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The Role of Microglia in the Effects of Steroid Hormones on Brain Inflammation

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THE ROLE OF MICROGLIA IN THE EFFECTS OF STEROID HORMONES ON BRAIN INFLAMMATION

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The conditions defining whether microglial activation is detrimental or beneficial to neuronal survival are still poorly understood. Better understanding of the factors regulating microglia activation may lead to improved therapies for neurodegenerative diseases. Clinical and animal studies point to the neuroprotective and anti-inflammatory effects of steroid hormones. However, our comprehension of the cellular targets and mechanisms of action of these hormones in the CNS is still unresolved. In view of these limitations, the main question addressed in this dissertation was the role that microglia play in the anti-inflammatory effects of steroid hormones in the brain, with particular emphasis on the neuroprotective hormone 17β-estradiol (E2), and the anti-inflammatory steroid, corticosterone.

To address this problem, microglia culture models were established using a microglia cell line and primary cultures from transgenic mice that facilitate the identification of microglia by EGFP expression. Collaborative studies were also done in mice *in vivo*. The expression of steroid hormone

receptors was studied as well as their function. This dissertation shows that microglia cells are not direct targets of estrogen actions, but respond profoundly to glucocorticoids, which exert anti-inflammatory effects on the production of cytokines like TNFα, IL-6 and NO. Steroid hormones can be produced within the brain. In this dissertation, microglia cells are shown to participate in the metabolism of steroids through expression of steroid-converting enzymes. Expression of 11βHSD1 in microglia mediated an autocrine re-activation of glucocorticoids, whereas, expression of enzymes like 17βHSD1 and 5αR catalyzed the conversion of active androgens and estrogens from steroid hormone precursors AD and DHEA. These microglia-derived hormones had estrogenic effects on neuronal cells, as described in the last section of this dissertation where the characterization and responsiveness of a neural progenitor cell line are presented.

In summary, microglia cells are highly susceptible to the action of glucocorticoids, but not estrogens. This specificity is dictated by the abundant expression of glucocorticoid receptors, and a minimal expression of estrogen receptors. A novel role of microglia is also presented. Microglia express steroid-metabolizing enzymes, which mediate the autocrine re-activation of glucocorticoids, or the production of active androgens and estrogens from steroid hormone precursors.

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I dedicate this work to my father and mother for their unconditional love and support; to my mentors, which without their trust and guidance I would of not come this far; and to my meditation teacher who showed me the greatness of serving and loving others.

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LIST OF ABBREVIATIONS

1°MG	primary microglia
3αHSD	3α hydroxysteroid dehydrogenase
3βHSD1,2,4,7	3β hydroxysteroid dehydrogenase isoforms 1,2,4,7
5αAD	5α-androstane-3,17-dione (5α-Adione)
5αR	steroid 5α reductase
11βHSD-1,2	11β hydroxysteroid dehydrogenase type 1 or type 2
11-DH-Cort	11-dehydro-corticosterone
17βHSD-1	17β hydroxysteroid dehydrogenase
AD	4-androstene-3,17-dione (androstenedione)
Adiol	5-androstene-3b,17b-diol (Δ^5-Adiol)
ANOVA	analysis of variance
AR	androgen receptor
Arsa	aryl sulfatase
BSA	bovine serum albumin
CREB	cyclic-nucleotide Regulatory Element Binding-protein
CNS	central nervous system
Cort	corticosterone
CSS	charcoal-stripped fetal calf serum
Cyc-D2	cyclin D2
EN-2	engrailed 2
ERE	estrogen response element
$ERK^{1/2}$	extracellular regulated mitogen activated protein kinase
DAB	diaminobenzidine
DHEA	3β-hydroxy-5-androstene-17-one (dehydroepi-androsterone)
DHT	5α-androstane-3-one-17β-ol (dihydrotestosterone)
DMEM	Dulbecco's modified Eagle's medium
DCX	doublecortin
E2	17β-estradiol
EGFP	enhanced green fluorescent protein

ERα, β	estrogen receptor alpha, beta
FACS	fluorescence activated cell sorting
FCS	fetal calf serum
FITC	fluourescein isothiocyanate
FMRP	fragile-X mental retardation protein
GFAP	glial fibrillary acidic protein
GM-CSF	granulocyte-macrophage colony stimulating factor
GR	glucocorticoid receptor
HPA	hypothalamic-pituitary-adrenal axis
HGP	hypothalamic-pituitary-gonadal axis
ICI	ICI 182780
IL-6	interleukin 6
INFγ	interferon gamma
L27A	ribosomal protein L27A
LPS	lipopolysaccharides
MAPK	mitogen activated protein kinase
Math-1	atonal homologue (Drosophila) 1
MCSF	macrophage colony stimulating factor
MR	mineralocorticoid receptor
NeuN	neuronal nuclear antigen
NO	nitric oxide
P	progesterone
P450c17	cytochrome p450 17-hydroxylase
P450c21	cytochrome p450 21-hydroxylase
p450Arom	cytochrome p450 aromatase
p450scc	cytochrome p450 side chain cleavage enzyme
Pax6	paired box gene 6
PE	phycoerithrin
PBR	peripheral benzodiazepine receptor
PBS	phosphate buffered saline
PFA	paraformaldehyde

PK	PK-11195
PR	progesterone receptor
Ro	Ro 5-4864
RT-PCR	real-time polymerase chain reaction
SERM	selective estrogen receptor modulator
SHR	steroid hormone receptor
StAR	steroidogenic acute regulatory protein
StS	steroid sulfatase
Sulft	DHEA sulfotransferase
T	4-androstene-3,17-diol (testosterone)
TGFβ1	transforming growth factor beta 1
TNFα	tumor necrosis factor alpha
TBS	tris-buffered saline
TLC	thin layer chromatography
Zic1-2	zinc finger protein of the cerebellum
Zipro-1	zinc finger protein 38

CHAPTER 1

INTRODUCTION

Microglia cells are the resident Central Nervous System macrophages

Early anatomical and cytological studies done by del Rio-Ortega (del Rio-Ortega, 1932) revealed a unique cell type differing in morphology from neurons and supporting glial cells designated as microglia (Fig.1A). These cells, which constitute approximately 12% of the brain, are distinguished by their small cell body and highly ramified morphology (Fig.1A-B). Microglia cells populate the brain early in development as bone marrow-derived monocytes with phagocytic functions, and eventually differentiate into resident parenchymal brain cells during the postnatal period [rev. by (Lawson, Perry et al. 1990)]. In the adult, two populations of microglia have been described: a slow turnover, resident population of cells within the brain parenchyma, and a high turnover population of cells that are found in the meninges, choroids plexus, walls of the ventricles, and other structures devoid of the blood brain barrier (Lawson, Perry et al. 1992).

Microglia are present in large numbers in all major regions of the brain but are not uniformly distributed (Lawson, Perry et al. 1990). The majority of microglia are found in gray matter (*vs* white matter); densely

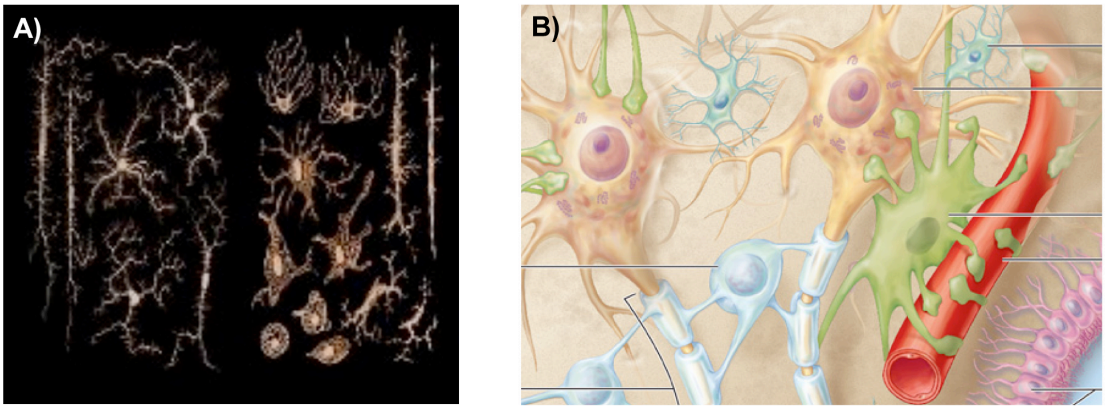

Figure 1. Microglia are distinguished by their small cell body and highly ramified morphology. **A)** Original drawings from Pio del Rio-Hortega depicting microglia cells (left) and cells from mesodermic origin (right) in the human brain. **B)** Cartoon* depicting the interrelations of the main cell types in the brain parenchyma: neurons (brown), oligodendrocytes (white), astrocytes (green) and microglia (blue). **Adapted from: McGraw-Hill Com., Inc. Kellog Community College 1999*

populated areas include the hippocampus, olfactory telencephalon, basal ganglia and substantia nigra, while less densely populated areas include fiber tracts, cerebellum and much of the brainstem. The cerebral cortex, thalamus and hypothalamus have intermediate cell densities relative to those discussed (Lawson, Perry et al. 1990). In the normal/resting (steady state) adult nervous system very little is known about the functions of microglia. Yet, emerging evidence indicates microglia actively scan their environment for any disturbances (Davalos, Grutzendler et al. 2005; Raivich 2005). Being the resident brain macrophages, microglia have important immunological and pathological functions (Aloisi 2001; Block and Hong 2005). As effector cells they are able to directly kill microorganisms and tumor cells, and they also partake in tissue remodeling processes controlling synaptogenesis and neuronal death (Marin-Teva, Dusart et al. 2004; Bessis, Bechade et al. 2007). However, microglia are best characterized for their involvement in brain inflammation.

Inflammation and Neurodegeneration

The inflammatory response is designed to activate local resident macrophages and attract circulating immune cells to the site of damage or infection, for the clearance of tissue debris or initiation of adaptive immune

responses, respectively. Microglia become rapidly activated in response to brain injury or immunological stimuli (Kreutzberg 1996; Raivich, Bohatschek et al. 1999). Activated microglia show characteristic morphological changes that include the shortening of processes and a hypertrophied cell body. Additionally, activated microglia release a number of soluble factors, like cytokines, which are pro-inflammatory in nature [rev. by (Raivich, Jones et al. 1999; Block and Hong 2005)]. These molecules activate surrounding microglia and astrocytes, change the permeability of endothelial cells, and induce the infiltration of blood leukocytes and lymphocytes to the inflamed tissue [rev. (Raivich, Bohatschek et al. 1999)]. Localized inflammatory responses are normally resolved by the late expression of anti-inflammatory cytokines and other tissue factors [rev. (Raivich, Bohatschek et al. 1999)], which in turn, resolve the production of inflammatory molecules and induce the clearance of activated cells. Inflammation is a normal physiologic response, but without appropriate regulation it causes damage to the otherwise, healthy tissue. This is particularly true in the central nervous system (CNS) due to the high metabolic state of neurons and their susceptibility to environmental changes.

An unregulated response by microglia leads to elevated and sustained levels of inflammatory mediators such as superoxide, nitric oxide and TNFα

(Colton and Gilbert 1987; Sawada, Kondo et al. 1989; Liu, Gao et al. 2002), which become highly neurotoxic (Block and Hong 2005). As demonstrated by the doubling of research articles about microglia during the last 6 years (119 titles in 2000 *vs* 226 in 2006 (NCBI, Pub Med)), an increased interest in the role microglia play in the initiation and maintenance of inflammation in the brain is evident. Increasingly, chronic or un-regulated inflammation is being recognized as a main contributing factor to the progression and etiology of neurodegeneration (Giovannini, Scali et al. 2003; Morale, Serra et al. 2006; Bonifati and Kishore 2007), as well as other body diseases like arthrosclerosis (Chait, Han et al. 2005), arthritis (Mizuno 2006), osteoporosis (Clowes, Riggs et al. 2005; Ginaldi, Di Benedetto et al. 2005).

Inflammation and steroid hormones

Systemic inflammation increases cytokine levels in the blood stream, and causes activation of a major neuro-endocrine circuit known as the hypothalamic-pituitary-adrenal (HPA) axis (Turnbull and Rivier 1995; Buckingham, Loxley et al. 1996; Beishuizen and Thijs 2003). HPA axis activation leads to the rapid production of adrenal steroids (glucocorticoids) and their release into circulation. Glucocorticoids are potent endogenous anti-inflammatory molecules (Dannenberg 1979; Umland, Schleimer et al.

2002), which affect cells involved in the inflammatory response by interfering with the activation of early inflammatory transcription factors like the p65 NFkB subunit as well as AP-1 (Smith, Burke et al. 1996; Sakurai, Shigemori et al. 1997; De Bosscher, Vanden Berghe et al. 2000; Gonzalez, Jimenez et al. 2000). Glucocorticoids also alter the distribution and trafficking, as well as the differentiation and maturation, of macrophages/monocytes and other blood leukocytes (Sorrells and Sapolsky 2007).

However, glucocorticoids can also act as immune-enhancing agents (Yeager, Guyre et al. 2004). For instance, moderate levels of corticosterone, induced by acute stress or administered directly, enhance skin immune reactions by increasing leukocyte trafficking, while chronic or high doses are inhibitory (Dhabhar and McEwen 1999). Thus, it becomes important to view steroid hormones not solely as immunosuppressant, but rather as modulators of inflammation.

This regulatory role has also been evident in a different class of steroid hormones, the sex hormones, where clinical and animal studies of inflammatory diseases have shown that they too can modulate inflammation (Whitacre, Reingold et al. 1999; Behl 2002; Wagner, Kaplan et al. 2002). One of the most widely studied steroids in this area has been the female sex

hormone 17β-estradiol (E2) (Cutolo, Sulli et al. 1995; Roof and Hall 2000; Garcia-Segura, Azcoitia et al. 2001; Rogers and Eastell 2001; Behl 2002; Hodgin and Maeda 2002; Pfeilschifter, Koditz et al. 2002; McCullough and Hurn 2003; Wise, Dubal et al. 2005).

Sex hormone status dictates severity and progression of inflammatory disease

Women generally present a more robust immune response than men: for example, they have more potent antigen presenting cells, higher circulating immunoglobulin levels, and stronger antigen specific humoral immune responses. In fact, females have 10 times higher propensity to develop autoimmune disorders compared to men [rev. by (Mor, Nilsen et al. 1999)]. E2 has been demonstrated as one of the sex hormones that influences the progression of different inflammatory pathologies such as cardiovascular disease, (Hodgin and Maeda 2002; Wagner, Kaplan et al. 2002), osteoporosis (Turner, Riggs et al. 1994; Watts 2002), and autoimmunity (Cutolo, Sulli et al. 1995).

While in the periphery E2 usually is pro-inflammatory, in the CNS it is considered as an anti-inflammatory hormone. Evidence from clinical trials and animal studies suggests that E2 is associated with a decreased incidence,

delayed onset and delayed progression of acute and chronic brain disorders, ranging from stroke and schizophrenia to Alzheimer's disease, multiple sclerosis, and Parkinson's disease (Chowen, Azcoitia et al. 2000; Roof and Hall 2000; Lee and McEwen 2001; Behl 2002; Bisagno, Bowman et al. 2003). The reduced susceptibility to acute brain injury, such as cerebral ischemia, neurotrauma and certain neurotoxic agents, in pre-menopausal females, both in humans and rodents, strongly suggests a correlation between the levels of E2 and the severity and progression of inflammatory diseases.

Steroid hormones affect brain injury and repair

Sex steroids have multiple effects in the CNS. Aside from reproduction, perhaps the most important role for these hormones is neuroprotection (McEwen 2001; Behl 2002). Estrogens, progesterone (P), and androgens, have potent neuroprotective actions (Wise, Dubal et al. 2001; Stein and Hoffman 2003; Schumacher, Guennoun et al. 2004; Hoffman, Merchenthaler et al. 2006; Singh 2006; Singh, Dykens et al. 2006). Animal studies show that these hormones protect against several types of injury, such as brain trauma, and enhance recovery after stroke and spinal cord injury (Kalimi, Shafagoj et al. 1994; Cardounel, Regelson et al. 1999; Stein

2001). In comparison to E2, the neuroprotective effects of androgens such as testosterone (T) are not as clear, although T deficiency has been associated with increased symptoms of Parkinson's Disease (Okun, McDonald et al. 2002; Okun, Walter et al. 2002).

The protective effects of E2 and other sex steroids on neurons have been extensively demonstrated in CNS damage models such as ischemia, glutamate excitotoxicity, oxidative stress, amyloid-β toxicity, and neuronal apoptosis (Wang, Santizo et al. 1999; Belcredito, Vegeto et al. 2001; Garcia-Segura, Azcoitia et al. 2001; Stein 2001; Behl 2002; Cordey, Gundimeda et al. 2003; McCullough and Hurn 2003; Rau, Dubal et al. 2003; Rau, Dubal et al. 2003; Sawada and Shimohama 2003; Guerra, Diaz et al. 2004; Wen, Yang et al. 2004; Corasaniti, Amantea et al. 2005; Heyer, Hasselblatt et al. 2005; Platania, Seminara et al. 2005; Wu, Wang et al. 2005; Morale, Serra et al. 2006). However, the mechanisms by which sex hormones affect CNS inflammation and cytokine production are less well understood. To address this issue, several studies have focused on microglia and astrocytes, given that these cells are pivotal in orchestrating the inflammatory response in the CNS (Mor, Nilsen et al. 1999; Raivich, Bohatschek et al. 1999; Raivich, Jones et al. 1999; Jankowsky and Patterson 2001; Hagberg and Mallard 2005; Ladeby, Wirenfeldt et al. 2005; Panickar and Norenberg 2005).

Evidence to date suggests microglia, like peripheral macrophages, are targets of direct steroid action through the expression of sex hormone receptors.

Multiple studies in mammalian species (rodents, and lower primates) document the expression of estrogen receptors (ERα, ERβ) and androgen receptors (AR) in glial cells (astrocytes and oligodendrocytes) (Gahr, Metzdorf et al. 1996; Finley and Kritzer 1999; Behl 2002). However reports describing expression of such receptors in microglia, *in vivo*, are scarce (Garcia-Ovejero, Veiga et al. 2002). It has also been shown that glial cells become increasingly responsive to the actions of these steroids in the injured CNS, as ERs and ARs are up-regulated after injury (Gahr, Metzdorf et al. 1996; Cooke, Hegstrom et al. 1998; Kruijver, Fernandez-Guasti et al. 2001; Garcia-Ovejero, Veiga et al. 2002). In the normal rat brain, astrocytes express low levels of ER and AR, which are transiently up-regulated in astrocytes following excitotoxic/chemical injury to the hippocampus, or stab wound to the parietal cortex and hippocampus (Garcia-Ovejero, Veiga et al. 2002). Under these circumstances AR is also induced in microglia (Garcia-Ovejero, Veiga et al. 2002). Likewise, progesterone receptor (PR) expression is up-regulated in neurons and glia, after CNS injury in the rat (Bulloch 2001; Meffre, Delespierre et al. 2005).

In the mouse CNS, E2 treatment decreases the recruitment of total inflammatory cells as well as TNFα positive macrophages and T cells at the onset of experimentally -induced auto-immune encephalitis (Ito, Buenafe et al. 2002). However, in this study microglia only show a moderate attenuation of the peak TNFα expression in response to E2 (Ito, Buenafe et al. 2002). Recently, a comprehensive review of steroid receptor expression in glial cells has been done by Garcia-Ovejero et al. (Garcia-Ovejero, Azcoitia et al. 2005), indicating that *in vivo,* microglia are limited in the expression of steroid receptors. These studies would suggest that E2 effects on neuroinflammation may be mediated through other cells expressing estrogen receptors.

Cytokine regulation by E2 and other hormones

In spite of the lack of evidence demonstrating estrogen receptor expression in microglia *in vivo*, E2 is reported to block a number of inflammatory mediators produced by activated microglia *in vitro*. Specifically, E2 pre-treatment of activated microglia in response to lipopolysaccharide (LPS), phorbol ester, or interferon-γ (IFNγ) stimulation has been noted in the literature to attenuate phagocytic activity, inducible nitric oxide synthase and matrix metalloproteinase-9 expression, as well as

production of superoxide, nitric oxide (NO), and prostaglandin-E2 (Bruce-Keller, Keeling et al. 2000; Drew and Chavis 2000; Vegeto, Bonincontro et al. 2001). Additionally, in an *in vitro* HIV inflammatory model, viral activation of microglia results in super oxide and NO production, increased phagocytosis, and tumor necrosis factor-α (TNFα) release. HIV microglial activation is suppressed by E2 pre-treatment through interference of viral-mediated MAPK activation (Bruce-Keller, Barger et al. 2001). Other reports indicate cytokines and surface receptors critical for adaptive immunity such as IL-10, TNFα, IFNγ, MHC Class I, CD40, and CD86, are also regulated by E2 in the microglia cell line, N9 (Dimayuga, Reed et al. 2005).

In contrast to the aforementioned studies, other studies have failed to demonstrate anti-inflammatory actions of E2 on microglia/macrophages (Dovio, Sartori et al. 2001; Suuronen, Nuutinen et al. 2005; Sierra in preparation). Additionally, studies on microglia activation have found no effects of E2, compared to other steroids such as glucocorticoids (Lieb, Engels et al. 2003; Fowler, Johnson et al. 2005). Suuronen et al. (2005) report anti-inflammatory effects of various selective estrogen receptor modulators (SERMs), but not of E2, and further suggest that SERM-induced modulation of LPS-activated pro-inflammatory signaling cascades is not estrogen receptor-mediated (Suuronen, Nuutinen et al. 2005).

In spite of the these apparently conflicting reports, several studies support a pure anti-inflammatory role of E2 on microglia (Mor, Nilsen et al. 1999; Bruce-Keller, Keeling et al. 2000; Vegeto, Bonincontro et al. 2001; Vegeto, Belcredito et al. 2003; Baker, Brautigam et al. 2004; Dimayuga, Reed et al. 2005; Ghisletti, Meda et al. 2005; Liu, Liu et al. 2005; Vegeto, Belcredito et al. 2006). Another set of studies with β-amyloid induction of NO in microglia, show E2 acts in a biphasic fashion, dependent on dose and milieu, in which high doses of E2 are inhibitory (Bruce-Keller, Keeling et al. 2000), and physiological doses of E2 stimulate NO secretion and toxicity (Harris-White, Chu et al. 2001). In fact, recent papers have shown ovary-derived E2 is essential for mounting a proper inflammatory response to bacterial lipopolysaccharides (LPS), suggesting that circulating E2 is exerting a permissive, pro-inflammatory effect (Soucy, Boivin et al. 2005).

The dichotomous effects of E2 on inflammation have been associated with the failure of clinical trials to prove the benefits of hormone replacement therapies (Stork, van der Schouw et al. 2004; Bushnell 2005; Wise, Dubal et al. 2005). A proposed compounding factor in these clinical trials could be responsiveness to E2, as Suzuki et al (2007) have suggested in a model of postmenopausal ischemic stroke. Here the neuroprotective and

anti-inflammatory actions of E2 were dependent on the extent of the hypo-estrogenic period (Suzuki, Brown et al. 2007).

Given the aforementioned studies, it is quite clear that there is controversy about the effects of estrogen on microglia activity. The view that sex hormones, like E2, are universally anti-inflammatory agents needs to be reconsidered given the lack of coherence in the literature. Steroid hormones could be described more accurately as conditional immuno-modulatory agents, exerting permissive, stimulatory, or inhibitory effects depending on hormone concentration, endpoints measured, cell types studied, and inflammation models used (Sternberg 2001; Dinkel, Ogle et al. 2002; Yeager, Guyre et al. 2004).

Sources of Steroid Hormones

The gonads and the adrenal glands are the principal sources of steroid hormone synthesis in the body. Steroid hormones are derived from the metabolism of cholesterol [rev. by (Pikuleva 2006)]. The first required step for steroidogenesis is the transfer of cholesterol into the mitochondria (Miller 1995), which is mediated by the steroid acute regulatory protein (StAR) and the peripheral benzodiazepine receptor (PBR) (Hauet, Liu et al. 2002). Within the mitochondrial lumen, cholesterol is converted by the p450

cytochrome side-chain cleavage (p450scc) into pregenenolone, which is further metabolized in the cell's endo-reticular system into progesterone, dehydroepiandrosterone (DHEA) and androstenedione (AD) by the p450c17 and the 3β-hydroxysteroid dehydrogenase (HSD) enzymes [rev by (Simard, Ricketts et al. 2005)]. The p450c21 enzyme can convert pregenenolone and progesterone into the precursors for glucocorticoids and mineralocorticoids, respectively (Simard, Ricketts et al. 2005). DHEA and AD are the precursors for the androgens and estrogens, which are synthesized by various enzymes, principally, 17βHSD, 3βHSD, p450-Aromatase (p450Arom), and 5α-reductase. The steroidogenic pathway and the enzymes mentioned are summarized in Figure 2.

Belief of the exclusive synthesis of steroid hormones in the gonads and adrenals was held for a long time. It was thought that hormones secreted into the circulation would then enter target tissues to exert their functions [rev. by (McEwen, Biegon et al. 1982)]. Currently, however, it is recognized that steroid synthesis/metabolism occurs in a number of tissues and cells, such as adipose tissue (Feher, Bodrogi et al. 1982), liver (Katagiri, Tatsuta et al. 1998), skin (Labrie, Luu-The et al. 2000), lungs (Kao, Crosswell et al. 1979), and notably, the brain (Tsutsui, Ukena et al. 1999; Baulieu, Robel et al. 2001; Stoffel-Wagner 2003). Steroidogenesis (the conversion of

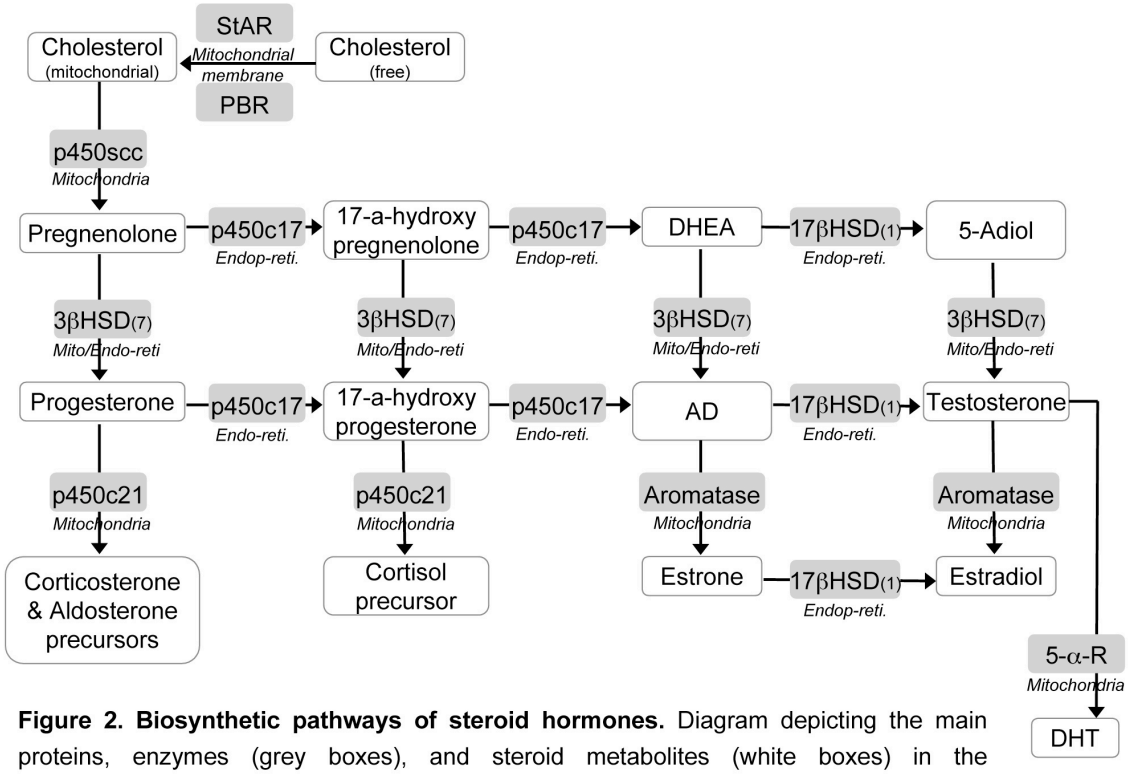

Figure 2. Biosynthetic pathways of steroid hormones. Diagram depicting the main proteins, enzymes (grey boxes), and steroid metabolites (white boxes) in the steroidogenesis pathway. Enzyme localization is indicated in small italics.

Adapted from: Herrmann M, et al. 2002

cholesterol into pregenenolone and downstream steroids) is more limited, but steroid conversion and metabolism appears to be much more widespread than previously believed.

End-organ regulation of steroid hormone action

Humans and certain primates secret into circulation large amounts of the steroid precursors DHEA and DHEA-sulfate (DHEA-S) from the adrenals. These steroids do not bind any receptors, yet exert estrogenic and androgenic actions after being converted into active estrogens and androgens in target tissues (Labrie 1991; Labrie, Luu-The et al. 2001). In fact, in post-menopausal women almost all sex steroids are produced from adrenal precursors, and in adult men, approximately half of androgens are made locally in target tissues (Labrie 2003; Labrie 2004).

Pathological conditions, such as breast cancer and chronic joint inflammation, have provided some of the best-studied cases of intracrine steroid synthesis/conversion, where local steroid production plays an active role in disease. Breast cancer cells can locally synthesize or regenerate estrogens, which serve as growth factors promoting tumor growth and persistence [rev by (Labrie 2003; Salhab, Reed et al. 2006)]. Therefore,

development of inhibitors for steroid-converting enzymes has become an active area of research (Purohit, Woo et al. 2001). In chronic inflammatory disease, such as osteoarthritis and rheumatoid arthritis, tissue synovial cells display increased production of estrogens with pro-inflammatory effects (Schmidt, Weidler et al. 2005). This pro-estrogen pathway can be blocked by the androgens, AD and T, which are converted into 5α-hydroxylated androgens that have anti-inflammatory properties (Schmidt, Weidler et al. 2005). In cases such as this, appreciation of the steroid-converting properties in the inflamed tissue can aid in the choice of therapeutic steroids to be used (Labrie 2003).

Not all intracrine steroid metabolism leads to pathological conditions. Local steroid synthesis can also contribute to the maintenance of homeostasis in tissues. Such is the case with the brain, as indicated by various reports on brain development (Tsutsui 2006), neurotransmission (Majewska 1992; Paul and Purdy 1992; Schumacher, Guennoun et al. 1997), and recovery from damage (Azcoitia, Sierra et al. 2001; Azcoitia, Sierra et al. 2003; Schumacher, Guennoun et al. 2004).

Neurosteroidogenesis: synthesis of steroids in the brain

In the early 1980s, Corpechot et al. (1981) found a high concentration of pregenenolone and DHEA in brains of adrenalectomized and castrated rats. They further demonstrated that these steroids were not affected by adrenocorticotropic hormone, or by suppression of circulating glucocorticoids (Corpechot, Robel et al. 1981). Soon after, the p450 side chain cleavage cytochrome (p450scc), which converts cholesterol into pregenenolone, was identified in the brain opening the possibility of *de novo* brain steroidogenesis (Schumacher, Guennoun et al. 2004). The term neurosteroid was coined, referring to steroids that are synthesized from cholesterol or another early precursor in the nervous system (Robel and Baulieu 1995).

In the CNS, DHEA is synthesized from pregenenolone by astrocytes and neurons. Astrocytes can further metabolize DHEA into sex hormones (Zwain and Yen 1999). Brain DHEA levels are higher than those in circulation, and have been negatively correlated with aging and neurodegeneration (Weill-Engerer, David et al. 2002). Additionally, DHEA has multiple effects when administered to the CNS, including modulating the activity of GABA and NMDA receptors [rev. by (Gibbs, Russek et al.

2006)], yet no specific receptor for this hormone has been detected (Regelson and Kalimi 1994; Majewska 1995). This has led investigators to suggest that in the brain DHEA may be metabolized into active steroid hormones, which then mediate the observed effects of DHEA (Zwain and Yen 1999; Schmidt, Kreutz et al. 2000; Jellinck, Lee et al. 2001; Jellinck, Kaufmann et al. 2006; Jellinck Submitted). Evidence for this model has been put forth by Veiga, et al. (2003) who have demonstrated that DHEA mediated neuroprotection in a model of kainic acid toxicity is dependent on the E2-synthesizing enzyme p450Arom (Veiga, Garcia-Segura et al. 2003). Moreover, the principal cell types in the brain, including astrocytes, neurons, and oligodendrocytes have been shown to convert DHEA into androstenedione (AD), and produce downstream estrogens and androgens (Zwain and Yen 1999). However, thus far, the participation of microglia in the metabolism and conversion of steroids in the CNS has not been addressed.

Neurosteroid levels correlate negatively with neurodegeneration

Neurosteroidogenesis has been described as an adaptive coping mechanism following brain damage (Garcia-Ovejero, Azcoitia et al. 2005), in agreement with the neuroprotective effects of DHEA (Mao and Barger

1998; Lapchak, Chapman et al. 2000; Kaasik, Kalda et al. 2001; Lapchak and Araujo 2001; Morfin and Starka 2001; D'Astous, Morissette et al. 2003; Veiga, Garcia-Segura et al. 2003; Charalampopoulos, Alexaki et al. 2006; Juhasz-Vedres, Rozsa et al. 2006; Wojtal, Trojnar et al. 2006) and estrogen (Behl 2002). As androgens and estrogens can be pro- or anti-inflammatory (Straub, Konecna et al. 1998; Jacobson and Ansari 2004; Cutolo, Capellino et al. 2005), the steroid profile in a tissue may determine the extent of inflammation (Schmidt, Naumann et al. 2006), and in the case of the brain, of neurodegeneration. Neurodegeneration has been negatively correlated with neurosteroid levels (Schumacher, Weill-Engerer et al. 2003). Measurements of various neurosteroids in Alzheimer's patients compared to aged healthy controls showed a trend towards less steroids in the diseased patients, as well as reduced DHEA metabolites; and a negative correlation was established between Alzheimer's biomarker proteins phospho-Tau and β-amyloid, and these neurosteroid levels (Weill-Engerer, David et al. 2002). These studies are highly suggestive of the neuroprotective role of steroids that are synthesized or metabolized within the brain.

Summary

Microglia cells are the resident brain macrophages, whose role in the initiation and maintenance of brain inflammation has been firmly established. Chronic or exacerbated inflammation can be deleterious to resident cells, particularly in the nervous system. The most common neurodegenerative diseases, such as Alzheimer's, Parkinson's, multiple Sclerosis, stroke induced neurodegeneration, etc., all share important inflammatory components, which have become a current therapeutic target. Steroid hormones are potent regulators of inflammation, particularly the glucocorticoids and sex hormones. Additionally, sex hormones, such as E2, have important neuroprotective effects. Neuroprotection afforded by E2 has been linked to the anti-inflammatory properties of this hormone, yet as previously stated, whether E2 directly affects microglia activation *in vivo* remains unclear.

For the initial stage of my thesis work I set out to test the hypothesis that estrogen exerts anti-inflammatory effects by acting directly on microglia. To this end, I developed a cell culture system using a microglia cell line, BV2, and primary microglia cultures from a transgenic mouse line. I then tested and compared the steroid hormones, estrogen and corticosterone, for their ability to modulate the activation of microglia with

LPS, and found that corticosterone is a potent inhibitor of pro-inflammatory cytokine production, while estrogen has no effects on microglia activation. My results were consistent with further studies done in the laboratory showing that microglia predominantly express glucocorticoid receptors and show minimal levels of ERα expression *in vivo*.

The brain can synthesize hormones *de novo* from cholesterol or metabolize circulating hormone precursors, like DHEA, into active androgens and estrogens, which have important roles in the homeostasis of the CNS. Steroid-converting enzymes are expressed in the various cell types of the brain, and although steroid metabolism has been implicated in the brain's response to injury, the participation of microglia in this process has not been described.

Given the protective properties of neurosteroids and the critical role that microglia play in neurodegeneration, in the latter part of this dissertation I formed the hypothesis that microglia are active contributors in the synthesis and metabolism of neurosteroids in the brain. To test this hypothesis I examined the expression and function of the major steroid converting enzymes in the steroidogenesis pathway through real-time PCR of *ex vivo* and *in vitro* microglia samples, and analyzed the function of these enzymes with metabolic assays in primary microglia cultures.

I further characterized a developing neuronal cell line to test its responsiveness to estrogen and other neurosteroids produced by microglia. My studies focused on the parenchymal, slow-turnover, resident microglia population. A novel role of microglia in relation to the effects of steroid hormones in the brain is presented in this dissertation. Microglia cells are shown to express various steroid-metabolizing enzymes, which mediate events such as the autocrine re-activation of glucocorticoids, or the production of active androgens and estrogens from steroid hormone precursors, which can influence neuronal and other brain cells.

CHAPTER 2

MODELS FOR STUDYING HORMONAL REGULATION OF MICROGLIA ACTIVATION

Steroid hormones simultaneously affect many cell types throughout the central nervous system (CSN) (Garcia-Segura, Chowen et al. 1994; McEwen 2001; Behl 2002; Bryant, Sheldahl et al. 2006). One cell type that has not been thoroughly evaluated for its susceptibility to steroid hormones is microglia. Microglia are related to the monocytic/macrophage lineage of immune cells (Raivich and Banati 2004); however they possess a distinctive morphology and characteristics which set them apart as CNS-specialized cells (Lawson, Perry et al. 1990; Walker 1999). To determine if microglia are targets of hormone action, they must be separated from other surrounding cell types. A widely used approach is the isolation and culture of enriched cell populations, which can also be transformed into permanently viable cell lines. Assay systems can then be developed on populations of these cells that allow for the systematic evaluation of end points, which may be potentially regulated by hormones in microglia.

The activation of the innate immune system in the brain leading to microglial expression of pro-inflammatory cytokines may be central to the

pathophysiology and etiology of neurodegenerative disorders (Nguyen, Julien et al. 2002; Lehnardt, Massillon et al. 2003). It is therefore, important to develop microglia models that can inform us of potential factors regulating the inflammatory response in these cells. In this section, I developed microglia cultures and characterized their activation by bacterial lipopolysaccharides (LPS). Granulocyte-monocyte colony stimulating factor (GM-CSF) cultured cells had the highest proliferation rates, maintained steady expression of macrophage/microglia markers, and showed a robust cytokine response after LPS stimulation.

For this thesis, I initially used the mouse microglia cell line, BV-2, as a model to determine whether the sex hormone 17β-estradiol (E2) could block production of inflammatory factors by activated microglia, since there were reports of such interactions in the literature (Mor, Nilsen et al. 1999; Bruce-Keller, Keeling et al. 2000; Drew and Chavis 2000; Vegeto, Bonincontro et al. 2001). The BV-2 cell line was originally generated by infecting primary microglial cell cultures with a v-raf/v-myc oncogene carrying retrovirus (Blasi, Barluzzi et al. 1990). This cell line will further be referred to as BV-2 cells. While cloned cell lines offer ease of experimentation, it is generally considered that microglia cell lines express partially activated phenotypes (Carson, Reilly et al. 1998; Nakamura, Si et

al. 1999; Aloisi 2001), and are not an authentic representation of the *in vivo* phenotype. Thus, to verify the authenticity of results found in BV-2 cells and to have a more reliable model, I also developed the use of primary microglia cultures. In this Chapter I describe the establishment of the assay systems used to evaluate hormonal regulation of microglia.

LPS stimulation as a model of neuro-inflammation in microglia

LPS is recognized as a potent activator of all macrophage cells, including microglia (Nakamura, Si et al. 1999). LPS acts through the innate immune pathway, comprised of evolutionarily conserved receptors called Toll-like receptors (TLRs), which detect and alert the immune system of invading pathogens (Iwasaki and Medzhitov 2004; Takeda and Akira 2005). TLR-4, coupled to the accessory protein MD2, and CD14, a myeloid phospholipid-anchored membrane protein, form the specific receptor for LPS (Palsson-McDermott and O'Neill 2004; Miller, Ernst et al. 2005). Upon ligand binding, TLR4-MD2-CD14 engages a signaling cascade that activates many transcription factors, especially NFkB, which mediate the expression of inflammatory genes (Doyle and O'Neill 2006). LPS has been used to characterize microglia activation (Nakamura, Si et al. 1999).

To establish LPS as a model for the activation of microglia, I characterized the response to LPS in the BV-2 cells. LPS stimulation of BV-2 microglia induced slight morphological changes, increased adherence, and proliferation of these cells (Fig.3), as well as the production of cytokines. I initially chose to measure 3 inflammatory cytokines and the generation of a reactive oxygen species as endpoints reflecting LPS-induced activation of microglia; these were: tumor necrosis factor-α (TNFα), interleukin-6 (IL-6), interleukin-1β (IL-1β), and nitric oxide (NO).

The results of these studies showed that LPS stimulation of BV-2 cells induced a time and dose-dependent production of TNFα, IL-6, IL-1β, and NO (Fig.4) into the cell culture supernatants. Based on these experiments I chose to use a dose of 100ng/ml LPS for subsequent stimulations. Each cytokine had a particular time course, TNFα being the earliest cytokine produced, followed by IL-1β, IL-6, and finally NO (Fig.4E). Secreted IL-1β levels were low (pg/ml range) (Fig.4C, E), maybe due to inadequate induction of caspase-1 activity required for its secretion (Thornberry, Bull et al. 1992). Thus, Western blot analysis was used to evaluate IL-1β production. These experiments showed that LPS induced a robust production of immature IL-1β of 33kDa by 3-6hr (Fig.5A). Overexposure of blots

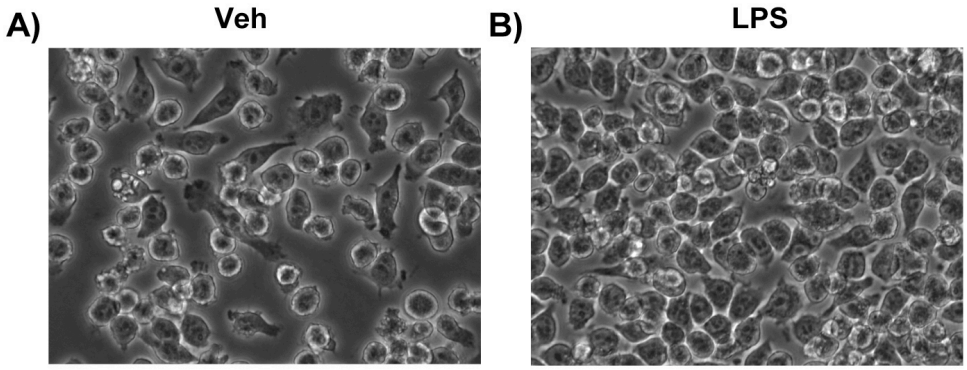

Figure 3. BV-2 microglia cells undergo proliferation and slight morphological changes following LPS stimulation. Light microphotographs of BV-2 cells. Cells were stimulated with **A)** vehicle (Veh) or **B)** 100ng/ml LPS for 24hr and then photographed with a Nikon inverted microscope using a 40X objective.

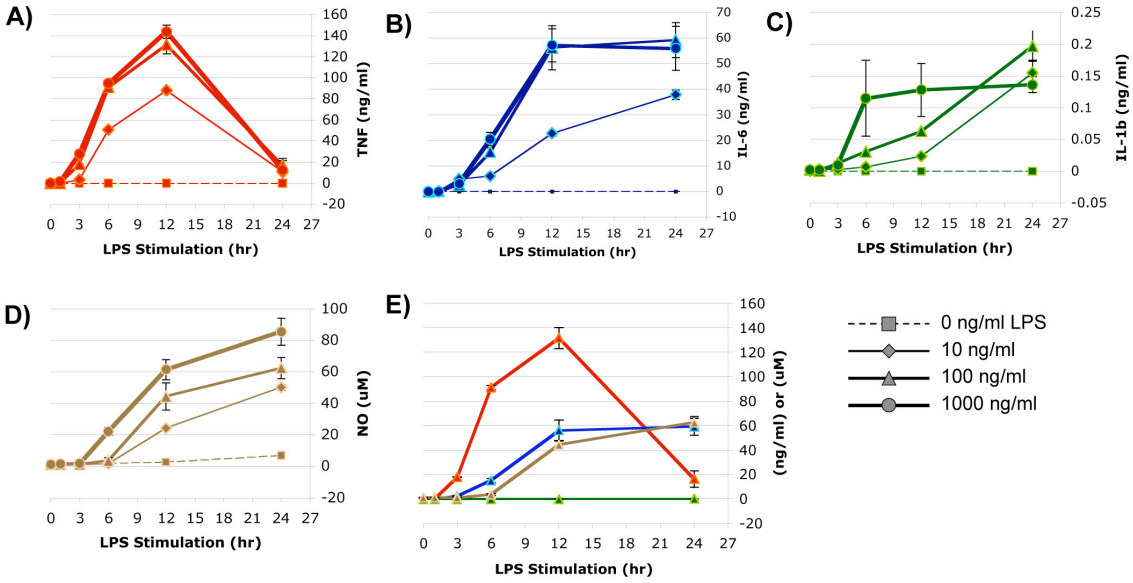

Figure 4. Cytokine responses to LPS in BV-2 microglia. LPS stimulation of BV-2 cells induces a time and dose-dependent production of inflammatory cytokines secreted into the cell-culture supernatant, such as **A)** TNFα, **B)** IL-6, **C)** IL-1β, and **D)** nitric oxide (NO). **E)** Time course comparison of the 3 cytokines and NO at the 100ng/ml LPS concentration.

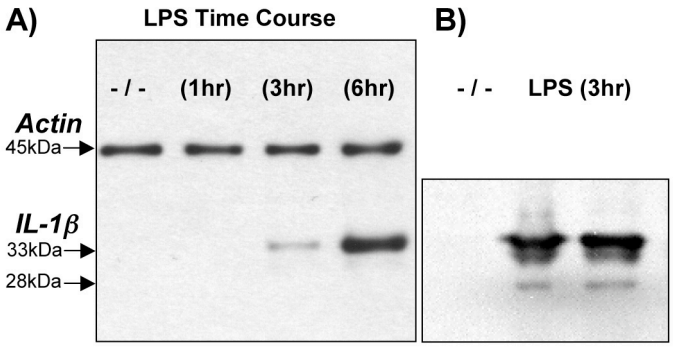

Figure 5. IL-1β expression in LPS-stimulated BV-2 cells. A) Cells were stimulated with 100ng/ml LPS and harvested at different time points for detection of IL-1β by Western blot. The immature form of IL-1β at 33kDa is observed. **B)** Overexposure of film reveals processing products of IL-1β at 28kDa.

revealed low levels of mature processed cytokine of 28kDa (Fig.5B). Resting BV-2 cells showed no detectable cytokine production.

Primary culture of microglia

To further extend my working model for the activation of microglia, and its potential regulation by steroid hormones, I developed primary microglia cultures from neonatal mouse brains. Primary cultures were derived from the transgenic mouse line p7.2*fms*-EGFP (C57BL6/6 X CBA background). This transgenic mouse expresses EGFP, a green fluorescent protein originally cloned from the jellyfish *Aequorea victoria*, controlled by the promoter and regulatory elements of the macrophage colony stimulating factor receptor gene, *cfms* (Sasmono, Oceandy et al. 2003). The *cfms* gene is exclusively expressed in the monocyte cell lineage, which includes all tissue macrophages and microglia (Sasmono, Oceandy et al. 2003; Sierra, Gottfried-Blackmore et al. 2007), providing a clear and reliable source for the isolation of highly enriched microglia cultures. Additionally, results from primary cultures of these mice can subsequently be validated *in vivo*, given that microglia are easily detected by immunofluorescence, or isolated by FACS from the tissue by the endogenous expression of EGFP (Sierra Submitted).

Protocols for primary culture of microglia were first developed by Giulian & Baker (1986) and Frei et.al. (1986) (Frei, Bodmer et al. 1986; Giulian and Baker 1986). *Ex vivo* isolated adult microglia have a tendency to undergo cell death after a dew days in culture and present low proliferative capacity *in vitro* (Fischer and Reichmann 2001). The use of early post-natal brains is favored because of high cell proliferation rates, and preparations at this stage of development present negligible levels of contaminating viable neuronal cells (McCarthy and de Vellis 1980). Therefore, I chose to use day-2 old neonatal mouse pups for primary cell cultures. Forebrains were extracted, meninges removed by micro-dissection, and tissue homogenized by mechanical dissociation and cell straining. Cells were then plated and cultured for up to 2 weeks. This method yields a stratified, mixed, culture of astrocytes, oligodendrocytes and microglia (Giulian and Baker 1986).

I tested two methods for isolating microglia cells from the mixed glial culture. The first was a method based on mild trypsinization of the astrocyte layer, yielding an enriched population of adherent microglia on the culture flask (Saura, Tusell et al. 2003). This method yielded an average of $0.05\pm0.01x10^6$ cells / mouse brain. The low yield of cells obtained from this method were mainly due to the fact that microglia attached to the tissue culture flasks after removal of the astrocyte layer were very difficult to

detach, even with prolonged trypsin incubations and vigorous shaking. The second, and most commonly used, is the shaking method in which loosely adherent microglia cells proliferating on top of the astrocyte layer are detached and collected in the culture media (Giulian and Baker 1986). This method yielded an average of $0.28\pm0.01x10^6$ cells / mouse brain, which proved to be greater than the mild trypsinization method.

Use of colony stimulating factors for primary microglia culture

Hematopoeitic cytokines, particularly the colony stimulating factors macrophage-colony stimulating factor (MCSF) and granulocyte-macrophage colony stimulating factor (GM-CSF) are able to induce proliferation and differentiation of microglia cells (Giulian and Ingeman 1988; Saura, Tusell et al. 2003; Ponomarev, Novikova et al. 2005). To determine the optimal culture conditions I examined the effects of these growth factors on primary microglia. In the mixed glial cultures microglia proliferation was augmented by MCSF or GM-CSF (Fig.6A-C), increasing the yields of isolated microglia, particularly by the shaking method (Fig. 6D).

A notable difference was observed in the morphology of MCSF and GM-CSF grown cells (Fig.6B, C). MCSF induced the elongation and ramification of adherent microglia (Fig.6C), where as GM-CSF induced an

amoeboid morphology (Fig.6B). These results are consistent with previous reports (Santambrogio, Belyanskaya et al. 2001). In spite of morphological changes, the expression of the monocyte/macrophage marker, CD11b, was not affected (Fig.7). GM-CSF is reported to induce a dendritic cell (DC)-like phenotype in primary microglia (Fischer and Reichmann 2001; Santambrogio, Belyanskaya et al. 2001; Ponomarev, Novikova et al. 2005). However, under my culture conditions, the use of GM-CSF did not induce a DC-like phenotype, as assessed by the expression of two DC-specific surface antigens, CD11c, which was negative, and DEC-205, which showed a marginal increase in expression (Fig.7B). The difference between my data and published reports may be due to multiple factors such as the mouse strain, source of GM-CSF (recombinant purified protein *vs* produced by a transformed cell line) and / or the dose and time of incubation. Longer incubations with GMCSF were not used because proliferation rates decreased (Fig.6D).

Purity of microglia preparations

Since microglia were cultured with astrocytes and oligodendrocytes, exclusion of these cells from the experiments in 1°MG cultures was vital. The purity of microglia preparations from the shaking protocol was verified

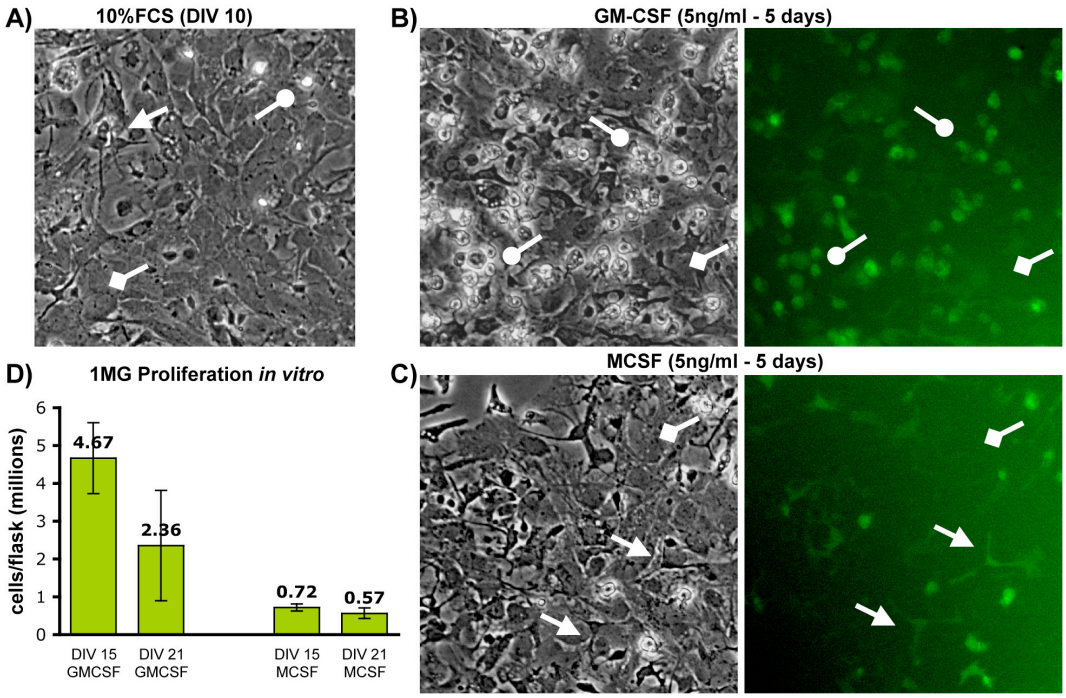

Figure 6. MCSF and GM-CSF induce morphological and proliferative changes in primary microglia. A) Light photomicrograph of mixed glial cells cultured for 10 days *in vitro*. Astrocytes *(rhomboid)* form a confluent layer, in which microglia grown. Proliferating microglia are observed as round, light-refringent cells *(circle)*, whereas attached microglia are flat, dark and with processes *(arrowhead)*. **B)** GM-CSF induces elevated microglia proliferation *(cirlces)*, while MCSF induces some proliferation and ramification of microglia. **C)** Quantification of cells obtained by the shaking method from cultures with GM-CSF or MCSF. DIV: days *in vitro*.

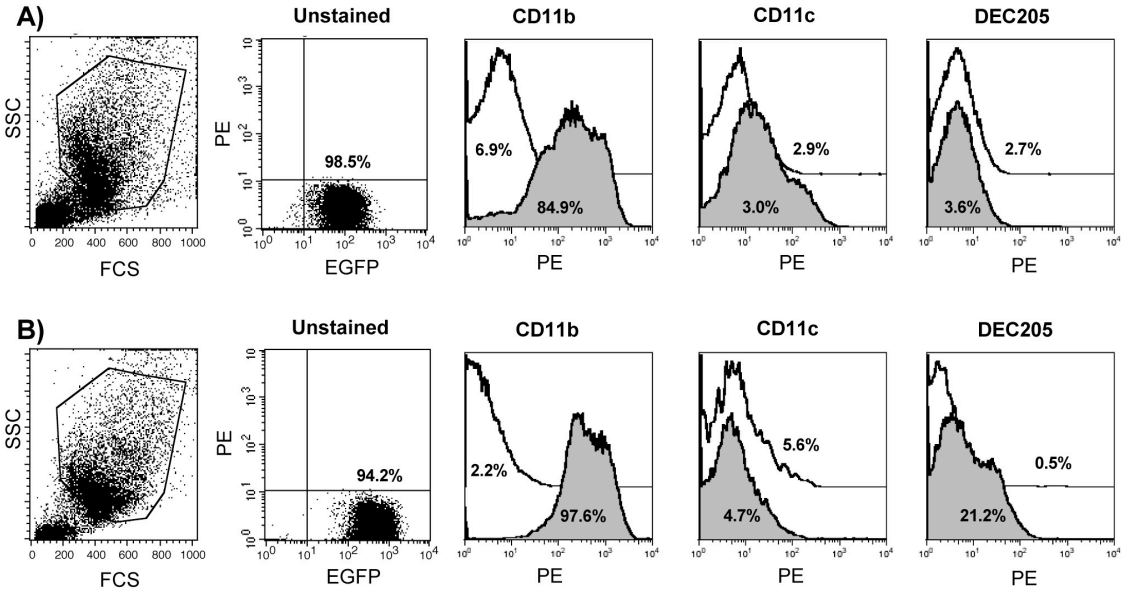

Figure 7. MCSF and GM-CSF do not induce phenotypic changes in CD11b, CD11c or DEC205. Flow cytometric analysis of micoglia obtained from mixed glial cultures grown for 2 weeks with colony stimulating growth factors: **A)** 5ng/ml MCSF or **B)** 5ng/ml GM-CSF. Microglia were separated by the shaking method and then stained with PE-conjugated antibodies for CD11b, CD11c, or DEC205 (grey histograms) with their respective isotype controls (open histograms). Percentage values in histograms reflect the % of double positive (EGFP and PE) cells for each staining condition.

by EGFP expression and by FACS analysis. Shaken 1°MG showed 99-98% purity by EGFP expression and FACS analysis (Fig.7). Therefore, the results obtained from the assays presented below can be attributed to microglia.

LPS activation of primary microglia

In this study, LPS stimulation of primary microglia led to the rapid (30 minutes) activation of NFkB, observed by its translocation to the nucleus by immunocytochemical analysis (Fig.8A). The stress-activated MAP kinase p38, another important transcription factor involved in inflammation (Lee and Young 1996), was also rapidly activated by LPS, through its phosphorylation and consequent translocation to the nucleus observed through immunofluorescence analysis and Western blotting (Fig.8B, C). I next analyzed the production of inflammatory cytokines and NO in primary microglia cultures.

MCSF and GM-CSF cultures stimulated with LPS alone showed a limited production of NO (3-5mM *vs* 60-80mM of BV-2 cells), consistent with previous reports of low NO responses to LPS alone in primary microglia (Saura, Tusell et al. 2003). Interferon-γ (INFγ) can synergize with LPS and stimulate the production of NO (Ding, Nathan et al. 1988; Chan and Riches 2001; Saura, Tusell et al. 2003; Powell, Boodoo et al. 2004).

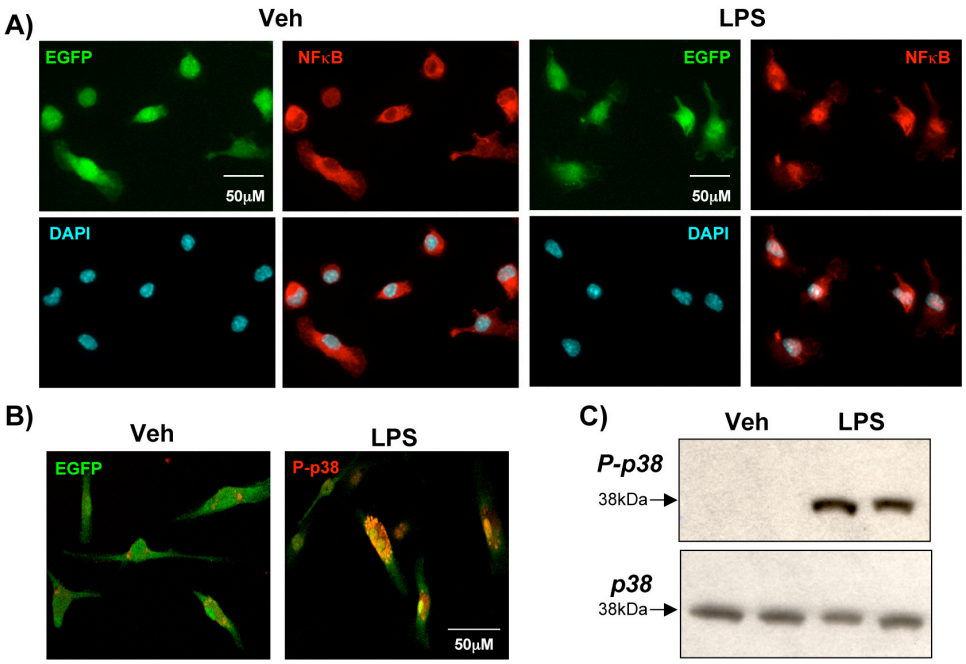

Figure 8. Acute LPS stimulation of *cfms*-EGFP primary microglia cells induces activation of NFkB and p38MAPK. A) Immunofluorescense confocal microscopy of *cfms*-EGFP primary microglia stimulated with vehicle (Veh) or 100ng/ml LPS (LPS) for 30 minutes, followed by fixation and immunocytochemical staining with anti-p65 NFκB and DAPI. **B)** Parallel cultures treated as in A) and stained with anti-phospho p38 MAPK (P-p38). **C)** Western blot analysis of cell lysates from cultures treated with Veh or LPS and probed with anti-P-p38 or the non-phosphorylated anti-p38 MAPK.

Therefore, INFγ was added to LPS for the activation of primary microglia. When stimulated with LPS+INFγ for 24hr, GM-CSF enriched microglia produced significantly higher levels of NO, and showed similar levels of TNFα and IL-6 compared to MCSF derived cells (Fig.9A).

The production of cytokines by primary microglia in response to 24hr LPS+INFγ was also dose-dependent, with a mid range 100ng/ml LPS + 10ng/ml INFγ (Fig.9B). Notably, primary microglia produced less TNFα and NO than BV-2 cells. The most reproducible and robust cytokine responses were obtained from GM-CSF cultured microglia.

In addition to TNFα, IL-6 and NO, LPS+INFγ stimulation of 1°MG caused an increase in other inflammatory cytokines and chemokines such as IL1β, interleukin-12 (IL-12), monocyte chemo-attractant protein-1 and -5 (MCP-1, MCP-5), the small chemokine RANTES (Regulated upon Activation, Normal T-cell Expressed, and Secreted), and the secreted TNF receptor-1 (sTNFR1) (Fig.10). Therefore GM-CSF cultured microglia obtained by the shaking method were used for the experiments presented in the following chapters of this thesis; namely, the effects of steroid hormones on microglia activation (Chapter 3), the autocrine involvement of microglia on the actions of glucocorticoids (Chapter 4), and the expression and activity of steroid metabolizing enzymes in microglia (Chapter 5).

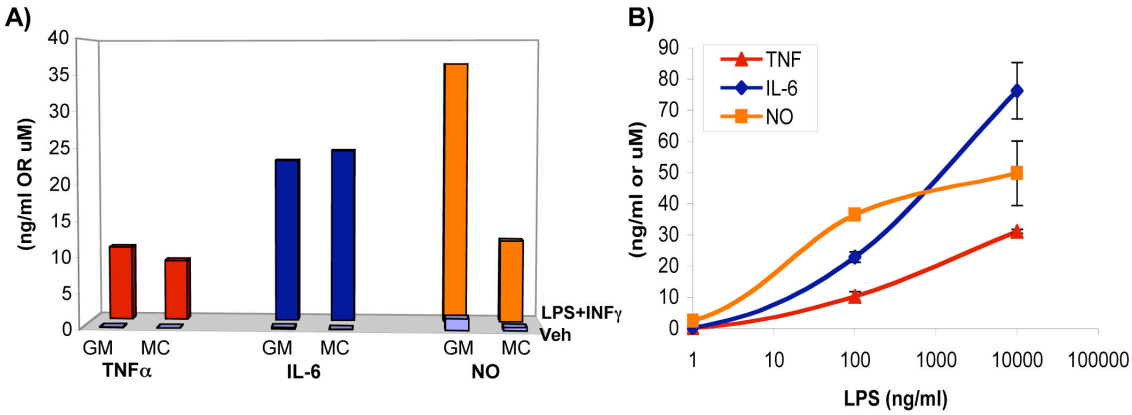

Figure 9. Cytokine responses in MCSF and GM-CSF cultured *cfms*-EGFP primary microglia. A) Comparison between GM-CSF (GM) and MCSF (MC) cultured microglia in their response to 24hr stimulation with 100ng/ml LPS+ 10ng/ml INFγ (LPS+INFγ). **B)** Dose dependence of LPS concentration on the production of cytokines and nitric oxide (NO) in GMCSF cultured microglia. INFγ was kept at 10ng/ml in this experiment. TNFα and IL-6 were measured by ELISA and NO by the Greiss Assay.

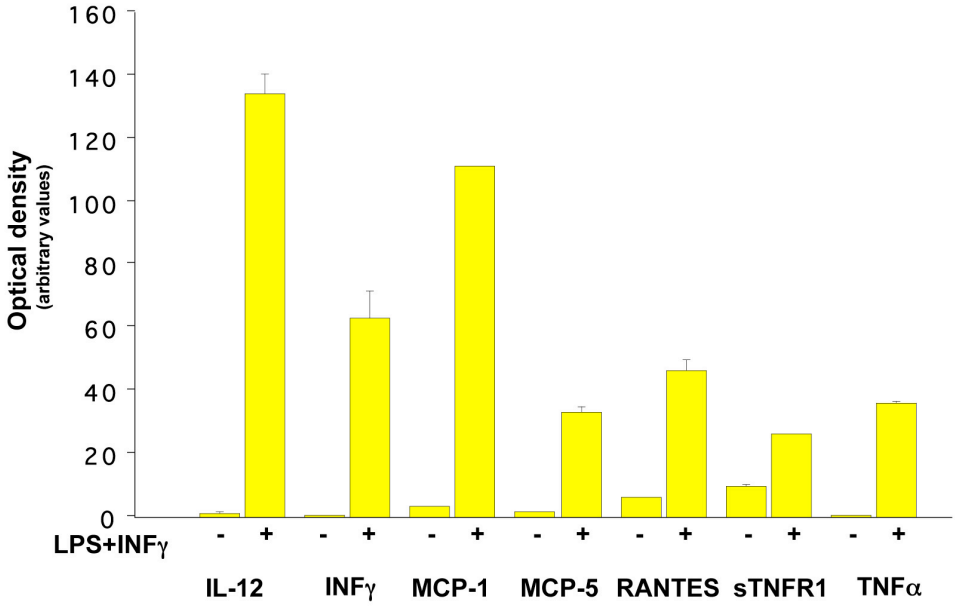

Figure 10. Production of various pro-inflammatory cytokines by *cfms*-EGFP primary microglia after 24hr stimulation with 100ng/ml LPS+ 10ng/ml INFγ (LPS+INFγ). Cytokines in cell supernatants were incubated on a RayBio Cytokine Array membrane and developed on film. Values represent optical density measurements of duplicate readings on the membrane array.

Discussion

Microglia cell cultures have been widely utilized for a variety of studies as they offer a way of assessing the specific effects of any given stimulus / manipulation, in the absence of compounding responses from other cell types. Different microglia cell lines have been established from mouse, rat and even human cells [examples can be noted in (Blasi, Barluzzi et al. 1990; Cheepsunthorn, Radov et al. 2001; Nagai, Mishima et al. 2005)], which have provided useful data for further validations in primary culture, and *in vivo*. One example is the initial description of purinergic receptors in microglia cell lines (Ferrari, Villalba et al. 1996). These studies led to further functional descriptions of these receptors in microglia cultures (Visentin, Renzi et al. 1999; Kaya, Tanaka et al. 2002; Gendron, Chalimoniuk et al. 2003) and in models of neuronal injury (Sasaki, Hoshi et al. 2003; Franke, Gunther et al. 2004); finally leading to *in vivo* imaging studies describing the chemo-attractant functions of these ATP receptors in microglia (Davalos, Grutzendler et al. 2005; Haynes, Hollopeter et al. 2006).

In this Chapter I have utilized BV-2 cells and primary microglia cultures to establish a model for studying microglia activation and establishing reliable end-points, which may be regulated by steroid hormones. The results presented here show that primary microglia cultures

can be reliably activated by LPS+INFγ. Additionally, the use of hematopoeitic growth factors such as GM-CSF, induced high proliferation rates in the mixed microglia cultures without changing the expression of the monocytes/macrophage marker CD11b nor inducing a dendritic cell phenotype. Moreover, GM-CSF enriched microglia showed steady cytokine responses after LPS+INFγ stimulation, which were similar to those of MCSF-cultured microglia with the exception of NO. These findings are in contrast to reports showing microglia exhibit different cytokine responses to LPS if grown in MCSF or GM-CSF (Fischer, Bielinsky et al. 1993). However, authors in this report used 10-fold higher concentrations of the growth factors to find cytokine differences, while my objective in using these growth factors was to increase proliferation without necessarily changing the microglia phenotype. For their increased proliferation and steady cytokine production, GM-CSF enriched microglia were selected as the optimal culture/activation conditions for primary microglia culture.

To monitor microglia activation in subsequent studies, I chose two main cytokines and a nitrogen radical, which have key roles in neuroinflammation: TNFα, IL-6, and NO. TNFα is implicated in the development of CNS inflammation, by causing expression of chemokines and its actions on the vascular endothelium leading to the infiltration of

blood leukocytes [rev by (Aloisi 2001)]. TNFα is also essential for the activation of parenchymal microglia during endotoxaemia, and can be detrimental to the brain by promoting apoptosis of oligodendrocytes and preventing remyelination, and by augmenting cell death in models of neurodegeneration [rev by (Rivest 2003)]. TNFα is also a potent inducer of IL-1β, PGE2 and IL-6. IL-6 induction is considered a ubiquitous and early marker of tissue damage in the brain and plays a central role in the activation of astrocytes [rev by (Raivich, Bohatschek et al. 1999; Raivich, Jones et al. 1999)]. Microglia derived IL-6 can interfere with production of new neurons in the adult brain (Monje, Toda et al. 2003), but has also many neurotrophic properties [rev by (Benveniste 1998)]. At low, physiological concentrations, NO has various roles in the homeostasis of the brain [rev. by (Ohkuma and Katsura 2001; Guix, Uribesalgo et al. 2005)]. However, overproduction of NO has been implicated in most neurodegenerative diseases (Murphy 2000) because of its neurotoxic properties (Dawson and Dawson 1996; Moreno-Lopez and Gonzalez-Forero 2006).

The activation of microglia and subsequent expression of pro-inflammatory cytokines may be central to the pathophysiology and etiology of neurodegenerative disorders (Nguyen, Julien et al. 2002; Lehnardt, Massillon et al. 2003). It is therefore, important to develop microglia models

that can inform us of potential factors regulating the inflammatory response in these cells. LPS produced a consistent cytokine response both in BV-2 microglia and in primary microglia cultures grown with MCSF or GM-CSF. These systems were used as a platform to study the potential anti-inflammatory effects of steroid hormones, namely the sex hormone E2 and the glucocorticoid corticosterone.

CHAPTER 3

MICROGLIA EXPRESS HORMONE RECEPTORS THAT CAN MODULATE THEIR LPS ACTIVATED RESPONSES

The neuroprotective and anti-inflammatory effects of E2 in the brain have been widely reported (Wise, Dubal et al. 2000; Behl 2002; Liao, Chen et al. 2002; Vegeto, Belcredito et al. 2003; Wen, Yang et al. 2004; Hoffman, Merchenthaler et al. 2006). *In vitro* studies have claimed that E2 can act directly on microglia cells to block synthesis of inflammatory molecules. Contrary to these reports, other findings indicate that E2 has no effects [rev. in Chapter 1]. To clarify whether microglia cells are direct targets of E2 and are active mediators of E2 effects in the brain, the expression of estrogen receptors in microglia was studied. These studies were done in collaboration with Dr. Amanda Sierra (Stony Brook, NY), a former postdoc in the McEwen laboratory, who developed a system for isolation of microglia from adult *cfms*-EGFP mice using fluorescence-activated cell sorting (FACS) to measure gene expression. Estrogen receptor studies were also conducted in

collaboration with Dr. Teresa Milner (Cornell Medical School, NY) with whom an ultrastructural analysis by electron microscopy was carried out.

I then proceeded to utilize BV-2 microglia and *cfms*-EGFP mouse-derived primary microglia cell cultures stimulated with LPS to determine whether microglia activation is affected by E2 and glucocorticoids. Glucocorticoids are well-recognized regulators of immune cell activation (Chapter 1) and were therefore, used as a positive control for comparison to the effects of E2.

Expression of Steroid hormone receptors in microglia

Microglia cell lines are reported to express mRNA and protein for both estrogen receptors (ERα and ERβ) (Bruce-Keller, Keeling et al. 2000; Vegeto, Bonincontro et al. 2001). However, few reports with primary microglia cultures, and no *in vivo* studies with microglia have corroborated that this cell type expresses estrogen receptors by conventional techniques such as immunocytochemistry and *in situ* hybridization (Garcia-Ovejero, Veiga et al. 2002; Sierra Submitted).

In collaboration with Dr. Sierra, microglia from adult *cfms*-EGFP mice were isolated by FACS, which enabled us to analyze *ex vivo* the expression of steroid receptor genes by real-time PCR (RT-PCR). Our RT-

PCR results demonstrated that *ex vivo* isolated microglia express glucocorticoid (GR) and mineralocorticoid (MR) receptors, as well as low levels of ERα mRNA. Additionally, we further described that *in vivo* activation of microglia by i.p. LPS injections led to a down-regulation of these receptors (Fig.11A) (Sierra Submitted). Furthermore, expression of ERβ, androgen receptor (AR), or progesterone receptor (PR) was not identified in resting or LPS-activated microglia (Sierra Submitted). Protein levels of GR were validated by Western blot, but no ERα protein was detected by this method, consistent with it's low mRNA levels (Fig.11B).

In agreement with previous reports, we were unable to detect ERα protein through conventional methods. For this reason, we established a collaboration with Dr. Milner, who has done extensive work on the detection of estrogen receptors in the brain at the ultrastructural level through electron microscopy. The expression of ERα protein in microglia was studied in dorsal hippocampal sections of *cfms*-EGFP female mice. Microglia were initially visualized by DAB immunohistochemistry using an anti-GFP antibody. Typical microglial processes were found in stratum radiatum of the CA1 region, juxtaposed to axons and dendrites and other elements of the brain's parenchyma (Fig.12).

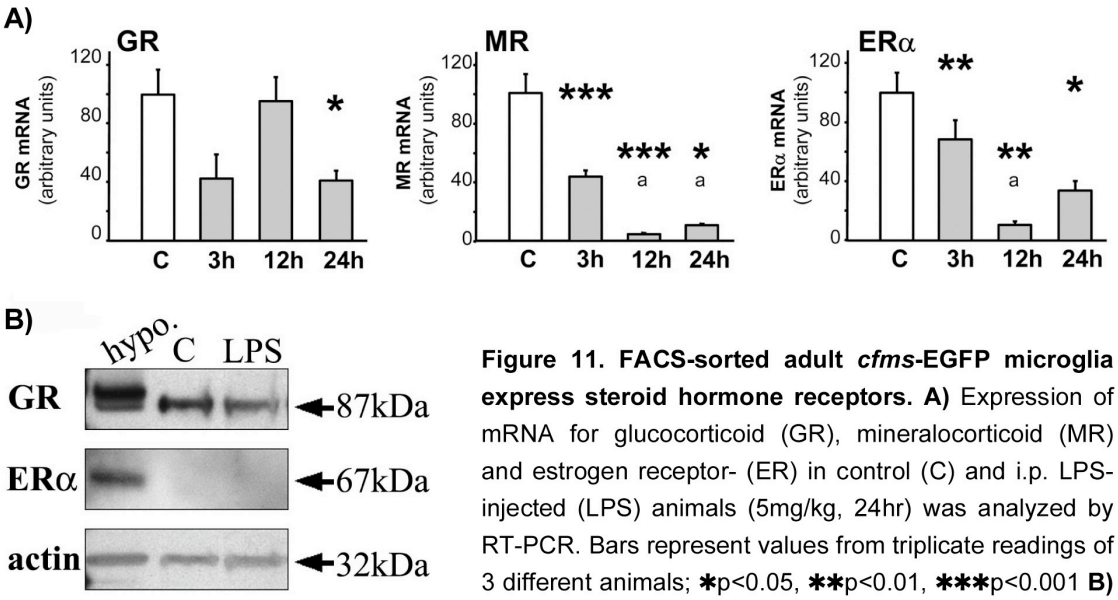

Figure 11. FACS-sorted adult *cfms*-EGFP microglia express steroid hormone receptors. A) Expression of mRNA for glucocorticoid (GR), mineralocorticoid (MR) and estrogen receptor- (ER) in control (C) and i.p. LPS-injected (LPS) animals (5mg/kg, 24hr) was analyzed by RT-PCR. Bars represent values from triplicate readings of 3 different animals; ✱p<0.05, ✱✱p<0.01, ✱✱✱p<0.001 **B)** Western blot analysis of GR and ER in FACS-sorted *cfms*-EGFP microglia.

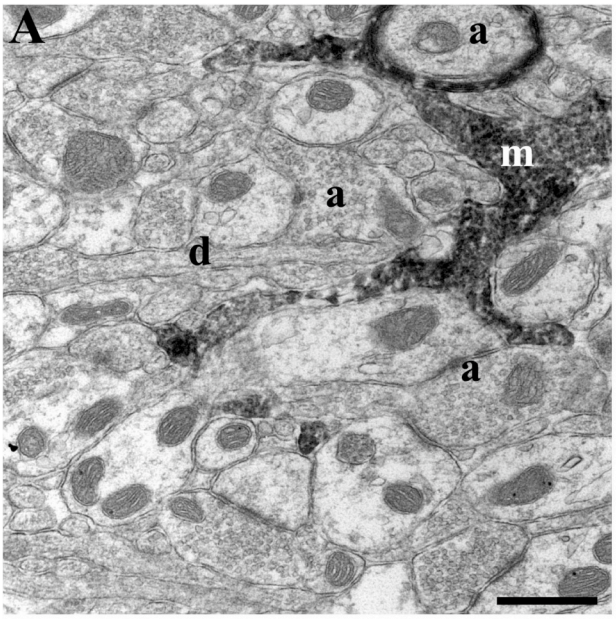

Figure 12. Electron microscopy of *cfms*-EGFP brain microglia. GFP-labeling by and visualization by DAB was observed in microglia processes (m) by electron microscopy in the stratum radiatum of the hippocampal CA1 region of *cfms*-EGFP mice; axon (a), dendrite (d). (Scale bar = 500nm)

Single labeling with a previously described anti-ERα antibody (Okamura, Yamamoto et al. 1992; Alves, Lopez et al. 1998; Milner, McEwen et al. 2001) revealed the presence of ERα protein in axons, dendrites and glial processes (Fig.13), as reported previously (Milner, McEwen et al. 2001).

For co-localization studies, microglia were identified with the anti-GFP antibody, which was then detected with a species-specific (chicken) secondary antibody conjugated to nano-gold particles; ERα was detected with an anti-ERα antibody and a species-specific (rabbit) secondary antibody conjugated to horseradish peroxidase revealed by diaminobenzidine (DAB) immunohistochemistry. Double staining of tissue sections revealed rare double-labeled microglia profiles, with most ERα labeling found on neuronal spines, terminals, and axons (Fig.13). Nevertheless, in some instances, we did observe rare instances of double-labeled microglia with ERα in the cytoplasm of cell processes (Fig.13C, D), and near the cell nucleus (data not shown). A parallel study was done with sections dually labeled for GFP and ERβ, in which no double-labeled processes were detected (data not shown). These ultrastructural results are consistent with our RT-PCR results showing that microglia express low levels of ERα and do not express ERβ.

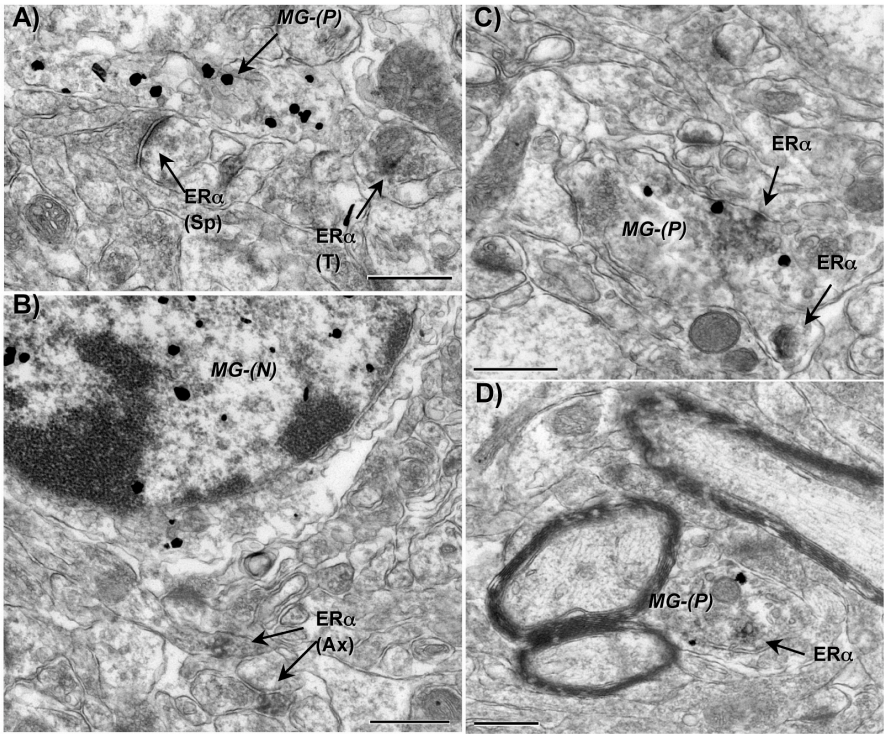

Figure 13. Double-labeling of ERα in microglia by electron microscopy in stratum radiatum of the hippocampal CA1 region of c*fms*-EGFP mice. A) GFP-labeled *(immuno-gold)* microglia process (MG-P) and **B)** nucleus (MG-N) in close apposition to an ERα-labeled *(DAB)* dendritic spine (Sp), terminal (T), and axon (Ax). **C-D)** Microglia processes with GFP immunogold labelling also contain immunoperoxidase labeling for ERα. (Scale bars= 500nm).

To assess the functional relevance of steroid hormone receptors expressed in microglia in the context of inflammation, BV-2 cells and primary microglia cultures were treated with corticosterone or E2, and then challenged with LPS+INFγ as detailed in Chapter 2.

E2 does not block the activation of p38 MAPK by LPS

An early event in the response of microglia to LPS is the activation of inflammatory transcription factors, such as the p38 MAPK (Lee and Young 1996). Microglia cells were pre-treated with 10nM E2 for 30 minutes and then stimulated with LPS for an additional 30 minutes. LPS-induced p38 MAPK phosphorylation was not significantly reduced by E2 in primary microglia (n=4) (Fig.14A), nor in BV-2 microglia (n=7) (data not shown) [Note: n= the number of independent experiments done in triplicate]. E2 also failed to prevent the nuclear translocation of phospho-p38 MAPK in primary microglia (Fig.14B). Preliminary studies with LPS-induced NFkB nuclear translocation in primary microglia showed a similar lack of E2 effects (not shown). Conversely, LPS-induced phosphorylation of p38MAPK was diminished by a 30 minute pre-treatment with 1μM corticosterone (52.69 ± 1.91%) (n=2) (p=0.05) (Fig.14A, C).

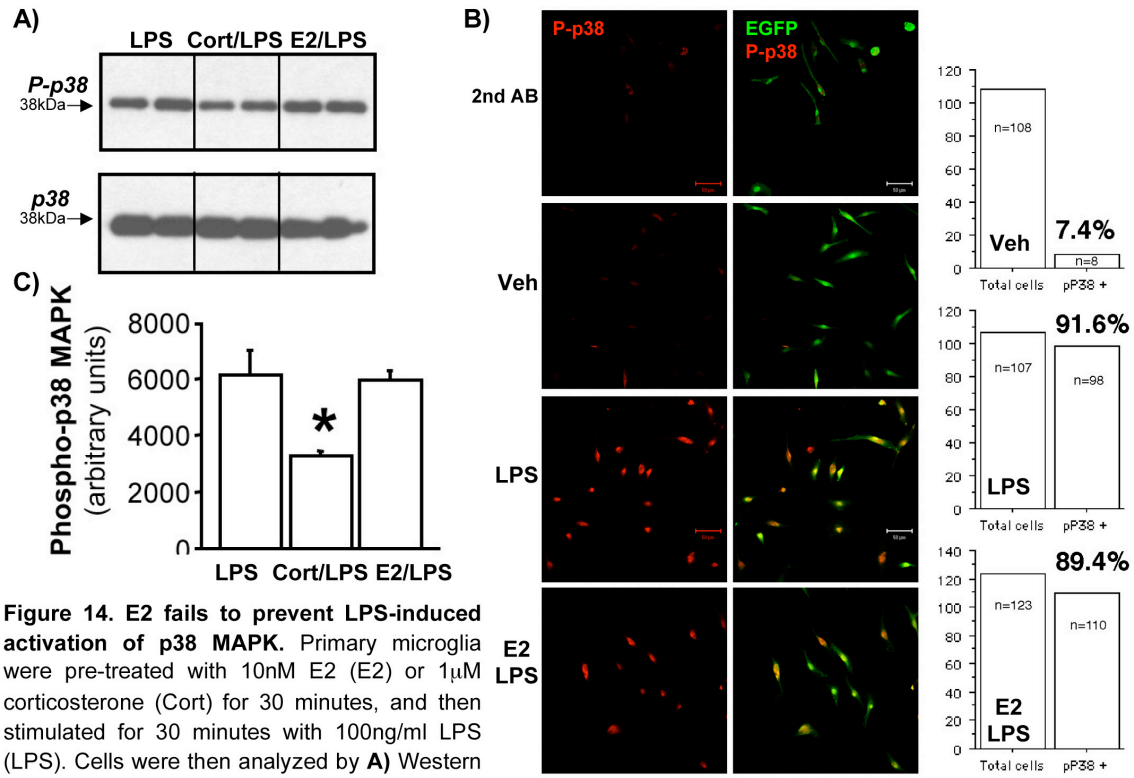

Figure 14. E2 fails to prevent LPS-induced activation of p38 MAPK. Primary microglia were pre-treated with 10nM E2 (E2) or 1μM corticosterone (Cort) for 30 minutes, and then stimulated for 30 minutes with 100ng/ml LPS (LPS). Cells were then analyzed by **A)** Western blot or **B)** immunofluorescence using phospho-specific anti-p38 AB. **C)** Western blot quantification, ✱p<0.05.

Corticosterone, but not E2, blocks the production of inflammatory cytokines in microglia

Cytokines are the principal mediators of microglia-induced inflammation, and therefore, a critical therapeutic target. Initial studies with LPS-stimulated BV-2 microglia showed that E2 pre-treatment had no consistent effect on production of TNFα, IL-6 or NO under various conditions tested [E2 10nM (n=3), 1uM (n=2), 10pM (n=2); pre-treatments 3hr (n=2), 24hr (n=1), 10 min (n=2)] (data not shown). Therefore, primary cultures of microglia cells were tested with corticosterone or E2 and then challenged with LPS+INFγ to elicit the expression of inflammatory cytokines (Chapter 2). Corticosterone was used as a positive control in these experiments, given its reported anti-inflammatory effects (McKay and Cidlowski 1999; Nadeau and Rivest 2003), and our findings showing predominant expression of GR in microglia compared to other steroid hormone receptors (Sierra Submitted).

We tested the effects of corticosterone and E2 over a wide range of concentrations (10pM, 10nM, and 1μM) (Bruce-Keller, Keeling et al. 2000; Ghisletti, Meda et al. 2005; Jacobsson, Persson et al. 2006). After 24hr, LPS+INFγ induced the secretion of the pro-inflammatory cytokines TNFα, IL-6 as well as NO, which were not detectable in the absence of LPS+INFγ

or with hormone treatment alone (Chapter 2). Corticosterone at the low dose (10nM) showed a trend to decrease the production of TNFα secreted by microglia (Fig.15A) by 16.91 ± 9.5% (n=8) (n.s.); while a higher concentration (1μM) of glucocorticoid reduced TNFα secretion by 53.72 ± 4.62% (n=15) ($p<0.001$). TNFα secreted by microglia remained unaffected by E2 over the entire range of concentrations tested (Fig.15).

The low concentration of corticosterone had no significant effect on IL-6 expression, while the high dose of corticosterone (1μM) significantly prevented the induction of IL-6 by 45.02 ± 3.75% (n=15) ($p<0.001$) (Fig.15B). The low concentration of E2 (10pM) produced a similar, but smaller decrease in IL-6 production of 14.55 ± 6.34% (n=4) ($p=0.01$). At a higher dose (10nM), E2 had no significant effect; yet at the highest dose (1μM) it produced a small but significant increase (16.91 ± 4.97% (n=7) ($p=0.004$) (Fig. 15B). The production of NO was reduced by corticosterone (1μM) by 25.46 ± 3.03% (n=15) ($p<0.001$) (Fig. 15C), whereas E2 was ineffective in modulating NO production at any of the concentrations tested (Fig. 15C).

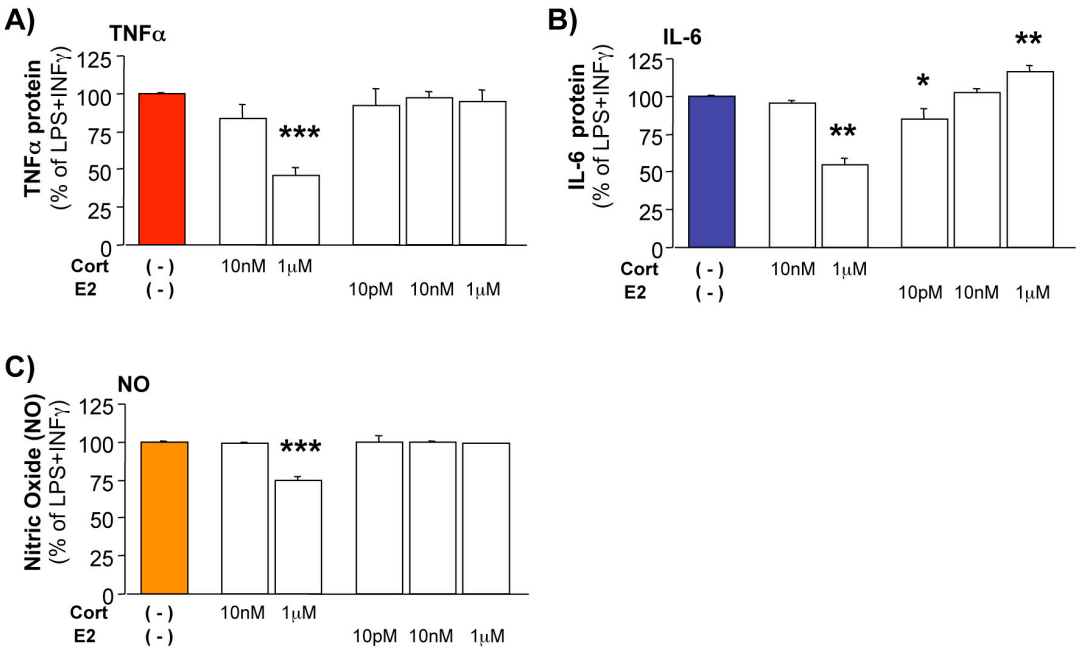

Figure 15. E2 does not block LPS-induced cytokine production in primary microglia. Primary microglia were pre-treated with E2 (E2) or corticosterone (Cort) for 10 minutes, and then stimulated for 24hr minutes with 100ng/ml LPS + 10ng/ml INFγ (LPS+INFγ). Supernatants were then analyzed by ELISA for **A)** TNFα and **B)** IL-6, or **C)** nitric oxide (NO) using the Greiss assay. ✱p<0.05, ✱✱p<0.01, ✱✱✱p<0.001

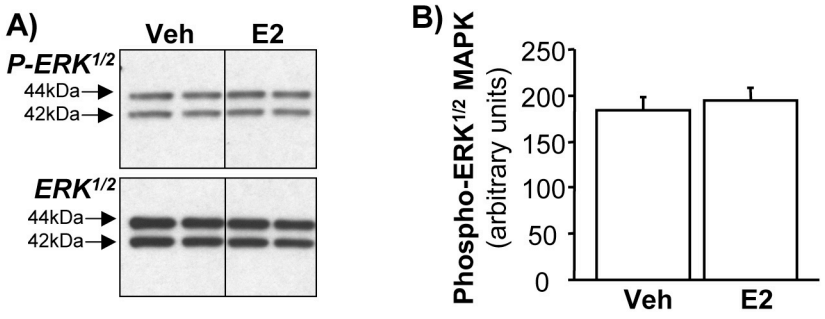

Figure 16. E2 does not regulate basal ERK$^{1/2}$ MAPK phosphorylation in primary microglia. Primary microglia were serum starved for 24hr and then treated with 10nM E2 (E2) for 30 minutes. Cell lysates were then analyzed by Western blot for total and phospho-ERK$^{1/2}$ MAPK. **A)** Representative Western blot. **B)** Densitometric quantification of phospho-ERK$^{1/2}$ MAPK normalized to non-phosphorylated MAPK protein.

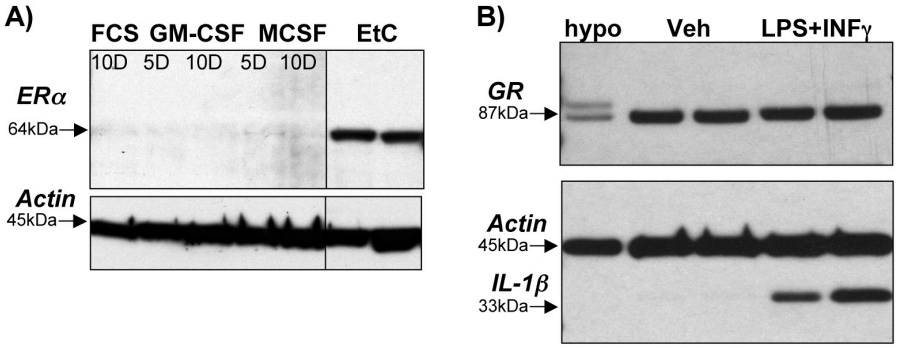

Figure 17. Primary microglia expression of ERα and GR. A) Protein samples from primary microglia cultures grown for 5-10 days (5D, 10D) in GM-CSF, MCSF, or regular media (FCS) were probed for ERα expression by Western blot. The EtC neuronal cell line (EtC) was used as a positive control. **B)** Immunoblot for glucocorticoid receptor (GR) from samples of GM-CSF cultured microglia stimulated with vehicle (Veh) or 100ng/ml LPS + 10ng/ml INFγ (LPS+INFγ) for 24hr. Brain hypothalamus (hypo) was used as a positive control. For A-B), samples were also probed for Actin as a loading control, and in the case of B) for IL-1β to show activation of microglia.

E2 does not induce rapid phosphorylation of p44/42 MAPK

The p44/42 extracellular regulated mitogen activated protein kinase (ERK MAPK), a mediator of multiple signaling pathways, plays an important role in brain responses to injury (Pearson, Robinson et al. 2001; Irving and Bamford 2002; Hetman and Gozdz 2004). In the brain, p44/42 MAPK is a main mediator of E2's neuroprotective effects (Bryant, Sheldahl et al. 2006). To determine if E2 could induce p44/42 MAPK activation in microglia, BV-2 cells or primary microglia cultures were acutely treated with E2 and p44/42 phosphorylation levels were measured by Western blot. E2 did not induce the phosphorylation of p44/42 MAPK in BV-2 microglia, even over a wide range of E2 concentrations [10pM (n=2), 1nM (n=2), 10nM (n=7), 1μM (n=2)] and time points of incubation (5 to 60 minutes) (data not shown). Some experiments showed a stimulatory effect of E2, but these were inconsistent, non-reproducible, and sometimes opposite. In primary microglia, E2 [10nM, 15min, (n=6)] consistently showed no effect on p44/42 phosphorylation levels (Fig. 16).

In view of the limited effects found with E2, and predominant effects exerted by corticosterone in primary microglia, I assessed the expression of ERα and GR in these cells by Western Blot. Primary microglia showed no detectable levels of ERα when cultured with regular media or in the

presence of MCSF or GM-CSF (Fig.17A). However, primary microglia had strong expression of GR (Fig. 15). GR protein levels were not regulated by LPS+INFγ stimulation (Fig. 17B). Primary microglia, both in resting state or after LPS+INFγ stimulation, were negative for ERβ (data not shown), as found in *ex vivo* microglia (Sierra in preparation).

Discussion

E2 anti-inflammatory effects are not mediated through MG

The data presented above indicate that microglia express glucocorticoid and mineralocorticoid hormone receptors. However, for the sex steroids, only ERα was expressed, whereas ERβ, androgen receptor and progesterone receptor were not. Recently, a membrane bound, G-protein coupled receptor for E2 has been described (GPR30) (Pedram, Razandi et al. 2006), yet no reports have examined its expression in microglia.

Expression of ERα was low in microglia, which was reflected by our inability to detect the protein by Western blot and immunohistochemistry. Indeed, the expression of sex hormone receptors in microglia has been a controversial issue, since their detection by conventional immunohistochemistry or *in situ* hybridization in the adult brain remained inconclusive (10, 19, 32). A main compounding factor in these studies may

be the low expression levels of these receptors coupled to their non-nuclear localization (Sierra, et al 2007). ERα detection was possible by electron microscopy ultrastructural analysis, which revealed only rare labeling of ERα in microglia profiles (Sierra, et al, 2007). Non-nuclear localization of ERα has been described for CA1 neurons of the hippocampus, which is also not detectable by light microscopy (Milner, McEwen et al. 2001).

Non-nuclear estrogen receptors are implicated in MAPK activation, yet in BV-2 cells and in primary microglia E2 failed to modulate basal p44/42 MAPK phosphorylation levels. My findings are both in agreement and in disagreement with previous reports with different microglia cell lines (Bruce-Keller, Keeling et al. 2000; Baker, Brautigam et al. 2004).

The low levels of ERα expression are consistent with my results showing the inability of E2 to exert significant anti-inflammatory effects on microglia, as assessed by p38 MAPK activation and the production of TNFα, IL6, and NO. As discussed in the Introduction, reports on E2 effects on activated microglia *in vitro* are inconsistent. Culture conditions, species from which primary cultures are obtained, and transformation of cell lines could be factors contributing to such discrepancies. However, we further have reported that *in vivo* E2 did not regulate the microglial expression of IL-6, TNFα, TGFβ1 mRNAs (Sierra Submitted). Therefore, the low

expression of ERα in microglia may play a yet unidentified role in these cells. Another possible explanation to the results presented here is that E2 may synergize with other, as of yet unidentified factors, to affect microglia cells. This could be explored by others in future studies.

E2 has widely reported neuroprotective and anti-inflammatory effects in the brain (Lee and McEwen 2001; Vegeto, Ciana et al. 2002). Based on the findings presented here, in which ERβ is not expressed and ERα is expressed at low levels in microglia, I propose that the anti-inflammatory effects of E2 observed in the brain *in vivo* are mediated by cell types other than microglia. Supporting this model, several groups have reported the expression of ERs, and corroborated the effects of E2, in cells involved in the brain's inflammatory response such as astrocytes (Azcoitia, Sierra et al. 1999; Sortino, Chisari et al. 2004; Dhandapani and Brann 2006), endothelial cells (Langub and Watson 1992; Galea, Santizo et al. 2002; Stirone, Duckles et al. 2003), and infiltrating leukocytes (Offner 2004; Shim, Gherman et al. 2006; Stygar, Westlund et al. 2006).

Glucocorticoids play a primary role in MG regulation

Microglia activation by LPS+INFγ was inhibited by corticosterone, supporting our findings that GR is the predominant steroid hormone receptor

expressed in these cells at both the mRNA and protein level (Sierra Submitted). Glucocorticoids play an essential role in protecting the brain against an inflammatory challenge (Sorrells and Sapolsky 2007). For instance, the GR agonist dexamethasone increases the survival of dopaminergic neurons after intranigral injection of bacterial lipopolysaccharides (LPS), while the GR/PR antagonist RU486 induces a dramatic degeneration (Castano, Herrera et al. 2002). The results presented in this Chapter support a model where glucocorticoids, and not E2s, are the main regulators of activated microglia. A more detailed description of the actions of glucocorticoids on brain inflammation is presented in the following chapter (Chapter 4).

CHAPTER 4

NEUROENDOCRINE MICROGLIA – LOCAL AMPLIFICATION OF GLUCOCORTICOIDS

In the previous chapter I presented evidence indicating that glucocorticoids, namely corticosterone, are the main steroid hormone regulating microglia activation. Glucocorticoids, cortisol in humans and corticosterone in rodents, are presumably the body's most effective regulators of inflammation, exerting permissive, stimulatory, and suppressive effects [rev by (Munck, Guyre et al. 1984; McEwen 1997; Yeager, Guyre et al. 2004)]. Systemic inflammation induces a surge in circulating glucocorticoids as a result of hypothalamic-pituitary-adrenal (HPA) axis activation by cytokines (Beishuizen and Thijs 2003). This surge of glucocorticoids is required for survival in experimental animals challenged with antigens, bacterial lipopolysaccharides (LPS), or tumor necrosis factor-α (TNFα) [rev by (Chapman, Gilmour et al. 2006)]. Glucocorticoids regulate inflammation by altering the distribution and trafficking, as well as differentiation and maturation, of monocytes/macrophages, neutrophils and granulocytes (McEwen 1997; Ashwell, Lu et al. 2000). Additionally, they suppress pro-inflammatory gene

transcription and promote expression of anti-inflammatory cytokines (Barnes 1998).

The effects of glucocorticoids follow a bell-shaped curve, showing permissive/stimulatory actions at physiological concentrations, while exerting suppressive effects at pharmacological doses (Munck and Naray-Fejes-Toth 1992; Sapolsky, Romero et al. 2000). In this way, physiological concentrations of glucocorticoids facilitate the initiation of inflammatory responses, and at supra-physiologic concentrations, achieved through HPA axis activation, glucocorticoids mediate anti-inflammatory actions to prevent an unregulated, and potentially lethal, inflammatory response [rev by (Chapman, Gilmour et al. 2006)].

For the inflammatory response to be effectively resolved, or perpetuated, the actions of glucocorticoids require specific targeting to the site of inflammation to exert their proper function. Regulatory targeting mechanisms include changing the affinity of glucocorticoid binding proteins, modulating the expression and/or affinity of glucocorticoid receptors (GR), and/or metabolically activating or inactivating glucocorticoids within the target cell [rev by (Rook, Baker et al. 2000)].

Glucocorticoids can enzymatically be activated or inactivated, between their C11-hydroxyl to their C11-ketone forms respectively, by two

distinct enzymes known as 11β-hydroxysteroid dehydrogenase (11βHSD) type 1 and type 2 [rev by (Seckl and Walker 2001; Tomlinson and Stewart 2001)]. 11βHSD-2 is a dehydrogenase, inactivating corticosterone (cortisol in humans) into 11-dehydro-corticosterone (cortisone in humans). 11βHSD-2 is predominantly expressed in mineralocorticoid target tissues, such as the kidney collecting tubules, colon and sweat glands, or tissues requiring protection from glucocorticoid actions, such as the placenta and developing brain (Brown, Diaz et al. 1996; Diaz, Brown et al. 1998). On the other hand, 11βHSD-1 acts predominantly *in vivo* and within cells, as an oxido-reductase, catalyzing the re-activation of corticosterone (cortisol in humans) from its 11-keto derivative (cortisone in humans) (Seckl and Walker 2001). Thus, cells expressing 11βHSD-1 increase the levels of available glucocorticoids for GR activation, whereas cells expressing 11βHSD-2 are glucocorticoid resistant.

In the 11βHSD-1 null mouse, glucocorticoid–mediated responses are attenuated (Kotelevtsev, Holmes et al. 1997), consistent with the model that 11βHSD-1 activity contributes significantly to the pool of intracellular active glucocorticoids (Seckl and Walker 2001; Morris, Brem et al. 2003). Although this role of 11βHSD-1 in the metabolic actions of glucocorticoids

has been clearly established, its role in the immune response has, until recently, remained unclear.

Initial studies with 11βHSD inhibitors suggested a role for these enzymes in immunity, yet it remained unclear whether the results pertained to 11βHSD-1 or 11βHSD-2 activity [rev. by (Chapman, Gilmour et al. 2006)]. Currently, 11βHSD-1 expression and activity has been detected in the principal cells of the immune system: monocytes, macrophages, dendritic cells, B and T lymphocytes, both in human and mouse [rev. by (Chapman, Gilmour et al. 2006)]. 11βHSD-1 null mice show a delay in acquisition of macrophage phagocytic capacity (Gilmour, Coutinho et al. 2006). Furthermore, a consistent pattern has emerged in which pro-inflammatory mediators, particularly TNFα and IL-1β, increase 11βHSD-1 expression to increase local glucocorticoid availability and hence, promote local anti-inflammatory action (Chapman, Gilmour et al. 2006; Hardy, Filer et al. 2006).

11βHSD-1 is predominantly expressed in metabolically active tissues such as the liver, adipose tissue, bone, gonads, and the brain (Moisan, Edwards et al. 1992; Seckl and Walker 2001). Many brain areas are rich in 11βHSD-1 expression, notably the hippocampus, cerebellum, and neocortex (Moisan, Seckl et al. 1990). It has been proposed that 11βHSD-1expression

in the brain plays roles in cognitive function, neuroprotection, and contributes to the negative feedback of glucocorticoids on HPA activity [rev by (Holmes, Yau et al. 2003)]. However, elevated or prolonged levels of glucocorticoids are neurotoxic to injured neurons (Reagan and McEwen 1997), and to our knowledge, no studies have addressed whether brain 11βHSD-1 expression plays a role in neuroinflammation.

Therefore, given that un-regulated microglia-mediated inflammation contributes greatly to neurodegeneration [rev in Chapter 1], and in view of the regulatory effects of glucocorticoids on microglia (Chapter 3), in the present Chapter of this thesis I tested the hypothesis that microglia express glucocorticoid converting enzymes 11βHSD type 1 and type 2, which modulate the actions of glucocorticoids in the CNS. To this end, I analyzed 11βHSD type 1 and type 2 mRNA expression by real-time PCR, and protein by immunofluorescence of brain tissue sections and Western blots of primary cultures. Additionally, the function of these enzymes was studied in primary microglia cultures using tritiated substrates. The expression and function of 11βHSDs were examined both in resting and activated microglia.

Microglia cells express functional 11βHSD-1

Expression of 11βHSD-1 and 11βHSD-2 mRNA was analyzed by real-time PCR (RT-PCR) in microglia isolated from adult *cfms*-EGFP mice by fluorescence-activated cell sorting (FACS) (*ex vivo* MG). 11βHSD expression was also assessed in primary cultures derived from neonatal mouse brains (1°MG) (Fig.18). Adrenal and ovary tissue samples were used as positive controls. Specific gene cycle threshold (C_t) values were normalized to C_t values of L27A, a ribosomal housekeeping gene, and converted to percentage using adrenal values as 100% for 11βHSD-1 (Fig.18A) and ovary as 100% for 11βHSD-2 (Fig.18B). *Ex vivo* MG and primary microglia expressed low levels of 11βHSD-1 compared to adrenals and ovary (Fig.18A), and no significant differences were found in the expression levels of 11βHSD-1 between e*x vivo* MG and primary microglia (Fig. 18A). On the other hand, 11βHSD-2 expression was almost undetectable in microglia samples (Fig.18B). Although both enzymes were expressed at low levels, further comparison of 11βHSD expression values in microglia showed that 11βHSD-1 is the main enzyme expressed (Fig.18C). This was further corroborated by Western Blot analysis of primary microglia showing a specific 11βHSD-1 immunoreactive band of predicted molecular weight (36kDa) (Fig.18D). 11βHSD-1 protein expression in microglia was

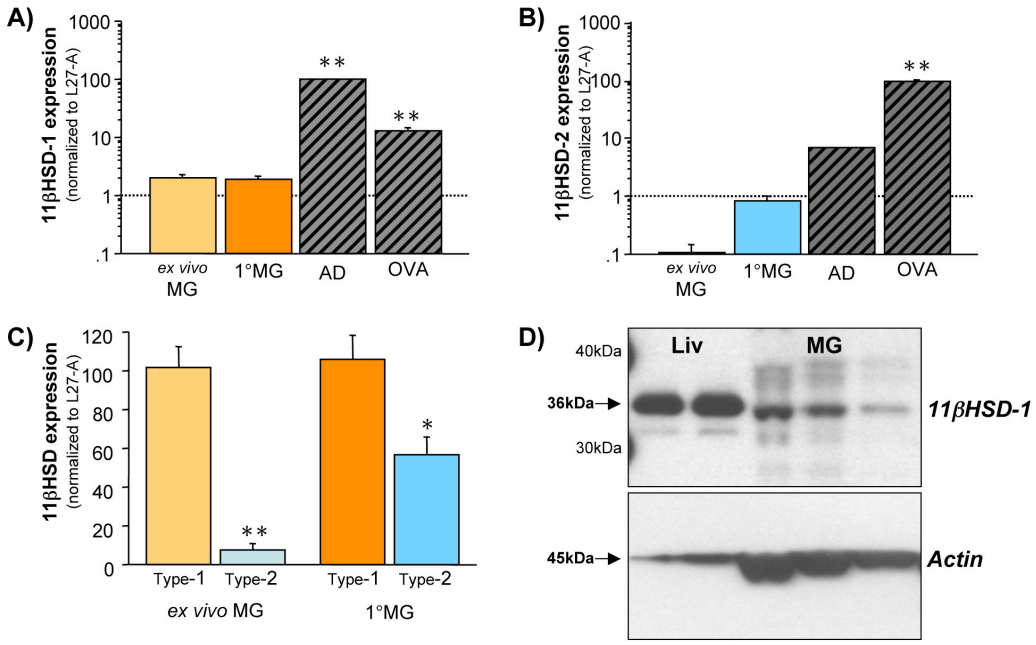

Figure 18. Microglia expression of 11βHSD enzymes. Real-Time PCR analysis of **A)** 11βHSD-1 and **B)** 11βHSD-2 expression in *ex vivo* FACS-sorted microglia (*ex vivo* MG) and primary microglia (1°MG). Adrenal (AD) and ovary (OVA) tissue was used as a positive control. **C)** Comparison of 11βHSD type-1 and type-2 in microglia. Bars represent 11βHSD expression normalized to the house-keeping gene L27-A, and expressed as % of AD (A), OVA (B), or Type-1 (C). **D)** Western blot analysis of 11βHSD-1 in 1°MG (MG; 50, 25, 5μg prot), and liver (Liv; 10ng prot). ✱✱ $p<0.01$; ✱ $p<0.05$ significantly different from 1°MG (A-B) or Type-1 (C).

low compared to liver (Fig.18C). 11βHSD-2 was not evaluated by Western blot, however the possibility of its presence was analyzed in the functional assays that follow.

To evaluate the possible function of 11βHSD-1 and 11βHSD-2, 1°MG cells were incubated with the tritiated substrates H^3-11-dehydro-corticosterone (11-DH-Cort) or H^3-corticosterone (Cort). The conversion of these steroids, from their 11-hydroxyl to 11-keto forms or vice versa, was analyzed by thin layer chromatography (Fig.19). 1°MG showed specific 11βHSD-1 conversion of 11-DH-Cort to Cort. Scanning of the TLC plate revealed only two peaks of radioactivity, indicating cells did not produce any other metabolites from 11-DH-Cort (Fig. 19A). To estimate the activity levels of 11βHSD-1, a time-course incubation with 11-DH-Cort was conducted with primary microglia. Conversion of 11-DH-Cort to Cort was time-dependent, observed as early as 3hr (57.8±2.3%, $p<0.05$), with substrate depletion by 12hr (85.6±0.2%, $p<0.05$) (Fig.19B). In contrast, 1°MG showed no 11βHSD-2 conversion of Cort to 11-DH-Cort (Fig.19C) suggesting that the 11βHSD-2 enzyme is absent in microglia. The specificity of 11-DH-Cort conversion to corticosterone was verified by co-incubation of the cells with an 11βHSD-1 inhibitor (11-keto-progesterone)

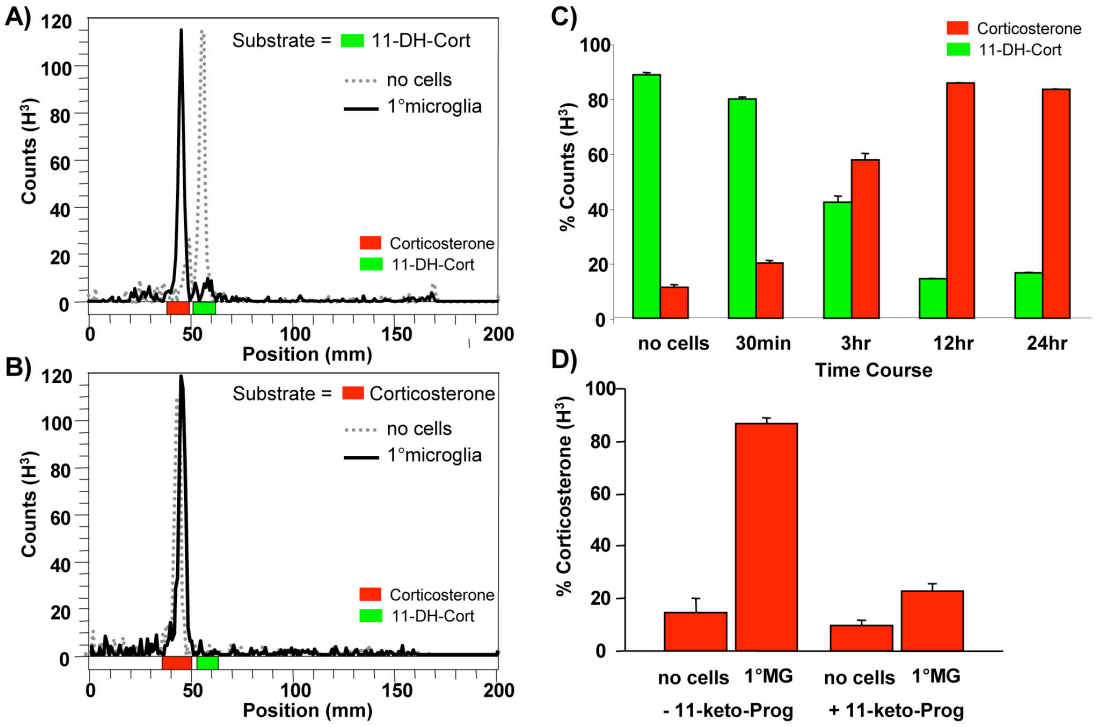

Figure 19. 11βHSD-1 activity in primary microglia. Thin layer chromatography analysis of steroids extracted from 1°microglia incubated with **A)** H^3-11-dehydro-corticosterone (11-DH-Cort) or **B)** H^3-Corticosterone for 24hr. **C)** Time course analysis of 1°microglia incubated with 11-DH-Cort. **D)** 24hr conversion of 11-DH-Cort into corticosterone by 1°microglia ± the 11βHSD-1 specific inhibitor, 11-keto-progesterone (11-keto-prog) (500nM).

(Latif, Pardo et al. 2005). Treatment of 1°MG with 11-keto-progesterone completely blocked the 11βHSD-1 conversion of 11-DH-Cort to Cort (Fig.19D).

11-dehydro-corticosterone blocks cytokine production via 11βHSD-1 activity in 1°MG

To assess the functional relevance of 11βHSD-1 expression in microglia, 1°MG cultures were stimulated with LPS to elicit the production of pro-inflammatory cytokines and the anti-inflammatory effects of 11-dehydro-corticosterone were compared to corticosterone. At a range of concentrations (5nM-5μM), 11-dehydro-corticosterone was equal to or even more effective than corticosterone in blocking the production of the pro-inflammatory cytokines TNFα ($IC50^{11C}$= 51nM *vs* $IC50^{C}$= 62.6nM, $p<0.0001$) (Fig.20A), IL-6 ($IC50^{11C}$= 201.8nM *vs* $IC50^{C}$= 232.4nM, $p<0.0001$) (Fig.20B), and NO ($IC50^{11C}$= 58.9nM *vs* $IC50^{C}$= 67.5nM, $p<0.0001$) (Fig.20C).

Activated microglia increase expression of 11βHSD-1

Activation of peripheral macrophages and monocytes by pro-inflammatory stimuli is reported to increase the expression of 11βHSD-1 (Chapman, Gilmour et al. 2006; Hardy, Filer et al. 2006). To establish if

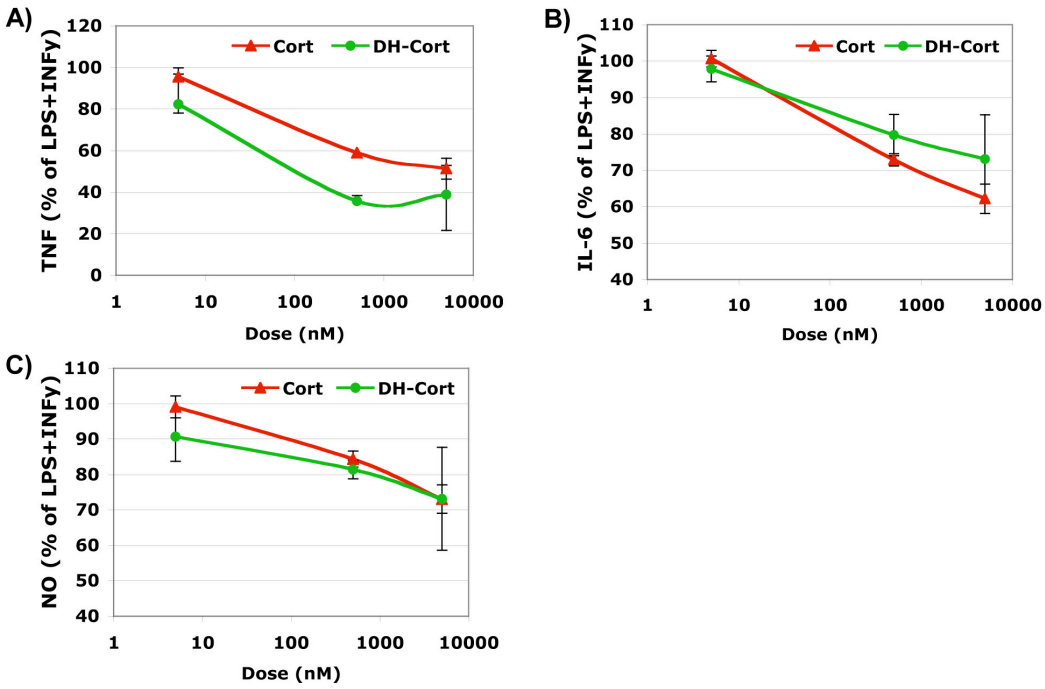

Figure 20. 11-DH-Corticosterone inhibits cytokine production in LPS-stimulated primary microglia. Primary microglia were pre-treated with increasing doses of corticosterone (Cort) or 11-DH-Cort for 10 minutes and then stimulated for 24hr with 100ng/ml LPS+10ng/ml INFγ (LPS+INFγ). Supernatants were then analyzed by ELISA for **A)** TNFα and **B)** IL-6, as well as by the Greiss Assay **C)** for nitric oxide (NO). Values are representative of one experiment done in triplicate.

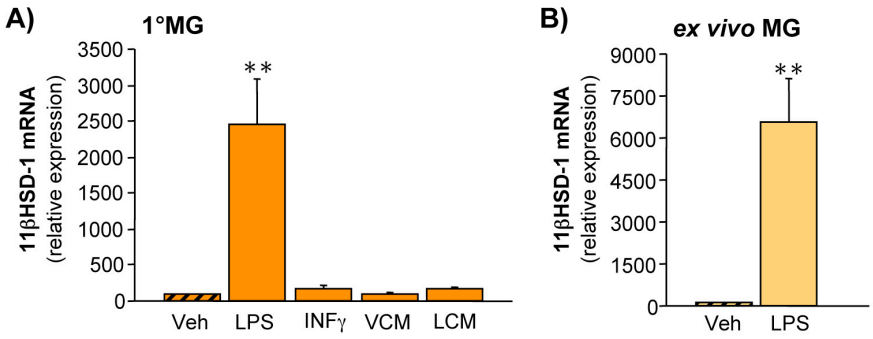

Figure 21. Microglia increase expression of 11βHSD-1 mRNA after LPS stimulation. A) RT-PCR quantification of 11βHSD-1 expression levels in primary microglia (1°MG) stimulated for 24hr with 100ng/ml LPS + 10ng/ml INFγ (LPS), 10ng/ml INFγ (INFγ), resting microglia conditioned media (VCM), or LPS-stimulated microglia conditioned media (LCM). **B)** RT-PCR quantification of 11βHSD-1 expression levels in FACS-sorted microglia from adult mouse brains (*ex vivo* MG) 24hr after i.p. injection with 5mg/kg LPS. Bars represent the means ± SEM of one representative experiment in triplicate (A) or 3 animals (B). ✱✱ $p \leq 0.01$.

activation of microglia also affected 11βHSD-1, mRNA expression of this enzyme was analyzed by RT-PCR in 1°MG cultures treated with LPS+INFγ. LPS+INFγ-activated microglia showed a 246±63-fold induction ($p<0.01$) in 11βHSD-1 expression (Fig.21A). To determine if 11βHSD-1 up-regulation could also be induced by downstream mediators of inflammation, primary microglia were stimulated with INFγ alone or microglia LPS-conditioned media (LCM). INFγ and LCM did not affect 11βHSD-1 expression in 1°MG (Fig.21A) indicating that LPS is a far more potent inducer of 11βHSD-1 mRNA expression than cytokines.

In spite of the increased mRNA levels of 11βHSD-1, there was no increase in enzymatic activity when LPS+INFγ activated primary cultures were incubated with H^3-11-DH-corticosterone (data not shown). These results suggest that other factors may be required for increased activity in activated microglia.

To validate my findings from primary microglia *in vivo*, I next analyzed by RT-PCR samples of FACS-sorted microglia from *cfms*-EGFP adult male mice that were intraperitoneally (i.p.) injected with saline or LPS for 24hr. This i.p. injection of LPS induces the activation of microglia as observed by morphological changes and the expression of inflammatory cytokines (Sierra, et al, 2007). RT-PCR analysis showed that LPS activation

of microglia induced a robust increase in the expression of 11βHSD-1 (655±154 fold induction, $p \leq 0.01$) (Fig.21B). This increase in 11βHSD-1 mRNA expression was consistent with my findings in primary microglia.

In a parallel study to the above experiment, 30 μm serial sections were collected from brains of perfused adult male *cfms*-EGFP mice following i.p. saline or LPS injections (24hr). Preliminary 11βHSD-1 immunofluorescence analyses indicated that in saline treated mice, 11βHSD-1 immunoreactivity was prominent in non-EGFP cells with little to no staining in microglia cells (assessed by co-localization with EGFP) (Fig.22A). Double-labeling experiments with anti-11βHSD-1 and a marker for neurons (anti-NeuN), astrocytes (anti-GFAP), or oligodendrocytes (anti-RIP) indicated that most 11βHSD-1 expression came from astrocytes (Fig.22B). After 24hr of LPS injection, 11βHSD-1 staining remained at low levels in microglia cells (Fig.22C). In sharp contrast, 11βHSD-1 immunoreactivity was greatly down-regulated in astrocytes (Fig.23D). This pattern of staining was observed at 48hr as well (data not shown), with an indication of returning levels of 11βHSD-1 in non-EGFP cells, and microglia returning to a resting phenotype, by day 5 after LPS challenge (data not shown).

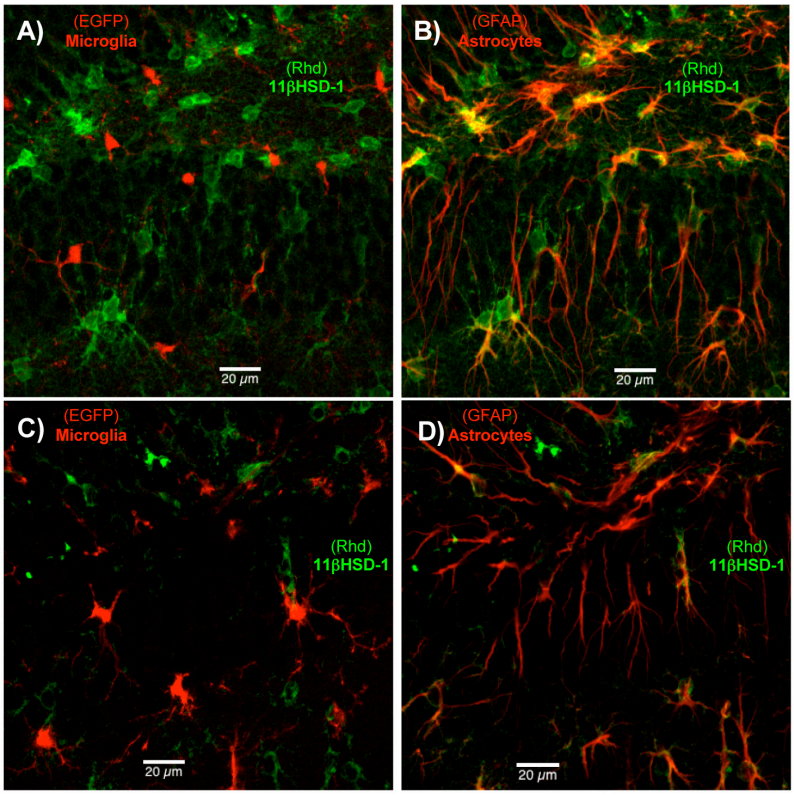

Figure 22. 11βHSD-1 expression in the brain after i.p. LPS challenge. Double -immunofluorescence analysis with anti-11βHSD-1 and anti-GFAP on Dentate Gyrus hippocampal sections from adult male *cfms*-EGFP mice i.p. injected with **A-B)** saline or **C-D)** 5mg/kg LPS for 24hr. **A,C)** EGFP-expressing microglia; **B,D)** GFAP-labeled astrocytes.

Glucocorticoids regulate 11βHSD-1 expression in microglia

In addition to inducing the activation of microglia, i.p. injection of LPS causes circulating corticosterone levels to rise (Beishuizen and Thijs 2003; Nadeau and Rivest 2003). Acute treatments of glucocorticoids are reported to increase 11βHSD-1 expression in the hippocampus. To assess whether microglia 11βHSD-1 expression is regulated directly by glucocorticoids, 1°MG cultures were treated with corticosterone. Preliminary experiments indicated that direct stimulation of 1°MG with corticosterone (1uM) did not significantly affect 11βHSD-1 expression (Fig.23). Corticosterone treatment significantly reduces the LPS+INFγ-activation of 1°MG, therefore, 1°MG cells were also treated with corticosterone, LPS+INFγ or both. LPS+INFγ induced an expected increase of 11βHSD-1 expression (406±63.4%, $p<0.05$) (Fig.23), and corticosterone had no effect on this up-regulation (442.3±9.0%, $p<0.01$) (Fig.23). These preliminary results indicate that acute, micromolar concentrations of corticosterone do not affect 11βHSD-1 mRNA expression in microglia, and also suggest they do not block the LPS+INFγ-induced increase in 11βHSD-1 expression.

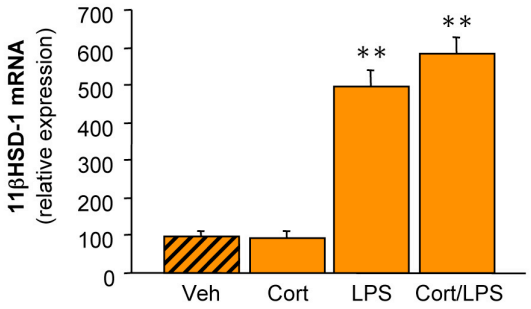

Figure 23. Corticosterone incubation does not change expression of 11βHSD-1. RT-PCR quantification of 11βHSD-1 expression levels in primary microglia cultured for 24hr with 1μM corticosterone (Cort) ± 100ng/ml LPS+10ng/ml INFγ (LPS). Bars represent the means ± SEM of 1 representative experiment in triplicate. ✱✱ $p<0.01$, significantly different from Veh.

Discussion

In the present Chapter of this thesis I have described the expression and activity of 11βHSD-1 in microglia, providing evidence that suggests an active role for 11βHSD-1 in regulating brain inflammation through the re-activation of glucocorticoids.

11βHSD-1 expression in Microglia

In the resting brain, preliminary immunofluorescence analysis indicated that 11βHSD-1 expression was predominantly found in EGFP-negative cells (possibly astrocytes and neurons), consistent with the proposed house-keeping roles of glucocorticoids in the homeostasis of the CNS [rev. by (McEwen 1997)]. In this chapter, 11βHSD expression and activity in microglia cells is described for the first time. Although preliminary findings suggest 11βHSD immunoreactivity is low in tissue microglia, mRNA levels assessed in *ex vivo* FACS-sorted microglia were high, and primary microglia cells showed the presence of mRNA, protein, and a functional enzyme. The expression levels and activity of 11βHSD-1 in microglia described here are comparable to other related cell types such as human monocyte-derived macrophages, which exhibit 95% conversion in 24hr (Thieringer, Le Grand et al. 2001). My results are also similar to those

found in other cells that participate in local tissue inflammation, such as TNFα stimulated synovial fibroblasts (Hardy, Filer et al. 2006) and skeletal muscle cells (Jang, Obeyesekere et al. 2006), which show reductase activity and substrate conversion with similar rates. However, the 11βHSD-1 activity in microglia is considerably lower than the activity observed in highly metabolic cells like hepatocytes, which exhibit 30% conversion in 30 minutes (Liu, Nakagawa et al. 2003), or that found in steroidogenic cells such as testicular Leydig cells, which exhibit 95% conversion of substrate within 30 minutes (private communication, Ge, et al.).

Although an increase in 11βHSD-1 mRNA was detected in activated microglia, 11βHSD-1 activity was not changed. This may be due to rate-limiting factors for 11βHSD-1 activity, which were not considered in the experimental design. The catalytic activity of 11βHSD-1 is determined by the redox potential set by the NADP+ to NADPH cofactor ratio, which is modulated by hexose-6-phosphate dehydrogenase (H6PD) activity. H6PD raises the intracellular level of NADPH favoring the reductase activity of 11ßHSD-1 observed in several tissues [rev by (Ge, Dong et al. 2005)]. Therefore, activity of H6PD and levels of NADPH in activated microglia should be considered, especially as lack of G6PD can abrogate 11ßHSD-1 activity (Lavery, Walker et al. 2006).

The adult brain presents expression of 11β-HSD-2 that is limited to mineralocorticoid sensitive areas involved regulation of blood pressure and salt appetite (Robson, Leckie et al. 1998). There is also low levels of 11β-HSD-2 expression reported in selected nuclei of the hypothalamus, as well as the amygdala, locus coeruleus and nucleus tractus solitarius (Robson, Leckie et al. 1998). Almost undetectable levels of 11βHSD-2 mRNA were observed in microglia, and no functional activity was present indicating these cells do not catalyze the de-activation of glucocorticoids and are not part of the brain population of cells expressing 11βHSD-2.

Modulation of 11βHSD-1 during neuroinflammation

High levels of glucocorticoids are deleterious to brain homeostasis, therefore tight regulation of active glucocorticoids is important (Sapolsky 1996). Acute stress, or glucocorticoid treatment in rats, induces a rise in hippocampal 11βHSD-1 expression (Jamieson, Fuchs et al. 1997; Jamieson, Nyirenda et al. 1999), which plays a role in mediating the necessary negative feedback on HPA activity [rev by (Holmes, Yau et al. 2003)]. However, chronic stress decreases 11βHSD-1 expression (Seckl 1997), as a likely neuroprotective mechanism to prevent the toxic metabolic effects of glucocorticoid excess (Rajan, Edwards et al. 1996). The decrease of

11βHSD-1 expression observed in non-EGFP cell in my studies may likely be induced by pro-inflammatory stimuli, such as cytokines secreted by microglia, and/or to the acute elevation of glucocorticoids following LPS injection.

On the other hand, 11βHSD-1 mRNA up-regulation in activated microglia following LPS challenge suggests these cells locally increase the availability of active glucocorticoids following their activation. However, previously we reported that under the same *in vivo* experimental paradigm, glucocorticoid receptors are down-regulated by LPS activation (Sierra Submitted). These apparently opposing effects of LPS in activated microglia are reminiscent of the effects of glucocorticoids on immune cells, in which cytokine production is inhibited, yet cytokine receptor expression is increased (Sorrell, 2006). Alternately, given that systemic LPS injection causes elevated corticosterone levels, the down-regulation of GR's observed in *ex vivo* MG could be due to autologous receptor down-regulation (Burnstein and Cidlowski 1992; Oakley and Cidlowski 1993). This prediction would be consistent with the fact that GR protein levels were unaffected by LPS+INFγ in primary microglia, and my preliminary findings showing that corticosterone does not down-regulate 11βHSD-1 mRNA expression in LPS+INFγ stimulated microglia.

Even with reduced GR expression, my results with primary microglia indicate that in spite of LPS+INFγ activation, 11-DH-Cort was equal to or more effective than Cort in blocking TNFα, IL-6 and NO production. This maybe due to the fact that 11-DH-Cort is being converted to Cort within the cells, where intracellular GRs are expressed. My results suggest that in activated microglia GR-mediated anti-inflammatory effects are not being compromised. Considering the down-regulation of 11βHSD-1 in astrocytes and the possible increased expression in microglia (because protein levels have not yet been seen to increase *in vivo*), my results indicate a role of 11βHSD-1 in gating glucocorticoid access to intracellular glucocorticoid receptors (GR) within specific cell types.

The results presented indicate that local amplification of glucocorticoids may be occurring at sites of neuro-inflammation through the expression and activity of 11βHSD-1 in microglia. 11βHSD-1 activity may be critical in boosting the anti-inflammatory effects of glucocorticoids locally and therefore, provide a mechanism for the safe resolution of inflammation and prevention of secondary neuronal toxicity. Future studies with 11βHSD-1 null mice in models of neuronal injury could validate these findings.

Following LPS challenge, preliminary immunofluorescent analyses noted a sharp decrease of 11βHSD-1 expression in non-EGFP (possibly astrocytes and neurons). This would suggest a neuroprotective response mediated by 11βHSD-1, given neuronal cell's susceptibility to high concentrations of glucocorticoids. Therefore, a model can be suggested where, as systemic or brain inflammation proceeds, 11βHSD-1 expression allows particular cell types in the brain to adapt and respond to elevated blood glucocorticoid levels resulting from HPA axis activation. This includes microglia, which engage in the conversion of inert 11-keto-glucocorticoids to active 11-hydroxl-glucocorticoids.

CHAPTER 5

MICROGLIA EXPRESS STEROID-CONVERTING ENZYMES AND METABOLIZE STEROID PRECURSORS INTO ACTIVE SEX HORMONES

The brain can synthesize steroids and metabolize circulating hormone precursors, like DHEA, into active androgens and estrogens. Neurosteroids have important roles in the homeostasis of the CNS, including neuroprotection, and their levels correlate negatively with neurodegeneration. Microglia play a critical role during brain damage responses, which can be ameliorated by the protective effects of neurosteroids. The enzymes that catalyze the conversion of cholesterol and the production of steroid hormones are expressed in the various cell types of the brain, and although steroid metabolism has been implicated in the brain's response to injury, the participation of microglia in this process has not been described. In view of my findings showing microglia are able to metabolize adrenal steroids, in this part of my thesis I set out to test the hypothesis that microglia contribute to the synthesis and metabolism of neurosteroids in the brain. To do so, I applied the RT-PCR gene expression approach, coupled to functional assays of steroid conversion for particular enzymes.

Protein(Gene)		Sequence	Bases	Exons
11βHSD-1	Fwd	TGCGAAGAGTCATGGAGGTCA	531-552	5-6
(Hsd11b1)	Rev	TTTCCCAGCCAAGGAGGAGAT	625-646	
11βHSD-2	Fwd	TGGCTGCTTCAAGACAGATGC	896-917	4-5
(Hsd11b2)	Rev	AGTCTTCACCATAGGCCTGGA	983-1005	
StAR	Fwd	GAGCTCTCTGCTTGGTTCTCAA	218-239	2-3
(StAR)	Rev	TTGAGTATGCCCAAGGCCTT	325-316	
PBR	Fwd	TGCAGAAACCCTCTTGGCATC	173-193	2-3
(Tspo)	Rev	TGAAACCTCCCAGCTCTTTCC	286-266	
p450scc	Fwd	CCTATTCCGCTTTTCCTTTGAGTCC	636-660	3-4
(Cyp11a1)	Rev	CGCTCCCCAAATATAACACTGCTG	686-663	
p450c21	Fwd	TGCCCCATCGTGCAACTAGG	1064-1083	8-9
(Cyp21a1)	Rev	AGCCGGAGATGCTGCTAGCC	1102-1083	
p450c17	Fwd	TCGGCCCCAGATGGTGACTC	416-435	1-2
(Cyp17a1)	Rev	TGGTCCGACAAGAGGCCTAGAG	454-433	
Arsa	Fwd	TTCACTGCAGATAACGGTCCTG	2038-2059	5-6
(Arsa)	Rev	AGGAGTAATGTGACCTGGCCA	2178-2158	
Sulft	Fwd	ACAGCTCTTTCCAAGCCATGA	614-634	5-6
(Sult2a2)	Rev	TCCCCAGTTGTGCCTTTTCT	722-703	
3βHSD-1	Fwd	TCTGAAAGGTACCCAGAACCTATTGG	433-458	3-4
(Hsd3b1)	Rev	TTGCTTGAACACAGGCCTCCA	476-456	
3βHSD-2	Fwd	AAAGGTACCCAGAACTTATTGGAGGC	417-442	3-4
(Hsd3b2)	Rev	GGCACACTGGCTTGGATACAGG	463-442	
3βHSD-4	Fwd	GGTCGAAAACAGGAAGAGGAATTGTC	118-144	1-2
(Hsd3b4)	Rev	TGGTCTTTGTCTGCAGCTTGGAC	140-163	
3βHSD-7	Fwd	CCATCCACAAAGTCAACGTGCAG	391-413	3-4
(Hsd3b7)	Rev	TCAATCACATTCTGTGTGCCCTG	433-411	
17βHSD-1	Fwd	AGTGTGGGAGGCTTGATGGGA	458-478	3-4
(Hsd17b1)	Rev	CACTTCGTGGAATGGCAGTCC	496-479	
p450Arom	Fwd	CTTTGGAGAACAATTCGCCCTTTC	418-441	3-4
(Cyp19a1)	Rev	GCCCGTCAGAGCTTTCATAAAGAA	462-439	
5αR	Fwd	TGTTTCCTGACAGGCTTTGCCC	436-457	2-3
(Srd5a1)	Rev	CCATGCCCACTAACCACAGGG	475-455	
3αHSD	Fwd	GCCATCGTGAAAAACAATGG	692-711	6-7
(Hsd3a1)	Rev	AATCAGCGCAGGAGTTCGA	795-777	
L27A	Fwd	TGTTGGAGGTGCCTGTGTTCT	442-462	1-1
(Rpl27a)	Rev	CATGGAGAGAAGGAAGGATGC	542-522	

Table 1. Primers for RT-PCR used to determine the expression of steroid-converting enzymes. The table shows the name of the protein analyzed (name of gene in parenthesis), specific oligonucleotide sequence, the number of bases, and the exons spanned by each amplicon.

Gene expression of steroid-converting enzymes in microglia

Although the steroid-metabolizing capacity of the principal cells in the CNS has been described to some extent, there are no reports addressing the contribution of microglia to brain steroidogenesis. To investigate the expression of the main steroid-converting enzymes in mouse microglia, I designed gene specific primers for Real Time PCR (RT-PCR) (Table 1). RT-PCR analysis of RNA from adult FACS-sorted microglia (*ex vivo* MG) and from primary microglia cultures (1°MG) revealed expression of the following genes: peripheral benzodiazepine receptor (PBR), steroid acute regulatory protein (StAR), aryl sulfatase (ArsaA), steroid sulfatase (StS) 3β-hydroxysteroid dehydrogenase type 7 (3βHSD7), 17β-hydroxysteroid dehydrogenase type 1 (17βHSD1), and 5α-reductase (5αR) (Table 2). On the other hand, cytochrome p450 side chain cleavage enzyme (p450scc), cytochrome p450 21-hydroxylase (p450c21), cytochrome p450 17-hydroxylase (p450c17), DHEA sulfotransferase (Sulft), 3βHSD type 1, 2, and 4, cytochrome p450aromatase (p450Arom), and 3α-hydroxysteroid dehydrogenase (3αHSD) were not detected (Table 2). The ratio of specific gene C_t values to the C_t value of L27A, a housekeeping gene, was calculated as a way of assessing the relative expression levels of each gene between *ex vivo* MG and 1°MG (Table 2).

	PBR	StAR	p450scc	p450c21	p450c17
InVivo MG	0.80 ±0.03	0.68 ±0.01	ND	ND	ND
1°MG	0.94 ±0.02	0.62 ±0.01	ND	ND	ND
Ovary	0.79 ±0.02	0.79 ±0.03	0.95 ±0.0	0.55 ±0.02	0.85 ±0.0

	Arsa	Sulft	3βHSD1	3βHSD2	3βHSD7
InVivo MG	0.85 ±0.04	ND	ND	ND	0.80 ±0.01
1°MG	0.83 ±0.02	ND	ND	ND	0.82 ±0.01
Ovary	0.69 ±0.02	0.54 ±0.01	0.73 ±0.03	0.69 ±0.01	0.69 ±0.02

	17βHSD	Arom	5αR	3αHSD	L27A (C_t)
InVivo MG	0.69 ±0.01	ND	0.69 ±0.01	ND	1.00 (21.5)
1°MG	0.70 ±0.02	ND	0.70 ±0.01	ND	1.00 (23.9)
Ovary	0.77 ±0.03	0.67 ±0.04	0.64 ±0.0	0.54 ±0.01	1.00 (14.6)

Table 2. Comparison of Steroidogenic Enzyme Expression in Microglia Expression levels of steroidogenic enzymes was evaluated by real-time PCR in microglia obtained from FACS-sorted cells from adult mouse brains (*ex vivo* MG), or from primary cultures (1°MG). Ovary tissue was used as a positive control. Values represent the mean (n=3) ± the SEM of the ratio of enzyme expression to the house-keeping ribosomal gene L27A.

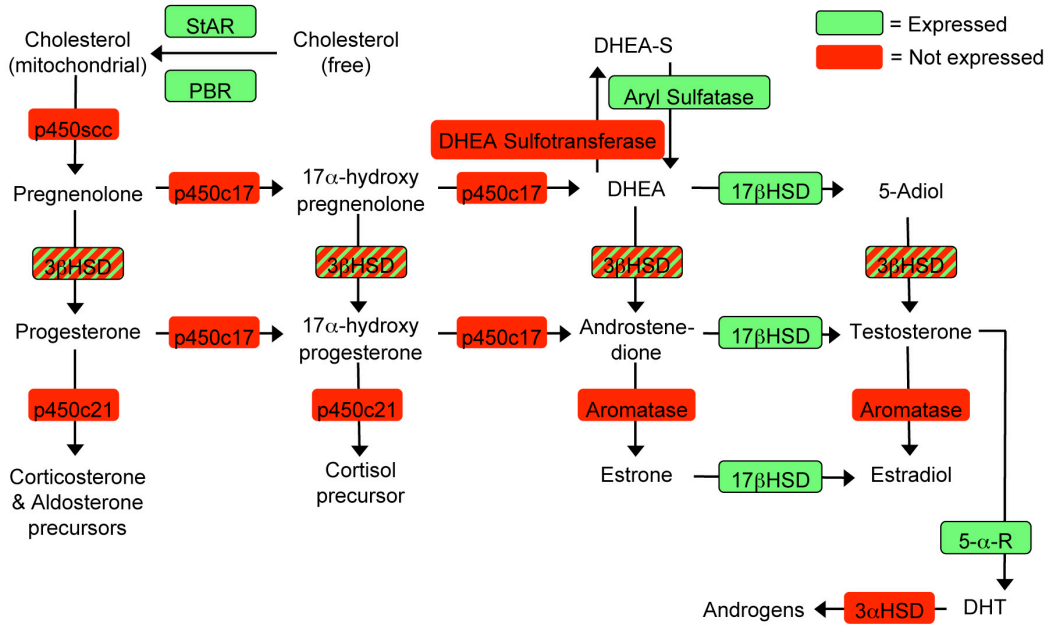

Figure 24. Expression profile of steroid-converting enzymes in resting microglia. Diagram of the steroidogenic pathway showing the main steroidogenic enzymes and their steroid substrates. The boxed enzymes are those expressed in resting microglia, the shadowed ones were not expressed.

Thus, the most abundant genes expressed were the PBR (ratio of 0.80; 0.94) and Arsa (ratio of 0.85; 0.83), while the lowest expression was found for StAR (ratio of 0.68; 0.62) (Table 2). No differences were found between the expression levels of *ex vivo* MG and 1°MG (Student T-test; p=0.16-0.64), except for the PBR ($p<0.0001$) and 3βHSD7 ($p<0.05$), which were expressed at higher levels in 1°MG (Table 2). A summary of the expression profile of steroid-converting enzymes in microglia is depicted in Fig.24.

LPS stimulation modulates expression of steroid-converting enzymes in microglia

To analyze the expression of steroid-converting enzymes in activated microglia, adult *cfms*-EGFP male mice were injected i.p. with saline or 5mg/kgLPS; 24hr later, brains were extracted and microglia were isolated by FACS-sorting (Chapter 4). This *in vivo* stimulation paradigm with LPS leads to the activation of brain microglia, indicated by changes in morphology and the expression of pro-inflammatory cytokines (Sierra, Gottfried-Blackmore et al. 2007; Sierra Submitted). *In vivo* stimulation with LPS led to an overall decrease in the expression of steroid-converting enzymes (Fig.25). StAR expression was decreased by 38.8±9.3% ($p<0.05$), Arsa by 26±10.6% (not significant), 3βHSD7 by 64.1±2.9% ($p<0.001$), 17βHSD1 by 71.4±0.8%

($p<0.05$), and 5αR by 66.6±1.8% ($p<0.05$) (Fig. 25). One notable exception was the PBR, whose expression was significantly increased 7.7±2.2-fold ($p<0.05$) (Fig.25). Furthermore, LPS stimulation did not induce the expression of p450scc, p450c21, p450c17, Sulft, 3βHSD1-2,4, p450Arom, or 3αHSD.

To further explore the effects of other pro-inflammatory stimuli on steroid-converting enzyme expression, primary microglia cultures (1°MG) were used. 1°MG were stimulated for 24hr with LPS+INFγ or INFγ alone and enzyme expression was assessed by RT-PCR. Stimulation with LPS+INFγ led to an increase in the expression of all the steroid-converting enzymes that were expressed in resting 1°MG (Fig.26). PBR expression was increased 9.6±1.0-fold ($p<0.01$) (Fig.26A), StAR increased 4.5±0.7-fold ($p<0.05$) (Fig.26B), Arsa increased 1.9±0.1-fold ($p<0.01$) (Fig.26C), 3βHSD7 increased 2.9±0.4-fold ($p<0.01$) (Fig.26D), 17βHSD1 increased 3.3±0.4-fold ($p<0.01$) (Fig.26E), and 5αR was increased 5.1±0.6-fold ($p<0.01$) (Fig.26F). INFγ stimulation alone only affected the expression of 3βHSD7 (increase of 2.9±0.4-fold ($p<0.05$)) (Fig.26D).

In vivo, i.p. injection of LPS induces high levels of circulating cytokines, which can indirectly activate brain MG and induce brain

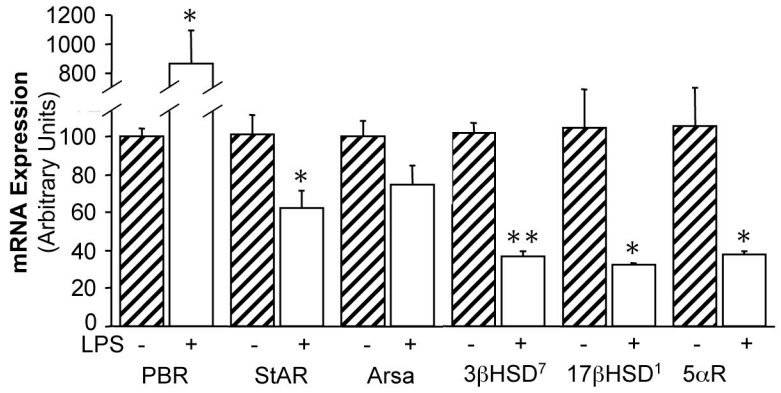

Fig 25. ***In vivo*** **LPS stimulation down-regulates expression of steroid-converting enzymes in microglia.** RT-PCR analysis of steroidogenic enzymes in FACS-sorted microglia from adult mice treated for 24hr with an i.p. injection of saline (hatched bars) or 5mg/kg LPS (open bars). Bars represent the % expression of each enzyme with respect to control samples (normalized to the house-keeping gene L27A). Values are the mean ± the SEM of three animals. ∗, $P < 0.05$; ∗∗, $P < 0.01$ *vs.* control, respectively.

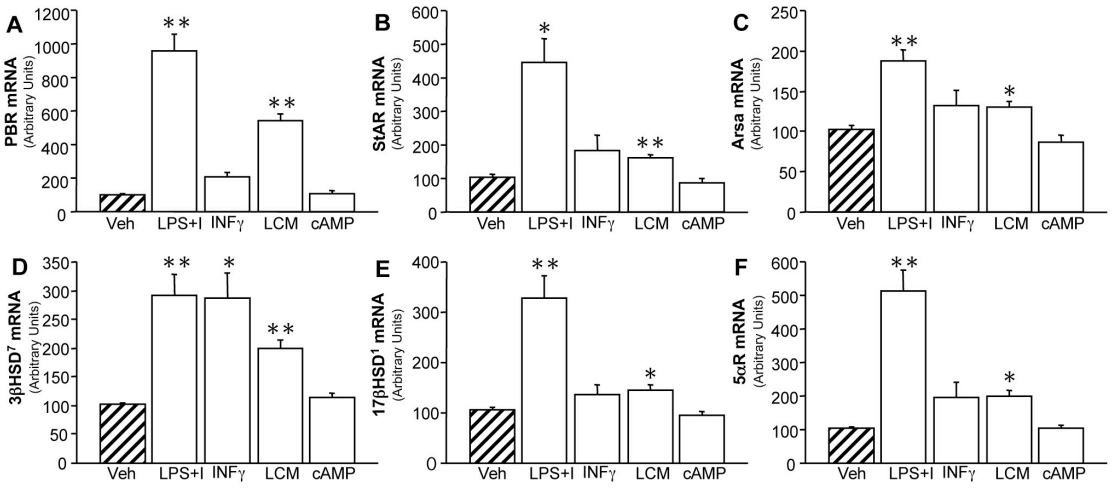

Fig 26. LPS increases the expression of steroid-converting enzymes in cultured 1°MG. Steroid-converting enzyme expression **A-F**) was analyzed by RT-PCR in cultured 1°MG stimulated for 24hr with 100ng/ml LPS+ 10ng/ml INFγ (LPS+I), 10ng/ml INFγ (INFγ), LPS-conditioned media (LCM), 1μM cAMP (cAMP), or vehicle (Veh)(hatched bars). Bars represent the % expression of each enzyme with respect to vehicle (normalized to the house-keeping gene L27A). Values are the mean ± the SEM of 2-3 independent experiments done in triplicate. ∗, ∗∗, $P < 0.05$, $P < 0.001$ *vs.* Veh, respectively.

expression of pro-inflammatory cytokines (Chakravarty and Herkenham 2005; Sierra, Gottfried-Blackmore et al. 2007). Our *in vivo* experiments with LPS yielded a down-regulation of steroid-converting enzyme expression. To determine whether an enriched milieu of inflammatory cytokines could mimic our *in vivo* results, 1°MG cultures were stimulated with microglia LPS-conditioned media, which contains LPS and high levels of several inflammatory cytokines such as TNFα, IL-6 and NO (Sierra Submitted), as well as IL1β, IL-12, MCP-1, MCP-5, and RANTES (Chapter 2). This LPS-conditioned media (LCM) was used to stimulate fresh cultures of 1°MG. 24hr stimulation with LCM did not induce a down-regulation of steroid-converting enzymes. Instead, it mimicked the LPS+INFγ effect on 1°MG, but to a lesser extent (Fig.26).

Finally, 24hr stimulation with cAMP, a known inducer of steroidogenesis in various endocrine cells, had no effect on the expression of steroidogenic enzymes in 1°MG (Fig.26). None of the treatments in 1°MG induced the expression of p450scc, p450c21, p450c17, Sulft, 3βHSD1-2,4, p450Arom, or 3αHSD (data not shown).

Steroid-converting activity in microglia

The results from our RT-PCR analysis suggested that microglia express the enzymes required for the conversion of DHEA into androgens and estrogens (Fig. 24). Steroid-converting activity of microglia was evaluated by incubating 1°MG with the steroid precursor dehydroepiandrosterone (DHEA) and measuring its metabolism. H^3-DHEA conversion of steroids was analyzed by thin layer chromatography (TLC) and beta counting of radioactive metabolites (Fig. 27). After 24hr incubation, 1°MG showed a 30±3.3% ($p<0.0001$) conversion of H^3-DHEA, with Adiol being the only product detected of this conversion, accounting for 15.5±%1.3 ($p<0.0001$) of H^3-radioactivity (Fig.27A). To determine if the activation of microglia would induce any change in enzyme activity, 1°MG were stimulated with LPS+INFγ and co-incubated with H^3-DHEA. Microglia stimulation had no effect on the conversion or profile of steroids produced from H^3-DHEA compared to resting cells (Fig.27B).

In steroidogenic glands, androstenedione (AD) is the main intermediary product between DHEA and the downstream synthesis of androgens and estrogens. 1°MG incubated with H^3-AD for 24hr showed a 36.7±1.7% ($p<0.0001$) conversion of this steroid (Fig.28A), with the main

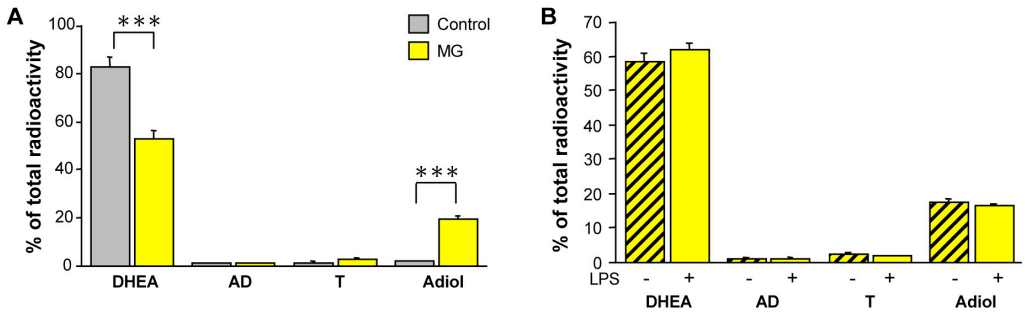

Fig 27. Metabolism of H^3-DHEA by 1°MG. 1°MG cells were incubated for 24hr with H^3-DHEA and metabolites extracted from culture supernatants were resolved by thin layer chromatography and counted on a scintillation counter. **A**) Resting microglia (MG, open bars) showed a significant conversion of DHEA compared to the no-cell controls (Control, grey bars), with the only product being Adiol. **B**) LPS (100ng/ml LPS+ 10ng/ml INFγ) stimulation of the cells (hatched bars) didn't alter the metabolic activity or profile of steroids produced from DHEA in microglia. Bars represent the % of total H^3 radioactivity collected from the TLC assay. Values are the mean ± the SEM of 2-3 independent experiments done in triplicate. ∗∗∗, $P <$ 0.0001 significantly different from control.

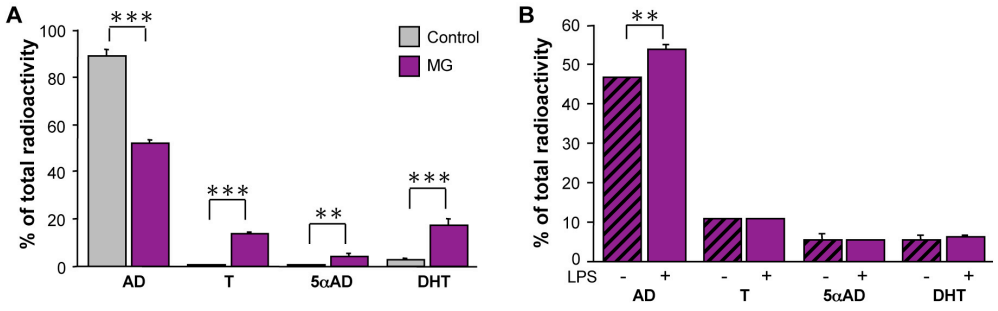

Fig 28. 1°MG convert H^3-AD into downstream steroids. 1°MG cells were incubated for 24hr with H^3-AD and metabolites extracted from culture supernatants were resolved by TLC and counted on a scintillation counter. **A**) Resting microglia (MG, open bars) showed a significant conversion of AD compared to the no-cell controls (Control, grey bars). The main products of AD were T/Adiol, estrone (E1), and DHEA/DHT. **B**) LPS (100ng/ml LPS+ 10ng/ml INFγ) stimulation (hatched bars) slightly decreased the conversion of AD compared to vehicle treated cells (open bars), but not the production of downstream steroids. Bars represent the % of total H^3 radioactivity collected from the TLC assay. Values are the mean ± the SEM of 2-3 independent experiments done in triplicate. ∗∗,∗∗∗, $P < 0.001$, $P < 0.0001$ *vs.* MG, respectively.

products of its conversion being T, 12.8±0.8% ($p<0.0001$); 5αAD, 3.8±0.8% ($p<0.01$); and DHT, 14.4±3.1% ($p<0.01$) (Fig.28A). Like in my DHEA experiments, LPS stimulation of the cells did not affect the conversion or the profile of steroids produced from H^3-AD (Fig.28B).

PBR role in microglia

PBR was the most abundant transcript detected in microglia; additionally, it showed a robust (7.7 to 9.6 fold) increase following microglia activation. The absence of cholesterol metabolizing enzymes, i.e. low levels of StAR and absence of p450scc and p450c17, suggest that PBR may play an alternate role in microglia. Stimulation of microglia with two selective ligands for the PBR, Ro and PK (10pM), led to a specific reduction of LPS-induced production of TNFα (16.2±10.8% decrease ($p<0.05$), and 40.9±8.9% ($p<0.05$) respectively), but had no effects on IL-6 and NO (Fig.29), except a 12.4±6.4% ($p<0.05$) increase by PK on NO (Fig.29). In contrast to these modulatory actions on cytokines, PBR ligands did not affect the metabolism of DHEA or AD (data not shown).

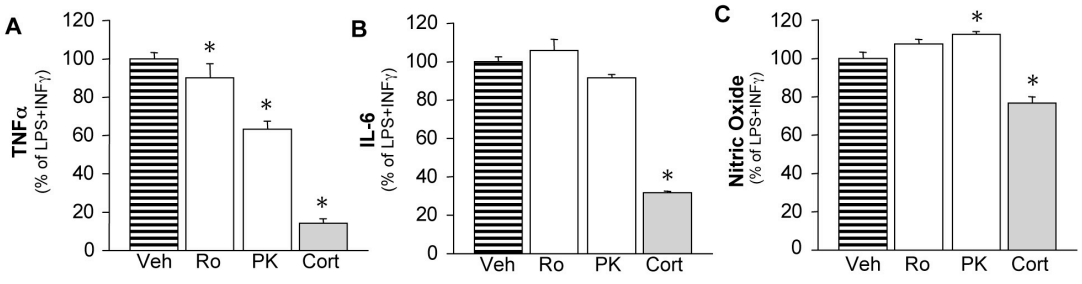

Fig 29. PBR ligands selectively modulate TNFα production in 1°MG. Cytokine secretion by 1°MG. Cells were incubated for 24hr with LPS (100ng/ml LPS+ 10ng/ml INFγ) with a 10 minute pre-treatment of the PBR ligands Ro (10pM) and PK (10pM), or 1μM corticosterone as positive control. Culture supernatants were processed by ELISA for the detection of **A)** TNFα and **B)** IL-6. Additionally, the Greiss assay was used for **C)** nitric oxide (NO) determination. Bars represent the % of total cytokines detected in the LPS controls. Values are the mean ± the SEM of 2-3 independent experiments done in triplicate. ∗ , $P < 0.05$ *vs.* LPS.

Adiol is an effective estrogen receptor agonist

The sole product of DHEA metabolism in 1°MG was Adiol. This steroid has been reported to have androgenic and estrogenic properties (Poortman, Prenen et al. 1975). To determine whether Adiol could function as a specific estrogen receptor (ER) agonist, we utilized an ER-expressing neuronal cell line, EtC.1 (Chapter 6) (Gottfried-Blackmore in preparation), transfected with a luciferase gene reporter coupled to 3 estrogen response elements (EREs). Incubation of these cells with Adiol induced the expression of the luciferase gene reporter at a high dose (1μM), but not at a lower dose (10nM) (Fig.30). Moreover, this induction was completely abrogated by pre-treatment of the cells with the specific ER-antagonist ICI-182,780 (100nM) (Fig.30). Microglia were not used for this assay because of their low expression of ERs and negligible responsiveness to E2 (Chapter 3) (Sierra Submitted).

Discussion

Neurosteroidogenesis and Microglia

Given the key role of microglia in neurodegeneration and the protective effects of neurosteroids, in this section of my thesis I attempted to verify whether microglia contribute to the synthesis and metabolism of

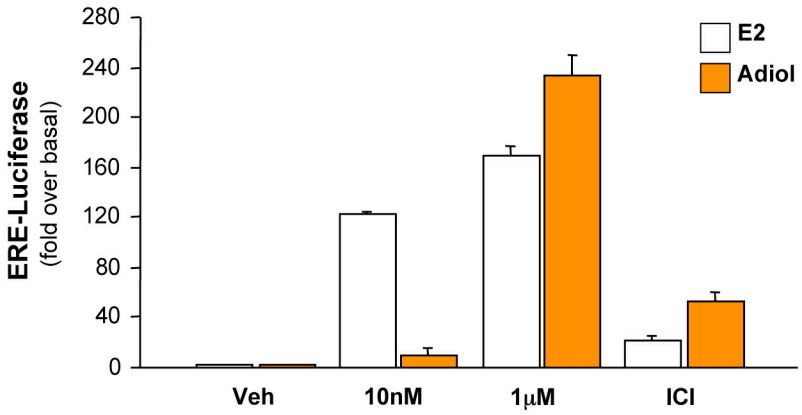

Fig.30 Adiol is an effective Estrogen Receptor Agonist. Estrogen receptor reporter assay, as reflected by luciferase expression from an ERE-Luciferase containing plasmid transfected into the neuronal cell line EtC.1. Bars represent luciferase activity determined by luminometry in cells treated with increasing doses of estrogen (E2) (open bars) or Adiol (grey bars). ICI 182,780 100nM (ICI), is a specific ER antagonist. Values are given in fold over basal ± the SEM from one representative experiment done in triplicate.

steroid hormones in the brain. Neuro-steroidogenesis involves the *de novo* synthesis of steroid hormone precursors, such as pregenenolone, progesterone and DHEA, from cholesterol within the brain. Some controversy still exists about this process occurring in the brain, mainly from conflicting results in the expression of p450c17 (Baulieu and Robel 1998; Zwain and Yen 1999), which yields DHEA. My results reveal that microglia do not express p450c17 nor p450scc, p450c21, or 3βHSD1-2, and therefore these cells do not have the capacity to synthesize neurosteroids from cholesterol.

Alternate roles for Steroidogenic Proteins

In spite of the lack of p450scc and p450c17, microglia expressed low levels of StAR mRNA, which is the main mediator for cholesterol import to the mitochondria for the initiation of steroidogenesis (Stocco 2000; Sierra 2004). StAR expression in microglia is likely to be playing a different role in these cells, such as cytosolic free sterol transfer, as has been suggested for other cholesterol binding proteins in macrophages (Rodriguez-Agudo, Ren et al. 2006).

Like StAR, the PBR also participates in the import of cholesterol into the mitochondria (Hauet, Liu et al. 2002). In the brain, PBR expression is

increased following nerve injury and can increase steroidogenesis locally (Lacor, Gandolfo et al. 1999). Aside from its role in steroidogenesis (Brown and Papadopoulos 2001), the PBR is widely expressed in monocytic cells (Carayon, Portier et al. 1996), and has been identified as a marker of activated microglia (Banati 2002). In my experiments with microglia PBR was the most abundant gene expressed. This is consistent with studies reporting that PBR is mainly expressed in glial cells, and its expression levels increase following glial activation induced by inflammation or neuronal damage [rev. by (Casellas, Galiegue et al. 2002)]. 1°MG and *ex vivo* MG challenged with inflammatory stimuli (LPS+INFγ or LPS injection respectively) responded with an increased expression of PBR.

In spite of enhanced PBR expression, PBR ligand stimulation, which increases steroidogenesis in other tissues (Lacor, Gandolfo et al. 1999; Brown and Papadopoulos 2001), had no effect on DHEA or AD conversion in microglia, probably because these PBR ligands affect the initial import of cholesterol into the mitochondria providing more substrate for p450scc, which is lacking in microglia. Yet, PBR ligands selectively decreased the TNFα response to LPS stimulation in 1°MG, in accordance with previous reports (Taupin, Gogusev et al. 1993; Taupin, Toulmond et al. 1993; Choi, Khoo et al. 2002). My data corroborate the proposed immuno-modulatory

role of PBR, which may account for this protein's expression in microglia and its reported neuroprotective effects (Leonelli, Yague et al. 2005; Veiga, Azcoitia et al. 2005).

LPS Activation and expression of Steroid-converting Enzymes in Microglia

Our results showed a significant reduction in all the steroid-converting enzymes expressed in *ex vivo* microglia after i.p. LPS activation. However, i*n vitro* in primary microglia, LPS+INFγ caused an increase in steroid-converting enzyme expression. These differences between *in vivo* effects of LPS and *in vitro* could be due to a number of reasons. An argument could be made for the developmental difference between adult FACS-sorted microglia and neonatal primary microglia, yet both cell populations showed similar expression levels of mRNA in the resting state, and when activated showed similar responses to genes like the PBR and 11βHSD-1 (Chapter 4).

Systemic injection of LPS *in vivo* causes a rapid induction of circulating cytokines and inflammatory mediators (Chensue, Terebuh et al. 1991), which can induce pro-inflammatory genes in microglia (Rivest 2003; Sierra, Gottfried-Blackmore et al. 2007). The down-regulation of steroid-converting enzyme expression *in vivo* due to cytokines could be ruled-out as well, given that *in vitro,* single cytokine stimulation (INFγ) or stimulation

with a combination of cytokines (LPS-conditioned media) showed only marginal effects. A caveat of these experiments is that the cytokines from microglia LPS-conditioned media may not mimic the cytokine milieu in the brain after i.p. LPS injections.

My results would suggest that other factors *in vivo* may be causing the down-regulation of steroid-converting enzymes in microglia, such as glucocorticoids. Following the systemic rise of cytokines, activation of the hypothalamic-pituary-adrenal (HPA) axis causes a 3-4 fold increase in circulating glucocorticoid levels (rev. by (Besedovsky and del Rey 1996)), which can block steroidogenesis in testicular Leydig cells (Gao, Shan et al. 1996; Badrinarayanan, Rengarajan et al. 2006). Similar effects of glucocorticoids may be occurring in brain microglia. Preliminary experiments indicate that corticosterone incubation can inhibit H^3-DHEA conversion in 1°MG (Fig.31). The effects of corticosterone on the expression of steroid-converting enzymes in microglia should be addressed in future studies.

In spite of the LPS-induced mRNA regulation of steroid converting enzymes, LPS+INFγ stimulation of 1°MG did not affect the rate or

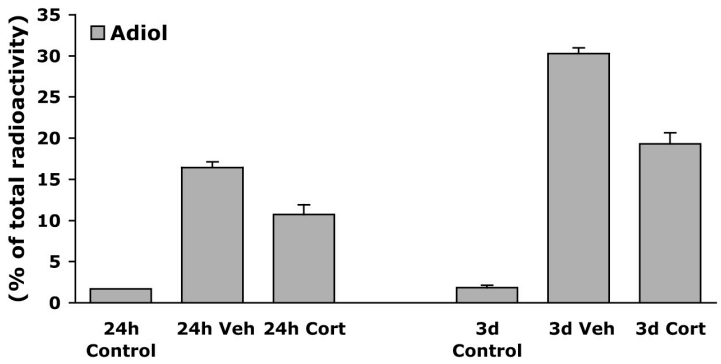

Fig 31. 1°MG conversion of H^3-DHEA to Adiol is reduced by Corticosterone. TLC analysis of H^3-DHEA metabolism in 1°MG ± corticosterone. 1°MG cells were incubated for 24hr or 3 days with H^3-DHEA ± 1μM corticosterone. Extracted metabolites were resolved by TLC and counted on a scintillation counter. Bars represent the % of total H^3 radioactivity collected from the TLC assay. Values are the mean ± the SEM of one representative experiment done in triplicate.

metabolism of H^3-DHEA or H^3-AD. These results are similar to what I found with 11βHSD-1 activity. Additional cofactors, such as NADP, may be required for observing effects of LPS treatment on actual steroid metabolism (Chapter 4).

DHEA metabolism and formation of active estrogens and androgens by microglia

In the CNS, DHEA has multiple effects reminiscent of sex hormones (Majewska 1995). The absence of a specific receptor for this hormone (Regelson and Kalimi 1994) has led investigators to suggest that DHEA is metabolized into active sex hormones that mediate the observed effects (Schmidt, Kreutz et al. 2000; Jellinck, Croft et al. 2005; Jellinck Submitted). Neurons, astrocytes and oligodendrocytes can metabolize DHEA into sex hormones (Zwain and Yen 1999). Recently, in collaboration with Dr. Jellinck (Queen's University, Canada), we reported that the microglial cell line, BV2, is able to convert DHEA into Adiol and validated the identity of this product by high-performance liquid chromatography (Jellinck In press). My current study corroborates these findings in 1°MG and shows the expression of 17βHSD1, required for this conversion, both in *ex vivo* MG and 1°MG.

In humans, DHEA levels are higher in brain than in circulation and have been correlated with aging and neurodegeneration (Weill-Engerer, David et al. 2002). Moreover, DHEA conversion in the brain into metabolites like Adiol may be reduced in patients with neurodegenerative disease (Weill-Engerer, David et al. 2003). Our data indicate that microglia can metabolize DHEA and specifically convert it to Adiol. This delta-5 steroid has reported androgenic and estrogenic properties in peripheral tissues (Poortman, Prenen et al. 1975; Adams 1985; Miyamoto, Yeh et al. 1998). Additionally, early studies describe Adiol binding pituitary estrogen receptors in the male rat brain and exerting estrogenic effects (Thieulant, Benie et al. 1983). Here I present evidence confirming that Adiol is an effective estrogen receptor agonist in ER-expressing neuronal cells (EtC.1). These data suggest that DHEA metabolism by microglia may be a source of active estrogens in the brain.

The results presented in this chapter indicate that microglia, like the other cells in the CNS, participate in the metabolism of steroid hormones through the expression and activity of steroid converting enzymes. Microglia do not participate in the initial utilization of cholesterol for generation of steroid hormone precursors, but rather show active metabolism of DHEA

and AD. The steroid converting capacity of microglia may be regulated by inflammatory stimuli, yet other factors need to be considered when performing *in vitro* studies. DHEA metabolism in microglia showed the exclusive formation of Adiol, which may function as a source of active estrogens in the brain.

CHAPTER 6

EFFECTS OF ESTROGEN ON CELLS FROM THE DEVELOPING CNS: EtC.1

E2 effects on microglia are marginal at best, compared to glucocorticoids, as indicated by my results (Chapter 3). However, further studies indicated that microglia may contribute to the formation of active estrogens (Chapter 5), which positively impact neuronal cells. Neurons are profoundly affected by estrogen, even in brain areas that are not related to reproductive functions (Priest and Pfaff 1995; McEwen, Alves et al. 1997; McEwen 2001). Most neuronal cells studied so far exhibit responses to estrogen that include modulation of their synaptic inputs (Woolley and McEwen 1992; Lewis, McEwen et al. 1995; Woolley, Weiland et al. 1997; Leranth, Hajszan et al. 2004), a higher resistance threshold to physiological stressors [rev. by (Behl 2002)], the ability to resist cell death or induction of apoptosis [rev. by (Amantea, Russo et al. 2005; Suzuki, Brown et al. 2006)], and even effects on neurogenesis and neuronal differentiation/maturation (Tanapat, Hastings et al. 1999; Brannvall, Korhonen et al. 2002; Kishi, Takahashi et al. 2005). However, the extent of E2's non-reproductive effects in the developing CNS is less well understood. As part of the laboratory's

efforts to further our understanding of estrogen's neurotrophic effects, and to investigate estrogen's effects on developing neurons, I undertook parallel studies to characterize a neuronal cell line from developing mouse brain cerebellum, EtC.1, and evaluate its responsiveness to estrogen. Additionally, throughout my studies with estrogen and microglia, I used elements of this neuronal system as a positive control of estrogen's effectiveness in my cell culture systems.

Characterization of the EtC.1 Cell line

Development of the mouse cerebellum occurs at a late embryonic stage and continues through the first few weeks of postnatal life. The EtC.1 cell line was originally cloned from embryonic mouse brain cerebellum and judged to be neuronal on the basis of its electrical excitability and characteristic surface antigens (Bulloch, Stallcup et al. 1977; Bulloch, Stallcup et al. 1978). The cells respond positively in a sodium flux assay that correlates well with the ability to generate action potentials, and also express one or more of three antigens previously found to be specific for nerve cells (Bulloch, Stallcup et al. 1977).

In the present study the EtC.1 cells were examined and analyzed for the expression of proteins commonly expressed in neural stem cells and

progenitor cells. This study was carried out jointly with Gist Croft M.S., a former research assistant in the laboratory. Additionally, the expression of specific cerebellar transcription factors was evaluated to determine the lineage and developmental stage of EtC.1 cells. These experiments were necessary for determining if the EtC.1 cell line could serve as a model for studying estrogen effects on developing cerebellar neurons.

EtC.1 cells were positive for the neuronal stem cell markers Nestin, Vimentin, Doublecortin (DCX), and Musashi (Fig.32A). However, EtC.1 were negative for the astrocyte marker GFAP, (Fig.32A), but expressed the neuronal marker NeuN (Fig.32B). These results confirm the early characterizations of the EtC.1 cells and substantiate their early developmental stage (Gottfried-Blackmore in preparation).

The developing cerebellum contains primarily progenitor cells of two principal lineages: the granule cell lineage and the Purkinje cell lineage. To distinguish to which lineage of cells the EtC.1 belong to, expression of lineage specific markers was performed by PCR. Zipro1, a defining transcription factor for the granule cell lineage (Yang, Zhong et al. 1996) was expressed in EtC.1 cells, as well as the granule cell precursor transcription factor Math-1 (Ben-Arie, Bellen et al. 1997), Pax-6 (Engelkamp, Rashbass et al. 1999), and En-2 (Liu and Joyner 2001) albeit

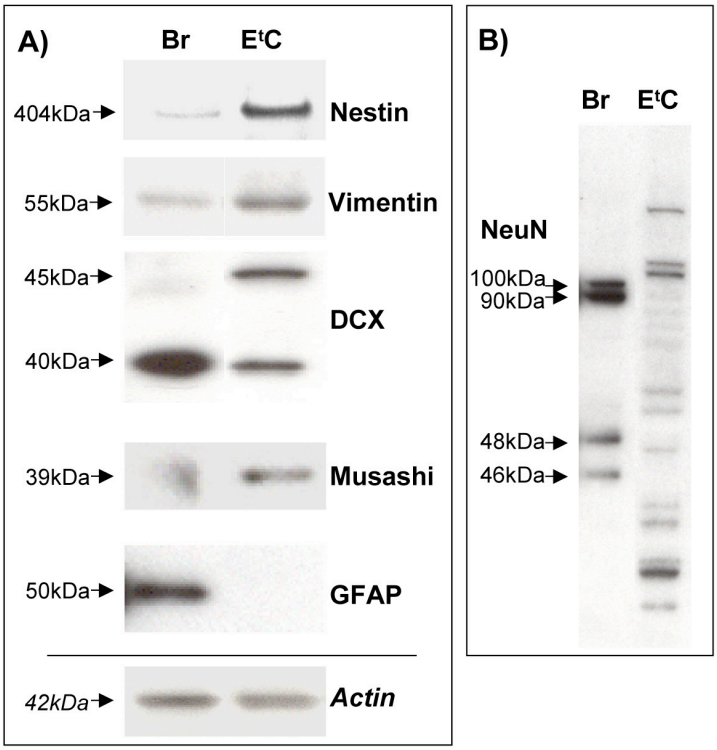

Figure 32. Western blot analysis of neural progenitor markers expressed in EtC.1. Protein samples were prepared from EtC.1 cells and processed by Western blot using specific antibodies for **A)** Nestin, Vimentin, doublecortin (DCX), Musashi, glial fibrillary acidic protein (GFAP), or **B)** neuronal nuclear antigen (NeuN). Actin was used for loading controls and brain tissue was used as a positive control for the neural markers.

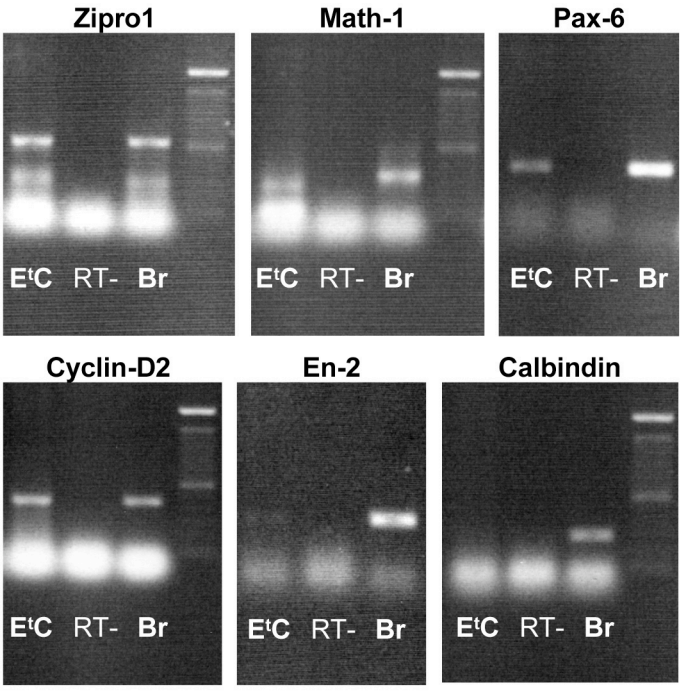

Figure 33. Immature granule cell transcription factors are expressed in EtC.1 cells. mRNA was extracted from EtC.1, reversed-transcribed and used for PCR amplification with specific primers for granule cell-specific transcription factor genes. PCR products were resolved on a 1% agarose gel stained with ethidium bromide, and posteriorly visualized under a UV-transilluminator.

at low levels (Fig. 33). In contrast, Calbindin, a commonly expressed marker in Purkinje cells, was not expressed in EtC.1 (Fig.33). In agreement with the precursor phenotype of the cells, Zic-1 and Zic-2, two transcription factors expressed in mature cerebellar granule cells (Aruga, Minowa et al. 1998; Aruga, Inoue et al. 2002), were not expressed in EtC.1 (data not shown), whereas the Cyclin-D2 gene present in dividing neural progenitor cells (Ross, Carter et al. 1996) was expressed (Fig.33).

Characterization of E2 receptors expressed in EtC.1 cells

Although estrogen receptors have been identified within the adult cerebellum (Litteria 1987; Jakab, Wong et al. 2001; Mitra, Hoskin et al. 2003), and during development (Belcher 1999; Price and Handa 2000; Guo, Su et al. 2001; Ikeda and Nagai 2006), the role of estrogen (E2) in the development of cerebellar neurons and their function in the adult has yet to be fully elucidated. Reminiscent of the effect seen on the principal cells of the adult mouse and rat hippocampus (Woolley and McEwen 1992), E2 is reported to affect the growth of dendritic spines in developing cerebellar Purkinje cells (Sakamoto, Mezaki et al. 2003).

Expression and function of ERs in EtC.1 was first assessed by WB analysis with specific antibodies against ERα and ERβ. ERα staining

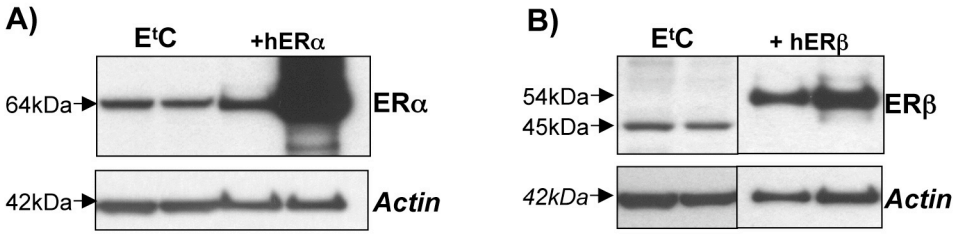

Figure 34. EtC express ERα and possibly ERβ. Western blot analysis of estrogen receptors expressed in EtC.1. Protein samples were prepared from control EtC.1 cells or from cells transfected with increasing concentrations of plasmids containing human estrogen receptors (hER). Samples were then processed by Western blot for **A)** ERα and **B)** ERβ. Actin was used for loading controls.

demonstrated an expected band (67 kDa) for EtC.1 protein extracts, which matched recombinant human ERα transiently expressed in EtC.1 (Fig.34A). ERβ staining yielded three bands (20kDa 45kDa and 100kDa) for EtC.1 protein samples; these did not match bands from mouse control tissues (ovary and prostate), nor from the human recombinant ERβ band (54kDa) (Fig.34B). Given some of the controversy that surrounds estrogen receptor antibodies, expression of ERα and ERβ transcripts was analyzed by qRT-PCR. Both ERα and ERβ transcripts were found in EtC.1. Additionally, ERα, but not ERβ, mRNA levels were reduced after E2 incubation (data not shown). These results indicate the predominant expression of ERα in EtC.1 cells.

The transcriptional activity of ERα and ERβ in the EtC.1 cell line was assessed in cells transiently transfected with an ERE-luciferase reporter gene construct. In this system, the detection of enzymatic luciferase activity in cell lysates is an indirect measure of transcription induced by activated ERs. After transfection, unstimulated cells showed minimal luciferase activity, while a physiological dose (10nM) of exogenous E2 induced a ca. 80-fold response over basal transcription (Fig.35). Luciferase induction reached a maximum at 12hr of E2 incubation and remained high at 24hr (data not shown). Subsequent experiments were performed using the 24hr time point.

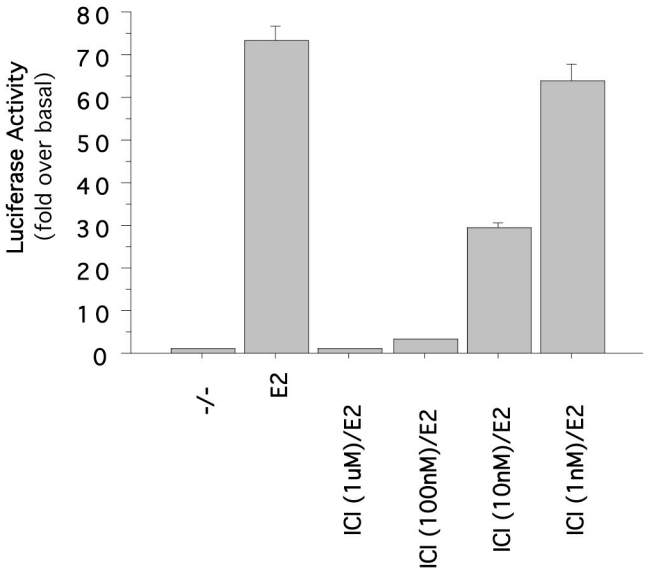

Figure 35. E2 induces luciferase activity in EtC.1, which is blocked by ICI. EtC.1 cells were transfected with the ERE-Luc gene reporter plasmid and incubated with E2 (10nM) in the presence or absence of increasing doses of the ER antagonist ICI 182,780. Luciferase activity was measured 24hr later. Data graphed reflect fold-over-basal luciferase activity (basal -/- was set to 1 unit).

E2-induction of reporter gene transcription was blocked by the ER antagonist ICI 182,780 (ICI) in a dose-dependent manner (Fig.35). ICI alone had no effect on reporter gene activity (data not shown). Together, these results show that EtC.1 cells express functionally active ERs.

To distinguish the participation of ERα and ERβ in the response elicited by E2, selective agonists for these receptors were compared, namely the ERα agonist PPT and the ERβ agonist DPN (Tocris). This work was jointly done with M.S. Croft. Incubation of the cells with the same dose (10nM) of E2 or the selective ER agonists revealed that the ERα-selective agonist PPT was significantly less active than E2, and that the ERβ-selective agonist DPN did not induce luciferase activity (E2 100%; PPT 46±5.7% $p<0.05$; DPN 1.2±0.6; $p<0.05$) (Fig.36A). These results were verified using a different set of ER agonists, MC1 for ERα and MC2 for ERβ (Merck) (E2 100%; MC1 62±2.16% $p<0.05$; MC2 2.4±0.44%; $p<0.05$) (Fig.36B). ERα and ERβ agonists were not additive, nor did they differ from the induction levels obtained from PPT alone (Fig.36). Like E2, both ERα-selective agonists PPT and MC1 were significantly blocked by ICI (Fig.36 $p<0.05$). The results suggest that ERβ is not functionally expressed, whereas ERα is the predominant receptor for E2 in EtC.1 cells.

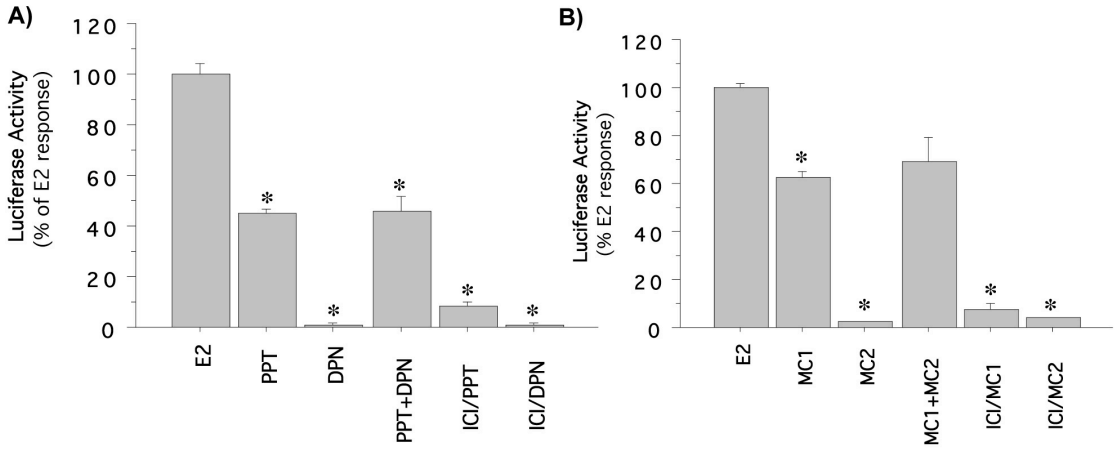

Figure 36. ER-selective agonists induce partial gene transcription in EtC.1 cells. Tocris ER-selective agonists **A)** PPT (ERα) and DPN (ERβ) or **B)** MC1 (ERα) and MC2 (ERβ), induce less reporter gene activity than E2, and are sensitive to ICI. Cells transfected with ERE-Luc were incubated with E2, PPT, MC1, and/or DPN, MC2 (10nM) in the presence or absence of the ER antagonist ICI (100nM). Luciferase activity was measured 24hr later. Data is graphed as % of E2 response. *$p<0.05$ (*vs* E2).

Estrogen is known to activate important second messenger signaling pathways in neuronal cells, such as those mediated by CREB and $ERK^{1/2}$ MAPK (Lee and McEwen 2001; Lee, Campomanes et al. 2004). To determine if E2 activated these pathways in EtC.1 cells, serum-starved EtC.1 cultures were stimulated with E2 (10nM) and phosphorylation of CREB and $ERK^{1/2}$ MAPK was assessed by WB. Both MAPK were constitutively phosphorylated, yet serum-starvation reduced phosphorylation levels (data not shown). $ERK^{1/2}$ phosphorylation was induced by fetal calf serum (FCS) stimulation, but not by E2 treatment (Fig.37A). CREB phosphorylation was induced by cAMP (1uM), yet was also not affected by E2 treatment (Fig.37b), indicating that ERs in the EtC.1 did not engage in these second messenger signaling pathways.

E2 does not affect maturation or development of EtC.1 cells alone

The cerebellum is rich in the expression of ERα and ERβ throughout development, where the ratio of these receptor subtypes changes as the cerebellum matures: ERβ seems to have a low constitutive expression in early development and becomes the predominant receptor in adulthood, where as ERα appears to play a role mostly in development (Belcher 1999; Price and Handa 2000; Guo, Su et al. 2001; Ikeda and Nagai 2006).

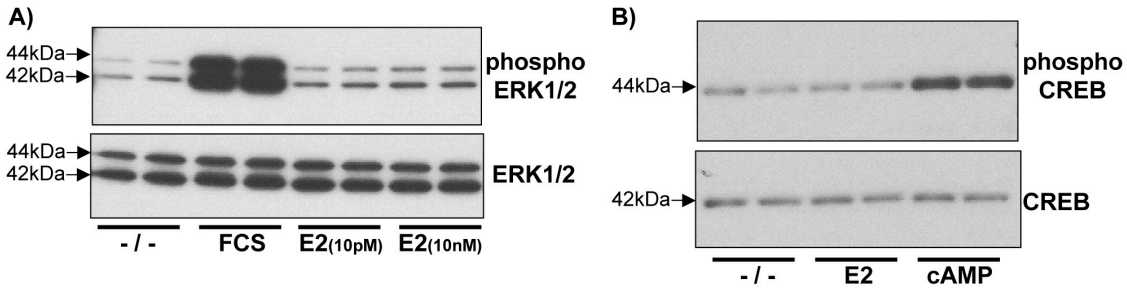

Figure 37. E2 fails to induce ERK1/2 MAPK or CREB signaling in EtC.1 cells. Western blot analysis of ERK$^{1/2}$ MAPK and CREB phosphorylation. EtC.1 cells were starved for 48hr in serum-free media and then stimulated with **A)** 30 minutes (ERK$^{1/2}$) or **B)** 3hr (CREB). Cells lysates were then analyzed by Western blot for total and phospho ERK$^{1/2}$ MAPK or CREB.

Our results showing the predominant expression of ERα in EtC.1 cells are consistent with this model.

To evaluate if estrogen plays a role in the maturation or differentiation of EtC.1, cells were incubated for 24hrs and 36hrs with E2 (10nM) and expression of the various neural and granule progenitor-cell markers was measured. E2 treatment had no effect on protein levels for the neural progenitor cell markers analyzed (Nestin, DCX, NeuN, Vimentin, Musashi), nor was the expression of GFAP induced (data not shown). Further, estrogen (10nM, 24hrs) did not affect expression levels of Pax-6 or Zipro1. Additionally, E2 treatment did not induce expression of Zic-1 and Zic-2 (data not shown). These results indicate that estrogen alone is not sufficient for the differentiation of the EtC.1 phenotype.

In our screen of neural proteins that might be regulated by E2, we found that EtC.1 cells expressed the fragile-X mental retardation protein (FMRP) (Fig.38A), which is involved in dendritic spine maturation (Feng, Gutekunst et al. 1997; Lu, Wang et al. 2004). Moreover, E2 treatment increased its expression (Fig.38B). To determine if E2 treatment could induce maturation of synaptic structures in EtC.1 cells, the expression of various pre- and post-synaptic proteins was assessed by Western blot. Resting EtC.1 cells were negative for Synaptophysin, Syntaxin, PSD-95,

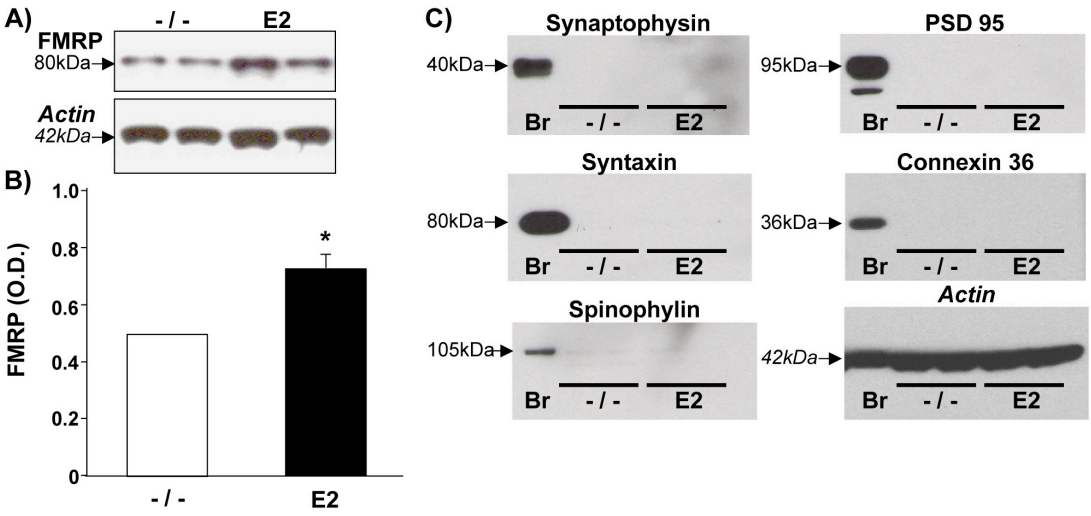

Figure 38. EtC.1 cells express FMRP but not mature synaptic markers. EtC.1 cells were starved for 24hr in serum-free media and then stimulated for 24hr with 10nM E2. Cells lysates were then analyzed by Western blot for **A)** FMRP, or **C)** 5 different synaptic markers. Blots were also probed for actin as a loading control. **B)** Densitometric analysis of FMRP protein. *p<0.05.

Spinophylin, and connexin-36. Further, 24hrs incubation with E2 (10nM) failed to induce expression of these proteins (Fig.38C). These data indicate that estrogen alone is not sufficient for the maturation of EtC.1 cells.

Discussion

EtC.1 cells expressed various protein markers characteristic of neuronal progenitor cells such as Nestin, Vimentin, DCX, and Musashi, which confirm their early developmental origin. Additionally, EtC.1 cells were identified as belonging to the granule cell lineage by their expression of Zipro1, Math1 and En-2, and the absence of the Purkinje cell marker Calbindin. To our knowledge, this would establish the EtC.1 cell line as the first available cell line of developing cerebellar granule cells. Further study of these cells could therefore, contribute to studies aimed at examining cerebellar granule cell development or maturation.

EtC.1 cells were positive for the expression of ERα and ERβ at the mRNA level, but at the protein level, expression of ERβ was inconclusive. Using an estrogen-sensitive gene reporter assay, I determined the function of these putative receptors. ERα, but not ERβ, was able to drive gene expression in EtC.1 cells. The expression of functional ERα, but not ERβ, in EtC.1 cells is in agreement with previous findings demonstrating the

predominant expression of ERα in the developing cerebellum (Belcher 1999; Price and Handa 2000; Guo, Su et al. 2001; Ikeda and Nagai 2006).

Despite the presence of functional ERα, our findings that estrogen stimulation alone was not sufficient to induce the differentiation (change in neural progenitor markers) or maturation (induction of synaptic proteins) of EtC.1 cells under the conditions tested. This developmental stage of EtC.1 cells may account for their inability to engage $ERK^{1/2}$ MAPK and CREB signaling after E2 stimulations. EtC.1 cells may require established signals to differentiate, such as bone morphogenic proteins (Angley, Kumar et al. 2003), and it may be likely that E2 could synergize with such differentiation factors or affect their actions. This line of experiments merits further attention and may be fruitful in future studies.

Using the ERE-Luciferase assay system in EtC.1 cells, I was able to test the effects of steroids metabolized by microglia, such as Adiol (Chapter 5). In addition, my experiments with microglia and EtC.1 utilized the same hormone stocks, providing an internal control for the quality of my reagents. ERα expression in EtC.1 provides a model to further study the neuroprotective effects of E2 in developing granule cells. A model could be set-up in which microglia derived Adiol and E2 could be compared for their neuroprotective, or other, properties in these cells.

CHAPTER 7

CONCLUSIONS AND FUTURE DIRECTIONS

Since the initial anatomic description of microglia in the first decade of the 20th century, and up until the 1980s, these cells were considered as mere by-standers in the steady state central nervous system (CNS), with a definite role in development, and proliferative and phagocytic capacity following brain injuries in the adult. However, our appreciation and understanding of microglia cells and their roles in brain homeostasis is steadily increasing. Today microglia are regarded as the main resident immune cells of the CNS, distinct from peripheral macrophages and monocytes, with key roles in the initiation and maintenance of inflammatory responses in the brain [rev in Chapter 1].

Microglia, once activated, become highly pro-inflammatory cells through their production of cytokines, chemokines, prostaglandins, and free radicals. Activated microglia exert beneficial effects following injury or an immune challenge, yet disregulation of the microglial response can be deleterious (Block, Zecca et al. 2007). Over-activated microglia have been implicated in basically all forms of neurodegenerative diseases due to the high susceptibility of neuronal cells to pro-inflammatory mediators, mainly

free radicals and TNFα [rev in Chapter 1]. Microglial involvement in neurodegeneration has given this cell type a new status in neuroscience research.

Given that the over-activation of microglia results in deleterious and neurotoxic effects, a considerable interest lies in determining whether activated microglia can be harnessed as effective targets of anti-inflammatory therapies in the treatment of neurodegenerative disease. The work presented in this thesis addresses this issue, and elucidates some of the regulatory processes involved in microglia activation with a focus on anti-inflammatory steroid hormones.

Clinical and animal studies have pointed to the neuroprotective and anti-inflammatory effects of steroid hormones [rev in Chapter 1]. The ample and varied literature on the effects of steroid hormones on inflammation indicates that diverse factors such as dose, timing and duration of exposure, receptor subtype and cell-type specific expression, can cause steroids to exert stimulatory, permissive, or inhibitory actions (Sternberg 2001; Dinkel, Ogle et al. 2002; Yeager, Guyre et al. 2004; Sorrells and Sapolsky 2007). Yet our comprehension of these issues is not complete, and in the case of the CNS, the cellular targets and mechanisms of action of these hormones are still unresolved. In view of these limitations, the main question addressed in

this dissertation was the role that microglia play in the well-documented anti-inflammatory effects of steroid hormones in the brain, with particular emphasis on the neuroprotective sex hormone 17β-estradiol (E2), and the anti-inflammatory adrenal steroid, corticosterone.

Steroid hormones and microglia activation

My studies clearly indicate that E2 does not have any profound effects on the LPS activation of microglia. These results are inconsistent with many published studies showing that E2 has an anti-inflammatory effect on microglia. However, on close examination of the literature, one can note that the field of E2 studies on microglia is riddled with contradictory reports, (Chapter 1, 3). These contradictions could be accounted by factors such as the use of microglia cell lines (Bruce-Keller, Keeling et al. 2000; Bruce-Keller, Barger et al. 2001; Baker, Brautigam et al. 2004), reports comparing macrophage cell lines to microglia cells (Vegeto, Ghisletti et al. 2004; Vegeto, Belcredito et al. 2006), species differences between rat and mouse-derived microglia (Drew and Chavis 2000; Vegeto, Bonincontro et al. 2001; Sierra Submitted), and the extrapolation of E2 anti-inflammatory effects *in vivo* on microglia (Vegeto, Belcredito et al. 2003; Vegeto, Belcredito et al. 2006). As described in this thesis, the absence of ERβ expression and low

levels of ERα *in vivo* in mouse microglia, coupled with the failure of E2 to regulate LPS-induced cytokines or MAPK activation in primary microglia cultures are strongly suggestive that E2 does not act directly on stimulated microglia (Chapter 3). Consistent with this data, we found a down-regulation of ERα mRNA in activated microglia from i.p. LPS-injected mice (Chapter 3).

Despite the claims of positive E2 effects on the pro-inflammatory state of microglia, my results do correspond with a lesser number of reports indicating no direct effects of this hormone on microglia activation (Chapter 1,3). However, it is likely that the literature does not reflect all the studies where no effects of E2 on inflammatory microglia have been found, given that negative results are often not published (based on private communications with at least three other laboratories during the annual, international, Society for Neuroscience Meetings 2004-2006).

These reports and the work presented here suggest a model whereby other cell types in the brain, such as oligodendrocytes, astrocytes and neurons, mediate most of E2's neuroprotective and anti-inflammatory effects (Fig.39). For example, NF-κB activation in astrocytes has recently been found to be a key regulator of inflammation in the CNS, and its inhibition has beneficial effects on tissue regeneration [rev by (Farina, Aloisi et al.

2007)]. ERs, which are expressed in astrocytes (Azcoitia, Sierra et al. 1999; Garcia-Ovejero, Veiga et al. 2002), could interfere with NFκB activation (Wen, Yang et al. 2004). Further, the anti-inflammatory effects of E2 could be due to indirect events that impact microglial responses, such as effects on astrocytes and neurons. Examples of these are the E2-induction of anti-inflammatory and neuroprotective cytokines like TGFβ by astrocytes (Wyss-Coray, Lin et al. 2001; Sortino, Chisari et al. 2004); and increased oligodendrocyte (Takao, Flint et al. 2004) and neuronal survival [rev. by (Behl 2002)] (Fig. 39). Another key mechanism of the anti-inflammatory effects of E2 in the brain may be the ability of E2 to abolish the autologous down-regulation of glucocorticoid receptors in the brain (Ferrini, Lima et al. 1995). Taken together, it is likely that the anti-inflammatory and neuroprotective effects of E2 are separate and complementary actions. A useful application of this distinction could be the combination of E2 with well-established anti-inflammatory drugs for future therapies in neurodegenerative diseases. An example of this approach is reported for lesioned retinal ganglion cells, where both neuroprotection and regeneration were enhanced by the combination of two compounds acting on different sites: a specific drug, aurintricarboxylic acid, acting directly on the neurons, and cortisol acting on the glial environment (Heiduschka and Thanos 2006).

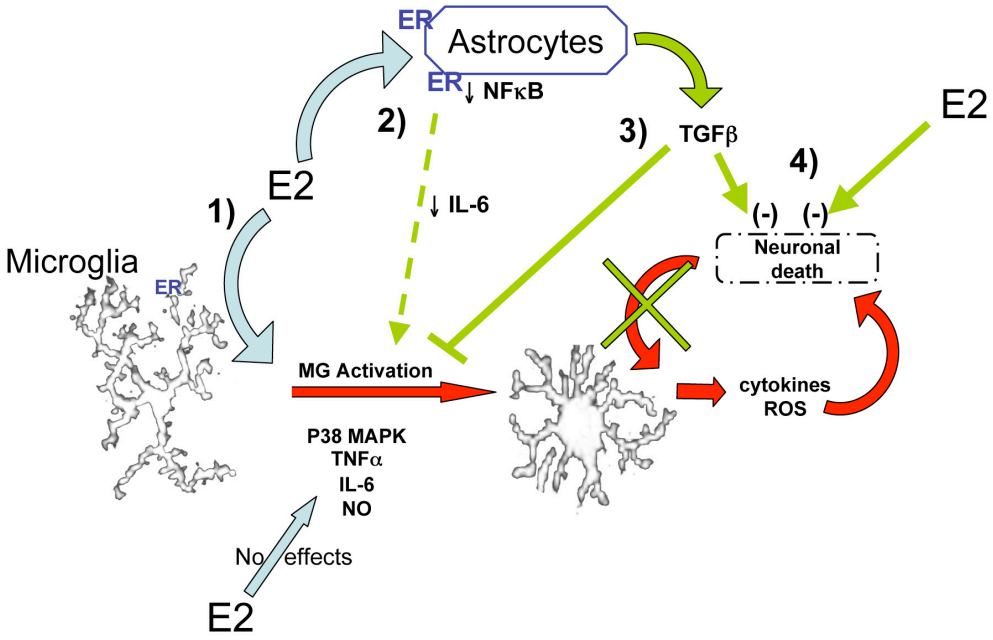

Figure 39. Diagram modeling the possible anti-inflammatory and neuroprotective mechanisms of E2 in the CNS. 1) E2 may directly affect microglia, however there is little to no effect on inflammatory mediators and microglia activation. Alternatively, E2 may act on other cells such as **2)** astrocytes, which express high levels of ERs. E2 can block NFκB and IL-6 production in these cells, which may lead to reduced activation of microglia cells. Alternatively, **3)** E2 induces expression of the anti-inflammatory cytokine TGFβ in astrocytes, which can block microglia activation. **4)** Both TGFβ and E2 also act as neurotrophic and neuroprotective factors, which diminish neuronal death, and therefore, reduce signals that activate microglia.

Using glucocorticoids as well-established anti-inflammatory compounds, I found that the adrenal steroid, corticosterone was an effective regulator of LPS-induced responses in microglia (Chapter 3,4), which is in agreement with previous reports. The work provided in this thesis supports the anti-inflammatory effects of elevated concentrations of glucocorticoids on microglial cells. However, glucocorticoids can act as a double-edged sword in the CNS, given that prolonged exposure to elevated glucocorticoid levels is neurotoxic (Sapolsky 1996), and that low levels of glucocorticoids are pro-inflammatory [rev by (Sorrells and Sapolsky 2007)].

The glucocorticoid receptor mediates most of the known anti-inflammatory effects of glucocorticoids, yet the mineralocorticoid receptor, which has a much higher affinity for corticosterone, is reported to have down-stream pro-inflammatory activity (Tanaka, Fujita et al. 1997). It has been postulated that early in the inflammatory response, initial low levels of corticosterone promote inflammation through the high-affinity mineralocorticoid receptor and unknown glucocorticoid receptor mechanisms, and then as glucocorticoid levels rise to supra-physiological levels they mediate anti-inflammatory effects predominantly through glucocorticoid receptor (Sorrells and Sapolsky 2007). Although the effects

of mineralocorticoids were not examined in this thesis, our studies indicate the expression of mineralocorticoid receptor mRNA in microglia (Sierra Submitted) suggesting that sub-nanomolar concentrations of corticosterone might be pro-inflammatory (Tanaka, Fujita et al. 1997). The pro-inflammatory role of the mineralocorticoid receptor in microglia needs further investigation, and could be a potentially interesting therapeutic target.

Glucocorticoids are the body's most effective regulators of inflammation, exerting permissive, stimulatory, and suppressive effects [rev by (Sorrells and Sapolsky 2007)]. In this dissertation I present evidence showing that microglia are not only responsive to glucocorticoids (Chapter 3), but also have the capacity of re-activating inactive glucocorticoids through 11βHSD-1 expression (Chapter 4). 11βHSD-1 converts 11-DH-Cort into active corticosterone, thus re-activating glucocorticoids. The local amplification of glucocorticoids through microglia 11βHSD-1 activity may be significant, particularly in the context of inflammation, as LPS-stimulation induced a robust increase of 11βHSD-1 expression in microglia, both *ex vivo* and *in vitro*. These results are suggestive of a model whereby following a systemic inflammatory challenge, and subsequent production of glucocorticoids by HPA-axis activation, microglia increase expression of

11βHSD-1 to amplify the anti-inflammatory effects of corticosterone, which regulates production of potentially neurotoxic cytokines and free radicals. It is interesting that in this setting, astrocytes down-regulated 11βHSD-1 expression, indicated by preliminary *in vivo* immunofluorescence analyses (Chapter4). This could be considered a protective response given the neuronal susceptibility to high concentrations of glucocorticoids (Sapolsky 1996).

It has been proposed that 11βHSD-1 expression in the brain plays roles in cognitive behavior, neuroprotection, and contributes to the negative feedback of glucocorticoids on HPA activity [rev by (Holmes, Yau et al. 2003)]. 11βHSD-1 activity during brain inflammation is an untapped area of research. The results I present in this thesis point to an active role of 11βHSD-1, expressed in microglia, in the re-activation of glucocorticoids. The existence of viable 11βHSD-1 KO mice could serve as a valuable model to study the participation of this enzyme following neuronal injury and microglia activation. Additionally, *in vivo* studies with selective 11βHSD-1 inhibitors could also be fruitful.

Brain steroid metabolism and microglia

In this thesis I present data that indicates an active role for microglia in the conversion of active steroids in the CNS (Chapters 4,5). Steroid hormones play numerous roles in maintaining the homeostasis of the CNS (Chapter 1). It is intriguing that the brain, like other tissues of the body, has evolved and acquired the capacity to metabolize and, in some cases, synthesize these hormones. Furthermore, it's been postulated that the appearance of steroid converting enzymes occurred concomitantly with the appearance of steroid hormone receptors to provide specificity and regulatory mechanisms as organs became more complex (Baker 2004). Intracrine production of hormones, that is synthesis or conversion of hormones within the target cell or tissue (Labrie 1991; Labrie, Luu-The et al. 2005), provides the brain with basal levels of steroids, which have already proven to play important roles in processes such as nerve regeneration, synaptic transmission, and notably responses to CNS injury (Chapter 1,5).

Up until now, no reports have described whether microglia participate in the metabolism of steroids in the brain. The conversion of glucocorticoids by microglia spurred me to characterize the participation of microglia in the broader metabolism of brain steroid hormones. In this dissertation I offer data suggestive of an active role for microglia in the conversion of steroids

in the CNS (Chapters 4,5). Microglia express steroid-converting enzymes, which mediated the conversion of steroid precursors DHEA and AD into downstream hormones. Therefore, microglia may contribute to the pool of active steroid hormones in the brain, as shown by the production of Adiol, an active estrogen in neuronal cells (Chapter 5).

It is unlikely that microglia are only causing deleterious effects to the injured CNS. In fact, it is becoming more widely accepted that microglial activation is necessary and crucial for host defense and neuronal survival [rev by (Block, Zecca et al. 2007)]. As proposed for 11βHSD-1 mediated amplification of anti-inflammatory effects of glucocorticoids, the steroid-converting capacity of microglia may be particularly significant during inflammation. Locally, astrocytic steroid metabolism is inhibited by inflammatory cytokines (Zwain and Yen 1999), whereas in my studies microglia steroid-converting activity occurred in spite of their activation state *in vitro* (Chapter 5). Although my studies also showed a down-regulation of mRNA expression in *ex vivo* microglia from i.p. LPS-injected mice, it remains to be determined what factors caused this down-regulation, and whether this would also be observed in the setting of a model of neuronal damage. Androgen production, particularly testosterone, from DHEA metabolism in microglia may be a substrate for astrocytic and

neuronal p450 Arom, which synthesizes E2 required for neuroprotection in various models of neuronal injury (Sierra, Azcoitia et al. 2003; Veiga, Garcia-Segura et al. 2003; Veiga, Azcoitia et al. 2005).

Inflammation is also known to suppress sex hormone production by interfering with neuroendocrine gonadal signaling. Within the brain, cytokines produced by microglia, like IL-2, IL-6, TNFα and IFNγ, affect the release of anterior pituitary hormones and block the hypothalamic-pituitary gonadal (HPG) axis (Jones and Kennedy 1993). Systemically, circulating cytokines can also disrupt HPG axis (Kalra, Fuentes et al. 1990). In addition to blocking the HPG-axis, inflammatory cytokines directly block steroidogenesis in the gonads. Macrophage secreted products, such as TNFα (Andreani, Payne et al. 1991; Xiong and Hales 1997), IL1β (Hurwitz, Payne et al. 1991) and NO (Pomerantz and Pitelka 1998), can inhibit production of DHEA and androgens in the testes via inhibition of p450c17 gene expression (Hales 1992; Li, Youngblood et al. 1995; Onami, Matsuyama et al. 1996). In contrast to the gonads, adrenal steroid synthesis is increased in response to inflammation (Chapter 1, 4). Therefore, after an inflammatory challenge or neuronal injury, DHEA originating from the adrenals could serve as a substrate for brain steroid conversion in cells like microglia and astrocytes in the absence of gonadal steroid production (Figure 40).

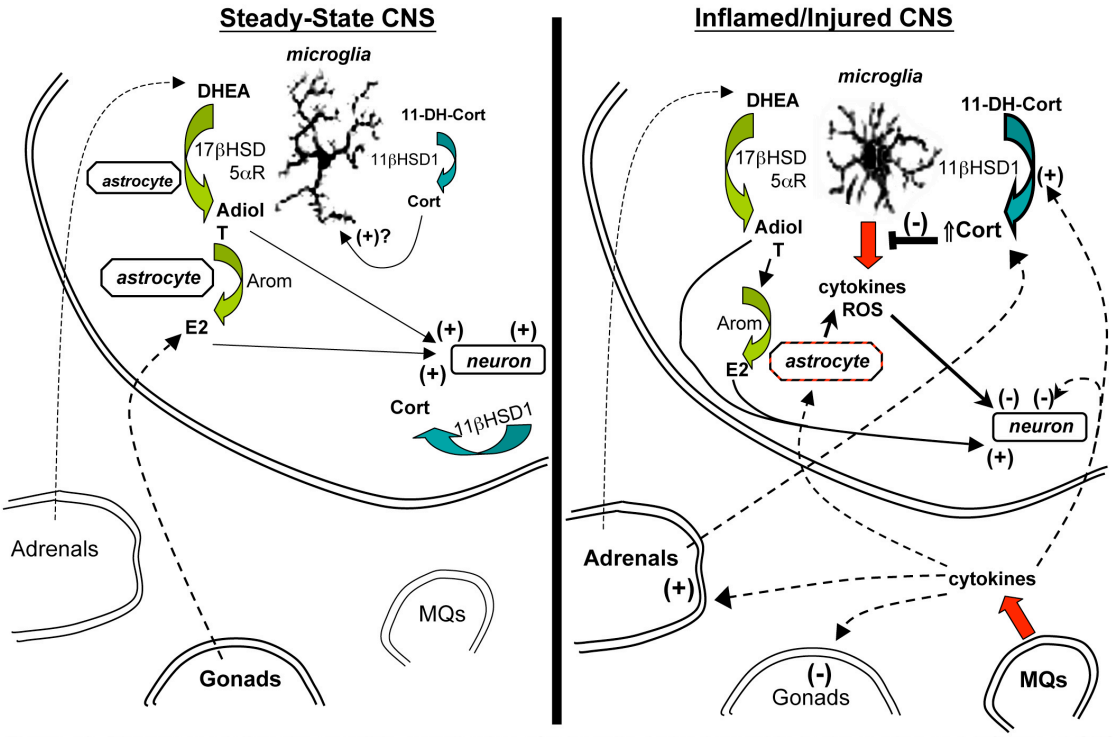

Figure 40. Diagram modeling the possible contribution of microglia-derived steroids in the resting and inflammed CNS. In the resting CSN, microglia and astrocytes convert DHEA into Adiol, testosterone (T), and estrogen (E2) respectively, which exert neurotrophic effects. Neurons and glia re-activate glucocorticoids (Cort) for normal CNS functions. In the inflamed CNS, cytokines block gonadal steroid production, possibly leaving microglia as the sole contributor of neuroprotective steroids, through DHEA conversion. Additionally, Cort anti-inflammatory effects are amplified by microglia.

Microglia, friend or foe?

In spite of our increased understanding, there are many questions in microglial biology that remain un-answered, particularly the role of microglia in the steady-state brain. Classically, ramified or resting microglia were considered to be inactive under physiological conditions, however, it is now known that microglia exhibit pinocytic activity and localized motility (Booth and Thomas 1991; Glenn, Booth et al. 1991). Recent studies have also shown that resting microglia are engaged in active surveillance of the brain tissue through their highly ramified protrusions, scanning the entire brain parenchyma every few hours (Davalos, Grutzendler et al. 2005; Nimmerjahn, Kirchhoff et al. 2005). Microglial processes directly contact neuronal cell bodies, astrocytes and blood vessels (Nimmerjahn, Kirchhoff et al. 2005), therefore it seems likely that microglia monitor the well-being of brain cells and also function to clear the extracellular milieu to maintain tissue homeostasis (Booth and Thomas 1991; Thomas 1992; Fetler and Amigorena 2005). Determining the effects of physiological concentrations of glucocorticoids on microglial surveillance or chemotaxis may help unravel more of microglia's "house-keeping" functions in the CNS.

Another question that remains unclear is: what are the differences between microglia populations in different brain regions? A great

heterogeneity of ramified microglia morphologies in different brain regions has been reported, which suggests that microglia may adapt to distinct micro-environments (Lawson, Perry et al. 1990). However, it is unknown whether this morphological variability reflects functional differences. One potential and appealing difference may be microglial responses to inflammatory stimuli or their receptivity to steroid hormones.

During CNS injury or pathology, it is still unclear to what extent resident microglia versus newly recruited cells from the bone marrow contribute to the resolution or the augmentation of neuronal cell death. In transgenic mouse models of Alzheimer's disease, it has been reported that blood-derived microglia and not their resident counterparts have the ability to eliminate β-amyloid deposits by a cell-specific phagocytic mechanism (Simard, Soulet et al. 2006). Additionally, impairing the accumulation of blood-recruited microglia at sites of plaque deposition causes increased β-amyloid load and leads to premature death (Khoury, Toft et al. 2007). However, β-amyloid is pro-inflammatory and activates microglia to release neurotoxic factors such as NO, TNFα, and superoxide, which potentiates neuronal damage and symptoms in Alzheimer's patients [rev by (Block, Zecca et al. 2007)]. It is likely that these neurotoxic effects are mediated by both resident and incoming microglia (Moore, El Khoury et al. 2002). Anti-

inflammatory drugs, like glucocorticoids, may be useful in slowing the progression of Alzheimer's disease (AD) through their effects on microglia activation. However, large-scale studies with anti-inflammatory drugs have produced negative results (Aisen 2002). Better understanding of the anti-inflammatory effects of E2 and glucocorticoids may result in beneficial therapies for progressive neurodegenerative diseases.

The conditions defining whether microglial activation is detrimental or beneficial to neuronal survival are still poorly understood. However, it is becoming more widely accepted that although microglial activation is necessary and crucial for host defense and neuron survival, the over-activation of microglia results in deleterious and neurotoxic consequences [rev by (Block, Zecca et al. 2007)]. A better understanding of the conditions regulating this cell type's activation will definitely lead to improved therapeutic approaches for neurodegenerative diseases.

Based on the work presented in this thesis, I propose that activation of microglia by innate immune pathways, such as LPS stimulation, is predominantly regulated by glucocorticoids, rather than E2. Under this model, microglia serve as key mediators of the anti-inflammatory effects of adrenal steroids, whereas the effects of E2 are mediated through other glial

cells and neurons. Additionally, microglia can be considered active contributors to the steroid-converting capacity of the brain; On one hand, amplifying the local anti-inflammatory effects of glucocorticoids in an autocrine manner, and on the other, potentially providing active androgens and estrogens that can affect neurons and astrocytes in view of non demonstrable effects of E2 on activated microglia and the absence of androgen receptors, ERβ, and low levels of ERα. Future studies aimed at elucidating the autocrine and paracrine anti-inflammatory and neuroprotective roles of microglia-derived steroids during CNS inflammatory responses to injury are promising and merit consideration.

MATERIALS AND METHODS

In vitro Culture (BV-2 microglia, 1°MG and EtC.1 cells)

BV-2 microglia cultures: Cells from an early passage (#3) were cryopreserved in liquid nitrogen according to standard tissue culture protocols. Cryoprotected cells were quickly thawed and seeded in 20 ml of Dulbecco's Modified Eagle Media with 4mM glutamine (DMEM, Gibco, Carlsbad, CA) containing 20% heat-inactivated Fetal Calf Serum (FCS) (Sigma, St. Louis, MO) and Penicillin, Streptomycin, Antimycotic (PSA) (Gibco). Cells were cultured in 75cm^2 tissue culture flasks (BD, Franklin Lakes, NJ) in a CO2 water jacketed incubator at 37ºC with 5% CO2. After initial plating, confluent cultures were trypsinized (0.25%, trypsin, Gibco), centrifuged, and re-suspended in standard culture media (SCM) comprised of DMEM containing 10% fetal calf serum plus PSA. All cells used in these experiments were derived from passages 4-10. The properties evaluated in this study remained stable throughout all passages. Tissue culture microscopy was performed with a Nikon inverted fluorescent microscope (Nikon, Melville, NY).

Primary microglia (1°MG) cultures: Microglia cultures were prepared following standard protocols (23). Briefly, day 2-old mouse pup brains were dissected on ice, and the meninges were carefully removed. The forebrains were minced in 5% FCS-PBS buffer, dissociated using fire polished Pasteur pipettes, and then passed through a 40μM nylon cell strainer (BD). Cells were washed once in buffer and seeded in culture media (10% FCS DMEM + PSA) at a density of roughly two forebrains per 75mm flask. Cells were grown at 37°C, 5 %CO_2, culture media was changed every 5 days, and, where indicated, supplemented with 5ng/ml macrophage-colony or granulocyte-monocyte colony stimulating factor (MCSF, Sigma, St. Louis, MO; or GM-CSF, Cell Sciences, Canton, MA). After 2 weeks in culture cells were shaken at 125rpm for 5hrs at 37°C to harvest detached microglia. Microglia were then counted and seeded in 10%FCS DMEM for different assays; for RNA-PCR: 6-well plates at a density of 1 million cells/well; or for hormone metabolism-TLC, and cytokine assays: in 24-well assay plates at a density of 0.25-0.3 million cells/well. After plating, microglia were allowed to adhere for 1hr and then rinsed with DMEM to remove non-adherent glial cells. Finally, 10%DMEM ± MCSF or GM-CSF was added to the cells and left overnight. The following day cells were rinsed with DMEM and treated as described below.

Microglia stimulations: For the activation of BV-2 cells, cells were rinsed and then incubated with 100ng/ml LPS diluted in DMEM alone. As LPS requires a specific serum binding protein for effective delivery to its cognate receptor (Hailman, Lichenstein et al. 1994), the LPS-induction of cytokines was dependent on media serum concentrations. Therefore, experimental conditions were adjusted to 1% FCS ($1/10^{th}$ of normal serum levels) for effective stimulation of the cells. To induce 1°MG activation, cells were incubated at 37°C with 1%FCS DMEM plus 100ng/ml LPS+ 10ng/ml INFγ. INFγ was supplemented to LPS to obtain a robust nitric oxide (NO) response.

LPS+ INFγ conditioned media (LCM) was obtained by stimulating 1°MG cultures with 1%FCS DMEM plus 100ng/ml LPS+ 10ng/ml INFγ, collecting the supernatants 24hr later, and centrifuging at 2000rpm for 5min to clear any debris. LCM contains elevated levels of several inflammatory cytokines such as TNFα, IL-6 and NO, as well as IL1β, IL-12, MCP-1, MCP-5, and RANTES (Chapter 2). This LCM was used to stimulate fresh cultures of 1°MG.

Estrogen, corticosterone and PBR ligand stimulation: Microglia cultures were pre-treated for 10 minutes (or other time points, see Chapter 2) in DMEM with 17β-estradiol (E2), corticosterone (Cort), 11-dehydro-corticosterone (11-DH-Cort), Ro 5-4864 (Ro) or PK-11195 (PK) (all compounds from Sigma), before 1%FCS LPS+INFγ stimulation. Stock solutions of all hormones were made in EtOH and stored at –20C. Final working dilutions were prepared with DMEM alone. Vehicle (EtOH) was always used as a control in non-treated cells at the same dilution.

Incubation with tritiated (H^3) glucocorticoids: The day after seeding, cells were rinsed and incubated in 0.25ml DMEM containing 3nM [1,2,6,7H^3]-Corticosterone (70 Ci/mmole) (NEN Life Science Products, Boston, MA) or 2nM [1,2,6,7 H^3]-11-dehydro-corticosterone (80 Ci/mmole). [H^3]-11-dehydro-corticosterone was synthesized from [H^3]-corticosterone and kindly provided by the laboratory of Dr. Hardy (Population Council, RU, NY). Radioactive steroids were incubated for various time points ± 11βHSD inhibitors, 11-keto-progesterone and 11-OH-progesterone (kind gift of Dr. Hardy). In experiments with activated microglia, cells were co-incubated with 100ng/ml LPS+10ng/ml INFγ in 1%FCS DMEM. All incubations were conducted in a 5% CO_2 atmosphere at 37°C. The incubations were stopped

by collecting the supernatant and vortexing with 2ml of diethyl-ether (Fisher, Carlsbad, CA). The organic phase extract was isolated and evaporated to dryness at room temperature, the residue was re-dissolved in 70µl of diethyl-ether, and separated by thin layer chromatography (TLC) for quantification of each product.

Incubation of tritiated (H^3) hormones and steroid extraction: The day after seeding, cells were rinsed and incubated in 0.2ml DMEM containing 16.7nM [1,2,6,7-H^3] DHEA (60 Ci/mmol) or 11nM [1,2,6,7-H^3] AD (105 Ci/mmol) (Perkin Elmer Life Science, Shelton, CT) for 22-24hr. All incubations were conducted in a 5% CO_2 atmosphere at 37°C. The reaction was stopped by vortexing the supernatant with acetone (0.2 ml) and ethyl acetate (0.5ml) (Fisher). A 0.2 ml portion of the organic phase extract was evaporated to dryness at room temperature, the residue was re-dissolved in methanol, and the yield of metabolites was determined after separation by thin layer chromatography (TLC).

EtC.1 cell culture: The EtC.1 cell line was cloned from embryonic day 17 (E17) mouse brain and tested for its neuronal properties (Bulloch et al., 1977; Bulloch et al., 1978). Cells from an early passage (#3) were

cryopreserved in liquid nitrogen for future use, according to standard tissue culture protocols. Cryoprotected cells were quickly thawed and seeded in 25 ml of DMEM with 4mM glutamine containing 20% heat-inactivated FCS and PSA in 75cm^2 tissue culture flasks in a CO2 water jacketed incubator at 37ºC with 5% CO2. After initial plating, confluent cultures were trypsinized, centrifuged, and re-suspended in standard culture media comprised of DMEM containing 10% FCS plus PSA. All cells used in these experiments were derived from passages 4-10. The properties evaluated in this study remained stable throughout all passages.

For experiments with E2 incubations, cells were either serum starved or cultured in Charcoal Stripped fetal calf serum (CSS, Hyclone, Logan, UT), as charcoal stripping removes endogenous bovine hormones and growth factors that could spuriously influence results. For ERK$^{1/2}$ and CREB phosphorylation experiments, cells were serum starved in DMEM without serum for 12hr and 48hr respectively, before stimulation with E2.

***In vivo* experiments (cfms-EGFP mice)**

Animals: For these studies, the transgenic mouse line p7.2fms-EGFP (C57BL6/6 X CBA background) was used (Sasmono, Oceandy et al. 2003).

Enhanced green fluorescent protein (EGFP) expression is driven by the promoter and the regulatory elements of the *c-cfms* gene that encodes the receptor for macrophage colony stimulating factor (CSF-1), resulting in EGFP expression in cells of the mononuclear phagocytic lineage, including microglia (Sasmono, Oceandy et al. 2003). The p7.2*fms*-EGFP mouse line was generously provided to the lab by Dr. Hume (Queensland, Australia) and Dr. Pollard (Albert Einstein, NY), and a colony was reared and maintained in the Rockefeller University Animal Facility for these studies. Animals were bred under 12:12 ligh:dark cycle and free access to chow and water. To induce inflammation, male mice received a single intraperitoneal (i.p.) injection with *Salmonella typhimurium* lipopolysaccharides (LPS; 1-5 mg/kg; Sigma, L2262). All experimental procedures were approved by the Rockefeller University Animal Care and Use Committee.

Ex vivo Microglia Isolation by fluorescence activated cell sorting (FACS): Previously reported methods to obtain a single population of microglia by FACS were used (Sierra, Gottfried-Blackmore et al. 2007). In brief, adult mice (2-3 months of age) were anaesthetized with pentobarbital (750mg/kg) and rapidly decapitated. Brains were removed and placed on ice in Hank's balanced salt solution (Gibco, Carlsbad, CA), and meninges, blood vessels

and choroid plexus were carefully removed under a dissecting scope. Brain cell suspensions, obtained after incubation with type II-S collagenase (600U; Sigma) and DNAse (450U; Invitrogen, Carlsbad, CA) for 30min at 37ºC in 15ml HBSS supplemented with 90 mM CaCl2, were homogenized by repetitive gentle pipetting with fire-polished Pasteur pipettes on ice followed by filtering through a 40μm cell strainer (BD).

Cells were washed by centrifugation and subject to percoll gradient centrifugation as described previously (Sierra, Gottfried-Blackmore et al. 2007). Cells collected from the 30/70 interphase, were washed and re-suspended in 5% FCS (fetal calf serum)-PBS containing 100ng/ml propidium iodide (PI), before sorting in a FACS Vantage SE Flow Cytometer (BD, Rockefeller University Flow Cytometry Facility), with smHighPurity precision. Post-sort analysis was performed to ensure the purity of the collection process.

Analytical Assays

FACS Analysis of 1ºMG: After shaking for 5hr, microglia were collected and washed in FACS buffer (5% FCS PBS). Cells were then blocked for 15 min at 4ºC with 5% mouse serum. Cells were then stained for 15 minutes at 4ºC with phycoerithryn (PE) conjugated primary antibodies: anti-CD11b (1:200)

(BD), or its corresponding PE-conjugated isotype, anti-rat IgG2b (1:200) (BD); anti-CD11c (1:200), or isotype anti-hamster IgG1 (1:200). Staining for DEC205 was done with a 15-minute incubation at 4ºC with biotin conjugated anti-DEC205 (2.8ng/µl), or its biotin conjugated isotype anti-III-10 (2.8ng/µl) (both antibodies kindly provided by Dr. Ralph Steinman's laboratory), and a secondary incubation with PE-conjugated strepavidin (1:500). Finally, cells were washed 3X in FACS buffer and then analyzed using a BD FACSCalibur system (BD) under the FITC and PE channels. Data was analyzed using FlowJo software (Tree Star Inc., OR).

Immunocytochemistry: Cells were seeded onto Poly-L coated glass cover-slips in a 24-well plate (2x104 cells/well). After treatments, cells were fixed in 4% paraformaldehyde PBS, permeabilized, and blocked in 5% goat or horse serum PBS, 0.5% Tween (Sigma) for 1hr at R.T. Primary antibodies incubations were done overnight at 4ºC in blocking buffer: anti-NFκB p65 (C-20, sc-372) (1:500) (Santa Cruz Biotech, Santa Cruz, CA), or anti-phospho-p38 MAPK (Thr180/Tyr182) (1:1000) (Cell Signaling Tech, Danvers, MA). Cells were washed 3X in 1% serum PBS, and then incubated for 1hr at room temperature with species-specific fluorescent secondary antibodies coupled to Alexa-594 (1:1000 Molecular Probes). Coverslips

were washed 5X and then mounted on glass slides using Dako fluorescent mounting media (Dako, Carpinteria, CA) for microscopy. Confocal images were acquired using a LSM510 confocal Zeiss Axioplan microscope with a kripton/argon laser and a HeNe laser (Rockefeller University Bioimaging Facility).

Cytokine and Nitric Oxide (NO) Measurements: 24hr after microglia stimulation with LPS or LPS+INFγ, supernatants were collected, cleared of cell debris by centrifugation at 4°C for 5 minutes at 2500 rpm (Eppendorph microfuge), and then frozen at –20°C until further analysis. Cytokines (TNFα and IL-6) were measured by enzyme linked immuno-absorbent assay (ELISA) following manufacturer's instructions (eBioscience, CA). NO was quantified using the Greiss assay (Promega).

Western Blotting: Cultured cells were rinsed in cold PBS supplemented with $Ca^{2+}Mg^{2+}$ and scraped in ice-cold protein lysis buffer (6M Urea, 20mM Tris-HCl pH7.5, 2%SDS, 10% glycerol, 1% protease inhibitor cocktail (Sigma)) supplemented with phosphatase inhibitor (1μM NaVO4; Sigma). Cell lysates were sonicated to homogeneity and then quantified using the BioRad Dc protein assay (BioRad, Hercules, CA). Samples were stored at –20°C

until processed by Western blot. Briefly, equal amounts of protein were mixed with Laemli loading buffer (Invitrogen), heated at 70°C for 10min, and separated by SDS-polyacrylamide gel electrophoresis performed under reducing conditions with 4-12% acrylamide NuPage gels according to manufacturer's instructions (Invitrogen). Resolved proteins were transferred to PDVF membranes (Invitrogen).

Membranes were rinsed in 0.1M Tris-Buffered Saline with 0.1% Tween-20 (TBS-T) and blocked with a solution of 5% non-fat dry milk in TBS-T for 1hr at room temperature on an orbital shaking platform. Membranes were then washed with TBS-T and incubated overnight at 4°C in 5% bovine serum albumin (BSA) (Sigma) in TBS-T solution with primary antibody.

Antibodies used for microglia studies included: anti-p38MAPK (1:2000) (Santa Cruz Biotech); anti-phospho-p38MAPK (1:2000) (Cell Signaling); anti-IL-1β (1:2000) (Chemicon, Temecula, CA); anti-Actin A5441 (1:40,000) (Sigma); anti-ERK$^{1/2}$ MAPK 9102 (1:1000) (Cell Signaling); anti-phosphoERK$^{1/2}$ MAPK 9101(Thr202/Tyr204) (1:2000) (Cell Signaling); anti-ERα 6F-11 (1:1000) (Novocastra); anti-ERβ 80424 (1:25,000) (Merck, Rahway, NJ); anti-GR antiserum (1:2000; M20, Santa

Cruz Biotechnology, Santa Cruz, CA); and anti-11βHSD-1 125-11 (1:1,000; Seckl Lab, UK).

Primary antibodies used for the EtC.1 studies included: anti-NeuN MAB377 (1:2000) (Chemicon); anti-GFAP MAB3402 (1:2000) (Chemicon); anti-Doublecortin SC-8067 (1:1000) (Santa Cruz Biotech); anti-Nestin MAB353 (1:2000) (Chemicon); anti-Musashi AB5977 (1:1000) (Chemicon); anti-Vimentin (1:250) (Hybridoma Bank, University of Iowa, #40E-C); anti-FMRP MAB2160 (1:2000) (Chemicon); anti-CREB 9192 (1:2000) (Cell Signaling); anti-phospho CREB 9198(Ser133) (1:2000) (Cell Signaling).

After overnight incubation in primary antibodies, membranes were washed and incubated with horseradish peroxidase-conjugated species-specific anti-antiserum (1:20,000) (Pierce, Rockford, IL) in blocking solution. After washing, membranes were developed with SuperSignal West Pico substrate (Pierce, Rockford, IL), and then exposed to X-Ray film (X-OMAT AR; Kodak, Rochester, NY). To control for protein loading, membranes were incubated in Restore Western Blot Stripping Buffer (Pierce), washed, and immunoblotted as described above using an anti-Actin antibody. Developed films were analyzed by densitometry using a computerized image analysis software (MCID-M4; Imaging Research, Inc,

St. Catherines, ON), and the data was normalized as follows: protein band (density*area)/actin (density*area). For phospho-proteins, their corresponding non-phosphorylated forms were for normalization.

Real-time PCR: Adult microglia were sorted by FACS into RNA lysis buffer (Absolute RNA Microprep kit (Stratagene, La Jolla, CA)), frozen in dry ice, and kept at -80ºC until processing. RNA was isolated using the Absolute RNA Microprep kit.

RNA from 1°MG cultures was obtained by rinsing the cell cultures with PBS and then lysing cells in 350µl RNA lysis buffer (RNeasy Mini kit (Quiagen, Valencia, CA)). RNA was extracted using the RNeasy Mini kit protocol (Qiagen). Both extraction methods included a step with DNAse incubation (Qiagen) to remove residual DNA.

RNA quality and concentration were then assessed by measurement of optical density at 260 and 280 nm (1°MG) or RNA was quantified with RiboGreen RNA Quantitation kit (Molecular Probes) following manufacturer instructions (*ex vivo* MG). 10ng of RNA were retrotranscribed with SuperScript II Reverse Transcriptase (Invitrogen) and 3µl of a 1:3 dilution of the cDNA were amplified by real-time PCR using SYBR Green master mix (AB) in a 7900HT SDS thermal cycler (AB). The gene

transcripts quantified were 11β hydroxysteroid dehydrogenase type 1 and type 2 (11βHSD-1, -2), peripheral benzodiazepine receptor (PBR), steroidogenic acute regulatory protein (StAR), cytochrome p450 side chain cleavage enzyme (p450scc), cytochrome p450 21-hydroxylase (p450c21), cytochrome p450 17-hydroxylase (p450c17), aryl sulfatase (Arsa), steroid sulfatase (StS), DHEA sulfotransferase (Sulft), 3β hydroxysteroid dehydrogenase type 1,2,4,7 (3βHSD-1, -2, -4, -7), 17β hydroxysteroid dehydrogenase (17βHSD-1), cytochrome p450 aromatase (p450Arom), steroid 5α reductase (5αR), 3α hydroxysteroid dehydrogenase (3αHSD), and ribosomal protein L27A (L27A). Primers sequences were designed using Primer Express Software (ABI) and are indicated in Table 1; amplicons were designed to span two exons in order to avoid potential contaminating DNA amplification. All primers were blasted on NCBI databases for target specificity and tested using appropriate positive control tissues such as ovary and adrenal glands. All samples were tested in triplicate in order to eliminate pipetting errors and the average Ct (threshold cycle) was used to calculate the relative amount of product by the -ΔΔCt- method (AB), using the ribosomal L27A as a housekeeping gene. The ratio of enzyme Ct to L27A Ct values was calculated as a way of assessing the relative expression levels of each enzyme comparing 1°MG and *ex vivo* MG.

In each experiment, both positive (1µg ovary mRNA/cDNA 1:3) and negative (RT minus and water) controls were included to ensure that the PCR reaction was working properly.

Thin Layer Chromatography (TLC) Identification of Glucocorticoids: Products from H^3-11-dehydro-corticosterone (11-DH-Cort) and H^3-corticosterone (Cort) incubations were separated by TLC on aluminum sheets pre-coated with silica gel containing a fluorescent indicator (Fisher). Re-constituted samples and non-radioactive steroids were spotted on TLC sheets and separated using chloroform/ethyl-acetate (60/40 % by vol.). Unlabeled steroids were purchased from Sigma. After steroid separation, 11-DH-Cort and Cort were visualized under UV light and pencil-marked. TLC plates were then scanned using a BioScan H^3 scanner (BioScan, Washington, DC). Radioactivity peaks were analyzed using WinScan software (BioScan). In parallel, quantification was also conducted by cutting the TLC aluminum sheet where 11-DH-Cort and Cort spots were located, and measuring radioactivity by scintillation counting. Data are presented as % radioactivity of initial substrate.

TLC Identification of Steroid hormone Metabolites: Products of tritiated steroid incubations were separated by TLC on silica gel containing a fluorescent indicator on pre-coated aluminum sheets (Fisher, Carlsbad, CA) using chloroform/ethyl-acetate/xylene (68/23/9 % by vol. for [^{3}H] DHEA) and (62/21/17 % by vol. for [^{3}H] AD). Non-radioactive steroids of known identity were added to the TLC sheets on lanes adjacent to the putative metabolites and were visualized and identified by their chromogenic properties after spraying with 5% (by vol.) sulphuric acid in methanol and heating on a hot plate. Unlabeled steroids used were 17β-estradiol (E2) and estrone (E1) (Sigma); and testosterone (T), androstenedione (AD), 5α-androstenedione (5αAD), dehydroepiandrosterone (DHEA), 5-androstenediol (Adiol), and dehydro-testosterone (DHT) (Steraloids Inc, New Port, RI). Purity of [^{3}H]-DHEA and [^{3}H]-AD (<98 %) was determined by TLC.

Immunofluorescence: 24hr, 48hr or 5 days after I.P. injection of LPS, mice were transcardially perfused with PBS, followed by 4% paraformaldehyde (PFA). Fixed brains were extracted, post-fixed overnight at 4°C in 4%PFA, and then stored at –20°C in cryoprotectant (sucrose %). Coronal sections (30M) were obtained using a Leica vibratome (Leica,). Sections were rinsed

in PBS and then washed 3x in TBS-Triton 1% (TBS-Tr), blocked in 3% goat serum for 1hr at room temperature, and then incubated overnight at 4°C in primary antibody dilutions in 3% BSA TBS-Tr: 11βHSD-1 (1:500); GFAP (1:1000); NeuN (1:1000). The following day, sections were washed 5x in TBS-Tr and then incubated for 1hr at room temperature with the appropriate Rhodamine-Red-X conjugated species-specific secondary antibodies. Sections were washed 5x in TBS-Tr and then rinsed in 0.1M PB before mounting on glass slides and cover-slipping with Dako aqueous fluorescent mounting media (Dako). Mounted sections were visualized by confocal microscopy using a Zeiss LSM confocal microscope. Z-stack image reconstructions and co-localization analysis were done using LSM software (Rockefeller University Bioimaging Facility) and images were labeled and marked in Adobe Photoshop (Adobe, San Jose, CA).

EtC.1 Transfection and Luciferase Assay: EtC.1 cells were seeded in 24-well plates, $2x10^4$ cells/well, in DMEM containing 10% charcoal stripped serum. 24hr later cells were transfected using Lipofectamine Plus (Invitrogen), following manufacturer's instructions, with 0.4μg of plasmid DNA/well. The 3X ERE-Luciferase plasmid was a generous gift of Don McDonnell

(Duke University Durham, NC), and the β-Galactosidase plasmid was from Promega (pSV-β-Gal control vector). 24hr after transfection, cells were incubated with various concentrations of E2 or Adiol for another 24hr. ICI pre-treatment was done for 30 minutes. Cell lysates were prepared and luciferase activity measured using Promega Luciferase Assay System according to manufacturer's instructions (Promega). β-Gal activity was measured from cell lysates to normalize for transfection efficiency.

Statistics: Statistical analysis was performed using StatView (SAS Institute Inc., Cary, NC). Experiments involving 2 groups were compared using a Student t-test. Experiments involving more than 2 groups were compared by Analysis of Variance (ANOVA), followed by posthoc analysis with Tukey-Kramer Honestly Significant Difference (HSD) when variances were homogeneous (using Equality of Variance F test); or with non-parametric Games-Howell test. Graphs show the mean ± the standard error of the mean (S.E.M.). $P<0.05$ was considered statistically significant. *, $p<0.05$, **, $p<0.01$ and ***, $p<0.0001$; (n.s.), non-significant.

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