CYTOKINE-MEDIATED REGULATION OF CATECHOLAMINE BIOSYNTHETIC ENZYMES IN ADRENAL CHROMAFFIN CELLS

by

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Abstract

The immune system is increasingly recognized for its role in the genesis and progression of hypertension. Catecholamines function in the neuro-hormonal regulation of blood pressure and have a well-established link to hypertension. It was hypothesized that cytokine signalling within the adrenal medulla influences catecholamine biosynthesis, thereby potentially impacting blood pressure. This study reports that IFN-α, IL-1β, IL-2, IL-6, IL-10, TNF-α, and CCL2 affected transcript levels of tyrosine hydroxylase, dopamine β-hydroxylase, and phenylethanolamine N-methyltransferase (PNMT) in PC12 cells. Further, simultaneous treatment with dexamethasone or forskolin produced context-dependent changes in the transcripts, some of which corresponded to alterations in PNMT promoter activation, but not to localization of glucocorticoid receptor. Thus, cytokines exert transcriptional control over catecholamine biosynthetic enzymes and cytokine signalling is integrated with hormonal and neural regulatory mechanisms in PC12 cells. Changes in adrenal cytokines during hypertension may promote blood pressure elevation by influencing catecholamine biosynthesis.

Keywords

Hypertension, Adrenal Medulla, Catecholamine, Immune, Cytokine, Glucocorticoid

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AADC	Aromatic amino acid decarboxylase
ACh	Acetylcholine
ACTH	Adrenocorticotropic hormone
Ang	Angiotensin
AP-1	Activator protein 1
AP-2	Activator protein 2
bp	Base pair
CA	Catecholamine
cAMP	Cyclic adenosine monophosphate
CBP	CREB-binding protein
CNS	Central nervous system
CRE	cAMP response element
CRH	Corticotropin-releasing hormone
CTM	Charcoal treated media
CVD	Cardiovascular disease
DA	Dopamine
DBH	Dopamine β-hydroxylase
DBP	Diastolic blood pressure
Dex	Dexamethasone
DMEM	Dulbecco's Modified Eagle's Medium

DNA	Deoxyribonucleic acid
DOCA	Deoxycorticosterone acetate
ECL	Enhanced chemiluminescence
Egr1	Early growth response 1
Epac	Exchange protein directly activated by cAMP
Epi	Epinephrine
ERK	Extracellular signal regulated kinases
Forsk	Forskolin
GC	Glucocorticoid
gp	Glycoprotein
GRE	Glucocorticoid response element
GRIP	Glucocorticoid receptor-interacting protein
HPA	Hypothalamic-pituitary-adrenal
HRP	Horseradish peroxidase
IFNAR	IFN-α receptor
IL-1R	IL-1 receptor
IL-6R	IL-6 receptor
IRF	Interferon regulatory factor
JAK	Janus kinase
LB	Luria Bertani
LDCV	Large dense core vesicle
L-DOPA	L-3,4-dihydroxyphenylalanine
MAPK	Mitogen-activated protein kinase

mRNA	Messenger ribonucleic acid
NE	Norepinephrine
NO	Nitric oxide
PACAP	Pituitary adenylate cyclase-activating peptide
PKA	Protein kinase A
PKC	Protein kinase C
PLC	Phospholipase C
PNMT	Phenylethanolamine N-methyltransferase
RAAS	Renin-angiotensin-aldosterone system
rpm	Revolutions per minute
RT-qPCR	Quantitative reverse transcription polymerase chain reaction
SA	Sympathetic-adrenal
SBP	Systolic blood pressure
SEM	Standard error of the mean
Ser	Serine
SHR	Spontaneously hypertensive rat
SNS	Sympathetic nervous system
Sp1	Specificity protein 1
SSV	Small synaptic vesicle
STAT	Signal transducer and activator of transcription
TH	Tyrosine hydroxylase
TNFR	TNF receptor

1 - Introduction

1.1 - Hypertension and the Roles of Catecholamines and Inflammation

1.1.1 - Hypertension

Approximately one in five Canadian adults live with hypertension (Wilkins et al., 2010). Hypertension is defined as chronically elevated arterial blood pressure that is greater than or equal to 140 mm Hg mean systolic blood pressure (SBP) or 90 mm Hg mean diastolic blood pressure (DBP). Elevated blood pressure can cause changes in arterial structure that can increase risk of stroke, heart disease, kidney failure, and other diseases (World Health Organization, 2009). Hypertension is a leading cause of renal failure, second only to diabetes (Rosamond et al., 2008). Globally, high blood pressure is attributed to causing 51% of stroke and 45% of coronary heart disease (World Health Organization, 2009). The significant health risks associated with hypertension have made it the world's leading risk factor for death, estimated as the cause of 13.5% of premature deaths (Lawes et al., 2008). A majority of adults in both developing and developed countries have blood pressure that is higher than is optimal and the risk of dying from high blood pressure is particularly great in lower income countries (Wilkins et al., 2010; World Health Organization, 2009). Globally, the cost attributed to blood pressure above optimal levels, including both prehypertension and hypertension, is estimated at US$370 billion, roughly 10% of money spent on healthcare (Gaziano et al., 2009). Most recent estimates affirm that the treatment of hypertension is one of the most cost-effective approaches available for increasing quality-adjusted life-years and decreasing preventable deaths (Myers, 2007).

Hypertension can be classified into the categories of essential (or primary) and non-essential (or secondary) hypertension. Hypertension is diagnosed as essential when there is no discernable underlying cause. Essential hypertension is often attributed to a combination of genetic and environmental factors. Non-essential hypertension is directly linked to a pre-existing medical condition such as sleep apnea, kidney damage or diseases that consist of abnormal hormone production. Only a small minority (5-10%) of hypertension diagnoses are classified as non-essential, leaving the remaining majority (90-95%) of diagnosis to be classified as essential hypertension (Rimoldi et al., 2014).

Like asthma, obesity, diabetes, and a multitude of other pathophysiological conditions, essential hypertension is a multigenic disease (see Fig. 1) that is highly influenced by environmental factors. Multigenic traits involve multiple genes and do not have a single recognizable pattern of inheritance as do single-locus Mendelian traits. Only a small proportion of cases of hypertension are directly caused by individual alleles which show distinct inheritance patterns within families. Most common genetic disorders arise as a complex interplay of intrinsic, extrinsic, and behavioral factors which together result in disease (O'Shaughnessy, 2001). Recent estimates of hypertension awareness and control have demonstrated increased proportions of people who are aware of their condition, who receive treatment, and who have controlled their blood pressure with medication (Wilkins et al., 2010). However, despite increased awareness and an abundance of available interventions, data from the 2007-2009 Canadian Health Measures Survey indicates that hypertension remains uncontrolled in 34% of Canadians (Wilkins et al., 2010).

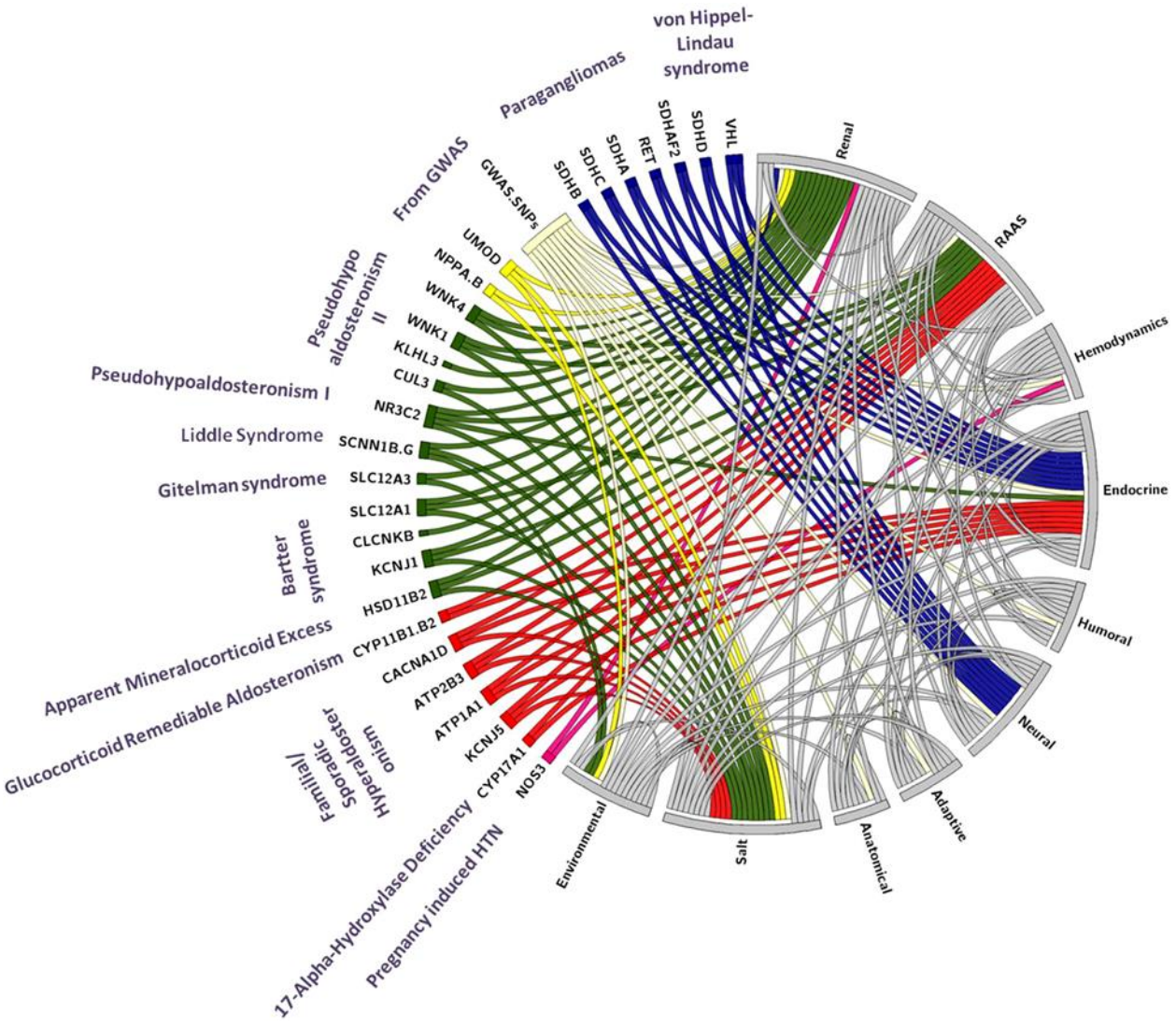

Figure 1: Genetic complexity of blood pressure regulation. Hypertension is multigenic and can be caused by disturbances in multiple physiological systems. Above is an updated version of the 1960 Paige mosaic model of blood pressure regulation, adapted from Padmanabha *et al.*, 2015. This model highlights some of the important factors identified from genetic studies, primarily from studies of monogenic forms of hypertension, and groups specific genes into broader physiological categories.

1.1.2 - Regulation of Blood Pressure

Currently there is a panoply of treatments available for reducing blood pressure and combating hypertension. This is, in part, due to the numerous physiological parameters that influence blood pressure and that are accessible targets for treatment. Blood pressure is the product of cardiac output and total systemic vascular resistance. These variables are dependent on parameters such as blood volume, extracellular fluid volume, arterial and venous compliance, and resistance to venous return (see Fig. 2). Changes in the structure and function of kidneys, blood vessels, and the heart are regulated by neuroendocrine feedback mechanisms and serve to control blood volume and vascular resistance (Coffman, 2011). Antihypertensive therapies must necessarily act upon the physiological mechanisms that control cardiac output and vascular resistance in order to effectively regulate blood pressure.

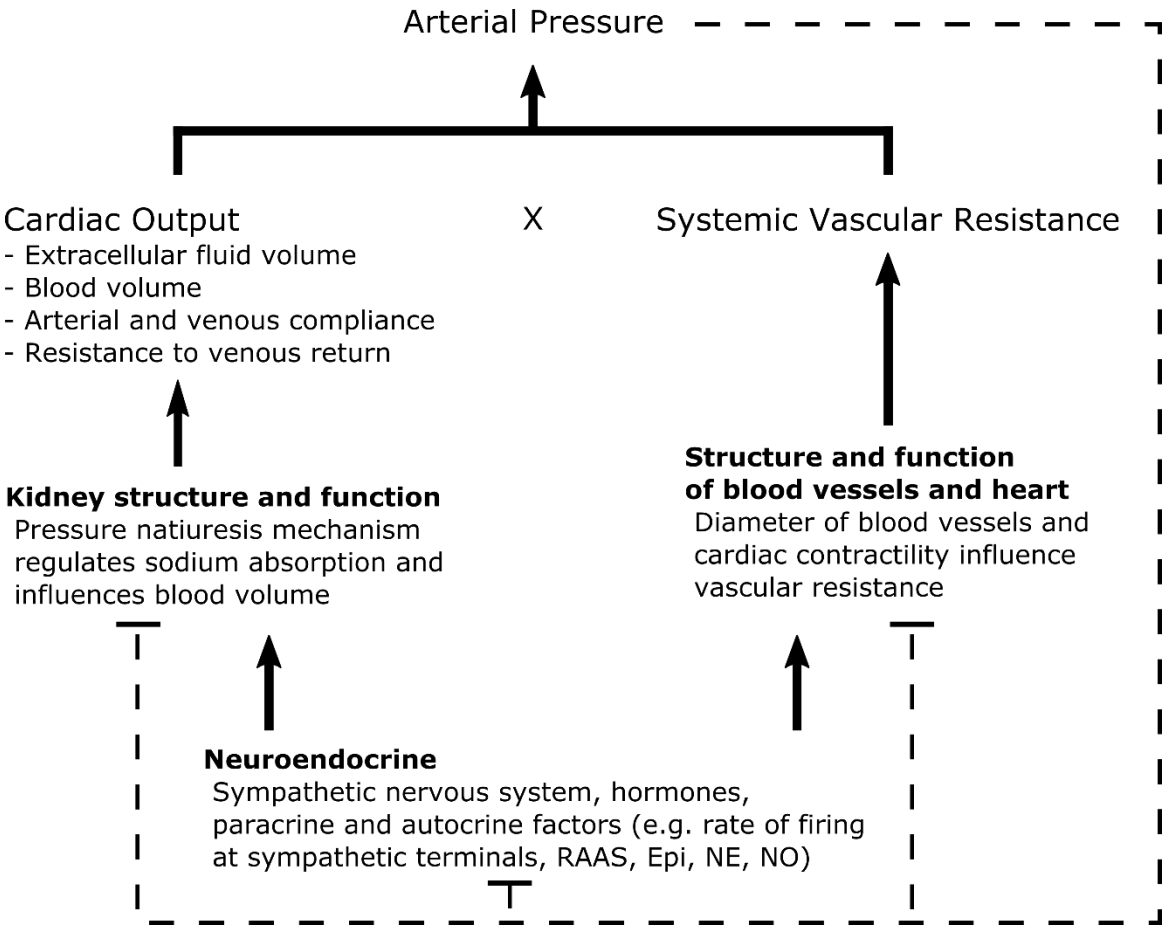

Figure 2: Schematic of the general mechanisms for blood pressure regulation. Arterial pressure is the product of cardiac output and systemic vascular resistance. This dynamic system is regulated by neural, hormonal, paracrine, and autocrine factors through changes in parameters such as vascular diameter, microvessel density, blood volume, etc. Control of fluid volume by the kidneys is an important means of regulating arterial pressure. Dashed lines depict negative feedback pathways important for the maintenance of homeostasis. For a comprehensive review, see Cowley (2006). RAAS = Renin-Angiotensin-Aldosterone System; NO = Nitric Oxide.

There are multiple drugs available for treating hypertension and they fall into 3 general categories: diuretics (including thiazides, loop diuretics, aldosterone blockers, and potassium sparers), adrenergic inhibitors (including peripheral inhibitors, β-blockers, central α_2-agonists, α_1-blockers, and combined α- β- blockers), and vasodilators (including direct vasodilators, angiotensin-converting enzyme [ACE] inhibitors, calcium channel blockers, and angiotensin [Ang] II receptor blockers) (Kaplan et al., 2010). Further, recent efforts have been made to develop surgical interventions for treating hypertension when other approaches prove inadequate. These include renal sympathetic denervation and the implantation of devices which electrically stimulate activation of the carotid baroreflex (Krum et al., 2014; Lohmeier & Iliescu, 2011). Changes in lifestyle, when possible, are also effective measures for treating hypertension and are recommended in current treatment guidelines by expert committees (Hypertension Canada, 2015; James et al., 2014; Krause et al., 2011; Mancia et al., 2013). Some of these lifestyle changes include the consumption of a healthy diet, engaging in regular physical activity, minimization of alcohol consumption, maintenance of healthy body weight (BMI 18.5-24.9 kg/m^2), maintenance of a moderate waist circumference (<102 cm for men, <88 cm for women), moderate sodium intake (<2000 mg/day), and living in a smoke-free environment. Treatment recommendations for hypertension generally include a combination of antihypertensive drugs and appropriate lifestyle modifications, together with careful self-monitoring (Hypertension Canada, 2015).

1.1.3 - Hypertension and Catecholamine Biosynthesis

Adrenergic inhibitors are among the most useful pharmacologic tools for treating hypertension. Adrenergic signalling via α- and β-adrenergic receptors can influence cardiac output and peripheral resistance. The catecholamines (CAs) dopamine (DA), norepinephrine (NE), and epinephrine (Epi) are involved in the regulation of the cardiovascular system. Because they are produced by neuroendocrine cells and have dual hormone and neurotransmitter functions, these CAs are classified both as neurotransmitters and/or neurohormones.

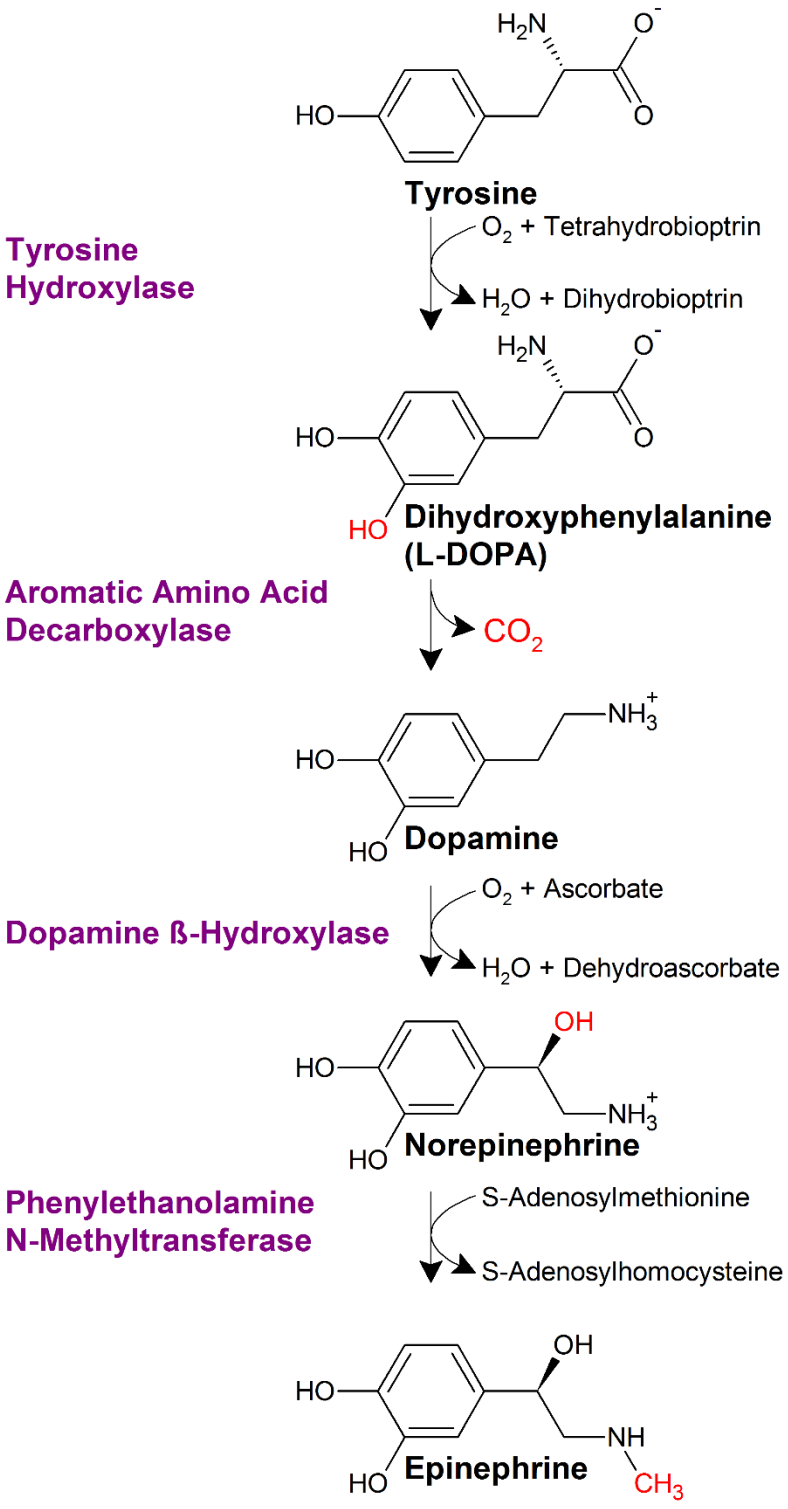

Figure 3: Catecholamine biosynthetic pathway. L-tyrosine is converted to L-DOPA by tyrosine hydroxylase, L-DOPA is converted to dopamine by aromatic amino acid decarboxylase, dopamine is converted to norepinephrine by dopamine β-hydroxylase, and norepinephrine is converted to epinephrine by phenylethanolamine N-methyltransferase.

The biosynthesis of CAs begins with the hydroxylation of tyrosine by the enzyme tyrosine hydroxylase (TH), producing L-3,4-dihydroxyphenylalanine (L-DOPA) (see Fig. 3) (Nagatsu et al., 1964). Next, L-DOPA is decarboxylated by the enzyme by L-aromatic amino acid decarboxylase (AADC), converting it to DA (Christenson et al., 1972). DA is then hydroxylated to produce NE; a reaction catalyzed by dopamine β-hydroxylase (DBH) (Weinshilboum & Axelrod, 1971). This sequence of reactions which converts tyrosine to NE is common in sympathetic and some brain neurons. In adrenal chromaffin cells one additional biosynthetic step occurs consisting of the methylation of NE by phenylethanolamine N-methyltransferase (PNMT) to produce Epi (Axelrod, 1962). Epi is the major secretory product of the adrenal gland, which is also the most abundant source of secreted Epi in the body (Hwang et al., 1994; Wong & Tank, 2007). Apart from being produced by the adrenal gland, DA and NE have important functions as neurotransmitters in the central and peripheral nervous systems. CAs in the adrenal medulla are sequestered in CA storage vesicles of chromaffin cells. When stimulated, chromaffin cells release CAs from their vesicles through Ca^{+2}-mediated exocytosis (Douglas, 1968; Smith et al., 1973).

Once released into circulation, CAs can interact with numerous adrenergic receptor types expressed in a variety of tissues. All CA receptors are G protein-coupled receptors (Caron & Lefkowitz, 1993). Adrenergic receptor subtypes include α_1-, α_2-, β_1-, β_2-, and β_3-adrenergic receptors, some of which can be divided into further subtypes. Adrenergic receptors are activated by the CAs Epi and NE, with each receptor having a distinct affinity for each ligand. Like the adrenergic receptors, there are multiple forms of DA receptor, and they can be categorized in at least five ($D_{1\text{-}5}$) different subtypes. Through these receptors, CAs can signal to numerous tissues throughout the body to produce a wide and coordinated

physiological response. The distribution and function of DA receptors suggests that DA may decrease BP by synergistically enhancing vasodilation, inhibiting synaptic NE release, decreasing circulating CAs, inhibiting aldosterone secretion and inhibiting sodium reabsorption in the kidney (Carey, 2001; Missale et al., 1998). β-adrenergic receptors are expressed in airway smooth muscle, epithelium, endothelium, immunocytes, and myocardium (Johnson, 2006). In cardiac tissue, although all three types are present, β_1-adrenergic receptors are the major β-adrenoceptor type expressed. β_1- and β_2-adrenoceptor-mediated actions in the heart include positive inotropic (increased contractility), chronotropic (increased heart rate), dromotropic (increased conductivity), and bathmotropic (increased threshold of excitation) effects (Adameova et al., 2009). β_3-adrenoceptors require higher concentrations of CAs for activation than other β-adrenoceptors, and β_3-adenoceptor signalling is suggested to counteract effects of β_1- and β_2-adrenoceptor activation, thus mediating a protective feedback loop to prevent adrenergic overstimulation. The α-adrenoceptors are important for the maintenance of vascular tone as well as promoting smooth muscle contraction in other parts of the body. Sympathetic stimulation of α_1-adrenoceptors is a major mechanism for sympathetic-mediated vasoconstriction (Guimarães & Moura, 2001).

Studies have shown elevated plasma levels of Epi and NE in patients with essential hypertension as well as in animal models of hypertension (Axelrod, 1976; Borkowski & Quinn, 1984; Bühler et al., 1982; Goldstein, 1983; Jablonskis & Howe, 1994). CA production is dependent upon and correlated with the activity of the CA biosynthetic enzymes (Axelrod, 1976). Most studies have focused on three key CA-synthesizing enzymes: TH, DBH, and PNMT. Relative to TH, DBH, and PNMT, AADC activity is

typically high and is not rate limiting under normal physiological conditions (Berry et al., 1996; Bowsher & Henry, 1986). In adrenal chromaffin cells, the expression of AADC is regulated to a lesser degree than the other CA biosynthetic enzymes (Kvetnansky et al., 1971, 1970; Thoenen, 1972). Control of CA biosynthesis occurs primarily through the enzymes TH, DBH, and PNMT. Regulation of TH, DBH, and PNMT is achieved through both transcriptional and post-transcriptional mechanisms (Hwang & Joh, 1993; Tai & Wong, 2003; Tekin et al., 2014; Unsworth et al., 1999). Transcript levels of TH and PNMT, and activities of TH and DBH are enhanced in the adrenal medullas of genetically hypertensive rat models (Grobecker et al., 1982; Nagatsu et al., 1977; Nguyen et al., 2009; Reja et al., 2002). Further, PNMT is one of the putative gene loci linked to hypertension in genetic studies (Hoehe et al., 1992; Kaneda et al., 1988; Koike et al., 1995). Prolonged elevation of plasma CA levels can contribute to cardiac dysfunction by the over activation of vascular smooth muscle cells, resulting in ischemia and functional hypoxia; and oxidative damage (through the formation of oxidized CAs and oxygen free radicals), resulting in ultrastructural changes and cellular damage in cardiomyocytes (Adameova et al., 2009). Oxidative damage may also lead to immune activation that contributes to the further progression cardiovascular dysfunction (*vide infra*). Taken together, the molecular mechanisms that regulate the CA biosynthetic enzymes and their activities are of interest for the development of interventions for controlling blood pressure and preventing hypertension-associated disease.

1.1.4 - Hypertension and Inflammation

The role of inflammation in the genesis of hypertension and accompanying organ damage is well established (Coffman, 2011). Inflammation is one of the most important factors contributing to cardiovascular risk; and it is a major part of the formation, progression and destabilization of atherosclerotic lesions (Hansson & Libby, 2006; Hollan et al., 2013; Libby, 2006). The link between immune and cardiovascular function is apparent in major immune diseases including rheumatic diseases, HIV, and psoriasis. Cardiovascular pathologies are the leading cause of premature mortality in patients with autoimmune rheumatic diseases (Zinger et al., 2009). Individuals with HIV infection have higher risk of cardiovascular disease (CVD), arterial stiffness, systolic and pulse pressures than matching uninfected individuals (Schillaci et al., 2013). A recent meta-analysis of observational studies concluded that psoriasis, a chronic inflammatory skin condition, is associated with increased prevalence and incidence of hypertension and that odds of hypertension increase with severity of psoriasis (Armstrong et al., 2013). Inflammation is an essential component of many diseases, and the connections between inflammation, hypertension, and CVD add support to its role in cardiovascular pathology.

A prospective cohort study of 20,525 women concluded that high plasma levels of the inflammatory biomarker C-reactive protein are predictive for the development of hypertension (Sesso et al., 2003). Several studies have supported immune involvement in the elevated blood pressure of spontaneously hypertensive rats (SHR). Purcell and Gattone (1992) found that young SHR have an elevated rate of nerve growth into the thymus, a primary lymphoid organ important in T-cell development, compared to their normotensive

Wistar Kyoto counterparts. Others have found that treatment of SHR with antithymocyte serum or with the immunosuppressant cyclophosohamide lowers blood pressure (Bendich et al., 1981; Dzielak, 1991). Later studies established the role of the adaptive immune response in hypertension after finding that mice with a genetic deletion in recombinase-activating protein (RAG-1 -/-), which lack T- and B-lymphocytes, experience blunted hypertension in response to both Ang II and deoxycorticosterone acetate (DOCA)-salt; adoptive transfer of T-cells restored the elevation in BP (Guzik et al., 2007). This study also identified the role of the cytokine TNF-α in BP elevation when mice treated with Ang II responded with both increased BP and increased production of TNF-α from T-cells; anti-TNF-α therapy with etanercept (a TNF-α inhibitor) blunted Ang II-mediated elevations in BP (Guzik et al., 2007). Taken together, these studies suggest the potential for enhanced neural activation of T cells in hypertension as well as a functional importance of cytokine signalling in blood pressure regulation. Also supporting the role of cytokines in hypertension, multiple studies have identified altered profiles of pro- and anti-inflammatory cytokines and cytokine production capacity in humans when comparing hypertensive or prehypertensive patients to control subjects (Chrysohoou et al., 2004; Peeters et al., 2001; Stumpf et al., 2005, 2011). Although results are sometimes conflicting, these studies implicate cytokines such as IL-1, IL-6, IL-4, IL-7, IL-13, TNF-α, and CCL2 in human hypertension. Other cytokines not analyzed or not changed in circulating concentration may also be important in hypertension because of the potential for local regulatory effects at important centers for blood pressure regulation.

Introduction of exogenous cytokine has been reported to induce changes in blood pressure. The cytokines IL-2 and IL-10 predominantly decrease blood pressure whereas the cytokine

IL-6 predominantly increases blood pressure. Other cytokines, such as TNF-α and IL-1, appear to have more intricate effects in relation to blood pressure regulation. In a screening experiment for changes in chemokine expression in DOCA/salt-induced hypertensive mice, transcript of the chemokine CCL2 and its receptor CCR2 were increased in aortas after the onset of hypertension. Treatment of mice with the CCR2 antagonist, INCB3344, substantially reduced DOCA/salt-induced infiltration of macrophages in aortic wall and DOCA/salt-induced elevations in blood pressure (Chan et al., 2012). Regulation of blood pressure by cytokines may be mediated by signalling to neural control centers or by direct actions on peripheral tissues. IL-1 can modulate blood pressure by influencing activity of neurohormonal control centers in the brain (McCann et al., 2000; Shi et al., 2010; Weidenfeld et al., 1989; Wilson et al., 1996). IL-1 can also modify vascular reactivity to NE (Baudry et al., 1996). Human cancer patients who receive high doses of IL-2 demonstrate hemodynamic changes which suggest decreased peripheral resistance but increased cardiac output, with an overall reduction in mean arterial pressure (Gaynor et al., 1988; Quan et al., 2005). Similar hemodynamic changes and hypotension are observed in experimental animals (Samlowski et al., 2003; Zeilender et al., 1989). Interestingly, IL-2 can decrease circulating Epi in mice, and reversal of IL-2-induced hypotension is associated with a rise in circulating CAs (Samlowski et al., 2003). Like IL-2, experimentally-induced elevations in IL-10 levels are associated with decreases in blood pressure in animal models (Shi et al., 2010; Tinsley et al., 2010; Viel et al., 2010). IL-2 and other cytokines may reduce CA-mediated vasoconstriction by enhancing superoxide production, a molecule that can interact with CAs and inhibit their signalling function (Faggioni et al., 1994; Macarthur et al., 2000; Suzuki et al., 1990). In contrast to many other cytokines, IL-6 appears to increase blood pressure. In a large cohort study of healthy men, after controlling for age and other

cardiac risk factors, baseline plasma IL-6 levels were positively associated with increased SBP, DBP, pulse pressure, and mean arterial pressure (Chae et al., 2001). In animal experiments, IL-6 is shown to contribute to Ang II-induced, but not salt-sensitive hypertension (Brands et al., 2010; Lee et al., 2006; Schrader et al., 2007; Sturgis et al., 2009). Pro-hypertensive effects of IL-6 may be mediated, in part, by vasoconstrictive effects of the cytokine that could result in increased peripheral resistance (Baudry et al., 1996; Iversen et al., 1999). The major proinflammatory cytokine TNF-α can induce hypotension; however, inhibition of TNF with etanercept can prevent elevations in blood pressure caused by Ang II infusion, high-fructose feeding, and chronic inflammation in animal models (Feinberg et al., 1988; Guzik et al., 2007; Saks & Rosenblum, 1992; Tran et al., 2009; Venegas-Pont et al., 2010). TNF-α inhibition does not influence blood pressure in salt-dependent hypertension (Elmarakby et al., 2008). TNF-α can directly modulate arterial tone, and TNF-mediated signalling is involved in cardiac remodelling (Baudry et al., 1996; Iversen et al., 1999). Prenatal exposure to elevated levels of cytokines may also contribute to hypertension during adulthood (Samuelsson et al., 2006). This may involve morphological changes in tissues, as suggested by findings of increased innervation by adrenergic nerve fibers in human thymus after IFN-α therapy (Cavallotti et al., 2002). The blood pressure-regulatory effects of cytokines are complex, affecting multiple physiological systems in combination with hormones, neurotransmitters, and other signalling molecules. Future investigations into the integration of cytokine signalling with the other blood pressure regulatory systems will provide a better understanding of the events responsible for blood pressure dysregulation in disease.

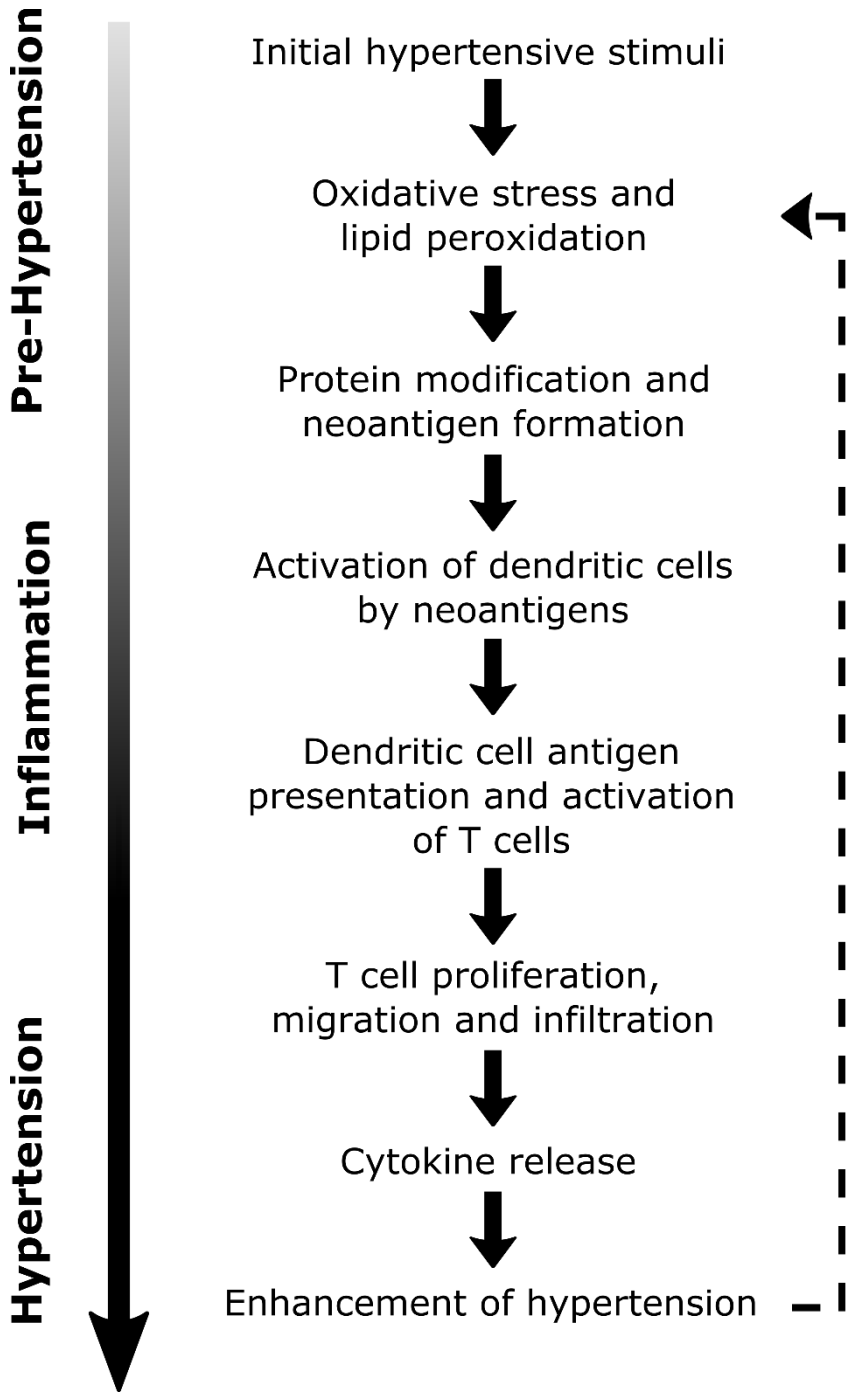

Figure 4: Possible inflammatory processes contributing to the progression of hypertension. Dashed line illustrates the positive feedback loop that may lead to further elevations in blood pressure.

In describing a new model for how inflammation and hypertension interact, Harrison *et al.* (2011) hypothesized that modest elevations in BP (to values of SBP ~135-140 mm Hg), such as in prehypertension, largely caused by activity of the CNS, trigger immune changes that lead to hypertension (see Fig. 4). In this model, initial elevations in BP are responsible for neoantigen formation from oxidation and altered mechanical forces in vasculature. Neoantigens then induce inflammatory responses in the kidneys, blood vessels and other tissues, where lymphocyte infiltration and expression of inflammatory mediators such as CCL2, IL-1, IL-6, IL-17, and TNF-α is increased (Blasi et al., 2003; Crowley et al., 2008; Guzik et al., 2007; Madhur et al., 2010). These cytokines and inflammatory mediators work in concert with CAs and other BP-elevating hormones leading to vascular and renal dysfunction and initiating a more severe hypertensive state (Harrison et al., 2011). This feed-forward model described by Harrison *et al.* (2011) was foretelling of recent findings by Kirabo *et al.* (2014), whose work outlined a mechanism for hypertension based on an autoimmune-like reaction. In this mechanism, initial increases in BP lead to oxidative stress and lipid peroxidation which results in neoantigen formation, immune cell activation, and initiation of T-cell proliferation and cytokine production, leading to further increases in BP. With the support of these and other findings, a new paradigm is being established that implicates inflammation in the elevation of BP and progression of hypertension.

1.2 - The Major Mechanisms of Adrenal Medullary Regulation

The adrenal gland is a key organ involved in the physiological adaptation to stress. The "fight-or-flight" response, first described by Cannon in the early 20th century, is characterized by increased blood pressure, increased heart rate, increased cardiac output, and changes in vascular and respiratory smooth muscle tone (Cannon & La Paz, 1911; Curtis & O'Keefe, 2002). The two major hormones secreted into circulation that facilitate the physiological stress response include cortisol and Epi, both being primarily products of the adrenal cortex and medulla respectively (Wong & Tank, 2007). There are two major effector circuits which are activated when the CNS perceives or anticipates a stress. They include the hypothalamic-pituitary-adrenal (HPA) axis, which stimulates the adrenal medulla through a hormonal mechanism, and the sympathetic-adrenal (SA) axis, which stimulates the adrenal medulla through a neural mechanism (Wong, 2006). These axes are in many ways physically distinct but they also have overlapping CNS components and physiological functions. Initiation of the physiological stress response, involving either HPA or SA axis, is primarily derived from structures of the limbic system. Termination of the stress response, caused by hormonal and neural feedback, also involves many of these same limbic structures. Integration of hormonal and neural signalling cascades allows the HPA and SA axis to function cooperatively while also tailoring individual responses to the specific initiating stimuli (Ulrich-Lai & Herman, 2009).

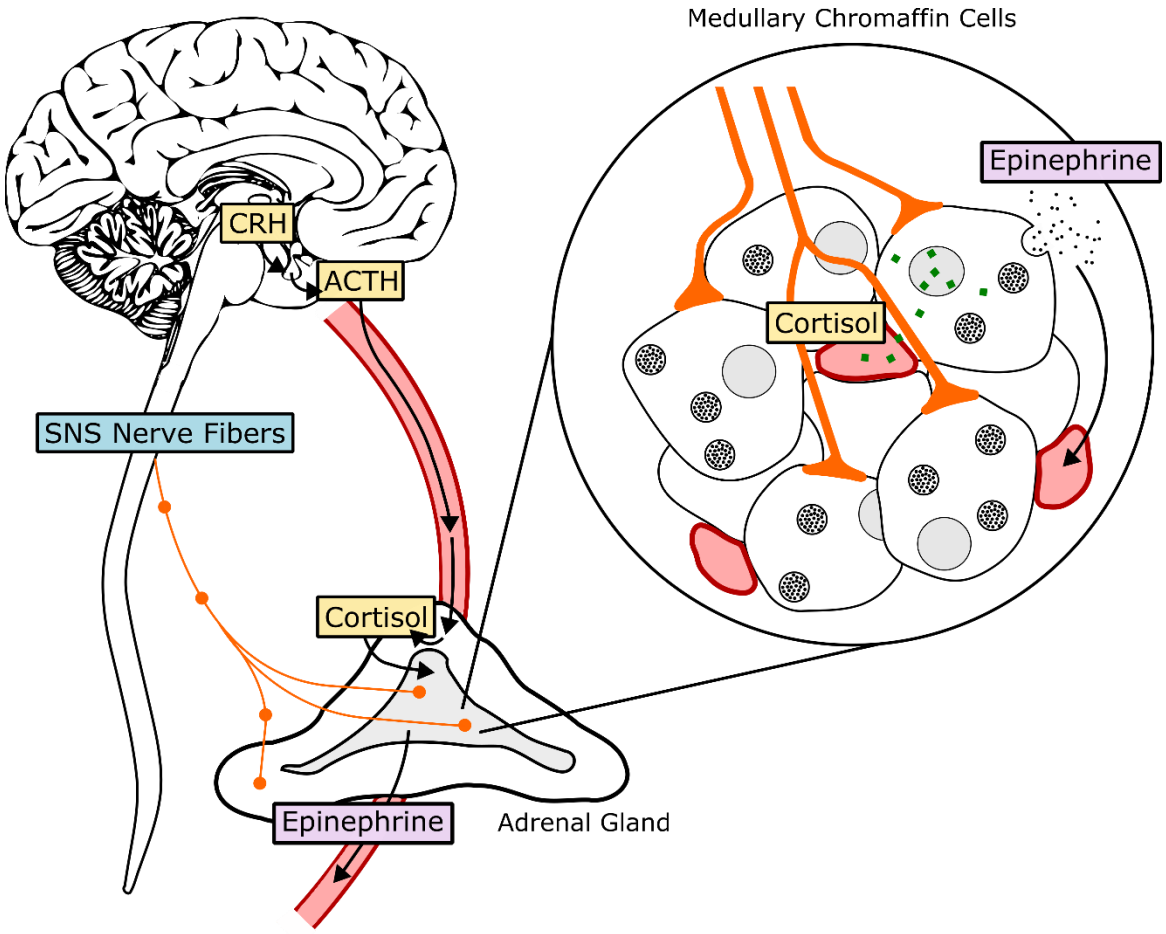

Figure 5: Hormonal and neural mechanisms regulating adrenal medullary chromaffin cells. The HPA-axis, comprised of the hormones corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH), and cortisol, is shown in yellow. The SA-axis, comprised of afferent preganglionic sympathetic nervous system (SNS) fibers, is shown in blue. Green squares represent glucocorticoid (cortisol) produced in the adrenal cortex and traveling to the adrenal medulla through vasculature. Neurotransmitters, including acetylcholine and pituitary adenylate cyclase-activating peptide are released from synaptic terminals. Both glucocorticoids and sympathetic input stimulate release of catecholamines, primarily epinephrine, from chromaffin cells by exocytosis. Epinephrine then enters into systemic circulation and travels to target tissues throughout the body.

1.2.1 - Hypothalamic-Pituitary-Adrenal Axis

The HPA axis consists of the paraventricular nucleus of the hypothalamus, the anterior pituitary gland and the adrenal gland (Smith & Vale, 2006). The HPA axis is activated when afferent neurons from multiple brain regions stimulate hypophysiotrophic neurons of the paraventricular nucleus and induce them to release corticotropin-releasing hormone (CRH) and vasopressin (Fig. 5). CRH and vasopressin then travel through hypophysial portal vessels to the anterior pituitary. CRH binds to receptors on pituitary corticotropes and promotes the secretion of adrenocorticotrophic hormone (ACTH) into systemic circulation. In the presence of CRH, vasopression has a synergistic effect, enhancing secretion of ACTH into circulation. ACTH then travels to parenchymal cells of the adrenocortical zona fasciculata, where it binds to plasma membrane receptors and initiates a rapid increase in the production and secretion of glucocorticoids (GCs). Once in systemic circulation, GCs bind to ubiquitously expressed intracellular glucocorticoid receptors (GRs) to induce physiological adaptations to the initial stressor. An intra-adrenal portal vascular system allows the exposure of adrenal medullary cells to especially high concentrations of GCs released from the adrenal cortex (Wurtman, 2002). GCs produce their cellular effects primarily by regulating transcription. Endogenous cortisol (corticosterone in rodents) is a lipid-soluble steroid hormone that binds to the cytoplasmic GR. Prior to ligand binding, GR is located in the cytoplasm as a multiprotein complex (Nicolaides et al., 2010). HSP90, one of the proteins in this complex, maintains the cytoplasmic retention of GR until binding of a ligand to GR causes dissociation from the complex and translocation into the nucleus. GR then homodimerizes and binds to glucocorticoid response elements (GRE) in the promoter regions of target genes directly,

or interacts with other transcription factor proteins causing transactivation or transrepression (Nicolaides et al., 2010). GCs are important regulators of homeostasis during basal conditions and during stress. They are critical for regulating cardiovascular, immune, metabolic, developmental, and reproductive processes (Smith & Vale, 2006). For example, the strong influence of GCs on immune function has allowed them to become some of the most commonly used compounds for therapeutic treatment of inflammatory, autoimmune, and lymphoproliferative disorders. In human leukocytes, approximately 20% of genes are regulated either positively or negatively by GCs (Galon et al., 2002). The effects of GCs on cardiovascular regulation are also important. Cortisol is a regulator of blood pressure in humans and can lead to hypertension when in excess (Kelly et al., 1998).

One way that GCs can influence blood pressure is by influencing CA production. GCs directly increase the release of CAs by sympathetic nerves and adrenal medullary cells (Pilipović et al., 2012; Sharara-Chami et al., 2010). To compensate for increased secretion of CAs, GCs must increase CA production by regulating CA biosynthetic enzymes. Early evidence of GC control over adrenal CA biosynthesis was demonstrated in experiments performed by Wurtman and Axelrod (1965), who reported that ablation of the pituitary gland decreases PNMT activity, which can be restored by addition of ACTH or GC (Wurtman & Axelrod, 1965). Later, *in vitro* and *in vivo* experiments confirmed that GCs are responsible for increasing PNMT mRNA expression (Evinger et al., 1992; Sharara-Chami et al., 2010; Wong et al., 1992), increasing the amount of functional intronless mRNA splice variant (Unsworth et al., 1999), increasing PNMT activity (Unsworth et al., 1999), and enhancing PNMT protein stability via regulation of the co-substrate S-adenosyl-methionine (Berenbeim et al., 1979; Wong et al., 1985, 1982) in adrenal chromaffin cells.

Studies in rat pheochromocytoma cells show that, in addition to PNMT, GCs regulate the other CA biosynthetic enzymes to produce parallel increases in their transcript level and activity (Kim et al., 1993b; Lewis et al., 1987, 1983; McMahon & Sabban, 1992; Tischler et al., 1983). Similar observations pertaining to regulation of enzyme transcript levels have also been made with primary cultures of bovine adrenal medullary cells; however, unlike rat pheochromocytoma cells, in bovine chromaffin cell primary cultures DBH transcript does not appear to be regulated by GC (Hwang & Joh, 1993; Stachowiak, Hong, et al., 1990).

Thus, GCs can increase transcript of TH, DBH, and PNMT. The site critical for GC responsiveness of the rat TH gene is located at about -5.7 kb and it closely resembles the activator protein 1 (AP-1) binding site (Rani et al., 2009). This finding is consistent with earlier observations the proximal promoter region (-773 to +27 bp) is not sufficient for GC regulation of the TH gene (Lewis et al., 1987, 1983). Another functional GRE has been identified at ~2.4 kb upstream in the mouse TH promoter (Hagerty et al., 2001). Several putative GREs have been identified in the first 1 kb of the upstream rat DBH gene, with corresponding sequences in the human DBH promoter (McMahon & Sabban, 1992). Although long exposure with GCs can increase transcript levels of DBH in PC12 cells, functionality of putative GREs in the DBH promoter has not yet been proven (McMahon & Sabban, 1992). In bovine chromaffin cells, GCs do not appear to regulate DBH transcription (Hwang & Joh, 1993). GCs are also important regulators of PNMT transcription (Wong et al., 1992). Three functional GREs have been identified in the proximal 1 kb rat PNMT promoter, and activation at these sites can be synergistically

regulated by the transcription factors early growth response 1 (Egr1) and activator protein 2 (AP-2) (Ross et al., 1990; Tai et al., 2002; Wong et al., 1998).

1.2.2 - Sympathetic-Adrenal Axis

Working alongside the HPA-axis, another effector circuit that signals the adrenal medulla to synthesize and secrete Epi, the SA-axis, consists of the direct innervation of adrenal medullary chromaffin cells by the sympathetic nervous system (Axelrod & Reisine, 1984). Stress signals, primarily originating from limbic structures, are transmitted to preganglionic sympathetic neurons in the intermediolateral cell column of the thoracolumbar spinal cord which project, via the splanchnic nerve, to adrenal chromaffin cells of the adrenal medulla (Ulrich-Lai & Herman, 2009). The projections of preganglionic neurons from the splanchnic nerve terminate with synapses on adrenal medullary chromaffin cells. The neural stimulus is delivered to each chromaffin cell by several synaptic boutons and compelling evidence now suggests that gap junctions also help to propagate electrochemical signals between neighboring cells (Colomer et al., 2012; Desarménien et al., 2013). A combination of neurotransmitters and neuropeptides are released from sympathetic nerve terminals and bind to plasma membrane receptors on chromaffin cells. These substances stimulate the release large amounts of stored CAs from chromaffin cell vesicles via Ca^{+2}-mediated exocytosis (Kvetnansky et al., 2009). Due to the direct innervations of adrenal chromaffin cells, the SA effector circuit has a short latency in comparison to excitation via the HPA axis, which is generally longer lasting and slower to respond (Droste et al., 2008). Stimulation of chromaffin cell activity by the SA axis may

contribute to hypertension through either an increase in sympathetic nerve firing or an unusually high sensitivity of chromaffin cells to sympathetic stimulation (Anderson et al., 1989; Fhaner et al., 2013; Schlaich et al., 2004; Segura-Chama et al., 2015).

Synaptic transmission at the SA synapse is mediated by the small molecule transmitter acetylcholine (ACh) and by neuroactive peptides. ACh is a transmitter for all preganglionic neurons of the autonomic nervous system and all postganglionic neurons of the parasympathetic nervous system (Kandel et al., 2012). The frequency of action potential firing at sympathetic nerve terminals influences the types of neurotransmitters released from the presynaptic nerve at the SA synapse. Stress is associated with high frequency splanchnic nerve firing, whereas basal sympathetic tone is characterized by lower frequency firing (Klevans & Gebber, 1970). In the preganglionic sympathetic nerves at the SA synapse, small synaptic vesicles (SSVs) contain ACh and large dense core vesicles (LDCVs) contain neuropeptides such as pituitary adenylate cyclase-activating peptide (PACAP). During high frequency firing both LDCVs and SSVs are released from the presynaptic nerve terminals. During basal conditions, only SSVs are released (Hökfelt et al., 2003). Both PACAP and ACh are integral at the SA synapse for promoting CA production and secretion (Wong, 2006). ACh is perhaps the best characterized molecule for synaptic transmission from the splanchnic nerve to the adrenal medulla. ACh binds to both nicotinic and muscarinic plasma membrane receptors on chromaffin cells. Cholinergic stimulation increases release of CAs from chromaffin cells (Evinger et al., 1994; Stachowiak, Hong, et al., 1990). Activation of nAChRs increases TH mRNA in chromaffin cells in a protein kinase A (PKA)-dependent manner (Gueorguiev et al., 1999; Hiremagalur et al., 1993; Stachowiak, Hong, et al., 1990). Cholinergic stimulation of chromaffin cells

also induces PNMT promoter-driven luciferase activity through a PKA-dependent mechanism (Wong et al., 2002). *In vitro* and *in vivo* evidence also supports the role of mAChRs in activating PNMT expression, via induction of the transcription factor Egr1 (Morita et al., 1996; Morita & Wong, 1996).

PACAP, primarily released from LDCVs during high frequency neuronal firing, is important for generating sustained increases in CA production by chromaffin cells (Wakade, 1988). PACAP is a ligand for the PAC1 receptor, which belongs to the subclass B1 GPCR. PAC1R signals through Gαs, which regulates adenylyl cyclase (Martin et al., 2005). Binding of PACAP to PAC1 can signal through the conventional cyclic adenosine monophosphate (cAMP)-PKA pathway and at least two other insulated, cAMP-sensitive signalling pathways involving the signal transduction proteins exchange protein directly activated by cAMP (Epac) and the extracellular signal regulated kinases (ERK) 1 and 2 (Emery & Eiden, 2012; Gerdin & Eiden, 2007; Kuri et al., 2009). The PAC1 receptor can also stimulate Gαq, which activates a phospholipase C (PLC)-protein kinase C (PKC) pathway (Yang et al., 2010). PACAP is capable of upregulating chromaffin cell expression of TH, DBH, and PNMT transcript (Stroth & Eiden, 2010; Tönshoff et al., 1997).

As mentioned above, signalling via cAMP is an important molecular mechanism induced by both ACh and PACAP, and is involved in the regulation of CA biosynthetic enzymes in adrenal chromaffin cells. In primary cultured bovine adrenomedullary chromaffin cells, cAMP signalling produces synchronized increases in both transcript and activity levels of TH, DBH, and PNMT (Hwang et al., 1994). Similar activation of the CA biosynthetic enzymes by cAMP signalling occurs in rat chromaffin cells (Cheng et al., 2008; Lewis et

al., 1987, 1983; McMahon & Sabban, 1992; Wong et al., 2002). It should be noted that in both rat and bovine models, the induction of PNMT by cAMP is relatively small compared to the induction of TH and DBH. Signalling by cAMP activates PKA and can lead to tissue-specific induction of other signalling pathways. For example, in PC12 cells, PACAP activates PKA signalling as well as signalling through the mitogen-activated protein kinases (MAPKs) p38 and ERK1/2 via a PKA dependent mechanism (Hansen et al., 2000; Vaudry et al., 2009). Signalling by ERK1/2, downstream of PKA activation, may contribute to PNMT transcriptional activation (Tai et al., 2010). The promoters of TH, DBH, and PNMT all contain motifs that can bind a number of common transcription factors. The transcription factors specificity protein 1 (Sp1), AP-2, and Egr1 all have functional consensus sequences in the promoters of TH, DBH, and PNMT rat genes. AP-1 and CRE motifs are also present within the rat TH and DBH promoters (Cheng et al., 2008; Kvetnansky et al., 2009). Induction of Egr1 in rat chromaffin cells occurs through a cAMP/PKA signalling mechanism (Ginty et al., 1991; Tai et al., 2001). In rat chromaffin cells, Egr1 regulates transcription of TH, DBH, and PNMT (Cheng et al., 2008; Ebert et al., 1994; Papanikolaou & Sabban, 2000). Splanchnic nerve activation, the cholinergic agonists nicotine and muscarine, and the neuropeptide PACAP are all inducers of Egr1 (Morita et al., 1996; Wong et al., 2002). Transcriptional activation of TH and DBH also occurs through cAMP induction of transcription factor binding to AP-1 and CRE motifs (Kim et al., 1993a; Lim et al., 2000; Stachowiak, Goc, et al., 1990; Swanson et al., 1998). Other transcription factors potentially involved in the activation of CA biosynthetic enzymes by neural stimuli include Sp1 and AP-2 (Tai et al., 2010; Wong et al., 2002). Taken together, these studies suggest that the neuronal regulation of chromaffin cells

involves a number of transcription factors which can act individually or cooperatively to regulate expression of the enzymes responsible for CA biosynthesis.

1.3 - Cytokine-Mediated Regulation of Catecholamine Biosynthesis

Investigations into the potential role of cytokines in regulating CA production by the adrenal gland were, in part, inspired by insights gained from studying depression. Depression can be induced by alterations in NE and other neurotransmitter levels, and sympathetic hyperactivity is a well characterized component of the condition (Raison et al., 2006). It has also been reported that a large proportion of patients receiving IFN-α therapy for treatment of cancer or infectious disease develop a behavioural syndrome that is very similar to major depression (Raison et al., 2006). This finding led to questions about the influence of cytokines on neurotransmitter production and the role of cytokines in regulating neural activity. Interestingly, depression is now associated both with elevations in plasma levels of proinflammatory cytokines and increased risk of hypertension, cardiovascular morbidity and mortality (Dowlati et al., 2010; Meng et al., 2012; Serrano et al., 2011). Although the causal relationships are not yet resolved, possible influences of inflammatory mediators such as cytokines on catecholaminergic cell function are now being investigated for their contribution to hypertension and CVD.

In humans, treatment with IFN-α increases circulating levels of NE and Epi (Corssmit et al., 1996; Pende et al., 1990). Both intravenous and intracerebroventricular administration

of IL-1 to rats has been reported to increase plasma levels of NE and Epi, along with increased renal sympathetic nerve activity, SBP and heart rate (Kannan et al., 1996; Rivier et al., 1989). Central administration of IL-1 to rats has also been reported to increase ACTH secretion (Hashimoto et al., 1993). These findings suggest that IL-1 can activate HPA and SA axes by direct stimulation of regulatory centers within the brain. In humans, peripheral administration of IL-6 increases plasma cortisol and NE but does not affect plasma Epi levels (van Hall et al., 2003; Steensberg et al., 2003; Stouthard et al., 1995; Torpy et al., 2000). Studies have suggested that peripherally, but not centrally administered, TNF-α elevates plasma CA levels in rats (Darling et al., 1989; De Laurentiis et al., 2002). Increased expression of IL-10 in the brain can inhibit elevations in plasma NE resulting from myocardial infarction in rats (Yu et al., 2007). Numerous cytokines, including IFNs, IL-1, IL-2, IL-6, and TNF-α induce changes in brain CA production or metabolism. Often, excitatory or inhibitory effects of cytokines in the brain are regionally dependent. Many of these same cytokines also modulate CA levels in the hypothalamus and influence function of the HPA axis (Dunn, 2006; Haddad et al., 2002). For example, central and peripheral administrations of IFN-α both alter levels of DA and NE in specific regions of the brain (Kamata et al., 2000; Kitagami et al., 2003; Kumai et al., 2000). The patterns of altered CA levels differ depending on the location, central or peripheral, of IFN-α administration. This suggests that direct and indirect sensing of cytokines by the brain induce unique responses in CA production by neural tissues. Numerous studies report similar regulatory effects for other cytokines in relation to brain cytokine production. In peripheral tissues, the effects of centrally or peripherally administered cytokines on CA levels and CA turnover is tissue-specific, suggesting that cytokines can influence sympathetic activity both directly and indirectly, and that modulation of sympathetic nerve activity is specific rather than global

(Akiyoshi et al., 1990; Bognar et al., 1994; Hurst & Collins, 1993; Saito et al.; Sterin-Borda et al., 1996; Terao et al., 1994; Vriend et al., 1993).

Cytokines have also been reported to regulate CA biosynthetic enzymes *in vivo*. *In vivo* studies using rats demonstrate that the cytokines IFN-α, IL-1β, and TNF-α regulate the CA biosynthetic enzyme TH in catecholaminergic cells of the brain and carotid body (Kang et al., 2009; Kumai et al., 2000; Sirivelu et al., 2012; Zhang et al., 2007). Interestingly, centrally administered cytokines can regulate CA biosynthetic enzymes in the adrenal medullas as well, likely through indirect mechanisms involving neural activation of level of the CNS and downstream effects mediated by the HPA or SA axes (Sim et al., 2012). For a more complete presentation of cytokine effects in the brain, HPA and SA axis, several reviews are available (Dunn, 2006; Elenkov et al., 2000; Haddad et al., 2002; Turnbull & Rivier, 1999). In sites of CA production outside the brain, the influence of cytokine signalling is only beginning to be understood.

1.3.1 - Cytokine Expression by Adrenal Chromaffin Cells

Adrenal cytokines can originate either systemically or locally; both situations have possible importance to cytokine-mediated regulation of adrenal function during hypertension. Numerous studies have identified unique profiles of circulating and tissue-expressed cytokines in hypertensive animal and human subjects (Chae et al., 2001; Chan et al., 2012; Chrysohoou et al., 2004; Shi et al., 2010). Even during normal physiological conditions, cytokines are expressed at detectable levels by adrenal medullary tissue. Cytokines are

expressed at varying levels throughout the adrenal gland (Call et al., 2000; González-Hernández et al., 1996). The highest levels of expression are most commonly observed in the cortex or steroid-producing cells within the medulla, although expression of cytokines by chromaffin cells themselves has also been demonstrated in a number of studies (see Table 1). In humans, as in many species, the adrenal medulla is contiguous with the adrenal cortex, meaning that chromaffin cells are in direct contact with steroidogenic cells (Willenberg et al., 2002). Chromaffin cells are also receptive to many cytokines that are produced locally in the adrenal gland (see Table 1). Receptiveness to cytokines is demonstrated either by expression of cytokine receptors or by response of isolated chromaffin cells to cytokines. In instances where the cytokine and its receptor are co-expressed, or when a locally produced cytokine can elicit a response in chromaffin cells, there is a possibility of autocrine or paracrine signalling that may influence endocrine function of the adrenal medulla (Douglas et al., 2010).

Table 1: Cytokine expression, responsiveness, and signalling observed in adrenal gland. JAK = Janus kinase; STAT = Signal Transducer and Activator of Transcription; NO = Nitric Oxide; GC = Guanylyl Cyclase; NPY = Neuropeptide Y; ZG = Zona Glomerulosa; ZF = Zona Fasciculata; ZR = Zona Reticularis.

Cytokine	**Cytokine Expression** (organism, tissue)	**Responsiveness** (organism, tissue, mode of exposure)	**Cytokine Receptors** (receptor name, organism, tissue)	**Supported Signalling Mechanisms**
IFN-α		Bovine (medulla and chromaffin cells, *in vitro*) (Douglas & Bunn, 2009; Tachikawa et al., 1997; Toyohira et al., 1998)	IFNAR2; Bovine (chromaffin cells) (Samal et al., 2013)	PKC, ERK1/2, STAT 1 and 2 (Douglas & Bunn, 2009) PKC (Toyohira et al., 1998)
IL-1 α/β	Human (cortex, medulla, and KAT45 cells) (González-Hernández et al., 1995; Venihaki et al., 1998) Rat and Mouse (chromaffin cells) (Schultzberg et al., 1989) Rat (cortex and medulla, chromaffin, and PC12 cells) (Alheim et al., 1991; Andersson et al., 1992; Engström et al., 2007; Nobel & Schultzberg, 1995; Schultzberg et al., 1995)	Human (chromaffin and KAT45 cells, *in vitro*) (Rosmaninho-Salgado et al., 2009; Venihaki et al., 1998) Rat (medulla, *in vivo*) (Engström et al., 2007) Rat (adrenal and PC12 cells, *in vitro*) (Gwosdow, 1995; Gwosdow et al., 1992; Joseph et al., 1995; Li et al., 1994; Liu et al., 2000; Venihaki et al., 1997) Mouse (primary chromaffin cells, *in vitro*) (Rosmaninho-Salgado et al., 2007) Bovine (medulla and chromaffin cells, *in vitro*) (Ait-Ali et al., 2004; Eskay & Eiden, 1992; Morita et al., 2004; Yanagihara et al., 1994)	IL1R1; Rat (medulla and PC12 cells) (Engström et al., 2007; Shu et al., 2007) IL1R2; Rat (medulla) (Liu et al., 2008) IL1RA; Rat (chromaffin cells) (Schultzberg et al., 1995)	MAPK, NO/PKC, NO/GC, NPY, PKA/NO (Rosmaninho-Salgado et al., 2009) NPY (Rosmaninho-Salgado et al., 2007) ERK1/2 (Morita et al., 2004) Ca^{2+} (Shu et al., 2007) PKA (Joseph et al., 1995) CRH (Venihaki et al., 1998)
IL-6	Human (cortex, medulla and chromaffin cells) (Kontogeorgos, Scheithauer, et al., 2002; Kontogeorgos, Messini, et al., 2002; Päth et al., 1997) Rat (medulla and PC12 cells) (Gadient et al., 1995; Liu et al., 2000; Möller et al., 2006; Sallmann et al., 2000) Bovine (ZG, ZF, ZR, medulla, and chromaffin cells) (Call et al., 2000; Samal et al., 2013)	Human (adrenal, *in vitro*) (Päth et al., 1997; Willenberg et al., 2002) Rat (PC12, *in vitro*) (Li et al., 2012; Satoh et al., 1988)	IL6R; Human (normal and macrophage-depleted adrenal) (Päth et al., 1997; Willenberg et al., 2002), Rat (medulla) (Gadient et al., 1995) Gp130; Human (adrenal) (Willenberg et al., 2002), Rat (medulla) (Gadient et al., 1995)	c-Fos (Satoh et al., 1988) STAT3 (Li et al., 2012)

IL-10	Rat (PC12) (Virmani et al., 2011)			
TNF-α	Human (ZR, medulla, chromaffin cells, and pheochromocytoma) (González-Hernández et al., 1996; Kontogeorgos, Scheithauer, et al., 2002; Tong et al., 2004) Rat (PC12) (Liu et al., 2000) Bovine (adrenal capsule, cortex, and medulla) (Call et al., 2000)	Rat (PC12, *in vitro*) (Goryo et al., 2011; Soeda et al., 2001; Trincavelli et al., 2003) Mouse (chromaffin cells, *in vitro*) (Ait-Ali, Stroth, et al., 2010) Bovine (chromaffin cells, *in vitro*) (Ait-Ali, Stroth, et al., 2010; Ait-Ali et al., 2004, 2008; Eskay & Eiden, 1992; Samal et al., 2013; Tamura et al., 2014; Xie et al., 1993)	TNFR1; Bovine (chromaffin cells) (Tamura et al., 2014) TNFR2; Bovine (chromaffin cells) (Ait-Ali et al., 2004, 2008; Tamura et al., 2014)	ERK 1/2, p38, AP-1, NFκB (Ait-Ali et al., 2004) NFκB (RelA, NFκB1, NFκB2) (Ait-Ali et al., 2008) NFκB (various Rel class members) (Samal et al., 2013)
CCL2	Rat (medulla, PC12_bPAC1hop cells) (Ait-Ali, Samal, et al., 2010; Liu et al., 2008) Bovine (chromaffin cells) (Samal et al., 2013)			

1.3.2 - Cytokine Signalling in Chromaffin Cells

Wherever they may originate, there is now strong evidence that cytokines profoundly influence the adrenal medulla by inducing changes in secretion, intracellular signalling, gene transcription, and translation (Bunn et al., 2012). The cytokines most studied for their influence on adrenal chromaffin cell function include IFN-α, IL-1β, IL-6, and TNF-α. These cytokines have likely received particular attention because they are prominent mediators of the systemic acute phase inflammatory response.

IFN-α is a type I interferon and signals via the IFN-α receptor (IFNAR) complex, which includes IFNAR-1 and IFNAR-2 subunits. Transcript expression of IFNAR2 has been reported to increase in response to TNF-α treatment of bovine adrenal chromaffin cells (Samal et al., 2013). In many cells, binding of ligand to IFNAR induces activation of janus kinase (JAK)/signal transducer and activator of transcription (STAT) signalling, with the phosphorylation of STAT1 and STAT2, which dimerize to form two different transcriptional activator complexes (a STAT1 homodimer and STAT1-STAT2-IRF9 heterotrimer). IFN-α can also activate other members of the STAT family (Caraglia et al., 2005). Treatment of bovine chromaffin cells with IFN-α induces phosphorylation, increased expression, and nuclear translocation of STAT1 and STAT2 (Douglas & Bunn, 2009). Further, IFN-α induces an increase in STAT3 phosphorylation but only increases nuclear STAT3 in a small proportion of cells (Douglas & Bunn, 2009). IFN-α also induces ERK1/2 signalling downstream of PKC activation in chromaffin cells (Douglas & Bunn, 2009; Toyohira et al., 1998). Similar to IL-1, IFN-α inhibits ACh-stimulated CA secretion from chromaffin cells (Tachikawa et al., 1997). IFN-α also suppresses NE uptake by

cultured bovine chromaffin cells (Toyohira et al., 1998). IFN-α induces PKC- and ERK1/2-dependent phosphorylation of TH at the serine (Ser) -31 site (no change in phosphorylation at Ser- 19 or 40), a post-translational modification that is linked to increased TH protein stability and activity (Douglas & Bunn, 2009; Moy & Tsai, 2004; Sutherland et al., 1993). ERK1/2 activation has also been reported to contribute to histamine and Ang II-induced increases in TH Ser 31 phosphorylation in bovine adrenal chromaffin cells (Bobrovskaya et al., 2001; Cammarota et al., 2003). Similar mechanisms of post-translational regulation of TH by ERK1/2 in adrenal chromaffin cells may be utilized by other ERK1/2-activating cytokines.

IL-1β increases protein levels of the CA biosynthetic enzyme TH and, like IFN-α, induces phosphorylation of TH, in this case at the Ser-40 site which decreases inhibitory feedback of CAs on TH activity (Daubner et al., 1992; Rosmaninho-Salgado et al., 2009). Induction of TH phosphorylation by either IL-1β or IFN-α is transient (lasting <30 min) (Douglas & Bunn, 2009; Rosmaninho-Salgado et al., 2009). Long-term (24 hour) incubation with IL-1β does increase total TH protein, while incubation with IFN-α has not yet been demonstrated to change TH protein level (Douglas & Bunn, 2009; Rosmaninho-Salgado et al., 2009). IL-1β-induced phosphorylation of TH at other Ser sites and the involvement of ERK1/2 signalling in IL-1β-induced TH regulation have not been investigated. IL-1 receptor (IL-1R)1 and IL-1R2 are both expressed by rat adrenal medullary cells (Engström et al., 2007; Liu et al., 2008; Shu et al., 2007). IL-1R1 is responsible for transmembrane signalling and IL1R2 is a decoy receptor that acts as an endogenous inhibitor, like IL-1RA, to IL-1 signalling (Peters et al., 2013). IL-1 exists in two forms, IL-1α and IL-1β. Although they are structurally very different, both IL-1α and IL-1β bind to the IL-1Rs and the

neurochemical effects of both forms are very similar (Dunn, 2006). The similarity in effects of IL-1α and IL-1β is observed in adrenal chromaffin cells as well (Ait-Ali et al., 2004; Gwosdow, 1995; Joseph et al., 1995; Morita et al., 2004; Rosmaninho-Salgado et al., 2009). Stimulation of chromaffin cells with IL-1 can induce PKA, ERK1/2, nitric oxide (NO)/PKC, and NO/guanylyl cyclase intracellular signalling mechanisms (Joseph et al., 1995; Morita et al., 2004; Rosmaninho-Salgado et al., 2009). Some IL-1-induced effects in chromaffin cells rely on intermediate autocrine signalling by factors such as NPY and CRH. IL-1 induction of NPY is responsible for downstream activation of PKA/NO, as well as ERK1/2, PKC and guanylyl cyclase pathways (Rosmaninho-Salgado et al., 2009). IL-1-induced CRH expression can trigger a signalling loop, where CRH stimulates chromaffin cells to produce more IL-1β (Venihaki et al., 1997, 1998). Exposure to IL-1 can also cause increased expression of IL-1R1 in PC12 cells (Shu et al., 2007). An autocrine signalling loop utilizing IL-1 is supported *in vivo*. Intravenous injection of IL-1β has been reported to increase IL-1β and IL-1R1 mRNA levels in the medulla of rats (Engström et al., 2007).

IL-1 alone has been reported to stimulate CA release from cultures of primary adrenal chromaffin cells and from pheochromocytomas (Gwosdow et al., 1992; Joseph et al., 1995; Rosmaninho-Salgado et al., 2009; Venihaki et al., 1998; Yanagihara et al., 1994). A significant portion of IL-1 induction of CA secretion relies on intermediate autocrine signalling by NPY (Rosmaninho-Salgado et al., 2009, 2007). In contrast to basal application of IL-1, when combined with ACh, IL-1 has an inhibitory effect on chromaffin cell CA release (Morita et al., 2004). IL-1 may function in the homeostatic control of CA production, where in the absence of stimulation by other sources, IL-1 enhances CA secretion, but when other activators are present IL-1 dampens their effects on CA secretion.

An auto-regulatory mechanism utilizing IL-1 is supported *in vivo*, as administration of cholinergic agonists increases IL-1 mRNA and decreases IL-1 protein stores in rat adrenals, suggesting enhanced IL-1 secretion in response to cholinergic stimulation (Andersson et al., 1992; Schultzberg et al., 1989). Interestingly, it has been reported that medullary expression of IL-R2 is increased by immobilization stress (Liu et al., 2008). If IL-1 is involved in homeostatic control of CA production, increased expression of the decoy receptor IL-1R2 may be a stress-specific response, whereby IL-1R2 prevents IL-1 from dampening CA release in response to a psychological stressor. Differential regulation of CA biosynthetic enzymes has been reported in response to long-term exposure to cold or immobilization stress (Kvetnansky, 2004; Kvetnansky et al., 2002, 2003).

IL-6 binds to a receptor complex consisting of IL-6 receptor (IL-6R) and glycoprotein (gp) 130 components. IL-6 binds to IL-6R, which can exist in either soluble or membrane-bound forms. The receptor-ligand complex then couples to the gp130 signal transducing component, promoting dimerization of gp130, facilitating downstream signalling (Kamimura et al., 2003). JAK/STAT3 and MAPK/ERK signalling are common IL-6 activated pathways and are induced in neurons exposed to IL-6 (Fang et al., 2013). IL-6-induced STAT3 signalling has been reported in PC12 cells, and both STAT3 and ERK1/2 signalling mechanisms are supported by preliminary investigations using bovine chromaffin cells (Bunn et al., 2009; Li et al., 2012). The signal transducing component of the IL-6R complex is shared with other IL-6 family cytokines which, like IL-6, bind to ligand-specific receptors which then complex with a gp130. Thus, gp130 expression in the adrenal chromaffin cells can be inferred by responsiveness to other IL-6 family cytokines (Gadient et al., 1995). In immune cells, IL-6 activates AP-1 via a Ras-dependent MAPK

signalling mechanism (Jones et al., 2001). Activation of this transcription factor may also occur in chromaffin cells, as IL-6 has been reported to induce increases in c-fos transcript in PC12 cells (Satoh et al., 1988). In PC12 cells, high concentrations of IL-6 were also found to have an inhibitory effect the basal CA-producing function, decreasing DA and NE release as well as TH protein (Li et al., 2012).

TNF-α binds to the plasma membrane situated receptors TNF receptor (TNFR) 1 and TNFR2. Binding of ligand to these receptors can initiate signalling cascades that utilize numerous protein kinases and lead to the activation of two major transcription factors, AP-1 and NFκB (Baud & Karin, 2001). Bovine chromaffin cells have been found to express TNFR1 and TNFR2; however, TNFR1 appears to be more consistently expressed of the two (Ait-Ali et al., 2004, 2008; Tamura et al., 2014). A detailed investigation by Ait-Ali *et al.* (2004) found that TNF-α signalling in bovine chromaffin cells relies on ERK1/2 and p38 signal transduction mechanisms. Further, this study determined that activation of the transcription factor AP-1 occurs downstream of ERK1/2 activation, also finding that TNF-α induces NFκB transcription factor activity in an ERK1/2-independent manner. In chromaffin cells, TNF-α-induced NFκB signalling appears to involve primarily the p65 subunit, and possibly p52 and p50 subunits. Thus, transcriptional regulation likely occurs primarily via the p65 homodimer (Ait-Ali et al., 2008). The inhibitor of NFκB, PDTC, blocks the enhancement of the transcript levels of some genes by TNF-α (Ait-Ali et al., 2008). Later experiments demonstrated that a variety of Rel family transcription factors are likely involved in the transcriptional regulatory effects of TNF-α (Samal et al., 2013). TNF-α has been reported to induce and to modulate neuropeptide transcript and protein in bovine adrenal chromaffin cells (Ait-Ali, Stroth, et al., 2010; Ait-Ali et al., 2004; Eskay & Eiden,

1992). The effects of TNF-α on CA production have not been thoroughly investigated. A study of cytokine modulation of hypoxic response found that TNF-α can inhibit hypoxic induction of TH transcript (Goryo et al., 2011). An oligonucleotide microarray analysis of TNF-α-induced changes in bovine chromaffin cell transcriptome identified upregulation of PNMT transcript after long (48 hour), but not short (6 hour) exposures to the cytokine (Ait-Ali et al., 2008).

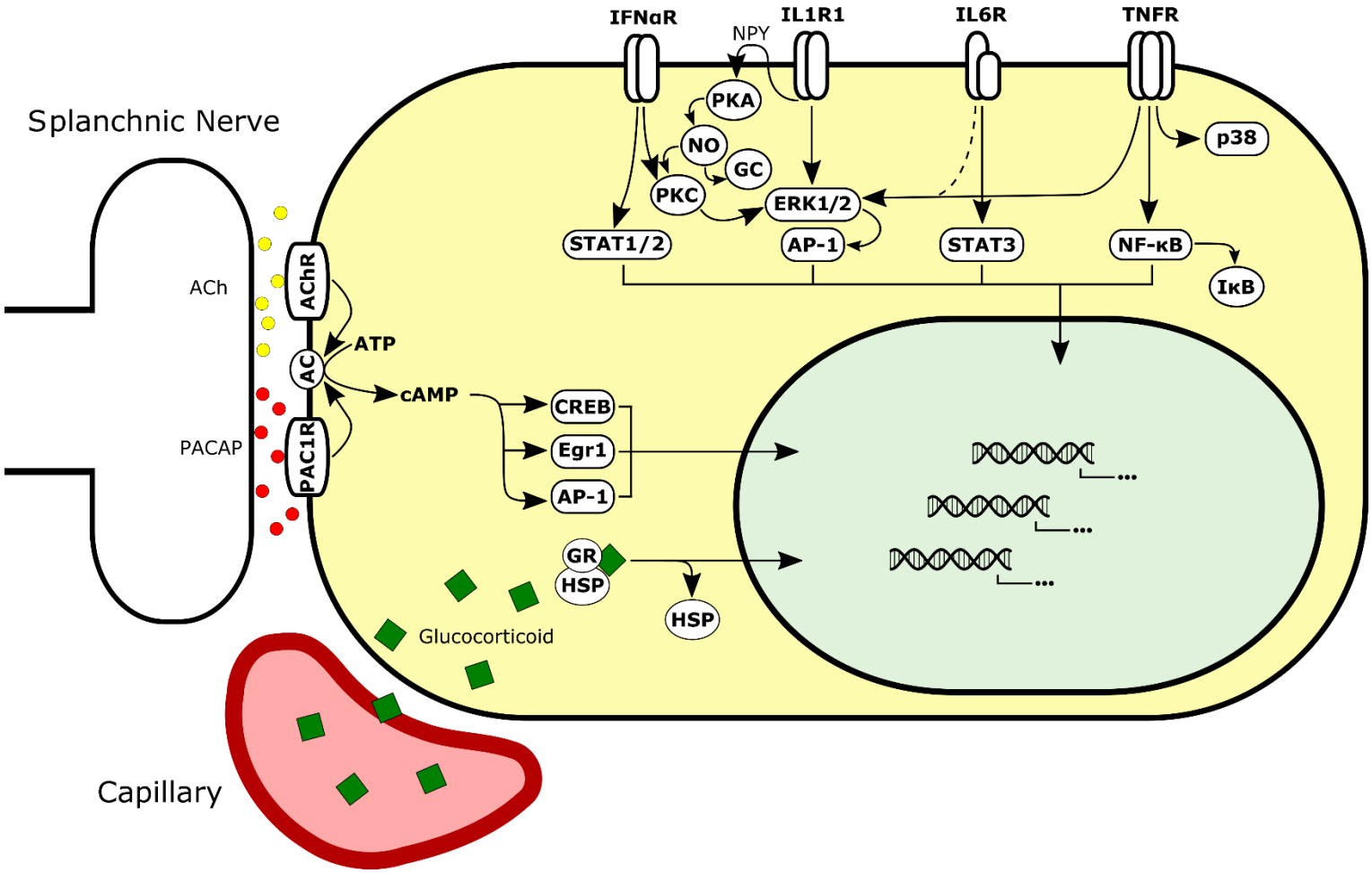

Figure 6: Simplified schematic of neural, hormonal, and immune signalling pathways activated in adrenal chromaffin cells. These intracellular signals may be integrated to regulate production of CAs by chromaffin cells during normal or pathological conditions. Dashed line represents signalling mechanism supported by unpublished findings. ACh = Acetylcholine; PACAP = Pituitary Adenylate Cyclase-Activating Peptide; AC = Adenylyl Cyclase; GR = Glucocorticoid Receptor; HSP = Heat Shock Protein; NPY = Neuropeptide Y; PKA = Protein Kinase A; NO = Nitric Oxide; PKC = Protein Kinase C; GC = Guanylyl Cyclase.

In addition to direct signalling mechanisms of cytokines (Fig. 6), evidence is now emerging that cytokines can induce long-term changes in chromaffin cells through the activation of autocrine signalling loops. It is a well-established phenomenon that in immune cells cytokines favour their own production and the production of other cytokines, resulting in the formation of autocrine signalling cascades (Cavaillon et al., 2003). Two long established examples are IL-1 and TNF-α, which can stimulate their own production, along with the production of numerous other cytokines and inflammatory mediators (DeForge et al., 1992; Descoteaux & Matlashewski, 1990; Dinarello et al., 1987; Sparacio et al., 1992). These autocrine signalling loops can be self-regulating by stimulating the production of anti-inflammatory molecules such as IL-10 (de Waal Malefyt et al., 1993; Wanidworanun & Strober, 1993). By inducing the production of autocrine signalling molecules, cytokines may initiate long-term signalling programs in the adrenal medulla (Ait-Ali, Stroth, et al., 2010; Call et al., 2000; Eskay & Eiden, 1992). For example, in primary cultures of bovine adrenal chromaffin cells, treatment with IL-1α has been reported to induce production of the cytokines IL-6 and TNF-α, as well as the neuropeptides VIP, NPY and Met-Enkephalin (Call et al., 2000; Eskay & Eiden, 1992). Intermediate autocrine signalling by NPY is critical for CA regulatory effects of IL-1 in chromaffin cells (Rosmaninho-Salgado et al., 2009, 2007).

Whether the responses of medullary cells to cytokines primarily functions for protective action against microbial challenge or if cytokines are a normal part of the diverse informational molecules that constantly regulate chromaffin cell homeostatic function, it is clear that local changes in cytokine signalling within the medulla has potential to exacerbate dysfunctional CA production. The regulation of adrenal function by cytokines and the

importance of changes in immune operation in contributing to the progression of hypertension and CVD are summarized above. The bi-directional relationship of the immune and neuro-endocrine systems conceivably provides fitness advantages to organisms in ways similar to the ‘neuro-immune circuit’ and may be a physiologically important part of maintaining health, dysfunction of which may result in pathology. The neuro-immune circuit has helped to explain perplexing phenomena such as the co-morbidity of neuropsychiatric symptoms and inflammatory disease (Irwin & Cole, 2011). Similarly, integration of immune and adrenal functions provides an explanation for the aetiology of inflammation-related hypertension and may help to elucidate mechanisms of essential hypertension.

1.3.3 - Cytokine Modulation of Glucocorticoid Signalling in Chromaffin Cells

GCs and transmitters released at splanchnic-adrenal medullary synapses are important informational molecules which control Epi biosynthesis during normal and stress conditions [see (Wong, 2006) and references therein]. Chromaffin cells must coordinate intracellular signalling pathways induced by these and other informational molecules in order to produce appropriate responses under diverse physiological conditions. Cytokines produced either systemically or locally may be significant modulators of adrenal CA biosynthesis by altering chromaffin cell response to GCs and neurotransmitters. How, and to what extent, chromaffin cells simultaneously process information from immune and stress circuits is not well understood.

A number of the cytokines, including IFN-α, IL-1, IL-2, and TNF-α, have been reported to have inhibitory effects either on GC-induced GR nuclear translocation, GR-GRE binding, or GR-mediated gene transcription in diverse cell types (Pace & Miller, 2009; Smoak & Cidlowski, 2004). In mouse hippocampal HT22 cells, inhibition of GR transcriptional activity by IFN-α is dependent on JAK/STAT signalling pathway. STAT5 (not STAT1 or STAT2) appears to be the major mediator of IFN-α inhibitory effects on GR function in HT22 cells. Co-immunoprecipitation revealed that phosphorylated STAT5 binds to GR within the nucleus, and IFN-α-induced repression of GR function does not rely on inhibition of GR protein expression or nuclear translocation (Hu et al., 2009). In bovine chromaffin cells, IFN-α does not induce phosphorylation of STAT5; instead, IFN-α primarily activates STAT1 and STAT2 (Douglas & Bunn, 2009). A trimeric complex consisting of STAT1, STAT2, and interferon regulatory factor (IRF) 9, induced by IFN-α/β receptor activation, is directly regulated by the nuclear coactivator of GR, glucocorticoid receptor-interacting protein (GRIP) 1 in murine macrophages (Flammer et al., 2010). Occupancy of target promoters by the STAT1-STAT2-IRF9 complex, pre-initiation complex assembly, and type I IFN stimulated gene expression were reported to be repressed by dexamethasone (Dex)-induced depletion of GRIP. Inhibition of IFN-induced gene expression by Dex appears to be caused by sequestration of GRIP1 from its functional activity as a coactivator for the STAT1-STAT2-IRF9 complex by GR. Conceivably, as long as GRIP1 protein levels are sufficiently low in adrenal chromaffin cells, this competitive mechanism could allow for the converse effect, whereby induction of the STAT1-STAT2-IRF9 complex by IFN-α leads to an inhibition of GR transcriptional effects through the repression of GRIP1 coactivation of GR.

In mouse fibroblast cells, inhibition of GR-GRE binding and GR-mediated promoter activation by IL-1 has been reported to depend on p38 signalling (Wang et al., 2004). The mechanism of p38 regulation of GR appears to involve direct phosphorylation of GR at the Ser-211 site (human) which modulates recruitment of GR coactivators to GRE-containing promoters (Kino et al., 2007; Miller et al., 2005). So far, p38 signalling has not been demonstrated as a significant contributor in IL-1 regulation of chromaffin cell function (Morita et al., 2004). Activation of p38 signalling pathway could possibly be a mechanism for inhibition of GR function by other cytokines, such as TNF-α, which have been reported to induce p38 signalling in chromaffin cells (Ait-Ali et al., 2004).

IL-2 inhibition of GR is dependent on signal transduction by p38 (reported in murine HT-2 T-helper cell line) and by JAK3 (reported in human PBMCs), both contributing to inhibition of GR nuclear translocation (Goleva et al., 2002; Irusen et al., 2002). In a murine T-lymphocyte cell line (CTLL-2) IL-2 represses GR transactivation through direct protein-protein interactions of IL-2-induced STAT5 with GR following translocation to the nucleus (Biola et al., 2001).

In human keratinocyte-derived HaCaT cells, TNF-α inhibits GC-induced transcriptional activation and GR nuclear translocation through a MEK-1/ERK-dependent mechanism (Onda et al., 2006). In human epithelial-derived HeLa cells, the inhibitory effect of MAPK signalling on GR function is demonstrated by inhibition of transcriptional activation of GR-responsive luciferase constructs in the presence of constitutively activated p38 or JNK (Szatmáry et al., 2004). In human colon carcinoma-derived HCT116 cells, TNF-α can suppress GR transactivation by induction of FLICE-associated huge protein, which

competes with GR to bind the nuclear coactivator GRIP1 (Kino & Chrousos, 2003). Additionally, mutual repression of GR and NFκB function may result from transrepression (also known as tethering), caused by direct protein-protein interactions within the nucleus, or from competition for the cofactors CBP and steroid receptor coactivator-1 (Caldenhoven et al., 1995; Ray & Prefontaine, 1994; Sheppard et al., 1998).

IL-6 enhances GR function through a STAT3-dependent mechanism in rat hepatoma H4IIE cells. Like STAT5, STAT3 forms complexes with GR in the nucleus; however, unlike STAT5, formation of a STAT3-GR complex appears to enhance promoter activation, both of IL-6 responsive elements and at GREs (Zhang et al., 1997).

IL-10 is another cytokine reported to have synergistic rather than inhibitory effects on GR function. In a study using human leukocytes (U937 cells and whole blood cell cultures), treatment with the anti-inflammatory cytokine IL-10 had opposite effects to TNF-α. Inhibition of IL-6 secretion in whole-blood cell cultures and promotion of IL-1R antagonist secretion by human monocyte-derived U937 cells are two effects of Dex treatment. TNF-α supresses, whereas IL-10 intensifies these Dex-induced activities (Franchimont et al., 1999). The mechanism of IL-10 and GR signalling crosstalk is not yet understood; however, the effect may be achieved through an increase in GR production (Franchimont et al., 1999). Other possible mechanisms include IL-10-induced activation of STAT3, or IL-10 induced inhibition of NFκB, a transcription factor which inhibits GR function through transrepression (Ray & Prefontaine, 1994; Wang et al., 1995; Williams et al., 2004).

The mechanisms of cytokine-induced GR regulation involve intermediate signalling molecules such as MAPKs and transcription factors including STAT1, STAT2, STAT3, STAT5, NFκB, and AP-1. In chromaffin cells, the most commonly reported signalling pathway by cytokines is that of the MAPK transduction molecules, particularly ERK1/2 (Bunn et al., 2012). The MAPKs JNK and ERK can inhibit GR-mediated transcriptional activation by direct and indirect phosphorylation of GR at Ser-246 (rats, Ser-226 in humans), which may prevent signalling by promoting nuclear export of the transcription factor (Galliher-Beckley & Cidlowski, 2009; I Rogatsky et al., 1998). ERK1/2 signalling can also lead to AP-1 transcription factor activity. In chromaffin cells, both IL-6 and TNF-α have been reported to induce activation of AP-1 subunits (Ait-Ali et al., 2004; Satoh et al., 1988). Treatment of bovine chromaffin cells with TNF-α increases transcriptional activation of AP-1 responsive promoter elements and increases binding of fos and jun proteins to TRE and CRE sequences through an ERK1/2-dependent mechanism (Ait-Ali et al., 2004). In humans, GC resistance is positively correlated with expression of c-fos in peripheral blood mononuclear cells *in vivo* (Takahashi et al., 2002). The interaction between GR and AP-1 is mutually repressive, involving tethering at the GR-DNA binding domain; thus, inhibition of GR function may occur after GR translocation to the nucleus (Heck et al., 1994; Kassel et al., 2004; Inez Rogatsky et al., 2002; Schüle et al., 1990). Activation of AP-1 signalling may be a component of chromaffin cell regulation by other ERK1/2-inducing cytokines, such as IFN-α and IL-1, and leading to insensitivity of chromaffin cells to GCs.

1.3.2 - Cytokine Modulation of Neurotransmitter and cAMP Signalling in Chromaffin Cells

Integration of neurotransmitters such as ACh and PACAP with some cytokines has been demonstrated in chromaffin cells. IL-1 inhibits ACh-induced CA release via ERK1/2-mediated suppression of Ca^{2+} influx in bovine chromaffin cells (Morita et al., 2004). Similarly, IFN-α has been reported to decrease ACh-stimulated CA secretion and Ca^{2+} influx in bovine chromaffin cells (Tachikawa et al., 1997). Chromaffin cell response to the neuropeptide PACAP is also modified by cytokine exposure. Combined treatment with PACAP and TNF-α synergistically upregulates VIP and galanin expression in bovine chromaffin cells (Ait-Ali, Stroth, et al., 2010).

The signalling pathways of cAMP and cytokines may be integrated through interactions at MAPK, JAK-STAT, and NFκB. A number of studies report effects of cAMP/PKA signalling on the function of these other signalling molecules. In a human T-lymphocyte cell line (Jurkat cells) cAMP increase ERK1/2 activity by inhibiting function of haematopoietic protein tyrosine phosphatase, a negative regulator of ERK1/2 and p38 (Saxena et al., 1999). In experiments using mouse (70Z/3) and human (Jurkat) lymphocyte cell lines, PKA was reported to contribute to the activation of NFκB by phosphorylating p65 following dissociation from IκB (Zhong et al., 1997). In human PBMCs, cAMP has an inhibitory effect on IL-6-induced STAT1 and STAT3 binding to DNA and prevents IL-6 induction of an IL-6-responsive gene harbouring a STAT1 and STAT3 binding sequence (Sengupta et al., 1996). Compared to cAMP regulatory effects on cytokine signalling pathways, the effects of cytokines on cAMP signalling are less well understood. This may

in part be because cAMP activation occurs early in intracellular signalling cascades and the cellular output of cAMP activation is highly tissue-dependent (Taskén & Aandahl, 2004; Zhang et al., 2005). In chromaffin cells, transcription factors involved in response to cAMP/PKA signalling include Egr1, AP-1, and CREB (Ginty et al., 1991; Swanson et al., 1998; Tai et al., 2001). Signalling via cAMP in chromaffin cells may also lead to activation of the MAPKs p38 and ERK1/2 (Hansen et al., 2000; Vaudry et al., 2009). In bovine chromaffin cells, the cytokines IL-1 and TNF-α have been reported to enhance induction of VIP and substance P expression by forskolin (Forsk) an activator of adenylyl cyclase (Eskay & Eiden, 1992). The mechanisms of cytokine modulation of cAMP signalling in chromaffin cells have not been defined.

1.4 - Hypothesis and Objectives

1.4.1 - Hypothesis

Current literature supports roles for numerous cytokines in influencing blood pressure and in regulating function of adrenal chromaffin cells. Cytokines are expressed locally in the adrenal medulla and changes in circulating cytokine levels have been reported in hypertensive patient cohorts. It was predicted that cytokines can directly regulate the CA biosynthetic pathway in adrenal chromaffin cells, thus, providing a mechanism for inflammation-mediated regulation of CA production during hypertension. Based on the current literature, it was predicted that the cytokines IFN-α, IL-1β, TNF-α, and CCL2 would increase, while IL-2, IL-6, and IL-10 would decrease CA biosynthetic enzyme

expression in PC12 cells. Further, it was hypothesized that cross-talk between immune and GC or cAMP signalling pathways would be observed in chromaffin cells. Changes in CA biosynthetic enzyme transcript levels were predicted to be regulated by changes in promoter activation. Cytokines were predicted to influence promoter activation by GCs, possibly by regulating nuclear localization of GR.

1.4.2 - Objectives

The objectives of this study were: (i) to analyze possible regulatory effects of cytokines on expression of the catecholamine biosynthetic enzymes TH, DBH and PNMT in PC12 cells.; (ii) to determine if hormonal or neural activation, mimicked by Dex and Forsk respectively, of TH, DBH and PNMT expression is modulated by cytokine signalling; and (iii) to investigate the mechanisms involved in the transcriptional regulatory effects of cytokines by analyzing PNMT promoter activation and GR nuclear localization.

2 - Methods

2.1 - Cell Culture

PC12 cells (ATCC; Manassas, Virginia, USA) were cultured in 100 mm tissue culture dishes (Biolite; Thermo Scientific; Waltham, Massachusetts, USA) in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 5% bovine calf serum, 5% equine serum (Hyclone Laboratories Inc.; Logan, Utah, USA) and 50 ng/ml gentamycin sulphate (Sigma-Aldrich; St. Louis, Missouri, USA). The cells were maintained in a humidified incubator at 37°C containing 5% CO_2 (Forma Series I Water Jacketed CO_2 Incubator, Thermo Scientific) and grown to a confluence of 80-90% before being planted in a subsequent passage for experimentation. In all experiments, cells (passage 14-20) were grown in DMEM containing 5% charcoal-treated serum (equal parts bovine calf and equine serum) and gentamycin sulphate. Charcoal treatment was required for the removal of endogenous hormones and growth factors that would otherwise affect the expression of the genes of interest. In all experiments, prior to planting, cells were dissociated using trypsin, counted and viability determined using a cell viability analyzer (Vi-cell XR, Beckman Coulter; Mississauga, Ontario, Canada). In all experiments, cells were planted in 5% charcoal treated media (CTM) and allowed to acclimate for 24 hours to the reduced serum environment prior to treatment with cytokines. At the end of the acclimation period, medium was changed to fresh 5% CTM just prior to the addition of cytokines or drugs.

For RNA extraction and RT-qPCR experiments, 0.7 x10 ^{6} cells were planted in 1 ml of medium per well in 12 well plates (Corning; Tewksbury, Massachusetts, USA). For luciferase assays, 0.35 x10 ^{6} cells were planted in 1 ml per well in 24 well plates (Corning). For nuclear protein extraction and western blotting, 2.5 x10 ^{6} cells were planted in 2 ml of medium per well in 6 well plates (Biolite dishes, Thermo Scientific). For microscopy, cells were planted using the same method as for nuclear protein extraction; however a coverslip was inserted into the bottom of the 6 well plates prior to planting cells.

2.2 - Cell Treatments

2.2.1 - Cytokine Sources, Preparation, and Application

The cytokines used in the study were: rat IL-1β, rat IL-2, rat IL-6, rat IL-10, rat TNF-α (PeproTech Inc.; Rocky Hill, New Jersey, USA); rat IFN-α (Sigma-Aldrich) and rat CCL2 (R&D systems; Minneapolis, Minnesota, USA). All cytokines were obtained as lyophilized powders and were resuspended in specific buffers prepared in 0.1% BSA (Table 2) as per manufacturer specifications, and further aliquoted for long term storage at -80°C.

Table 2: Buffer solutions used for resuspending cytokines and cytokine doses.

Cytokine	Buffer	Dose(s) Used
IFN-α	20 mM Citric Acid and 150 mM NaCl, pH 5.0	1000 U/ml
IL-1β	1 mM Sodium Acetate, pH 6.5	10 ng/ml and 50 ng/ml
IL-2	1 mM Sodium Citrate, pH 4.0	100 ng/ml
IL-6	1 mM Tris, pH 8.0 +0.01% SDS	10 ng/ml and 100 ng/ml
IL-10	2 mM Sodium Phosphate, pH 7.0	50 ng/ml
TNF-α	0.05x PBS, pH 7.1	100 ng/ml
CCL2	No Additives (MilliQ H_2O)	10 ng/ml and 100 ng/ml

Doses of cytokines and period of treatment were determined from prior screening experiments (data not shown) or were selected based on previously published literature (see Table 1 for papers). Two doses were selected for some cytokines when literature indicated multiple effective doses or bimodal response patterns (see Table 2). For the initial screening experiments, two time points (6 and 24 hours) of treatment with cytokine were used. All remaining experiments described below were performed with a 24-hour cytokine treatment. In all experimental designs, vehicle treated groups were included to account for effects from buffers.

2.2.2 - Dexamethasone and Forskolin Sources, Preparation, and Application

In all experiments analyzing the effect of cytokine on modifying responses to GC or cAMP signalling, samples were treated with Dex (Cayman Chemicals; Ann Arbor, Michigan, USA) or Forsk (L.C. Laboratories; Boston, Massachusetts, USA) respectively. Drugs were dissolved according to manufacturer's instructions and stored at -80^{0}C. Immediately prior to treating cells, drugs were diluted in 5% CTM to concentrations of 10 mM and 100 µM for Forsk and Dex respectively. Drugs were then applied to cells to obtain final concentrations of 10 µM and 1 µM for Forsk and Dex respectively. Cells were treated with either Dex of Forsk 6 hours prior to harvesting. Drug concentrations and treatment times were based on previous studies from our laboratory (Tai et al., 2002; Tai & Wong, 2003).

2.3 - RNA extraction, cDNA Synthesis and RT-qPCR

2.3.1 - RNA Extraction

At the end of treatment period, media was aspirated and RNA extracted with Trizol reagent (Sigma-Aldrich) as per the manufacturer's protocol. In brief, cells were lysed with Trizol (500 µl) by pipetting several times before transferring to fresh tubes. Subsequently, 100 µl of chloroform was added, mixed thoroughly by vortexing, and incubated for 10 minutes at room temperature, then the samples were centrifuged (12,000 x g) for 20 minutes at 4˚C. The aqueous phase containing the RNA was transferred to a fresh tube and RNA precipitated with isopropanol; 125 µl of isopropanol was added per sample and incubated for 5-10 minutes, followed by centrifugation at 12,000 x g for 8 minutes at 4°C. The RNA pellet was washed with 500 µl of 70% ethanol and centrifuged (7,500 x g) for 5 minutes at 4˚C. Ethanol was then removed, RNA pellets air-dried, then dissolved in RNAse-free, diethyl pyrocarbonate-treated water. RNA concentration was determined at 260 nm using a NanoDrop ND-1000 Spectrophotometer (Nanodrop Technologies; Wilmington, Delaware, USA).

2.3.2 - DNAse Treatment and cDNA Synthesis

Prior to cDNA synthesis, 2 µg of RNA was treated with Amplification Grade DNAse I (1 U/µL; Sigma Aldrich) for 15 minutes at room temperature in the DNAse Reaction buffer. The reaction was terminated by the addition of a stop solution (50 mM EDTA; Sigma-

Aldrich) followed by heating at 70°C for 10 minutes. For cDNA synthesis, M-MLV Reverse Transcriptase (M-MLV RT, Promega; Madison, Wisconsin, USA) was used as per the manufacturer's instructions. Briefly, random primers (1 μg) were added to the DNAse-treated RNA and heated for 5 minutes at 70°C. After cooling on ice, a master mix containing reaction buffer, dNTPs and M-MLV RT was added to the reaction mix and samples were reverse transcribed at 37°C for 1 hour using a Mini Thermal Cycler (BioRad). cDNAs were stored at -20^{0}C for up to two weeks or -80^{0}C for extended storage.

2.3.3 - Quantitative Real Time PCR

The transcripts for RPL29, TH, DBH, and PNMT were quantified by quantitative PCR using the GoTaq qPCR mastermix with SYBR Green (Promega) as per the instructions from the manufacturer. Briefly, for each gene, a standard curve using serial dilutions (0.78 ng to 50 ng) of cDNA from control cells prepared in parallel with the experimental samples was used to determine the efficiency of PCR reactions and to validate primers. Following this, the lowest cDNA input that yielded high efficiency (95-100%) with Ct value between 16-32 cycles was selected for subsequent qPCR analysis of target genes. The qPCRs were performed in a BioRad Chromo 4 machine and analyzed using the Opticon Monitor program (BioRad). The cycling conditions, primers, and cDNA input for each gene target is summarized Table 3. Specificity of the product was analyzed by assessing the melting curve. The Ct value for each sample was determined and fold changes were analyzed using the

$ratio = (E_{target})^{\Delta CT_{target}(control-sample)} / (E_{ref})^{\Delta CT_{ref}(control-sample)}$ method (Pfaffl, 2001). For each experimental condition in a set, three biological replicates were included. All samples were run in duplicate in the qPCR (technical replicates), and the experiment was performed for n=3 unless otherwise stated in the legend.

Table 3: Primer specification and polymerase chain reaction set-up.

Gene target	Primer Sequences (5'-3')	Amplicon Size (bp)	Amplification Conditions	cDNA Input (ng/µl)	Primer Input (nM)
PNMT X75333	**F**: CATCGAGGAC AAGGGAGAGTC **R**: GCAGCGTCGT GATATGATAC	219	1. 95°C, 2 min 2. 95°C, 1 min 3. 60°C, 1 min 4. 72°C, 1 min 5. Plate read 6. Go to line 2, 39 more times 7. Melting Curve from 55-95^{0}C, read every 1°C, hold 10 sec	1	300
TH L22651	**F**: GCGACAGAGTC TCATCGAGGAT **R**: AGAGCAGGTT GAGAACAGCATT	150	1. 95°C, 2 min 2. 95°C, 1 min 3. 58°C, 1 min 4. 72°C, 1 min 5. Plate read 6. Go to line 2, 29 more times 7. Melting Curve from 55-95^{0}C, read every 1°C, hold 10 sec	0.5	600
DBH NM_013158	**F**: TTCCCCATGTT CAACGGACC **R**: GCTGTGTAGTG TAGACGGATGC	240	1. 95°C, 2 min 2. 95°C, 1 min 3. 58°C, 1 min 4. 72°C, 1 min 5. Plate read 6. Go to line 2, 29 more times 7. Melting Curve from 55-95^{0}C, read every 1°C, hold 10 sec	0.5	600
Beta Actin NM_031144	**F**: TCTGTGTGGAT TGGTGGCTCT **R**: GACTCATCGTA CTCCTGCTTG	83	1. 95°C, 2 min 2. 95°C, 1 min 3. 58°C, 1 min 4. 72°C, 1 min 5. Plate read 6. Go to line 2, 29 more times 7. Melting Curve from 55-95^{0}C, read every 1°C, hold 10 sec	0.5	600
RPL29 NM_017150	**F**: CAGAAATGGC ATCAAGAAACCC **R**: TCTTGTTGTGC TTCTTGGCAAA	105	1. 95°C, 2 min 2. 95°C, 1 min 3. 58°C, 1 min 4. 72°C, 1 min 5. Plate read 6. Go to line 2, 29 more times 7. Melting Curve from 55-95^{0}C, read every 1°C, hold 10 sec	0.5	600

2.5 - Western Blot

2.5.1 - Protein Extraction and Quantification

After completion of experimental treatments, samples were washed twice with cold PBS, scraped from the dish and transferred into a 1.5 ml tube in 1 ml of PBS. Next, samples were centrifuged (15,700 x g) for 10 seconds at 4 ºC and the supernatant discarded. The cells were resuspended with 150 µl of lysis buffer A (10 mM Hepes-KOH, 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT, 0.2 mM PMSF, 20 µl protease inhibitor cocktail) and incubated on ice for 10 min. To enhance cell lysis, each sample was force ejected 10 times through a 22-gauge needle using 1ml syringes. Next, samples were vortexed for 10 seconds, centrifuged (15,700 x g) for 10 seconds at 4 ºC, and the supernatant containing the cytosolic protein fraction was collected. For nuclear protein extraction, the pellet was resuspended in 50 µl of lysis buffer C (20 mM Hepes KOH, 1.5 mM $MgCl_2$, 420 mM NaCl, 25% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.2 mM PMSF, 20 µl protease inhibitor cocktail) and incubated on ice for 20 min. Finally, samples were centrifuged (15,700 x g) for 2 minutes at 4°C and the supernatant containing nuclear proteins was collected into a new 1.5 ml microcentrifuge tube. The samples were kept at -80°C for long-term storage.

Protein concentration was determined using the Bradford assay employing a protein standard made with purified IgG ranging from 1-20 µg. The standards and sample dilutions were plated in a total volume of 20 µl in a clear 96 well flat-bottomed microplate (Corning). Next, 250 µl of 1x Bradford reagent (BioRad) was added to each well and incubated on a microplate shaker at room temperature for 5 minutes. The absorbance was measured at a

wavelength of 595 using a spectrophotometer (Bio-Tek Instruments Inc.; Winooski, Vermont, USA). The data was analyzed with KC-4 software to determine total amount of protein using the standard curve.

2.5.2 - Sample Preparation

From each sample, 30 μg of total nuclear protein was transferred to a fresh tube, and their volume adjusted using lysis buffer C so that all samples have identical volumes of at least 20 μl (to facilitate accurate pipetting). Sample loading dye with β-mercaptoethanol was added and sample preparations were heated to 100°C for 5 minutes and then immediately loaded onto gels.

2.5.3 - Gel Casting

Polyacrylamide gels for protein gel electrophoresis were prepared using 10% polyacrylamide resolving gel and 4% polyacrylamide stacking gel in 1.5 mm spaced plates (BioRad) and 15-well combs (BioRad). 10 μl of PageRuler Prestained protein ladder (FroggaBio; North York, Ontario, Canada) was loaded as a molecular weight marker. Gels were run at 75 V until the dye front migrated from the stacking gel into the resolving gel. The voltage was then increased to 100 V and resolved until the dye front migrated to the base of the gel.

2.5.4 - Protein Transfer

Gels were transferred onto 0.2 μm pore size nitrocellulose membranes (Pall Laboratories; Port Washington, New York, USA) using transfer cassettes and the immersed tank system (BioRad) with 1x Transfer buffer (250mM Tris, 1.92M glycine with 20% methanol) for 1hr at 100 V.

2.5.4 - Western Blot

The membranes were incubated in blocking buffer made with 5% skim milk in Tris Buffer Saline with Tween (TBS-T; 10 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween-20) for a minimum of 30 minutes on a rocker. Membranes were then rinsed 4 times in TBS-T for 10 minutes each at room temperature. The blots were incubated with primary antibody diluted in blocking buffer overnight at 4°C on a rocker (1:1000 anti GR; clone M-20, Santa Cruz Biotechnology; Dallas, Texas, USA). Next, membranes were washed 4 times in TBS-T (10 min each), and then incubated in horseradish peroxidase (HRP) conjugated anti-rabbit secondary antibody diluted in blocking buffer (1:2000 goat anti rabbit-HRP, Santa Cruz Biotechnology) for 1 hour. Finally, the membranes were washed again in TBS-T (10 min, 4 times) were blotted dry and proteins were detected by enhanced chemiluminescence (ECL) and visualized on film processed on a developer. After the films were developed, membranes were washed twice with TBS-T and stained with Ponceau S. Images of the

Ponceau S stained membrane were acquired using the BioRad Molecular FX Imager with the Sypro Ruby red module (BioRad).

2.5.5 - Densitometry

For densitometric analysis, the band intensities were quantified using Image J software. For quantification of variation in GR under the various experimental conditions, the intensity of GR was normalized to the intensity of a band visualised by Ponceau S staining.

2.4 - Analysis of PNMT Promoter Activation

2.4.1 - Transformation and Plasmid Preparation

PC12 cells were transfected with a PNMT promoter driven luciferase reporter construct (pGL893PNMT), consisting of the 893 bp proximal region of the rat PNMT promoter sequence (-863 to +18 bp) cloned into the pGL3Basic vector (Promega) (for details see Her et al., 1999). 1 µg of the stock plasmid was used to transform competent DH5α bacteria as per the manufacturer's protocol (Invitrogen; Carlsbad, California, USA). Bacteria harbouring the plasmid were selected on Luria Bertani (LB) agar plates supplemented with ampicillin; selected colonies were then grown in LB broth with ampicillin and plasmid extracted using the PureLink HiPure Plasmid Filter MaxiPrep kit (Invitrogen). The plasmid was resuspended in Tris-EDTA (TE) buffer, DNA concentration determined by NanoDrop

ND-1000 Spectrophotometer (Nanodrop Technologies) and adjusted to a concentration of 1 μg/μl before being aliquoted for storage at 4 ºC.

2.4.2 - Transfection and Luciferase Assay

PC12 cells were seeded in 24 well plates at a density of 0.35 x10 ^{6} cells per well as described above. After the 24 hour acclimation period which followed planting, the PNMT promoter-luciferase reporter gene construct pGL3RP893 was transfected into the cells using Turbofect transfection reagent (Promega). Briefly, for each well to be transfected, 1 μg of plasmid DNA was mixed with 100 μl of basal serum free DMEM and 1 μl of Turbofect reagent. The mixture was vortexed and allowed to incubate for 20 minutes at room temperature before adding to the cells. For multiple wells, a master mix of transfection solution was prepared. Six hours post-transfection, the cells were treated with cytokines or vehicle as per the experimental design for a total of 24 hours. 18 hours post cytokine treatment, Dex (1 μM) was added to appropriate sample groups for the remaining 6 hours prior to harvesting.

At the end of treatments, the cells were washed twice with cold PBS and lysed with 100 μl of cell lysis reagent supplemented with DTT before freezing at -80°C. The plates were thawed on ice and the lysate, transferred to a V-shaped 96 well plate (Corning). The lysates were then centrifuged at 1000 g for 10 min at 4°C, a fraction of the supernatant (40 μl) was transferred into a 96-well white microplate (Corning). The luciferase activity was measured by direct injection of 50 μl Luciferase assay buffer into the wells using the injection needle

and pump assembly of a microplate reader (FLUOstar OPTIMA; BMG Labtech; Ortenberg, Baden-Württemberg, Germany). The protein concentration was determined using a Bradford assay as described above. Finally, the luciferase activity for each experimental condition was expressed relative to protein concentration (Relative light units or RLU/µg protein).

2.6 - Microscopy

PC12s were seeded on coverslips in 6 well plates and treated with cytokine or Dex or a combination of the two as outlined above. At the end of the treatments, cells were washed with PBS, fixed with 3.7% p-formaldehyde, permeabilized with 0.2% Triton X100 and stained with anti-GR for 1 hour at 37°C (1:20 dilution, 50 µl per coverslip; Clone, M-20, Santa Cruz Biotechnology). The coverslips were rinsed twice with PBS and incubated with 1:2000 Alexa 488 conjugated secondary and 1:1000 DAPI (Invitrogen) prepared in 1% BSA for 30 minutes in the dark at room temperature. The coverslips were washed twice with PBS and mounted on slides using PermaFluor Mountant (Thermo Scientific). The slides were visualized on a confocal microscope (Nikon TE2000, Nikon Instruments Inc.; Melville, New York, USA). A minimum of 10 fields and 50 cells were visualised.

2.7 - Data Analysis

All data are presented as the mean ± SEM (n = 3-9). Statistical significance between control and experimental groups was determined by unpaired, two-tailed t-tests or one-way ANOVA followed by a Dunnett test for post hoc analyses. Statistical analysis was performed using GraphPad Prism 5.00. Results were considered statistically significant with values of $p \leq 0.05$.

3 - Results

3.1 - Effects of Cytokines on Expression of Catecholamine Biosynthetic Enzymes

PC12 cells were treated for 6 or 24 hours with the cytokines IFN-α, IL-1β, IL-2, IL-6, IL-10, TNF-α, or CCL2 and transcript levels of TH, DBH, and PNMT were measured using RT-qPCR in order to determine the effects of cytokines on basal expression of the CA biosynthetic enzymes in adrenal chromaffin cells.

TH: Results from the RT-qPCR analysis of PC12 cells treated for 6 or 24 hours with IFN-α, IL-1β, IL-2, IL-6, or TNF-α show specific changes in TH mRNA level. IFN-α (1000 U/ml) decreased TH mRNA levels after either 6 hour (0.78-fold; $p<0.05$) of 24 hour (0.74-fold; $p<0.001$) incubation period (Fig. 7-A). IL-1β (10 ng/ml for 6 hours) decreased TH mRNA (0.74-fold; $p<0.05$) (Fig. 7-B). IL-2 (100 ng/ml for 6 hours) decreased TH mRNA to 0.73-fold ($p<0.05$) (Fig. 7-C). IL-6 decreased TH mRNA with both 10 ng/ml (0.73-fold; $p<0.01$) and 100 ng/ml (0.81-fold; $p<0.05$) treatments after 24 hour exposure (Fig. 7-D). 6 hour TNF-α treatment decreased TH expression to 0.69-fold ($p<0.001$) and 24 hour treatment decreased expression to 0.75-fold ($p<0.001$) (Fig. 7-F). Neither IL-10 nor CCL2 significantly changed TH mRNA levels with any of the conditions tested (Fig. 7- E and G). TH was significantly responsive to 5 of the 7 cytokines tested, all producing decreases in mRNA levels relative to respective control group.

DBH: Results from the RT-qPCR analysis of PC12 cells treated for 24 hours with IL-6 or TNF-α show specific changes in DBH mRNA level. 24 hour treatment with IL-6 (100 ng/ml) increased DBH expression to 1.20 fold ($p<0.05$) (Fig. 8-D). 24 hour treatment with TNF-α decreased DBH expression to 0.42-fold ($p<0.001$) (Fig. 8-F). The cytokines IFN-α, IL-1β, IL-2, IL-10, and CCL2 did not significantly modify DBH mRNA levels in any of the conditions tested.

PNMT: Results from RT-qPCR analysis of PC12 cells showed no significant changes in PNMT mRNA levels following incubation with either IFN-α, IL-1β, IL-2, IL-6 IL-10, TNF-α or CCL2 with any of the conditions tested (Fig. 9).

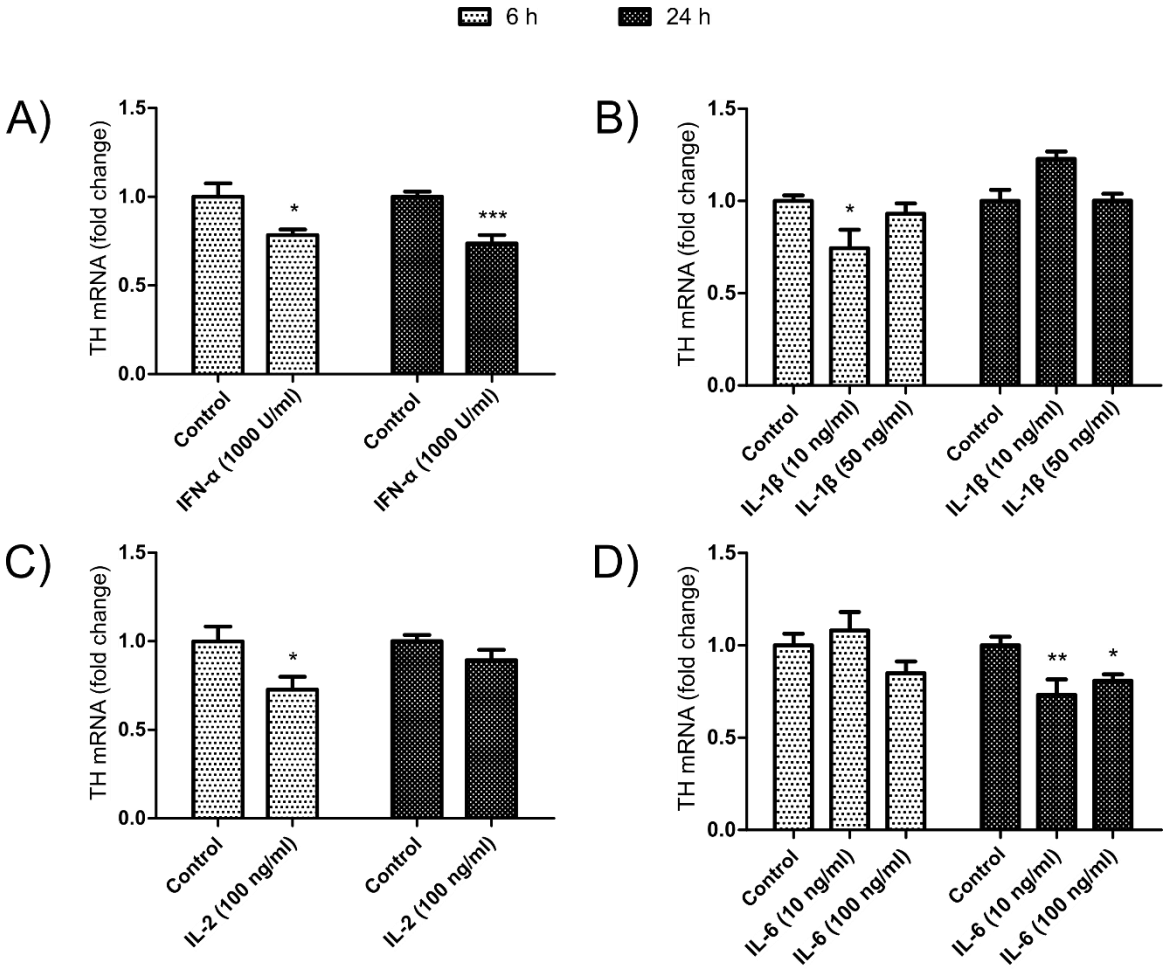

Figure 7 (A-D): Cytokine regulation of basal tyrosine hydroxylase (TH) expression in PC12 cells. Cells were treated with either cytokine suspension buffer (control) or with IFN-α (A), IL-1β (B), IL-2 (C), IL-6 (D), IL-10 (E), TNF-α (F) or CCL2 (G) for 6 hours (left) or 24 hours (right). RNA was extracted, cDNA synthesised and RT-qPCR performed for detecting TH expression. The experiment was repeated two to three times with each treatment condition made in triplicate. Fold changes were calculated using the ΔΔCt method described by Pfaffl (2001) for qPCR quantification and all genes were normalized to RPL29 expression of each sample; cytokine-treated groups were then compared to the buffer-treated control group. The fold changes of cytokine-treated groups relative to respective control group are represented graphically; error bars display mean ± SEM. Statistical significance between control and cytokine treatment was determined with either unpaired two-tailed t-test or one-way ANOVA followed by Dunnett's multiple comparison test. Significant differences between cytokine-treated and control group are denoted by *$p<0.05$ or ***$p<0.001$.

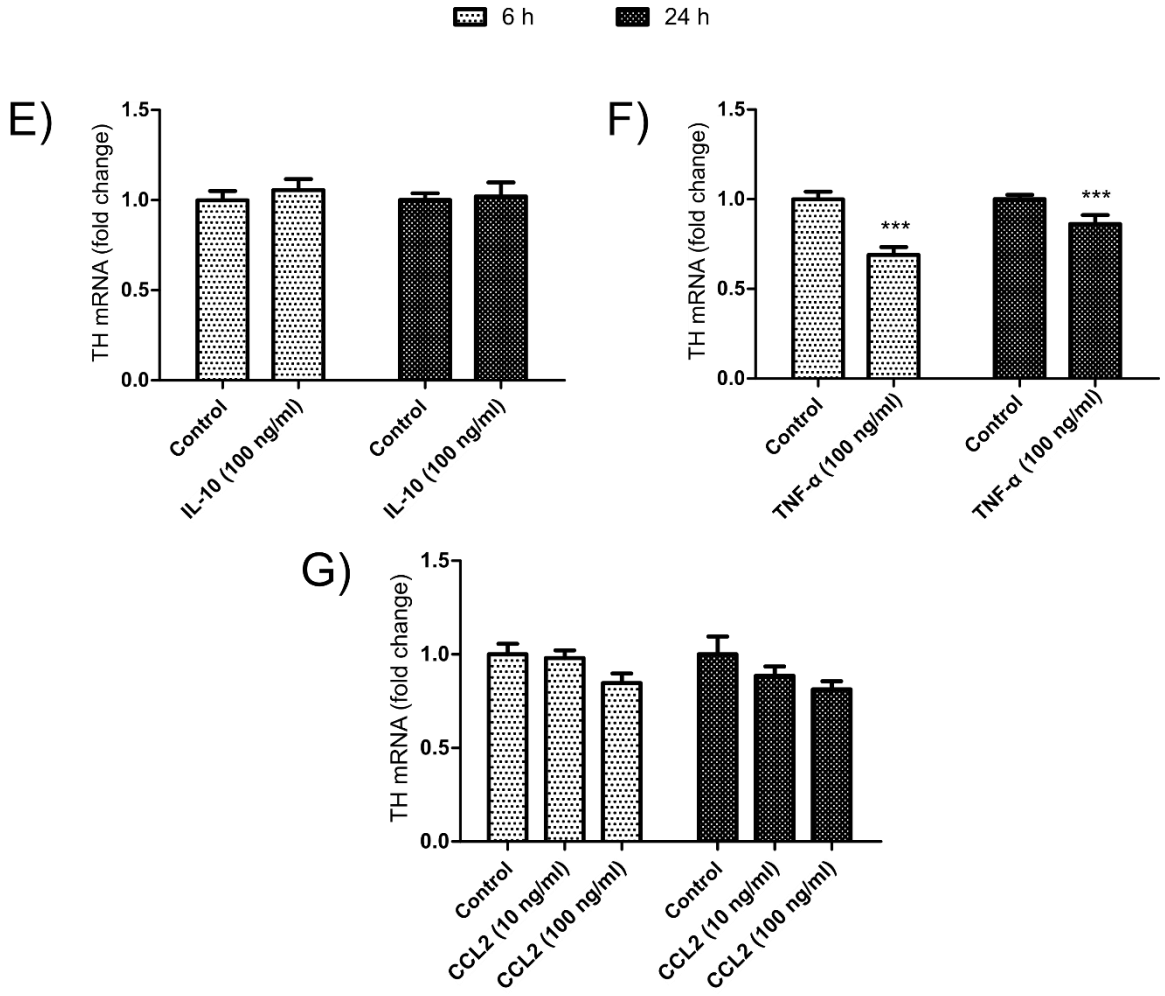

Figure 7 (E-G): Cytokine regulation of basal tyrosine hydroxylase (TH) expression in PC12 cells. Cells were treated with either cytokine suspension buffer (control) or with IFN-α (A), IL-1β (B), IL-2 (C), IL-6 (D), IL-10 (E), TNF-α (F) or CCL2 (G) for 6 hours (left) or 24 hours (right). RNA was extracted, cDNA synthesised and RT-qPCR performed for detecting TH expression. The experiment was repeated two to three times with each treatment condition made in triplicate. Fold changes were calculated using the ΔΔCt method described by Pfaffl (2001) for qPCR quantification and all genes were normalized to RPL29 expression of each sample; cytokine-treated groups were then compared to the buffer-treated control group. The fold changes of cytokine-treated groups relative to respective control group are represented graphically; error bars display mean ± SEM. Statistical significance between control and cytokine treatment was determined with either unpaired two-tailed t-test or one-way ANOVA followed by Dunnett's multiple comparison test. Significant differences between cytokine-treated and control group are denoted by *$p<0.05$ or ***$p<0.001$.

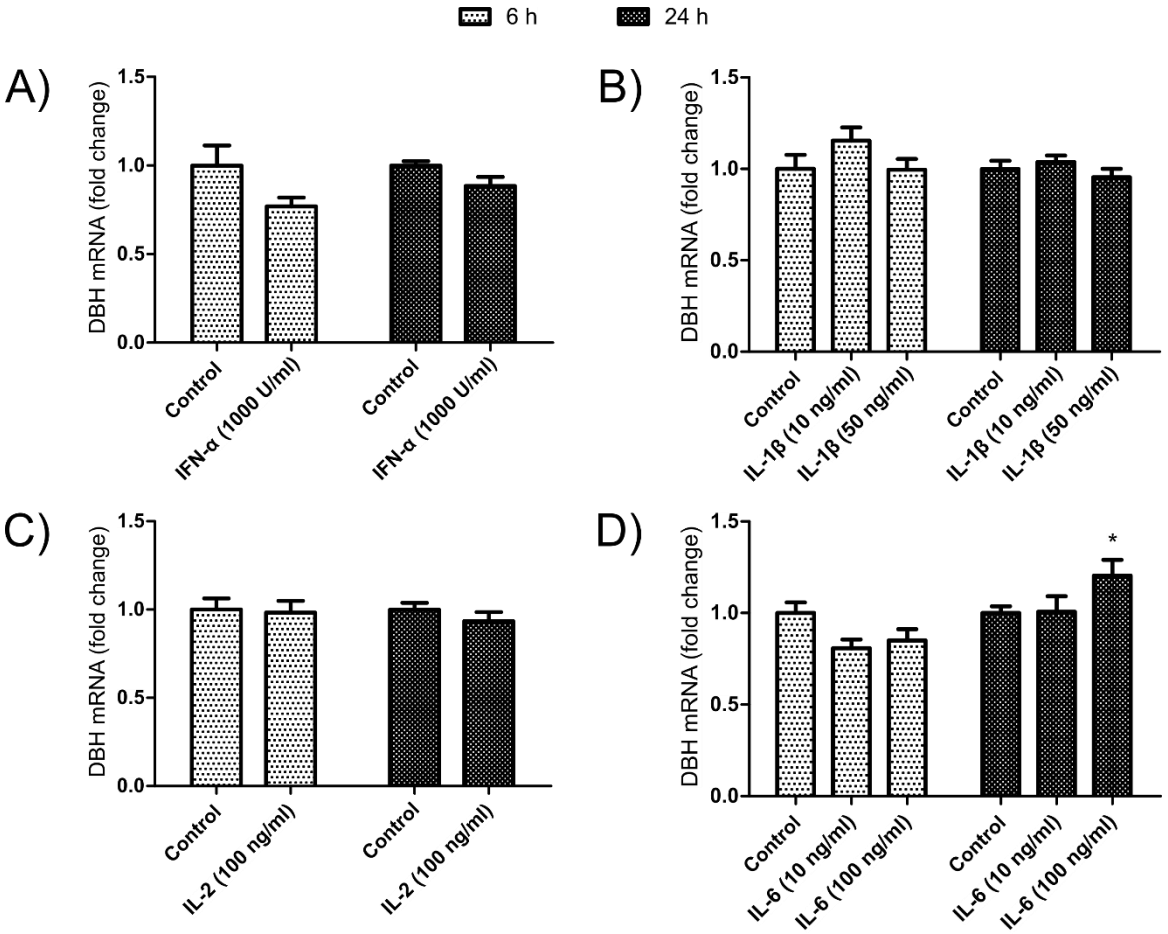

Figure 8 (A-D): Cytokine regulation of basal dopamine β-hydroxylase (DBH) expression in PC12 cells. Cells were treated with either cytokine suspension buffer (control) or with IFN-α (A), IL-1β (B), IL-2 (C), IL-6 (D), IL-10 (E), TNF-α (F) or CCL2 (G) for 6 hours (left) or 24 hours (right). RNA was extracted, cDNA synthesised and RT-qPCR performed for detecting DBH expression. The experiment was repeated two to three times with each treatment condition made in triplicate. Fold changes were calculated using the ΔΔCt method described by Pfaffl (2001) for qPCR quantification and all genes were normalized to RPL29 expression of each sample; cytokine-treated groups were then compared to the buffer-treated control group. The fold changes of cytokine-treated groups relative to respective control group are represented graphically; error bars display mean ± SEM. Statistical significance between control and cytokine treatment was determined with either unpaired two-tailed t-test or one-way ANOVA followed by Dunnett's multiple comparison test. Significant differences between cytokine-treated and control group are denoted by ***$p<0.001$.

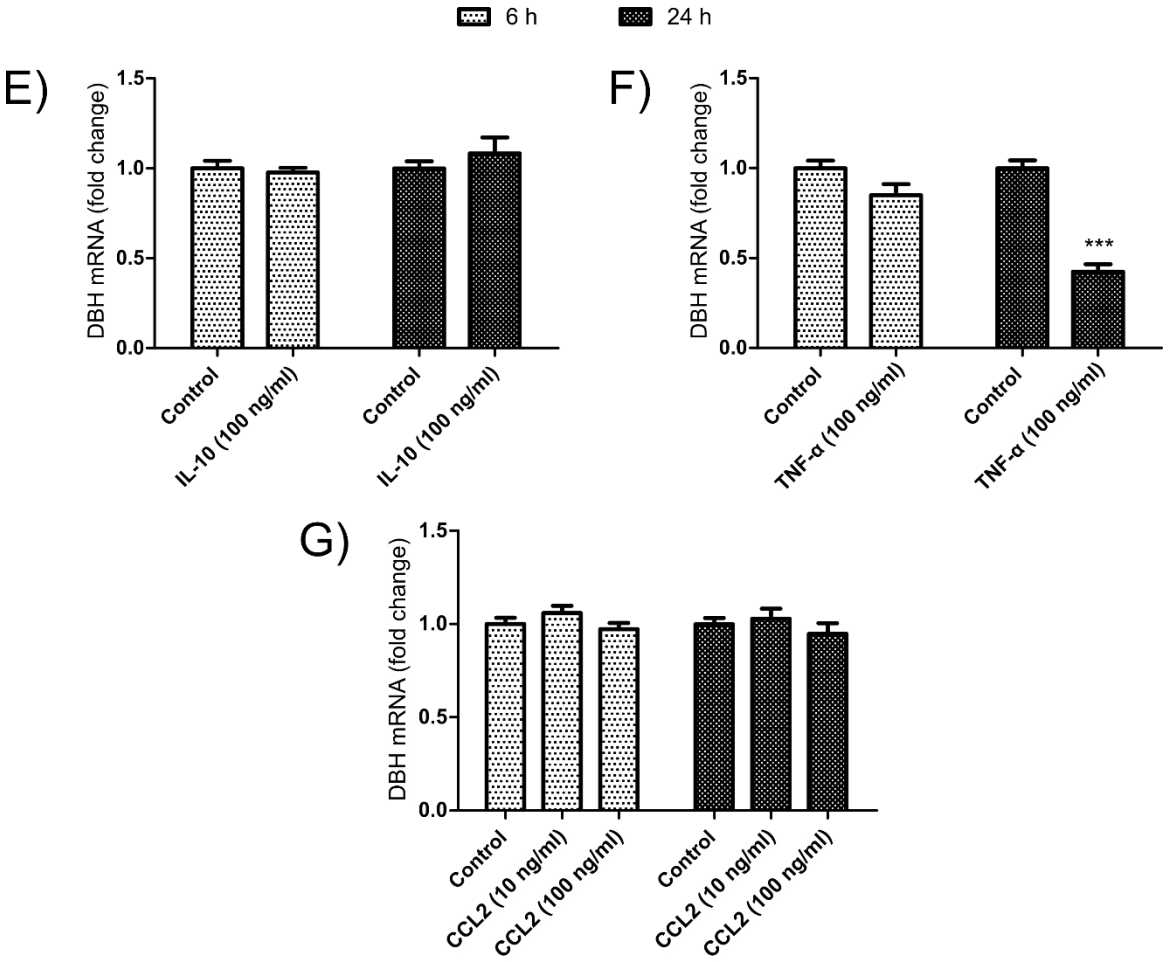

Figure 8 (E-G): Cytokine regulation of basal dopamine β-hydroxylase (DBH) expression in PC12 cells. Cells were treated with either cytokine suspension buffer (control) or with IFN-α (A), IL-1β (B), IL-2 (C), IL-6 (D), IL-10 (E), TNF-α (F) or CCL2 (G) for 6 hours (left) or 24 hours (right). RNA was extracted, cDNA synthesised and RT-qPCR performed for detecting DBH expression. The experiment was repeated two to three times with each treatment condition made in triplicate. Fold changes were calculated using the ΔΔCt method described by Pfaffl (2001) for qPCR quantification and all genes were normalized to RPL29 expression of each sample; cytokine-treated groups were then compared to the buffer-treated control group. The fold changes of cytokine-treated groups relative to respective control group are represented graphically; error bars display mean ± SEM. Statistical significance between control and cytokine treatment was determined with either unpaired two-tailed t-test or one-way ANOVA followed by Dunnett's multiple comparison test. Significant differences between cytokine-treated and control group are denoted by ***$p<0.001$.

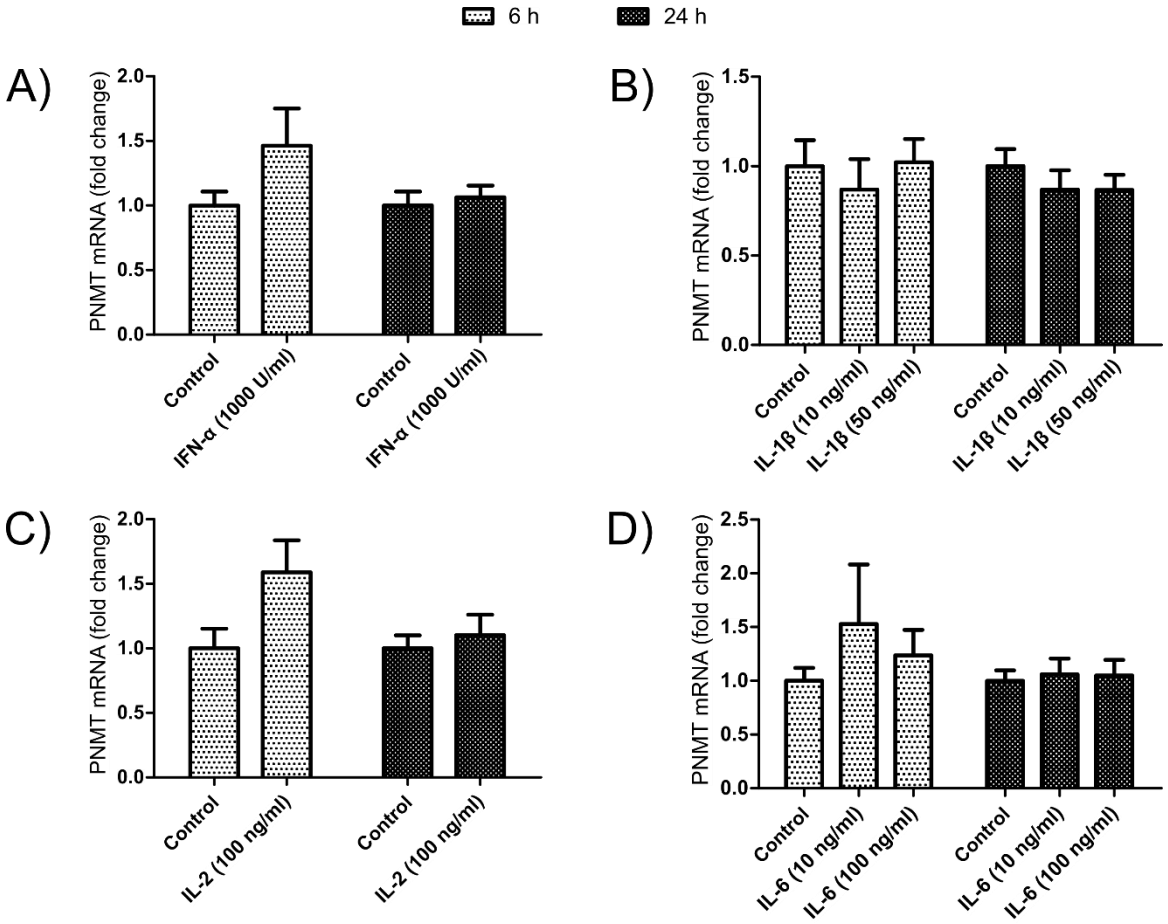

Figure 9 (A-D): Cytokine regulation of basal phenylethanolamine N-methyltransferase (PNMT) expression in PC12 cells. Cells were treated with either cytokine suspension buffer (control) or with IFN-α (A), IL-1β (B), IL-2 (C), IL-6 (D), IL-10 (E), TNF-α (F) or CCL2 (G) for 6 hours (left) or 24 hours (right). RNA was extracted, cDNA synthesised and RT-qPCR performed for detecting PNMT expression. The experiment was repeated two to three times with each treatment condition made in triplicate. Fold changes were calculated using the ΔΔCt method described by Pfaffl (2001) for qPCR quantification and all genes were normalized to RPL29 expression of each sample; cytokine-treated groups were then compared to the buffer-treated control group. The fold changes of cytokine-treated groups relative to respective control group are represented graphically; error bars display mean ± SEM. Statistical significance between control and cytokine treatment was determined with either unpaired two-tailed t-test or one-way ANOVA followed by Dunnett's multiple comparison test.

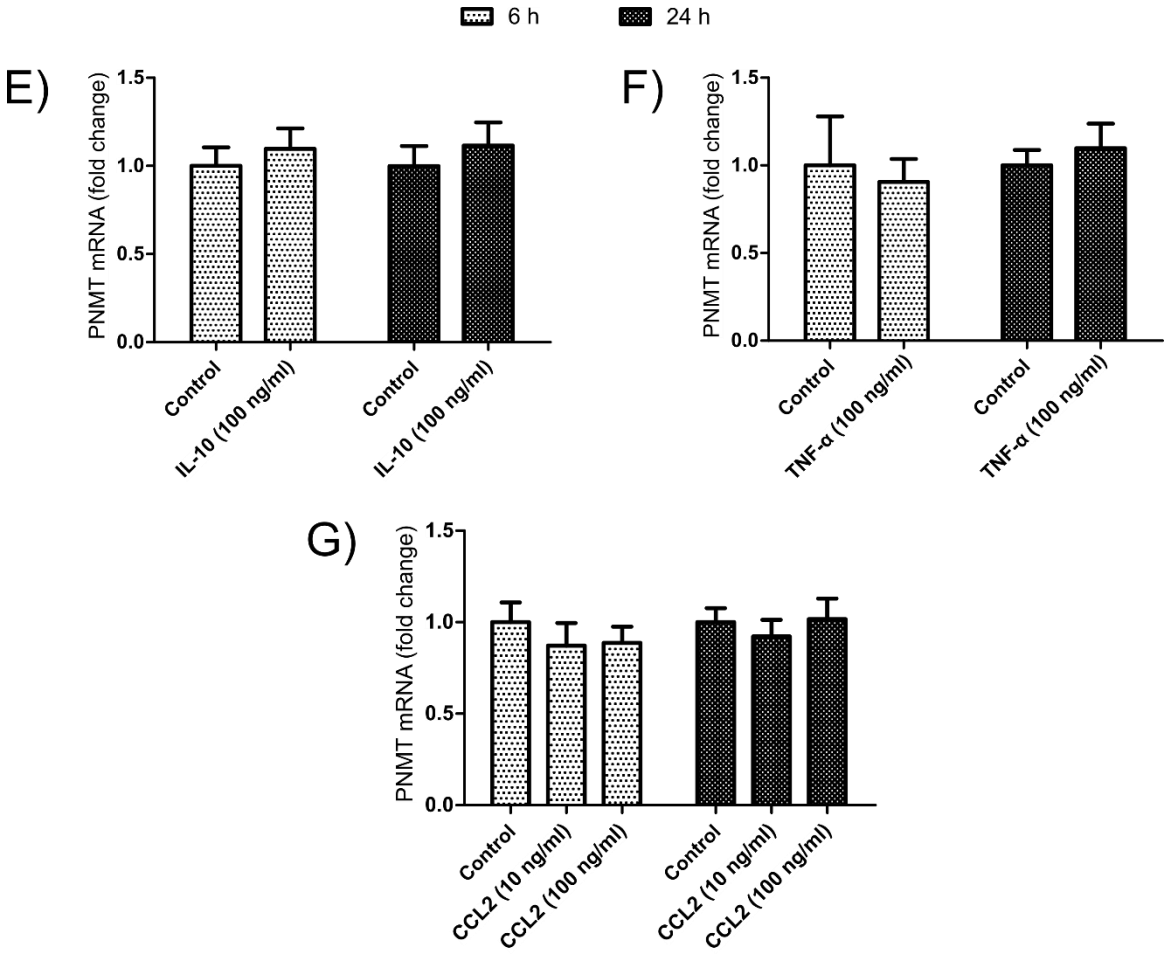

Figure 9 (E-G): Cytokine regulation of basal phenylethanolamine N-methyltransferase (PNMT) expression in PC12 cells. Cells were treated with either cytokine suspension buffer (control) or with IFN-α (A), IL-1β (B), IL-2 (C), IL-6 (D), IL-10 (E), TNF-α (F) or CCL2 (G) for 6 hours (left) or 24 hours (right). RNA was extracted, cDNA synthesised and RT-qPCR performed for detecting PNMT expression. The experiment was repeated two to three times with each treatment condition made in triplicate. Fold changes were calculated using the ΔΔCt method described by Pfaffl (2001) for qPCR quantification and all genes were normalized to RPL29 expression of each sample; cytokine-treated groups were then compared to the buffer-treated control group. The fold changes of cytokine-treated groups relative to respective control group are represented graphically; error bars display mean ± SEM. Statistical significance between control and cytokine treatment was determined with either unpaired two-tailed t-test or one-way ANOVA followed by Dunnett's multiple comparison test.

3.2 - Cytokines as Modifiers of Hormonal and Neural Activation of Catecholamine Biosynthetic Enzyme Gene Expression

To investigate modulatory effects of cytokines on hormonal or neural activation of chromaffin cells, PC12 cells were treated for 24 hours with a combination of cytokine (IFN-α, IL-1β, IL-2, IL-6, IL-10, TNF-α, or CCL2) and either Dex or Forsk. Dex is a potent GR agonist and was used to simulate chromaffin cell activation by adrenal cortical GC. Forsk is commonly used to experimentally activate adenylyl cyclase and increase intracellular cAMP levels. cAMP is a major component of the signalling mechanisms activated by the splanchnic-adrenal neurotransmitters ACh and PACAP. Dex (1 μM) and Forsk (10 μM) treatments were performed 6 hours prior to harvesting. Regulation of chromaffin cell CA biosynthetic enzyme expression was observed using RT-qPCR to measure changes in TH, DBH, and PNMT mRNA levels. The patterns of TH, DBH, and PNMT expression in response to activation by cAMP or GC were consistent with previous reports (Kim et al., 1993b; Lewis et al., 1983; McMahon & Sabban, 1992).

TH: There were significant increases in TH transcript when cells were treated with either Dex alone (2.7 fold; $p<0.001$) or Forsk alone (1.7 fold; $p<0.001$) (Fig. 10-A-G). Only IFN-α and TNF-α significantly changed TH mRNA levels when combined with drug treatments. IFN-α inhibited Forsk-induced elevation of TH transcript (0.77 fold; $p<0.001$) when compared to Forsk alone (Fig. 10-A). TNF-α combined with drug treatment decreased TH transcript when compared to either Dex (0.85; $p<0.01$) or Forsk (0.65; $p<0.001$) alone. The inhibitory effect of TNF-α on TH expression was proportionally similar in both drug-

treated and basal conditions (~25%). The cytokines IL-1β, IL-2, IL-6, IL-10, and CCL2 did not significantly change TH mRNA levels with either Dex or Forsk treatments.

DBH: Treatment of PC12 cells with Dex or Forsk resulted in increases in DBH transcript of 1.7 fold ($p<0.001$) and 4.3 fold ($p<0.001$) respectively, relative to untreated control (Fig 11-A-G). The cytokines IL-6, TNF-α, and CCL2 significantly changed DBH mRNA levels when combined with drug treatments. IL-6 potentiated the response to Dex by increasing DBH mRNA to 1.33 fold ($p<0.01$), and the response to Forsk by increasing DBH mRNA to 1.22 fold ($p<0.05$) when compared to either drug alone (Fig. 11–D). TNF-α decreased DBH mRNA expression when combined with either Dex (to 0.24 fold; $p<0.001$) or Forsk (to 0.39 fold; $p<0.01$) relative to DBH expression with either drug alone (Fig. 11– F). CCL2 had no effects on DBH transcript under either basal conditions or when combined with Dex; however, CCL2 significantly increased DBH transcript (to 1.16 fold; $p<0.05$) in Forsk-treated cells when compared to Forsk treatment alone (Fig. 11-G). The cytokines IFN-α, IL-1β, IL-2, and IL-10 did not significantly change DBH mRNA levels with either Dex or Forsk treatments.

PNMT: Expression of PNMT was increased ~25 fold ($p<0.001$) following treatment with Dex, while Forsk did not significantly change PNMT expression compared to control samples (Fig. 12-A-G). Decreased Dex-induced expression of PNMT mRNA was observed with IFN-α (to 0.59 fold; $p<0.05$; Fig. 12-A), IL-6 (to 0.31 fold; $p<0.001$; Fig. 12-D) or TNF-α (to 0.51 fold; $p<0.001$; Fig. 12-F) treatment. IL-10 (Fig. 12-E) was the only cytokine observed to potentiate (to 1.35 fold, $p<0.01$) the Dex-mediated increase in PNMT transcript

when compared to Dex alone. The cytokines IL-1β, IL-2, and CCL2 did not significantly change PNMT mRNA levels with Dex treatments.

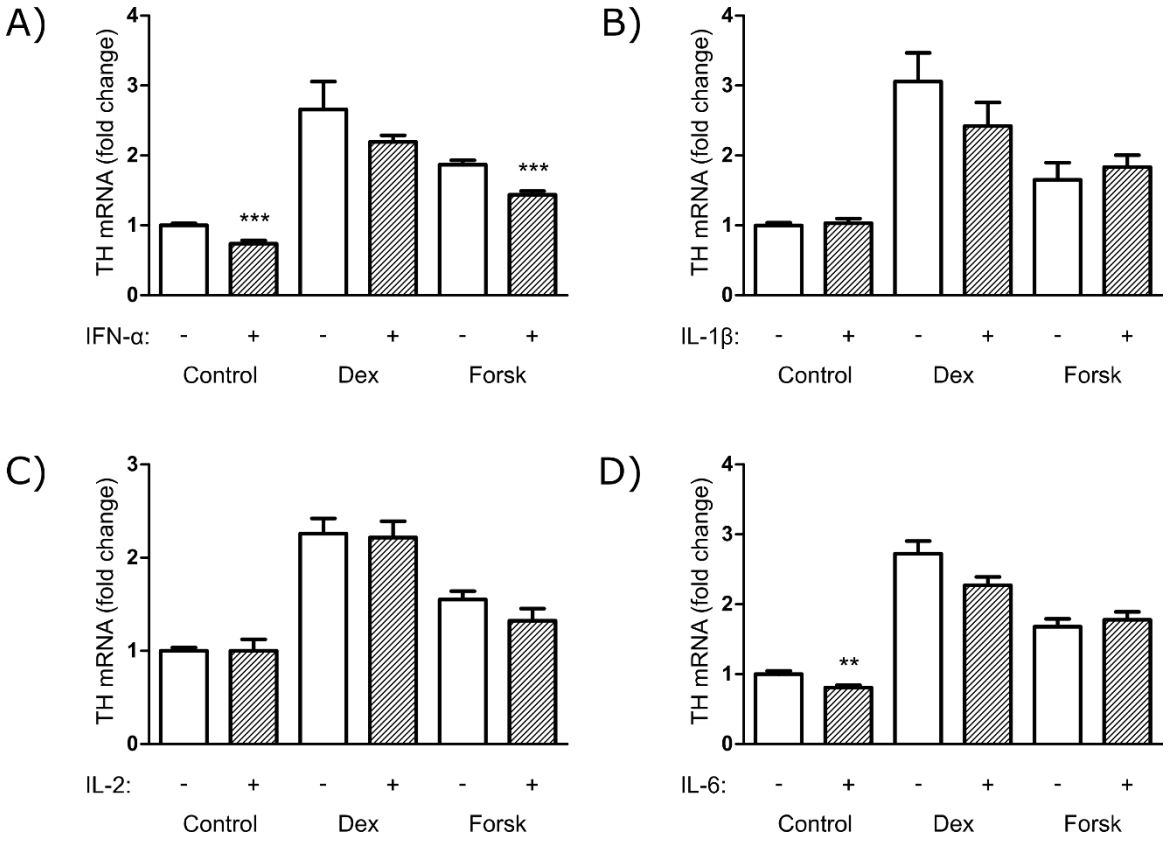

Figure 10 (A-D): Cytokine regulation of glucocorticoid or cAMP-activated tyrosine hydroxylase (TH) expression. PC12 cells were treated with either cytokine suspension buffer (control) or with IFN-α (A), IL-1β (B), IL-2 (C), IL-6 (D), IL-10 (E), TNF-α (F) or CCL2 (G) for 6 hours (left) or 24 hours (right). Dexamethasone (1 μM) or forskolin (10 μM) were added during the last 6 hours of incubation. RNA was extracted, cDNA synthesised and RT-qPCR performed for detecting TH expression. Each treatment was performed in triplicate. Fold changes were calculated using the ΔΔCt method described by Pfaffl (2001) for qPCR quantification and all genes were normalized to RPL29 expression of each sample; cytokine-treated groups were then compared to the buffer-treated control group. The fold changes of cytokine-treated groups relative to respective control group are represented graphically; error bars display mean ± SEM. Statistical significance between control and cytokine treatment was determined with either unpaired two-tailed t-test. Significant differences between cytokine-treated and control group are denoted by **p<0.01 or ***p<0.001.

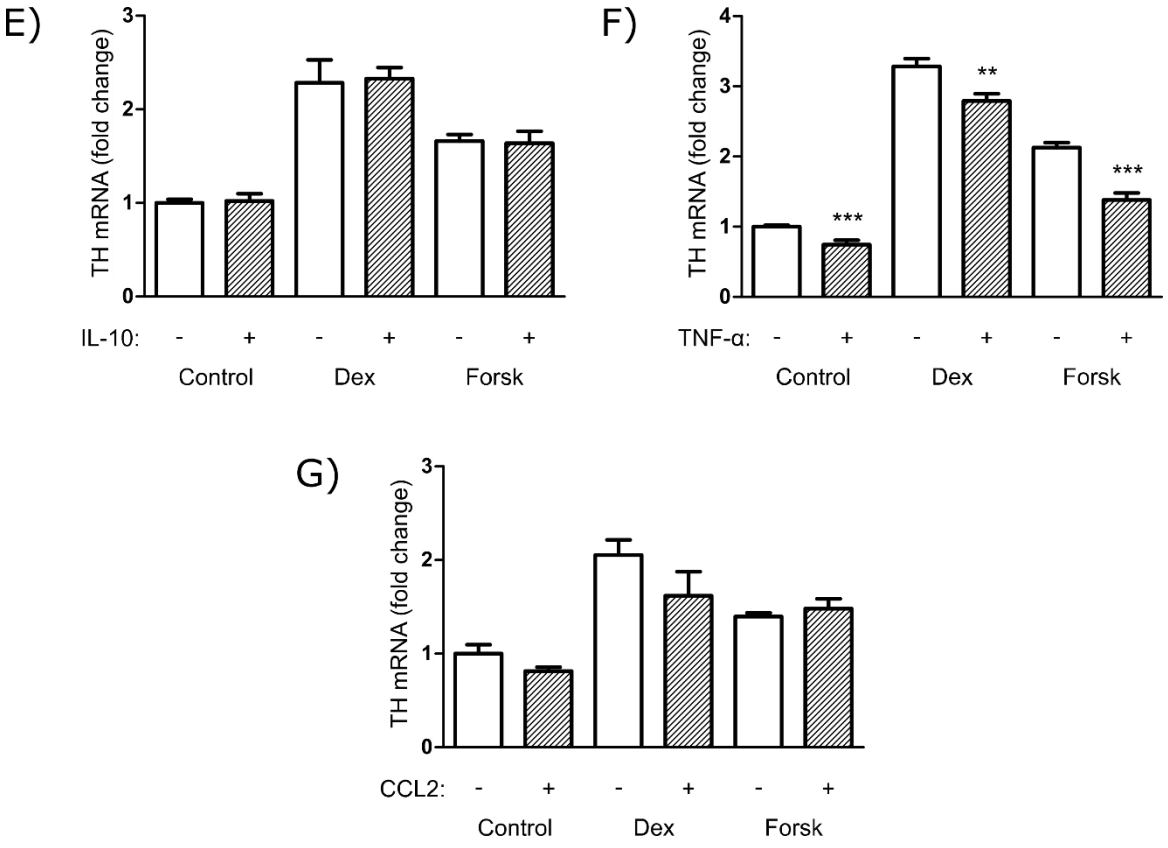

Figure 10 (E-G): Cytokine regulation of glucocorticoid or cAMP-activated tyrosine hydroxylase (TH) expression. PC12 cells were treated with either cytokine suspension buffer (control) or with IFN-α (A), IL-1β (B), IL-2 (C), IL-6 (D), IL-10 (E), TNF-α (F) or CCL2 (G) for 6 hours (left) or 24 hours (right). Dexamethasone (1 μM) or forskolin (10 μM) were added during the last 6 hours of incubation. RNA was extracted, cDNA synthesised and RT-qPCR performed for detecting TH expression. Each treatment was performed in triplicate. Fold changes were calculated using the ΔΔCt method described by Pfaffl (2001) for qPCR quantification and all genes were normalized to RPL29 expression of each sample; cytokine-treated groups were then compared to the buffer-treated control group. The fold changes of cytokine-treated groups relative to respective control group are represented graphically; error bars display mean ± SEM. Statistical significance between control and cytokine treatment was determined with either unpaired two-tailed t-test. Significant differences between cytokine-treated and control group are denoted by **$p<0.01$ or ***$p<0.001$.

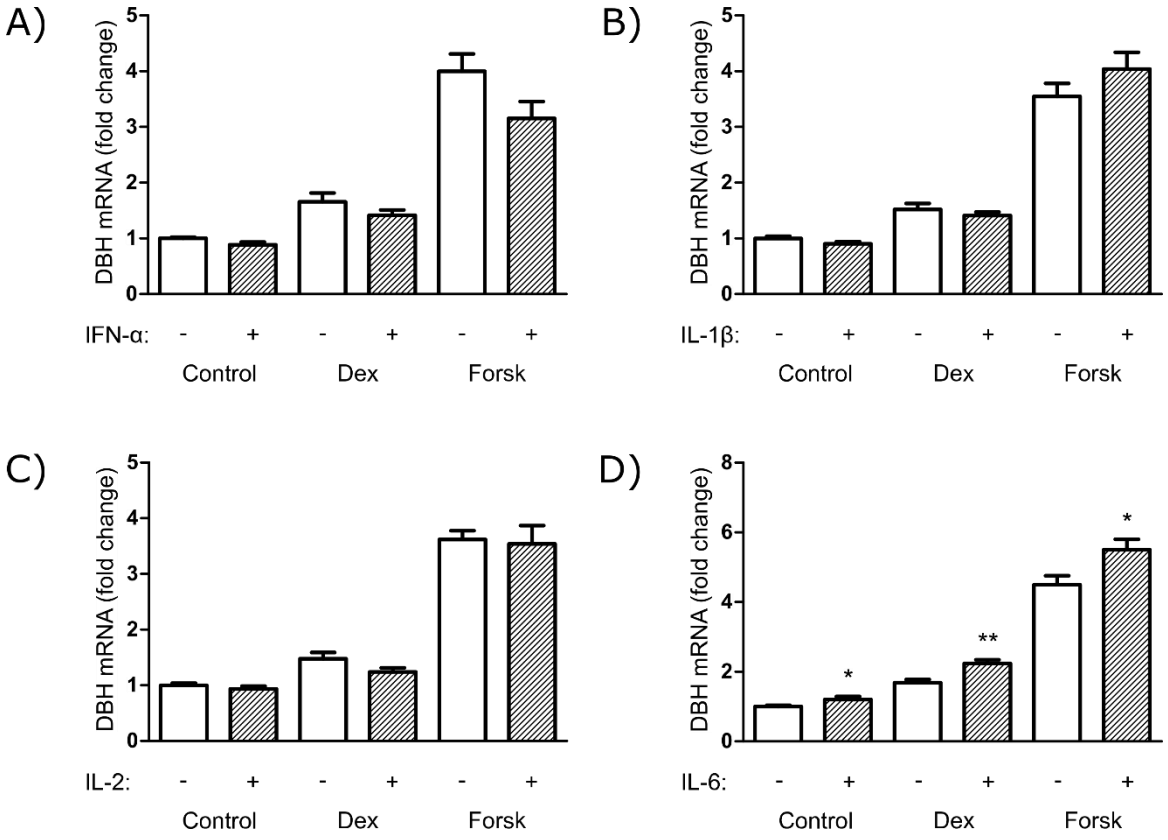

Figure 11 (A-D): Cytokine regulation of glucocorticoid or cAMP-activated dopamine β-hydroxylase (DBH) expression. PC12 cells were treated with either cytokine suspension buffer (control) or with IFN-α (A), IL-1β (B), IL-2 (C), IL-6 (D), IL-10 (E), TNF-α (F) or CCL2 (G) for 6 hours (left) or 24 hours (right). Dexamethasone (1 μM) or forskolin (10 μM) were added during the last 6 hours of incubation. RNA was extracted, cDNA synthesised and RT-qPCR performed for detecting DBH expression. Each treatment was performed in triplicate. Fold changes were calculated using the ΔΔCt method described by Pfaffl (2001) for qPCR quantification and all genes were normalized to RPL29 expression of each sample; cytokine-treated groups were then compared to the buffer-treated control group. The fold changes of cytokine-treated groups relative to respective control group are represented graphically; error bars display mean ± SEM. Statistical significance between control and cytokine treatment was determined with either unpaired two-tailed t-test. Significant differences between cytokine-treated and control group are denoted by *$p<0.05$, **$p<0.01$, or ***$p<0.001$.

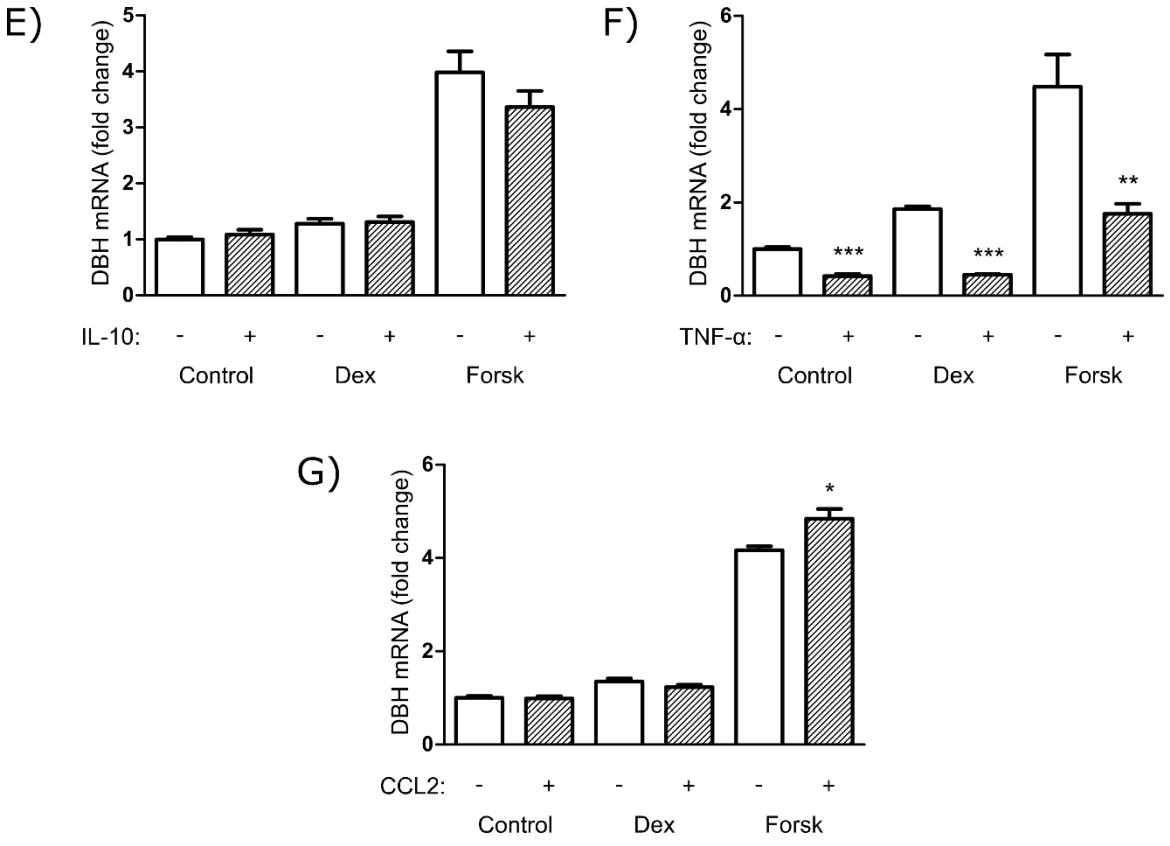

Figure 11 (E-G): Cytokine regulation of glucocorticoid or cAMP-activated dopamine β-hydroxylase (DBH) expression. PC12 cells were treated with either cytokine suspension buffer (control) or with IFN-α (A), IL-1β (B), IL-2 (C), IL-6 (D), IL-10 (E), TNF-α (F) or CCL2 (G) for 6 hours (left) or 24 hours (right). Dexamethasone (1 μM) or forskolin (10 μM) were added during the last 6 hours of incubation. RNA was extracted, cDNA synthesised and RT-qPCR performed for detecting DBH expression. Each treatment was performed in triplicate. Fold changes were calculated using the ΔΔCt method described by Pfaffl (2001) for qPCR quantification and all genes were normalized to RPL29 expression of each sample; cytokine-treated groups were then compared to the buffer-treated control group. The fold changes of cytokine-treated groups relative to respective control group are represented graphically; error bars display mean ± SEM. Statistical significance between control and cytokine treatment was determined with either unpaired two-tailed t-test. Significant differences between cytokine-treated and control group are denoted by *$p<0.05$, **$p<0.01$, or ***$p<0.001$.

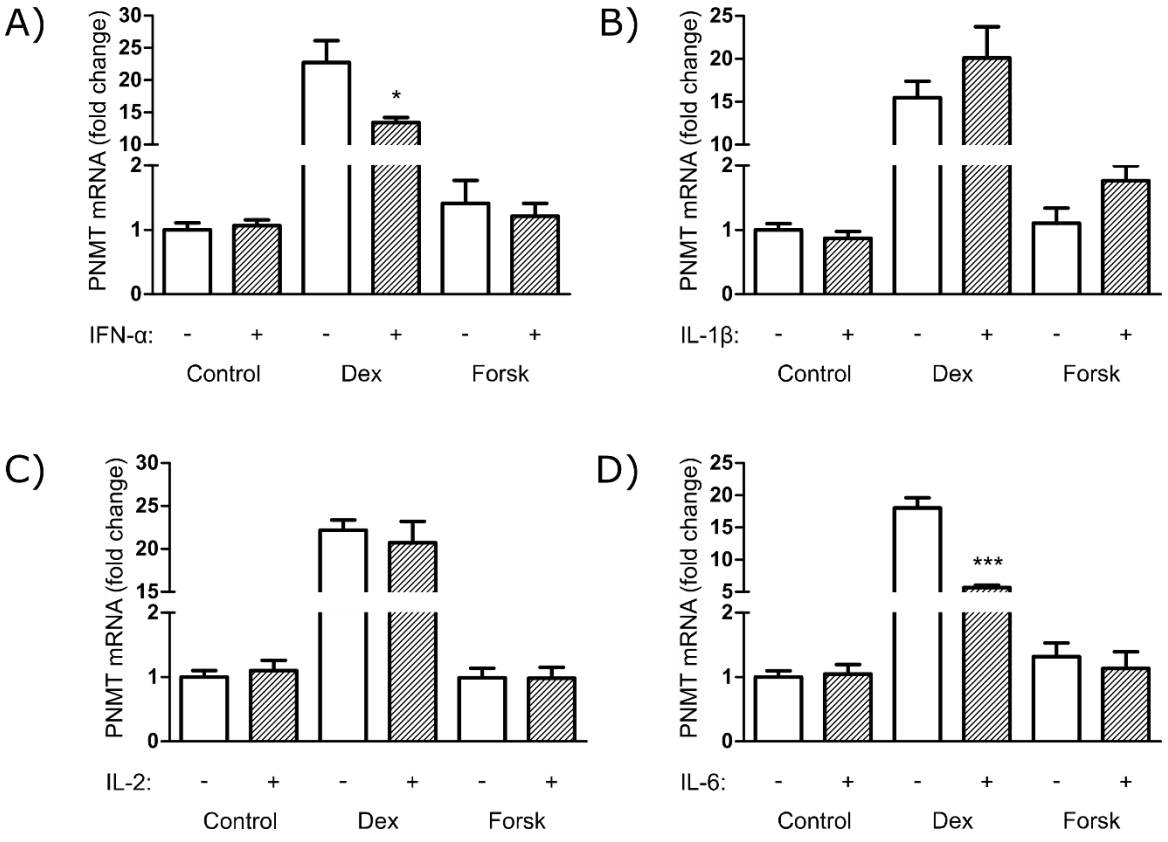

Figure 12 (A-D): Cytokine regulation of glucocorticoid or cAMP-activated phenylethanolamine N-methyltransferase (PNMT) expression. PC12 cells were treated with either cytokine suspension buffer (control) or with IFN-α (A), IL-1β (B), IL-2 (C), IL-6 (D), IL-10 (E), TNF-α (F) or CCL2 (G) for 6 hours (left) or 24 hours (right). Dexamethasone (1 μM) or forskolin (10 μM) were added during the last 6 hours of incubation. RNA was extracted, cDNA synthesised and RT-qPCR performed for detecting PNMT expression. Each treatment was performed in triplicate. Fold changes were calculated using the ΔΔCt method described by Pfaffl (2001) for qPCR quantification and all genes were normalized to RPL29 expression of each sample; cytokine-treated groups were then compared to the buffer-treated control group. The fold changes of cytokine-treated groups relative to respective control group are represented graphically; error bars display mean ± SEM. Statistical significance between control and cytokine treatment was determined with either unpaired two-tailed t-test. Significant differences between cytokine-treated and control group are denoted by *$p<0.05$, **$p<0.01$, or ***$p<0.001$.

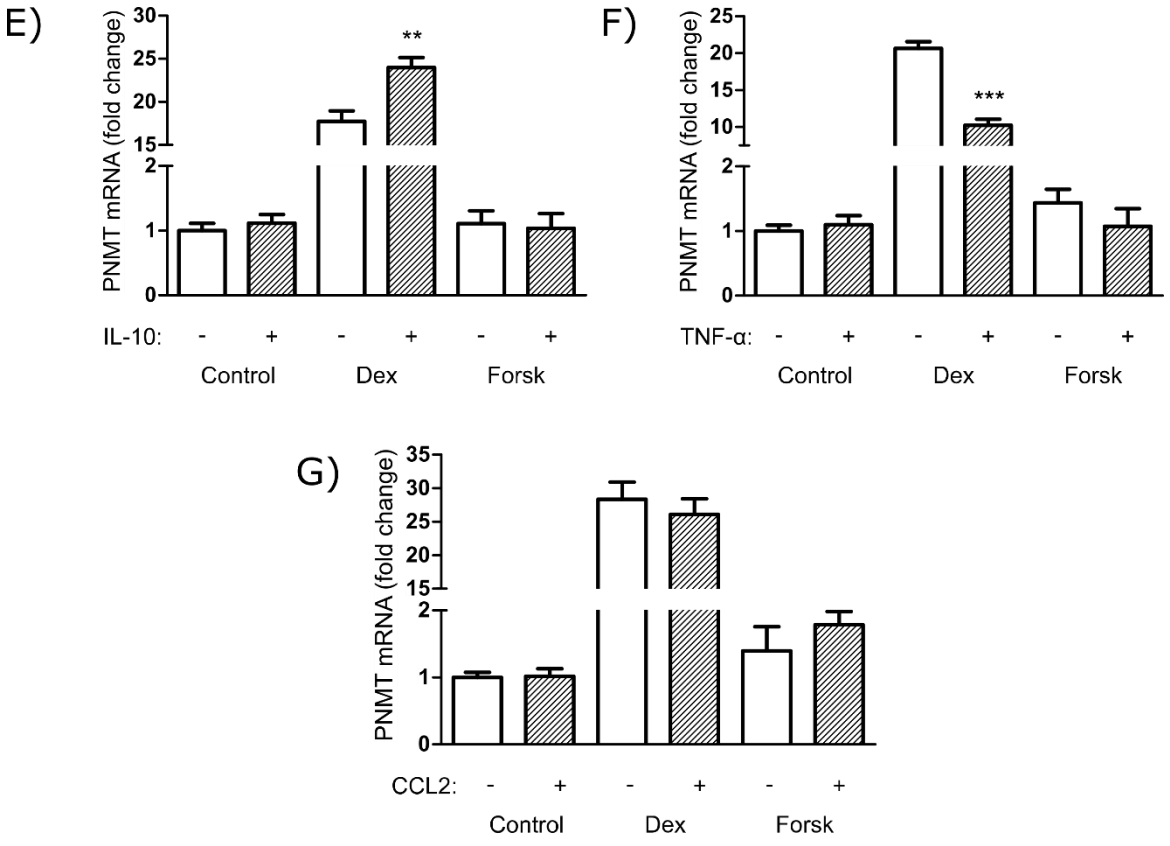

Figure 12 (E-G): Cytokine regulation of glucocorticoid or cAMP-activated phenylethanolamine N-methyltransferase (PNMT) expression. PC12 cells were treated with either cytokine suspension buffer (control) or with IFN-α (A), IL-1β (B), IL-2 (C), IL-6 (D), IL-10 (E), TNF-α (F) or CCL2 (G) for 6 hours (left) or 24 hours (right). Dexamethasone (1 μM) or forskolin (10 μM) were added during the last 6 hours of incubation. RNA was extracted, cDNA synthesised and RT-qPCR performed for detecting PNMT expression. Each treatment was performed in triplicate. Fold changes were calculated using the ΔΔCt method described by Pfaffl (2001) for qPCR quantification and all genes were normalized to RPL29 expression of each sample; cytokine-treated groups were then compared to the buffer-treated control group. The fold changes of cytokine-treated groups relative to respective control group are represented graphically; error bars display mean ± SEM. Statistical significance between control and cytokine treatment was determined with either unpaired two-tailed t-test. Significant differences between cytokine-treated and control group are denoted by *$p<0.05$, **$p<0.01$, or ***$p<0.001$.

3.3 - Effects of Cytokines on Glucocorticoid Activation of the PNMT Promoter

Results from this study show that the cytokines IFN-α and TNF-α have strong regulatory effects on basal and Dex-induced PNMT promoter activation (Fig. 13). To determine if cytokine-induced regulation of PNMT transcript levels are caused by changes in transcription rate or by post-transcriptional effects, a PNMT promoter-driven luciferase vector was used to examine promoter activation. PC12 cells were transfected with the PNMT promoter-driven luciferase vector prior to treatment with cytokines for 24 hours, followed by Dex treatment in the last 6 hours. Identical Dex treatment conditions have previously been reported to increase rat PNMT promoter activation in chromaffin cells *in vitro* (Tai et al., 2002). As expected, treatment with Dex alone increased PNMT promoter activity (2.6-fold, $p<0.001$), determined from increased luminescence (Fig. 13 A-D). Further, as seen in Fig. 13-A and 13-D, basal PNMT promoter activation was decreased by IFN-α (to 0.68 fold; $p<0.05$) or TNF-α (to 0.46 fold; $p<0.001$) treatments. IFN-α and TNF-α both decreased PNMT promoter activation with Dex, reducing expression to 0.54 fold ($p<0.001$) and 0.45 fold ($p<0.001$) respectively. IL-6 and IL-10 had no effect on PNMT promoter activation, either alone, or in combination with Dex (Fig. 13-B and 13-C).

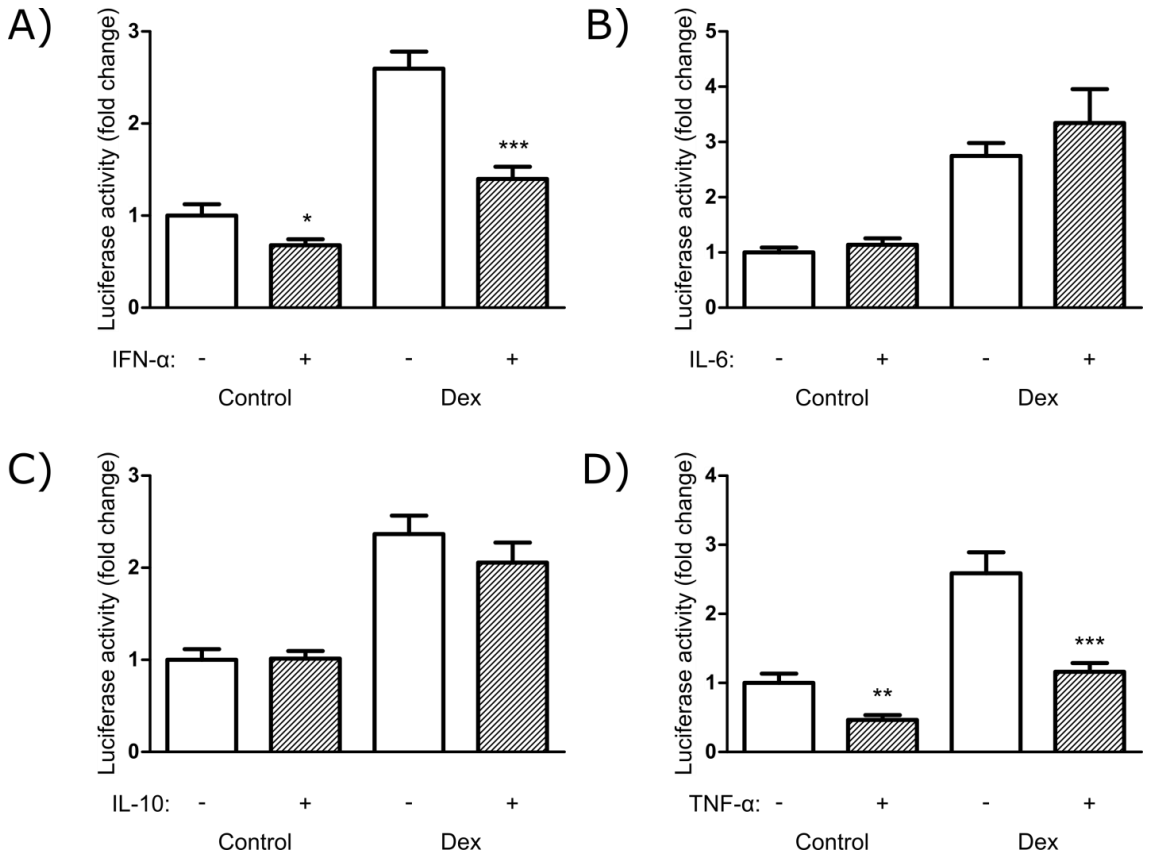

Figure 13: Cytokine regulation of phenylethanolamine N-methyltransferase (PNMT) promoter activation. PC12 cells were transfected with pGL3-PNMT 893 luciferase vector prior to treatment with IFN-α (A), IL-6 (B), IL-10 (C) or TNF-α (D) for 24 hours. Dex (1 μM) was added during the last 6 hours of incubation. Luciferase activity was calculated relative to protein expression and normalized to the untreated control (first bar). The fold changes of cytokine-treated groups relative to respective control group are represented graphically; error bars display mean ± SEM. Statistical significance between control and cytokine treatment was determined by unpaired two-tailed t-test. Significant differences between cytokine-treated and control group are denoted by ($p<0.05$, $**p<0.01$, or $***p<0.001$.

3.4 - Effect of Cytokines on Localization of Glucocorticoid Receptor

3.4.1 - Western Blot Analysis

To further analyze the mechanism by which cytokines can inhibit PNMT promoter activation, the nuclear translocation of GR in PC12 cells upon treatment with Dex was investigated. GR is a major regulator of PNMT promoter activation; the PNMT promoter contains three functional GREs which can bind GR and enhance PNMT expression. Further, as discussed above, numerous cytokines have been reported to suppress GC signalling by influencing the nuclear localization of GR. Regulation of Dex-mediated PNMT promoter activation by IFN-α and TNF-α may be mediated by inhibition of GR nuclear translocation.

PC12 cells were treated with cytokines for 24 hours and Dex was added in the last 6 hours of treatment. At the end of the treatment period cells were harvested and nuclear extracts prepared as described in Materials and Methods. Nuclear extracts were resolved using PAGE, and membranes developed by ECL to detect GR in the nuclear extracts.

Treatment of PC12 cells with Dex results in a 2.1 fold average increase in nuclear GR compared to untreated control (Fig. 14-A-D). The amount of nuclear GR protein did not change when cells were treated with IFN-α, IL-6, IL-10, or TNF-α in either the Dex-treated or the untreated groups. This suggests that none of the cytokines tested regulate PNMT promoter activation by influencing GR nuclear translocation in the experimental conditions tested.

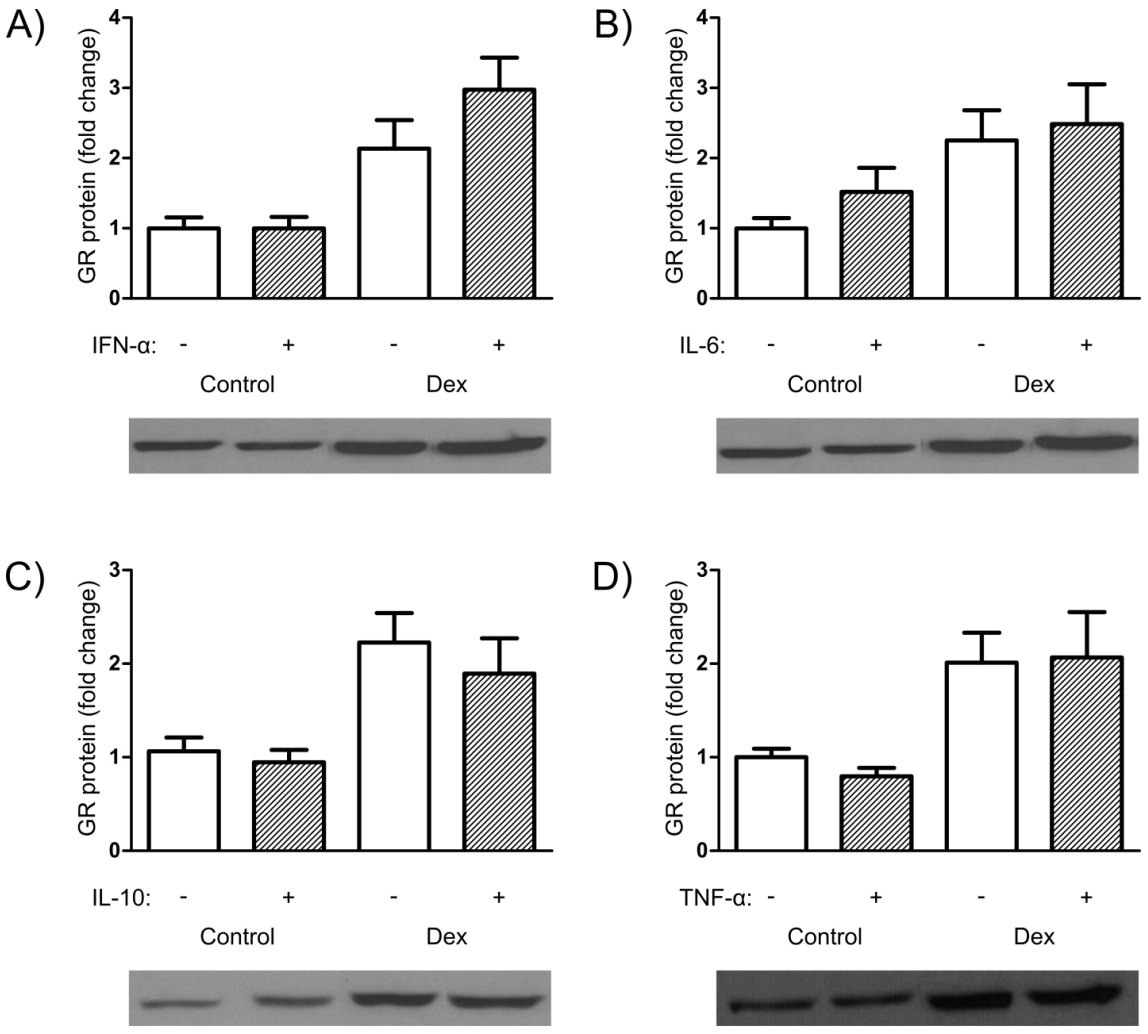

Figure 14: Analyses of glucocorticoid receptor in presence of cytokines by western immunoblotting. PC12 cells were treated with IFN-α (A), IL-6 (B), IL-10 (C) or TNF-α (D) or respective suspension buffers for 24 hours. Dex (1 μM) was added during the last 6 hours of incubation. Nuclear extracts were collected as outlined in Materials and Methods, resolved by PAGE, and GR detected by ECL. The experiment was performed two times in triplicates. Bottom panels show representative gel images for each cytokine treatment. Densitometric analysis of the GR band was done using Image J software. GR expression was normalized to the density of a random band detected by Ponceau S staining of the membrane. The graph in the upper panel depicts fold changes relative to untreated control (first bar). Error bars show mean ± SEM. Statistical analysis was performed using unpaired two-way t test.

3.4.2 - Microscopy

Observation of GR nuclear translocation by Western analyses revealed that IFN-α and TNF-α did not inhibit GR nuclear translocation. Fluorescence microscopy was used to visualize changes in cytoplasmic and nuclear GR. The experimental design was the same as for the western analyses with the exception that cells were grown on coverslips. At the end of the treatment period, cells were fixed, permeabilized and stained with an antibody against GR and with DAPI to visualize the nucleus.

As expected, when compared to untreated controls, cells treated with Dex showed markedly higher nuclear localization of GR (green channel) (Fig. 15-A). Cytokine treatment with IFN-α, IL-6, IL-10 or TNF-α alone had no effect on the localization of GR as compared to untreated cells (green channel in upper panels of Fig. 15-A-E). Further, in cells treated with cytokine and Dex, there was no difference evident in the localization of GR when compared to Dex treatment alone (green channel in lower panels of Fig. 15-A-E). Taken together, these findings are in agreement with the observations made from the Western blotting experiments, suggesting that none of the cytokines tested influence localization of GR under the experimental conditions tested.

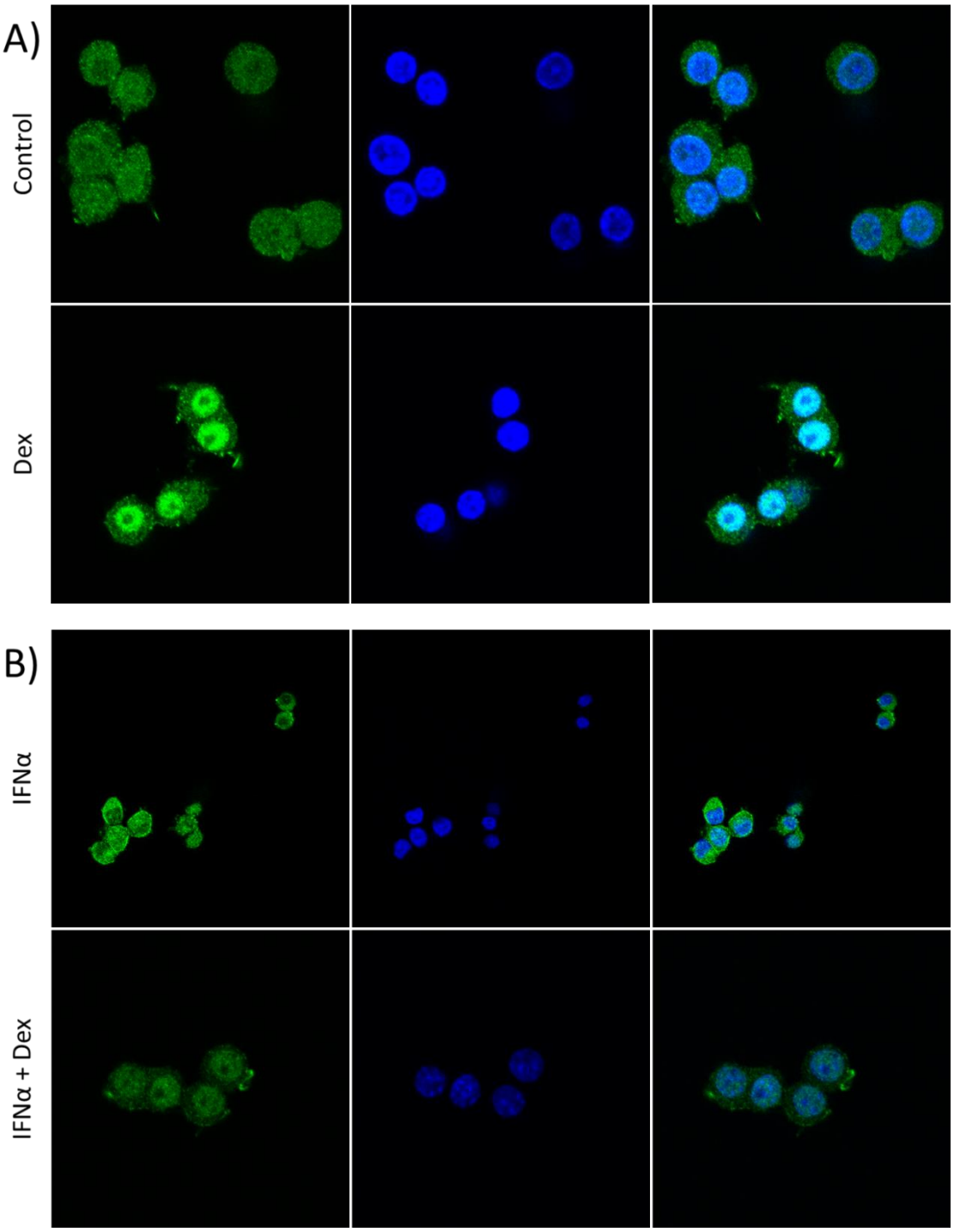

Figure 15 (A-B): Effect of cytokines on glucocorticoid receptor localization visualized by immunofluorescent microscopy. Representative untreated and Dex-treated (1 μM) controls (A) show enhanced nuclear translocation following incubation with Dex. PC12 cells were treated with IFN-α (B), IL-6 (C), IL-10 (D) or TNF-α (E) for 24 hours, with or without Dex treatment during the last 6 hours of incubation. Cells were fixed, permeabilized and stained with anti-GR followed by secondary antibody conjugated with Alexa Flour 488 (green channel; left column in a panel). Nuclei were revealed with DAPI staining (blue channel; middle column in a panel).

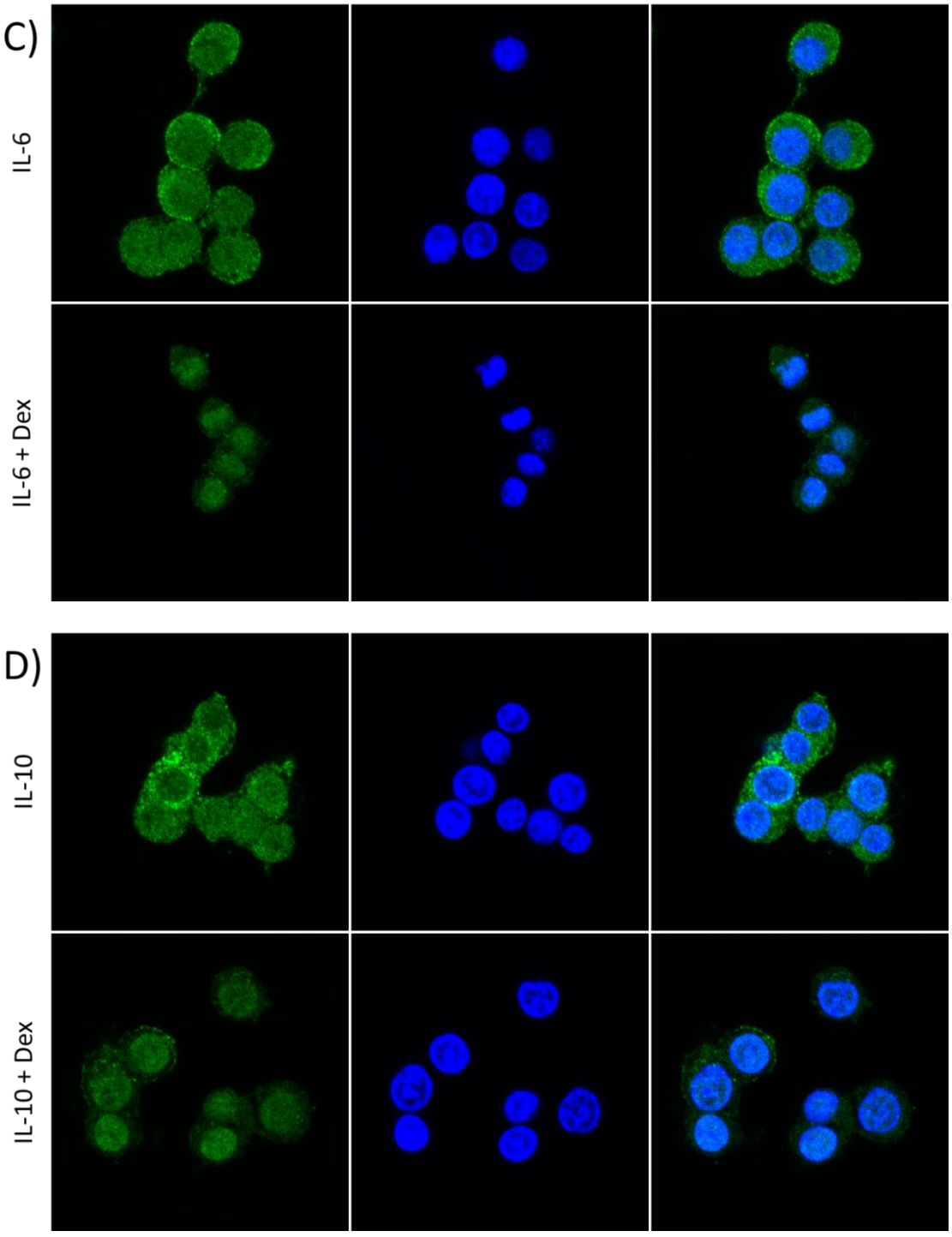

Figure 15 (C-D): Effect of cytokines on glucocorticoid receptor localization visualized by immunofluorescent microscopy. Representative untreated and Dex-treated (1 μM) controls (A) show enhanced nuclear translocation following incubation with Dex. PC12 cells were treated with IFN-α (B), IL-6 (C), IL-10 (D) or TNF-α (E) for 24 hours, with or without Dex treatment during the last 6 hours of incubation. Cells were fixed, permeabilized and stained with anti-GR followed by secondary antibody conjugated with Alexa Flour 488 (green channel; left column in a panel). Nuclei were revealed with DAPI staining (blue channel; middle column in a panel).

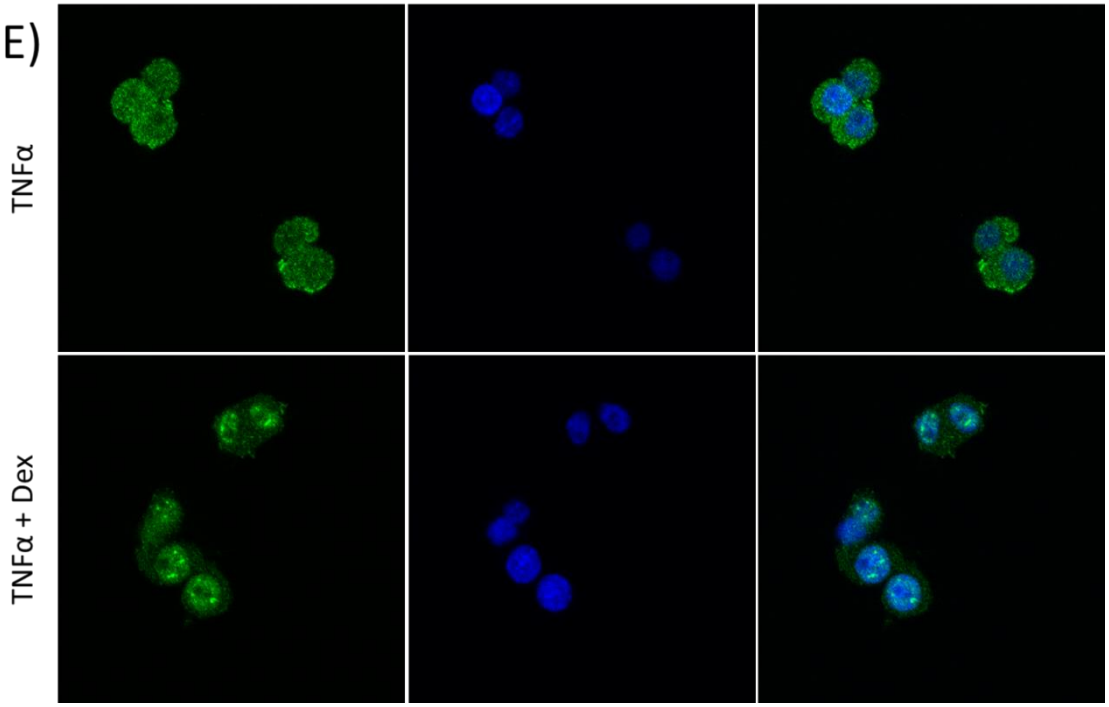

Figure 15 (E): Effect of cytokines on glucocorticoid receptor localization visualized by immunofluorescent microscopy. Representative untreated and Dex-treated (1 μM) controls (A) show enhanced nuclear translocation following incubation with Dex. PC12 cells were treated with IFN-α (B), IL-6 (C), IL-10 (D) or TNF-α (E) for 24 hours, with or without Dex treatment during the last 6 hours of incubation. Cells were fixed, permeabilized and stained with anti-GR followed by secondary antibody conjugated with Alexa Flour 488 (green channel; left column in a panel). Nuclei were revealed with DAPI staining (blue channel; middle column in a panel).

4 - Discussion

The present study provides compelling evidence that adrenal medullary chromaffin cells are capable of integrating signals from immune signalling molecules with those of the HPA and SA axes. These findings extend the current understanding of adrenal function in several important ways. This study supports, for the first time, adrenal chromaffin cell responsiveness to the cytokines IL-2, IL-10, and CCL2, and the potential regulatory effects of these cytokines on CA biosynthetic function. Moreover, robust inhibitory effects of IFN-α or TNF-α on expression of TH transcript and on PNMT promoter activation were observed. This study also is the first to determine that chromaffin cells have the capacity to integrate cytokine and GC signalling, resulting in context-specific regulation of CA biosynthetic enzyme expression. This integration of cytokine and GC signalling occurs independently of GR nuclear translocation and may rely on specific inhibitory mechanisms within the nucleus.

As shown in Table 1, many cytokines are constitutively expressed in the adrenal medulla, and can be derived locally from chromaffin cells themselves, from infiltrating immune cells, or from adjacent steroid-producing cells (Call et al., 2000; Engström et al., 2007; González-Hernández et al., 1994; Kontogeorgos, Messini, et al., 2002; Schober et al., 1998). Based on the links between cytokines and hypertension, regulation of plasma CA levels, or direct effects on adrenal chromaffin cells, seven cytokines were selected to determine their influence on CA biosynthetic enzyme transcription. The concentrations of

cytokines used in our experiments were higher than concentrations reported in circulation during hypertension; however, local adrenal cytokine concentrations may differ greatly from those measured in circulation. Indeed, it has long been known that, compared to peripheral sources, locally derived signalling molecules are particularly potent regulators of adrenal chromaffin cell function (Wurtman, 1966). It was expected to that the cytokines IFN-α, IL-1β, TNF-α, and CCL2 would increase, while IL-2, IL-6, or IL-10 would decrease in CA biosynthetic enzyme expression in PC12 cells.

Our experiments demonstrated that IFN-α decreases basal TH transcript level. Signalling by IFN-α in chromaffin cells may inhibit TH expression through activation of STAT1 and STAT2. IFN-α increases phosphorylation and nuclear translocation of STAT1 and STAT2 in bovine chromaffin cells (Douglas & Bunn, 2009). STAT1 and STAT2 directly interact with the CREB-binding protein (CBP)/p300 family of transcriptional coactivators (Bhattacharya et al., 1996; Wojciak et al., 2009; Zhang et al., 1996). Competition of multiple transcription factors for CBP/p300 has been demonstrated as a mechanism for signal-induced transcriptional repression (Hottiger et al., 1998). CBP/p300 are also coactivators of CREB, a major mediator of CRE-dependent transcription of TH in PC12 cells (Lewis-Tuffin et al., 2004; Vo & Goodman, 2001). The cAMP response element (CRE) at -45 in the proximal rat TH promoter is important for basal TH expression and cAMP response (Kim et al., 1993a; Trocmé et al., 1998). Preferential interaction of STAT1 and/or STAT2 with CBP/p300 could potentially explain IFN-α-induced decreases in TH expression in basal conditions and in combination with Forsk. Like TH, the DBH promoter also contains CRE-like motifs. Two adjacent CRE-like motifs are located in the DB1 element at approximately -180 bp in the DBH promoter; however, they appear to

preferentially bind Jun rather than CREB in the rat adrenal medulla (Sabban & Nankova, 1998). This may be why IFN-α did not have the simultaneous effect of inhibiting DBH expression. Another change observed with IFN-α treatment was an inhibition of Dex-induced PNMT mRNA expression. This finding was supported by a simultaneous reduction in PNMT promoter driven luciferase activity. The IFN-α-induced decreases in luciferase activity and PNMT mRNA with Dex treatment were proportionally similar, resulting in approximately 45% inhibition. Inhibition of Dex-induced PNMT promoter activation by IFN-α may be caused by transrepression of GR through STAT1 and STAT2 signalling. STAT1-STAT2-IRF9 complex may prevent GRIP1 coactivation of GR if concentrations of GRIP1 in chromaffin cells are limiting. Functional antagonism between STAT1-STAT2-IRF9 complex and GR signalling has been reported previously in macrophages, which express GRIP1 protein at low levels (Flammer et al., 2010). IFN-α treatment also inhibited basal PNMT promoter expression in PC12 cells in absence of Dex, suggesting that the inhibition could also occur by an alternate mechanism, independent of direct interaction between IFN-α and GR signal transduction mechanisms. The inhibitory effects of IFN-α on CA biosynthetic enzyme expression were not expected. IFN-α has been reported to increase TH Ser-31 phosphorylation and inhibit NE uptake from chromaffin cells i*n vitro*, suggesting that under basal conditions this cytokine promotes the production and release of CAs (Douglas & Bunn, 2009; Toyohira et al., 1998). Moreover, IFN-α treatment has been reported to increase circulating NE and Epi levels in humans (Corssmit et al., 1996; Pende et al., 1990). It is important to note, however, that IFN-α-induced TH Ser-31 phosphorylation is transient (returning to baseline after 20 min), and long-term (48 hour) incubation with IFN-α does not increase TH protein levels in chromaffin cells (Douglas & Bunn, 2009). A study of patients receiving acute treatment with IFN-α reported enhanced

HPA axis response, with elevated plasma ACTH and cortisol (Capuron et al., 2003). Central or peripheral administration of IFN-α has also been reported to increase electrical activity of splenic sympathetic nerve filaments in rats (Katafuchi et al., 1991). Therefore, it is plausible that increases in circulating CA levels following injection of IFN-α *in vivo* result from indirect regulatory effects on adrenal CA production. Moreover, in line with our observation of IFN-α-induced inhibition of TH expression following Forsk treatment, IFN-α has been reported to inhibit ACh-stimulated CA secretion in bovine chromaffin cells (Tachikawa et al., 1997).

IL-1β treatment decreased TH mRNA levels after short term (6 hour) incubation, but IL-1β did not significantly alter expression of TH, DBH, or PNMT in any other conditions tested, including combined treatment with Dex or Forsk. IL-1β does not appear to be an important regulator of CA biosynthetic enzymes at the transcript level. IL-1β treatment has been reported to induce increases in total TH protein concentration (beginning at 6 hours), TH Ser-40 phosphorylation, and CA release from adrenal chromaffin cells (Gwosdow et al., 1992; Joseph et al., 1995; Rosmaninho-Salgado et al., 2009; Venihaki et al., 1998; Yanagihara et al., 1994). Conversely, IL-1α/β has been reported to inhibit ACh-induced CA release in bovine chromaffin cells (Morita et al., 2004). The increases in TH protein concentration in chromaffin cells reported in the literature may result from regulation of TH translation. In the absence of increases in TH transcript, induction of TH protein may occur either through an enhanced rate of TH translation or an increase in TH protein stability. Induction of TH protein and activity without concurrent elevation of TH mRNA has been reported in rodent midbrain dopaminergic neurons through a mechanism regulating TH mRNA translation rate (Chen et al., 2008).

After 6 hour incubation with IL-2, TH mRNA was decreased and after 24 hour incubation with IL-10 Dex-induced elevation in PNMT mRNA was increased. Regulatory effects of IL-2 were not observed in any other experimental condition tested. IL-10 did not significantly change basal or Dex-induced PNMT promoter activation. IL-2 and IL-10 have both been reported to decrease blood pressure and plasma Epi concentration. It was expected that these cytokines would decrease expression of the CA biosynthetic enzymes in adrenal chromaffin cells. Observations from the present study suggest that the suppressive effect of IL-10 on plasma Epi concentration result from indirect action of the cytokine on adrenal CA biosynthesis. IL-10 potentiation of PNMT transcriptional activation by Dex may rely on post-transcriptional mechanisms, whereby integration of IL-10 and GC signalling pathways results in altered PNMT mRNA splicing or stability. The expression ratio of two distinct PNMT mRNA splice variants is subject to regulation and has been correlated with PNMT enzyme activity (Unsworth et al., 1999). IL-10 could plausibly increase the relative expression of the functional intronless PNMT transcript compared to the intron-retaining transcript without changing overall PNMT promoter activation.

The present study demonstrates that treatment with IL-6 (beginning at 24 hours) decreases basal TH transcript in PC12 cells. Treatment of PC12 cells with IL-6 has previously been reported to decrease TH protein levels (beginning at 24 hours) as well as DA and NE release (Li et al., 2012). The IL-6-induced decrease in TH protein expression reported by Li *et al.* (2012) may thus result from regulatory effects at the level of gene expression which lead to reduced TH translation. Another possible mechanism may be gp-130-mediated

destabilization of TH protein, which has been reported in experiments using sympathetic neurons treated with the IL-6 family cytokines ciliary neurotrophic factor and leukemia inhibitory factor (Shi & Habecker, 2012). Increased expression of DBH with IL-6 treatment was also observed. This increased DBH expression was present in control, Dex-, and Forsk-treated groups, suggesting that this effect is independent GC or cAMP signalling mechanisms. IL-6 signalling may cause repression of nuclear factors that bind to negative-acting *cis*-regulatory elements in the DBH promoter (Kim et al., 1998; Shaskus et al., 1995). Another possibility is that IL-6 signalling may inhibit basal Egr1 function, which could conceivably result in reduced expression of TH and PNMT, and increased DBH expression, which is similar to the overall effects observed in the present experiments (Cheng et al., 2008). Because TH functions at an earlier stage in CA biosynthesis than DBH, the inhibitory effects of IL-6 on TH expression may still limit activity of DBH, resulting in the reported IL-6-induced decreases in DA and NE release from chromaffin cells (Li et al., 2012). IL-6 also had a pronounced inhibitory effect on Dex-induced elevation of PNMT transcript in PC12 cells. IL-6 did not produce a corresponding decrease in PNMT promoter activation, suggesting that the effects of IL-6 on PNMT mRNA expression rely on post-transcriptional mechanisms. Induction of STAT3 signalling has been reported in PC12 cells treated with IL-6 (Li et al., 2012). STAT3 has also been reported to function synergistically with GR signalling in rat hepatoma H4IIE cells, forming complexes with GR in the nucleus and enhancing activation of IL-6 responsive elements and GREs (Zhang et al., 1997). Our data suggest that synergistic interaction of IL-6 signalling pathways and GR must be tissue-specific, as the effect was not observed in PC12 cells. Potential synergistic interaction of GR and STAT3 may be inhibited by other IL-6-induced signalling events in chromaffin

cells, or IL-6 may fail to induce nuclear coactivators important in the function of the STAT3-GR complex.

Incubation of PC12 cells with TNF-α produced significant changes in gene expression of all three CA biosynthetic enzymes analyzed. TNF-α significantly decreased mRNA levels of TH and DBH but did not decrease PNMT transcript level, although a decrease in PNMT promoter activation was observed in luciferase experiments. A previous study using oligonucleotide microarray analysis of TNF-α-induced changes in bovine chromaffin cell transcriptome identified upregulation of PNMT transcript after long (48 hour), but not short (6 hour) exposures to the cytokine (Ait-Ali et al., 2008). Preliminary experiments from the present study did not reveal similar increases in PNMT mRNA levels with 48 hour exposure to TNF-α; however, the concentration reported to increase PNMT was slightly higher than our maximum tested concentration of 100 ng/ml (data not shown). The difference in observations may be due to differences in the model systems used to assess cytokine regulation of chromaffin cells. Differences between transcriptomes of bovine chromaffin cells and PC12 cells following exposure to the same stimuli has been reported in the literature (Ait-Ali, Samal, et al., 2010). Our observations from luciferase assay suggest that TNF-α signalling in chromaffin cells does influence PNMT promoter activation, presumably affecting transcription factor interaction with *cis*-regulatory elements. TNF-α has been previously reported to have a suppressive effect on transactivation by the transcription factor Sp1. Experiments using mouse hepatic and adipose cells demonstrated that TNF-α signalling suppresses Sp1 binding to *cis*-regulatory elements and reduces activity of an Sp1-responsive promoter-driven luciferase construct (Barth et al., 2002; Denson et al., 2001). Promoters of TH, DBH, and PNMT all contain Sp1 binding sites.

Sp1binds to a *cis*-regulatory motif in the rat TH promoter that is critical for promoter activation (Yang et al., 1998). Sp1 has also been reported to bind to the DBH promoter, although its functional role is not fully understood (Kim et al., 1998; Seo et al., 1996). There are two functional Sp1 binding sites in the PNMT promoter and Sp1contributes to basal PNMT expression in chromaffin cells (Her et al., 1999, 2003). Taken together, TNF-α-mediated inhibition of transactivation by Sp1 could explain the observed attenuation of CA biosynthetic enzyme expression. Because TNF-α-induced decreases in gene expression with Dex or Forsk were observed in tandem with decreases in basal expression, it is not clear if the reduction in gene expression with combined application of TNF-α and either Dex or Forsk is caused by functional interaction of the signalling pathways. TNF-α-mediated inhibition of CA biosynthetic enzyme expression with Dex or Forsk may result from inhibition of a factor, such as Sp1, that is involved in constitutive PNMT expression. Such a mechanism for the repressive action of TNF-α on CA enzyme expression is supported by the observation that GR localization following Dex treatment was not significantly altered by TNF-α. Contradictory to the present observations, it was expected that TNF-α would promote expression of the CA biosynthetic enzymes. Circulating TNF-α concentrations are elevated in both mild hypertension and essential hypertension patient cohorts relative their normotensive counterparts (Chrysohoou et al., 2004; Stumpf et al., 2005). Further, it has been reported that peripherally administered TNF-α elevates plasma CA levels in rats (Darling et al., 1989). The expected potentiation of CA biosynthetic function in chromaffin cells by TNF-α was not observed, either under basal conditions or when combined with stimulators of GR or cAMP signalling. Elevation of circulating CAs by TNF-α may involve adrenal regulation through an intermediate mechanism similar to the HPA and sympathetic activation reported with IFN-α. The present study is the first

detailed investigation of the direct regulatory effects of TNF-α on the CA biosynthetic pathway in adrenal chromaffin cells. TNF-α has a marked inhibitory effect on CA biosynthetic enzyme expression. This cytokine has been reported to be constitutively expressed in the adrenal medulla (see Table 1). Thus, TNF-α may be an important local regulator of chromaffin cell CA biosynthetic function *in vivo*, perhaps functioning to decrease CA production during the progression of hypertension or other inflammatory pathologies.

Though the chemotactic cytokine CCL2 may be a promising therapeutic target for reducing blood pressure in hypertension, it does not appear to be an important regulator of chromaffin cell CA biosynthetic enzyme expression. CCL2 has been suggested to contribute to the progression of hypertension, with CCR2 antagonist reducing DOCA/salt-induced elevations in blood pressure in mice. Elevated CCL2 expression is also reported in mildly hypertensive humans and in tissues of hypertensive animals models, including mouse aorta and rat renal cortex (Blasi et al., 2003; Chan et al., 2012; Stumpf et al., 2005). This cytokine contributes to monocyte infiltration in the aortic wall in DOCA/salt hypertensive mice (Chan et al., 2012). CCL2 expression in the adrenal medulla may be increased in hypertension, originating either from the dense networks of medullary blood vessels or from the chromaffin cells themselves. Indeed, CCL2 transcript expression has been reported in bovine and rat chromaffin cells, with mRNA levels increasing *in vitro* following incubation with TNF-α (>10-fold; bovine chromaffin cells) or PACAP (3-fold; PC12_bPAC1hop cells), and *in vivo* in response to 2 hour immobilization stress (8-fold, rat medulla) (Ait-Ali, Samal, et al., 2010; Liu et al., 2008; Samal et al., 2013). Inflammation and enhanced SA activation may produce similar elevations in medullary CCL2 expression

during hypertension. Rather than directly influencing blood pressure through regulation of chromaffin cell CA biosynthetic function, the blood pressure regulatory effects of CCL2 may be mediated by macrophage infiltration and the effects of macrophages on physiological mechanisms of blood pressure control.

The cytokines IFN-α, IL-1, IL-2, IL-6, IL-10, TNF-α, and CCL2 can regulate expression of enzymes responsible for production of CAs, the major secretory product of chromaffin cells and important regulators of blood pressure homeostasis. The cytokines exerting the strongest regulatory effects included IFN-α, IL-6, and TNF-α, which exhibited predominantly inhibitory effects on CA biosynthetic enzyme expression. Constitutively expressed cytokines may have an important function in homeostatic control of CA biosynthesis by suppressing CA production during inflammation. This could be an important innate mechanism for preventing the progression of hypertension, by dampening CA production as inflammation increases with elevations in blood pressure. The inhibitory effect of cytokines on GC-induced activation of chromaffin cells may also be part of an autoregulatory loop to prevent medullary over-stimulation. This could be particularly important in inflammatory conditions where inflammation induces a compensatory increase GC secretion (an important endogenous anti-inflammatory molecule) (McEwen et al., 1997). Increased concentration of GCs in the adrenal medulla, in the absence of such an inhibitory mechanism, would result in increased CA release (Wurtman & Axelrod, 1966). Thus, immune changes that coincide with hypertension could potentially help to signal an adaptive inhibition of CA biosynthetic function of the adrenal medulla. Cytokine-mediated antagonism of GC-induced chromaffin cell activation could also help to prevent adrenal medullary over-activation in situations when inflammation leads to increased

secretion of anti-inflammatory GCs. Both effects may be protective mechanisms against the development of hypertension; disturbance of such mechanisms, either by changes in local adrenal cytokine levels or by disruption of chromaffin cell sensitivity to cytokines could be a contributing factor to the progression of hypertension in some individuals.

5 - Conclusion

Findings from the present study suggest that: (i) cytokines including IFN-α, IL-1β, IL-2, IL-6, and TNF-α have direct regulatory effects on basal expression of the CA biosynthetic enzymes TH and DBH; (ii) adrenal chromaffin cells have the capacity to integrate signals from immune, hormonal, and neural origins to produce unique regulatory effects on CA biosynthetic machinery and that these effects are particularly prominent in the regulation of PNMT by IFN-α, IL-6, IL-10, and TNF-α; (iii) the regulatory effects of some cytokines, such as IFN-α and TNF-α, involve direct control of PNMT promoter activation, whereas other cytokines, such as IL-6 and IL-10, regulate PNMT transcript levels without affecting promoter activation; and, (iv) cytokines do not regulate promoter activation by influencing GR nuclear translocation in chromaffin cells. These findings support the possibility of a novel mechanism, whereby cytokines could function as potent endogenous regulators of CA biosynthetic function in the adrenal with the possibility of contributing to the progression of hypertension.

6 - Future Directions

A potential role for multiple cytokines in regulating CA biosynthetic function of adrenal chromaffin cells has now been identified *in vitro.* It is unknown if medullary expression of cytokines that have robust regulatory effects on chromaffin cells, such as IFN-α, IL-6, or TNF-α, is changed during hypertension. Future investigations to determine changes in local cytokine concentrations in the adrenal medulla during prehypertension and overt hypertension will provide better insight into the relevance of cytokine-chromaffin cell signalling in this disease. The particular molecular mechanisms involved in integrating hormonal, neural, and immune inputs to the adrenal medulla also remain to be elucidated. The patterns of cytokine regulatory effects on CA enzyme expression provide insight into potential points of interaction. Because cytokines inhibited PNMT transcript in presence of Dex, and also altered promoter activation under these conditions, it would be interesting to elucidate if these effects could be mediated by changes in GR binding at GREs or by changes in other transcription factors that mediate PNMT transcription. Future investigations into converging immune, hormonal, and neural signalling mechanisms may also include: competition studies between STAT1/2 with CREB and GR for coactivators CBP/p300 and GRIP1 respectively, the influence of IL-6 on binding of negative acting *cis*-regulatory elements in the DBH promoter, regulation of Egr1-mediated promoter activation by IL-6, suppression of Sp1 binding to *cis*-regulatory elements or Sp1-responsive promoter-driven luciferase activity by TNF-α.

Appendices

Appendix A: Summary of significant results. Significant changes induced by cytokine treatments are expressed as ↑ for increase and ↓ for decrease relative to the applicable non-cytokine treated group. Genes analyzed included tyrosine hydroxylase (TH), dopamine β-hydroxylase (DBH), and phenylethanolamine N-methyltransferase (PNMT). Changes in transcript were determined using RT-qPCR and changes in PNMT promoter activation were determined using luciferase assay. GC = Glucocorticoid; cAMP = Cyclic Adenosine Monophosphate.

Cytokine Treatment	**Gene**	**Basal Transcript**		**GC- or cAMP-Induced Transcript**		**Promoter Activation**	
		6h	**24h**	**Dex**	**Fsk**	**Ctl**	**Dex**
IFN-α	**TH**	↓	↓		↓	-	-
	DBH					-	-
	PNMT			↓		↓	↓
IL-1β	**TH**	↓				-	-
	DBH					-	-
	PNMT					-	-
IL-2	**TH**	↓				-	-
	DBH					-	-
	PNMT					-	-
IL-6	**TH**		↓			-	-
	DBH		↑	↑	↑	-	-
	PNMT			↓			
IL-10	**TH**					-	-
	DBH					-	-
	PNMT			↑			
TNF-α	**TH**	↓	↓	↓	↓	-	-
	DBH		↓	↓	↓	-	-
	PNMT			↓		↓	↓
CCL2	**TH**					-	-
	DBH				↑	-	-
	PNMT					-	-

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