fetal calf serum (Bio Whittaker)). Cells were plated on uncoated flasks and incubated one week. Media was changed at day 8, 10, and 12 post-plating. On day 15 post-plating flasks were paraffin sealed and shaken at 100 rpm for 1 hr at 37°C. Unattached cells were collected and spun at 2000 rpm for 5 min. Microglia in the cell pellet were resuspended in growth media (DMEM high glucose media (Gibco) with 2mM L-glutamine, 1mM sodium pyruvate, 1x pen/strep and 10% fetal horse serum (Sigma)). Microglia were plated at 200,000 cells per ml onto uncoated coverslips. Purity of cultures was confirmed to be greater than 98% by Ox-42 immunostaining.

**Lipopolysaccharide (LPS) activation of microglia:** LPS (Calbiochem) stock solution was dissolved in distilled water at a concentration of 5 µg/ml. 24 hrs after plating cells, LPS was added to the media at a final concentration of 50 ng/ml. 24 hrs after treatment, cells were fixed in 4% PFA. These LPS treatment experiments were done in triplicate and were repeated on three separate microglial cell isolations.
**Cyp46 analysis:** The human Cyp46 cDNA as well as wildtype and Cyp46 knockout mouse brain lysate were the generous gifts of Dr. David W. Russell (University of Texas Southwestern Medical Center). Cyp46 cDNA was transferred into the pExchange-6a vector (Stratagene) which contains a myc-tag; identity to the published sequence (AF094480) was confirmed by sequencing. COS-7 cells were either plated onto coverslips for immunohistochemistry or onto 6 well plates for western blot and maintained in Opti-Mem media (Gibco) containing 10% fetal bovine serum (Gibco). When cells were 80% confluent, they were transfected with Fugene 6 (Roche) according to manufacturer’s directions, with either the myc-tagged human Cyp46 sequence or the pExchange-6a vector as control. The polyclonal anti-Cyp46 antibody (Nkk2) was produced in the rabbit against the sequence GKDWWQRRREALKRGED which are amino acids 254-270 of the mouse Cyp46 sequence. This antibody was the generous gift of Dr. Suzana Petanceska (Nathan Kline Institute).

**Western Blot:** Cells in culture were washed in PBS and then lysed in RIPA buffer (Pierce Biotechnology) with protease inhibitor (Roche) for protein extraction for 10 min on ice. Lysates were spun at 14,000 rpm for 10 min at room temperature (RT). For rat cortical injury or sham tissue, cortex at the location of the procedure was dissected out and homogenized in RIPA containing protease inhibitor cocktail. Lysates were measured for protein concentration using the Bradford method according to manufacturer’s directions (Biorad). Lysates were mixed 1:1 with loading buffer
(Biorad), heated for 5 min at 95°C, spun at 14,000 rpm for 1 min and loaded at 20 µg/well onto polyacrylamide gels. Protein was then transferred onto PVDF membrane and blocked for 1 hr at RT in 5% milk in PBS. Membranes were probed with anti-Cyp46 antibody (1:1000), ApoE antibody (Abcam, 1:1000), or ABCA1 antibody (Novus, 1:1000) overnight at 4°C. Membranes were washed 3 times for 10 min in PBS and then incubated with goat anti-rabbit HRP tagged antibody at 1:10,000 dilution. Membranes were washed 3 times for 10 min at RT and incubated with DURA (Pierce) for 5 min and exposed to Kodak MR film. Membranes were reprobed with anti-beta-actin antibody (1:5000) to control for protein loading. Bands were quantified using Scion Image. Local background levels were subtracted and levels were adjusted for minor variations in loading controls. ANOVA statistical analysis was performed using Graphpad Prism 4. P values were calculated using the Newman-Keuls Multiple Comparison Test.

**Immunohistochemistry:** Coronal sections were rinsed in PBS twice and incubated in 0.03% hydrogen peroxide for 30 min. Sections were washed 10 times in PBS and blocked 1 hr in 10% fetal bovine serum (Gibco) in PBS with 0.01% Triton X-100 (Fisher) (PBST-100). Slides were incubated overnight with anti-Cyp46 antibody (1:1000). DAB staining was performed using the rabbit ABC kit (Vector Lab). All washes were done 10 times with PBST-100 unless specified otherwise. Slides were washed and incubated for 3 hr at RT with anti-rabbit antibody (1:1000). Slides were
washed, incubated with AB solution for 30 min, washed again, and incubated with DAB solution for 5 min. Finally, they were washed with PBST-100 and then 10 times in PBS. Slides were allowed to dry overnight and then mounted with Permount (Fisher Scientific). Images were taken with a Zeiss Axiophot Microscope.

**Fluorescence Immunostaining:** Cells on coverslips were fixed in 4% PFA in PBS for 10 min at RT, rinsed twice in PBS and stored at 4ºC until further use. Rat brain sections (16 µm) or fixed cells were incubated in PBST-100 for 10 min at RT, washed 10 times with PBS and blocked for 1 hr at RT in 10% fetal bovine serum plus 0.01% sodium azide. Tissue was incubated overnight at 4ºC with anti-Cyp46 antibody (1:1000), washed 10 times in PBS and counterstained with mouse monoclonal antibodies against neuronal marker MAP-2 (1:100, Chemicon), microglial marker Ox42 (1:100, Serotec), astrocytic marker GFAP (1:100, Sigma) or the nuclear marker ToPro-3 (1:1000, Molecular Probes). Tissue was washed 10 times in PBS and incubated 1 hr with Alexa 488 anti-rabbit antibody (1:100, Molecular Probes) and Alexa 546 anti-mouse antibody (1:100, Molecular Probes). Tissue was washed 10 times in PBS and mounted with Fluoramount G (Electron Microscopy Sciences). Staining was visualized with a Zeiss 510 Met confocal laser scanning microscope using an argon ion laser emitting at 488 nm and helium-neon lasers emitting at 543 and 633 nm.
**Relative Cyp46 levels:** For analysis of Cyp46 fluorescent immunostains, a single 1 micron slice at the greatest intensity was obtained for a field of cells using Zeiss LSM software. Signal from Alexa 488 (Cyp46) was converted to grayscale and inverted using Adobe Photoshop. Relative intensity levels at the cell body were measured using a constant sampling area for all cells within an experimental set. An equal number of fields were obtained for each condition. For primary microglial experiments all cells in the field were measured regardless of activation level. For *in vivo* measurements following TBI an equal number of fields were obtained from each animal (n=3). Cell type specific grayscale measurements were guided using a grid and identical fields in color including both Cyp46 signal (Alexa 488, green) and cellular marker (Alexa 546, red). For each cell type measurement all cells of that type were measured. Statistical analysis was performed using Graphpad Prism 4 and Students t-test.

**RESULTS**

To test whether our anti-Cyp46 antibody recognized Cyp46 in immunostains and western blots, we examined COS-7 cells transfected with either control vector or human Cyp46 cDNA. We used western blot to examine whole rat brain lysate as well as COS-7 cells transfected with control vector or myc-tagged Cyp46. Cyp46 is 57 kDa while the myc tagged version of human Cyp46 is expected to be an additional 2 kDa. A Cyp46-reactive band was found at the expected size in rat brain lysate with additional smaller bands (Figure 2-1A, 1st lane). No bands were seen in untransfected COS-7 cells
(Figure 2-1A, 2\textsuperscript{nd} lane), but Cyp46 immunoreactivity was observed at the expected size in Cyp46-myc transfected cells (Figure 2-1A, 3\textsuperscript{rd} lane). We also examined antibody specificity in western blot looking at wildtype versus Cyp46 knockout mouse brain lysates (Figure 2-1B). The expected 57 kDa band was found in wildtype but not Cyp46 knockout brain indicating that this band is authentic Cyp46. Smaller bands similar to those found in rat brain lysate were observed in both wildtype and knockout mouse brains, indicating these bands are unrelated to Cyp46. To test specificity of anti-Cyp46 antibody in immunohistochemistry, we transfected COS-7 cells with either control vector or Cyp46 cDNA. Control cells showed little Cyp46 immunostaining (Figure 2-2A), while Cyp46-transfected cells showed strong immunostaining in over 20% of cells (Figure 2-2B). In order to test whether the anti-Cyp46 antibody recognized endogenous Cyp46, we cultured primary neurons \textit{in vitro} since Cyp46 is primarily expressed in neurons (Lund et al., 1999; Ramirez et al., 2008). In DIV16 primary neurons, we found strong expression of Cyp46 especially at the soma but also continuing throughout the processes (Figure 2-2C). Similar results were seen at DIV9 (data not shown). MAP-2 neuronal staining indicated that there were also non-neuronal cells in the cultures that expressed Cyp46 at much lower levels (Figure 2-2C, arrow). Thus, the anti-Cyp46 antibody displayed specificity in both immunoblots and immunohistochemistry.
Figure 2-1. Cyp46 Antibody recognizes Cyp46 in immunoblots. (A) Cyp46 expression in whole rat brain lysate was compared to COS-7 cells following transfection for 48 hr with control vector or myc-tagged human Cyp46 using immunoblot. (arrow: endogenous Cyp46 57 kD band); (asterix: myc tagged huCyp46 59 kDa band). Molecular weight markers are shown on the right. (B) Cyp46 expression was compared in wildtype mouse versus Cyp46 knock-out mouse whole brain lysate. (arrow: endogenous Cyp46 57 kD band) Molecular weight markers are shown on the right. Both mouse and rat brain lysate show non-specific band below the 57 kD Cyp46 band while these bands were not present in COS-7 cell lysate.
Figure 2-2. Cyp46 Antibody recognizes Cyp46 by immunohistochemistry. Cos7 cells were transfected with either control vector (A) or human Cyp46 (B) for 48 hrs. Immunostaining was performed using anti-Cyp46 antibody and Alexa 488 secondary antibody (green). Cell nuclei were counterstained with Topro-3 (blue), 20x, bars = 100 µm. (C) In rat DIV16 hippocampal neurons, Cyp46 levels (Green) were most pronounced in large, well connected neurons with very high expression in the cytoplasm. Neuronal cells were identified using anti-MAP-2 antibody and Alexa 546 secondary (red). Some MAP-2 negative cells show low levels of Cyp46 expression (arrow), 63x, bar = 20 µm.
Using this anti-Cyp46 antibody, we examined whether Cyp46 expression was altered following an acute brain trauma, using moderate fluid percussion injury of the cortex. Previous studies of microglial phagocytosis of lipids have shown that lipid debris begins to co-localize with microglia 2 days post-injury and reaches a maximum at 7 days post-injury (Gasparovic et al., 2001; Kamada et al., 2003). We therefore investigated possible changes in Cyp46 levels 3 and 7 days after TBI. Sham animals received a craniotomy but no percussion injury. Either 3 days or 7 days after injury animals were sacrificed and perfusion with 4% PFA for immunohistochemistry. Brains were frozen and sectioned coronally at 16 µm. There were three animals in each group.

At 3 days post-injury Cyp46 immunostaining was increased in the cortex at the site of injury in comparison to the contralateral cortex in all three rats at this time point (Figure 2-3A-B). At 7 days post-injury, the site of injury included more cortical area than at 3 days post-injury (data not shown) and Cyp46 levels were again increased in comparison to contralateral cortex, again in all three animals (Figure 2-3C-D). Sham tissue 7 days after craniotomy showed cortical Cyp46 expression consistent with neuronal staining and comparable to that seen in sham tissue 3 days after craniotomy and in contralateral cortex of injured animals (data not shown). Control staining lacking primary antibody showed no signal in sham tissue (data not shown) or at the injury site 7 days post-injury (Figure 2-3F).
Figure 2-3. Cyp46 levels increased at site of injury by immunohistochemistry.
DAB immunostaining against Cyp46 (A) contralateral cortex 3 days post-injury, 20x, bar=100 µm. (B) site of injury 3 days post-injury, 20x, bar=100 µm. (C) Contralateral cortex 7 days post-injury, 20x, bar=100 µm. (D) site of injury 7 days post-injury, 20x, bar=100 µm. Cyp46 levels appeared to be upregulated at both 3 days and 7 days post-injury in comparison to contralateral cortex. (E) Sham cortex 7 days after craniotomy, 20x, bar=100 µm. Expression patterns and levels appeared similar to that seen in contralateral cortex of injured animals. (F) secondary control staining, site of injury 7 days post-injury, 20x, bar=100 µm. No staining was visible with secondary antibody alone.
To quantify cortical changes in Cyp46 levels following TBI, we analyzed lysate of parietal cortex on the side of injury compared to parietal cortex on the side of craniotomy from sham controls. Separate sets of animals (N=3 for each condition) were treated and sacrificed for these western blots. Shams at 3 days and 7 days showed no signs of cortical injury by immunohistochemical staining of coronal sections and were comparable (data not shown). Therefore western blot analysis included only those sham animals sacrificed 3 days after surgery. Cyp46 levels were clearly increased 7 days after injury compared to sham controls (Figure 2-4A). Lower molecular weight bands were observed but showed no changes with brain injury (data not shown). Quantification of band intensity following correction for small variability in loading demonstrated that Cyp46 levels were significantly increased 28% by 7 days post-injury (Figure 2-4B, p<0.05) but that there was no significant change in Cyp46 levels 3 days post-injury.
Figure 2-4. Cyp46 levels increase with injury by immunoblot. (A) 3 days and 7 days after TBI, Cyp46 protein levels increased in comparison to sham controls (upper panel). Beta-actin levels were unchanged (lower panel). (B) Quantification of levels seen in (A) following correction for each beta-actin loading control indicated a significant increase in Cyp46 levels at 7 days (28%) post-injury compared to sham levels according to Newman-Keuls Post-hoc Multiple Comparison test (* p<0.05). Changes between sham and 3 days post injury or 3 days versus 7 days post-injury did not reach significance (overall ANOVA: p=0.0505, F=5.116, df (treatment)= 2, n=3). Error bars (standard error of the mean).
Cyp46 levels were increased locally at 3 and 7 days post-injury (Figure 2-3A-B) and showed significant regional increases at 7 days post-injury (Figure 4B). However, increased Cyp46 levels do not necessarily indicate increased activity. In fact, hepatic P450 enzymes have been shown have decreased activity with increased levels of inflammatory cytokines (Bleau et al., 2000). To determine whether these increases translated into increased LXR activity downstream, we examined levels of ABCA1 and ApoE, two LXR regulated cholesterol homeostasis proteins. Levels of ApoE were increased at 3 days and 7 days post-injury while ABCA1 levels were increased 7 days post-injury (Figure 2-5), indicating that increased Cyp46 enzymatic expression correlates with increased LXR activity following TBI.
**Figure 2-5. ABCA1 and ApoE levels increase with cortical injury.** A) ABCA1 protein levels (upper panel) with corresponding β-actin levels (lower panel). ABCA1 levels increased 7 days (7d) after TBI in comparison to sham controls (S). Each lane represents a separate animal (n=2). B) ApoE protein levels (upper panel) with corresponding β-actin levels (lower panel). ApoE protein levels increased at 3 days (3d) and 7 days (7d) after TBI in comparison to sham controls (S).
Cyp46 expression has been shown to be unaffected by most regulatory axes known to influence cholesterol homeostasis but has been shown to be regulated by oxidative stress and the inflammatory signals *in vitro* (Ohyama et al., 2006). These same signals would also lead to the microglial and astrocytic activation seen following TBI (Morganti-Kossmann et al., 2007). Although Cyp46 expression in normal brain is primarily neuronal, we have found non-neuronal expression of Cyp46 in primary cell culture (Figure 2-2C) and other studies have indicated that astrocytes may express Cyp46 in AD brains (Bogdanovic et al., 2001; Brown III et al., 2004) or following kainate injury (He et al., 2006). In a model of multiple sclerosis (MS) Cyp46 expression was seen in macrophages (Teunissen et al., 2007). To determine whether changes seen with immunohistochemistry may be limited to a subset of cells, we performed co-staining of Cyp46 and cellular markers for neurons, microglia, and astrocytes on coronal sections from rat brain 3 days post-injury. As expected, Cyp46 expression colocalized with Map2 positive neurons in contralateral cortex and at the site of injury (Figure 2-6, row A). In the contralateral cortex, Cyp46 positive cells were neuronal in morphology and were generally evenly spaced, and orientated in alignment with the cortical layers (Figure 2-6, row A, left panel). However, this alignment was disrupted at both the edge and even more at the center of the injury site, while Map2 expression decreased (Figure 2-6, row A, right panel). Very few Ox42 positive microglia showed Cyp46 expression in contralateral cortex (Figure 2-6, row B, left panel). However there was clear colocalization of Cyp46 with microglia at the site of
injury (Figure 2-6, row B, right panel). In addition, the microglia at the site of injury appeared larger and more numerous indicating the activation and proliferation of this cell type at the site of injury. Faint Ox42 expression was seen in contralateral cortex with the star-like staining pattern characteristic of resting microglia (Figure 2-6, row B, left panel). Increased levels of Ox42 staining were seen at the center of the injury site, indicating an increase in microglial activation (Figure 2-6, row B, right panel). There was minimal colocalization of Cyp46 to GFAP positive astrocytes in contralateral cortex (Figure 2-6, row C, left panel). At the site of injury the number of GFAP positive astrocytes was increased indicating activation and proliferation. However, only occasional astrocytes showed Cyp46 positive staining at the site of injury (Figure 2-6, row C, right panel), indicating that astrocytes are only a minor source of Cyp46 expression following injury.
Contralateral Cx  Injury Site

A

B

C
Figure 2-6. Cyp46 expression in neurons, microglia, and astrocytes 3 days following cortical injury. Cyp46 levels in MAP-2 positive neurons (row A), Ox42 positive microglia (row B) or GFAP positive astrocytes (row C) in contralateral cortex (left column) and at the injury site (right column); Cyp46 (Alexa 488, green), MAP-2 (row A), Ox42 (row B) and GFAP (row C) (Alexa 546, red), 63x, bars = 50 µm. While cyp46 expression occurred in neurons in both contralateral cortex and at the site of injury, there was minimal microglial expression of Cyp46 in contralateral cortex but clear colocalization of expression at the site of injury (arrow). Cyp46 expression was not present in contralateral cortex astrocytes. At the site of injury some astrocytes showed Cyp46 positive staining but most did not.
In order to compare cell type specific changes in Cyp46, we measured Cyp46 immunoflorescence intensity levels at the soma of cells in contralateral cortex and central to the site of injury at 3 days post-injury. Because the soma size varied, a constant size sample measurement at the soma was taken rather than a measurement of total Cyp46 expression for the cell. Cells from an equal number of fields (n=4) were measured from each animal (n=3). Although levels tended to decrease, relative levels of Cyp46 did not change significantly in Map2 positive neurons (Figure 2-7A). An 84% increase in relative Cyp46 levels was observed in Ox42 positive microglia in comparison to microglia in contralateral cortex (Figure 2-7B). In GFAP positive astrocytes there was no significant change in relative levels of Cyp46 with injury, although levels tended to increase (Figure 2-7C).
Figure 2-7. Cyp46 levels increase in microglia and astrocytes but not neurons 3 days following cortical injury. Relative levels of Cyp46 at the cell body of cells were compared to those in the contralateral cortex. (A) MAP-2 positive neurons at the site of injury did not show a significant change in Cyp46 levels although levels tended to decrease (contralateral cortex n=139, injury site n=88). (B) Ox42 positive microglia at the site of injury showed an of increased 84% in Cyp46 levels (*, students t-test: p<0.0001, contralateral cortex n=62, injury site n=130). (C) GFAP positive astrocytes at the site of injury did not show a significant change in Cyp46 levels although levels tended to increase (contralateral cortex n=55, injury site n=57). Error bars (standard error of the mean).
We have shown that microglia are a significant source of Cyp46 levels following TBI. However, because one of the main roles of activated microglia is the phagocytosis of cellular debris, we wanted to determine whether activated microglia increase expression of Cyp46 or whether results seen in vivo were due to phagocytosis of the neuronally derived protein. To determine this we activated primary microglial cells with LPS in vitro. In untreated resting microglia we found little Cyp46 immunostaining (Figure 2-8A). After a 24 hour treatment of 50 ng/mL LPS, many microglia showed enlarged soma indicating microglial activation, although not all cells in a field appeared activated with this relatively mild LPS treatment. Based on Topro-3 nuclear staining, cells appeared to cluster and there were more cells in a given field, indicating LPS induced proliferation. An increase in Cyp46 expression was seen following 24 hr LPS treatment, appearing not only in soma of microglia but also continuing into processes (Figure 2-8B). Quantification of relative intensity levels at the soma indicated a 27% increase in relative Cyp46 levels. Because all cells in a field were measured regardless of apparent activation levels this measurement may underestimate increases in Cyp46 with microglial activation.
Figure 2-8. Cyp46 levels increase in activated microglia. Primary rat microglia were either left untreated (A) or treated with 50 ng/ml LPS for 24 hrs (B). LPS resulted in a strong increase in Cyp46 expression (Alexa 488, green), as well as a proliferation of microglial cells. Cell nuclei were counterstained with Topro-3 (blue), 20x, bars = 100µm. (C) Relative levels of Cyp46 at the cell body showed a 28% increase according to Students t-test (*, students t-test: p<0.0001, control n=197, LPS treatment n=269). Error bars (standard error of the mean).
DISCUSSION

In the normal brain, Cyp46 expression is a mechanism for maintaining cholesterol homeostasis (Jurevics and Morell, 1995; Turley et al., 1996; Turley et al., 1998; Lund et al., 2003; Xie et al., 2003). Previous research identified Cyp46 as a neuron specific enzyme under normal conditions (Lund et al., 1999; Bretillon et al., 2007; Ramirez et al., 2008). Neuronal Cyp46 has stable expression, perhaps performing a role in maintaining stable cholesterol levels within the neuronal membrane (Lund et al., 1999; Joseph et al., 2003; Ohyama et al., 2006). However, following brain injury there is significant neuronal loss (Cernak et al., 2004; Di Giovanni et al., 2005; Zhang et al., 2005). We expected that with neuronal death, lipid debris would increase while neuronally expressed Cyp46 would be decreased. Instead, we observed that Cyp46 levels increased after TBI at the site of damage (Figure 2-4). This finding is in line with other models of CNS injury showing increases in Cyp46 within lesion sites (He et al., 2006; Teunissen et al., 2007) and led us to examine Cyp46 in non-neuronal cells.

Previous studies looking at Cyp46 following CNS damage have seen expression in glia, especially in astrocytes. Post-mortem AD brains do not exhibit gross increases in Cyp46 levels but have been shown to have Cyp46 expression in GFAP positive astrocytes not seen in control tissue (Bogdanovic et al., 2001; Brown III et al., 2004). Following hippocampal kainate injury Cyp46 expression was seen in astrocytes at the lesion site but not elsewhere (He et al., 2006). In TBI we did not observe a strong up-
regulation of Cyp46 in neurons or astrocytes but we did observe Cyp46 levels increased 84% in activated microglia (Figure 2-6 and 2-7). Microglia are the immune cells of the CNS (Streit, 2002). At a time when neurons are dying, microglia are activated and proliferating (Kamada et al., 2003; Cernak et al., 2004; Di Giovanni et al., 2005). In addition, a function of microglia following injury is the removal of lipid debris (Gasparovic et al., 2001; Kamada et al., 2003). Thus, increased expression of Cyp46 in microglia may be a necessary step in the processing of phagocytosed lipids. In a rat model of MS, Cyp46 staining has been shown to colocalize to ED1 positive macrophages within brainstem lesions (Teunissen et al., 2007). This expression was attributed to infiltrating peripheral macrophages, however the activation of peripheral peritoneum derived macrophages with LPS in vitro suppressed Cyp46 expression (Teunissen et al., 2007). In contrast, we have shown that brain derived microglia increase Cyp46 expression following LPS activation (Figure 2-8). The consensus based on previous studies and our findings is that both astrocytes and microglia are capable of upregulating Cyp46 expression following neuronal injury. However, after TBI this upregulation appears to be primarily in microglia and not in astrocytes.

There are some indications that very high levels of 24S-hydroxycholesterol might be detrimental. Human control subjects show levels of CSF levels of 24S-hydroxycholesterol levels averaging at 6.7 µM while AD subjects showed levels averaging 8.7 µM (Papassotiropoulos et al., 2002). While in vitro treatments of 50 µM
24S-hydroxycholesterol were found to be toxic to neurons in hippocampal slices, 15 µM treatment, still significantly higher than endogenous levels, did not cause damage (He et al., 2006). In co-cultures of primary human neurons and glia 10 µM 24S-hydroxycholesterol treatments increase inflammatory signals (Alexandrov et al., 2005), possibly due to the induction of changes in cholesterol homeostasis. However, after TBI neuronal membranes have already been disrupted by damage leading to neuronal death and lipid debris (Faden, 1996; Gasparovic et al., 2001; Kamada et al., 2003). Although we did not directly measure Cyp46 activity, we did see an increase in Cyp46 expression in microglia (Figure 2-6) which would be expected to promote the removal of cholesterol debris following TBI.

There are plenty of reasons to believe that increases in Cyp46 expression may be beneficial following brain damage. The effects of Cyp46 are threefold, including clearance of cholesterol by hydroxylation (Lund et al., 2003), clearance of cholesterol via ABCA1 export to lipoproteins (Rebeck, 2004b), and anti-inflammatory and antioxidant effects attributed to LXR regulated genes (Joseph et al., 2003; Walcher et al., 2006), especially ApoE (Hoane et al., 2007; Laskowitz et al., 2007). LXR activation has been shown to suppress inflammatory cytokine production including IL-6 (Joseph et al., 2003; Walcher et al., 2006) as well as iNOS and Cox-2 production (Joseph et al., 2003). ApoE has been shown to have anti-inflammatory effects (Stannard et al., 2001; Guo et al., 2004), possibly due to cholesterol clearance. Peptides
containing the receptor binding domain of ApoE have been shown to be anti-inflammatory (Hoane et al., 2007; Laskowitz et al., 2007), indicating that ApoE may have additional anti-inflammatory properties unrelated to cholesterol clearance. Since ApoE secretion is necessary for cholesterol efflux through ABCA1 (Fagan et al., 1998; Huang et al., 2001; Hirsch-Reinshagen et al., 2005), efflux of cholesterol to lipoproteins would be most effective when both ABCA1 and ApoE are upregulated. We have found that both ApoE and ABCA1 levels increase after TBI indicating that effective lipid efflux is possible (Figure 2-5).

From our studies, we hypothesize that microglial expression of Cyp46 following injury may increase microglial cholesterol efflux following phagocytosis of cellular debris and also may act to reduce inflammatory and oxidative signals regionally. Our results demonstrate that changes in Cyp46 expression are not a marker of any specific neurodegenerative disease; rather, Cyp46 increases are a marker of CNS injury. The signals controlling Cyp46 expression have yet to be fully discerned, but increasing Cyp46 expression would be potentially beneficial following TBI. The effects of these endogenous mechanisms, although positive, may come only after significant neuronal loss has occurred. In addition, oxidative stress, inflammatory events, and glial activation all contribute to neuronal death (Fitch et al., 1999; Langley and Ratan, 2004). By treating TBI early on with moderate levels of 24S-hydroxycholesterol or a synthetic LXR agonist (such as TO901317 or GW-3965), it may be possible to confer
the benefits of cholesterol efflux as well as anti-inflammatory and anti-oxidative properties in advance of microglial activation.
CHAPTER III: 24S-HYDROXYCHOLESTEROL EFFECTS ON LIPID METABOLISM GENES ARE MODELED IN TRAUMATIC BRAIN INJURY.

ABSTRACT

Cholesterol 24S-hydroxylase (Cyp46) is normally expressed in neurons but is increased in microglia after traumatic brain injury (TBI). In this study we take a systematic look at the effects of the enzymatic product of Cyp46, 24S-hydroxycholesterol on SREBP-1 and 2 levels, their post-translational regulation, and the downstream effects on fatty acid and cholesterol synthesis enzymes in cell cultures. This work also examines the effects that TBI induced increases in 24S-hydroxycholesterol may have on these pathways in vivo. 24S-hydroxycholesterol is a potent agonist of the nuclear hormone receptor LXR, which promotes transcription of SREBP-1 but not SREBP-2. Indeed, 24S-hydroxycholesterol increased full length protein and mRNA levels of LXR regulated SREBP-1 but did not change cleavage levels in 293 and SY5Y cells. 24S-hydroxycholesterol decreased levels of SREBP-2 mRNA, full-length protein, and its active cleavage product. In the periphery, SREBP-1 has been shown to primarily regulate transcription of fatty acid synthesis enzymes while SREBP-2 primarily regulates transcription of cholesterol synthesis enzymes. In SY5Y neuroblastomas cells, 24S-hydroxycholesterol decreased mRNA levels of the cholesterol synthesis enzymes HMG CoA reductase, squalene synthase, and FPP synthase, but did not alter levels of acetyl CoA carboxylase or fatty acid synthase. In
contrast, in BV2 microglia, 24S-hydroxycholesterol had no significant effect on HMG CoA reductase or squalene synthase but significantly increased mRNA levels of acetyl CoA carboxylase and fatty acid synthase. After TBI, as after 24S-hydroxycholesterol treatment \textit{in vivo}, we found that SREBP-1 mRNA levels were increased while SREBP-2 mRNA levels were decreased. mRNA levels of HMG CoA reductase and squalene synthase were significantly decreased. Fatty acid synthase mRNA levels were not altered but acetyl CoA carboxylase mRNA levels were significantly decreased. Thus, changes to transcription of cholesterol synthesis genes after TBI are consistent with increases in Cyp46 activity, but changes to fatty acid synthesis genes must be regulated by other mechanisms.

\textbf{INTRODUCTION}

Although much is known about regulation of cholesterol in cells in the periphery, less is known about its control in the central nervous system. Cholesterol 24S-hydroxylase (Cyp46) is a brain enriched enzyme expressed primarily in neurons and is responsible for converting cholesterol from the plasma membrane into 24S-hydroxycholesterol (Lund et al., 1999; Ramirez et al., 2008). Expression of Cyp46 in the brain is altered during normal development but is relatively stable in normal adult brain (Lund et al., 1999; Ohyama et al., 2006). Cyp46 is also increased after traumatic brain injury (TBI), specifically in microglia (Cartagena et al., 2008). Other models of injury including hippocampal kainate injury (He et al., 2006) and acute experimental autoimmune
encephalomyelitis (Teunissen et al., 2007) have shown increases in Cyp46 expression at lesion sites. The enzymatic product of Cyp46, 24S-hydroxycholesterol, is increased in the CSF of Alzheimer’s disease patients compared to control subjects (Papassotiropoulos et al., 2002). These findings suggest that while Cyp46 activity in neurons plays a critical role in normal cholesterol homeostasis, increased Cyp46 activity may be a characteristic of the brain response to injury.

24S-hydroxycholesterol is an activator of the nuclear transcription factors liver X receptor (LXR) α and β (Lehmann et al., 1997; Janowski et al., 1999) and can induce the upregulation of genes involved in cholesterol efflux (Rebeck, 2004a; Tall, 2008). LXR activation has been shown to increase expression of genes important for cholesterol efflux such as ATP-binding cassette transporter (ABC) A1 in both neurons and glia (Fukumoto et al., 2002) and expression of Apolipoprotein (Apo) E in astrocytes (Liang et al., 2004). We have shown increases in both ApoE and ABCA1 coinciding with increased Cyp46 expression after TBI (Cartagena et al., 2008). When both ApoE and ABCA1 are present, cholesterol can be passed from the cell to extracellular lipoproteins by cholesterol efflux (Huang et al., 2001; Hirsch-Reinshagen et al., 2005). These cholesterol efflux mechanisms are important for lowering cholesterol levels when cellular cholesterol is in excess. In addition, the conversion of cholesterol to 24S-hydroxycholesterol makes the molecule more soluble and easier to clear into the extracellular space and across the blood brain barrier into the blood
(Bjorkhem, 2006). Thus, 24S-hydroxycholesterol allows for two separate mechanisms for clearing cholesterol from the cell and from the brain overall.

In addition to its roles in cholesterol efflux, 24S-hydroxycholesterol potentially plays a role in regulating the cholesterol and fatty acid synthesis pathways in the brain. Oxysterols alter cholesterol synthesis mechanisms by acting on sterol regulatory element binding proteins (SREBPs). SREBPs are expressed as inactive 120 kDa cytosolic precursors (cSREBP) which are integral to the endoplasmic reticulum (ER) membrane. cSREBPs are translocated from the ER to the Golgi by SREBP cleavage-activating protein (SCAP) where they are cleaved into a 67 kDa active transcription factor which is not membrane bound. This shorter nuclear SREBP (nSREBP) alters transcription of genes containing a sterol regulatory element in the promoter region. These genes are responsible for critical enzymes in both the cholesterol synthesis pathway and the fatty acid synthesis pathway. Peripheral oxysterols such as 25-hydroxycholesterol have been shown to suppress the cleavage of cSREBP to nSREBP (Thewke et al., 1998; Adams et al., 2004; Du et al., 2004), perhaps by retaining SCAP in the ER and thus preventing cSREBP translocation to the Golgi for cleavage (Radhakrishnan et al., 2007).
There are two genes for SREBPs giving rise to the three isoforms: 1a, 1c, and 2. Isoform SREBP-1c differs from SREBP-1a only in a small portion of exon 1 but they are otherwise identical (Brown and Goldstein, 1997). SREBP-1a is constitutively expressed (Raghow et al., 2008). SREBP-2 has been shown to contain a sterol regulatory element (SRE) in its promoter region which when bound increased transcription of SREBP-2 (Sato et al., 1996). Interestingly, isoform SREBP-1c has been shown to be under LXR control (Whitney et al., 2002) and also contains a SRE in its promoter region leading to positive feedback regulation (Cagen et al., 2005). While all three isoforms bind sterol regulatory elements when cleaved to their active nuclear form, nSREBP-1a preferentially activates fatty acid synthesis while nSREBP-2 preferentially activates cholesterol synthesis in the liver (Horton et al., 1998; Horton and Shimomura, 1999).

Various oxysterols differ in their biological effects (Gill et al., 2008). For example, 27-hydroxycholesterol is an LXR agonist (Fu et al., 2001), while 25-hydroxycholesterol has only minimal LXR activity (Janowski et al., 1999). 24S-hydroxycholesterol is the main oxysterol in the brain (Dietschy and Turley, 2004; Karu et al., 2007). In glia, 24S-hydroxycholesterol decreased expression of the rate limiting step in cholesterol synthesis, HMG CoA reductase, and increased LXR regulated ApoE expression (Abildayeva et al., 2006). In cortical neurons, 24S-hydroxycholesterol decreased expression of cholesterol synthesis enzymes including HMG CoA synthase and
squalene synthase (Wang et al., 2008). In this study we take a systematic look at 24S-hydroxycholesterol effects on both SREBP-1 and 2 levels, post-translational regulation and downstream effects on fatty acid and cholesterol synthesis enzymes. This work also examines the effects TBI induced increases in 24S-hydroxycholesterol may have on these two pathways in vivo.

METHODS

Chemicals

Stock solutions of 2 mM TO-901317 (Tocris), 10 mM 24S-hydroxycholesterol (Steraloids), 10 mM 27-hydroxycholesterol (Steraloids) and 10 mM Cholesterol (Sigma) were all made in ethanol and stored at -20 °C. These were diluted in media at 0.5µl/mL immediately before treating cells.

Cell Culture

293 cells were cultured in Opti-MEM (Gibco) containing 10% fetal bovine serum (FBS) (Gibco). SY5Y cells were cultured in DMEM (ATCC) containing 10% FBS. BV2 cells were cultured in Opti-MEM containing 5% FBS. Cells were plated into 6 well plates. After 24 h cells media was removed and replaced with fresh media containing either 5 µM 24S-hydroxycholesterol or 0.5 µl/mL ethanol (vehicle). Cells were treated for 48 h and then collected for either protein or mRNA isolation. Cell culture experiments were performed in triplicate on at least two separate occasions.
Animals

Ten male 4 month old C57BL/6J mice were used in these experiments. All animal protocols were approved by the Georgetown University Institutional Animal Use and Care Committee, and were in compliance with the standards stated in the Committee on Care and the Use of Laboratory Animals Publication [NIH] 85-23 1985.

Mouse Controlled Cortical Impact (CCI) Trauma Model

The CCI-injury device was designed and built at the Georgetown University, and consists of a microprocessor-controlled pneumatic impactor with a 3.5 mm diameter tip (Fox et al., 1998). Moderate injury was induced by the impactor velocity of 6 m/s and deformation depth of 2 mm. Mice were anaesthetized with isoflurane (induction at 4% and maintenance at 2%) evaporated in a gas mixture containing 70% N$_2$ and 30% O$_2$ and administered through a nose mask. Depth of anesthesia was assessed by monitoring respiration rate and pedal withdrawal reflexes. The mouse was placed on a heated pad, and a core body temperature was maintained at 37°C. The head was mounted in a stereotaxic frame, and the surgical site was clipped and cleaned with Nolvasan scrubs. A 10-mm midline incision was made over the skull, the skin and fascia were reflected, and a 4-mm craniotomy was made on the central aspect of the left parietal bone. The impounder tip of the injury device was then extended to its full stroke distance (44 mm), positioned to the surface of the exposed dura, and reset to
impact the cortical surface. After injury, the incision was closed with interrupted 6-0 silk sutures, anesthesia was terminated, and the animal was placed into a heated cage to maintain normal core temperature for 45 min post-injury. All animals were monitored carefully for at least 4 h after surgery and then daily. Five animals underwent CCI-injury. An additional five animals underwent sham injury where a 4-mm craniotomy was made but the dura was not disturbed. At day 7 after surgeries, animals were sacrificed with CO$_2$ and brains were removed. Isolated cortex was collected from the injury site as well as from the contralateral side. In sham animals, cortex at the site of craniotomy was collected. Tissue was snap frozen in isopentane on dry ice and stored at -80°C until later RNA isolation.

*Western blot*

Cells were washed in phosphate buffered saline (PBS), lysed in RIPA buffer (Pierce) containing protease inhibitor (Roche). Protein concentrations in lysates were measured by the Bradford method (Biorad). 20 µg total protein from each sample were loaded onto polyacrylamide gels. Protein was transferred to PVDF membranes and blocked 1 h at room temperature (RT) in 5% milk in PBS. Membranes were probed with anti-SREBP-1 (Abcam, 1:100) or anti-SREBP-2 (BD Biosource, 1:100) overnight at 4°C. Membranes were washed in PBS and incubated with goat anti-mouse HRP tagged antibody at 1:10:000. Membranes were washed in PBS and incubated with DURA (Pierce) and exposed to Kodak MR film. Membranes were reprobed with anti-β-actin antibody (1:5000) for protein loading controls. Bands were quantified using Quantity 1
software (Biorad). Local backgrounds were subtracted and levels were adjusted for small variations in loading controls. Statistical analysis was performed using Graphpad Prism 4 and either one way ANOVA with Newman-Keuls multiple comparison post-hoc test or Student’s t-test.

mRNA isolation

Total RNA was isolated using the Absolute RNA Miniprep purification kit (Stratagene) according to manufacturer’s directions from either cell culture or mouse cortical brain tissue. Briefly, cultured cells in 6-well plates were lysed in 400 µl/well manufacturer’s lysis buffer and mouse hemi-cortex was lysed in 1 mL lysis buffer. Lysate was loaded onto a prefilter column and spun at 14,000 rpm. Filtrate was mixed 1:1 with 70% ethanol, loaded onto a RNA binding column and spun at 14,000 rpm. The column was washed with low salt buffer and treated for 15 min with DNase at 37ºC. The column was washed in high salt and then low salt buffers. 60ºC elution buffer was added to the column and spun at 14,000 rpm to collect purified RNA. Total RNA concentrations and purity were calculated from spectrophotometer optical density measurements at 260 and 280 nm. RNA was stored at -80ºC until further use.

cDNA synthesis

cDNA was synthesized using the AffinityScript QPCR cDNA Synthesis kit (Stratagene). Briefly, 20 µl reactions contained 3 µg total RNA, 0.3 µg oligo(dT)
primers, 10 µl 2x mastermix and 1 µl AffinityScript RT/RNAsen Block enzyme mixture. The reaction was incubated at 25ºC for 5 min, 42ºC for 45 min, 95ºC for 5 min, 4ºC for 5 min, and then stored at -20ºC until further use.

**Primer design**

All primers were designed using Geneious Pro 3.0.6 software (Biomatters). All primers were designed to cross at least two exons. Primers used in semi-quantitative PCR are listed in Table 1. Primers to human sequences used in real-time PCR are listed in Table 2 while primers to mouse sequences are listed in Table 3. Real-time primers were designed to have zero primer-dimers and to produce product sizes between 120-150 base pairs. Real-time primers were tested using 1:2, 1:4, and 1:8 serial dilutions of cDNA to confirm efficiency of the primer pair. A dissociation curve was performed to confirm the absence of any primer-dimer pair products.
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Table 3-1. Primers used in semi-quantitative PCR.
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<td>GAACCTGTCGGGCTATTCAGG</td>
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<td>CCTCCTTCCTGGCCGTAATTT</td>
</tr>
<tr>
<td>acetyl CoA carboxylase</td>
<td>TGACAGAGGAGGTGGTGTTCC</td>
<td>GCTGAGTGGGTATATGTGTGCT</td>
</tr>
<tr>
<td>fatty acid synthase</td>
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<td>GTGGATGATGCTGATGATG</td>
</tr>
<tr>
<td>β-actin</td>
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Table 3-2. Human primers used in real-time PCR.
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Table 3-3. Murine primers used in real-time PCR.
**Semi-quantitative PCR**

Amplifications of cDNA were performed with a thermocycler using GoTaq Green (Promega) mastermix. Reactions were incubated at 95°C for 2 min, then 30 cycles of 95°C for 15 sec, 58°C for 30 sec and 72°C for 30 sec, and then 72°C for 5 min. Product was run on 2% agarose gels and bands were digitally imaged, inverted and quantified using Quantity 1 (BioRad) software. Statistical analysis was performed using Graphpad Prism 4.

**Real-time PCR**

Relative mRNA quantities were measured by real-time PCR using PowerSYBR green (Applied Biosystems) mastermix and primers listed in Table 2 and Table 3. Amplifications were performed with an ABI 7900 HT sequence detection system and were incubated at 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 sec, 58°C for 1 min and 72°C for 30 sec. Each sample was tested in triplicate. β-actin was used as an endogenous control to calculate ΔΔCt levels and relative quantities for each sample. Analysis of real-time amplification data was done using SDS 2.3 (Applied Biosystems) and relative quantities were calculated using RQ Manager software (Applied Biosystems). Statistical analysis was performed using Graphpad Prism 4 and either one-way ANOVA with Newman-Keuls multiple comparison post-hoc test or Student’s t-test.
RESULTS

Although oxysterols are generally considered to inhibit cleavage of cSREBPs to produce nuclear transcription factors (Gimpl et al., 2002; Radhakrishnan et al., 2007; Gill et al., 2008) they have differential effects on mechanisms of gene regulation through LXR activation (Janowski et al., 1999; Fu et al., 2001).

We sought to demonstrate what effects the brain-derived 24S-hydroxycholesterol may have on gene transcription mechanisms. We began by analyzing levels of full-length and cleaved SREBP-1 and 2 in human 293 cells. We treated cells with 5 µM 24S-hydroxycholesterol for 48 hr and analyzed cell lysate by western blot (5 µM is approximately the concentration of 24S-hydroxycholesterol in the CSF (Papassotiropoulos et al., 2002)). The antibody to SREBP-1 demonstrated bands at 120 kD and 67 kD corresponding to the expected sizes of cSREBP-1 and nSREBP-1; similar sized bands were seen in membranes probed for SREBP-2 (Figure 3-1A). 24S-hydroxycholesterol increased cSREBP-1 levels while nSREBP-1 levels remained unchanged (Figure 3-1A, 1st and 2nd panel). The SREBP-1 antibody recognizes both SREBP-1a and 1c. However, since SREBP-1a is thought to be constitutively expressed (Raghow et al., 2008), we attribute the observed increases in SREBP-1 levels to upregulation of cSREBP-1c. In contrast to SREBP-1, both cSREBP-2 and nSREBP-2 were suppressed by 24S-hydroxycholesterol treatment (Figure 3-1A, 3rd and 4th panel). Quantification of cSREBP-1 levels showed an increase of 38% after 24S-hydroxycholesterol treatment while there were no significant changes in nSREBP-1
levels (Figure 3-1B). Quantification of cSREBP-2 levels showed a decrease of 51% after 24S-hydroxycholesterol treatment while nSREBP-2 levels showed a decrease of 91% (Figure 3-1C).
A.  
\begin{align*}
\text{cSREBP-1} & \quad \text{C} \quad \text{24} \quad \text{C} \quad \text{24} \\
\text{nSREBP-1} & \quad \text{120 kDa} \\
\text{cSREBP-2} & \quad \text{67 kDa} \\
\text{nSREBP-2} & \quad \text{120 kDa} \\
\end{align*}

B.  
\begin{align*}
\text{cSREBP-1} & \quad \% \text{ Control} \\
\text{Control} & \quad 100 \\
\text{5 } \mu \text{M 24S} & \quad 150 \quad \ast \\
\text{nSREBP-1} & \quad \% \text{ Control} \\
\text{Control} & \quad 100 \\
\text{5 } \mu \text{M 24S} & \quad 50 \\
\end{align*}

C.  
\begin{align*}
\text{cSREBP-2} & \quad \% \text{ Control} \\
\text{Control} & \quad 150 \quad \ast \\
\text{5 } \mu \text{M 24S} & \quad 50 \\
\text{nSREBP-2} & \quad \% \text{ Control} \\
\text{Control} & \quad 150 \\
\text{5 } \mu \text{M 24S} & \quad \ast \\
\end{align*}
Figure 3-1. 24S-hydroxycholesterol increases SREBP-1 protein levels while suppressing SREBP-2 levels in immunoblot. 293 cells were treated for 48 hr with 5µM 24S-hydroxycholesterol (24) or control vehicle (C). A) Levels of cytosolic SREBP-1 (cSREBP-1) and nuclear SREBP-1 (nSREBP) as well as cytosolic SREBP-2 (cSREBP-2) and nuclear SREBP-2 (nSREBP-2) were measured in cell lysate by immunoblot. B) Quantification of cSREBP-1 levels showed an increase of 38% after 24S-hydroxycholesterol treatment (Student’s t-test, *p<0.05, n=8) while there were no significant changes in nSREBP-1 levels. C) Quantification of cSREBP-2 levels showed a decrease of 51% after 24S-hydroxycholesterol treatment (Student’s t-test, *p<0.0001, n=7) while nSREBP-2 levels showed a decrease of 91% (Student’s t-test, *p<0.0001, n=7). Error bars (standard error of the mean).
We sought to determine whether the observed effects of 24S-hydroxycholesterol could be at least partially due to changes in mRNA levels of both SREBP-1 and 2. Thus, we treated 293 cells with 5 µM 24S-hydroxycholesterol or 1 µM TO-901317 (an LXR agonist) for 48 hr and isolated RNA. Levels of SREBP-1 mRNA showed an increase following 24S-hydroxycholesterol and a greater increase following TO-901317 treatment (3-2A). Semi-quantitative measurements of bands showed an increase of 188% in SREBP-1 mRNA levels following 24S-hydroxycholesterol treatment and an increase of 331% following TO-90137 treatment (Figure 3-2B, left panel). Levels of SREBP-2 showed no significant change following either 24S-hydroxycholesterol or TO-901317 treatment (Figure 3-2B, right panel). We used the more quantitative real-time PCR to test these findings. Analysis of SREBP-1 mRNA levels showed significant increases of 280% with 24S-hydroxycholesterol treatment and 370% with TO-901317 treatment (Figure 3-2C). Analysis of relative quantities of SREBP-2 mRNA levels showed a small but significant decrease of 18% with 24S-hydroxycholesterol treatment and no significant change with TO-901317 treatment (Figure 3-2C).
Figure 3-2. 24S-hydroxycholesterol and LXR agonist TO-901317 both increases SREBP-1 mRNA levels but not SREBP-2 levels in semi-quantitative PCR. 293 cells were treated for 48 hr with 5 μM 24S-hydroxycholesterol (24), 1 μM TO-901317 (TO) or control vehicle (C). mRNA from these cells was converted to cDNA and amplified using primers against either SREBP-1 or SREBP-2. A) Relative levels of SREBP-1 mRNA showed a clear increase following 24S-hydroxycholesterol and a greater increase following TO-901317 treatment. B) Semi-quantitative measurements of bands seen in A showed an increase of 188% in SREBP-1 mRNA levels following 24S-hydroxycholesterol treatment (Newman-Keuls, *p<0.05, n=6) and an increase of 331% following TO-901317 treatment (left panel) (Newman-Keuls, *p<0.05, n=6). Semi-quantitative measurements of SREBP-2 mRNA levels (right panel) by showed no significant change following 24S-hydroxycholesterol or TO-901317 treatment (n=6). C) Real-time PCR quantification of SREBP-1 mRNA levels showed an increase of 280% with 24S-hydroxycholesterol treatment (Newman-Keuls, *p<0.001, n=6) and an increase of 370% with TO-901317 treatment (Newman-Keuls, *p<0.001, n=6). Real-time PCR quantification of SREBP-2 mRNA levels showed a decrease of 18% with 24S-hydroxycholesterol treatment (Newman-Keuls, *p<0.05, n=6) but no significant change with TO-901317 treatment. Control relative quantity=1. Error bars (standard error of the mean).
To determine whether the effects of 24S-hydroxycholesterol on SREBP-2 are specific to 24S-hydroxycholesterol, we treated 293 cells with 1 µM TO-901317, 5 µM 24S-hydroxycholesterol, 5 µM 27-hydroxycholesterol, or 5 µM cholesterol for 48 hr. Levels of both cSREBP-2 and nSREBP-2 were measured by western blot (Figure 3-3A).

cSREBP-2 levels showed a decrease of 50% with 24S-hydroxycholesterol treatment while 27-hydroxycholesterol caused a 36% but non-significant decrease in cSREBP-2 levels (Figure 3-3B). Neither cholesterol nor TO-901317 had a significant effect on cSREBP-2 levels. nSREBP-2 levels showed significant decreases of 84% with 24S-hydroxycholesterol treatment and 80% with 27-hydroxycholesterol treatment (Figure 3-3C). TO-901317 decreased nSREBP-2 levels by 36%, a significantly smaller decrease than seen with the oxysterols (Figure 3-3C). Cholesterol did not have a significant effect on either levels of nSREBP-2 levels or cSREBP-2 (Figure 3-3B and C).
A

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<th></th>
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<th>T</th>
<th>24</th>
<th>27</th>
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B

![cSREBP-2 Bar Graph](image)

C

![nSREBP-2 Bar Graph](image)
Figure 3-3. 24S-hydroxycholesterol but not cholesterol or TO-901317 suppresses SREBP-2 protein levels while oxysterols and TO-901317 suppress SREBP-2 cleavage. 293 cells were treated for 48 hr with 5 µM 24S-hydroxycholesterol (24), 5 µM 27-hydroxycholesterol (27), 5 µM cholesterol (Ch), 1 µM TO-901317 (T) or control vehicle (C). A) Levels of cytosolic SREBP-2 (cSREBP-2) and nuclear SREBP-2 (nSREBP-2) were measured in cell lysate by immunoblot. B) Quantification of cSREBP-2 levels showed a decrease of 50% with 24S-hydroxycholesterol treatment (Newman-Keuls, *p<0.001, n=6) while 27-hydroxycholesterol failed to significantly decrease cSREBP-2 levels. Neither cholesterol nor TO-901317 had a significant effect on cSREBP-2 levels. C) nSREBP-2 levels showed a decrease of 84% with 24S-hydroxycholesterol treatment (Newman-Keuls, *p<0.001, n=6) and a decrease of 80% with 27-hydroxycholesterol treatment (Newman-Keuls, *p<0.001, n=6). TO-901317 also decreased nSREBP-2 levels by 36% (Newman-Keuls, *p<0.05, n=6). Decreases in nSREBP-2 levels by 24S-hydroxycholesterol and 27-hydroxycholesterol were significantly lower than those seen with TO-901317 (Newman-Keuls, #p<0.01, n=6). Cholesterol did not have a significant effect on nSREBP-2 levels. Error bars (standard error of the mean).
24S-hydroxycholesterol is produced primarily by neurons (Lund et al., 1999; Lund et al., 2003; Ramirez et al., 2008). Although 293 cells display many properties of immature neurons (Shaw et al., 2002), we wanted to determine the effects of 24S-hydroxycholesterol on SREBP-1 and 2 levels in a brain derived cell line with more established neuronal characteristics. We therefore treated SY5Y cells with 5 µM 24S-hydroxycholesterol. Levels of SREBP-1 and 2 showed similar patterns as those seen in 293 cells (Figure 3-4). cSREBP-1 levels were increased with 24S-hydroxycholesterol treatment (Figure 3-4, row 1) while there were no clear changes in nSREBP-1 levels (Figure 3-4, row 2). cSREBP-2 levels were suppressed with 24S-hydroxycholesterol treatment while nSREBP-2 levels were virtually eliminated. We also measured mRNA levels of SREBP-1 and SREBP-2 by real-time PCR. 24S-hydroxycholesterol significantly increased SREBP-1 mRNA levels by 210% and significantly decreased SREBP-2 mRNA levels by 31% (Figure 3-5A).
Figure 3-4. 24S-hydroxycholesterol increases SREBP-1 protein levels while suppressing SREBP-2 levels in SY5Y cells in immunoblot. SY5Y cells were treated for 48 hr with 5µM 24S-hydroxycholesterol (24S) or control vehicle (C). Results seen in this representative blot were similar to those seen in 293 cells. cSREBP-1 levels were clearly increased while nSREBP-1 levels remained relatively stable with 24S-hydroxycholesterol treatment. Both cSREBP-2 and nSREBP-2 were suppressed by 24S-hydroxycholesterol treatment.
Figure 3-5. Effects of 24S-hydroxycholesterol on cholesterol and fatty acid synthesis enzymes in human neuroblastoma SY5Y cells. SY5Y cells were treated for 48 hr with 5 µM 24S-hydroxycholesterol or vehicle control and mRNA levels were measured by real-time PCR. A) 24S-hydroxycholesterol increased SREBP-1 mRNA levels by 210% (student’s t-test, *p<0.001, n=6) and decreased SREBP-2 mRNA levels by 31% (student’s t-test, *p<0.001, n=6) in comparison to controls. B) 24S-hydroxycholesterol decreased mRNA levels of the rate limiting cholesterol synthesis enzyme HMG CoA reductase by 18% (student’s t-test, *p<0.005, n=6), decreased squalene synthase by 31% (student’s t-test, *p<0.005, n=6) and FPP synthase by 17% (student’s t-test, *p<0.01, n=6). C) 24S-hydroxycholesterol did not significantly change mRNA levels fatty acid synthesis enzymes acetyl CoA carboxylase or fatty acid synthase in comparison to controls. Control relative quantity=1. Error bars (standard error of the mean).
SREBPs have been shown to regulate the transcription of enzymes in both the cholesterol and fatty acid synthesis pathways. Previous studies have indicated nSREBP-2 preferentially upregulates enzymes in the cholesterol synthesis pathway while nSREBP-1 has greater control over fatty acid synthesis enzymes (Horton et al., 1998). Because 24-hydroxycholesterol has effects on both SREBP-1 and 2, we wanted to determine its overall effects on the cholesterol synthesis and fatty acid synthesis pathways. To do this, we used real-time PCR to measure relative quantities of mRNA for cholesterol synthesis enzymes (HMG CoA reductase, squalene synthase, and FPP synthase) and fatty acid synthesis enzymes (acetyl CoA carboxylase and fatty acid synthase). 24S-hydroxycholesterol significantly decreased mRNA levels of all three cholesterol synthesis genes: HMG CoA reductase by 18%, squalene synthase by 31%, and FPP synthase 17% (Figure 3-5B). In contrast, 24S-hydroxycholesterol did not significantly change mRNA levels of either of the fatty acid synthesis genes, acetyl CoA carboxylase or fatty acid synthase (Figure 3-5C). Thus, in SY5Y cells, 24S-hydroxycholesterol decreased transcription of cholesterol synthesis genes but did not affect genes involved in fatty acid synthesis.

We previously found that levels of Cyp46, which produces 24S-hydroxycholesterol, is strongly upregulated in microglia following TBI (Cartagena et al., 2008). In order to determine what effects 24S-hydroxycholesterol may have in microglial regulation of cholesterol and fatty acid synthesis, we treated a mouse microglial cell line, BV2, with
5 µM 24S-hydroxycholesterol and measured mRNA levels by real-time PCR. As in 293 and SY5Y cells, 24S-hydroxycholesterol significantly increased SREBP-1 mRNA levels by 20% and significantly decreased SREBP-2 mRNA levels by 15% (Figure 3-6A). In contrast to SY5Y cells, 24S-hydroxycholesterol did not significantly alter either HMG CoA reductase or squalene synthase levels in BV2 cells (Figure 3-6B) while 24S-hydroxycholesterol treatment increased acetyl CoA carboxylase mRNA levels by 70% and fatty acid synthase mRNA levels by 60% (Figure 3-6C).
Figure 3-6. Effects of 24S-hydroxycholesterol on cholesterol and fatty acid synthesis enzymes in mouse microglial BV2 cells. BV2 cells were treated for 48 hr with 5 µM 24S-hydroxycholesterol or vehicle control and mRNA levels were measured by real-time PCR. A) 24S-hydroxycholesterol increased SREBP-1 mRNA levels by 20% (student’s t-test, *p<0.0001) and decreased SREBP-2 mRNA levels by 15% (student’s t-test, *p<0.0005) in comparison to controls. B) 24S-hydroxycholesterol failed to decrease mRNA levels of HMG CoA reductase or squalene synthase. Control relative quantity=1. Error bars (standard error of the mean). C) 24S-hydroxycholesterol increased mRNA levels of the rate limiting fatty acid synthesis enzyme acetyl CoA carboxylase by 70% (student’s t-test, *p<0.0005) and fatty acid synthase by 60% (student’s t-test, *p<0.01).
Thus, in vitro, 24S-hydroxycholesterol strongly suppresses SREBP-2 levels and the expression of cholesterol synthesis genes but fails to suppress SREBP-1 levels and fatty acid synthesis genes. In fact, in microglia, 24S-hydroxycholesterol upregulated genes involved in fatty acid synthesis, acetyl CoA carboxylase and fatty acid synthase. Since Cyp46 is upregulated following TBI (Cartagena et al., 2008), subsequent changes in 24S-hydroxycholesterol levels may alter both cholesterol and fatty acid synthesis levels. We examined mRNA in cortex of TBI mice and found that SREBP-1 mRNA levels were significantly increased by 86% at the site of injury (Figure 3-7A) and SREBP-2 mRNA levels were significantly suppressed by 43% at the site of injury (Figure 3-7B) in comparison to sham control cortex. There were no differences in either SREBP-1 or 2 between samples from brain contralateral to the TBI (Figure 3-7).
Figure 3-7. Regulation of SREBP-1 and SREBP-2 mRNA levels 7 days after TBI. SREBP-1 and SREBP-2 mRNA levels were measured by real-time PCR in cortex ipsilateral to the site of injury 7 days after TBI (tbi icx), contralateral cortex (tbi ccx) and sham cortex ipsilateral to craniotomy (sham icx). A) SREBP-1 mRNA levels at the site of injury were significantly increased 86% in comparison to sham controls (Newman-Keuls, *p<0.01, n=5). B) SREBP-2 mRNA levels at the site of injury were significantly decreased 43% in comparison to sham controls (Newman-Keuls, *p<0.05, n=5).
As we had previously done *in vitro*, we wanted to determine what overall effects of altered SREBP-1 and 2 mRNA levels may be on the cholesterol synthesis and fatty acid synthesis pathways *in vivo*. To do this, we used real-time PCR to measure relative quantities of mRNA for the cholesterol synthesis enzymes HMG CoA reductase and squalene synthase and fatty acid synthesis enzymes acetyl CoA carboxylase and fatty acid synthase. Following TBI, we found that mRNA levels of HMG CoA reductase were significantly decreased by 38% and squalene synthase mRNA levels were significantly decreased by 33% at the site of injury in comparison to sham cortex (Figure 3-8A). Despite the increase in SREBP-1 mRNA (Figure 7A), found that acetyl CoA carboxylase mRNA levels were significantly decreased by 26% following TBI, and fatty acid synthase mRNA levels were not changed (Figure 3-8B).
Figure 3-8. Regulation of HMG CoA reductase and acetyl CoA carboxylase mRNA levels 7 days after TBI. mRNA levels of acetyl CoA carboxylase, fatty acid synthase, HMG CoA reductase and squalene synthase were measured by real-time PCR in cortex ipsilateral to the site of injury 7 days after TBI (tbi icx), and sham cortex ipsilateral to craniotomy (sham icx). A) HMG CoA reductase mRNA levels were significantly decreased 38% (Student’s t-test, *p<0.005, n=5) and squalene synthase mRNA level were significantly decreased 33% (student’s t-test, *p<0.05) at the site of injury in comparison to sham controls. B) acetyl CoA reductase mRNA levels at the site of injury were significantly decreased 26% (Student’s t-test, *p<0.05, n=5) while there was no change in fatty acid synthesis mRNA levels at the site of injury in comparison to sham controls. Error bars (standard error of the mean).
DISCUSSION

In order to maintain cholesterol homeostasis, the brain must not only regulate cholesterol efflux but also cholesterol synthesis, since cholesterol in not imported from the periphery but synthesized in the brain (Jurevics and Morell, 1995; Turley et al., 1996; Turley et al., 1998; Lund et al., 2003; Xie et al., 2003). Cyp46 is an enzyme expressed primarily in neurons that converts cholesterol to the oxysterol, 24S-hydroxycholesterol (Lund et al., 1999; Ramirez et al., 2008). Oxysterols have previously been shown to affect a key step in cholesterol homeostasis, the cleavage of the inactive cSREBPs to produce the transcription factor nSREBP (Thewke et al., 1998; Adams et al., 2004; Du et al., 2004). nSREBPs upregulate transcription of multiple enzymes in the cholesterol synthesis pathway, including the rate limiting enzyme, HMG CoA reductase (Saucier et al., 1989; Horton et al., 2002). Previous studies have shown that nSREBP-2 has more control over transcription of genes encoding cholesterol synthesis enzymes while nSREBP-1 has more control over transcription of genes encoding fatty acid synthesis enzymes (Horton et al., 1998; Horton and Shimomura, 1999; Horton et al., 2002). In our study, 24S-hydroxycholesterol suppressed nSREBP-2 levels cleavage by 80-91% (Figures 3-1, 3-3 and 3-4). Previous research has shown that cholesterol can also inhibit cSREBP cleavage (Gimpl et al., 2002; Gill et al., 2008). However, in our system, cholesterol failed to suppress cleavage of cSREBP-2 (Figure 3-3). This is consistent with the idea that while exogenously applied oxysterols can cross membranes to have intracellular
signaling effects, exogenous cholesterol does not have an effect on intracellular SREBP signaling. We show that another oxysterol found in the brain, 27-hydroxycholesterol, significantly inhibited nSREBP-2 levels by 84% (Figure 3-3). Interestingly, the LXR agonist TO-901317 induced a small but significant decrease (36%) in cSREBP-2 cleavage (Figure 3-3), indicating that the cSREBP-2 cleavage inhibition seen with 24S-hydroxycholesterol and 27-hydroxycholesterol may be due to a combination of direct inhibition of cleavage as well as potential indirect effects from LXR activity (Janowski et al., 1999; Fu et al., 2001).

In addition to its suppression of nSREBP-2, 24S-hydroxycholesterol significantly decreased cSREBP-2 expression. 24S-hydroxycholesterol decreased protein levels of the precursor cSREBP-2 by 50-51% (Figures 3-1, 3-3, and 3-4). This is in contrast to the effects of the oxysterol 25-hydroxycholesterol where suppression of cleavage coincided with increased cSREBP-2 levels (Thewke et al., 1998; Adams et al., 2004; Du et al., 2004). Neither 27-hydroxycholesterol nor the synthetic LXR agonist TO-901317 significantly altered cSREBP-2 levels (Figure 3-3), indicating that the effects on cSREBP-2 levels are relatively specific to 24S-hydroxycholesterol and are not related to its LXR activity. 24S-hydroxycholesterol significantly decreased SREBP-2 mRNA levels (Figure 3-2C). However, while 24S-hydroxycholesterol reduced cSREBP-2 levels by at least 50%, SREBP-2 mRNA levels were decreased by only 18-31% (Figure 3-2 and 3-5). Because the promoter region contains a sterol regulatory
element, and is regulated by positive feedback mechanisms (Sato et al., 1996), a small suppression at the mRNA transcription level may be amplified to give a greater effect in protein expression and this may account for greater changes in cSREBP protein levels relative to changes in mRNA levels with 24S-hydroxycholesterol treatment.

While nSREBP-2 has more control over transcription of genes involved in cholesterol synthesis enzyme, nSREBP-1 has greater control over transcription of genes involved in fatty acid synthesis enzymes transcription (Horton et al., 1998; Horton and Shimomura, 1999; Horton et al., 2002). We found that, as expected for an LXR agonist, 24S-hydroxycholesterol increased mRNA levels of SREBP-1 by over 200% (Figure 3-2 and 3-5) and that this translated into increases of about 40% in cSREBP protein levels (Figure 3-1). The LXR agonist TO-901317 also strongly increased SREBP-1 mRNA levels (Figure 3-5), Unlike our finding with nSREBP-2 levels and in contrast to the effects of 25-hydroxycholesterol (Thewke et al., 1998; Adams et al., 2004), nSREBP-1 levels were not decreased with 24S-hydroxycholesterol. One plausible explanation is that while 24S-hydroxycholesterol increased cSREBP-1 expression, it also inhibits its cleavage and these two competing functions lead to a lack of change in nSREBP-1 levels overall.

Although levels of nSREBP-1 and nSREBP-2 give some indication of downstream transcriptional activity, direct measurement of mRNA levels is a better indicator of
24S-hydroxycholesterol effects on the cholesterol and fatty acid synthesis pathways.

We show here 24S-hydroxycholesterol treatment suppressed many of the enzymes in the cholesterol synthesis pathways including HMG CoA Reductase, squalene synthase, FPP synthase in neuroblastomas cells (Figure 3-5B). Coinciding with our finding that nSREBP-1 levels were relatively stable in these cells after 24S-hydroxycholesterol treatment, we found no significant changes in mRNA level of the fatty acid synthesis enzymes fatty acid synthase or the rate-limiting acetyl CoA reductase. These data suggest that in neuronally derived cells, 24S-hydroxycholesterol strongly suppresses cholesterol synthesis but has minimal effect on fatty acid synthesis despite being a strong LXR agonist. These findings are in agreement with a recent study in cortical neurons showing decreased protein levels of cholesterol synthesis enzymes HMG CoA synthase and squalene synthase but no significant changes in acetyl CoA carboxylase or fatty acid synthase levels after 24S-hydroxycholesterol treatment (Wang et al., 2008) and a study in STTG glia where 24S-hydroxycholesterol decreased mRNA levels of HMG CoA reductase (Abildayeva et al., 2006).

We have previously shown that, although Cyp46 is a neuronally expressed enzyme under normal conditions, expression is upregulated in microglia by 3 days after brain injury and also after LPS activation (Cartagena et al., 2008). Here we show that, similar to effects in neuronally derived cells, 24S-hydroxycholesterol suppresses SREBP-2 mRNA levels and increases SREBP-1 levels in mouse microglial cells.
(Figure 3-6A). In contrast to our findings in neuronally derived cells as well as previous studies in cortical neurons (Wang et al., 2008) and STTG glia (Abildayeva et al., 2006) where 24S-hydroxycholesterol decreased cholesterol synthesis enzyme levels, we found that 24S-hydroxycholesterol failed to suppress mRNA levels of cholesterol synthesis rate-limiting enzyme HMG CoA reductase or squalene synthase (Figure 3-6B) and significantly increased mRNA levels of acetyl CoA reductase and fatty acid synthase (Figure 3-6C). Thus, while regulation of cholesterol and fatty acid synthesis is related in the brain, there are likely to be cell-type specific differences.

Because Cyp46 expression is strongly increased after traumatic brain injury, it is possible that increased conversion of lipid debris into 24S-hydroxycholesterol would cause regional alterations in both the cholesterol and fatty acid synthesis pathways. Following brain injury CSF cholesterol levels are increased resulting from cellular loss and subsequent lipid debris (Fagan et al., 1998; Gasparovic et al., 2001; Kamada et al., 2003). While increases in ABCA1 and ApoE after TBI would facilitate increased cholesterol efflux either for neuronal regeneration in other areas or export from the brain (Cartagena et al., 2008), oxysterol suppression of cholesterol synthesis would also assist in bringing the brain back into cholesterol homeostasis after injury. Here we show that similar to what was seen in vitro, 7 days after TBI SREBP-1 mRNA levels are increased and SREBP-2 levels are decreased at the site of injury (Figure 3-7). While increased expression of Cyp46 after injury may be localized to the lesion site
(Teunissen et al., 2007; Cartagena et al., 2008), 24S-hydroxycholesterol can enter the CSF (Papassotiropoulos et al., 2002) and thus have more of a gradient effect on the brain as a whole. We found decreases in HMG CoA reductase and squalene synthase mRNA levels after TBI (Figure 3-8B), indicating that cholesterol synthesis at the site of injury is suppressed.

After brain injury levels of both endogenously produced fatty acids such as palmitic acid as well as those derived from polyunsaturated fatty acids (PUFAs) such as arachidonic acid are released aspects of membrane debris (Dhillon et al., 1994; Dhillon et al., 1999; Farooqui et al., 2007). The oxidation of arachidonic acid leads to the production of pro-inflammatory prostaglandins and leukotrienes, which can lead to increased cell death (Phillis et al., 2006; Farooqui et al., 2007). PUFAs have also been shown to inhibit transcription of fatty acid synthesis mRNA (Worgall et al., 1998; Xu et al., 1999; Sampath and Ntambi, 2005). Incorporation of palmitic acid, the final product on acetyl CoA carboxylase and fatty acid synthase activity has been linked positively to cellular recovery and axonal regeneration after brain injury (Tone et al., 1987; Robinson and Rapoport, 1989). However, this end-product of fatty acid synthase activity also leads to inhibition of acetyl CoA carboxylase activity (Mabrouk et al., 1990; Brownsey et al., 2006) and may promote its degradation. Our in vitro finding in neuroblastomas cells and microglia on SREBP-1 and 2 regulation as well as transcription of cholesterol synthesis genes after 24S-hydroxycholesterol treatment
correspond positively with \textit{in vivo} regulation of these same genes after TBI. In contrast, we show that after TBI, despite elevated SREBP-1 levels, fatty acid synthase mRNA levels remain stable and acetyl CoA carboxylase mRNA levels are in fact reduced (Figure 3-8A). Other factors, such as infiltrating peripheral oxysterols, feedback inhibition from palmitate derivatives, or increasing levels of PUFAs, may contribute to the overall decreases in fatty acid synthesis seen after TBI.

After TBI, increased Cyp46 expression is accompanied by upregulation of cholesterol efflux factors ABCA1 and ApoE (Cartagena et al., 2008). Here we show that TBI is also accompanied by suppression of cholesterol synthesis, which we attribute to 24S-hydroxycholesterol activity, as well as suppression of fatty acid synthesis which is unrelated to 24S-hydroxycholesterol activity.
CHAPTER IV: EFFECTS OF CYP46 AND 24S-HYDROXYCHOLESTEROL ON APP PROCESSING: POSSIBLE RELEVANCE TO ALZHEIMER’S DISEASE.

INTRODUCTION

Amyloid plaques are one of the hallmarks of Alzheimer’s disease (AD). The amyloid protein found in these plaques is a cleavage product of the amyloid precursor protein (APP) (Rocchi et al., 2003). APP is a membrane protein which can be cleaved by several secretase enzymes (Dewachter and van Leuven, 2002). Cleavage of APP by either α- or β-secretase releases a soluble form of APP (APPs), which is released into the extracellular space while the fragment containing the transmembrane and intracellular domains remain bound at the membrane (Sambamurti et al., 2002). This remaining portion of APP can be cleaved within the transmembrane domain by a protein complex known as γ-secretase. If APP is first cleaved by β-secretase, this second cleavage by γ-secretase results in the release of the β amyloid (Aβ) fragment. There are two sites at which γ-secretase may cleave, resulting in Aβ fragments 40 and 42 amino acids in length (Sambamurti et al., 2002). The enzymatic processing of APP has been illustrated in Figure 4-1. Mutations in APP and γ-secretase components are responsible for much of the early onset familial AD (Dewachter and van Leuven, 2002; Kowalska, 2003; Rocchi et al., 2003).
The risk of AD onset at an earlier age is increased with the APOE4 genotype in comparison to APOE2 and APOE3 (Rocchi et al., 2003). In addition, individuals with the APOE2 genotype seem to have a reduced risk of AD (Corder et al., 1994). Since ApoE is involved in cholesterol efflux, this finding suggests a role for cholesterol efflux in the development of AD. There is additional evidence that cholesterol metabolism has an effect on the risk of developing AD since subjects with high plasma cholesterol levels have higher susceptibility to AD (Jarvik et al., 1995; Kuo et al., 1998). 24S-hydroxycholesterol has also been shown to be elevated in the central nervous system of AD patients (Lutjohann et al., 2000; Papassotiropoulos et al., 2002). This indicates that CYP46 and cholesterol efflux may be involved in the disease process. Two retrospective studies showed statins, which reduce serum levels of cholesterol by inhibiting cholesterol production, are protective against AD (Jick et al., 2000; Wolozin et al., 2000). This suggests that lowering cholesterol production, and possibly reducing cholesterol efflux due to homeostatic mechanisms, may decrease amyloid plaque formation.

Several in vivo studies of cholesterol and Aβ have supported this association of cholesterol with AD. Brain Aβ levels increased in transgenic mice expressing familiar AD mutations after being fed a high cholesterol diet (Refolo et al., 2000). In contrast, brain Aβ levels decreased when transgenic APP mice are treated with BM15.766, which inhibits the conversion of desmosterol into cholesterol (Refolo et al., 2001). Simvastatin, another cholesterol-lowering drug, reduced Aβ levels in guinea pig
plasma (Fassbender et al., 2001) and avorvastatin treatment of transgenic APP mice reduced Aβ deposits (Petanceska et al., 2002).

Several *in vitro* studies also support the association of cholesterol and AD. Treatment of Neuro2A cells with either 22R-hydroxycholesterol or TO-901317 increased Aβ levels without changing APP or APPs levels (Fukumoto et al., 2002). Neuro2A cells treated with 22R-hydroxycholesterol have significantly higher Aβ levels than cells pretreated with an RNAi inhibitor of ABCA1 (Fukumoto et al., 2002). These findings indicate that ABCA1 activity may allow for greater Aβ formation and that reductions in cholesterol efflux through decreased ABCA1 may limit Aβ formation. In contrast to these findings, treatment of APP over-expressing neuronal cells with either methyl-β-cyclodextrin, which extracts cholesterol from the cell, or lovastatin, which inhibits cholesterol production, increased α-secretase cleavage of APP and decreased Aβ levels (Kojro et al., 2001). When cholesterol levels were lowered by 70% in hippocampal neurons treated with a combination of methyl-β-cyclodextrin and lovastatin, Aβ levels were inhibited completely while levels of secreted APP did not change. These effects were reversed when cholesterol was re-applied to depleted cells (Simons et al., 1998).

Based on this research we wanted to determine what effect Cyp46 over-expression or 24S-hydroxycholesterol treatment had on APP processing and generation of Aβ peptide fragments.
Figure 4-1. Enzymatic cleavage of amyloid precursor protein leads to Aβ peptide.
METHODS

Cell Culture

Chinese hamster ovary cells overexpressing the human familial Swedish mutation of APP (swAPP) and wildtype presenilin 1 (PS70 cells) were cultured in 6 well plated with Opti-MEM media containing 5% FBS. 293 cells were cultured in 6 well plates with Opti-MEM media containing 10% FBS.

Cell Treatment

Stock solutions of 24S-hydroxycholesterol and TO-90137 were prepared and 293 cells and treated with ethanol (vehicle), 5 µM 24S-hydroxycholesterol or 1 µM TO-901317 in media as described on page 79 and incubated for 48 hr.

Cell Transfections

PS70 cells were transfected with Cyp46 cDNA in the CMV6 vector or the pEGFP-N1 (Promega) control vector, while 293 cells were transfected with wild-type APP (wtAPP) cDNA in the pExchange-6a vector or the pExchange-6a control vector using Fugene 6 (Roche Applied Science) according to the manufacturer’s directions.
**Aβ ELISAs**

In cells overexpressing swAPP (PS70 cells), Aβ levels were measured with either a human Aβ40 or Aβ42 ELISA kit (Biosource) according to manufacturer’s directions. Serial dilution standards for either Aβ40 or Aβ42 were included on each plate. Optical densities were measured using a Victor 2 plate reader (Perkin Elmer) and sample concentrations were calculated using optical density measurements of the corresponding plate standards. In cells overexpressing wtAPP (293 cells), Aβ40 levels were measured using an ELISA developed by Dr. Yasuji Matsuoka. Briefly, A 96-well Maxisorp plate (Nunc, Rochester, NY) was coated overnight with clone 1A10 (IBL), which is specific for Abeta1-40. After blocking with Block Ace (Serotec, Raleigh, NC), 100uL of conditioned media was loaded, in duplicate, and incubated overnight at 4°C. The plate was then incubated with an HRP-coupled Aβ N-terminal end-specific antibody, clone 82E1 (IBL), for 4 hrs, and visualized using TMB (Pierce) as a substrate. Statistical analysis was done using Student’s t-test with Graphpad Prism 4.

**Western Blot**

Media was collected and equal volumes were loaded into wells or cells were lysed in RIPA buffer and equal protein levels were loaded into wells as described on page 45. APP levels were determined by western blot as described on page 45 using the primary antibody C1/6.1 against the C-terminal epitope of APP or using the primary antibody 6E10 against an epitope within the Aβ domain of APP. Band density was measured.
using Scion Image and statistical analysis was done using ANOVA Newman-Keuls multiple comparison post-hoc analysis with Graphpad Prism 4.

**RESULTS**

Based on some previous studies, we predicted that promoting cholesterol efflux via LXR activation would decrease production of Aβ (Koldamova et al., 2003; Sun et al., 2003), possibly by decreasing total cholesterol levels. We hypothesize that TO-901317 would increase cholesterol efflux and that this would lead to a decrease in Aβ42 levels. Thus we treated cells with 5 μM TO-901317 or vehicle control in serum free media for 48 hr. However, we observed that treatment of PS70 cells with 5 μM TO-901317 for 48 hrs significantly increased Aβ42 levels by 120% in comparison to vehicle controls.
Figure 4-2. Aβ42 levels 48 hours after treatment with LXR agonist TO-901317. LXR activation of PS70 cells significantly increased Aβ42 levels by 120% in comparison with controls (student’s t-test, *p<0.001, n=3). Error bars (standard error of the mean).
Increasing levels of Cyp46 in cell culture should increase the conversion of cholesterol to 24S-hydroxycholesterol, thereby lowering cholesterol levels by oxysterol diffusion. Thus, we hypothesize that increasing Cyp46 levels will lead to a decrease in Aβ42 levels. PS70 cells in serum free media were transfected with Cyp46 cDNA or control vector. 24 hr later media was collected and Aβ40 levels were measured by ELISA. Over-expression of Cyp46 in PS70 cells did not alter Aβ40 levels (Figure 4-3). 48 hr later media was collected and Aβ42 levels were measured. Over-expression of Cyp46 cDNA significantly decreased Aβ42 levels by 24% compared to controls (Figure 4-4).
Figure 4-3. Aβ40 levels 24 hours after transfection with CYP46. PS70 cells were transfected with either Cyp46 cDNA or a control vector containing no cDNA. After 24 hr media was collected and Aβ40 levels were measured by ELISA. No significant changes were observed in Aβ40 levels in comparison to controls. (n=3).
Figure 4-4. Aβ42 levels 48 hours after transfection with CYP46. Increasing levels of Cyp46 significantly decreased Aβ42 levels by 24% in comparison to controls (student’s t-test, *p<0.001, n=3). Error bars (standard error of the mean).
Although Aβ levels are more easily measured with overexpression of swAPP, only a small percentage of Alzheimer’s patients carry this familial mutation. In addition, the Swedish mutation causes altered processing of APP in the ER/Golgi (Goate, 1998) and this may not be relevant to sporatic AD. We wished to determine whether 24S-hydroxycholesterol would alter levels of Aβ derived from wtAPP. Accordingly, 293 cells were transfected for 24hrs with wtAPP and then treated with 5 µM 24S-hydroxycholesterol or vehicle control. Aβ40 levels were measured 48 hr later by ELISA. There were no significant changes in Aβ40 levels with 24S-hydroxycholesterol treatment (Figure 4-5).
Figure 4-5. Aβ40 levels after 24S-hydroxycholesterol. Amyloid beta levels in the media of 293 cells transfected with wtAPP were analyzed by ELISA following 48hr treatment with vehicle control or 5 µM 24S-hydroxycholesterol (24S). 24S-hydroxycholesterol had no significant effects on Aβ40 levels in comparison to vehicle controls (n=6). Error bars (standard error of the mean).
While Aβ40 levels were not altered by Cyp46 overexpression or 24S-hydroxycholesterol treatment, Aβ42 levels were decreased by Cyp46 overexpression. In order to determine what effects 24S-hydroxycholesterol had on processing of endogenous human APP and whether these alterations are related to increased LXR activity, 293 cells were treated with 5 µM 24S-hydroxycholesterol, 1 µM TO-901317, or vehicle control for 48 hr. Full length APP and APP C-terminal fragments (CTFs) were analyzed by western blot. 24S-hydroxycholesterol treatment increased full length APP levels by 20% while there was no significant change with TO-901317 treatment (Figure 4-6). 24S-hydroxycholesterol treatment dramatically decreased APP CTF levels by 96% but no change in APP CTF levels was seen with TO-901317 treatment (Figure 4-6).
**Figure 4-6. APP increases full length APP levels and modifies APP processing.**

A) Full length APP (flAPP) and APP CTF levels were analyzed following 48hrs treatments in 293 cells with vehicle (C), 5 µM 24S-hydroxycholesterol (24S) or 1 µM TO-901317 (TO). B) Quantification of full length APP levels showed an increase of 20% with 24S-hydroxycholesterol treatment (Newman-Keuls, p<0.05, n=3). There was no significant change in levels with TO-901317 treatment. C) Quantification of APP CTF levels showed a decrease of 96% with 24S-hydroxycholesterol treatment (Newman-Keuls, p<0.001, n=3) but no change with TO-901317 treatment.
We have shown that 24S-hydroxycholesterol leads to dramatic decreases in APP CTF levels and increases in full length APP levels. In order to determine whether 24S-hydroxycholesterol treatment leads to changes in the \( \alpha \)-secretase activity, 293 cells were treated with ethanol (vehicle) or 5 \( \mu \)M 24S-hydroxycholesterol for 48 hr, after which media was collected and secreted APP (sAPP) levels were analyzed by western blot. Although some samples treated with 24S-hydroxycholesterol appeared to have lower levels of sAPP\( \alpha \) compared to controls, there were no consistent differences between treatments (Figure 4-7).
Figure 4-7. Secreted APPα levels after 24S-hydroxycholesterol treatment. Secreted APPα (sAPPα) levels were analyzed following 48hrs treatments in 293 cells with vehicle (C) or 5 µM 24S-hydroxycholesterol (24S). 24S-hydroxycholesterol had no consistent effect on sAPPα levels.
DISCUSSION

Increased cholesterol levels in vitro increased Aβ levels (Askanas and Engel, 2002; Fukumoto et al., 2002) while statin treatment in vitro decreased Aβ levels (Simons et al., 1998; Kojro et al., 2001). However, a direct link between these cholesterol mechanisms and APP processing has not been established. Here we show that direct LXR activation by TO-901317 in Chinese hamster ovarian (CHO) cells over-expressing swAPP increased Aβ42 levels by 120% in (Figure 4-2), supporting previous findings in the Rebeck laboratory (Fukumoto et al., 2002). TO-901317 upregulates expression of LXR regulated cholesterol efflux genes including ABCA1 and ApoE and thus may increase cholesterol efflux. However, by affecting cholesterol efflux without also suppressing cholesterol synthesis, TO-901317 may not have lowered overall cholesterol levels. Thus, TO-901317 treatment of PS70 cells in culture may have resulted in a redistribution of cellular cholesterol. In addition, TO-901317 has been shown to directly affect γ-secretase, indicating that TO-901317 induced changes in APP processing may be unrelated to LXR activity (Czech et al., 2007).

While the effects of LXR activation on APP processing is an interesting question, we were more concerned with the effect of the endogenous brain LXR activator, 24S-hydroxycholesterol. Cyp46 can reduce cholesterol by several methods including removing cholesterol from the membrane as it converts it to 24S-hydroxycholesterol, increasing cholesterol efflux through LXR activation, and suppressing cholesterol synthesis through the effects of this oxysterol on SREBP activation. Thus, Cyp46 may
more effectively lower overall cholesterol levels than an LXR agonist alone, leading to decreased Aβ42 levels. We show here that increased Cyp46 expression resulted in decreased Aβ42 levels (Figure 4-4) although Aβ40 levels were not altered by Cyp46 overexpression (Figure 4-3) or by 24S-hydroxycholesterol treatment (Figure 4-5). It is possible shifting cholesterol levels preferentially effects the formation of Aβ42 levels. Effects of Cyp46 on Aβ42 are particularly important because Aβ42 is more likely to aggregate and is thought to facilitate plaque formation in Alzheimer’s patients.

24S-hydroxycholesterol dramatically decreased not only APP CTF levels but also levels of other undefined intermediate size APP cleavage products (Figure 4-6). This suggests that either α- or β- secretase activity has been decreased or γ- secretase has been increased (See Figure 4-1 for illustration of secretase cleavage of APP). Increases in γ-secretase should also lead to increases in Aβ levels. In previous experiments Aβ42 levels decreased with Cyp46 over-expression (Figure 4-4), indicating that 24S-hydroxycholesterol is unlikely to be increasing γ-secretase levels. Decreases in β-secretase should hypothetically increase levels of α-CTF, which is not what we observed with 24S-hydroxycholesterol treatment (Figure 4-6). Thus, a probable conclusion is that 24S-hydroxycholesterol is decreasing α-secretase activity. However this hypothesis conflicts with another study showing that 24S-hydroxycholesterol increased α-secretase activity and decreased β-secretase activity in SY5Y cells (Famer et al., 2007).
Increased in α-secretase activity should lead to increases in the sAPPα cleavage product of APP. While previous work in SY5Y cells showed increased α-secretase activity with 24S-hydroxycholesterol (Famer et al., 2007) in our system we saw no change in sAPPα levels (Figure 4-7) indicating that α-secretase activity is not altered. Several factors may contribute to these differences including different cell lines and different media conditions. Interestingly, in the previous study no change was found in sAPPα when cells were treated with both 24S-hydroxycholesterol and 27-hydroxcholesterol in combination, indicating that the presence of other oxysterols alters the effects of 24S-hydroxycholesterol on APP processing. Since our measurements of sAPPα levels were done with 10% FBS present, it is possible that the other oxysterol in the media are influencing the effects of 24S-hydroxycholesterol seen in this system.

The experiments described above raise some interesting questions about possible interactions between Cyp46 induced cholesterol mechanisms and APP processing. Clearly, both Cyp46 overexpression and direct treatment of cells with 24S-hydroxycholesterol alter APP processing. These effects do not seem to be due to increased LXR activity and may be indirect effects of lowering overall cholesterol levels in the cell. Our previous findings in traumatic brain injury, as well as other studies of CNS injury, support the idea that increases in Cyp46 in not a marker of Alzheimer’s disease but a more general marker of CNS injury. Although the experiments shown here are very preliminary, the results indicate that increased Cyp46
expression may in fact affect the development of Alzheimer’s disease. A systematic study of the effects of 24S-hydroxycholesterol on the processing of APP is needed to better determine the mechanisms involved in this process.
CHAPTER V: OVERALL CONCLUSIONS AND SIGNIFICANCE

Traumatic brain injury is characterized by the cellular damage caused by the primary injury seen within minutes to a few hours after impact as well as the many secondary injury effects which occur within hours to days after the impact (Menon and Wheeler, 2005; Morganti-Kossmann et al., 2007). Primary effects include cellular loss from the impact itself as well as loss of ATP, a necessary energy source for many cellular mechanisms, and failure of ionic pumps (Menon and Wheeler, 2005). Secondary effects include swelling of the astrocytic foot processes, contributing to the breakdown of the blood brain barrier (Fitch et al., 1999; Menon and Wheeler, 2005; Morganti-Kossmann et al., 2007), peripheral macrophage infiltration (Fitch and Silver, 1997) and local astroglia and microglia proliferation and activation (Cernak et al., 2004; Di Giovannoni et al., 2005). Microglia and macrophages release proinflammatory cytokine and promote oxidative stress (Delgado and Ganea, 2003; Langley and Ratan, 2004; Menon and Wheeler, 2005; Morganti-Kossmann et al., 2007), while astogial neurotransmitter reuptake is reversed leading to toxic levels of the excitatory amino acid glutamate (Faden and Stoica, 2007; Park et al., 2008). This leads to excitotoxic neuronal death from depolarization of cells via activation of AMPA and NMDA receptors and the resulting high intracellular calcium levels (Faden and Stoica, 2007; Park et al., 2008). Although cellular necrosis is the cause of primary neuronal loss after TBI, apoptotic mechanisms contribute to secondary neuronal loss (Langley and Ratan,
Neuronal death from both primary and secondary injury leads to lipid debris, releasing excess cholesterol and fatty acids into the surrounding area (Dhillon et al., 1994; Faden, 1996; Dhillon et al., 1999; Gasparovic et al., 2001; Kamada et al., 2003; Kay et al., 2003). Cholesterol is normally under strict homeostatic control (Horton et al., 2002; Ory, 2004). After TBI, excess cholesterol from lipid debris must be cleared to regain cholesterol homeostasis. We propose that the enzyme Cyp46 plays a critical role in re-establishing cholesterol homeostasis after TBI. Cyp46 converts cholesterol into the more hydrophilic, and therefore more soluble, 24S-hydroxycholesterol (Lund et al., 1999). The effects of 24S-hydroxycholesterol on cholesterol homeostasis after TBI are potentially four-fold.

First, because 24S-hydroxycholesterol is more soluble than cholesterol and can cross membranes, it can be cleared from the cell and from the brain overall by diffusion across the blood brain barrier (Lund et al., 1999; Papassotiropoulos et al., 2002; Xie et al., 2003; Bjorkhem, 2006). Studies on the Cyp46 knock-out mouse indicate that this is the primary mechanism for clearing cholesterol from the brain under normal circumstances (Xie et al., 2003). Thus, increased Cyp46 activity would promote
clearance of cholesterol from the site of injury through increased production of 24S-hydroxycholesterol.

Second, 24S-hydroxycholesterol is a potent LXR agonist (Lehmann et al., 1997; Janowski et al., 1999). We found increased levels of the two LXR-responsive gene products ABCA1 and ApoE. Both ABCA1 and ApoE are necessary for cholesterol and phospholipid efflux from cells to lipoproteins (Huang et al., 2001; Smith et al., 2004; Hirsch-Reinshagen et al., 2005), and allows cholesterol to be cleared from the brain (Xie et al., 2003) presumably through arachnoid granulations into the superior sagittal sinus and subsequently the venous system. By increasing expression of both ABCA1 and ApoE protein levels, 24S-hydroxycholesterol promotes a second path to clearing cholesterol, as well as phospholipids, out of cells and ultimately out of the brain. Increased export of cholesterol and phospholipid laden lipoproteins may in part account for increased CSF lipid levels seen after TBI (Kay et al., 2003).

Third, LXR activation has been shown to have anti-inflammatory effects (Stannard et al., 2001; Joseph et al., 2003; Guo et al., 2004; Walcher et al., 2006). This may be due to a decrease in the cholesterol load since increases in cholesterol have pro-inflammatory effects and decreases in cholesterol have anti-inflammatory effects in the periphery (Hansson et al., 2006; Ansell et al., 2007; Navab et al., 2007; Sanossian et al., 2007). Additionally, some LXR responsive gene products, such as ApoE, may be
directly anti-inflammatory. Peptides derived from the ApoE receptor binding domain alone have been shown to have anti-inflammatory effects (Hoane et al., 2007; Laskowitz et al., 2007; Pocivavsek et al., 2008). Thus, increasing 24S-hydroxycholesterol at the injury site promotes multiple anti-inflammatory processes. This in turn may decrease neuronal death and thus prohibit additional release of cholesterol containing lipid debris.

Finally, 24S-hydroxycholesterol suppresses cholesterol synthesis by decreasing levels of nSREBP-2. Here we show that 24S-hydroxycholesterol decreases nSREBP-2 levels by multiple mechanisms. First, 24S-hydroxycholesterol, like other oxysterols, effectively inhibits cleavage of cSREBP-2 to nSREBP-2. However, unlike other oxysterols such as 25-hydroxycholesterol or 27-hydroxycholesterol, 24S-hydroxycholesterol also decreases mRNA levels of cSREBP-2 resulting in lower cSREBP-2 protein levels. Thus 24S-hydroxycholesterol decreases availability of this precursor protein and then further inhibits its cleavage and availability as a transcription factor for cholesterol synthesis enzymes. The availability of nSREBP-2 regulates the transcription not just of the rate limiting enzyme HMG CoA reductase, but also several other enzymes in the cholesterol synthesis pathway. Here we show that 24S-hydroxycholesterol decreases transcription of HMG CoA reductase, squalene synthase, as well as FPP synthase, and thus decreasing cholesterol synthesis.
After TBI we observed increased Cyp46 levels. One function of microglia following injury is the removal of lipid debris (Gasparovic et al., 2001; Kamada et al., 2003) and the increased expression of Cyp46 in these cells may be a necessary step in the processing of phagocytosed lipids. The resulting 24S-hydroxycholesterol would not only affect signaling in microglia, but since 24S-hydroxycholesterol passes membranes and diffuses in the extracellular space (Lutjohann et al., 2000; Papassotiropoulos et al., 2002; Xie et al., 2003), it can potentially affect other neighboring cell, including neurons and astroglia. Corresponding with this idea of a TBI-induced 24S-hydroxycholesterol gradient, we saw significant increase in SREBP-1 and decreases in SREBP-2 at the site of injury in comparison to shams and we saw intermediate level non-significant changes in the contralateral cortex of injured animals.

Release of lipid debris after TBI-induced neuronal death also leads to excess fatty acids, the building blocks of membrane phospholipids. This includes PUFA’s such as arachidonic acid, which lead to pro-inflammatory eicosanoids (Fritsche, 2006) as well as endogenously synthesized palmitic and oleic acids (Dhillon et al., 1994; Dhillon et al., 1999). As with cholesterol, fatty acids are under homeostatic control. This is primarily through regulation of cSREBP-1a or cSREBP-1c to their nuclear forms. By increasing ABCA1 and ApoE facilitated phospholipids efflux into lipoproteins, 24S-hydroxycholesterol can promote clearance of fatty acids after TBI. 24S-hydroxycholesterol also potentially plays a dual role controlling fatty acid synthesis.
First, 24S-hydroxycholesterol upregulates mRNA levels of cSREBP-1c, which is under LXR regulation, potentially leading to increased fatty acid synthesis. Second, based on studies of other oxysterols, 24S-hydroxycholesterol potentially inhibits the cleavage of both cSREBP-1a and cSREBP-1c to their nuclear forms. This would inhibit fatty acid synthesis. Our findings suggest that these competing effects of 24S-hydroxycholesterol lead to little change in the combined nSREBP-1a and 1c levels.

Based on studies in transgenic mice, the nuclear forms of SREBP-1a and 1c, both upregulate the enzymes in the fatty acid synthesis pathway, acetyl CoA carboxylase and fatty acid synthase (Horton et al., 1998; Horton and Shimomura, 1999). However, nSREBP-1a appeared to be a more effective transcription factor than nSREBP-1c and led to greater upregulation of fatty acid synthesis enzymes (Horton et al., 1998; Horton and Shimomura, 1999). When looking at the downstream transcription effects of 24S-hydroxycholesterol on fatty acid synthesis, we found that in 293 cells and SY5Y cells there was no significant net effect on either acetyl CoA carboxylase or fatty acid synthase. However, when we looked at similar downstream outcomes in BV2 microglia, we found that 24S-hydroxycholesterol significantly upregulated both acetyl CoA carboxylase and fatty acid synthase. It may be that the relative influence of nSREBP-1c in comparison to nSREBP-1a is greater in BV2 cells than in 293 or SY5Y cells, leading to increases fatty acid synthesis enzymes in these cells.
Contrary to the regulatory effect of 24S-hydroxycholesterol on fatty acid synthesis seen in vitro, we show that after TBI transcript levels of the rate limiting enzyme acetyl CoA carboxylase are decreased while levels of fatty acid synthase did not change. This is despite coinciding significant increases in SREBP-1 mRNA levels which indicate increased LXR activation. Based on findings in vitro which indicate that 24S-hydroxycholesterol has either no effect or a positive effect on fatty acid synthesis, we attribute these results to factors other than 24S-hydroxycholesterol. Both palmitic acid and PUFAs are upregulated after TBI (Dhillon et al., 1994; Dhillon et al., 1999; Pilitsis et al., 2003). When fatty acid levels are high after TBI due to accumulating lipid debris, increasing fatty acid synthesis would work against homeostatic mechanisms rather than facilitating them. While cholesterol synthesis is regulated primarily by SREBP mechanisms, fatty acid synthesis has other regulatory mechanisms that may be factors after TBI (Horton et al., 2002). Palmitoyl CoA, a derivative of the end product palmitic acid, causes the disassembly of ACC filaments and inhibits the transport of citrate, a facilitator of ACC filaments, from the mitochondria to the cytoplasm (Rubink and Winder, 2005; Berg et al., 2007). In addition, PUFAs have been shown to decrease SREBP-1a and 1c as well as acetyl CoA carboxylase mRNA levels, although the mechanisms for this inhibition have not been well defined (Xu et al., 1999). Thus PUFAs as well as increased levels of palmitic acid after TBI may counteract any effects of 24S-hydroxycholesterol. The role of Cyp46 and 24S-hydroxycholesterol has been modeled in Figure 4-1.
Figure 4-1. Model of Cholesterol and Fatty Acid Mechanisms after TBI. Factors that are increased are indicated in red while factors that are decreased are indicated in blue.
Based on these findings, we hypothesize that Cyp46 expression and increased 24S-hydroxycholesterol levels following injury may increase microglial cholesterol efflux following phagocytosis of cellular debris, and reduce inflammatory and oxidative signals regionally. We hypothesize that microglia at the injury site may utilize 24S-hydroxycholesterol to upregulate internal fatty acid synthesis for membrane expansion during activation and proliferation but that this does not translate into overall increases in fatty acid synthesis. Similar changes in Cyp46 expression have been seen in other models of injury including in acute experimental autoimmune encephalomyelitis (Teunissen et al., 2007), hippocampal kainate injury (He et al., 2006), and in post-mortem AD tissue (Bogdanovic et al., 2001; Brown III et al., 2004), while increased levels of 24S-hydroxycholesterol, indicating increased levels or activity of Cyp46, has been seen in the CSF and serum of AD patients (Bretillon et al., 2000; Papassotiropoulos et al., 2002). Our results demonstrate that increases in Cyp46 expression are a marker of CNS injury, not a marker of any particular neurodegenerative condition. Its activity is an endogenous mechanism that achieves many of the biological goals attempted in research of pharmaceuticals after brain injury or inflammation. For instance, statins have been used to lower cholesterol synthesis by inhibiting HMG CoA reductase and have been linked to a decrease in the risk of developing AD (Wolozin et al., 2000). LXR agonists have been proposed as a way to promote cholesterol efflux after amyloid
induced neurodegeneration, potentially reducing neuronal damage (Riddell et al., 2007). Peptides of the ApoE receptor binding domain have been examined as possible clinical treatments to reduce inflammatory effects after brain injury (Hoane et al., 2007; Laskowitz et al., 2007). Through its enzymatic activity and upregulation of 24S-hydroxycholesterol, Cyp46 accomplishes all these functions. Although the mechanisms controlling Cyp46 expression have yet to be defined, it is possible that future basic research may determine a mechanism to upregulate Cyp46 expression, possibly through gene therapy. Induction of these positive Cyp46 effects would be potentially beneficial following TBI.
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APPENDIX A: ADDITIONAL EXPERIMENT RELATING TO CHAPTER II:

Cortical injury increases cholesterol 24S hydroxylase levels in the rat brain.

Experiment A-1. Cyp46 expression in mixed primary cell cultures

**Hypothesis:** Previous published literature describes Cyp46 as being expressed exclusively in neurons except for the occasional astrocyte or oligodendrocyte in postmortem AD tissue. We hypothesize that cells other than neurons express Cyp46 in primary culture.

**Methods:** Cortices were taken from P1 Sprague-Dawley rats and cells were mechanically dissociated, suspended in DMEM containing 10% FBS, and plated in T75 flasks. Media was changed to 10% FBS at day 3 and every other day after that. At day 14 **in vitro** cells were rinsed with PBS and fixed with 10% paraformaldehyde. Cells were stained for Cyp46 and costained using antibodies against the cell markers NeuN for neurons (Chemicon, 1:100), O4 for oligodendrocytes (Chemicon, 1:100), Ox42 for microglia (Chemicon, 1:100), and GFAP for astrocytes (Chemicon, 1:200) using the method for florescent staining and confocal imaging described previously (page 41).
**Results:** Most neurons express Cyp46 at high levels. Oligodendrocytes showed occasional positive staining for Cyp46. Microglia also showed occasional positive staining for Cyp46. Astrocytes showed did not show positive staining for Cyp46.

**Conclusions:** The cell culture conditions of this experiment were not optimized for any particular cell type. However, under these conditions, it is clear that Cyp46 is mostly neuronal, but not entirely.
Figure A-1. Cyp46 expression colocalizes with neurons and some glia in rat primary mixed cell culture. Cortical cells from rat P1 pups were cultured for 14 days. Cells were stained for Cyp46 (green, column 2) and cell specific markers (column 1) A) Staining of neurons with NeuN (red) showed that most neurons express Cyp46 at high levels. B) Staining of oligodendrocytes with O4 (red) occasional positive staining for Cyp46. C) Staining of microglia with Ox42 (red) also showed occasional positive staining for Cyp46. D) Staining of astrocytes with GFAP (red) showed no positive staining for Cyp46.
Experiment A-2. Cyp46 expression in primary neuronal cultures

Hypothesis: In mixed primary cell cultures we saw that cell types other than neurons can express Cyp46. We hypothesize that in hippocampal primary cultures Cyp46 will show strong expression in neurons primarily at the soma but that non-neuronal cells also express some Cyp46.

Method:
Hippocampal neurons were cultured from E18-19 Sprague-Dawley rats at 150 cells/mm2 as described previously (Page 42). Cells were stained for Cyp46 and MAP-2 and image taken by confocal microscopy as described previously (Page 44).

Results: In rat hippocampal neurons, Cyp46 levels were most pronounced in large, well connected neurons with very high levels in the cytoplasm. Smaller, less connected neurons showed lower Cyp46 levels. Although highest levels were at the cell body, Cyp46 continues throughout the neuronal processes. Processes not containing Map-2 still contain Cyp46. Highest levels of expression are consistently in neuronal cells. However, some MAP-2 negative cells show low levels of Cyp46 expression (Figure A-2D).
Conclusions: In agreement with previously published findings (Lund et al., 1999; Ramirez et al., 2008), we found that Cyp46 is primarily in neurons. In addition we found that levels may depend on the maturity of neurons since large mature neurons with many processes showed more intense expression. In addition we showed clear evidence that non-neuronal cells express Cyp46 (Figure A-2D). Previous studies have show that Cyp46 is associated with the ER in neurons (Ramirez et al., 2008). Here we show that neuronal and non-neuronal Cyp46 expression appeared in a pattern also consistent with ER association.
Figure A-2. Cyp46 expression highest in mature primary neurons. (A) In rat DIV16 hippocampal neurons, Cyp46 levels (Green) were most pronounced in large, well connected neurons with very high levels in the cytoplasm. Smaller, less connected neurons showed lower Cyp46 levels. Neuronal cells were identified using anti-MAP-2 antibody and Alexa 546 secondary (red). 20x (B-D) Although highest levels of Cyp46 is in the cell body, expression continues throughout the network of neuronal processes. Processes without Map-2 still have Cyp46. 63x (D) Highest levels of Cyp46 are consistently in neuronal cells as indicated by MAP-2 positive staining. However, some MAP-2 negative cells show low levels of Cyp46 (arrows).
Experiment A-3. Cyp46 expression in oligodendrocytes.

Hypothesis: In initial experiments looking at mixed glia we saw occasional Cyp46 positive staining in oligodendrocytes (Figure A-1). We hypothesize that in oligodendrocyte enriched primary cultures, Cyp46 expression will be present.

Method:
Cortices were taken from E20 Sprague-Dawley rats and cells were mechanically dissociated, suspended in DMEM containing 12% FBS, and plated in T75 flasks. Media was changed to 10% FBS at day 3 and every other day after that. After 10-12 days in culture, the flasks were shaken at 100 rpm for an hour to remove the microglia. Oligodendrocyte progenitor cells (OPCs) growing on top of the astrocytes were detached by further shaking the flasks overnight at 200 rpm and 37C. OPCs were plated on Poly-L-Ornithine coated coverslips in N1 media. After 2 hours, cultures were treated with PDGF (10 ng/ml) for 3 days. Cells were then fixed for 10 min in 4% paraformaldehyde. O4 staining demarked mature oligodendrocytes.

Conclusions:
PDGF stimulates maturation of oligodendrocytes from the progenitor state. Levels of Cyp46 were strongest in cells with lower O4 levels (less mature) and lower in cells that
had strong O4 expression (more mature). This may indicate that as oligodendrocytes mature they decrease Cyp46 expression.
Figure A-3. Cyp46 expression in oligodendrocytes. Primary rat oligodendrocyte progenitor cell (OPC) cultures treated with PDGF for 3 days were stained for O4 (red) and Cyp46 (green). Images were taken at magnifications of 20x (A) and 63x (B). OPCs lacking O4 staining appeared to express more Cyp46 than more mature oligodendrocytes expressing high levels of O4.
**Experiment A-4. Development of myc-tagged Cyp46 expression vector**

**Purpose:** To develop an expression vector for a myc-tagged Cyp46 protein in a commercially available plasmid.

**Method:** The human Cyp46 cDNA was PCR amplified out of the CMV6 vector (a gift of Dr. David Russell, UT Southwestern) using forward primer CGCGCGGCCGCGGAGCCATGAGCCCCGGGCT and the reverse primer CCGGAATTCGCAGGGGGGTGGTGGGGTGGTGGGCTG. Amplification was performed using 0.4 µg of the original Cyp46 cDNA, 1.5 mM MgCl₂, 200 µM dNTPs, 1 µM of each primer, and 5 units Taq (Promega) in a 20 µl reaction. This reaction was incubated for 3 min at 96 ºC followed by 35 cycles at 94 ºC for 30 sec, 77 ºC for 1 min, 72 ºC for 2 min 30 sec. This was followed by incubation at 72 ºC for 5 min. The PCR product was purified using the QIAquick PCR purification kit (Qiagen) according to manufacturer’s directions. The resulting cDNA insert and the pExchange-6a vector (Stratagene) were digested with the restriction enzymes Not I and EcoR I. The cDNA insert and linearized vector were ligated together using T4 DNA ligase and incubating for 16 hr. This cDNA transfer has been illustrated in Figure A-4.

The resulting Cyp46-myc cDNA was transformed into JM109 competent cells. Briefly, 50 µl competent cells and 1 µg Cyp46-myc cDNA were combined and incubated 10
min on ice followed by incubation at 42 ºC for 45 sec and then 2 min on ice. 750 µl LB media was added and the cells were incubated 1 hr 30 min at 37 ºC and shaking at 200 rpm. Cells were then cultured on LB agar plates containing 100 µg/mL ampicillin.

Clones from ten isolated colonies were cultured in 5 mL of LB media containing 100 µg/mL ampicillin. Plasmid DNA was purified from each clone using a plasmid mini-prep purification kit (Quiagen) according to manufacturer’s directions. A sample of purified plasmid DNA for each clone was sequenced (Retrogen) using primers against the T3 and T7 promoters present in the pExchange-6a vector backbone. The resulting sequences were compared to the published sequence for Cyp46 (Genebank accession #AF094480).

We found that all resulting cDNA clones contained a mismatch at base pair 355 corresponding to amino acid 119 and resulting in a change in that amino acid from the published arginine to a threonine. To determine whether this mismatch was present in the original cDNA from the CMV6 vector we designed three primers to the Cyp46 sequence. The two forward primers were CAATCCAAAAACACATCTTG and TACCTCTAACCACACACTGA while the reverse primer was GGCTAAGAAGTATGGACCTG. These primers were used to sequence the original Cyp46 cDNA in the CMV6 vector (Retrogen). The resulting sequences were compared
to the Genebank sequence AF094480). We found that the mismatch at base pair 355 was present in the original Cyp46 cDNA from the CMV6 vector.

To correct the Cyp46 cDNA sequence, we designed the primer TTGGCCGAAGAGTCTCTCTCACAAACACAG corresponding to the surrounding non-coding sequence of genebank sequence AF094480. We corrected the sequence of the Cyp46 cDNA in the pExchange-6a vector using a PAGE purified form of this primer, and the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene) according to manufacturer’s directions. The resulting Cyp46 cDNA expression vector was transformed into ultracompetent XL10-Gold cells provided in this kit. Cells were cultured in NZY+ media for 1 hr and then cultured on a NZY+ agar plate containing 100 µg/mL ampicillin.

Ten clones were obtained from isolated colonies from this agar plate and cultured in NZY+ media containing 100 µg/mL ampicillin. Plasmid DNA was isolated from these cultures as described above. Samples of cDNA from each clone were sequenced using primers to the T7 and T3 promoters in pEnchange-6a. All clones sequenced contained the corrected sequence corresponding to genebank sequence AF094480 and were identical to each other. This corrected Cyp46-myc expression vector was used in experiments in this thesis.
Figure A-4. Extraction of Cyp46 cDNA from CMV6 and insertion into p-Exchange 6a vector.
APPENDIX B: ADDITIONAL EXPERIMENTS RELATING TO
CHAPTER III: 24S-hydroxycholesterol effects on regulation of lipid metabolism
genes are modeled in traumatic brain injury.

Experiment B-1: 24S-hydroxycholesterol regulation of ApoE in BV2 microglia

Hypothesis: 24S-hydroxycholesterol is an agonist of the LXR nuclear receptor. A key
LXR regulated gene important for cholesterol efflux is ApoE. We hypothesize that
24S-hydroxycholesterol will upregulate mRNA levels of ApoE.

Method: BV2 cells were treated with 24S-hydroxycholesterol or vehicle for 48 hr as
described on page 80. mRNA from these cells was isolated and converted to cDNA
and measured by real-time PCR as described on pages 84-86. Primers to the ApoE
sequence for use in real-time PCR were designed as described on page 84 and are
listed in Table B-1.

Results: Contrary to our initial hypothesis, 24S-hydroxycholesterol treatment of BV2
cells led to a small but significant decreased ApoE mRNA levels (12%).
**Conclusion:** Recent research from our laboratory (Pocivavsek et al., 2008) has shown that LPS activation of BV2 cells through the toll receptor leads to significant decreases in ApoE protein levels and that this is related to phosphorylation of c-jun N-terminal kinase (JNK). We have shown that 24S-hydroxycholesterol upregulates LXR controlled SREBP-1 in BV2 cells (Figure 3-6), indicating that 24S-hydroxycholesterol is indeed activating LXR. Perhaps 24S-hydrocholesterol treatment is leading indirectly to microglial activation and the decreases in ApoE mRNA seen after 24S-hydroxycholesterol treatment are a combined effect of limited upregulation through LXR and downregulation through some yet to be defined pathway involving JNK. Future studies looking into the effects of 24S-hydroxycholesterol on microglial activation, examining the time course effects of 24S-hydroxycholesterol, or using inhibitors to JNK, could help address this question.
<table>
<thead>
<tr>
<th>Target</th>
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<tbody>
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<tr>
<td>ApoE</td>
<td>TCGGAAGGAGCTGACTTG</td>
<td>CCAAGGTTGGTTGCTTTG</td>
</tr>
<tr>
<td>APP</td>
<td>TCTGGGCTGACAAACATCAA</td>
<td>CACATCTTCAGCAAAGAACACC</td>
</tr>
<tr>
<td>BACE1</td>
<td>GCTGCCGTCAAGTCCATC</td>
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<tr>
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<tr>
<td>β-actin</td>
<td>TGACAGGATGCAGAAGGAGA</td>
<td>ACATCTGCTGGAAGGTGGAC</td>
</tr>
</tbody>
</table>

Table B-1. Primers designed against mouse sequences for real-time PCR. Cyp46: cholesterol 24S-hydroxylase; ApoE: apolipoprotein E; APP: amyloid precursor protein; BACE1: Beta-site amyloid precursor cleaving enzyme; PS1: presenilin 1.
Figure B-1. Effects of 24S-hydroxycholesterol on ApoE mRNA levels in mouse microglial BV2 cells. BV2 cells were treated for 48 hr with 5 µM 24S-hydroxycholesterol or vehicle control and mRNA levels were measured by real-time PCR. 24S-hydroxycholesterol decreased ApoE mRNA levels by 12% (student’s t-test, * p<0.05, n=6). Error bars (standard error of the mean).
**Experiment B-2: Cyp46 mRNA levels 7 days after TBI.**

**Hypothesis:** Because Cyp46 protein levels are significantly upregulated 7 days after TBI, we hypothesize that Cyp46 mRNA levels are also increased after TBI.

**Method:** Cyp46 mRNA levels were measured by real-time PCR in cortex ipsilateral to the site of injury 7 days after TBI. Descriptions of the injury model can be found on page 80. mRNA from these brain samples was isolated and converted to cDNA and measured by real-time PCR as described on pages 84-86. Primers to the Cyp46 sequence for use in real-time PCR were designed as described on page 84 and are listed in Table B-1.

**Results:** Cyp46 mRNA levels at the site of injury were not significantly different from contralateral cortex or sham cortex 7 days after TBI.

**Conclusion:** We have shown by western blot and immunohistochemistry that Cyp46 protein levels are significantly increased 7 days after TBI (Cartagena et al., 2008). In addition, significant increases in SREBP-1 mRNA, decreases in SREBP-2 mRNA, and decreases in mRNA levels of downstream cholesterol synthesis enzymes HMG CoA reductase and squalene synthase all indicate the presence an oxysterol with LXR activity, presumably 24S-hydroxycholesterol. However, we do not see any changes in
Cyp46 mRNA at this time point. It is possible that changes in protein levels at one week after TBI are due to earlier changes in mRNA which have returned to baseline by that time point. Analysis of mRNA at various time points after injury could address this issue.
Figure B-2. Cyp46 mRNA levels 7 days after TBI. Cyp46 mRNA levels were measured by real-time PCR in cortex ipsilateral to the site of injury 7 days after TBI (tbi icx), contralateral cortex (tbi ccx) and sham cortex ipsilateral to craniotomy (sham icx). Cyp46 mRNA levels at the site of injury were not significantly different from contralateral cortex or sham cortex (n=5). Error bars (standard error of the mean).
From: "Ballen, Karen" <KBallen@liebertpub.com>
Sent: Thursday, October 2, 2008 11:58 am
To: Casandra Marie Cartagena <cmc66@georgetown.edu>
Cc: Bcc
Subject: RE: including published work in thesis

Dear Casandra:
Permission granted.
Karen Ballen
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-----Original Message-----
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Sent: Wednesday, October 01, 2008 9:47 PM
To: Ballen, Karen
Subject: RE: including published work in thesis

Dear Ms Ballen,

Per our phone conversation I am writing to you to request permission to use my work published this September in Journal of Neurotrauma (v25, pgs 1087-1098, 2008) as part of my PhD Thesis. If you could please send written permission I would be very grateful.

Sincerely,

Casandra Cartagena