THE ROLE OF RENALASE IN CATECHOLAMINE BIOSYNTHESIS: A NEW PROTECTIVE MECHANISM AGAINST HYPERTENSION?

by

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Abstract

Renalase is a secretory protein that may protect against hypertension by reducing levels of circulating catecholamines (CAs). Renalase knockout mice are hypertensive with elevated CA levels. There are no known physiological mechanisms that fully describe renalase's influence on plasma CAs. The adrenal medulla is a major site for CA regulation, however, the role of renalase in this tissue has not been investigated. The purpose of this study was to determine a potential role of renalase in this tissue using three objectives: 1) Examine the regulation of renalase by signal activators of CA biosynthesis; 2) Determine the effects of renalase on the regulation of CA-biosynthesizing enzymes; 3) Examine the expression of renalase in hypertensive rats. For objective 1, renalase expression was analyzed in pheochromocytoma-derived PC12 cells after treatment with 10 signal activators (e.g. phorbol 12-myristate 13-acetate (PMA), forskolin, cobalt chloride ($CoCl_2$), and dexamethasone (Dex)). For objective 2, PC12 cells were treated with recombinant renalase and mRNA levels of CA-biosynthesizing enzymes were quantitated. For objective 3, renalase expression was examined in adrenal glands from spontaneously hypertensive rats (SHRs) and compared with normotensive Wistar-Kyoto rats (WKYs). RT-PCR and western blotting were performed to quantify mRNA and protein levels. Three signal activators significantly altered renalase expression; changes in renalase expression were observed after PMA, $CoCl_2$, and Dex treatment. Recombinant renalase treatment did not change the expression of CA-biosynthesizing enzymes. SHRs compared to WKYs had significantly higher levels of renalase mRNA, but not protein. Overall, this study is a first step to determine if renalase's role in the adrenal medulla is to regulate CA biosynthesis and protect against hypertension.

Keywords: Renalase, catecholamine biosynthesis, adrenal medulla, PC12 cells, hypertension, spontaneously hypertensive rat (SHR)

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2DHNAD(P)	2-Dihydronicotinamide adenine dinucleotide (with or without phosphate)
5/6Nx	5/6 Subtotal nephrectomy
6DHNAD(P)	6-Dihydronicotinamide adenine dinucleotide (with or without phosphate)
$\alpha_{1/2}$	Type 1/2 alpha adrenergic receptor
AADC	Aromatic L-amino acid decarboxylase
AC	Adenylate cyclase
ACE	Angiotensin-converting enzyme
ACh	Acetylcholine
ADH	Anti-diuretic hormone
AGT	Angiotensinogen
AKI	Acute kidney injury
Ang II	Angiotensin II
ANOVA	Analysis of variance
ANS	Autonomic nervous system
ARB	Angiotensin type-1 receptor blocker
AP1	Activator protein-1
AP1AR	Activator protein-1 activating region
AP2	Activator protein-2
AP2AR	Activator protein-2 activating region
ApoE	Apolipoprotein E
APS	Ammonium persulphate
Asp	Aspartic acid
AT1R	Angiotensin type-1 receptor
ATP	Adenosine triphosphate
β	Beta adrenergic receptor
β_2	Type 2 beta adrenergic receptor
BCA	Bicinchoninic acid
BH_4	Tetrahydrobiopterin
BME	β-Mercaptoethanol

β-NAD(P)$^{+}$	Oxidized beta nicotinamide adenine dinucleotide
BP	Blood pressure
bp	Base pair
BTG2	B-cell translocation gene protein 2
CA	Catecholamine
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
CCB	Calcium channel blocker
CD	Collecting duct
cDNA	Complementary deoxyribonucleic acid
cGMP	Cyclic guanosine monophosphate
CGRP	Calcitonin gene-related peptide
CNP	C-type natriuretic peptide
CNS	Central nervous system
CO	Cardiac output
CO_2	Carbon dioxide
$CoCl_2$	Cobalt chloride
COMT	Catechol-O-methyltransferase
CRE	Cyclic adenosine monophosphate response element
CREB	Cyclic adenosine monophosphate response element-binding protein
CSF	Cerebrospinal fluid
CTM	Charcoal-treated media
Cu^{2+}	Copper (cupric ion)
CVD	Cardiovascular disease
D1-D5	Type 1-5 dopaminergic receptor
D1R	D1-like receptor
D2R	D2-like receptor
DA	Dopamine
DAG	Diacylglycerol
DAT	Dopamine transporter
DBH	Dopamine β-hydroxylase

DEPC	Diethyl pyrocarbonate
Dex/D	Dexamethasone
DMEM	Dulbecco's modified eagle's medium
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphate
DOPA	Dihydroxyphenylalanine
DTT	Dithiothreitol
E-box	Enhancer-box
EBS	Early growth response protein 1 binding site
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGR-1	Early growth response protein 1
EMT	Extraneuronal monoamine transporter
ENaC	Epithelial sodium channel
Epac	Exchange protein directly activated by cyclic adenosine monophosphate
Epi	Epinephrine
ERK	Extracellular signal-regulated kinase
ESRD	End-stage renal disease
ET	Endothelin
EtBr	Ethidium bromide
FAD	Flavin adenine dinucleotide
Fe^{2+}	Iron (ferrous ion)
FIH	Factor inhibiting HIF1
Fsk	Forskolin
$G_{\alpha}i$/Gi	Guanine nucleotide binding protein inhibitory alpha subunit
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
$G_{\alpha}q$/Gq	Guanine nucleotide binding protein q/11 alpha subunit
$G_{\alpha}s$/Gs	Guanine nucleotide binding protein stimulatory alpha subunit
G_{β}	Guanine nucleotide binding protein beta subunit
G_{γ}	Guanine nucleotide binding protein gamma subunit

GFR	Glomerular filtration rate
Glu	Glutamic acid
GnRH	Gonadotropin-releasing hormone
GPCRs	G protein-coupled receptors
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
H^+	Proton
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HEK-293	Human embryonic kidney-293
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid
HIF1α/β	Hypoxia-inducible factor 1α/β
HOXB9	Homeobox protein B9
HPA	Hypothalamic-pituitary-adrenal
HPLC	High-performance liquid chromatography
HRE	Hypoxia response element
HRP	Horseradish peroxidase
HSP	Heat shock protein
HVA	Homovanillic acid
IgG	Immunoglobulin G
IP_3	Inositol 1,4,5-trisphosphate
IP_3R	Inositol 1,4,5-trisphosphate receptor
IQR	Interquartile range
K^+	Potassium ion
KCl	Potassium chloride
kDa	Kilodalton
KH_2PO_4	Monopotassium phosphate
KKR	Lysine-lysine-arginine
KO	Knockout
KOH	Potassium hydroxide
mAChR	Muscarinic acetylcholine receptor

MAO-A/B/C	Monoamine oxidase A/B/C
MAPK	Mitogen-activated protein kinase
Max	Myc-associated factor X
MEK	Mitogen-activated protein kinase/extracellular signal-regulated kinase kinase
Mg^{2+}	Magnesium ion
$MgCl_2$	Magnesium chloride
min	Minute
miRNA	Micro-ribonucleic acid
mmHg	Millimetres of mercury
M-MLV	Moloney Murine Leukemia Virus
mRNA	Messenger ribonucleic acid
Na^+	Sodium ion
nAChR	Nicotinic receptor
NaCl	Sodium chloride
NAD(P)H	Reduced nicotinamide adenine dinucleotide (with or without phosphate)
Na_2HPO_4	Sodium hydrogen phosphate
NaN_3	Sodium azide
NaOH	Sodium hydroxide
NE	Norepinephrine
NET	Norepinephrine transporter
NGF	Nerve growth factor
NHE	Sodium-proton exchanger
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
Nic	Nicotine
nNOS	Neuronal nitric oxide synthase
NO	Nitric oxide
NOS	Nitric oxide synthase
NOX	Nicotinamide adenine dinucleotide phosphate oxidase
NP-40	Nonyl phenoxypolyethoxylethanol 40
NPY	Neuropeptide Y

O_2	Molecular oxygen
ODD	Oxygen-dependent domain
OH	Hydroxide
P2Y	Purinergic receptor type 2 subclass Y
PAC1	Pituitary adenylate cyclase-activating polypeptide receptor 1
PACAP	Pituitary adenylate cyclase-activating polypeptide
PAGE	Polyacrylamide gel electrophoresis
PAH	Phenylalanine hydroxylase
PBS	Phosphate-buffered saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PG	Prostaglandin
PHD	Prolyl-4-hydroxylase
Phe	Phenylalanine
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PKD	Protein kinase D
PKG	Protein kinase G
PLC	Phospholipase C
PLP	Pyridoxal phosphate
PMA	Phorbol 12-myristate 13-acetate
PMCA1/4b	Plasma membrane calcium ATPase 1/4b
PMSF	Phenylmethylsulphonyl fluoride
PNMT	Phenylethanolamine N-methyltransferase
PTP	Protein tyrosine phosphatase
pVHL	Von Hippel-Lindau protein
RAAS	Renin-angiotensin-aldosterone system
Rap1	Ras-related protein 1
RIPA	Radioimmunoprecipitation assay

RKIP	Raf kinase inhibitory protein
RNA	Ribonucleic acid
Rnls/R	Renalase
ROS	Reactive oxygen species
RP-220/P	Renalase peptide 220
rpm	Revolutions per minute
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction
s	Second
SA	Sympatho-adrenal
SAM	S-adenosyl methionine
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
Ser-40	Serine-40
SHR	Spontaneously hypertensive rat
SNP	Single nucleotide polymorphism
Sol.-AC	Soluble adenylate cyclase
SP1	Specificity protein 1
SP1RE	Specificity protein 1 response element
SQR	Succinate-coenzyme Q reductase
SRC	Simple renal cyst
SSAO	Semicarbazide-sensitive amine oxidase
STAT3	Signal transducer and activator of transcription 3
T3	Triiodothyronine
TAE	Tris-acetate-EDTA
TBE	Tris-borate-EDTA
TBS-T	Tris-buffered saline with tween-20
TEMED	Tetramethylethylenediamine
TF	Transcription factor
TH	Tyrosine hydroxylase
TM-AC	Transmembrane adenylate cyclase

TrkA	Tropomyosin receptor kinase A
TSS	Transcriptional start site
Tyr	Tyrosine
Ub	Ubiquitin
UV	Ultraviolet
VGCC	Voltage-gated calcium channel
VIP	Vasoactive intestinal peptide
VMA	Vanillylmandelic acid
VMAT	Vesicular monoamine transporter
VPAC1	Vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide receptor 1
WKY	Wistar-Kyoto rat
ZBP-89	Zinc-finger binding protein-89

1 – Introduction

1.1 – Overview

Catecholamine (CA) regulation is an important physiological process that can be used to help explain the complex etiologies of hypertension. Patients with hypertension, when compared with normotensive individuals, commonly have altered CA regulatory processes throughout the body, resulting in higher levels of CAs in the blood (Currie, Freel, Perry, & Dominiczak, 2012; Goldstein, 1983). The CAs adrenaline and noradrenaline are potent vasoconstrictors, which is why they are regulated by several intricate mechanisms of homeostatic control (Guimarães & Moura, 2001; Kumer & Vrana, 1996). Many of these regulatory mechanisms have not been fully characterized and it is likely that there are others still to be discovered.

One example of a potentially novel regulator of CAs is the protein, renalase. Renalase knockout (KO) mice have elevated plasma CA levels, lower body mass, higher blood pressure (BP), and tachycardia (Y. Wu et al., 2011). Additionally, recombinant renalase administration can decrease systolic and diastolic BP for up to 24 hours (Desir, Tang, et al., 2012). Nevertheless, there are no complete explanations for renalase's regulation of BP and CA levels. Therefore, the following review will serve to summarize the current understanding of the physiological connections between hypertension, CAs, and renalase.

1.2 – The Relationship Between Catecholamines and Hypertension

1.2.1 – *Modern Issues of Hypertension on Human Health*

Hypertension, or high BP, is frequently described as one of the biggest medical challenges of the twenty-first century. This disease is defined as a systolic and/or diastolic BP greater than or equal to 140mmHg and 90mmHg, respectively (J. A. Whitworth & World Health Organization, International Society of Hypertension Writing Group, 2003). One major medical issue of hypertension is the positive correlation between BP and the risk of developing various diseases such as cardiovascular disease (CVD), renal disease, Alzheimer disease, gout, and age-related maculopathy (Carretero & Oparil, 2000; Kivipelto et al., 2002; Roubenoff et al., 1991; Sperduto & Hiller, 1986). In 2010, it was estimated that hypertension affects 1.39 billion people, or 31.1% of the adult human population (Mills et al., 2016). Considering the health care expenditures in developed countries, it is surprising to know that 22.6% of adult Canadians were reported to have hypertension in 2013 (Padwal, Bienek, McAlister, & Campbell, 2016). A reason for this finding is that approximately 95% of cases of hypertension have unknown causes (Cowley, 2006). Such cases are named essential hypertension (also known as primary/idiopathic hypertension), while cases with known causes are named secondary hypertension (Carretero & Oparil, 2000). When considering a cause of hypertension, there are countless potential dysregulations of BP, carried out via genetic mutations and/or environmental factors, and they have required the use of many categories of anti-hypertensive medications. These include diuretics, angiotensin-converting enzyme (ACE) inhibitors, calcium-channel blockers (CCBs), angiotensin type-1 receptor blockers (ARBs), β-blockers, and several other groups that are less-commonly used (Weber et al., 2014). The cost of these medications, along with the cost of hospitalizations, physician claims, and ambulatory care, were approximated to be $13.9 billion in 2010 for Canada alone, thus placing a

large burden on the overall economy (Weaver et al., 2015). Despite our current efforts to treat hypertension, studies have estimated 10-30% of cases are categorized as resistant hypertension (Calhoun et al., 2008; Viera, 2012; Yaxley & Thambar, 2015). Resistant hypertension is defined as BP that remains above a treatment goal, under the condition that the patient has tried three different classifications of anti-hypertensive medications concurrently (Calhoun et al., 2008). Taken together, essential and resistant hypertension reveal that our understanding of this disease is limited. Considering the high risk, prevalence, and cost of hypertension, researching new pathophysiological mechanisms of hypertension remains an important component of healthcare. A key to revealing these mechanisms is to increase our understanding of BP homeostasis.

1.2.2 – *Understanding Blood Pressure Regulation*

The magnitude of one's BP can be generalized as relating to two hemodynamic factors: cardiac output (CO) and vascular resistance (Cowley, 2006). CO is defined as the volume of blood pumped by the left or right heart in a specific time, while vascular resistance is a measurement of the resistance of blood flow through the systemic or pulmonary circulations (Bigatello & George, 2002). Many physiological variables are known to influence CO and/or vascular resistance such as heart rate (chronotropic effects), cardiac contractility (inotropic effects), blood and interstitial fluid volumes, arterial and venous diameters, blood viscosity, and vascular compliance (Bigatello & George, 2002; Eisner, Caldwell, Kistamás, & Trafford, 2017; Forconi, Wild, Munzel, & Gori, 2011; A. Y. Hwang, Dietrich, Pepine, & Smith, 2017). Interactions between these variables and BP are summarized in Figure 1. In this figure, it is demonstrated that the regulation of these variables is a responsibility that is shared across multiple organs and tissues.

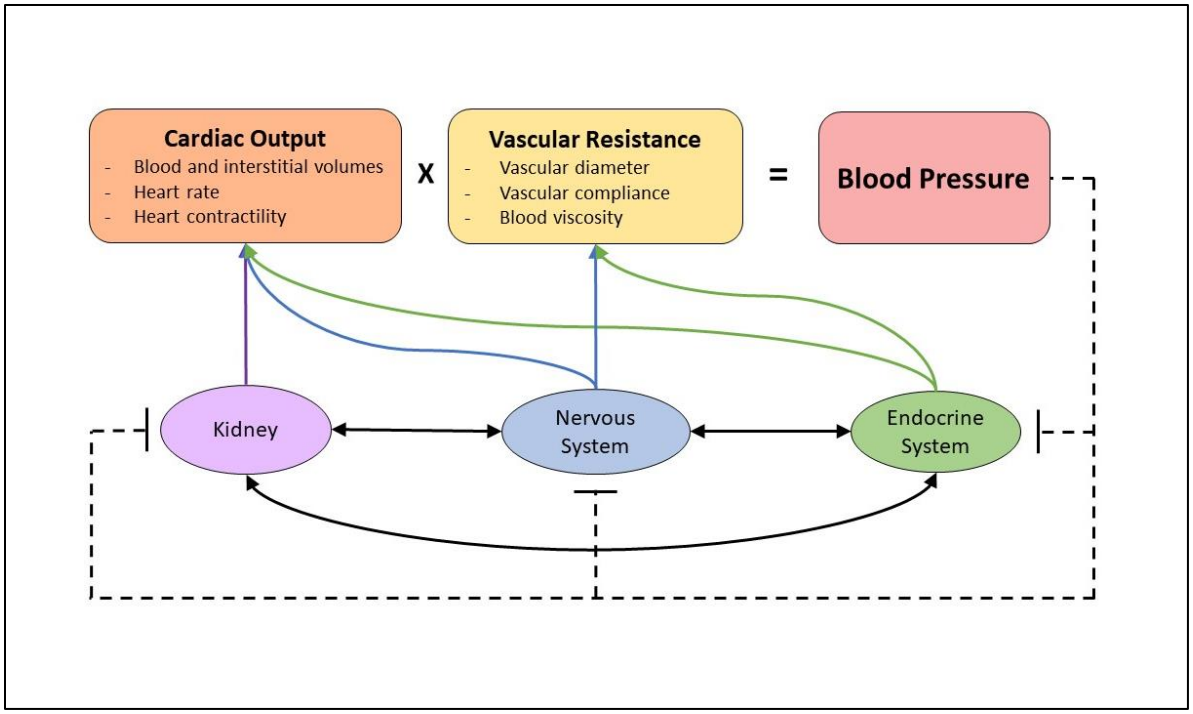

Figure 1: Flow chart of blood pressure regulation. Measurements of blood pressure are attained from the product of cardiac output (CO) and vascular resistance. Renal, nervous, or endocrine tissues regulate both physiological factors directly or indirectly via cross-talk between tissue types. Dashed lines indicate inhibition from negative feedback mechanisms. Image is modified from Cowley, 2006.

The nervous system uses several communication mechanisms that alter BP. Such mechanisms include the activities of the sympathetic and parasympathetic nervous systems. Sympathetic efferent fibers innervate the heart, blood vessels, adrenal medulla, and kidney (Chopra, Baby, & Jacob, 2011; Guyenet, 2006). In these tissues, they release norepinephrine (NE) or acetylcholine (ACh) from post-ganglionic terminals as a signal to increase BP (Purves et al., 2001). On the other hand, parasympathetic efferent fibers innervate the heart and only release ACh from post-ganglionic terminals as a signal to decrease BP (Guyenet, 2006; Purves et al., 2001). The action to either increase or decrease BP is partly determined by continuous afferent sensory signaling from baroreceptors and chemoreceptors. The baroreceptors sense changes in BP and intra-renal pressure and are found in various areas across the cardiovascular system (e.g. the carotid sinus and aortic arch) and the kidney (Guyenet, 2006; Kougias, Weakley, Yao, Lin, & Chen, 2010). Chemoreceptors are located in the carotid artery and kidney and sense changes in O_2, CO_2, or particular ion concentrations (Booth, May, & Yao, 2015; Purves et al., 2001). Other areas of the nervous system, especially the hypothalamus, can create indirect effects on BP by regulating the release of hormones (Ayada, Toru, & Korkut, 2015).

The kidney is another vital regulator of BP. Its actions consist of blood volume control, renin-angiotensin-aldosterone system (RAAS) activation, and electrolyte homeostasis (Wadei & Textor, 2012). The kidney receives signals to perform these actions from sympathetic nerves, the RAAS, and the pituitary hormone, anti-diuretic hormone (ADH) (Johns, 2013). Nephrons are capable of self-regulation by sensing components in the blood and filtrate by the juxtaglomerular complex, tubules, and collecting duct (CD) (Wadei & Textor, 2012). Blood volume control is mainly carried out through changes in the glomerular filtration rate (GFR), afferent arteriole vasoconstriction, and water reabsorption (Johns, 2013). These changes influence BP through a

direct relationship between CO and blood volume (Cowley, 2006). RAAS activation is initiated by juxtaglomerular cells with the secretion of renin (Atlas, 2007). Renin secretion is a necessary step to the formation of angiotensin II (Ang II) and subsequently, aldosterone, which together increase BP through numerous system-wide effects (Atlas, 2007). Additionally, the role of electrolyte reabsorption can be considered as a major influence on BP as there are many studies showing a positive correlation between blood concentrations of electrolytes (usually sodium – Na^+) and BP (Jones, 2004). These correlations are not fully understood because of the general involvement of electrolytes in most physiological mechanisms across the body. However, they can be partially explained by the notion that salt increases blood volume and left ventricular mass (Meneton, Jeunemaitre, de Wardener, & Macgregor, 2005).

Myriad other contributions to BP regulation come from the endocrine system. These contributions involve the traditional endocrine glands, as well as tissues with specialized endocrine function. Regarding sex hormones, there is evidence that estrogens can decrease BP in several ways, including a direct signaling action on the vasculature to elicit vasodilation (Dubey, Oparil, Imthurn, & Jackson, 2002). Further, the thyroid hormone, triiodothyronine (T3), can induce increased systolic BP when T3 is high, or increased diastolic BP when T3 is low (Danzi & Klein, 2003). A notable tissue with specialized endocrine function is the endothelium. This tissue can sense variable conditions in the blood and consequently secrete vasoconstrictive peptides, such as endothelins (ETs), or vasodilatory compounds, such as prostaglandins (PGs), nitric oxide (NO), adenosine, C-type natriuretic peptide (CNP), and endothelium-derived factors (Inagami, Naruse, & Hoover, 1995). The adrenal glands reputably create stress-related BP changes by producing steroids (e.g. aldosterone and cortisol) and the CAs, epinephrine (Epi), and NE (Ayada et al., 2015). Two important neuroendocrine mechanisms for stress-related changes in BP are the

hypothalamic-pituitary-adrenal (HPA) axis and sympatho-adrenal (SA) axis, which results in cortisol (a glucocorticoid) and CA secretion into circulation, respectively (Ayada et al., 2015). In this research project, it is this process of CA regulation that will be focused on in further detail.

While these physiological mechanisms are effective at controlling BP, there are countless environmental and genetic factors that disturb this homeostasis. For example, Mendelian inheritance can enhance or reduce the activity of candidate genes for hypertension. Genes with mutations that alter their activity include sodium reabsorption genes (e.g. aldosterone synthase and the epithelial Na^+ channel – ENaC) and RAS genes (e.g. angiotensinogen – AGT, and ACE; Jeunemaitre et al., 1992; Lifton, Gharavi, & Geller, 2001). Millions of single nucleotide polymorphisms (SNPs) have been associated with high or low BP too (Delles, McBride, Graham, Padmanabhan, & Dominiczak, 2010). SNPs have been identified in genes responsible for calcium channels (e.g. plasma membrane calcium channel 1 – PMCA1), steroid-biosynthesizing enzymes (e.g. cytochrome P450 17A1), and transcription factors (TFs) involved in the cardiovascular differentiation process (Delles et al., 2010). Many gender differences in BP regulation exist, indicating the importance of X and Y-linked genes. It is likely that this finding is partly due to the effect of estrogens and possibly, androgens (Reckelhoff, 2001). With regard to one's environment, diet, exercise, and drug use are all contributing factors to BP. For example, consumption rates of electrolytes (e.g. magnesium – Mg^{2+}, potassium – K^+, calcium – Ca^{2+}, and Na^+), fiber, and antioxidants (e.g. polyphenols) can all affect BP (Hügel, Jackson, May, Zhang, & Xue, 2016). Exercise can alter BP over long periods of time by protecting against left ventricular hypertrophy and by modulating lipid profiles to decrease vascular resistance (Kannan et al., 2014; Lou, Zong, & Wang, 2017). Drugs can affect many areas of BP regulation, such as sympathetic activation by nicotine and endothelial function by alcohol (Husain, Ansari, & Ferder, 2014; Pickering, 2001).

Over time, collective effects of environmental factors usually lead to increased BP with age (Lifton et al., 2001). Environmental effects start as early as the fetal development stage *in utero*. Environmental influences like diet and stress can change the expression levels of BP-regulating genes in offspring and eventually lead to adult hypertension (Barker et al., 1993; Tain et al., 2014). Finally, these genetic and environmental factors are a large reason why mRNA and protein levels are commonly upregulated or downregulated in hypertensive patients and animals. Another area of BP regulation that is commonly altered by such influences is CA regulation. We will now see why alterations in CA regulation can have large effects on many physiological systems and BP.

1.2.3 – *The Physiological Effects of Catecholamines*

The CAs, dopamine (DA), Epi, and NE, each have their own set of signaling roles across the body. Epi and NE bind to adrenoceptors while DA binds to dopaminergic receptors. These receptors are further divided into subclassifications with independent signaling mechanisms. The expression pattern of CA receptors is largely tissue-dependent, allowing for CAs to have unique effects on specialized cell-types. Because CAs are localized in the circulation, nervous tissue, and interstitial tissues, their physiological effects are expansive and difficult to fully characterize.

Dopaminergic activation is carried out through two types of receptors: D1-like receptors (D1Rs) and D2-like receptors (D2Rs). D1Rs consist of D1 and D5 receptors and interact with Gs to activate adenylate cyclase (AC), protein kinase A (PKA), and Ca^{2+} release from endoplasmic reticulum stores (Missale, Nash, Robinson, Jaber, & Caron, 1998). The D2Rs consist of D2, D3, and D4 receptors and provide opposite effects via Gi interaction and AC inhibition (Missale et al., 1998).

The effects of DA mainly apply to nervous, endocrine, and cardiovascular systems. In the nervous system, central neurons, especially in the limbic system, use DA as a neurotransmitter

(Nieoullon, 2002). The dopaminergic neural pathways typically have a role in memory, emotion, learning, attention, and motor function (Nieoullon, 2002). In the kidney, DA is synthesized and secreted by tubule cells of the nephron (mostly in proximal tubules; M. R. Lee, 1993). Here, DA usually signals for increased natriuresis and diuresis via regulation of transporters such as the sodium-proton exchanger (NHE; Bobulescu et al., 2010). Both D1Rs and D2Rs can be differentially expressed in the tubules and CDs, making the effects of DA largely dependent on the prevalence of each receptor (Missale et al., 1998). Moreover, D2Rs are present in the pituitary gland and adrenal gland, allowing for DA to inhibit the secretion of certain hormones like prolactin, melanocyte-stimulating hormone, aldosterone, and CAs (as a feedback mechanism; Baek, Seo, & Lim, 2008; Fitzgerald & Dinan, 2008; McKenna, Island, Nicholson, & Liddle, 1979; Missale et al., 1998). DA also increases RAAS activity (via D1Rs of juxtaglomerular cells), but also modifies RAAS's effect by inhibiting aldosterone and various Ang II receptors (Gildea, 2009; McKenna et al., 1979; Missale et al., 1998). In the cardiovascular system, the localization of DA receptors in the tunica media of various arteries (e.g. in the renal, femoral, coronary, and mesenteric arteries) results in an overall effect of vasodilation (Amenta, Ricci, Tayebati, & Zaccheo, 2002). In contrast, highly concentrated DA in the blood can increase CO by a specialized DA binding pattern in the heart (Clark & Menninger, 1980). Therefore, the effect of circulating DA is usually a decrease in BP, but in exceptional cases, an increase in BP can be observed.

Adrenergic receptors are grouped into two categories: α and β receptors (Guimarães & Moura, 2001). α receptors can be further divided into α_1 and α_2 receptors, each with different signaling activities (Guimarães & Moura, 2001). α_1, α_2, and β receptors have three additional subtypes, all having high affinities to NE and Epi, with the exception of the β_2 subtype, which has a low affinity for NE (Molinoff, 1984). Like DA receptors, adrenergic receptors are all coupled to

G proteins. Generally, α_1 receptors activate protein kinase C (PKC), α_2 receptors inhibit PKA, and β receptors activate PKA (Tank & Lee Wong, 2015).

The effects of NE and Epi are usually summarized as a mediator of the "fight-or-flight" response. This response is an evolutionary tactic used to temporarily combat or evade predators. NE and Epi are localized in the central nervous system (CNS), where part of their function is to learn and memorize stressful events (Tank & Lee Wong, 2015). α_1 receptors are found in smooth muscles of the eye and vasculature and their stimulation contracts muscles by increasing Ca^{2+} in the cytosol (Guimarães & Moura, 2001). Tubular cells can have α_1 receptors, which leads to increased sodium reabsorption during stimulation (Ritz, Amann, & Fliser, 1998). α_2 receptors, on the other hand, are expressed in presynaptic terminals of post-ganglionic neurons and inhibit AC activation to reduce the effect of excess NE during neurotransmission (Giovannitti, Thoms, Crawford, & Crawford, 2015). Another inhibitory action of α_2 receptors occurs in the islets of Langerhans; stimulation of α_2 receptors can inhibit insulin secretion and reduce glucose storage/uptake from the blood (Hsu, Xiang, Rajan, & Boyd, 1991). Because chromaffin cells (the secretory cells of the adrenal medulla) are derived from post-ganglionic neurons, they also express α_2 receptors, which may produce a negative feedback mechanism when CA secretion occurs (Moura, Afonso, Hein, & Vieira-Coelho, 2009). Regarding β receptors, β_2 subtypes are vasodilatory in the vasculature, while β_1 subtypes produce positive chronotropic and inotropic effects on the heart (Guimarães & Moura, 2001). This feature of subtype distribution creates a characteristic increase in blood flow to skeletal muscle and a decrease in blood flow to digestive organs. To add, specialized localization of the β receptors results in a concentration-dependent effect of Epi on BP (Tank & Lee Wong, 2015). Low concentrations of Epi activate β_2 receptors, leading to vasodilation, thus lowering BP. With high concentrations of Epi, α_1 receptor activation

predominates, leading to vasoconstriction and increased BP. NE's low affinity for β_2 receptors allows for it to increase BP at a range of concentrations. β receptors are present in adipocytes, the juxtaglomerular complex, and hepatocytes too (Erraji-Benchekroun et al., 2004; Louis, Jackman, Nero, Iakovidis, & Louis, 2000; Torretti, 1982). Hence, the "fight-or-flight" response is also known for increasing lipolysis, renin secretion, and glycogenolysis and gluconeogenesis.

1.3 – Mechanisms of Catecholamine Regulation

1.3.1 – *Tissue-Specific Regulation of Catecholamines*

The mechanisms of CA regulation are largely determined by their tissue localization. Tissues regulate CAs either by the rate of their synthesis, storage, secretion, or breakdown.

The majority of CA biosynthesis occurs in the adrenal medulla and specialized neurons (Kvetnansky, Sabban, & Palkovits, 2009). To a limited degree, the kidney, heart, liver, immune cells, mesenteric organs, and other tissues may synthesize CAs (Armando, Villar, & Jose, 2011; Eisenhofer, Kopin, & Goldstein, 2004; Elayan, Kennedy, & Ziegler, 1990; Flierl, Rittirsch, Huber-Lang, Sarma, & Ward, 2008; Peltsch et al., 2016; Uhlen et al., 2015; Ziegler, Bao, Kennedy, Joyner, & Enns, 2002). The CA biosynthesis pathway is a highly regulated process that can convert three amino acid precursors to any of the CAs using a system of five enzymes (Figure 2).

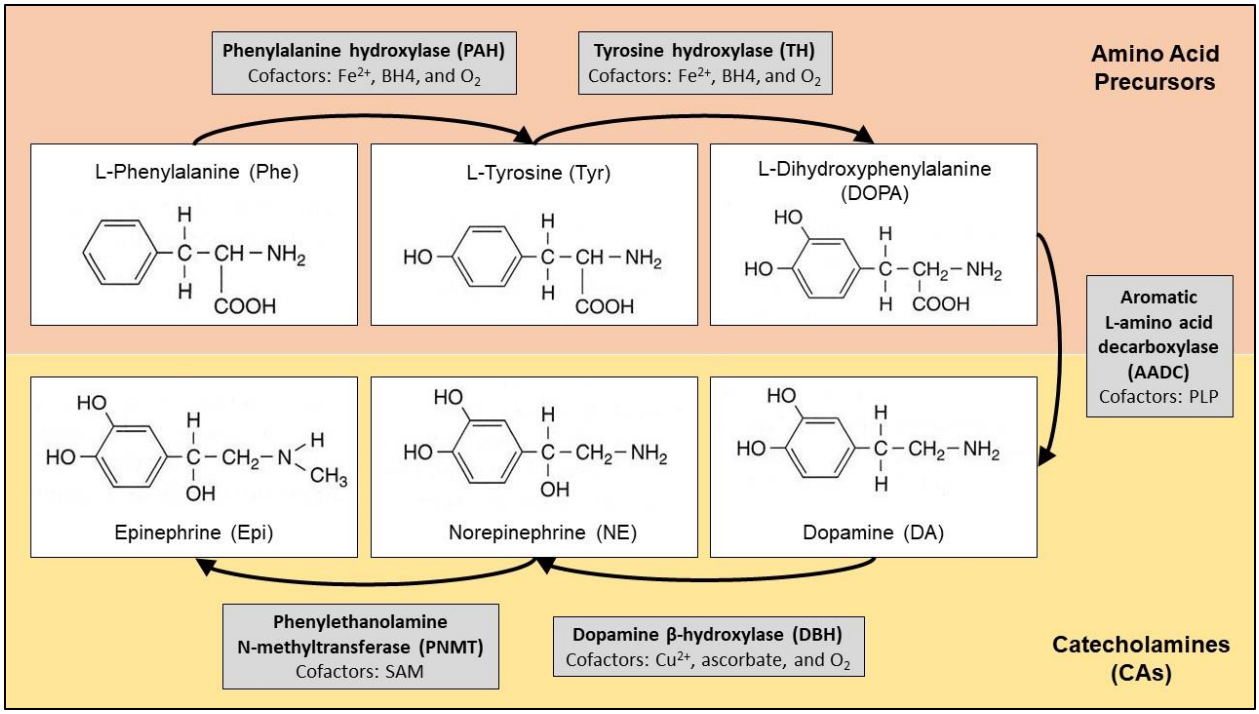

Figure 2: The catecholamine biosynthesis pathway. The five enzymes and cofactors are boxed in grey. Amino acid precursors are in the region highlighted in red and CAs are in the region highlighted in yellow.

The first step of this pathway is the conversion of L-phenylalanine (Phe) to L-tyrosine (Tyr) by the enzyme phenylalanine hydroxylase (PAH). This step is mostly performed by the liver, but there is evidence showing it can occur in CA-synthesizing tissues (Uhlen et al., 2015). Because Tyr can be consumed in the diet, PAH's actions are not the rate-limiting step of CA synthesis. Instead, a rate-limiting step occurs with Tyr's conversion to L-dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase (TH; Kuhar, Couceyro, & Lambert, 1999a). As a rate-limiting enzyme, it is appropriate that many forms of regulation have evolved, including phosphorylation (e.g. Ser-40 modification), allosteric binding, transcriptional regulation, and translational regulation (Kumer & Vrana, 1996). Both PAH and TH require oxygen, tetrahydrobiopterin (BH_4), and iron for their enzymatic mechanisms (Flydal & Martinez, 2013; Kumer & Vrana, 1996). After this step, DA is produced from DOPA with the enzyme, aromatic L-amino acid decarboxylase (AADC), and its cofactor, pyridoxal phosphate (PLP). Evidence suggests that AADC does not have complex regulatory systems similar to TH (Waymire & Haycock, 2002). Next, DA can be hydroxylated to form NE by dopamine β-hydroxylase (DBH) and its cofactors, oxygen, copper, and ascorbate (Kvetnansky et al., 2009). Several regulatory mechanisms of DBH exist, many of which being tissue-dependent. Tissue-based regulation is important as it allows the secretory cells to release DA or NE, which have largely different physiological roles. One notable regulatory mechanism of DBH is the ability of cyclic adenosine monophosphate (cAMP) to increase the level of DBH gene transcription (O. Hwang & Joh, 1993). The last reaction of the biosynthesis pathway is the conversion of NE to Epi by phenylethanolamine N-methyltransferase (PNMT) and its cofactor, s-adenosyl methionine (SAM; Kuhar et al., 1999a). Epi has different receptor affinities from NE and therefore, PNMT is also regulated by numerous mechanisms. Following the transcription of PNMT, two splicing variants are produced: intron-retaining and intronless mRNA (Unsworth,

Hayman, Carroll, & Lelkes, 1999). The intron-retaining mRNA contains an early stop codon and translates to an inactive form of PNMT (Wong, Tai, Wong-Faull, Claycomb, & Kvetnanský, 2008). Increased transcriptional regulation of PNMT can alter the levels of intron-retaining mRNA, intronless mRNA, or both splice variants (Ansell, Grandbois, & Tai, 2014; Unsworth et al., 1999). *In vivo* studies of adrenal tissue suggest that intron-retention may be important for cellular differentiation, but its overall role is still unclear (Unsworth et al., 1999). A well-accepted regulatory mechanism of PNMT is the robust effect of glucocorticoids to decrease intron-retaining mRNA and increase intronless mRNA. Consequently, for tissues to synthesize CAs *de novo*, they require extracellular regulatory conditions to activate the biosynthesis enzymes, which is why CA synthesis is generally restricted to nervous and adrenal tissues.

Another form of CA regulation is to change the rate of CA uptake and storage into secretory vesicles. CA products must be transported to secretory vesicles because biosynthesis enzymes are mostly localized in the cytosol (the exception being DBH, which is found in secretory vesicles; Kuhar, Couceyro & Lambert, 1999b). CA uptake into secretory vesicles is performed by a family of H^+-ATPase transporters known as vesicular monoamine transporters (VMATs). These transporters can be regulated transcriptionally and post-translationally via phosphorylation and protein-protein interactions (German, Baladi, McFadden, Hanson, & Fleckenstein, 2015). On the cell membrane of secretory cells, they express other CA transporters like norepinephrine transporters (NETs) and dopamine transporters (DATs; Uhlen et al., 2015). These transporters are important for reusing excess CAs in the cerebrospinal fluid (CSF), blood, and interstitial spaces. Studies have shown that these transporters may be downregulated by increased oxidative stress (Mao et al., 2004). Storage regulation is also highly dependent on the expression of chromogranins. Chromogranins are proteins important for the formation of secretory vesicles, the inhibition of

secretory particle degradation, and their fragmentation into negative feedback peptides (e.g. catestatin and vasostatin; D'amico, Ghinassi, Izzicupo, Manzoli, & Di Baldassarre, 2014; T. Kim, Tao-Cheng, Eiden, & Loh, 2001; Koshimizu, Cawley, Kim, Yergey, & Loh, 2011; Kvetnansky et al., 2009). Transcriptional upregulation of chromogranins may be stimulated by protein kinase D (PKD), PKA, and extracellular signal-regulated kinase (ERK)-dependent mechanisms (Mahapatra, Mahata, O'Connor, & Mahata, 2003; von Wichert et al., 2008).

When CAs are localized to their vesicles, they are ready for signal-mediated exocytosis. Most signals for exocytosis depolarize the cell membrane and potentiate the influx of Ca^{2+} via voltage-gated channels. This Ca^{2+} influx then structurally modifies vesicle fusion proteins and facilitates exocytosis (Rizo & Rosenmund, 2008). In neurons, action potential depolarization is sufficient for voltage-gated Ca^{2+} channels (VGCCs) to open (Jackson, Trout, Brain, & Cunnane, 2001). In chromaffin cells, VGCC activation occurs when ACh binds to nicotinic receptors (nAChRs), resulting in Na^{+} influx and K^{+} efflux (Sala, Nistri, & Criado, 2007). Aside from these main stimuli for secretion, many receptors function to potentiate CA secretion by increasing depolarization, while other receptors function to inhibit CA secretion by hyperpolarization (Lodish et al., 2000). Further, the activity of nAChRs can be modulated at allosteric sites by certain compounds like substance P and calcitonin gene-related peptide (CGRP; Sala et al., 2007).

The last primary method to regulate CAs is their rate of breakdown into metabolites. There are only three types of enzymes that initiate the metabolism of CAs: monoamine oxidases (MAO-A and MAO-B), catechol-O-methyltransferases (COMTs), and for DA and NE alone, semicarbazide-sensitive amine oxidases (SSAOs; Axelrod, 1959; Boomsma, Bhaggoe, van der Houwen, & van den Meiracker, 2003). Through a series of succeeding metabolic reactions, the final metabolite of NE and Epi is mostly vanillylmandelic acid (VMA), while the final metabolite

of DA is mostly homovanillic acid (HVA; Eisenhofer et al., 2004). An alternative metabolic mechanism is the conjugation of CA metabolites with sulfate or glucuronide. Conjugation reactions appear to be important for the excretion of metabolites, but not for the overall rate of CA metabolism (Boobis, Murray, Jones, Reid, & Davies, 1980). MAOs and COMTs are localized intracellularly in most tissues, while SSAOs can be localized to the cell membrane, cytosol, or in the circulation (Boomsma et al., 2003; Uhlen et al., 2015). However, most CA metabolism is catalyzed by MAOs and COMTs and occurs at the site of synthesis (i.e. pre-synaptic terminals and chromaffin cells (Eisenhofer et al., 2004). Pre-synaptic terminals predominantly metabolize CAs via MAOs, and chromaffin cells metabolize CAs with both MAOs and COMTs (Kvetnansky et al., 2009). In cells expressing the extraneuronal monoamine transporter (EMT) gene, CAs can be transported intracellularly to be metabolized in different tissues as well (Kvetnansky et al., 2009). MAOs are flavoenzymes and use a flavin adenine dinucleotide (FAD) cofactor to deaminate CAs, producing hydrogen peroxide (H_2O_2) in the process (Edmondson, Binda, Wang, Upadhyay, & Mattevi, 2009). COMTs require SAM as a cofactor for their O-methylation of CAs (Tsao, Diatchenko, & Dokholyan, 2011). SSAOs require copper and a quinone cofactor and produce aldehyde metabolites, H_2O_2, and ammonia (Boomsma et al., 2003; Holt et al., 1998). The transcription of MAOs and COMTs can both be altered by steroid hormones, specificity protein 1 (SP1) activation, and epigenetic methylation in their promoters (Abdolmaleky et al., 2006; Schendzielorz, Rysa, Reenila, Raasmaja, & Mannisto, 2011; Tenhunen, 1996; Zwart, Verhaagh, Buitelaar, Popp-Snijders, & Barlow, 2001). Recently, it was demonstrated that hypoxia can induce the expression of SSAOs (Y. Zhang et al., 2017). Regulators like SP1 activation and hypoxia also stimulate increases in CA activity, making the metabolizing enzymes part of a negative feedback mechanism.

1.3.2 – *Adrenomedullary Cell Signals for Catecholamine Regulation*

Interestingly, chromaffin cells express all major proteins that regulate CA synthesis, storage, secretion, and metabolism. This is one reason why the adrenal medulla is the area of focus for this current study.

There are several key hormones that signal to the adrenal medulla from the extracellular environment. This tissue is innervated by a sympathetic splanchnic nerve that releases ACh and an assortment of other signaling molecules. Besides ACh, nerve terminals can release pituitary adenylate cyclase-activating polypeptide (PACAP), vasoactive intestinal peptide (VIP), neuropeptide Y (NPY), enkephalins, substance P, somatostatin, and CGRP (Livett & Marley, 1993). Additionally, the extracellular environment contains signaling elements that originate from chromaffin granule exocytosis. Along with CAs, these granules release substances like adenosine triphosphate (ATP), Ca^{2+}, adrenomedullin, chromogranin metabolites, natriuretic peptides, NPY, VIP, enkephalins, substance P, and CGRP (Crivellato, Nico, & Ribatti, 2008). Other chemical signals are from blood vessels that pass through the adrenal cortex (E. J. Whitworth, Kosti, Renshaw, & Hinson, 2003). From the blood, chromaffin cells can be affected by signals like cortisol, nerve growth factor (NGF), Ang II, low oxygen (hypoxia), and reactive oxygen species (ROS) like peroxides, superoxides, NO, and hydroxyl radicals (Crispo, Ansell, Ubriaco, & Tai, 2011; K. T. Kim, Park, & Joh, 1993; Lillien & Claude, 1985; H.-J. Lim, Lee, & Lim, 2013). Frequently, these factors change the membrane voltage potential, bind allosterically to receptors, or alter TF expression via intracellular signaling pathways. Five commonly activated signaling pathways involved in CA regulation are the PKC, ERK/MAPK, PKA, glucocorticoid, and hypoxia pathways (Figure 3).

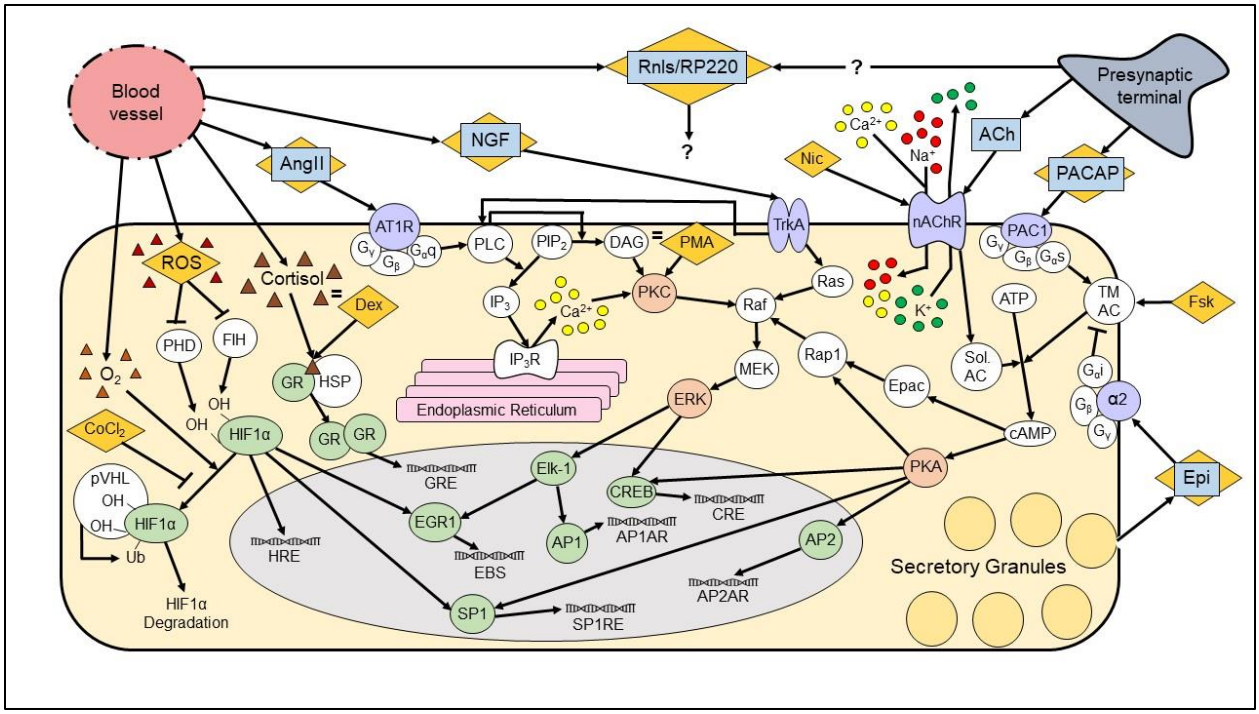

Figure 3: Common chromaffin cell signals and transcription factor activations. Transcription factors are marked in green. Signals used for the treatment of cells in this study are marked as gold diamonds. Equal signs show when two signals have an equivalent function. Extracellular signals that do not cross the outer membrane are marked as blue rectangles. Membrane receptors are highlighted with light purple. Signals that can cross plasma membranes are shown as triangles. Circles indicate ion transport. Ubiquitination and hydroxylation are abbreviated as Ub and OH, respectively. The central signaling kinases, PKC, PKA, and ERK, are highlighted with light red. All other signaling mediators are shown in white. For the full names of these signaling molecules, see the List of Abbreviations.

G protein-coupled receptors (GPCRs) that interact with $G_{\alpha}q$ (e.g. angiotensin type-1 receptors – AT1Rs, muscarinic acetylcholine receptors – mAChRs, and α_1 adrenergic receptors) propagate their signal by activating particular phospholipase C subtypes (PLC; Guimarães & Moura, 2001; Gwathmey, Alzayadneh, Pendergrass, & Chappell, 2012; Harada, Matsuoka, Miyata, Matsui, & Inoue, 2015). PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP_2), producing inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol (DAG; Wu-Zhang & Newton, 2013). IP_3 releases Ca^{2+} from the endoplasmic reticulum via the IP_3 receptor, which activates various isoforms of PKC (Wu-Zhang & Newton, 2013). DAG also interacts with certain isoforms of PKC and another regulatory pathway, PKD (Rozengurt, 2011; Wu-Zhang & Newton, 2013). Through the inhibition of Raf kinase inhibitory protein (RKIP), PKC indirectly activates the ERK/MAPK pathway via Raf-1 (Corbit et al., 2003). ERK activation indirectly phosphorylates TFs like cAMP response element-binding protein (CREB) and directly phosphorylates TFs such as Elk-1 (Zhang & Liu, 2002). Elk-1 further propagates this signal by increasing mRNA levels for the TFs, early growth response protein 1 (EGR-1) and c-Fos (a subunit of activator protein 1 – AP1; Monje, Hernández-Losa, Lyons, Castellone, & Gutkind, 2005; Zhang & Liu, 2002). Interestingly, the NGF tyrosine receptor kinase, TrkA, can strongly activate both PLC/PKC and ERK directly through protein interactions, and indirectly via the protein kinase B/Akt (PKB) pathway (Cosgaya & Shooter, 2008; Powers, Shahsavari, Tsokas, & Tischler, 1999). Cell survival or apoptotic pathways can be induced by another NGF receptor, p75, but this mechanism is stress-dependent (Cosgaya & Shooter, 2008).

Other extracellular signals can induce PKA activation or inhibition for the regulation of CAs. For example, nAChRs, β-adrenergic receptors, and the PACAP/VIP family of receptors (particularly PAC1 and VPAC1) are all activators of PKA (Conconi, Spinazzi, & Nussdorfer, 2006; Guimarães & Moura, 2001; Marley, Thomson, & Bralow, 1995). In contrast, neuropeptide

Y receptors, somatostatin receptors, and P2Y purinergic receptors inhibit PKA (Ben-Shlomo & Melmed, 2010; Powell, Teschemacher, & Seward, 2000; Zheng, Zhang, & Hexum, 1997). With the exception of nAChRs, all these receptors are GPCRs that interact with $G_{\alpha}s$ to activate adenylate cyclase or $G_{\alpha}i$ to inhibit adenylate cyclases. G proteins bind to transmembrane adenylate cyclases (TM-ACs), while nAChRs most likely activate a soluble form of adenylate cyclase (Sol.-AC; Hollenhorst, Lips, Kummer, & Fronius, 2012). When ACs are activated, they produce cAMP from ATP, which then activates PKA (Yan, Gao, Cui, Zhang, & Zhou, 2016). This activation is proceeded by the phosphorylation and stimulation of TFs like SP1, activator protein 2 (AP2), and CREB (Delghandi, Johannessen, & Moens, 2005; García, Campillos, Marina, Valdivieso, & Vázquez, 1999; Rohlff, Ahmad, Borellini, Lei, & Glazer, 1997). Like the PKC pathway, PKA can activate ERK and its related TFs. Instead of Raf1 phosphorylation, cAMP and PKA trigger this pathway through a Ras-related protein 1 (Rap1)-Braf mechanism (Emery, Eiden, Mustafa, & Eiden, 2013; Takahashi, Li, Dillon, & Stork, 2017). Finally, cAMP has the ability to increase the stability of glucocorticoid receptor (GR) mRNA, and SP1 can increase GR transcription (Peñuelas, Encío, López-Moratalla, & Santiago, 1998; Suehiro et al., 2004).

GR is a unique TF that plays a role in glucocorticoid signaling and the regulation of CAs. Without the stimulation of glucocorticoids, GR is bound to heat shock proteins (particularly HSP90), which inhibits its TF function (Drouin et al., 1992). When glucocorticoids passively cross the plasma membrane, they can bind to GR and inhibit HSP binding. This inhibition then leads to GR homodimerization and TF activation (Drouin et al., 1992).

The hypoxia-inducible factor 1α (HIF1α) is another TF that is important for CA regulation. In normoxic conditions, von Hippel-Lindau protein (pVHL) can bind to an oxygen-dependent domain (ODD) on HIF1α (Pugh & Ratcliffe, 2003). pVHL then ubiquitinates HIF1α and targets it

for proteasome degradation (Pugh & Ratcliffe, 2003). This process only occurs if the ODD is hydroxylated by the oxygen-dependent specific proteins, prolyl-4-hydroxylase (PHD) and factor inhibiting HIF1 (FIH; Marxsen et al., 2004). Under hypoxic conditions, hydroxylation and pVHL binding are inhibited and HIF1α is stabilized, allowing for it to dimerize with HIF1β and act as a TF (Dengler, Galbraith, & Espinosa, 2014). This HIF1 heterodimer binds to hypoxia response elements (HREs), and also increases the expression of other important TFs in CA regulation, notably SP1 and EGR-1 (Tai, Wong-Faull, Claycomb, & Wong, 2010). Another effect of HIF1α activation is an increase in the level of certain ROS, which damage macromolecules (e.g. proteins, lipids, and nucleic acids) and modulate the activity of signaling proteins like tyrosine phosphatases (PTPs) and PKB (Crispo et al., 2011; Ray, Huang, & Tsuji, 2012). In many cases of elevated ROS, HIF1α hydroxylases are inhibited, further stabilizing the activity of this TF (Qutub & Popel, 2008). In other cases, such as those involving NO, these hydroxylases are activated (Hagen, Taylor, Lam, & Moncada, 2003). To add, NO can alternatively function to activate MAPKs like ERK1/2 and p38 through cyclic guanosine monophosphate (cGMP) and protein kinase G (PKG) mechanisms (Browning, McShane, Marty, & Ye, 2000; Francis, Busch, Corbin, & Sibley, 2010). Therefore, depending on the activity of ROS-generating mechanisms (e.g. hypoxia, membrane oxidases, NO synthases – NOSs, and mitochondrial respiration), HIF1α activity and CA regulation can be altered in various ways.

Most of these TFs are especially important for regulating CA biosynthesis enzymes. The TH promoter contains many regulatory elements comprised of a SP1/EGR-1 binding site, AP1 and AP2 activating regions (AP1ARs and AP2ARs), a HRE, a CREB response element (CRE), and in the case of rats and mice, glucocorticoid response elements (GREs; Kvetnansky et al., 2009; J. Lim, Yang, Hong, & Kim, 2000). An enhancer box (E-box), which binds dimers of variable TFs,

is another activating site in the promoter of the TH gene (Desbarats, Gaubatz, & Eilers, 1996; Kvetnansky et al., 2009). Like the TH promoter, the DBH promoter contains an AP1AR, an AP2AR, a CRE, an SP1 response element (SP1RE), an E-box, an EGR-1 binding site (EBS), and a GRE (Kobayashi, Kurosawa, Fujita, & Nagatsu, 1989; Kvetnansky et al., 2009; Seo, Yang, Kim, & Kim, 1996). While most of these regulatory elements increase the expression of DBH, the EGR-1 binding to the promoter inhibits DBH expression (Cheng, Serova, Glazkova, & Sabban, 2008). As for the PNMT promoter, it contains GREs, EBSs, SP1REs, AP2ARs, and a low-affinity HRE (Kvetnansky et al., 2009; Tai, Wong-Faull, Claycomb, & Wong, 2009). The characteristics of these promoters indicate that PKA, PKC, ERK, glucocorticoid, and hypoxia signaling are all important for the regulation of these biosynthesis enzymes. Therefore, stimulating or inhibiting these signaling mechanisms at the cellular level is an effective way to determine new regulators of CA biosynthesis.

1.3.3 – *The PC12 Cell Model of Catecholamine Synthesis*

Many studies involving the regulation of CA biosynthesis use the PC12 cell model. These cells are derived from undifferentiated rat pheochromocytoma cells (Greene & Tischler, 1976). Pheochromocytoma cells express the biosynthesis enzymes at an elevated level (with the exception of PNMT, which is unchanged) and have a depressed level of MOAs and COMTs (Jarrott & Louis, 1977). Pheochromocytoma cells also have a notable decrease in the level of succinate-coenzyme Q reductase (SQR), the second complex of the electron transport chain and an enzyme of the citric acid cycle (Jarrott & Louis, 1977). PC12 cells do not express the E-box-binding TF, myc-associated factor X (Max), possibly altering CA regulatory pathways (Hopewell & Ziff, 1995). While chromaffin cell are specialized to contain mostly NE or Epi in their vesicles, the majority of CAs in PC12 cell vesicles is DA (Westerink & Ewing, 2008). A signaling process of PC12 cells

to produce Epi or NE can be carried out by treating them with NGF, PACAP, or the synthetic glucocorticoid, dexamethasone (Dex; Beaujean et al., 2003; Das, Freudenrich, & Mundy, 2004; Vaudry et al., 2002). When signaled by NGF, PC12 cells differentiate by proliferation and the formation of neurite extensions (Greene & Tischler, 1976). NOS induction is another important change that occurs during NGF differentiation (Schonhoff, Bulseco, Brancho, Parada, & Ross, 2001; Sheehy, Phung, Riemer, & Black, 1997). PACAP-induced differentiation can occur as well, which increases neurite outgrowth but inhibits proliferation (Vaudry et al., 2002). Stimulation of PC12 cells by Dex increases the production of Epi and can attenuate the NGF-provoked neurite outgrowth, while increasing proliferation (K. T. Kim et al., 1993; Terada et al., 2014). PC12 cells are valuable for *in vitro* studies because there are many cell treatments that are used to replicate increases in CA biosynthesis in chromaffin cells. For example, cobalt chloride ($CoCl_2$) binds HIF1α's ODD and inhibits pVHL ubiquitination, resulting in hypoxic-like conditions (Crispo et al., 2011; Yuan, Hilliard, Ferguson, & Millhorn, 2003). Treatment of PC12 cells with forskolin (Fsk) activates AC and PKA/ERK regulatory mechanisms (Seamon & Daly, 1981). Moreover, the use of phorbol 12-myristate 13-acetate (PMA) on PC12 cells reproduces DAG's activation of specific subtypes of PKC (i.e. Ca^{2+}-independent subtypes; Wu-Zhang & Newton, 2013). Using PC12 cells, with control of these intracellular pathways, allows for the detection of new genes that are involved with CA biosynthesis. If a gene is regulated in a similar manner to the biosynthesis enzymes, it is possible that this gene is involved with CA regulation. In the present study, this cell model will be used to determine if the novel protein, renalase, is related to CA biosynthesis in chromaffin cells.

1.4 – The Physiological Importance of Renalase

1.4.1 – *A Novel Player in Catecholamine Regulation*

Renalase was first discovered in 2005 and was proposed to catalyze the oxidation of circulating CAs (Xu et al., 2005). In this same study, renalase was shown to oxidize DA, NE, and Epi in the presence of FAD. This oxidation was detected by measuring H_2O_2 and fluorescence using a MAO assay kit. Unlike MAOs or COMTs, renalase converts CAs to aminochrome products (Desir, Tang, et al., 2012). Its highest affinity was towards DA and its lowest affinity was towards NE (Xu et al., 2005). Later studies confirmed these oxidation reactions using high-performance liquid chromatography (HPLC)-based methodologies, but claimed that renalase's highest affinity was towards Epi, and its weakest affinity was towards DA (Quelhas-Santos et al., 2015). Xu et al. also administered recombinant renalase to rats and observed negative inotropic and chronotropic effects, as well as a reduction in BP within 30s of injection (2005). Two renalase knockout (KO) models in mice have been generated, demonstrating increased BP, HR, and circulating CA levels, and decreased body mass (Quelhas-Santos et al., 2014; Y. Wu et al., 2011). If these studies are correct, and renalase's function *in vivo* is to degrade CAs, renalase would be one of the most important proteins for the discovery of new medical treatments for hypertension, and pathophysiological mechanisms of this disease.

1.4.2 – *Potential Roles in Disease*

Shortly after the discovery of renalase, a study of 2586 individuals from a northern Chinese Han population found eight SNPs in the human renalase gene (Zhao et al., 2007). In the same study, two of these SNPs were associated with hypertension: an rs2576178 GG genotype in the 5' flanking region, and an rs2296545 CC genotype coding for a Glu37Asp substitution in exon 2. Further, a meta-analysis study has confirmed the association of the rs2296545 SNP and

hypertension, but not for the rs2576178 SNP (Lv et al., 2016). Because there is a common comorbidity of hypertension and diabetes, 892 diabetes patients of Polish origin with or without hypertension were genotyped for three renalase SNPs (Buraczynska, Zukowski, Buraczynska, Mozul, & Ksiazek, 2011). The rs2296545 SNP was significantly more prevalent in hypertensive diabetic patients compared to normotensive patients and healthy controls (Buraczynska et al., 2011). Interestingly, Buraczynska et al. also found that the rs2576178 SNP was significantly more prevalent in all diabetes patients compared to healthy controls (2011). More recently, the two major renalase SNPs have also been associated with ischemic stroke and coronary heart disease in a northern Chinese Han population (X. Li et al., 2014; R. Zhang et al., 2013). Other studies including populations from Sweden, Bosnia & Herzegovina, and China did not show an association between hypertension and these SNPs (Fava et al., 2012; Kiseljakovic et al., 2016; Shi & Wang, 2015). Overall, renalase SNPs can be associated with hypertension and several diseases, but this finding appears to be population-dependent.

Altered renalase activity has been shown in a genetic animal model of hypertension, the spontaneously hypertensive rat (SHR). SHR plasma compared to normotensive rats (Wistar-Kyoto rats – WKYs) had a reduced level of renalase (Jiang et al., 2012). In the kidney of SHRs, authors found increased renalase mRNA in rats with severe hypertension, but not with moderate hypertension (Fedchenko, Globa, Buneeva, & Medvedev, 2013). Yet, another study observed a decrease in renalase protein in kidneys of SHRs (Jiang et al., 2012). In the heart and brain, SHRs had increased and decreased renalase mRNA, respectively (Fedchenko et al., 2013). These studies demonstrate that renalase regulation is genetically regulated, but also by context and tissue-dependent variables that are yet to be discovered.

The activity of renalase is also altered in many pathological states. In circulation, renalase levels have been shown to negatively correlate with systolic BP in patients with resistant hypertension (Schlaich et al., 2010). Shortly after its discovery, renalase plasma levels in end-stage renal disease (ESRD) patients was found to be significantly lower (Xu et al., 2005). In kidney and heart transplant recipients, renalase plasma levels were increased (Przybylowski, Koc-Zorawska, Malyszko, Mysliwiec, & Malyszko, 2010; Zbroch, Małyszko, Małyszko, Koc-Żórawska, & Myśliwiec, 2012). Lower GFR in kidney transplant individuals is associated with increased markers for NO metabolism, which may be an effect from the increased plasma renalase levels that were also observed (Stojanovic et al., 2015). Generally, renalase plasma levels are increased in hemodialysis and peritoneal dialysis patients (Malyszko et al., 2015; Zbroch, Koc-Zorawska, Malyszko, Malyszko, & Mysliwiec, 2013; Zbroch, Malyszko, Malyszko, Koc-Zorawska, & Mysliwiec, 2012). In subtotal nephrectomized rats (5/6Nx) and several forms of acute kidney injury (AKI), renalase plasma levels are decreased, making renalase a potential biomarker for kidney function (Desir & Peixoto, 2014; H. T. Lee et al., 2013; Wybraniec & Mizia-Stec, 2015). Another renal pathology that may involve renalase is the production of simple renal cysts (SRCs); patients with SRCs were found to have lower circulating renalase levels (Elcioglu et al., 2015). Alternative to kidney disorders, renalase has been implicated in other pathologies across the body like atherosclerosis, cancer (specifically melanoma and pancreatic cancer), lupus nephritis, and preeclampsia (Guo et al., 2016; Hollander et al., 2016; Qi et al., 2015; Yılmaz, Akkaş, Yıldırım, Yılmaz, & Erdem, 2016; Zhou et al., 2015). Because of renalase's involvement with these pathologies, administration of recombinant renalase for medical purposes is a frequently researched topic. Renalase has the ability to lower BP and provides protective effects for ischemic or cisplatin-induced AKI, cardiac ischemia, proteinuria, glomerular hypertrophy, and interstitial

fibrosis (Desir & Peixoto, 2014; Du et al., 2015; H. T. Lee et al., 2013; L. Wang et al., 2014; Y. Wu et al., 2011; Yin et al., 2016).

1.4.3 – *Localization of Renalase*

As a secretory protein, renalase can be isolated from plasma and urine (Xu et al., 2005). Urinary renalase appears to be consistently active and plasma renalase is only activated by certain signals like Epi (G. Li et al., 2008). Therefore, the activity of renalase may be highly variable and dependent on its localization.

The name, renalase, stems from the idea that the kidney provides a large fraction of the circulating protein. In the kidney, renalase is mostly expressed in podocytes, mesangial cells, and tubule cells (F. Wang et al., 2012). In this same study, Wang et al. detected renalase protein in the supernatant of tubule cells, but not from the supernatants of mesangial cells or podocytes (2012). Protein localization in the glomerular cells appears to be more punctiform than the tubule cells, suggesting that renalase is localized to intracellular vesicles. If renalase is indeed localized to vesicles, it may be secreted by glomerular cells in a more transient and signal-dependent manner.

It is now evident that there are alternative sources of circulating renalase throughout the body. Renalase is expressed in the heart, vasculature, liver, pancreas, lung, thyroid, adipocytes, immune cells, small intestine, skeletal muscle, brain, peripheral nerves, reproductive tissues, mouse embryo (12.5 days), and adrenal glands. (Guo et al., 2016; Hennebry et al., 2010; Hollander et al., 2016; Ramanjaneya et al., 2014; J. Wang et al., 2008; Xu et al., 2005; Zhou et al., 2013). In the adrenal glands, it is strongly expressed in the cortex and moderately expressed in the medulla (Zhou et al., 2013). Despite these findings, renalase expression has yet to be analyzed in various other tissues and cell types. For example, its expression has not been observed in undifferentiated cell types like the PC12 cell model.

1.4.4 – *Structure and Isoforms of Renalase*

Initial studies on renalase's structure identified that it contained an N-terminal signal sequence, a Rossmann-fold FAD-binding site, and a monoamine oxidase domain (Figure 4; Xu et al., 2005). In 2011, these structures were verified by Milani et al., who first observed the crystal structure of renalase. These features are highly conserved between species, with its signal sequence being identified in a range of animals like rats, zebrafish, and even sea urchins (J. Wang et al., 2008). Although renalase's structure appears to resemble MAOs, their sequences of amino acids are only 13.2% similar (Xu et al., 2005). Because of renalase's differential activity in plasma and urine, it has been hypothesized that its protein also exists as prorenalase (G. Li et al., 2008). It has been thought that the activation of prorenalase possibly involves dimerization, which is observed when renalase is collected from urine (Xu et al., 2005). However, mass spectrometry later identified there to be only a single monomeric form in urine (Fedchenko et al., 2012). Therefore, the structural changes and/or modifications that activate prorenalase have yet to be identified. The presence of an overlapping signal sequence with the FAD-binding site indicates that the active site of the enzyme may be partially cleaved after secretion. It is possible that renalase secretion involves an alternative pathway that evades cleavage, but recent studies discovered that urinary renalase lacks its signal sequence (Desir, Wang, & Peixoto, 2012; Fedchenko et al., 2015). If extracellular renalase lacks this functional domain, its mechanism of action in the circulation must be reconsidered.

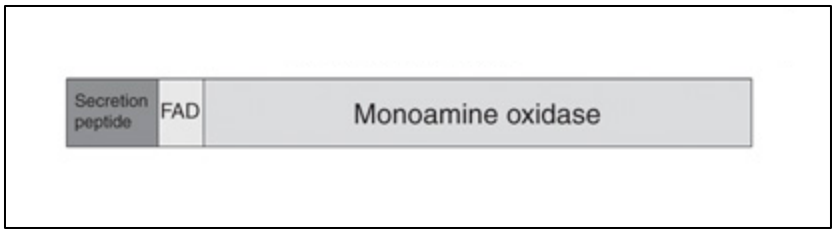

Figure 4: Domain layout of renalase. Renalase contains an N-terminal signal sequence, FAD-binding site, and monoamine oxidase domain. Image is modified from Hennebry et al., 2010.

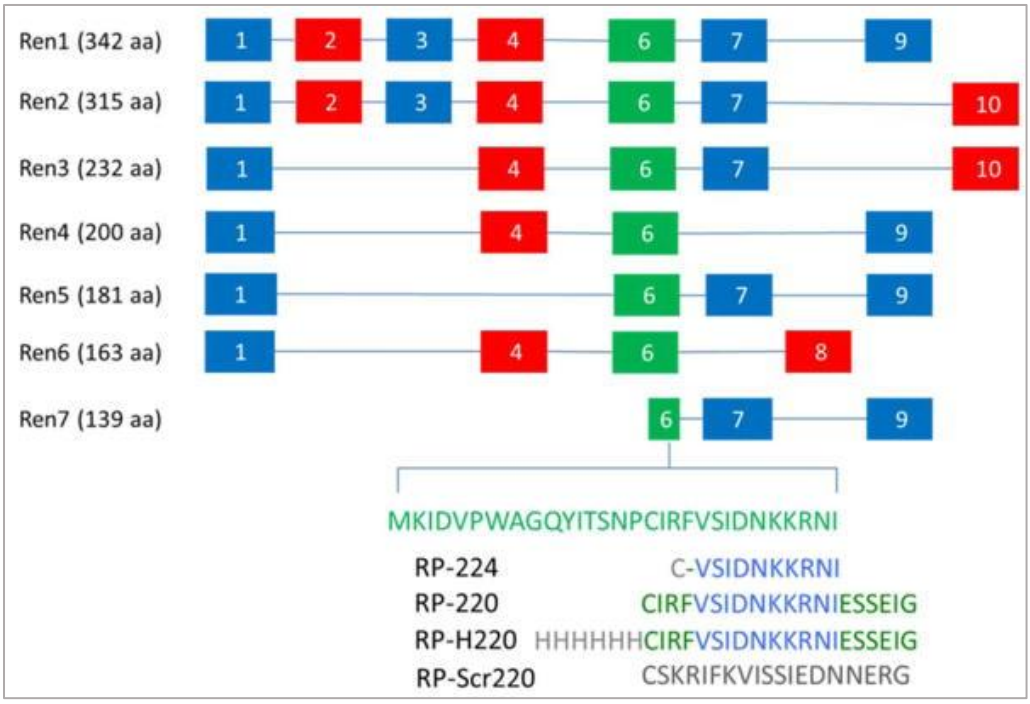

Figure 5: Transcript variants of renalase. The ten exons of the renalase gene are numbered in blue, red or green. All transcript variants include exon 6. Image is modified from L. Wang et al., 2014. In that study, three active peptides were synthesized from exon 6 and one scrambled peptide.

In humans, seven transcript variants for renalase have been identified thus far (Figure 5). Of these seven variants, only two protein products have been identified in humans. These two proteins are named hRenalase1 and hRenalase2 (Fedchenko, Kaloshin, Mezhevikina, Buneeva, & Medvedev, 2013). hRenalase1 is 342 amino acids long and has a molecular weight of 37.85kDa (Xu et al., 2005). hRenalase2, on the other hand, contains 315 amino acids and has a molecular weight of 34.95kDa (Fedchenko et al., 2013). While the function of each transcript variant is currently unknown, there are some important similarities between them. For instance, all but one variant (variant 7) contain the signal sequence (Hennebry et al., 2010; L. Wang et al., 2014). Another similarity is the inclusion of exon 6 in all variants (L. Wang et al., 2014). The three-dimensional confirmation of renalase allows for a particular set of amino acids, coded by exon 6, to be solvent exposed (Milani et al., 2011; L. Wang et al., 2014). Hence, this area is now under investigation to determine if renalase can function as a hormone.

1.4.5 – *Recent Insights into Mechanisms of Activity*

Such structural details have led to the discovery of several signaling peptides derived from renalase's 6th exon (Figure 5; L. Wang et al., 2014). Further studies of the renalase peptide 220 (RP-220) have revealed that it can bind to PMCA4b on proximal tubule cells (L. Wang, Velazquez, Chang, Safirstein, & Desir, 2015). When recombinant renalase or RP-220 bind to this receptor, they stimulate small changes in the phosphorylation status of ERK1/2 but larger changes in the status of p38 phosphorylation (L. Wang et al., 2015). When a lysine-lysine-arginine (KKR) sequence in RP-220 is replaced with alanine, the protective effects of renalase signaling are inhibited (L. Wang et al., 2015). Nevertheless, this signaling mechanism does not complete our understanding of renalase's regulation of BP and CAs. For this, further analysis should be performed on PMCA4b receptor structural changes upon substrate binding. One possible

mechanism that would allow for this receptor to regulate BP and CAs is a downstream activation of NOS. Such an activation is possible because PMCA4b commonly binds and interacts with neuronal NOS (nNOS; Schuh, Uldrijan, Telkamp, Röthlein, & Neyses, 2001). Moreover, activation of PMCA4b by renalase could potentiate a further reduction in intracellular Ca^{2+}, resulting in membrane hyperpolarization in tissues like the adrenal medulla. PMCA subtype expression is highly tissue-dependent and therefore, more research should be done to determine if renalase similarly signals PMCAs in other cell types (Brandt, Neve, Kammesheidt, Rhoads, & Vanaman, 1992).

In 2015, Beaupre et al. discovered another alternative function of renalase. Using its FAD-binding domain, renalase was found to be capable of oxidizing isomeric forms of reduced nicotinamide adenine dinucleotide with or without phosphate (NAD(P)H; Beaupre, Hoag, Roman, Försterling, & Moran, 2015). These isomers were 6-dihydroNAD(P) and 2-dihydroNAD(P) (6DHNAD(P) and 2DHNAD(P)). In an enzymatic reaction that consumes oxygen and produces H_2O_2, these isomers are converted to β-$NAD(P)^+$ (Figure 6; Beaupre, Hoag, Roman, et al., 2015).

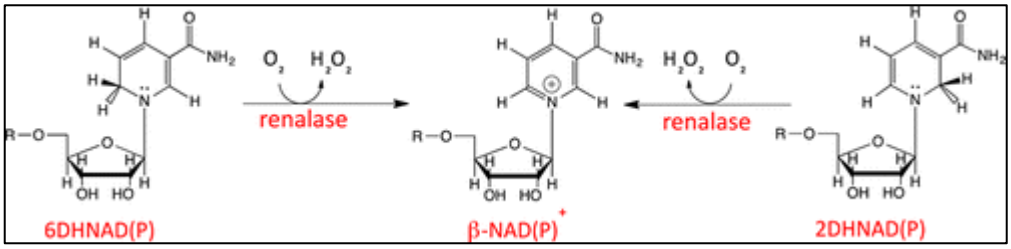

Figure 6: Enzymatic reaction mechanism of renalase. It is proposed that renalase oxidizes isomers of reduced nicotinamide adenine dinucleotide (NAD(P)H), producing hydrogen peroxide (H_2O_2) in the process. Image is taken from Beaupre, Hoag, Roman, et al., 2015.

If the FAD-binding site is cleaved during secretion, this reaction most likely takes place intracellularly. The isomers that were shown to react with renalase could be physiologically relevant because of their ability to inhibit the NAD(P)H-dependent enzymes malate and lactate dehydrogenase (Beaupre, Hoag, Roman, et al., 2015). It is important to note that other ROS, in addition to H_2O_2, might be produced by renalase. Elevated ROS would occur if ROS-producing NADPH oxidases (NOXs) are also inhibited by the NAD(P)H isomers (Panday, Sahoo, Osorio, & Batra, 2015). NOSs are dependent on NADPH too, and this could make renalase an activator of NO via this enzymatic mechanism (Förstermann & Sessa, 2012). Unfortunately, there is only a limited amount of information on the relationship between ROS and renalase. This area should be further investigated for a better understanding of the physiological implications of renalase.

1.4.6 – *The Regulation of Renalase*

Revealing regulatory mechanisms of renalase is a crucial step towards understanding its function. Circulating CAs (Epi, NE, and DA) all regulate the synthesis, secretion, and activity of renalase (G. Li et al., 2008). Activity and secretion were quickly upregulated (within 15min), and synthesis was only upregulated after 12 hours (G. Li et al., 2008). In this case, activity was measured by CA metabolism, and not hormonal function or NADPH oxidation. If the prorenalase theory is true, CAs would be partly responsible for the activation of enzymatic renalase. Increased renalase expression by CAs has also been verified by other studies, where SP1 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) were the mediators of increased expression (Sonawane et al., 2014; F. Wang et al., 2014). Additionally, renalase may be regulated by the D5 receptor in proximal tubule cells through an alternative signaling mechanism that involves PKC instead of PKA (S. Wang et al., 2014). An issue with this finding is that it only applied to the dimerized form of renalase, which was previously identified as albumin, and not

renalase (Fedchenko et al., 2012). The minimal treatment time for these D5-based regulations was 12 hours. As previously stated, renalase expression is also dependent on kidney function. In addition to renalase's relationship with factors like AKI and GFR, salt intake may be a regulator of renalase expression (Desir & Peixoto, 2014; Stojanovic et al., 2015). Evidence for this claim was found when rats were fed a high-salt diet and renalase expression in the kidney and plasma was decreased (Y. Wang et al., 2015). This decrease was not altered by the use of an ARB or an ACE inhibitor (Y. Wang et al., 2015). In white muscle fibers, it was found that renalase protein and mRNA is decreased in untrained rats subjected to endurance training exercises, possibly as a function to redistribute blood flow (Czarkowska-Paczek, Zendzian-Piotrowska, Gala, Sobol, & Paczek, 2013). The influence of fat metabolism on renalase expression is beginning to be examined as well. In apolipoprotein E (ApoE) knockout mice, renalase expression is robustly increased in fat tissue (Zhou et al., 2015). So far, research on miRNA regulation of renalase has found the involvement of miR-29 and miR-146 in the post-transcriptional downregulation of renalase (Dziedzic et al., 2017; Kalyani et al., 2015). These miRNA could play a role in other regulators of CAs like COMT, NET, and D1Rs (Kalyani et al., 2015). The influence of gonadotropin-releasing hormone (GnRH) on renalase expression is also a new finding; GnRH antagonism decreased renalase expression in reproductive tissues (Zhou et al., 2013). Therefore, the transient activity of GnRH might help explain our finding that renalase mRNA is lower in female kidneys (Appendix A; Tsutsumi & Webster, 2009). Reproductive tissues also demonstrate higher expression of renalase in leptin-deficient mice (Zhou et al., 2013). Uninvestigated areas that should be analyzed for renalase regulation include apoptotic/stress, differentiation, and cellular remodeling mechanisms.

A greater understanding of renalase regulation requires the analysis of the renalase promoter. Currently, there are only four regulatory elements that have been identified in the renalase promoter (Figure 7). So far, there have been no inhibitory binding sites located on the renalase promoter. Three of these elements, which bind signal transducer and activator of transcription 3 (STAT3), SP1, and zinc-finger binding protein-89 (ZBP-89), were verified by Sonawane et al. in 2014. In this same study, other putative binding sites were found, but not investigated. Du et al. also identified four HREs in the renalase promoter, one of which being verified as a strong activator of transcription (2015). This finding is consistent with the notion that hypoxia has been shown to increase renalase expression in the renal cortex for up to 48 hours (F. Wang et al., 2015). STAT3's function is usually to mediate cell survival and inflammatory signals, but it has been demonstrated that nicotine (Nic) can also induce STAT3 activation in several cell lines (Chen, Ho, Guo, & Wang, 2008; Sonawane et al., 2014). Similar to STAT3, ZBP-89 has a role in inflammatory signaling (Borghaei, 2009). However, the physiological implications of its regulation of renalase remain to be elucidated. So far, only one study has revealed epigenetic regulation of the renalase promoter. In that study, the promoter was found to be inhibited by CpG island methylation in Epstein-Barr virus-infected keratinocytes (Birdwell et al., 2014). Ultimately, these regulatory mechanisms provide additional evidence that renalase has a homeostatic function in the body.

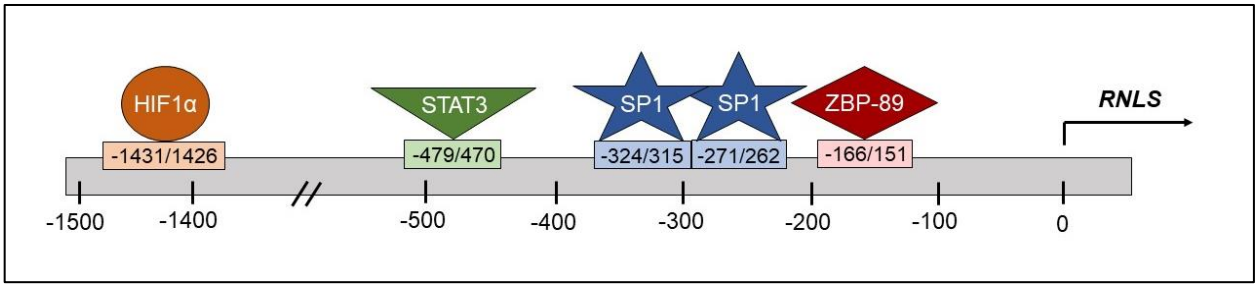

Figure 7: Established regulatory elements in the renalase promoter. The scale indicates the number of nucleotides upstream from the transcriptional start site (TSS). The exact locations of the binding sites are also noted below the transcription factors (TFs). TFs that bind to renalase's promoter include hypoxia-inducible factor 1α (HIF1α), signal transducer and activator of transcription 3 (STAT3), specificity protein 1 (SP1), and zinc-finger binding protein-89 (ZBP-89).

1.4.7 – *Current Issues Regarding the Function of Renalase*

The alternative name for renalase, monoamine oxidase C (MAO-C), is likely to be a misnomer. As previously mentioned, only 13.2% of renalase's amino acid sequence is shared with MAO-A (Xu et al., 2005). This partially explains why inhibitors of MAOs (e.g. clorgyline and pargyline) do not decrease the activity of renalase (Xu et al., 2005). Since renalase's discovery, there have been speculations about its ability to degrade CAs. Boomsma and Tipton were first to criticize renalase's CA oxidation assay from from Xu et al. in 2005 (Boomsma & Tipton, 2007). In their paper, they mention that the production of H_2O_2 was too slow and corresponded to only 0.25% of the CA substrates. Antioxidants were not added to these reactions and it has been proposed that the production of H_2O_2 originated from autoxidation of CAs (Boomsma & Tipton, 2007). HPLC-based techniques have added evidence that renalase does not contribute to the breakdown of CAs (Beaupre, Hoag, & Moran, 2015). Renalase's production of adrenochrome incites other questions about its catalytic function. A small number of studies suggest that adrenochrome is not a CA metabolite in normal physiology (Axelrod, 1959). Lastly, the secretion mechanism of renalase should be analyzed to confirm cleavage of the FAD-binding site within its signal sequence. If cleavage does occur in all tissues, renalase could metabolize CAs intracellularly in cell types like podocytes and mesangial cells, where the secretion of renalase is likely reduced or prevented.

If renalase does not oxidize CAs, there is still much to be known about its mechanism of action. Intracellularly, the inactivation of NAD(P)H isomers is a possible function (Beaupre, Hoag, Roman, et al., 2015). The inhibitory quality of 6DHNAD(P) and 2DHNAD(P) could apply to hundreds of enzymes and hence, more research is needed in this area. Renalase's highly conserved signal sequence indicates it is an important signaling molecule too. Once secreted, renalase could

act as a hormone by binding to PMCA4b (L. Wang et al., 2015). With only proximal tubules being analyzed so far, this signaling ability should be investigated in other tissues and with other types of PMCAs. Nevertheless, there is no complete connection between this signaling mechanism and the elevated CAs found in the renalase KO mice. Although p38 and ERK phosphorylation are consequences of renalase signaling, it is unknown if PMCA4b binding also increases Ca^{2+} efflux and nNOS activation. Together, these intracellular signaling pathways could alter CA biosynthesis, storage, secretion, and breakdown. The role of renalase in the adrenal glands should be investigated because they express renalase at a level that is among the highest in the body (Zhou et al., 2013). In this tissue, the cortex, medulla, and the splanchnic nerve could secrete renalase and create high extracellular concentrations of renalase in the adrenal medulla. Yet, it is still only a question whether autocrine, paracrine, and endocrine signals of renalase affect the largest producer of circulating CAs in the body.

1.5 – Hypothesis & Objectives

The purpose of this study is to examine the potential role of renalase in the regulation of CA biosynthesis in the adrenal medulla. It is hypothesized that renalase is part of a negative feedback mechanism in the adrenal medulla that functions to reduce CA biosynthesis, thereby regulating circulating CAs and consequently, BP. In this study, there are 3 objectives to test this hypothesis:

Objective 1: Examine the regulation of renalase by signal activators of CA biosynthesis. This objective was performed using PC12 cells and renalase mRNA and protein levels were both analyzed. Signaling molecules in this study were either activators of CA-biosynthesis or Epi itself (highlighted in Figure 3). As part of a negative feedback mechanism, it was predicted that renalase expression will increase after such treatments.

Objective 2: Determine if renalase, or its peptide, RP-220, regulates CA-biosynthesizing enzymes. mRNA levels in PC12 cells were used to detect changes in CA biosynthesis. Because renalase can act as a hormone with PMCA4b, it is possible that a similar or alternative membrane protein can induce intracellular signals that regulate CA biosynthesis (e.g. MAPK or NO signaling mechanisms; Figure 3). Following renalase treatment, it was predicted that CA-biosynthesizing enzyme mRNA levels will decrease.

Objective 3: Examine the expression of renalase in hypertensive rats. Renalase mRNA and protein levels were both compared in the adrenal gland between adult normotensive WKYs and hypertensive SHRs. Because of the anti-hypertensive effects of renalase, it was predicted that SHRs will have a lower expression of renalase.

2 – Materials and Methods

2.1 – Animal Handling and Tissue Collection

Male SHRs and WKYs (n = 4) were purchased from Charles River (Wilmington, MA). All protocols regarding the handling of animals have been previously described (Nguyen et al., 2009). Animals were cared for in accordance with the Canadian Council on Animal Care guidelines, and were approved by the Laurentian University Animal Care Committee. Adrenals were collected at 12 weeks of age and stored at -80^{o}C until use.

2.2 – Cell Culture

2.2.1 – *Cell Splitting and Counting*

All experiments were performed using rat PC12 cells (Dr. Daniel O'Connor, University of California, San Diego, CA). Cells were cultured in Nunclon Delta-treated dishes (ThermoFisher, Waltham, MA) using Dulbecco's Modified Eagle's Medium (DMEM; Hyclone, Logan, UT) containing 5% bovine calf serum (Hyclone), 5% equine serum (Hyclone), and 0.05µg/mL of gentamycin sulfate (Sigma-Aldrich, St. Louis, MO). Growth of cells took place in a humidified incubator (Forma Scientific model 3130; ThermoFisher) at 37^{o}C supplied with 5% CO_2. Cell growth and division was monitored and cells were split at a confluency of 80-90%. Before splitting, reagents were warmed to 37^{o}C and the cell culture hood was exposed to ultraviolet (UV) light for 20min. First, cells were washed twice with autoclaved phosphate-buffered saline (PBS; pH = 7.4; 137 mM sodium chloride (NaCl), 2.7 mM potassium chloride (KCl), 8 mM sodium hydrogen phosphate (Na_2HPO_4), and 2 mM monopotassium phosphate (KH_2PO_4). All reagents for PBS were purchased from Sigma-Aldrich, except for NaCl, which was from Thermo-Fisher. Washing was followed by a 5min incubation with trypsin-ethylenediaminetetraacetic acid (EDTA; 1mL per 100mm dish; Gibco, Waltham, MA). Cells were neutralized by adding DMEM (10mL

per 100mm dish) and clumps were disturbed by pipetting the cell mixture. Cell viability was performed using a Vi-CELL cell counter (Beckman Coulter, Mississauga, ON) as per the manufacturer's instructions.

2.2.2 – *Cell Treatments*

Before cell treatment, cell viability was confirmed to be greater than 90%. 2.75 million viable cells (or lower depending on treatment duration) were placed in 35mm dishes (Corning, Tewksbury, MA) with DMEM containing charcoal-treated serum (CTM). Cells were grown in CTM for 16-24 hours before drug treatment. Treatment concentrations and durations were used because of their signaling effects established in previous studies (Table 1). Prior to treatment, all signaling substances were diluted to a working concentration in CTM. For the recombinant renalase treatments, urea and glycerol (each at 0.1%; ThermoFisher) were used to control for trace amounts in the renalase solvent. For the RP-220 treatments, acetonitrile (0.05%; Sigma-Aldrich) was used to control for trace amounts in the peptide solvent. PMA, $CoCl_2$, Nic, PACAP, and Ang II were purchased from Sigma-Aldrich. Dex, Fsk, H_2O_2, and Epi were acquired from Cayman Chemicals (Ann Arbor, MI), LC Laboratories (Woburn, MA), ThermoFisher, and Alfa Aesar (Haverhill, MA), respectively. NGF was provided by Dr. G. Ross (Medical Sciences Division, Northern Ontario School of Medicine, Sudbury, ON). Recombinant renalase and RP-220 were purchased from Abcam (ab134535 and ab45730, respectively; Cambridge, UK). Treatments for mRNA quantitation were completed by aspirating media, washing cells once in warm 1x PBS, and adding 500µL of TRI Reagent (Sigma-Aldrich) per 35mm dish. Plates were then stored at -80^{o}C until RNA extraction. Cells used for protein quantification were immediately extracted after treatment.

Table 1: Conditions used for the treatment of PC12 cells.

Signaling Molecule	Treatment Concentration(s)	Treatment Duration(s)	Reference with Similar Treatment Conditions
Phorbol 12-myristate 13-acetate (PMA)	8, 40, 80, 400, & 800nM for 6h	1, 6, 12, & 24h at 80nM	Tai & Wong, 2003
Dexamethasone (Dex)	0.01, 0.1, 1, 10, & 100µM for 6h	1, 6, 12 & 24h at 1µM	Wong, Siddall, Ebert, Bell, & Her, 1998
Cobalt chloride ($CoCl_2$)	200 & 500µM for 6h	1, 6, 12, & 24h at 200µM	Tai et al., 2009; Crispo et al., 2011
Forskolin (Fsk)	10µM	1, 6, 12, & 24h	Tai & Wong, 2003
Epinephrine (Epi)	200µM	24h	Sonawane et al., 2014
Nicotine (Nic)	500µM	24h	Sonawane et al., 2014
Pituitary adenylate cyclase-activating polypeptide (PACAP)	10 & 100nM	45m & 6h	Tai, Wong-Faull, Claycomb, Aborn, & Wong, 2010; Taupenot, Mahata, Mahata, & O'Connor, 1999
Hydrogen peroxide (H_2O_2)	500µM	30m	Ito, Nakashima, & Nozawa, 1997
Angiotensin II (Ang II)	100nM	6h	Yu, Lu, Rowland, & Raizada, 1996
Nerve growth factor (NGF)	500pM	1, 6, 12, & 24h	Tai, Wong-Faull, Claycomb, & Wong, 2006
Renalase (Rnls)	50 & 250nM	24h	L. Wang et al., 2014
Renalase peptide 220 (RP-220)	1.5 & 8.5µM	24h	L. Wang et al., 2015

2.3 – RNA Extraction, cDNA Synthesis, and RT-PCR

2.3.1 – *RNA Extraction*

For tissue analysis, whole adrenals were placed in 1mL of TRI Reagent and homogenized with a Qiagen TissueLyser (Newtown, PA). Extraction from adrenals was performed using Qiagen's AllPrep DNA/RNA/Protein Mini Kit as per the manufacturer's instructions. For RNA extraction from cells, plates from -80^{o}C were thawed and samples were transferred to tubes. Then, 100µL of chloroform (per 500µL of TRI Reagent; Sigma-Aldrich) was added, vortexed for 10s, and incubated at room temperature for 10min. Samples were then placed in an Eppendorf 5415 R centrifuge (Hamburg, Germany) for 20min at 4^{o}C with a speed of 12 000 x g. After centrifugation, the top aqueous layer was carefully removed and added to 125µL of isopropanol (per 500µL of TRI Reagent; Sigma-Aldrich). This mixture was incubated for 8min at room temperature and centrifuged for another 8min at 12 000 x g and at 4^{o}C. The supernatant was removed and the pellet was washed in 500µL of 70% ethanol (Commercial Alcohols, Toronto, ON) per 500µL of TRI Reagent. A last centrifugation was performed for 5min at 7500 x g and 4^{o}C. Pellets were air-dried for no longer than 10min and resuspended in 20-40µL of diethyl pyrocarbonate water (DEPC water; reagent and protocol from Fluka/Honeywell, Morris Plains, NJ). Pellets were dissolved in DEPC water using an Eppendorf thermomixer R for 10min at 37^{o}C at 1000rpm. Samples were stored at -20^{o}C for short-term storage and -80^{o}C for long-term storage.

2.3.2 – *Spectrophotometry and RNA Integrity Analysis*

RNA yield, quality, and purity were quantified using a NanoDrop ND-1000 spectrophotometer (Wilmington, DE). All samples were confirmed to have a 260/280 ratio between 1.90 and 2.05. Visual analysis of RNA integrity was carried out with the electrophoresis on a 1% gel containing 0.5µg/mL of ethidium bromide (EtBr; Sigma-Aldrich) and agarose

(FroggaBio, Toronto, ON) dissolved in a TBE (Tris-borate-EDTA) buffer. TBE buffer was made using MilliQ water (EMD/MilliPore, Billerica, MA) containing 90mM Tris base, 90mM boric acid, and 2mM EDTA. These reagents for TBE buffer were purchased through ThermoFisher. 500ng of each RNA sample was added to 2µL of 6x gel-loading dye and a difference of DEPC water to provide a total load volume of 12µL. A water control was prepared using 10µL of DEPC water and 2µL of loading dye (30% glycerol and 0.25% bromophenol blue (Sigma-Aldrich)). 5µL of 100 base pair (bp) DNA ladder (FroggaBio) was also loaded. Electrophoresis was completed using a horizontal electrophoresis apparatus and PowerPac power supply from BioRad (Hercules, CA). After electrophoresis for 1 hour at 100V, RNA integrity gels were visualized using a Bio-Rad UV ChemiDoc analyzer and the Quantity One Software. Samples were considered to have acceptable integrity if the 28s band was approximately twice the density of the 18s band. No other major bands were visualized during RNA integrity analysis.

2.3.3 – *DNase Treatment and cDNA Synthesis*

2µL of 10x DNase reaction buffer and amplification grade DNase I (Sigma-Aldrich) were added to 2µg of RNA for each sample. A reaction volume of 20µL was achieved by adding extra volume of DEPC water. One sample was duplicated for a negative reverse-transcriptase (RT) control. Samples were sealed and incubated at room temperature for 15min. 2µL of a stop solution (Sigma-Aldrich) was added to each sample, followed by enzyme denaturation for 10min at 70^{o}C. The RT reaction protocol was initiated by adding 0.5µg of random primers (Roche, Basel, Switzerland) to 1µg of RNA and heating samples for 5min at 70^{o}C. Each sample was mixed with 2.5µL of a 10mM deoxynucleotide triphosphate (dNTP; Promega, Madison, WI) solution, 10µL of 5x reaction buffer for Moloney Murine Leukemia Virus (M-MLV) RT (Promega), 12.5µL of DEPC water, and 2µL of M-MLV RT (200U/µL; Promega). RT was replaced by DEPC for the

negative RT control. The RT reaction was carried out at 37ºC for 1 hour. All temperature incubations were performed using a Bio-Rad Thermal Cycler (S1000 or MJ Mini).

2.3.4 – *RT-PCR and Gel Electrophoresis*

Polymerase chain reaction (PCR) reactions had a total volume of 25µL and contained 4ng/µL cDNA, autoclaved MilliQ water, 5µL of 5x Green GoTaq flexi reaction buffer (Promega), 1.5mM magnesium chloride ($MgCl_2$; Promega), 200µM dNTPs, 2ng/µL of forward and reverse primers (Sigma-Aldrich), and GoTaq flexi DNA polymerase (5U/µL; Promega). Negative RT, and the no template (water) controls were also included in the PCR runs by substituting the cDNA volume with the respective control input. Primers were resuspended in 10mM Tris (pH = 8.0). Temperature changes were achieved using a Bio-Rad Thermal Cycler (S1000 or MJ Mini). Renalase primers were designed to bind its two main transcript variants (hRenalase1 and hRenalase2). Primer design was carried out using the National Center for Biotechnology Information (NCBI) primer-BLAST software and the Integrated DNA Technologies (IDT) oligoanalyzer tool. All primer sequences, cycle numbers, and annealing temperatures are shown in Table 2. The target genes of this study correspond to renalase (*Rnls*), EGR-1 (*Egr1*), GR (*Nr3c1*), HIF1α (*Hif1a*), PAH (*Pah*), TH (*Th*), DBH (*Dbh*), intronless and intron-retaining PNMT (*Pnmt*), and 28s RNA (*Rn28s1*). Visualization of PCR products was completed using the same electrophoresis conditions as the RNA integrity protocol except that a 2% TAE (tris-acetate-EDTA) gel was immersed in TAE buffer. TAE buffer was made using MilliQ water containing 40mM Tris base, 20mM acetic acid, and 1mM EDTA. 10µL of PCR product, negative RT control, or water control were loaded into each well.

2.3.5 – *Densitometry*

PCR products were visualized using a Bio-Rad UV ChemiDoc analyzer and the Quantity One software. Flat-field correction was enabled for all gels and exposure times were maximized until brightest bands were just under saturated density. Adjusted volumes using the "Local" function were used for the final density measurements. All measurements were normalized to the 28s gene.

Table 2: Primer specifications and conditions for RT-PCR.

Gene Name; Accession Number	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')	# of Cycles	Annealing Temperature (°C)	Amplicon Size (bp)
Rnls; NM_001014167.1	GATAACAAGTGGGAAGTCTC	CATAAAAGAGGCCCAGAGC	30	54	187
Egr1; NM_012551.2	TTTCCACAACAACAGGGAGAC	CTCAACAGGGCAAGCATACG	35	58	261
Nr3c1; NM_012576.2	CTCTGGAGGACAGATGTACCA	GCTTACATCTGGTCTCATTCC	28	58	232
Hif1a; NM_024359.1	GGTGCTAACAGATGATGGTGA	CTGGGCCATTTCTGTGTGTAAGC	27	58	161
Pah; NM_012619.2	GCTGCTAAGCTAGACACCTCA	CTTGTTTCCTGCCCAAAGTCT	31	58	105
Th; NM_012740.3	GCGACAGAGTCTCATCGAGGAT	GGCACCTCGAAGCGCACAAAA	20	52	303
Dbh; NM_013158.2	TTCCCCATGTTCAACGGACC	AGCTGTGTAGTGTAGACGGATGC	28	58	240
Pnmt; NM_031526.1	CAGACTTCTTGGAGGTCAACCG	AGCAGCGTCGTGATATGATAC	35	58	426/316
Rn28s1; NR_046246	AGGGATAACTGGCTTGTGGC	TAAACCCAGCTCACGTTCCC	18	58	143

2.4 – Western Blotting

2.4.1 – *Protein Extraction*

Immediately after treatment, cells were washed twice in ice-cold PBS. 250µL of cold radioimmunoprecipitation assay (RIPA) buffer was added per 35mm well. RIPA was made using 25 mM Tris base, 150 mM NaCl, 1% sodium deoxycholate (Sigma-Aldrich), 1% nonyl phenoxypolyethoxylethanol 40 (NP-40; Sigma-Aldrich), 0.1% sodium dodecyl sulphate (SDS; ThermoFisher), 2 mM EDTA, 0.5 mM phenylmethylsulphonyl fluoride (PMSF; Sigma-Aldrich), cOmplete Mini protease inhibitor cocktail tablet (1 tablet per 10mL; Roche), and hydrochloric acid (HCl)/sodium hydroxide (NaOH) to make a pH of 7.6. Cells were incubated on ice on a shaker for 10min and then centrifuged for 20min at 12 000 x g at 4^{o}C. Supernatants were collected and stored at -20^{o}C for short-term storage or -80^{o}C for long term storage. In the single case for HIF1α protein, a nuclear extraction was performed instead of RIPA lysis. For nuclear extraction, cells were first washed twice with cold PBS immediately after treatment. Subsequently, cells were scraped, collected into microcentrifuge tubes with 1mL of cold PBS, and centrifuged at 13 000 x g for 10s at 4^{o}C. The pellet was incubated for 10min on ice in 200µL of lysis buffer A, which contained 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulphonic acid-potassium hydroxide (Hepes-KOH; EMD/MilliPore), 1.5mM $MgCl_2$ (Sigma-Aldrich), 10mM KCl, 0.5mM dithiothreitol (DTT; ThermoFisher), 0.2mM PMSF (Pierce, Waltham, MA), and 20µL of 1x protease inhibitor cocktail (Sigma-Aldrich). Next, samples were force-ejected 10 times through a 22-gauge needle, vortexed, and centrifuged at 13 000 x g, as before. The following pellet was placed on ice for 20min in 50µL of lysis buffer C, which contained 20mM Hepes-KOH, 1.5mM MgCl2, 420mM NaCl, 25% glycerol, 0.2mM EDTA, 0.5mM DTT, 0.2mM PMSF, and 20µL of protease inhibitor cocktail. Another centrifugation at 13 000 x g (4^{o}C) was carried out for 2min

and nuclear extracts were collected from the supernatant. Finally, protein extraction from WKY/SHR adrenals was performed using Qiagen's AllPrep DNA/RNA/Protein Mini Kit as per the manufacturer's instructions.

2.4.2 – *Protein Quantification*

Protein concentrations were estimated using standard Bradford or bicinchoninic acid (BCA) assay procedures. BCA assays were performed for adrenal protein and PC12 protein from time-variable treatments with PMA, Dex, Fsk, and NGF. Bradford standards were prepared using immunoglobulin G protein (IgG; Sigma-Aldrich) and MilliQ water. 250µL of 1x Bradford reagent (BioRad) was added to each well and incubated at room temperature for 5min. For the BCA assays, all sample and standard preparation were performed as per the manufacturer's instructions (Pierce). Bradford assay samples/standards were read at a wavelength of 595nm, while BCA assay samples/standards were read at 562nm. Absorbance readings were measured using a PowerWave XS spectrophotometer and KC-4 software by BioTek (Winooski, VT). Two technical replicates were completed for the quantification of all standards and samples.

2.4.3 – *Gel Casting*

Polyacrylamide gel electrophoresis (PAGE) was performed using the Mini-PROTEAN Tetra cell kit and instructions from BioRad. All solutions were made according to the available protocol, with the exception that a 29:1 acrylamide/bis-acrylamide ratio was used instead of 37.5:1. Acrylamide/bis-acrylamide, ammonium persulphate (APS), tetramethylethylenediamine (TEMED), and glycine were purchased from ThermoFisher. Spacer plate thickness in all cases was 1.5mm. 10% resolving gels were layered with 200µL of butanol (Sigma-Aldrich) saturated with water and left to polymerize for 30min. Water-saturated butanol was thoroughly washed out with distilled water, 4% stacking gels were poured, 15-well combs were set up, and polymerization took place for 25min.

2.4.4 – *Sample Loading and Gel Electrophoresis*

A load volume of 30µL was used to help prevent sample loss. RIPA (or lysis buffer C) was used to adjust sample volume. Samples contained 20µg of protein and 5µL of 6x loading dye (made from 300mM Tris base, 12% SDS, 12mM EDTA, 6% β-mercaptoethanol (BME; Sigma-Aldrich), 60% glycerol, and 6% bromophenol blue). Prior to loading, samples were denatured at 95^{o}C for 5min using a Digital Dry Block Heater from VWR (Radnor, PA). After assembly of the gel in the apparatus with running buffer, excess salt in the newly-formed wells was washed and cleared using a 23-gauge needle. 10µL of the 100kDa protein ladder from Promega was also loaded. Using a BioRad's PowerPac, electrophoresis through the stacking gel was completed at 75V. After the protein reached the resolving gel, the voltage was increased to 100V and remained for 2.5 hours.

2.4.5 – *Electrotransfer*

All transfers were achieved using BioRad's Mini Trans-Blot Cell System and the manufacturer's instructions were followed. Methanol (Sigma-Aldrich) was added to the transfer buffer at a concentration of 20%. Transfer buffers were cooled to 4^{o}C before use. Polyacrylamide gels were equilibrated in cold transfer buffer for 5min before transfer. Proteins were transferred onto a nitrocellulose membrane with a pore size of 0.2µm (Pall, Port Washington, NY). 3 filter papers were added outside and adjacent to the gel and membrane (6 papers total). Potential bubbles within the transfer sandwich were removed by rolling a pipette across the outer surface four times. After assembly of the apparatus, a PowerPac was set to 100V for 1 hour. Activation of the stir bead was performed using a VWR magnetic stirrer.

2.4.6 – *Blocking and Antibody Incubation*

Washing procedures were performed using tris-buffered saline with tween-20 (TBS-T; 20mM Tris HCl, 465mM NaCl, and 0.1% tween-20 (MP Biochemicals, Santa Ana, CA); pH = 7.4). A blocking buffer was made using 5% instant skim milk powder (Carnation, Markham, ON) in TBS-T. Immediately after transfer, protein transfer quality was verified by incubating membranes with Ponceau S stain (0.1% Ponceau S (Sigma-Aldrich) and 5% acetic acid in MilliQ water) for 2min and destained with 0.1M NaOH and subsequent washes in distilled water. After destaining, membranes were placed in blocking buffer for 1 hour on a shaker with a medium speed setting and at room temperature. Before primary antibody incubation, membranes were washed in TBS-T four times (5min each) on the shaker with the same conditions as before. Primary antibodies were diluted in blocking buffer (containing 0.01% sodium azide (NaN_3; Sigma-Aldrich) after first use) using the conditions provided in Table 3. Membranes were placed in excess primary antibody mixture and left on a Rocker II (Boekel, Feasterville, PA) at 4^{o}C overnight. Between primary and secondary antibody incubations, four washes of the membrane were completed at 10min each. Secondary antibodies conjugated to horseradish peroxidase (HRP) were also diluted in blocking buffer (without NaN_3) using the conditions listed in Table 3. Membranes were immersed in this mixture, placed on a rocker at room temperature, and left for 1 hour. Finally, before the enhanced chemiluminescence (ECL) reaction, membranes were washed (4 x 10min) on a shaker at room temperature.

Table 3: Antibody specifications and conditions for western blotting.

Protein Specificity	Antibody Type	Host Animal	Company; Catalogue #	Lot #	Dilution Ratio (Antibody: Buffer)
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	Primary	Mouse	Abcam (Cambridge, UK); ab8245	GR232949-3	1:10 000
Proliferating cell nuclear antigen (PCNA)	Primary	Mouse	Santa Cruz (Dallas, TX); sc-56	A1113	1:500
Hypoxia-inducible factor 1α (HIF1α)	Primary	Rabbit	Novus (Littleton, CO); NB100-134	M-3	1:1000
Early growth response protein 1 (EGR-1)	Primary	Rabbit	Santa Cruz; sc-189	G2114	1:500
Phospho-ser40 tyrosine hydroxylase (TH)	Primary	Rabbit	Novus; NB300-173	AJ0514y	1:4000
Renalase (Rnls)	Primary	Rabbit	Abcam; ab178700	GR153391-1	1:2500
Mouse IgG	HRP-conjugated secondary	Donkey	Santa Cruz; sc-2005	B2014	1:2000
Rabbit IgG	HRP-conjugated secondary	Goat	Abcam; ab97051	GR249836-5	1:5000

2.4.7 – *Enhanced Chemiluminescence*

A 1:1 ratio of ECL A and ECL B solutions was made immediately before the ECL reaction. ECL A solution contained 2.5mM luminol (Sigma-Aldrich), 0.4mM p-coumaric acid (Sigma-Aldrich), and 0.1M Tris-HCl (from a solution with pH = 8.5) dissolved in MilliQ water. ECL B solution contained 8.2mM H_2O_2, and 0.1M Tris-HCl (pH = 8.5) dissolved in MilliQ water. Membranes were blot-dried on a clean plastic sheet and approximately 1.5mL of the ECL mixture was added to each membrane for 2min. The ECL mixture was quickly blot-dried after incubation and membranes were stored in clear plastic sheets for film exposure. Membranes were stored in a sealed plastic film at room temperature.

2.4.8 – *Film Exposure, Development, and Densitometry*

In a darkroom, ThermoFisher CL-XPosure films were placed over membranes using an x-ray cassette. Exposure times were chosen to allow bands to be just under saturation. Films were developed using a SRX 101A Konica Film Processor and reagents (Mississauga, ON) as per the manufacturer's instructions. Band densities were analyzed using the Bio-Rad UV ChemiDoc analyzer and the Quantity One software as before. Protein quantities were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for cytosolic extractions or proliferating cell nuclear antigen (PCNA) for nuclear extractions.

2.4.9 – *Stripping Membranes*

Membranes were stripped and reprobed up to 2 times after the first western blot procedure. Harsh stripping buffer was made with MilliQ water containing 2% SDS, 62.5mM Tris-HCl (from a solution with pH = 6.8), and 0.8% BME (as per Abcam's instructions). Membranes were incubated with harsh stripping buffer in a sealed container for 30min. This incubation took place in a heated shaking water bath (Precision Series 51221080 from ThermoFisher) on low speed at 50^{o}C. Membranes were then rinsed 10 ten times with distilled water and washed 6 times (10min

each) in TBS-T on a shaker with moderate speed. Membranes were transferred to a new container and blocking, washing, and reprobing were carried out as before (starting with the first blocking step after Ponceau staining).

2.5 – Statistical Analyses

All statistical analyses were performed using the GraphPad Prism software (La Jolla, CA). In all cases, mean, standard error of the mean (SEM), and interquartile range (IQR) were calculated. Outliers were considered when measurements were lower than the first quartile or higher than the third quartile by a magnitude greater than 1.5 x IQR. Differences between treatment durations and doses were analyzed using a one-way ANOVA with a post-hoc Tukey test. All other statistical analyses were performed using an unpaired, two-tailed t-test. Significant differences were considered where $p \leq 0.05$.

3 – Results

3.1 – Objective 1: Regulation of Renalase by Signal Activators of Catecholamine Biosynthesis

To complete the first objective of this study, renalase expression was analyzed after treatments of PC12 cells with Epi and 9 signal activators of CA biosynthesis. For most treatments, positive control genes (i.e. EGR-1, HIF1α, TH, and PNMT) were also analyzed to confirm activation of the particular intracellular signaling pathways. In all PCR analyses, two negative controls were included by substituting RT or cDNA with MilliQ water. As expected, all negative controls did not show DNA amplification following PCR.

3.1.1 – *Protein Kinase C Signaling*

Time-variable and dose-variable treatments of PC12 cells with PMA were performed for the activation of PKC mechanisms. Because EGR-1 expression is normally upregulated by PMA, it was measured to confirm PMA signals (Tai & Wong, 2003). EGR-1 mRNA and protein were upregulated after 1 hour (1.81-fold and 22.24-fold, respectively; $p \leq 0.001$) and began reduction to baseline levels after 6 hours (Figures 8A and 8B). mRNA levels of EGR-1 were still significantly upregulated after 6 hours (1.38-fold; $p \leq 0.05$) but not after 12 or 24 hours. EGR-1 protein was still elevated after 6, 12, and 24 hours, but did not reach significance. Renalase mRNA and intracellular protein were detected in all treatment groups and controls, indicating that PC12 cells express renalase. In Figure 8C it is shown that PMA signal activation reduced renalase mRNA after 6-hour treatments (0.71-fold; $p \leq 0.05$) and significantly increased to normal levels from 6 to 24 hours (1.35-fold; $p \leq 0.01$). Renalase protein was unchanged by time-variable treatments of PMA. All PMA doses did not alter renalase mRNA levels.

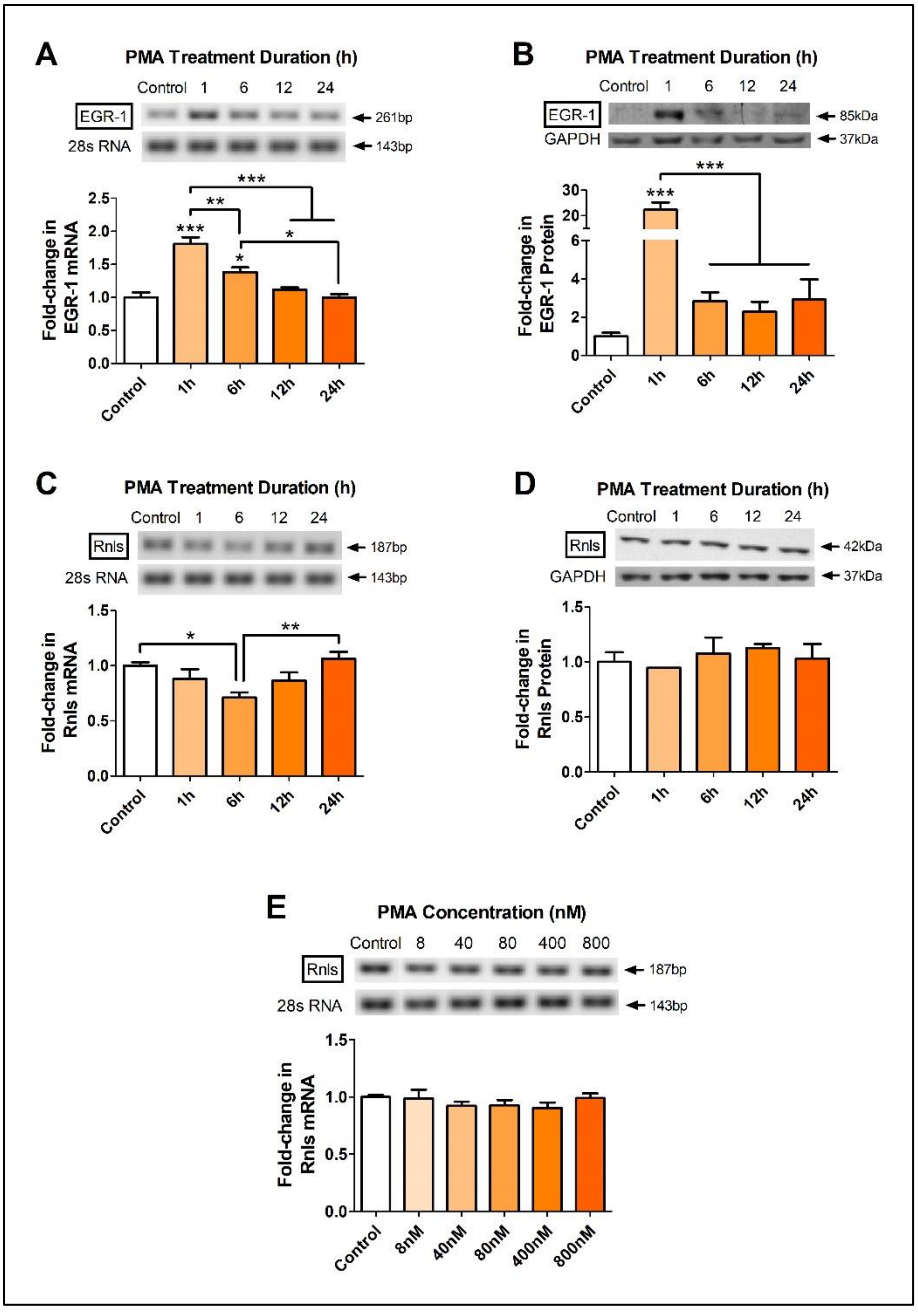

Figure 8: Gene expression after time-variable and dose-variable treatments of PC12 cells with phorbol 12-myristate 13-acetate (PMA). Image shows mRNA and intracellular protein levels of early growth response protein 1 (EGR-1; A and B) and renalase (Rnls; C, D, and E) detected by RT-PCR and western blotting. Time-variable treatments used 80nM PMA and dose-variable treatments were 6 hours long. Images shown are representative gel bands of each treatment. Fold-changes were normalized to untreated controls. mRNA and protein data were normalized to 28s RNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), respectively. Data are presented as mean ± SEM (n = 3). *, **, and *** directly above bars indicate significance from untreated controls where $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively (using ANOVA with a post-hoc Tukey test). All other significant differences between groups are bracketed.

3.1.2 – *Glucocorticoid Signaling*

To activate glucocorticoid signaling processes, Dex treatments were also performed on PC12 cells in a time-variable and dose-variable manner. The intronless form of PNMT mRNA is normally increased by Dex, and here, it was quantitated to confirm Dex activity (Figure 9A; K. T. Kim et al., 1993). This mechanism was confirmed, as intronless PNMT mRNA was upregulated after 6, 12, and 24 hours of Dex treatment (12.56-fold, 13.95-fold, and 9.34-fold, respectively; $p \leq 0.001$ in all cases). Dex did not show significant differences in intronless PNMT between 1-hour treatments and controls. Instead, the 1-hour treatment group was significantly different compared with the 6, 12, and 24-hour time points. TH activity is also increased by Dex signaling (Williams, Sandquist, Black, & Williams, 1981). Active TH protein (pSer40) was therefore analyzed after time-variable treatments with Dex (Figure 9B). This analysis revealed significantly increased pSer40 TH after 24 hours (2.78-fold; $p \leq 0.05$). Renalase mRNA levels were unchanged after time-variable or dose-variable treatments (Figures 9C and 9E). In contrast, renalase protein level (Figure 9D) was higher in 24-hour treatments compared to 6 hours. (1.35-fold; $p \leq 0.01$). However, no time-points had significantly different protein levels compared to controls.

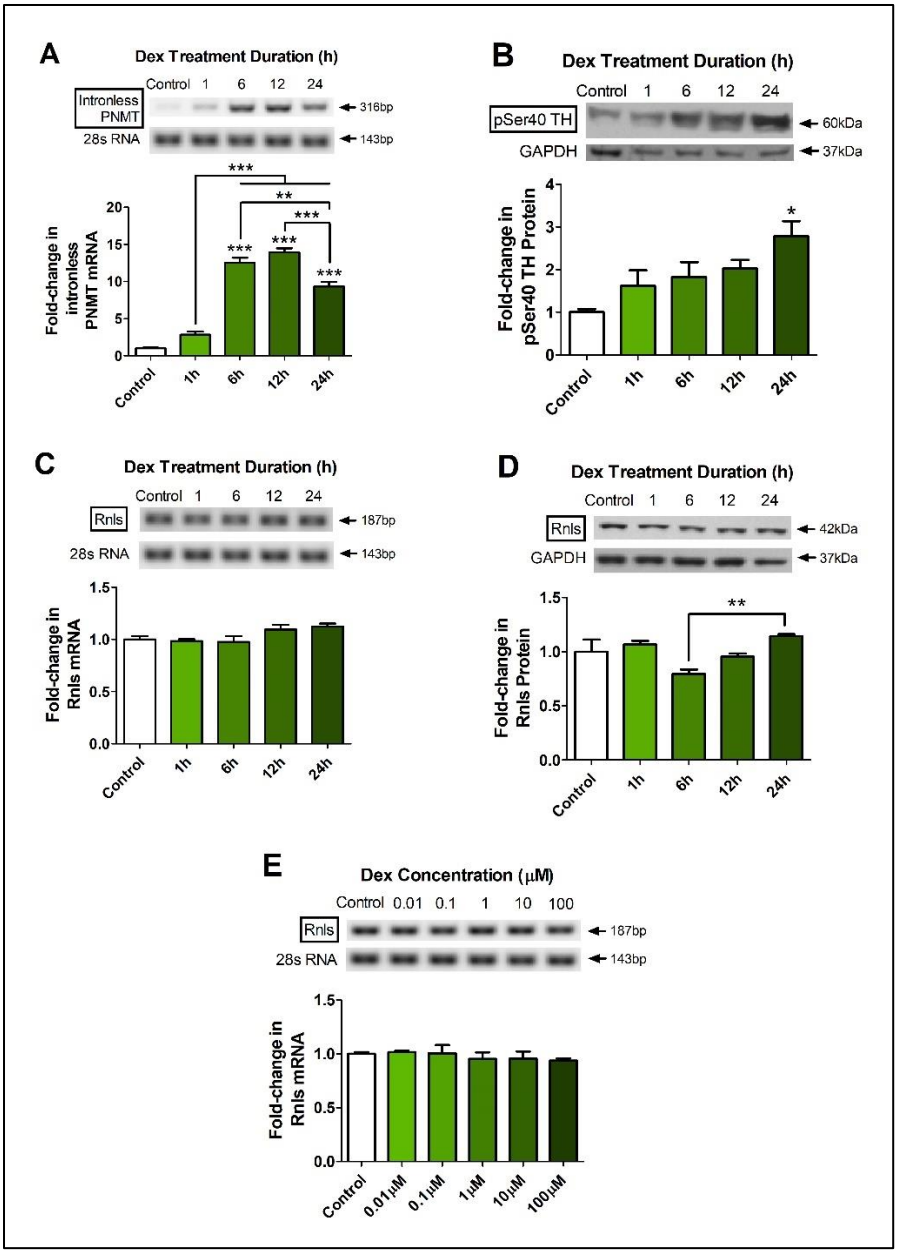

Figure 9: Gene expression after time-variable and dose-variable treatments of PC12 cells with dexamethasone (Dex). Image shows mRNA levels of intronless phenylethanolamine N-methyltransferase (PNMT; A) and renalase (Rnls; C). Image also shows intracellular protein levels of pSer40 tyrosine hydroxylase (TH; B) and renalase (Rnls; D and E) detected by RT-PCR and western blotting. Time-variable treatments used 1μM Dex and dose-variable treatments were 6 hours long. Images shown are representative gel bands of each treatment. Fold-changes were normalized to untreated controls. mRNA and protein data were normalized to 28s RNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), respectively. Data are presented as mean ± SEM (n = 3). *, **, and *** directly above bars indicate significance from untreated controls where $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively (using ANOVA with a post-hoc Tukey test). All other significant differences between groups are bracketed.

3.1.3 – *Hypoxia-inducible Factor Signaling*

To observe the influence of hypoxia signaling on renalase expression in PC12 cells, time-variable and dose-variable treatments with $CoCl_2$ were completed. Signal activation was confirmed by analyzing mRNA and protein levels of HIF1α. Cobalt binds directly to HIF1α and inhibits its degradation (Yuan et al., 2003). Without degradation, it localizes to the nucleus and acts as a TF. Therefore, an increase in HIF1α protein in the nucleus is characteristic of the signaling activities of $CoCl_2$. Under different conditions of hypoxia, HIF1α mRNA has been shown to be unchanged or increased following cell treatment (Huang, Arany, Livingston, & Bunn, 1996; Tai et al., 2009). In this study, its mRNA was unchanged (Figure 10A), but nuclear protein levels were increased after 6 hours (3.46-fold; $p \leq 0.01$; Figure 10B). Nevertheless, HIF1α mRNA was not analyzed after 24 hours and nuclear protein was not analyzed with time-variable treatments. Renalase mRNA levels were quantified after three separate experiments of $CoCl_2$ treatments (one time-variable, one dose-variable, and one set of 24-hour treatments; Figures 10C, 10E, and 10D, respectively). No changes in renalase mRNA were observed in all treatments other than the 24-hour treatment. After 24 hours of $CoCl_2$ treatment, renalase mRNA was moderately downregulated (0.77-fold; $p \leq 0.05$). Renalase protein was also quantified after 24-hour treatments with $CoCl_2$ (not shown). Due to high variability in the normalization protein, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), no conclusive analysis could be performed on renalase protein regulation after $CoCl_2$ treatment.

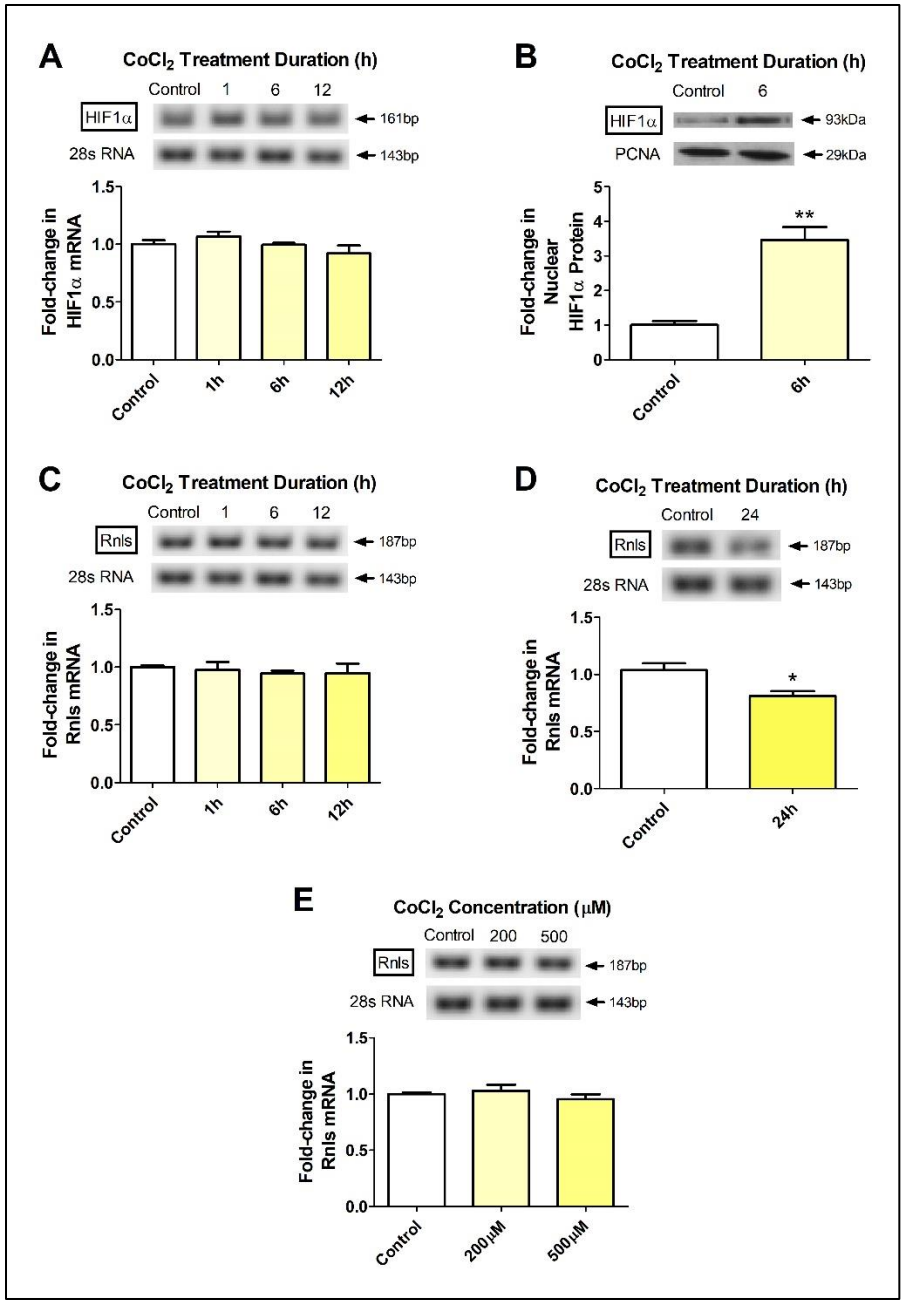

Figure 10: Gene expression after time-variable and dose-variable treatments of PC12 cells with cobalt chloride ($CoCl_2$). Image shows mRNA and intracellular protein levels of nuclear hypoxia-inducible factor 1α (nuclear HIF1α; A and B) and mRNA levels of renalase (Rnls; C, D, and E) detected by RT-PCR and western blotting. Time-variable treatments used 200μM $CoCl_2$ and dose-variable treatments were 6 hours long. Images shown are representative gel bands of each treatment. Fold-changes were normalized to untreated controls. mRNA and protein data were normalized to 28s RNA and proliferating cell nuclear antigen (PCNA), respectively. Data are presented as mean ± SEM (n = 3). * and ** directly above bars indicate significance from untreated controls where $p \leq 0.05$ and $p \leq 0.01$, respectively (using ANOVA with a post-hoc Tukey test).

3.1.4 – *Protein Kinase A Signaling*

Next, renalase expression was determined following PKA activation by Fsk treatments. Confirmation of intracellular signaling was completed by analyzing the expression of EGR-1, which was previously shown to be quickly increased after Fsk treatment (Tai & Wong, 2003). Indeed, following 1-hour treatments with Fsk, EGR-1 mRNA was significantly upregulated by a factor of 1.50 ($p \leq 0.01$; Figure 11A). There were no significant differences between controls and 6, 12, or 24-hour Fsk treatments. EGR-1 protein was quantified after the same time-points and treatment conditions. There was no detectable EGR-1 protein in all treatment groups other than 1-hour treatments. Despite this signal activation, neither renalase mRNA or protein were altered at the time-points investigated (Figures 11B and 11C).

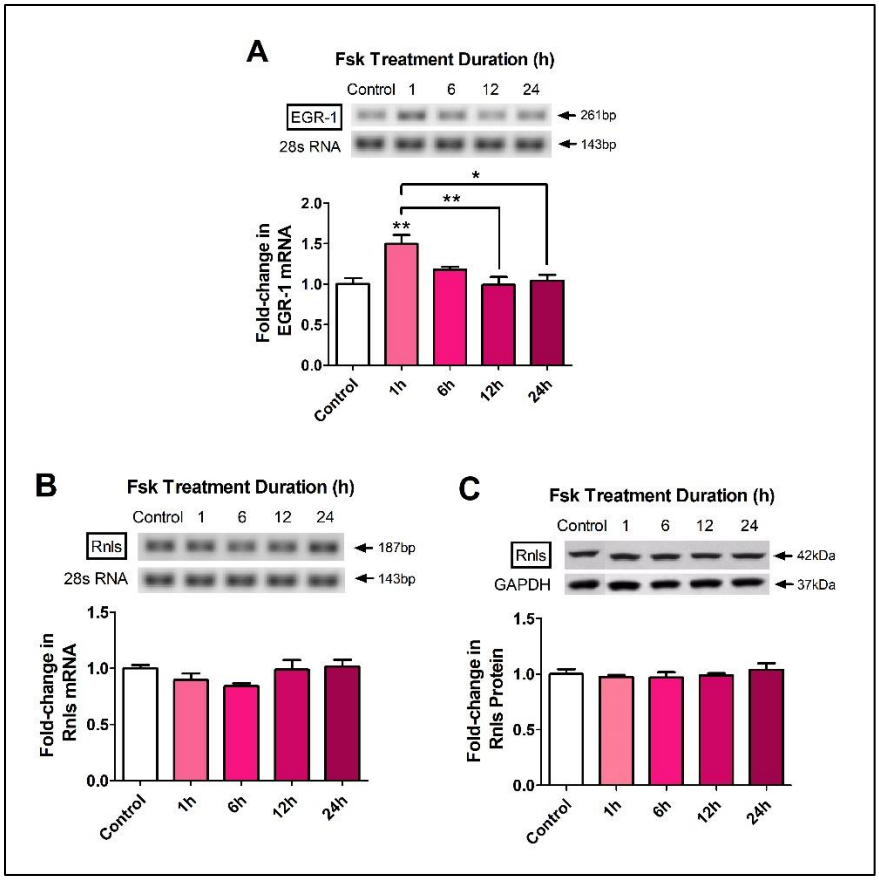

Figure 11: Gene expression after time-variable treatments of PC12 cells with forskolin (Fsk; 10µM). Image shows mRNA of early growth response protein 1 (EGR-1; A) and mRNA and intracellular protein levels of renalase (Rnls; B and C). Expression levels were detected by RT-PCR and western blotting. Images shown are representative gel bands of each treatment. Fold-changes were normalized to untreated controls. mRNA and protein data were normalized to 28s RNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), respectively. Data are presented as mean ± SEM (n = 3). * and ** directly above bars indicate significance from untreated controls where $p \leq 0.05$ and $p \leq 0.01$, respectively (using ANOVA with a post-hoc Tukey test). All other significant differences between groups are bracketed.

3.1.5 – *Epinephrine and Nicotine Treatment*

24-hour treatments with Epi or Nic were performed for two reasons: to activate common signals of chromaffin cells, and to examine previously established regulating signals of renalase in PC12 cells (Sonawane et al., 2014). Because Epi autocrine and paracrine signaling pathways aren't fully characterized in PC12 cells, mRNA levels of TH, intron-retaining PNMT, and intronless PNMT were analyzed. These genes were also used to confirm signal activations of Nic. After Epi treatments, mRNA levels showed downregulated TH (0.79-fold; $p \leq 0.05$; Figure 12A), upregulated intron-retaining PNMT (2.56-fold; $p \leq 0.05$; Figure 12B), and unaltered intronless PNMT (Figure 12C). Nic slightly increased TH mRNA (1.18-fold; $p \leq 0.05$; Figure 12A), but didn't change the level of PNMT transcript variants (Figures 12B and 12C). Likewise, renalase mRNA was unchanged after Nic and Epi treatments.

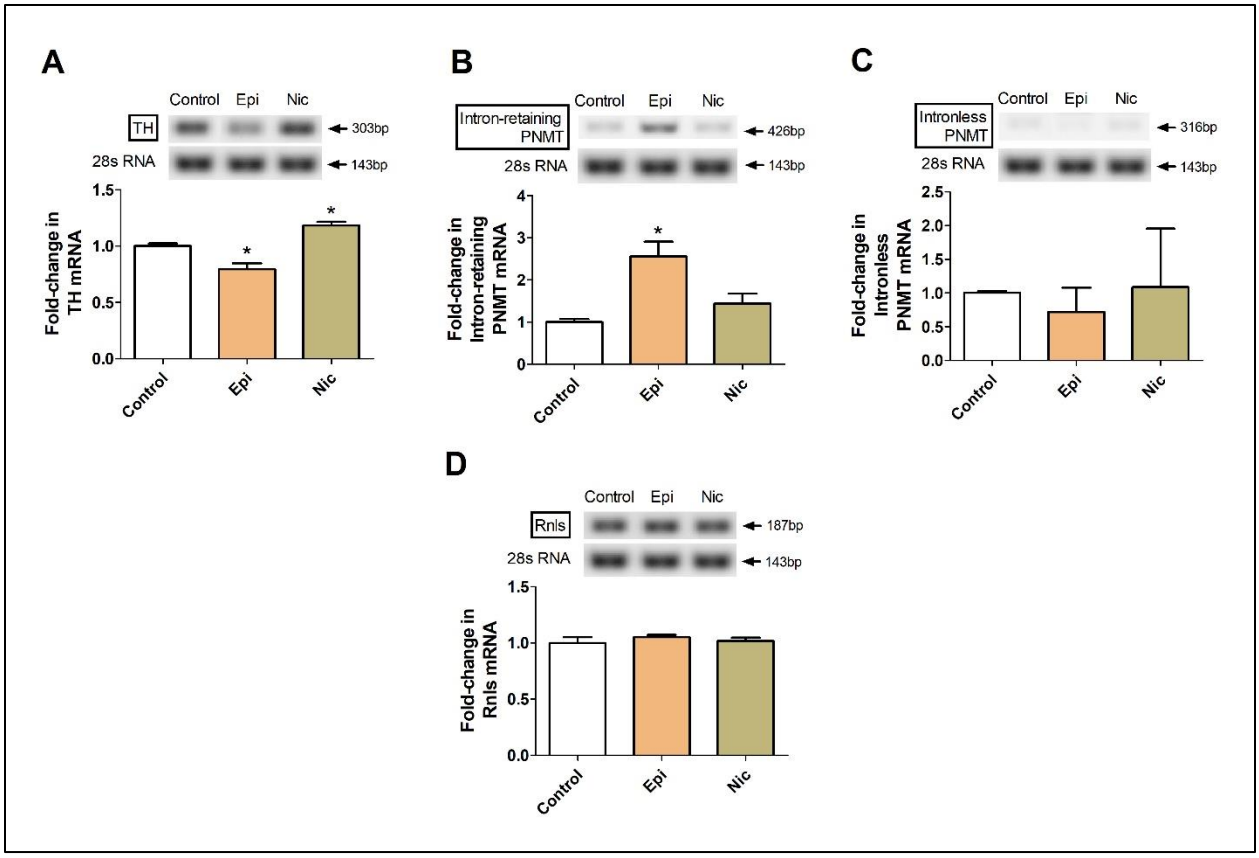

Figure 12: mRNA levels after 24-hour treatments of PC12 cells with epinephrine (Epi; 200µM) and nicotine (Nic; 500µM). Top row shows mRNA levels of tyrosine hydroxylase (TH; A), intron-retaining phenylethanolamine N-methyltransferase (PNMT; B) and intronless PNMT (C). Bottom row (D) shows mRNA levels of renalase (Rnls). Expression levels were detected by RT-PCR. Images shown are representative gel bands of each treatment. Fold-changes were normalized to untreated controls. mRNA levels were normalized to 28s RNA. Data are presented as mean ± SEM (n = 3). * indicates significance from untreated controls where $p \leq 0.05$ (using a two-tailed, unpaired t-test).

3.1.6 – *Pituitary Adenylate Cyclase-Activating Polypeptide, Hydrogen Peroxide, and Angiotensin II Treatment*

Additional activators of CA biosynthesis, PACAP, H_2O_2, and Ang II, were investigated to determine if their intracellular signaling pathways also regulate renalase. Two treatment conditions were used for PACAP to stimulate different intracellular signals that include PKA and PKC activation (Tai et al., 2010; Taupenot, Mahata, Mahata, & O'Connor, 1999; Vaudry et al., 2002). One gene that is regulated by PACAP and Ang II is PNMT (Cahill, Eertmoed, Mangoura, & Perlman, 2002; Tai et al., 2010). H_2O_2 could potentially regulate PNMT by inhibiting HIF1α degradation (Qutub & Popel, 2008). Thus, intron-retaining and intronless PNMT mRNA levels were quantified following treatments. There were no significant differences in either of the PNMT mRNA variants (not shown). No changes in renalase mRNA or protein were observed for all treatment conditions analyzed (Figures 13A – 13D).

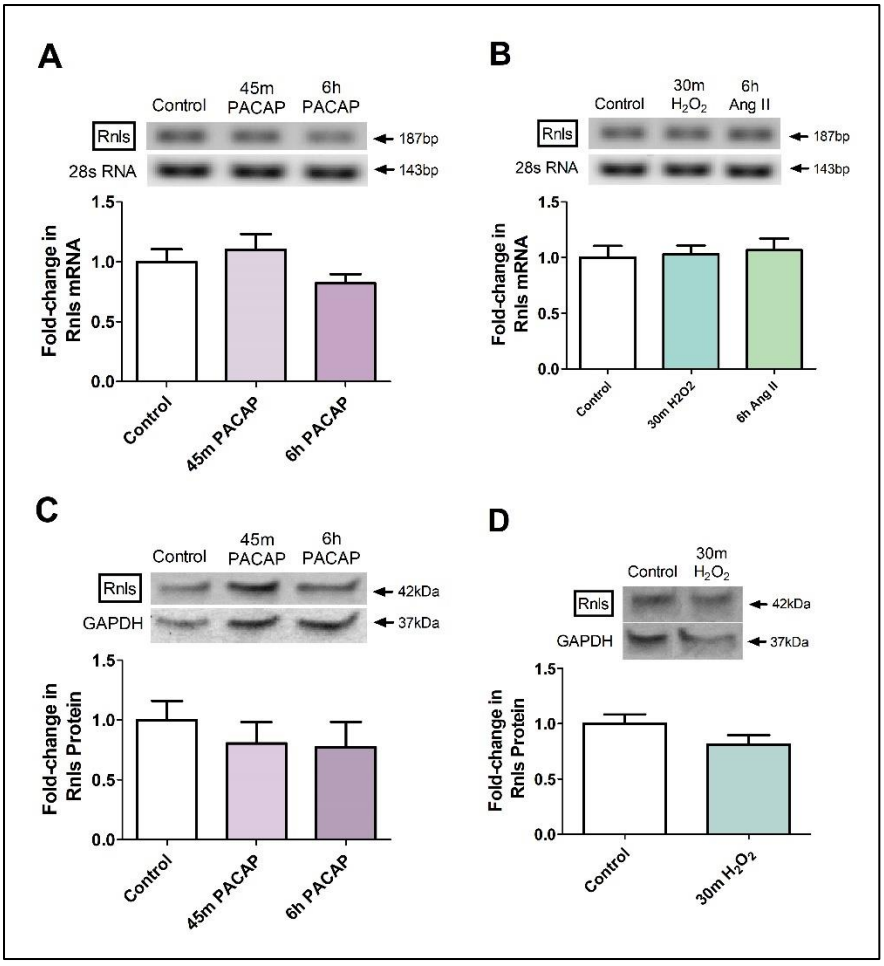

Figure 13: Renalase (Rnls) gene expression after treatments of PC12 cells with pituitary adenylate cyclase-activating polypeptide (PACAP), hydrogen peroxide (H_2O_2), and angiotensin II (Ang II). Top row shows mRNA levels detected by RT-PCR and bottom row shows intracellular protein levels detected by western blotting. PACAP treatments (A and C) were performed for 45min at 10nM or 6 hours at 100nM. H_2O_2 treatments (B and D) were performed for 30min at 500µM. Ang II treatments (B) were performed for 6 hours at 100nM. Images shown are representative gel bands of each treatment. Fold-changes were normalized to untreated controls. mRNA and protein levels were normalized to 28s RNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), respectively. Data are presented as mean ± SEM (n = 3). No significant differences between groups were revealed using a two-tailed, unpaired t-test.

3.1.7 – *Nerve Growth Factor Treatment*

The last activator of CA biosynthesis that was used for the treatment of PC12 cells was NGF. Because NGF induces time-dependent increases in EGR-1 expression, this gene was used to confirm intracellular signal activation (Tai et al., 2006). In the present study, time-variable treatments with NGF increased EGR-1 mRNA levels at all time points investigated. The highest increase in EGR-1 mRNA occurred with 1-hour treatments (1.79-fold; $p \leq 0.001$) and significant decreases from 1-hour treatments were observed with 6, 12, and 24-hour NGF incubations (Figure 14A). Nevertheless, all NGF treatment durations resulted in upregulated EGR-1 mRNA. A similar pattern was observed with EGR-1 protein. The highest increase in protein occurred after 1 hour (5.54-fold; $p \leq 0.001$) and levels were significantly reduced from the 1-hour time-point with 6, 12, and 24-hour treatments (Figure 14B). When analyzing renalase expression, there were no significant changes after NGF treatment at the mRNA or protein level (Figures 14C and 14D).

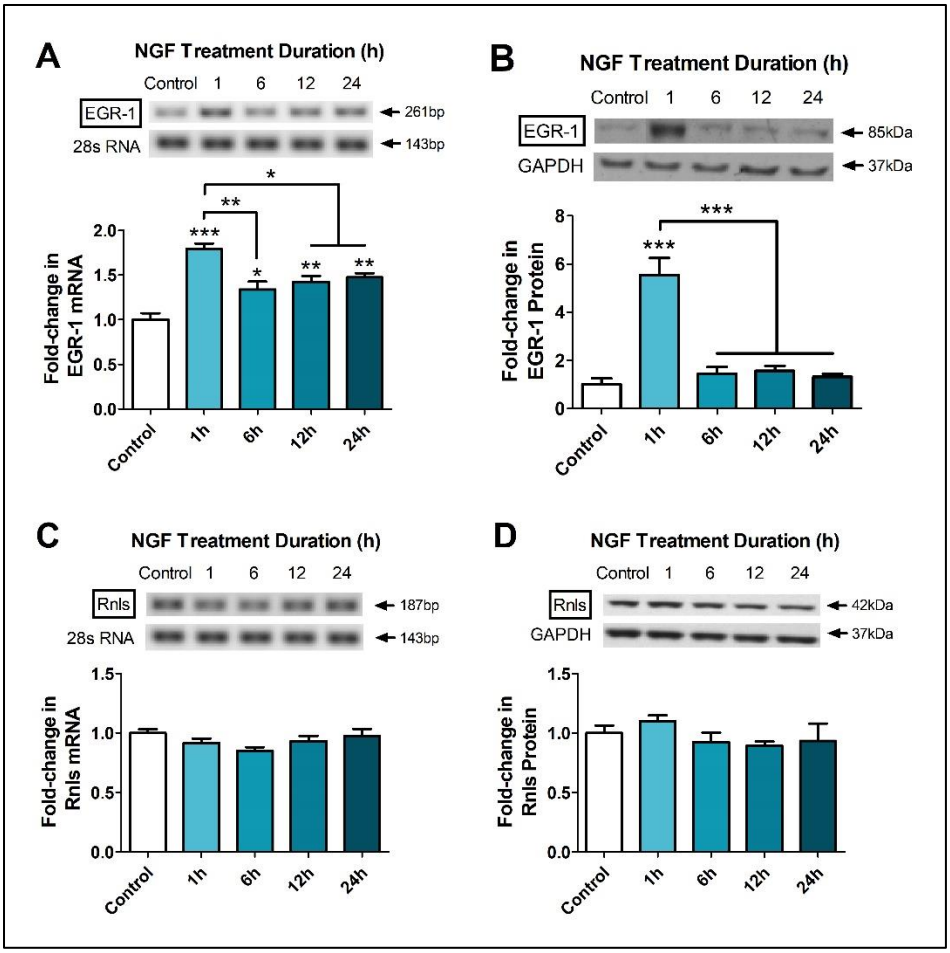

Figure 14: Gene expression after time-variable treatments of PC12 cells with nerve growth factor (NGF; 500pM). Image shows mRNA and intracellular protein levels of early growth response protein 1 (EGR-1; A and B) and renalase (Rnls; C and D) detected by RT-PCR and western blotting. Images shown are representative gel bands of each treatment. Fold-changes were normalized to untreated controls. mRNA and protein data were normalized to 28s RNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), respectively. Data are presented as mean ± SEM (n = 3). *, **, and *** indicate significance from untreated controls where $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively (using ANOVA with a post-hoc Tukey test). All other significant differences between groups are bracketed.

3.2 – Objective 2: Regulation of Catecholamine Synthesis by Renalase

3.2.1 – *Renalase Peptide-220 Treatment*

For the completion of the second objective, mRNA levels of CA-biosynthesizing enzymes were quantitated following dose-variable treatments of PC12 cells with the renalase signaling peptide, RP-220. Dex treatment was also included with or without the RP-220 treatments. Dex alone was used as a positive control to detect changes in CA biosynthesizing enzymes. Dex treatment, added in combination with RP-220, was performed to find potential protective effects of RP-220 on the Dex response. GR mRNA was also analyzed to determine if potential reductions to the CA-biosynthesis enzymes would be due to the expression of this central TF in the Dex response. To account for trace amounts of acetonitrile in the RP-220 solvent, extra control groups were included that contained acetonitrile alone, or acetonitrile with Dex. For all genes, there were no significant changes with the addition of acetonitrile. The TF, GR, as well as PAH did not show significant alterations in mRNA levels for all treatment groups (Figures 15A and 15B). TH mRNA analysis revealed a characteristic Dex-dependent upregulation (1.55-fold; $p \leq 0.001$; Figure 15C). Dex, in combination with RP-220, did not show altered TH regulation when compared with Dex alone. RP-220 itself did not induce any changes to TH regulation. All three treatment groups containing Dex had significantly higher TH mRNA levels compared with untreated controls and RP-220 groups ($p \leq 0.001$). For DBH, RP-220 alone did not alter mRNA levels at both concentrations (Figure 15D). mRNA levels, when compared to the control group, were significantly increased with Dex treatment (1.22-fold; $p \leq 0.05$) and with Dex and 8.5μM RP-220 (1.23-fold; $p \leq 0.05$). All groups containing Dex had significantly higher DBH mRNA compared to the 1.5μM RP-220 group. 8.5μM RP-220 treatment was not significantly different to the Dex groups. Regarding PNMT, Dex treatment, with or without RP-220, showed complete loss of

intron-retaining mRNA and significant increases in intronless mRNA, when compared to controls (Figures 15E and 15F). Both RP-220 concentrations resulted in a slight increase in intron-retaining mRNA, but these changes were not significant. mRNA levels of intron-retaining and intronless PNMT were significantly different between the RP-220 groups and all three Dex groups ($p \leq 0.01$). No differences were observed between Dex and Dex with RP-220 for both PNMT variants.

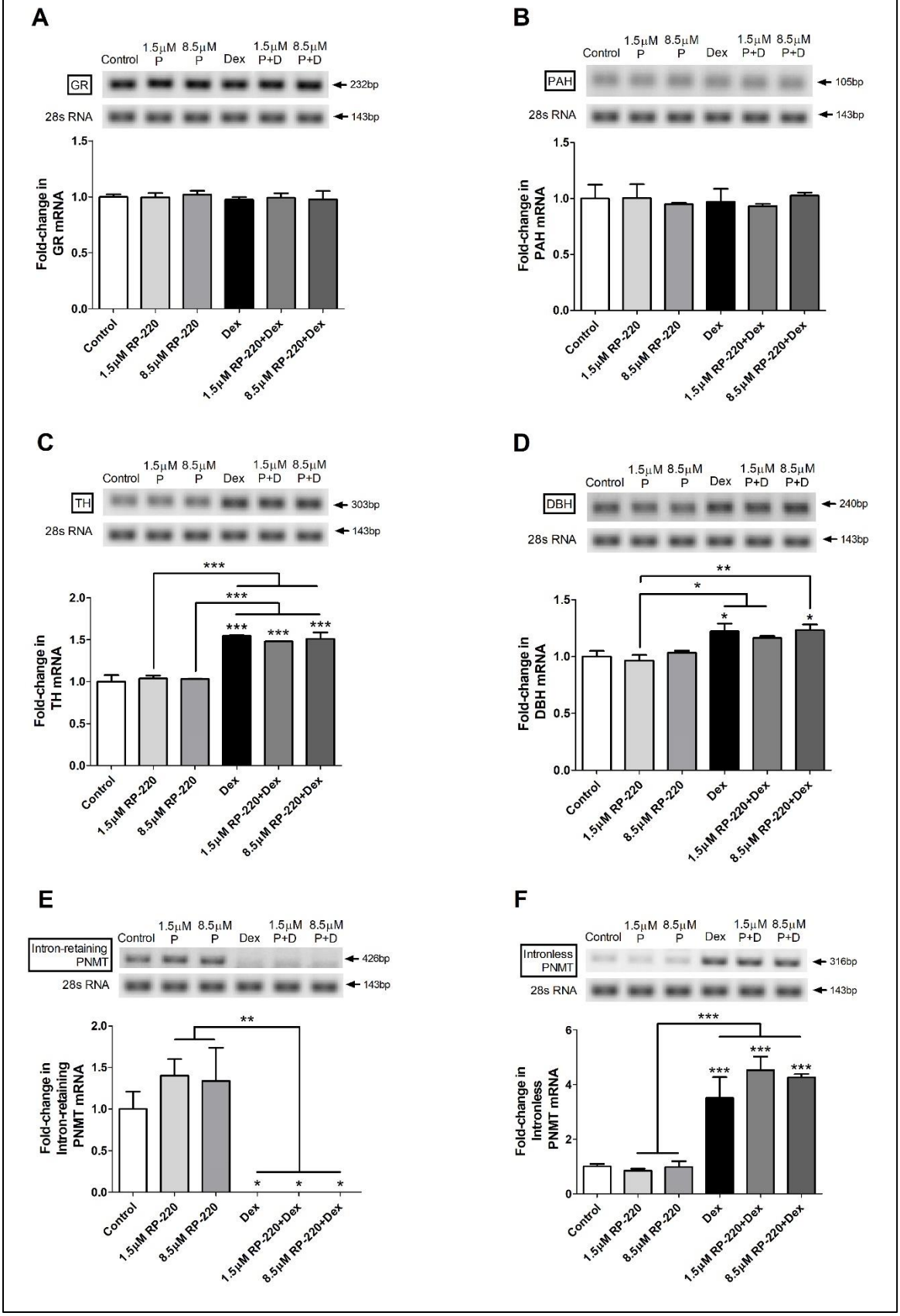
A
Control
1.5μM P
8.5μM P
Dex
1.5μM P+D
8.5μM P+D
GR
232bp
28s RNA
143bp
Fold-change in GR mRNA
Control
1.5μM RP-220
8.5μM RP-220
Dex
1.5μM RP-220+Dex
8.5μM RP-220+Dex
B
PAH
105bp
Fold-change in PAH mRNA
C
TH
303bp
Fold-change in TH mRNA
D
DBH
240bp
Fold-change in DBH mRNA
E
Intron-retaining PNMT
426bp
Fold-change in Intron-retaining PNMT mRNA
F
Intronless PNMT
316bp
Fold-change in Intronless PNMT mRNA

Figure 15: mRNA levels of catecholamine-biosynthesizing proteins after renalase peptide (RP-220; P) treatments with and without dexamethasone (Dex; D). Image shows mRNA levels of glucocorticoid receptor (GR; A), phenylalanine hydroxylase (PAH; B), tyrosine hydroxylase (TH; C), dopamine β-hydroxylase (DBH; D), intron-retaining phenylethanolamine N-methyltransferase (PNMT; E) and intronless PNMT (F). mRNA levels were detected by RT-PCR. RP-220 treatments were 24 hours long at variable doses. Dex treatments were performed 6 hours before the end of RP-220 treatments and at a concentration of 1μM. Images shown are representative gel bands of each treatment. Fold-changes were normalized to untreated controls. mRNA data were normalized to 28s RNA. Data are presented as mean ± SEM (n = 3). *, **, and *** indicate significance from untreated controls where $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively (using ANOVA with a post-hoc Tukey test). All other significant differences between groups are bracketed.

3.2.2 – *Renalase Treatment*

Furthermore, mRNA levels of CA-biosynthesizing enzymes were quantified following dose-variable treatments with recombinant renalase in PC12 cells. Like the RP-220 treatments, Dex was used as a treatment alone, or in combination with two concentrations of recombinant renalase. Trace amounts of urea and glycerol in the renalase solvent were accounted for by including these in control groups +/- Dex. The inclusion of urea and glycerol did not have any effects on gene mRNA. GR and PAH did not show any changes in mRNA levels with all treatment conditions (Figures 16A and 16B). TH mRNA was upregulated with Dex treatment (1.55-fold; $p \leq 0.001$; Figure 16C). There were no differences between Dex in combination with renalase and Dex alone. TH regulation was unchanged by both concentrations of renalase. The three groups containing Dex showed significantly increased TH mRNA when compared with controls or renalase treatments ($p \leq 0.01$). In contrast with RP-220 DBH mRNA levels were not significantly different between groups (Figure 16D). A partial factor for this finding is that the variability within groups was slightly higher than with the RP-220 treatments, thus making the ANOVA just under significance ($p = 0.087$). Very similar patterns of PNMT mRNA regulation were observed between RP-220 and renalase treatments. Again, Dex alone and Dex in combination with renalase revealed a loss of intron-retaining mRNA and increases in intronless mRNA, compared to controls (Figures 16E and 16F). 50nM and 250nM renalase treatments appeared to increase intron-retaining mRNA, but these changes were not significant. For intron-retaining and intronless PNMT mRNA, there were significant differences between both renalase groups and all three Dex groups ($p \leq 0.01$). No differences were observed between Dex and Dex with renalase for both PNMT variants.

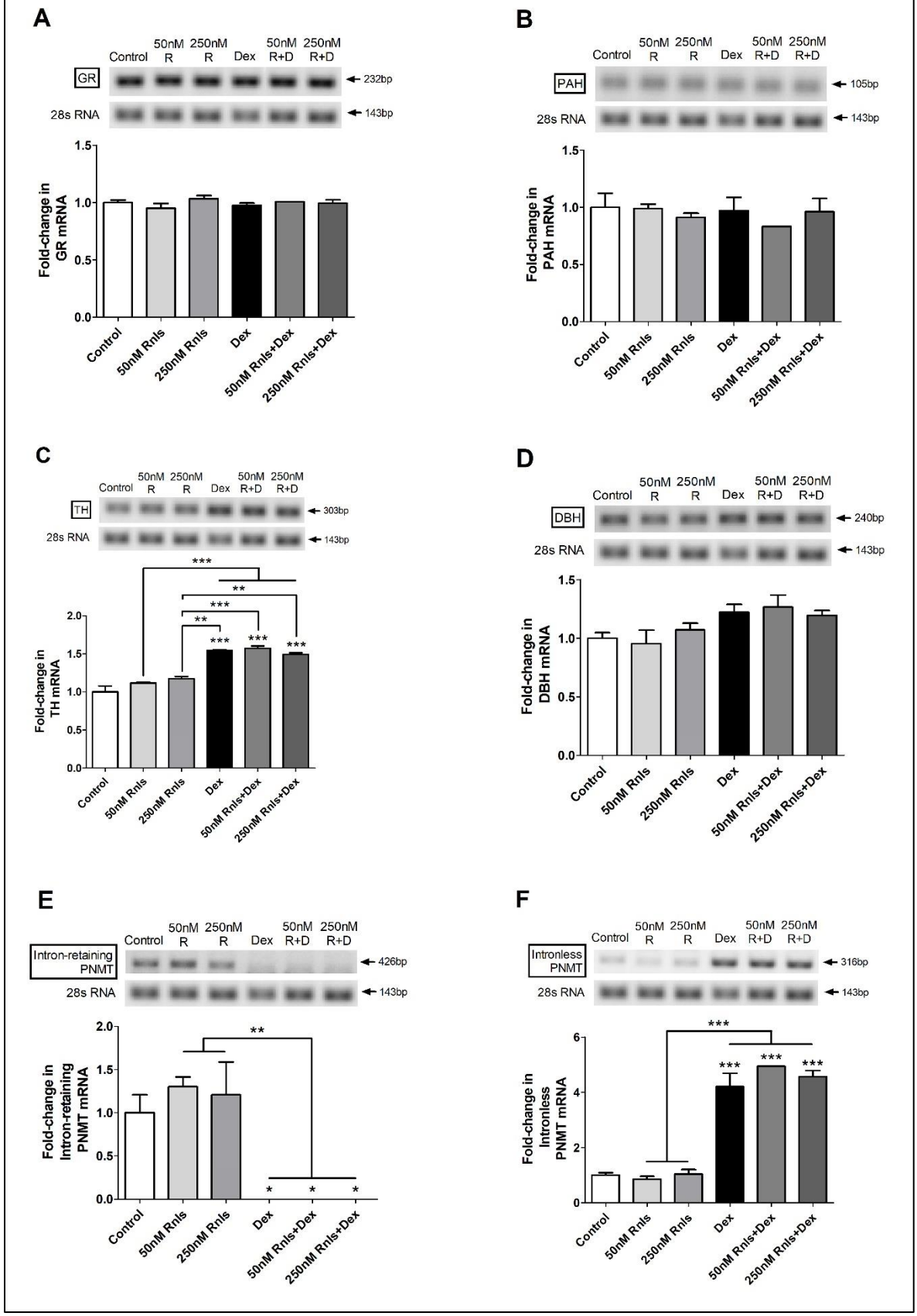
A
Control
50nM R
250nM R
Dex
50nM R+D
250nM R+D
GR
232bp
28s RNA
143bp
Fold-change in GR mRNA
Control
50nM Rnls
250nM Rnls
Dex
50nM Rnls+Dex
250nM Rnls+Dex
B
PAH
105bp
Fold-change in PAH mRNA
C
TH
303bp
Fold-change in TH mRNA
D
DBH
240bp
Fold-change in DBH mRNA
E
Intron-retaining PNMT
426bp
Fold-change in Intron-retaining PNMT mRNA
F
Intronless PNMT
316bp
Fold-change in Intronless PNMT mRNA

Figure 16: mRNA levels of catecholamine-biosynthesizing proteins after recombinant renalase (Rnls; R) treatments with and without dexamethasone (Dex; D). Image shows mRNA levels of glucocorticoid receptor (GR; A), phenylalanine hydroxylase (PAH; B), tyrosine hydroxylase (TH; C), dopamine β-hydroxylase (DBH; D), intron-retaining phenylethanolamine N-methyltransferase (PNMT; E) and intronless PNMT (F). mRNA levels were detected by RT-PCR. Rnls treatments were 24 hours long at variable doses. Dex treatments were performed 6 hours before the end of Rnls treatments and at a concentration of 1μM. Images shown are representative gel bands of each treatment. Fold-changes were normalized to untreated controls. mRNA data were normalized to 28s RNA. Data are presented as mean ± SEM (n = 3). *, **, and *** indicate significance from untreated controls where $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively (using ANOVA with a post-hoc Tukey test). All other significant differences between groups are bracketed.

3.3 – Objective 3: Expression of Renalase in the Adrenal Gland of the SHR

The third objective of this study investigated the expression of renalase in adrenal glands from WKYs and SHRs. At the mRNA level, there was a clear upregulation of renalase in adrenal glands from SHRs (1.43-fold; $p \leq 0.001$; Figure 17). There was also a notably small variability between animals (SEM = 0.022 for WKYs; SEM = 0.031 for SHRs). Surprisingly, the variability of renalase protein levels was exceptionally higher than that of mRNA (SEM = 0.42 for WKYs; SEM = 0.27 for SHRs). The main contributing factor of this large variability was the robust increases of renalase protein in the adrenals of certain animals (Figure 18). These animals with robust increases were not limited to a particular animal model. This variability was only observed with renalase, and not with the normalization protein, GAPDH.

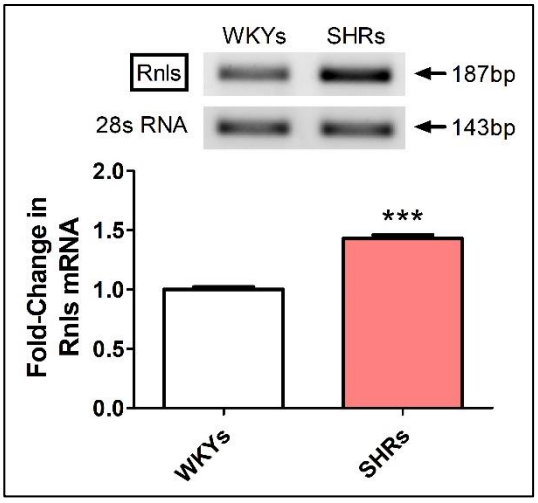

Figure 17: Renalase (Rnls) mRNA levels in the adrenal glands of Wistar-Kyoto rats (WKYs) and spontaneously hypertensive rats (SHRs). mRNA levels were detected by RT-PCR. Images shown are representative gel bands of each animal group. Fold-changes were normalized to WKY controls. mRNA data were normalized to 28s RNA. Data are presented as mean ± SEM (n = 4). *** indicates significance from WKYs where $p \leq 0.001$ (using a two-tailed, unpaired t-test).

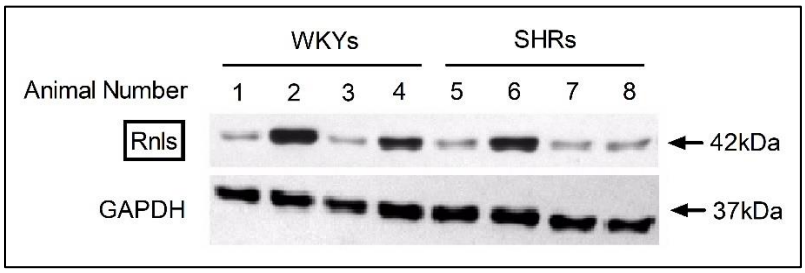

Figure 18: Renalase (Rnls) protein levels in individual adrenal glands from Wistar-Kyoto rats (WKYs) and spontaneously hypertensive rats (SHRs). Protein levels were detected by western blotting. Images shown are representative gel bands from each animal. Protein levels are compared to glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

4 – Discussion

Although there is opposing evidence to renalase's ability to oxidize CAs, many studies have shown protective effects of renalase on CA levels and hypertension (Desir, Wang, et al., 2012). These findings suggest that renalase regulates CAs with an alternative regulatory mechanism. Since its discovery in 2005, no studies have investigated the role of renalase in the adrenal medulla, which is the largest producer of circulating CAs. Yet, renalase's expression in the adrenal gland is among the highest in the body (Zhou et al., 2013). Hence, it is a strong possibility that renalase signaling in the adrenal medulla is regulated by paracrine, autocrine, and endocrine mechanisms. This study presents the first investigation of the potential role of renalase in the adrenal medulla.

These results present several novel findings. First, the PC12 cell model continuously expresses the renalase gene, with its mRNA and protein being easily detectable in all treatment groups and controls. With this finding, it is now known that the gene expression profile of undifferentiated pheochromocytoma cells do not strongly suppress renalase expression (Hopewell & Ziff, 1995; Jarrott & Louis, 1977). Second, renalase is not robustly regulated by the cell signals and conditions tested in objective 1 of this study (Figures 8-14). Despite there being time-dependent variations in renalase expression by PMA, Dex, and $CoCl_2$, all fold-changes were low and possibly have only minor effects at the physiological level (Figures 8C, 9D, and 10D). Another novel finding was that renalase and its peptide, RP-220, do not alter the expression of CA-biosynthesizing enzymes in PC12 cells (Figures 15 and 16). Still, it is possible that renalase is part of a negative feedback mechanism that acts upon other areas of CA regulation (i.e. CA storage/reuptake, secretion, or COMT and MAO-dependent metabolism). The last major finding of this study was the identification of altered regulations of renalase *in vivo*. In the adrenal glands

of SHRs, renalase mRNA was significantly upregulated (Figure 17). At the protein level, renalase could have been increased in certain animals from WKYs and SHRs (Figure 18), but the mechanism for this regulation remains to be elucidated.

This experiment presents the first comprehensive examination of renalase regulation in PC12 cells. Its regulation was examined following 10 known extracellular and intracellular signal activators within this cell line. Renalase regulation, in any cell or animal model, has not been previously examined following Dex, PACAP, ROS, NGF, or Fsk treatment. The other 5 cell signals have been previously used in other cell lines, but mostly showed different results than the present study.

The results of objective 1 show the novel finding that PKC signaling in PC12 cells does not alter renalase expression. Previously, S. Wang et al. showed that in renal proximal tubule cells (RPTs), the activator of PKC, PMA, increased renalase protein after 24 hours (2014). This detection may or may not have been renalase because of the molecular weight of the protein that was analyzed (70kDa, instead of the normal 42kDa for renalase). If renalase is altered by PMA in RPTs, the present study suggests that renalase regulation is likely different in PC12 cells and possibly chromaffin cells, suggesting a tissue-specific response. Our study demonstrated that renalase expression was not altered by PMA using similar concentrations and time points as the RPT treatments. Renalase mRNA could be temporarily reduced after 6 hours of PMA treatment (Figure 8C), but the same dose and time point from the dose-variable treatments did not replicate this reduction (Figure 8E). PMA only activates DAG and a limited number of PKC subtypes (Figure 3; Wu-Zhang & Newton, 2013). From this experiment, it can only be stated that DAG-activated PKC isoforms do not regulate renalase independently in PC12 cells. To account for more isoforms of PKC, Ang II treatment was performed. AT1R receptors can activate more PKC

subtypes than PMA (via IP3 and intracellular Ca^{2+} signaling; Gwathmey et al., 2012; Wu-Zhang & Newton, 2013). In a previous study, it was suggested that the RAS may not play a role in regulating renalase (Y. Wang et al., 2015). Results of the present study supported this previous finding as Ang II did not alter mRNA levels of renalase (Figure 13B). Taken together, these findings provide evidence that most PKC mechanisms do not regulate renalase mRNA levels independently.

Alternatively, renalase regulation might require PKA activation. The effects of PKA activation were investigated by treating PC12 cells with Fsk, which activates this pathway by direct interaction with AC (Seamon & Daly, 1981). Nevertheless, Fsk did not alter renalase expression (Figures 11B and 11C). Nic is another activator of the PKA pathway, in addition to its ability to increase intracellular Ca^{2+} (Madhok, Matta, & Sharp, 1995; Sala et al., 2007). Because renalase was unchanged following Nic treatment (Figure 12D), this study demonstrates that PKA in combination with calcium signaling is another pathway that does not regulate renalase in this cell type. Similar to CA-biosynthesizing enzymes, renalase might require both PKC and PKA signaling for increased expression in PC12 cells (Tai & Wong, 2003). Evidence that does not support this idea was provided by treatments with PACAP and NGF, which have complex signaling pathways that can activate PKA and PKC (Kalman, Wong, Horvai, Cline, & O'Lague, 1990; Tai et al., 2006). Results of our study show that time-variable and dose-variable treatments of PC12 cells with PACAP or NGF did not induce changes to renalase expression (Figures 13 and 14). The findings from NGF treatments additionally indicate that renalase expression changes are not involved with PKB pathways and the neuronal differentiation process. Overall, these results show that the PKA pathway, whether independently activated or in combination with other protein kinase pathways, does not regulate renalase in PC12 cells.

Another novel finding was that HIF1α activation and ROS signaling in PC12 cells could have a small influence on renalase mRNA levels. One previous study showed that 200μM $CoCl_2$ treatment of cardiomyocytes for 24 hours induced increases in renalase mRNA via HIF1α activation (Du et al., 2015). Our results show that PC12 cells treated with $CoCl_2$ can increase HIF1α activation, but do not alter renalase mRNA levels at early time points or with higher concentrations (Figures 10B, 10C, and 10E). Using the same treatment conditions as Du et al., renalase mRNA levels were slightly decreased, instead of being increased (Figure 10D). Analysis of renalase protein levels after 24-hour treatments with $CoCl_2$ should be performed to validate the effects of $CoCl_2$ on renalase expression. Since ROS is produced by $CoCl_2$, H_2O_2 treatments were included to detect potential changes in renalase expression (Crispo et al., 2011). Following this treatment, no changes in renalase expression were observed (Figures 13B and 13D). Therefore, H_2O_2-mediated ROS, like $CoCl_2$, may not regulate renalase in this cell line. AT1Rs can also increase H_2O_2, indicating that our results from Ang II treatment support the notion that this form of ROS does not alter renalase expression in this cell model at the times and concentrations analyzed (Anupama et al., 2016). Other ROS like superoxides, nitric oxides, and hydroxyl radicals should also be investigated for potential regulation of renalase. Although these results provide evidence that $CoCl_2$ downregulates renalase mRNA in PC12 cells, it is still unknown whether HIF1α, or ROS species (other than H_2O_2) are responsible for this regulation.

Moreover, increases in renalase mRNA after Epi and Nic treatment of human embryonic kidney-293 (HEK-293) cells were not replicated in PC12 cells using the same treatment conditions (Figure 12D; Sonawane et al., 2014). Under certain conditions, Epi can activate SP1, and Nic can activate STAT3 and SP1. Both of these TFs have binding sites in the renalase promoter (Figure 7). Previous studies have established that Nic induces PKA activation, suggesting that SP1 is also

activated (Madhok et al., 1995). If renalase expression was unchanged, SP1-mediated renalase regulation could require a coactivator. No change with Epi treatment is most likely due to a specialized adrenoceptor expression profile in PC12 and chromaffin cells (one that enhances α_2 activity). On the other hand, HEK-293 cells abundantly express α_1 adrenoceptors (specifically, the α_{1B} subtype), which could be why Sonawane et al. observed upregulation of renalase following Epi treatment (Atwood, Lopez, Wager-Miller, Mackie, & Straiker, 2011).

Finally, the glucocorticoid signaling pathway was induced by treating PC12 cells with the synthetic glucocorticoid, Dex. This pathway is one of the most important signaling mechanisms in chromaffin cells because of the proximity of these cells to the adrenal cortex. Under the conditions of the present study, it does not appear that Dex can robustly regulate renalase mRNA or protein levels (Figures 9C and 9D). Although no studies have analyzed the effects of Dex treatment on renalase regulation, Dex could be a regulator of miRNA-29b, which can downregulate renalase mRNA (Deng, Tufan, Raza, Jones, & Zhu, 2016; Kalyani et al., 2015). Although renalase mRNA levels were unchanged after Dex treatment, miRNA-29b could be a potential mechanism for why renalase protein was slightly altered in a time-dependent manner.

The minimal renalase regulation observed in this study provides insight for its regulation across numerous tissues. PC12 cells, being derived from rat pheochromocytoma tissue, are among the few *in vitro* cell models of chromaffin cells (Greene & Tischler, 1976). If the PC12 cell model accurately represents regulation of renalase in chromaffin cells, these results suggest that renalase's regulation in the adrenal medulla is different from other tissues. One explanation for renalase's sustained expression is because of the implications of autocrine or paracrine signaling by renalase. If renalase is increased by synthesis-activating signals, as the hypothesis suggests, its autocrine or paracrine signaling could immediately reduce the effect of the original signal. Because

the findings in this study suggest that renalase is not increased in chromaffin cells by these synthesis-activating signals, it could be that other endocrine tissues are responsible for the regulation of renalase in the blood. In other words, the negative feedback mechanism could still exist, but the regulation of renalase would occur in tissues other than chromaffin cells. Restricting renalase expression in chromaffin cells could allow for stronger SA activity, while still allowing for a trans-activated negative feedback mechanism.

If renalase does regulate CAs in the adrenal medulla, the findings of this study indicate that the inhibition of CA-biosynthesizing enzymes may not be an effect of renalase signaling. Using both the signaling peptide, RP-220, and recombinant renalase to treat PC12 cells in a dose-variable manner, no changes were detected in the mRNA levels of PAH, TH, DBH, and intron-retaining or intronless PNMT (Figures 15 and 16). As a positive control, Dex treatments were performed and expected increases in TH, DBH, and PNMT mRNA were observed. Because renalase's mechanism of action could be to attenuate Dex/cortisol-induced CA biosynthesis upregulation, additional groups of cells were exposed Dex during the last 6 hours of RP-220 or recombinant renalase treatment. The results of this study demonstrate that renalase does not attenuate the Dex/cortisol-induced increases of TH, DBH, or PNMT mRNA (Figures 15 and 16). Without any downregulations in these enzymes being found, it is expected that GR mRNA was unchanged after renalase treatment (Figures 15A and 16A). The analysis of this TF was performed because it would also strongly inhibit the Dex/cortisol response if downregulated. Dex did not induce upregulation of GR because it mainly acts as a dissociating factor for the inactive HSP-GR complex (Drouin et al., 1992). PAH was also unchanged by renalase or Dex (Figures 15B and 16B). However, this enzyme is not necessary for *de novo* CA biosynthesis and its tissue expression levels suggest that this step is mainly performed by the liver (Uhlen et al., 2015). Future experiments should

investigate if renalase attenuates the actions of other mechanisms that increase CA activity, such as CA vesicular storage, uptake, and secretion. Renalase treatment could also be performed to determine if renalase increases the expression of CA-metabolizing enzymes (e.g. MAOs and COMTs).

As for the expression of renalase in SHR and WKY adrenals, there were several important findings that provide a clue for the role of renalase in this tissue. First, it appears that an unknown genetic factor results in renalase mRNA being consistently elevated in the SHR (Figure 17). SHRs have elevated CAs in the blood, partially due to upregulated CA-biosynthesizing enzymes in the adrenal gland (Nguyen et al., 2009). Thus, renalase mRNA could be increased directly by CAs or by similar genetic factors that increase CA-biosynthesis in the SHR. Because renalase mRNA was stable in PC12 cells, the increase in renalase mRNA could have occurred in the adrenal cortex by the same signal activators used in objective 1. It is also possible renalase regulation was due to other unknown phenotypic differences in the SHR. Upregulated renalase mRNA cannot be generalized to other tissues because it was previously shown that SHRs also have lower renalase mRNA in the brain and lower renalase protein in plasma (Fedchenko et al., 2013; Jiang et al., 2012). Our investigation of renalase protein levels in the adrenal glands provides the first set of evidence that renalase could play a large role in the adrenal gland. When protein levels were normalized to GAPDH, large differences in renalase protein levels were observed in various WKYs and SHRs (Figure 18). Renalase protein, when compared with its respective mRNA, suggests a potential post-translational or proteolytic mechanism of renalase regulation. If such a mechanism exists, its regulation of renalase could be part of normal physiology, because both normotensive and hypertensive animals exhibited high variation in renalase protein levels. Considering that our methods were designed to reduce extraneous variables between rats, the

unknown signal activator of renalase regulation is likely difficult to keep consistent between animals. Therefore, the unknown regulator of renalase could be a fast-acting or circadian signal. Adrenocorticotropic hormone (ACTH) is an example of such signal that can be fast-acting and involved with circadian rhythms (Mohn et al., 2005; Park et al., 2013).

Despite the novelty of these findings, the central hypothesis was not supported. Originally, it was proposed that renalase downregulates CA-biosynthesizing enzymes as part of a negative feedback mechanism in the adrenal medulla. For renalase to be part of this feedback mechanism, its expression would have to be increased after the upregulation of CA biosynthesis. Because there are many ways for CA biosynthesis to be regulated, multiple signaling pathways were used to detect this increase (e.g. PKC, PKA, cortisol, and hypoxia signaling). So far, there is no evidence to show that renalase is upregulated by the same signals that activate CA biosynthesis. However, the number of signaling mechanisms analyzed in this study is limited and there are other cell treatments still to be performed that could regulate CA biosynthesis and renalase together. One example of a treatment that should be investigated is the use of multiple signal activators in combination. Another test for this hypothesis was to analyze the expression of CA-biosynthesizing enzymes after treatment of PC12 cells with renalase, or its signaling peptide, RP-220. Following these treatments, no changes to the expression of CA-biosynthesizing enzymes were observed. If the hypothesis is correct, the anti-hypertensive effects of renalase may be downregulated in animals with elevated CA biosynthesis, like the SHR (Nguyen et al., 2009). Instead, renalase was upregulated at the mRNA level, and its protein level was regulated by other factors still to be determined.

Because this study attempts to detect regulatory mechanisms of a novel gene, it is important to mention many small details that could have influenced our results. For example, no positive

control genes verified intracellular activity for PACAP, H_2O_2, Ang II, Epi, Nic, renalase, and RP-220. PNMT mRNA levels from PACAP, Ang II, and Nic treatments were quantitated and showed high variability with no significant changes compared to controls. In other studies, these signal activators increased PNMT expression (Cahill et al., 2002; Evinger et al., 2005; Tai et al., 2010). It is unclear why these treatments did not induce PNMT upregulation as shown in other studies. Next, Epi induced a downregulation of TH and an increase in the inactive form of PNMT. It is likely that this effect is characteristic of Epi because of the abundance of inhibitory α2 receptors in PC12 cells (Moura et al., 2009). Downregulation of TH and upregulation of intron-retaining PNMT mRNA has not been previously shown before and it is unknown if these regulatory patterns can be used as a positive control of Epi signaling in PC12 cells. If further studies validate this signaling activity, intron-retaining PNMT mRNA could be used as a positive control of Epi signaling in the future. Of course, it is important to mention that renalase, RP-220, or H_2O_2 could not be verified for intracellular signaling activity without a proper positive control. Due to proper storage and handling, it is unlikely, but not guaranteed, that their degradation occurred over time. Other treatments including $CoCl_2$ and Dex had some confirmation of signal activity, but not for all experiments. For example, $CoCl_2$ activity was confirmed by nuclear HIF1α, but only in separate cells that were not analyzed for renalase expression. Finally, the use of pSer40 TH, although a good measurement of active TH, should have been performed on protein samples with the addition of sodium fluoride (NaF) in their extraction buffer. This addition would have decreased the activity of serine phosphatases (Sharma & Carew, 2002). Although an increase in pSer40 TH protein was observed after 24 hours (Figure 9D), this increase could have been due to different phosphatase activity between samples. In these cases of cell treatment where positive control genes were not completely analyzed, the unchanged renalase expressions could have been due to a lack of

intracellular activity. Unchanged renalase expression could also be due to the experimental design, which does not fully represent human physiology.

Specifically, the use of PC12 cells could have hindered the analysis of renalase's role in the adrenal medulla. As undifferentiated pheochromocytoma cells, their intracellular responses to the treatments of this study are slightly different than chromaffin cells (Qin et al., 2014).

There are several reasons why the PC12 cell model could have prevented the detection of renalase expression changes. Renalase was previously shown to have higher expression in particular cancer cell types (Guo et al., 2016; Hollander et al., 2016). If renalase expression in pheochromocytoma cells is similarly upregulated, it could be that renalase transcription is consistently "switched on" with no ability for additional increases in mRNA. Because three established TFs that bind the renalase promoter (i.e. HIF1α, SP1, and STAT3) are all capable of being induced in PC12 cells, it is not likely that these TFs could consistently upregulate renalase (Tai et al., 2010; Y. Y. Wu & Bradshaw, 2000). If ZBP-89, which has not been fully analyzed in PC12 cells, has a low activity in this cell line, it could potentiate renalase transcription. In previous studies, it was shown that ZBP-89 can repress gene expression both independently, and through SP1 interaction (X. Zhang, Diab, & Zehner, 2003). Because a ZBP-89 binding site is located beside a SP1RE on the renalase promoter (Figure 7), downregulated ZBP-89 activity could increase renalase expression and reduce the range of renalase regulation. There is a possibility that ZBP-89 is altered in PC12 cells because ZBP-89 is ultimately a tumour suppressor with pro-apoptotic effects (To et al., 2011). Being a cancer cell line, it is probable that tumour suppressors like ZBP-89 are downregulated. Other putative binding sites in the renalase promoter could be responsible for consistent upregulation or downregulation (Sonawane et al., 2014). The putative homeobox protein B9 (HOXB9) binding site in the renalase promoter can sometimes be associated with the

repression of gene transcription (Sonawane et al., 2014; Wan et al., 2016). Interestingly, PC12 cell differentiation by NGF increases the expression of B-cell translocation gene protein 2 (BTG2), which has been shown to enhance HOXB9 promoter binding (el-Ghissassi et al., 2002; Prévôt et al., 2000). This increased HOXB9 activity by NGF could be another reason why the combined effects of PKA, PKC signaling, and cell differentiation did not induce changes in renalase expression. Other evidence that supports the idea of HOXB9 repression of renalase transcription is that estrogen increases HOXB9 transcription and female rats were found to have lower renal renalase mRNA levels (Appendix A). Therefore, any changes in BTG2 or HOXB9 in PC12 cells could alter the regulation of renalase from normal chromaffin cell physiology. PC12 cells could also affect renalase promoter activity via its putative E-box element (Sonawane et al., 2014). PC12 cells do not express the E-box-binding TF, Max (Hopewell & Ziff, 1995). Although the particular renalase E-box sequence is not entirely specific to Max TFs, it is still possible that variable forms of Max activation bind to the renalase promoter (Adhikary & Eilers, 2005). Another mechanism that could consistently repress renalase expression is CpG island or histone methylation. Unfortunately, there is only a limited understanding of the epigenetic regulation of renalase. PC12 cells could have a different intracellular localization of renalase compared to chromaffin cells as well. It has been shown that RPTs, but not podocytes or mesangial cells secrete renalase (F. Wang et al., 2012). Finally, if PC12 cells localize renalase differently than chromaffin cells, the regulation experiments in this study cannot be generalized to actual normal physiology. Also, all quantifications of renalase protein for objective 1 could have been underestimated by not accounting for secreted protein.

The use of the PC12 cell model could have prevented the effects of renalase or RP-220 signaling as well. PC12 cells express the renalase receptor, PMCA4b, but levels of NOS are only

normally expressed after differentiation (Garcia, Usachev, Thayer, Strehler, & Windebank, 2001; Poluha et al., 1997; Sheehy et al., 1997). If renalase's main intracellular signal is to alter NOS activity via PMCA4b, then the PC12 cell model would need to be differentiated before the effects of renalase could be detected. In contrast, chromaffin cells can express NOS, but only in certain subgroups of cells that secrete VIP and enkephalins (Heym, Colombo-Benckmann, & Mayer, 1994). Therefore, renalase signaling in the adrenal medulla might only occur with these subgroups. However, it is still possible that other receptors of renalase exist that have yet to be identified. Another potential reason why renalase did not induce changes to CA biosynthesis is that its receptor's expression and activity is lower in PC12 cells compared to chromaffin cells. It should also be considered that the recombinant renalase and the peptide used in this experiment were human. Because PC12 cells are derived from rats, potential surface receptors might not bind human renalase. Nevertheless, alignment of rat and human renalase reveals an amino acid similarity of 98%. This is one reason why human renalase, when administered to rats, still reduces circulating CA levels (H. T. Lee et al., 2013). If the primary receptor of renalase is PMCA4b, human renalase should still have signaling activity because both rat and human renalase have the necessary KKR sequence for receptor binding (L. Wang et al., 2014). Even with the presence of these signaling residues in our experiment, other experiments should confirm the effects of renalase in the adrenal medulla using other cell lines like bovine chromaffin cells.

5 – Conclusion

To summarize, this study presents the first set of evidence that renalase is not part of an autocrine/paracrine negative feedback mechanism in the adrenal medulla. Out of the 10 signal activators that were used to analyze renalase expression, none of them resulted in a large change in renalase mRNA or protein levels. Renalase was also incapable of reducing the expression of CA biosynthesizing enzymes. Still, there could be signaling activity of renalase towards the adrenal medulla because of its regulation *in vivo*. Whether this regulation occurs in the adrenal cortex or medulla has yet to be determined. The details and contexts of these experiments indicate that our results can only be applied in a specific manner to the physiological systems of humans. This study has added plenty of new findings, but questions remain regarding renalase's role in the adrenal medulla. If renalase does act upon the adrenal medulla, genetic or environmental changes to this process could be a new pathophysiological mechanism of hypertension.

6 – Future Directions

To add to the results of this experiment, several experiments can be performed. One helpful experiment would be to perform immunohistochemistry on the adrenal glands from WKYs and SHRs. With this method, the localization of renalase should be determined to confirm the findings in this experiment, and to see how renalase is regulated in the cortex and medulla. Another experiment that could be done is to use an ELISA-based catecholamine assay to detect changes in the cell media from renalase/RP-220-treated cells. From this method, it would first be necessary to confirm that CAs are upregulated by Dex. If this positive control is successful, it can be observed if renalase or RP-220 lower the levels of secreted CAs from PC12 cells (with or without Dex treatment). If renalase does lower CAs or protects against increased CAs, other methods should be performed to detect how this effect is achieved by renalase. For example, the membrane receptor (which may or may not be PMCA4b) should be confirmed, and intracellular pathways should be analyzed for activation. Because it is possible that renalase increases ROS regulation (by inactivating NAD(P)H isomers), ROS assays should be performed after a renalase treatment of PC12 cells as well. Particularly, levels of H_2O_2 and NO should be observed because of the mechanisms proposed by Beaupre et al. 2015 and Wang et al. 2015, respectively (Beaupre, Hoag, Roman, et al., 2015; L. Wang et al., 2015). Plenty of other cell signals should be used to treat PC12 cells and determine if they alter renalase expression. Such cell signals include estrogen, because of the regulation shown in Appendix A, and cytokines, which may activate TFs that bind to the renalase promoter (i.e. STAT3; Y. Y. Wu & Bradshaw, 2000). Of course, it would be helpful to perform the cell treatments in this study in alternative cell lines like bovine chromaffin cells or the adrenocortical cell line, H295R.

As a newly discovered protein, more work should be done to understand the overall influence of renalase on normal physiology and pathophysiology. For instance, it is still unknown if renalase is secreted from all cell types. If renalase is not secreted from all cell types, an intracellular function should be established. Further, the role of renalase is still to be determined in tissues that strongly express renalase (e.g. the adrenal cortex and the glomerulus). Currently, there are no studies that have examined why renalase's expression is remarkably higher in these tissues. To identify potential receptors of renalase in other cell types, cross-linking experiments followed by mass spectrometry can be performed as in L. Wang et al., 2015. There are many topics to be explored regarding renalase regulation too. These topics include regulatory mechanisms involving miRNAs, epigenetics, post-transcriptional processes, post-translational modifications, and putative promoter binding sites (e.g. validating the HOXB9 binding site). To conclude, the mystery of renalase remains and it is important for us to research these areas to reveal its potential uses in health and medicine.

7 – References

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8 – Appendix

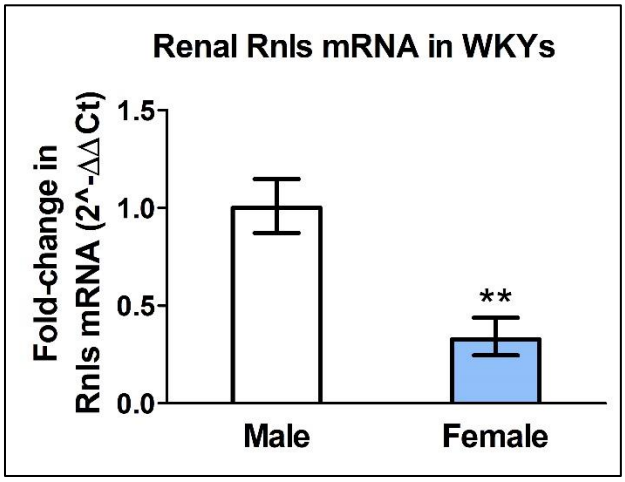

Figure A-1: Renalase (Rnls) mRNA levels in the kidney of adult male and female Wistar-Kyoto rats. RNA was extracted from coronal sections approximately 2mm thick through the hilus and homogenized with TRI Reagent. mRNA levels were detected by quantitative real-time RT-PCR (qRT-PCR) using a BioRad Chromo4 detector and Opticon 3 software. cDNA mastermixes were made using SensiFAST SYBR green Hi-ROX reagents as per the manufacturer's instructions (FroggaBio). Fold-changes were compared to male rats using the 2^-ΔΔCt method. Renalase mRNA data were normalized to the GAPDH gene. Group means are presented along with their respective up and down standard errors (n = 4). ** indicates significance from males where $p \leq 0.01$ (using a two-tailed, unpaired t-test).