

REGULATION OF O₂- AND CO₂-CHEMOSENSITIVITY IN CHROMAFFIN CELLS

Molecular Mechanisms Regulating Ontogeny of O₂- and CO₂-Chemosensitivity in Rat Adrenomedullary Chromaffin Cells: Role of Nicotinic ACh and Opioid Receptor Signalling

By

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ABSTRACT

Catecholamine (CAT) secretion from adrenomedullary chromaffin cells (AMCs) is essential for survival of the fetus and for adaptation of the newborn to extrauterine life. CAT secretion protects the fetus from intrauterine hypoxia (low O₂) and is required for maintaining cardiac conduction and preparing the lungs for air breathing. Asphyxial stressors (e.g. hypoxia, hypercapnia (high PCO₂), and acidosis (low pH)) arising from labor contractions and postnatal apneas, are the main stimuli for the ‘non-neurogenic’ CAT release from perinatal AMCs. In the rat, the mechanisms of hypoxia chemosensitivity in AMCs involve inhibition of a variety of K⁺ channels, leading to membrane depolarization, voltage-gated Ca²⁺ entry, and CAT secretion. The magnitude of this depolarization is regulated by the simultaneous activation of ATP-sensitive K⁺ (K_{ATP}) channels, which tends to hyperpolarize the membrane potential during hypoxia. Interestingly, chemosensitivity of rat AMCs and CAT secretion in response to asphyxial stressors are markedly reduced postnatally following the development of functional innervation of these cells by the splanchnic nerve.

The primary purpose of this thesis was to delineate molecular mechanisms involved in the suppression of hypoxia and hypercapnia chemosensitivity following splanchnic innervation in neonatal rat AMCs. Experiments were designed to test the general hypothesis that the ontogeny of O₂ and CO₂ sensitivity in AMCs is regulated by the activation of postsynaptic nicotinic ACh and opioid receptor signalling pathways following innervation. Previous studies in this laboratory showed that exposure of perinatal rat AMCs to nicotine *in utero* and *in vitro* resulted in the selective blunting of hypoxia (but *not* hypercapnia) chemosensitivity. The underlying mechanism was attributable to the increased membrane hyperpolarization caused by the functional upregulation of K_{ATP} channels. In Chapter 2, I report the results of investigations of molecular mechanisms involved in the nicotine-induced upregulation of K_{ATP} channels, using a rat fetal-derived, O₂- and CO₂-sensitive immortalized chromaffin cell line (MAH cells), as a model. Exposure of MAH cells to chronic nicotine (50 μM) for 7 days in culture caused an increase in the expression of the K_{ATP} channel subunit, Kir6.2. This effect was blocked by α-bungarotoxin, a blocker of homomeric α7 nicotinic acetylcholine receptors (α7 nAChRs). The upregulation of Kir6.2 in MAH cells was also dependent on the transcription factor, hypoxia inducible factor (HIF)-2α. First, whereas the upregulation of Kir6.2 was present in wild type and scrambled control

MAH cells, it was absent in HIF-2 α -deficient (shHIF-2 α) MAH cells. Second, chronic nicotine caused a progressive, time-dependent increase in HIF-2 α accumulation that occurred in parallel with the increase in Kir6.2 expression. Third, chromatin immunoprecipitation (ChIP) assays revealed the binding of HIF-2 α to a hypoxia response element (HRE) in the promoter region of the Kir6.2 gene. These data suggest that chronic nicotine causes the accumulation of HIF-2 α which results in the transcriptional upregulation of the Kir6.2 gene. These observations were validated in an *in vivo* model where rat pups were exposed to nicotine *in utero*. Western blot analysis of adrenal gland tissues from nicotine-exposed (relative to saline-exposed) pups revealed a significant increase in Kir6.2 subunit expression and HIF-2 α accumulation, and both were restricted to the medullary (but not cortical) tissue.

Chapter 3 tested the hypothesis that postnatal innervation causes the suppression of O₂- and CO₂-chemosensitivity in neonatal AMCs via opioid receptor signalling. It was found that chronic μ - and δ -opioid agonists (2 μ M) *in vitro* led to the suppression of both O₂- and CO₂-chemosensitivity; this was correlated with the upregulation of K_{ATP} channel expression and the downregulation of carbonic anhydrase (CA) I and II respectively. The underlying molecular and signalling mechanisms were further investigated in Chapter 4. Using the MAH cell model, it was found that exposure to a combination of μ - and δ -opioid agonists for 7 days resulted in the naloxone-sensitive upregulation of Kir6.2 subunit and the downregulation of CAII. Similar to chronic nicotine exposure, the effects of chronic opioids on the upregulation of Kir6.2 and downregulation of CAII were HIF-2 α -dependent. Western blot analysis revealed that HIF-2 α accumulation in opioid-treated MAH cells occurred along a time-course that paralleled the upregulation of Kir6.2 subunit. ChIP assays demonstrated the binding of HIF-2 α to the promoter region of the Kir6.2 subunit gene in opioid-treated MAH cells. Moreover, PKA activity (but not PKC or CaMK) was found to be required for the effects of opioids on Kir6.2 and CAII expression, but not HIF-2 α accumulation. In complementary *in vivo* studies, adrenomedullary tissues from morphine-exposed rat pups showed an increased expression of both HIF-2 α and Kir6.2, and decreased expression of CA1 and II protein. These findings have uncovered novel mechanisms by which postnatal innervation contributes to the ontogeny of O₂- and CO₂-chemosensitivity in rat adrenal chromaffin cells. They also suggest mechanisms by which exposure of the fetus to nicotine in cigarette smoke or opioids from drug abuse might contribute to abnormal arousal reflexes, and pathophysiological conditions such as Sudden Infant Death Syndrome (SIDS).

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LIST OF ABBREVIATIONS

AMC	adrenomedullary chromaffin cell
BK _{Ca}	large (big) conductance calcium (Ca²⁺)–activated potassium (K⁺) channel
BSA	Bovine Serum Albumin
bHLH	Basic Helix-Loop-Helix
CAT	catecholamine
ChIP	Chromatin Immunoprecipitation
CREB	cAMP response-element binding protein
DNA	Deoxyribonucleic Acid
E14.5	embryo day 14.5
HIF	hypoxia inducible factor (transcription factor; subtypes HIF1,2,3)
Hox	Hypoxia
HRE	Hypoxia Response Element
HRP	Horse Radish Peroxide
HNK–1+	human natural killer–1 (marker for neural crest cells)
IgG	immunoglobulin G (most abundant class of antibody in blood)
K _{ATP}	ATP –sensitive (or dependent) K⁺ channel
Kir	K⁺ inward rectifier (subunit of K _{ATP} channel)
K _v	voltage–gated, delayed rectifier, K⁺ channel
MAH	<i>v-myc</i> , adrenal–derived, HNK–1+ cell
NSERC	National Sciences and Engineering Research Council of Canada
Nox	Normoxia
PBS	Phosphate Buffered Saline
PC12	pheochromocytoma 12 (cancer cell line derived from rat adrenal medulla)
PCR	Polymerase Chain Reaction
PHD	Prolyl Hydroxylase Domain Enzymes
PNMT	phenylethanolamine N–methyltransferase
PO ₂	(partial) pressure of oxygen (O₂)
PVDF	Polyvinylidene Difluoride
QPCR	quantitative reverse transcription–polymerase chain reaction
RNA	Ribonucleic Acid
RNAi	RNA interference
ROS	Reactive O₂ Species
RT	Reverse Transcriptase
ScCont	Scrambled Control MAH cells
SDS	Sodium Dodecyl Sulphate
shRNA	short hairpin RNA
SH–SY5Y	third generation human neuroblastoma cell line
SUR	sulfonylurea receptor (subunit of K _{ATP} channel)
TASK	TWIK–related acid–sensitive K⁺ channel
TBS	Tris Buffered Saline
VGCaC	voltage–gated Ca²⁺ channel
<i>v-myc</i>	viral oncogene for myelocytomatosis
VEGF	Vascular Endothelial Growth Factor
VHL	von Hippel Lindau
w.t.	Wild Type

CHAPTER 1: GENERAL INTRODUCTION

1. Introduction

Mammals have developed a variety of mechanisms by which specialized organs sense and respond to changes in oxygen (O_2) tension in order to maintain metabolic homeostasis. A constant O_2 supply is needed for proper function of mammalian cells. Exposure of specialized ‘receptor’ cells in specific organs to a decrease in O_2 tension or partial pressure of O_2 (PO_2), i.e. hypoxia, induces a physiological response so as to restore PO_2 homeostasis in different organs and tissues in the body. In adult mammals, chemoreceptor cells, i.e. type I or glomus cells, of the carotid body are specialized in sensing local chemical factors in arterial blood that include not only PO_2 , but also partial pressure of CO_2 (PCO_2) and pH as well (Gonzalez *et al.*, 1994; Kumar, 2007). Thus, a decrease in arterial PO_2 , or an increase in CO_2/H^+ (i.e. acid hypercapnia) causes membrane depolarization in glomus cells and release of excitatory neurotransmitters (e.g. acetylcholine and ATP) onto sensory afferent terminals of the carotid sinus nerve (Fitzgerald, 2000; Iturriaga & Alcaayaga, 2004; Nurse, 2005; Prabhakar, 2006). A reflex pathway is thereby activated, resulting in a compensatory increase in ventilation so as to maintain arterial PO_2 , PCO_2 and pH constant (Gonzalez *et al.*, 1994).

In neonatal mammals on the other hand, the carotid body is not fully developed and arousal responses to asphyxial stressors such as hypoxia and acid hypercapnia originate in ‘chromaffin’ cells located in the medullary region of the adrenal gland (Lagercrantz & Bistoletti, 1977; Lagercrantz & Slotkin, 1986; Wasicko *et al.*, 1999). These cells, referred to as neonatal adrenomedullary chromaffin cells (AMCs), are similar to glomus cells of the carotid body in their ability to respond directly to hypoxia and acid hypercapnia (Nurse *et al.*, 2009). Unlike

glomus cells, however, the direct chemosensitivity of human and rodent AMCs is restricted to the perinatal period when their innervation is immature. Interestingly, chemosensitivity is lost or suppressed postnatally as the AMCs acquire functional innervation via the splanchnic nerve (Seidler & Slotkin, 1985). Moreover, denervation of the adult adrenal gland results in the restoration of direct chemosensitivity in AMCs (Slotkin & Seidler, 1988; Cheung, 1990). When the research described in this thesis began, the molecular mechanisms underlying the loss of direct chemosensitivity in neonatal AMCs following innervation were poorly understood. A major goal of this thesis was to uncover at the molecular level some of the fundamental mechanisms by which innervation of AMCs regulates their sensitivity to hypoxia and hypercapnia. The physiological importance of direct chemosensing mechanisms in neonatal AMCs, and the critical role of catecholamine secretion during the transition of the neonate to extra-uterine life, is reviewed below.

2. Role of catecholamine secretion in the transition of the neonate to extrauterine life

Catecholamine (CAT) secretion from adrenomedullary chromaffin cells (AMCs) is a crucial physiological response during the transition of the fetus to extra-uterine life (Slotkin & Seidler, 1988; Van Woudenberg *et al.*, 2012). CAT secretion is required in the newborn for a number of physiological adaptations such as preparation of the lungs for air breathing, activation and maintenance of cardiac conduction, glucose homeostasis, temperature control, and eating (Van Woudenberg *et al.*, 2012). For example, as delivery approaches, CAT secretion decreases lung fluid production by shutting down the active chloride-mediated fluid production and activating the passive Na^+ transport with water and other electrolytes (via Na^+/K^+ -ATPase activation in type II epithelial cells) to clear the airways (Grotberg, 2001). In addition, CAT secretion stimulates

the production of surfactant in the lungs (via β_2 -receptor activation) which helps to reduce the fluid surface tension to keep the lung inflated (Faridy & Thliveris, 1987; Burri, 2006). In the developing fetus, CAT secretion protects the fetus from intra-uterine hypoxia-induced bradycardia and maintains fetal O_2 homeostasis (Ream *et al.*, 2008). Moreover, it directs cardiac output to supply vital organs (e.g. heart, brain and adrenal gland) at the expense of non-essential organs (e.g. kidney and lungs) (Giussani *et al.*, 1993).

Evidence that emerged in the 1960s showed that CAT secretion in the perinatal period in response to asphyxial stimuli originated in the adrenal gland (Comline & Silver, 1961). Impaired CAT secretion as a result of adrenalectomy in neonatal mammals (e.g. sheep and rat) leads to abnormal arousal reflexes, and increased mortality and morbidity rates (Seidler & Slotkin, 1985; Padbury *et al.*, 1987). Prior to maturation of the preganglionic sympathetic input to the adrenal gland via the splanchnic nerve, CAT secretion is initiated in response to asphyxial stressors that the neonate experiences during, and soon after birth (Comline & Silver, 1961; Lagercrantz & Slotkin, 1986; Seidler & Slotkin, 1986). These stressors arise as a result of uterine contractions during the birthing process and postnatal apneas due to intermittent cessations of breathing in the newborn (Lagercrantz & Slotkin, 1986; Poets *et al.*, 1994; Nock *et al.*, 2004). However, CAT secretion from AMCs in response to direct asphyxial stressors is diminished postnatally following the maturation of splanchnic innervation (Lagercrantz & Slotkin, 1986; Seidler & Slotkin, 1986; Thompson *et al.*, 1997). These and other studies (see later) suggest that innervation *per se* plays a key role in regulating the direct O_2 and CO_2/H^+ chemosensitivity of developing AMCs.

3. Structural organization of the adrenal gland

The adrenal gland was first described and localized in 1563 by Bartolomeo Eustachius though it was not generally accepted at that time. The presence of the adrenal gland in the human body was subsequently established in the early 17th century and various theories were then proposed to explain its physiological function (Richard, 1990). In the mid-19th century, upon the emergence of histochemical techniques, Kölliker was able to distinguish the medulla and cortex as distinct regions of the adrenal gland (Kölliker, 1854). It is now generally accepted that the structure of the adrenal gland consists of the outer steroid-producing cortex (arising from the mesoderm) and the inner catecholamine-producing medulla (arising from the neuroectoderm). In general, the adrenal gland releases a number of hormones into the blood stream that help maintain homeostasis during stress.

3.1.The adrenal cortex

The adrenal cortex consists of three distinct layers. The outermost area is called the zona glomerulosa and is the site of mineralocorticoid synthesis such as the hormone, aldosterone, which plays a key role in Na⁺ homeostasis. The central area is referred to as zona fasciculata which is responsible for the release of glucocorticoids (i.e. cortisol and corticosterone) during stress (Boron, 2011). Glucocorticoids influence the expression of phenylethanolamine-*N*-methyltransferase (PNMT) in epinephrine-producing chromaffin cells (Betito *et al.*, 1992). In addition, they trigger gluconeogenesis and inhibit the uptake of glucose into muscle and adipose tissues to ensure enough energy is available during stress (Boron, 2011).

3.2.The adrenal medulla

In mature mammals, the sympathetic nervous system and adrenal medulla contribute to the ‘fight-or-flight’ response during stressful conditions. The main cell types in the adrenal medulla are the chromaffin cells which synthesize and release stress hormones, i.e. catecholamines (e.g. norepinephrine, epinephrine and dopamine), when they are stimulated. This catecholamine secretion is triggered by the activation of the sympathetic nervous system whose preganglionic axons make cholinergic synapses with adrenal chromaffin cells (Schinner & Bornstein, 2005). Thus, activation of the sympathetic nervous system leads to acetylcholine (ACh) release from preganglionic splanchnic nerve terminals and stimulation of mainly nicotinic neuronal acetylcholine receptors (nAChRs) expressed on chromaffin cells. Stimulation of these nAChRs increases cell excitability, leading to voltage-dependent Ca^{2+} entry and catecholamine release.

3.2.1. Adrenomedullary chromaffin cells (AMCs)

Catecholamine-producing endocrine cells of the adrenal medulla were named ‘chromaffin’ cells due to their affinity for chromic acid or chromate salts, as first observed in histological studies by Bertholdus Werner in the mid-1960s (Richard, 1990). Similar to sympathetic neurons, chromaffin cells are derived from pluripotent precursor cells in the embryonic neural crest (Anderson, 1993b). These precursor cells migrate ventrally and eventually differentiate into their ultimate fate following interactions with environmental factors. The differentiation of chromaffin cells occurs around embryonic day 14.5 in the rat (Fernandez-Espejo *et al.*, 2005), and is facilitated by glucocorticoids produced by the adrenal cortex. In addition, glucocorticoids also promote the expression of phenylethanolamine *N*-methyltransferase (PNMT), the enzyme that catalyzes the conversion of norepinephrine to epinephrine (Anderson, 1993a). There are two functionally distinct populations of chromaffin cells in the adrenal medulla, i.e. epinephrine- and

norepinephrine-synthesizing cells. This distinction was made based on electron microscopy and immunohistochemical studies that labelled the glucocorticoid receptor and PNMT-containing chromaffin cells (Ceccatelli *et al.*, 1989). PNMT expression in chromaffin cells is induced by corticotropin, as well as cholinergic (nicotinic and muscarinic), and angiotensin II stimulation. Glucocorticoids are thought to be the signal required for differentiation and maintenance of the chromaffin phenotype (Doupe *et al.*, 1985).

In order to study factors that regulate the differentiation of sympathoadrenal precursors, the use of cell lines has attracted much attention. Perhaps the most commonly-used cell line is the pheochromocytoma or PC12 line, which originated from a tumor of the adult rat adrenal gland (Greene & Tischler, 1976). For this thesis, however, another immortalized chromaffin cell line played a critical role in addressing the main aims. A brief history and relative advantages of this cell line, and a comparison to PC 12 cells, are discussed below.

3.2.2. The immortalized fetal adrenomedullary chromaffin cell line (MAH cells) versus PC12 cells

The use of continuous cell lines is a common practice in molecular and biochemical studies. Such cell lines can provide a suitable surrogate model for studying molecular mechanisms since they are easily propagated and maintained, and can be readily manipulated for molecular applications. Although primary cell cultures offer a more relevant model for physiological studies, it is often difficult to obtain enough material for molecular analyses (e.g. western blotting and PCR), as well as a relatively pure cell population. Therefore, the use of continuous cell lines, for example the O₂-sensitive PC 12 cell line, has proved advantageous. Indeed, the PC 12 line has been widely utilized to study O₂-sensing mechanisms and the effects of acute,

chronic, and intermittent hypoxia on K^+ channel activity and regulation of gene expression (Conforti & Millhorn, 1997; Taylor & Peers, 1998; Conforti *et al.*, 2000; Prabhakar, 2001; Kumar & Prabhakar, 2008). Similar to primary neonatal chromaffin cells, hypoxia causes K^+ channel inhibition coupled to membrane depolarization and catecholamine secretion in PC 12 cells (Taylor & Peers, 1998). However, the validity of this model has been questioned since neonatal rat chromaffin cells respond to hypoxia via inhibition of a different subset of K^+ channels when compared to PC 12 cells. For example, in PC12 cells, hypoxia causes inhibition of Shaker-type K^+ channels containing the Kv1.2 α -subunit (Conforti & Millhorn, 1997; Conforti *et al.*, 2000). In contrast, hypoxia inhibits the large conductance, voltage and Ca^{2+} -activated K^+ (BK) channels and/or the small conductance Ca^{2+} -activated K^+ (SK) channels in both rat and ovine chromaffin cells (Thompson & Nurse, 1998). These considerations have led to the search for other models to study the O_2 sensitivity of chromaffin cells.

A more recent and attractive model used by our laboratory for studying the ontogeny of O_2 chemoreception in chromaffin cells is the immortalized *v-myc* adrenal- derived **H**NK1⁺ (MAH) cell line (Fearon *et al.*, 2002; Nurse *et al.*, 2009). This cell line was derived from sympathoadrenal progenitor cells at embryonic day 14.5 of the fetal rat adrenal medulla and was originally utilized to study the development of neural crest-derived sympathetic neurons (Birren & Anderson, 1990). The cells were immortalized using a *v-myc*-expressing retrovirus and identified by the cell surface marker HNK1, expressed by neural crest progenitors (Birren & Anderson, 1990). Similar to primary neonatal AMCs, MAH cells respond to asphyxial stressors, i.e. hypoxia (low O_2), hypercapnia (high CO_2), and hypoglycemia (low glucose), and express similar O_2 -sensitive K^+ (SK, BK) channels (Thompson & Nurse, 1998; Fearon *et al.*, 2002; Johnson *et al.*, 2004; Buttigieg *et al.*, 2008a; Buttigieg *et al.*, 2008b; Piskuric *et al.*, 2008;

Livermore *et al.*, 2011). Consequently, this cell line appears as an attractive model for studying factors that regulate the development of O₂ and CO₂/H⁺ chemosensitivity in perinatal chromaffin cells, as demonstrated later in this thesis.

4. Innervation of chromaffin cells and ontogeny of O₂ and CO₂/H⁺ chemosensitivity

The mature adrenal gland receives both an extrinsic and intrinsic innervation. The extrinsic innervation is supplied mainly by cholinergic sympathetic preganglionic fibers via the splanchnic nerve, with additional inputs from preganglionic parasympathetic and afferent sensory fibers (Coupland & Weakley, 1970; Toth, 1998). The intrinsic innervation is supplied by two types of ganglionic neurons distributed throughout the cortex and the medulla (Parker *et al.*, 1993; Dagerlind *et al.*, 1995). Type I ganglionic neurons express a number of neuropeptides including neuropeptide tyrosine, substance P, pituitary adenylate cyclase activating peptide (PCAP), and pro-enkephalin, in addition to catecholamine- synthesizing enzymes except PNMT (North & Egan, 1983; Dagerlind *et al.*, 1995; Mravec, 2005). Type II ganglionic neurons contain vasoactive intestinal peptide (VIP) (Holgert *et al.*, 1996).

In adult animals, stressful events cause activation of preganglionic sympathetic fibers, leading to release ACh, and excitation of chromaffin cells via nicotinic receptors. The resulting catecholamine secretion causes a number of adaptive cardiovascular responses, e.g. increased blood pressure, heart rate, and contractility that enable the animal to cope with the stress. By contrast, the preganglionic sympathetic innervation is immature at birth in a number of species including humans and rodents (Seidler & Slotkin, 1986; Cooper *et al.*, 1990; Naidoo *et al.*, 2001). In neonatal rodents, functional splanchnic innervation does not become fully mature until around postnatal day 10. However, in response to asphyxial stressors (e.g. low oxygen (hypoxia)

and high CO₂ (hypercapnia)) experienced at birth, chromaffin cells release catecholamines by a ‘non-neurogenic’ mechanism and this is critical for proper transition of the newborn to extrauterine life (Seidler & Slotkin, 1985; Lagercrantz & Slotkin, 1986).

5. Electrophysiological mechanisms of O₂ and CO₂ sensing in neonatal rat chromaffin cells

Neonatal rat chromaffin cells express a number of ion channels that participate in the transduction pathway leading to catecholamine secretion in response to asphyxial stressors. Since the molecular mechanisms by which chromaffin cells respond to these stressors is central to this thesis, a basic understanding of the role of ion channels in O₂ and CO₂ sensing is necessary. It is generally accepted that hypoxia and hypercapnia cause inhibition of several K⁺ channel subtypes that facilitate membrane depolarization, voltage-dependent Ca²⁺ entry, and CAT secretion (Mochizuki-Oda *et al.*, 1997; Thompson *et al.*, 1997; Thompson & Nurse, 1998; Keating *et al.*, 2001; Thompson *et al.*, 2002; Rico *et al.*, 2005; Bournaud *et al.*, 2007; Livermore *et al.*, 2011; Livermore *et al.*, 2012). Importantly, and as discussed in more detail below, hypoxia also simultaneously activates ATP-sensitive K⁺ (K_{ATP}) channels, which are a major focus of this thesis.

5.1. Hypoxia inhibits Ca²⁺-dependent K⁺ and delayed rectifier K⁺ channels

Excitable cells in general express a number of voltage-dependent Ca²⁺-activated K⁺ channels that help control cell excitability. In perinatal chromaffin cells, two types of these channels are known to be inhibited by hypoxia. These are the large-conductance, voltage- and Ca²⁺-activated K⁺ (Maxi K⁺ or BK) channels (Mochizuki-Oda *et al.*, 1997; Thompson *et al.*, 1997), and the

small conductance calcium-activated K^+ (SK) channels (Lee *et al.*, 2000; Keating *et al.*, 2001; Keating *et al.*, 2005). The SK channels are open at the resting membrane potential in neonatal rat and fetal ovine chromaffin cells, and their inhibition by acute hypoxia leads to membrane depolarization (i.e. receptor potential) (Lee *et al.*, 2000; Keating *et al.*, 2001; Keating *et al.*, 2005). Delayed rectifier-type (K_v) K^+ channels have also been shown to be inhibited by hypoxia in a number of O_2 sensitive cells, including neonatal rat and mouse chromaffin cells (Thompson & Nurse, 1998; Lopez-Barneo *et al.*, 2001; Thompson *et al.*, 2002). Inhibition of the voltage-dependent K^+ channels is thought to contribute to the broadening of the action potential duration, increased Ca^{2+} entry, and enhanced catecholamine secretion (Thompson & Nurse, 1998; Nurse *et al.*, 2009).

5.2. Hypoxia activates ATP-sensitive K^+ (K_{ATP}) channels in perinatal chromaffin cells

Chromaffin cells express another subtype of K^+ channels, i.e. ATP-sensitive K^+ channels (K_{ATP}), that are *activated* during hypoxia (Thompson & Nurse, 1998; Bournaud *et al.*, 2007). Generally, these channels are proposed to link metabolic function in the cell to membrane excitability (Miki *et al.*, 1999; Ashcroft & Gribble, 2000; Miki *et al.*, 2001). Historically, K_{ATP} channels were first discovered in the heart and other organs including the brain, skeletal and smooth muscle cells, kidney, and pancreatic β -cells, where they play a role in glucose-induced insulin release (Ashford *et al.*, 1994; Miki *et al.*, 1999). Structurally, the K_{ATP} channels are hetero-octameric proteins containing 4 inwardly rectifying K^+ channel (Kir6.1 or Kir6.2) pore-forming subunits and 4 sulfonylurea receptor (SUR1 or SUR2A/B) subunits, in varying combinations depending on cell type (Miki *et al.*, 1999; Nichols, 2006; Craig *et al.*, 2008).

Functionally, K_{ATP} channels are activated during exposure to stimuli that inhibit metabolism and lower intracellular ATP levels (e.g. hypoxia and hypoglycemia) (Trauner & Kramer, 2004). In neonatal rat chromaffin cells, physiological levels of ATP under normoxic conditions result in K_{ATP} channel closure at the resting membrane potential (Thompson & Nurse, 1998). During exposure to hypoxia or hypoglycemia, the lower intracellular ATP concentration results in channel activation that increases K^+ efflux and favours membrane hyperpolarization (Thompson & Nurse, 1998; Bournaud *et al.*, 2007; Piskuric *et al.*, 2008; Livermore *et al.*, 2011). Similarly, pharmacological studies using the K_{ATP} channel blocker, glibenclamide, have shown that activation of K_{ATP} channels during aglycemia decreases excitability in the immortalized chromaffin cell line, i.e. MAH cells (Fearon *et al.*, 2002; Piskuric *et al.*, 2008). In general, activation of K_{ATP} channels during such stimuli is thought of as an “excitatory brake” that limits membrane depolarization, rise in intracellular Ca^{2+} , and catecholamine secretion.

Relevant to this thesis, previous work in this laboratory demonstrated that chronic exposure of neonatal AMCs to nicotinic nAChR agonists (i.e. nicotine) *in vitro* caused the functional upregulation of K_{ATP} channel expression and blunting of hypoxia sensitivity (Buttigieg *et al.*, 2008a; Buttigieg *et al.*, 2009). Also, neonatal AMCs derived from rat pups exposed to nicotine *in utero* showed functional K_{ATP} channel upregulation and diminished membrane depolarization and CAT secretion during acute hypoxia (Buttigieg *et al.*, 2008a; Buttigieg *et al.*, 2009). K_{ATP} channel expression also plays a key role in the blunting of hypoxia sensitivity in chromaffin cells following chronic opioid exposure, as will be discussed in detail in Chapter 4 of this thesis.

5.3. Mechanisms of CO₂ sensing in chromaffin cells

Similar to hypoxia, sensitivity of neonatal AMCs to isohydric hypercapnia (10% CO₂; pH=7.4) involves inhibition of outward K⁺ current, membrane depolarization, voltage-gated Ca²⁺ entry and CAT secretion (Munoz-Cabello *et al.*, 2005; Buttigieg *et al.*, 2008a; Buttigieg *et al.*, 2009). However, in contrast to hypoxia, the receptor potential induced by high CO₂ appears to be mediated by activation of a resting Na⁺-permeable, cation conductance that has not been well characterized (Munoz-Cabello *et al.*, 2005). In addition, responsiveness to hypercapnia is dependent on the activity of the CO₂ hydrating enzyme, i.e. carbonic anhydrase (CA). This is supported by electrophysiological observations that hypercapnia sensitivity in neonatal chromaffin cells is prevented when the membrane-permeable CA inhibitor, methazolamide, is present (Munoz-Cabello *et al.*, 2005). According to this schema, CO₂ diffuses into the cell where intracellular CA catalyzes the reversible hydration of CO₂ as follows:



The resulting increase in H⁺ lowers intracellular pH, which in turn leads to ion channel regulation, e.g. inhibition of voltage-gated K⁺ channels (Lahiri *et al.*, 1996; Putnam *et al.*, 2004; Munoz-Cabello *et al.*, 2005). *In situ* hybridization studies revealed the presence of two CA isoforms (CAI and II), localized to chromaffin cells in tissue sections of the neonatal rat adrenal gland; interestingly, expression of both isoforms was markedly reduced or absent (>1000-fold lower) in the adult gland (Munoz-Cabello *et al.*, 2005). This apparent loss in expression of the two CA isoforms during postnatal development appeared to be specific for the adrenal gland as it was not observed in the kidney or carotid body. These observations highlight the important role of CAI and II in the mechanisms mediating hypercapnia sensitivity in neonatal rat AMCs (Munoz-Cabello *et al.*, 2005). Chapter 4 of this thesis examines the regulation of these two enzymes in chromaffin cells exposed to chronic opioids *in vivo* and *in vitro*.

6. Signalling mechanisms via postsynaptic receptors on chromaffin cells

This thesis addresses the hypothesis that chronic activation of specific neurotransmitter receptors on chromaffin cells plays a key role in the developmental regulation of O₂ and CO₂ sensitivity. In Chapter 2, the focus is on nicotinic ACh receptor signalling, whereas Chapters 3 and 4 focus on opioid receptor signalling. A brief account of the relevant signalling mechanisms associated with these receptors in the context of chromaffin cell biology is presented below.

6.1. Nicotinic acetylcholine receptors (nAChR)

In 1968, the term "stimulus-secretion coupling" was coined by William Wilton Douglas and his colleagues following the discovery that calcium ions were required for catecholamine secretion from chromaffin cells in the adrenal medulla (Douglas, 1968). It was later recognized that release of ACh from preganglionic sympathetic nerve endings activated postsynaptic nicotinic neuronal AChRs (nAChRs) on chromaffin cells, leading to membrane depolarization and CAT secretion (Kidokoro *et al.*, 1982). The nAChR is a ligand-gated ion channel with a pentameric structure that spans the cell membrane and is permeable to Na⁺, K⁺ and Ca²⁺ ions (Ifune & Steinbach, 1992). Cloning techniques have identified several nAChR subunits that includes ten α -subunits and four β -subunits (Sala *et al.*, 2008). Molecular studies indicate that chromaffin cells express mRNA for about seven of these nAChR subunits including α 2, α 3, α 4, α 5, α 7, β 2, and β 4 (Mousavi *et al.*, 2001; Di Angelantonio *et al.*, 2003). Mature nAChRs expressed on chromaffin cells can have both homomeric and heteromeric structures. The predominant forms consist of two α 3 and three β 4 subunits, though the α 3 subunit can also associate with β 2 and constitute functional nAChRs in chromaffin cells (Mousavi *et al.*, 2001). These heteromeric receptors are

insensitive to the snake toxin, α -bungarotoxin (α -Btx), and are involved in mediating membrane depolarization and CAT release (Free *et al.*, 2002). The homomeric nAChRs on chromaffin cells are mainly formed by the $\alpha 7$ subunit and are highly permeable to Ca^{2+} ; they are inhibited by α Btx but do not contribute significantly to the physiological response of chromaffin cells during stress (Garcia-Guzman *et al.*, 1995). However, their existence on neonatal chromaffin cells raises questions about their physiological significance. Chapter 2 of this thesis partly addresses this point.

6.2. Opioid peptides and opioid receptors in the adrenal gland

Opioid peptides such as met-enkephalin, have been localized within splanchnic nerve terminals and in adrenal chromaffin cells (Schultzberg *et al.*, 1978; Chang *et al.*, 1982). Moreover, immunohistochemical studies revealed a postnatal, age-dependent increase in the expression of these opioid peptides that coincides with the loss of direct hypoxic chemosensitivity in chromaffin cells (Holgert *et al.*, 1998). Chapters 3 and 4 of this thesis will test the hypothesis that activation of postsynaptic opioid receptors during splanchnic innervation contributes to the suppression of direct O_2 and CO_2 chemosensitivity in neonatal rat AMCs. Opioid receptors are known to be members of the G-protein coupled receptor (GPCR) superfamily containing seven hydrophobic transmembrane domains that are connected to each other by few intracellular and extracellular loops. Molecular cloning of opioid receptors and pharmacological studies have identified three major types of opioid receptors, μ , δ , and κ (Law *et al.*, 2000). Opioid receptors are known to be promiscuous when activated, in the sense that they interact with multiple G-protein subunits. Upon activation, opioid receptors interact with multiple G-proteins to regulate a number of effectors such as adenylyl cyclase (AC), phosphatidylinositol-3 kinase (PI3K) and the

MAP kinase pathways (Carter & Medzihradsky, 1993; Laugwitz *et al.*, 1993; Prather *et al.*, 1994; Burt *et al.*, 1996). Receptor activation by opioid drugs is known to be involved in a number of effects such as analgesia, euphoria and reward, respiratory depression, constipation, progression of drug tolerance, and dependence (Law *et al.*, 2000).

In the rat adrenal gland, mRNA analysis showed the presence of all three main classes of opioid receptors, μ , δ and κ (Wittert *et al.*, 1996). Radioligand binding assays show specific κ -opioid binding sites predominantly localized in the adrenal cortex (Quirion *et al.*, 1983; Wittert *et al.*, 1996). Opioid receptors are also activated by the synthetic alkaloid opioids including heroin and its metabolic derivative, morphine. Binding of opioid ligands such as morphine, [D-Ala², NMe-Phe⁴, Gly⁵-ol]-enkephalin (DAMGO), and [D-Pen², D-Pen⁵]-enkephalin (DPDPE) shows some specificity for the three major classes of opioid receptors. These three synthetic opioids were used in Chapters 3 & 4 of this thesis to stimulate the different opioid receptors on chromaffin cells.

6.2.1. Signalling pathways induced by opioid receptor stimulation

Stimulation of the three G-protein coupled opioid receptors activates signalling pathways that regulate a similar range of effectors including adenylyl cyclase (AC) (Fukuda *et al.*, 1993; Meng *et al.*, 1993), phospholipase C (Johnson *et al.*, 1994), mitogen-activated protein kinases (Fukuda *et al.*, 1996), ion channels including Ca²⁺ channels (Piros *et al.*, 1996), and G-protein gated inward rectifying K⁺ channels (Henry *et al.*, 1995). The presence of at least 9 isoforms of mammalian ACs with different sensitivities to G-protein subunits allows opioid receptors to regulate multiple cell-specific signalling pathways (Tang & Hurley, 1998). Recently, a number of proteins and signalling pathways have been proposed to be regulated by chronic opioid

exposure. Of particular relevance to Chapter 4 of this thesis is a recent study demonstrating that chronic exposure to opioid agonists (i.e. fentanyl) induced the transcription factor HIF-1 α via μ - and δ - opioid receptors in neuroblastoma (SH-SY5Y) cells (Daijo *et al.*, 2011). The next section contains a brief review of HIF transcription factors which were found to play a critical role in the effects of both chronic nicotine and chronic opioid exposure on perinatal chromaffin cells (Chapters 2 and 4 of this thesis).

7. Hypoxia inducible factors (HIFs)

The hypoxia inducible factors (HIFs) are master regulators of gene expression in response to low O₂ (i.e. hypoxia) and were first discovered in 1993 by Semenza's group (Wang & Semenza, 1993). Cellular adaptations to hypoxia involves the regulation of expression of genes (e.g. glycolytic enzymes) that lead to activation of alternative metabolic processes with lower demand for molecular O₂. Adaptations to hypoxia also involve increased O₂ delivery at the tissue level via upregulation of erythropoietin (EPO) to increase red blood cell production (Jiang *et al.*, 1996b), and vascular endothelial growth factor (VEGF) to increase angiogenesis (Forsythe *et al.*, 1996). The HIF family of transcription factors, i.e. HIF-1 α , HIF-2 α and HIF-3 α , bind to DNA as a heteromeric structure consisting of two subunits, HIF- α and HIF-1 β (Wang *et al.*, 1995; Rocha, 2007; Kenneth & Rocha, 2008; Semenza, 2009). Both subunits contain two well- conserved domains, i.e. the basic helix-loop-helix (bHLH) and the Per-AHR-ARNT-sim (PAS) domains (Wang *et al.*, 1995). The bHLH domain is present in most transcription factors and is required for the dimerization of the two subunits and binding to DNA (Jiang *et al.*, 1996a). HIF-1 α and HIF-2 α are closely related and they contain additional domains known as the oxygen-dependent degradation domain (ODD) and N-terminal and C-terminal transactivation domains (N-TAD and

C-TAD). HIF-3 α is less closely related and lacks the C-TAD domain (Wiesener *et al.*, 1998). While HIF transcripts are stable, products of the α -subunit (unlike HIF-1 β subunit) are constitutively degraded in normoxia via mechanisms involving O₂-dependent proline hydroxylation (at the ODD domain, residues 400-600) and ubiquitination (Wang *et al.*, 1995). In normoxia, HIF degradation is mediated via the protein von Hippel-Lindau tumor suppressor (pVHL) which recruits the E3 ubiquitin ligase complex (Kamura *et al.*, 2000). Proline hydroxylation in the ODD signals the binding of pVHL which recruits the ubiquitin ligase complex to facilitate HIF ubiquitination (Baek *et al.*, 2007). Hydroxylation of HIF proline residues occurs via prolyl hydroxylase domain enzymes (PHD) 1-3. PHD2 is known to hydroxylate principally HIF-1 α whereas PHD1 and 3 hydroxylate HIF-2 α (Appelhoff *et al.*, 2004; Baek *et al.*, 2005). During hypoxia, HIF-1 α and HIF-2 α subunits are stabilized via a decrease in proline hydroxylation, ubiquitination and proteasomal degradation. Following stabilization, the α -subunit translocates to the nucleus where it dimerizes with the β -subunit and binds to its consensus/core binding site located within the hypoxia response element (HRE) [(A/G)CGTG] so as to regulate gene transcription (Semenza *et al.*, 1996; Wenger *et al.*, 2005).

Another level of HIF regulation involves transactivation domains, C-TAD (residues 786-826) and N-TAD (located at the ODD, residues 531-575) (Hirota & Semenza, 2005; Schofield & Ratcliffe, 2005). TADs bind to coactivators such as p300 and CBP (cAMP response-element binding protein (CREB)-binding protein) to form the transcription activation complex involved in initiating gene transcription. Factor inhibiting HIF-1 (FIH-1) binds to asparagine (Asn) residues (residue 803 for HIF-1 α and residue 847 for HIF-2 α) in the C-TAD and hydroxylates them in normoxia. Asparagine hydroxylation of the TAD blocks the binding of coactivators CBP

and p300 (Lando *et al.*, 2002). Therefore, O₂-dependent regulation of HIF may occur via changes in stability and/or transcriptional activity.

7.1. Oxygen-independent HIF regulation

A number of “non-hypoxic” stimuli has been reported to induce HIF accumulation in some cells. For example, VEGF expression can be induced by angiotensin II (a vasoactive hormone) via HIF-1 α accumulation (Richard *et al.*, 2000). Similarly, insulin and insulin-like growth factors induce HIF-1 α accumulation under normal O₂ levels (Wenger, 2002). Mechanical stress has also been reported to induce HIF-1 α accumulation in cardiac myocytes under normoxic conditions (Kim *et al.*, 2002). Furthermore, chronic exposure to neurotransmitter receptor agonists such as nicotine and opioids has also been shown to induce HIF accumulation in non-small cell lung cancer (NSCLC) cells and SH-SY5Y cells respectively (Zhang *et al.*, 2007; Daijo *et al.*, 2011). Interestingly, as discussed in Chapters 2 and 4 of this thesis, HIF-2 α accumulation is also induced in perinatal chromaffin cells during chronic nicotine and opioid exposure, and is critically involved in the regulation of O₂ and CO₂ chemosensitivity in these cells.

8. Thesis objectives and organization

The overall objective of this thesis is to elucidate the cellular and molecular mechanisms involved in the postnatal suppression of the hypoxic and hypercapnic chemosensitivity in neonatal rat adrenomedullary chromaffin cells (AMCs). The central hypothesis is that tonic release of factors (i.e. neurotransmitters) by the preganglionic splanchnic nerve during development, results in the stimulation of postsynaptic receptors on AMCs and the initiation of a signalling cascade that ultimately regulates the expression of specific markers that control O₂ and

CO₂ sensitivity. In these studies, the use of dissociated cell cultures of primary neonatal adrenomedullary chromaffin cells (AMCs), an immortalized embryonic chromaffin-derived MAH cell line, a HIF-2 α -deficient MAH cell line, and *in vivo* models where adrenal gland tissues were sampled from rat pups exposed *in utero* to nicotine and morphine, aided in testing the main hypotheses.

The main body of this thesis is written in a “sandwich style” that consists of three papers, two of which are published, and one is in preparation for submission. A short preface at the beginning of each chapter will outline author contributions.

In Chapter 2, I examined the molecular mechanisms involved in the nicotine-induced suppression of hypoxic chemosensitivity in neonatal AMCs and the role of HIF-2 α . In this study, control (wild type) MAH cells, HIF-2 α -deficient (>90% knock-down) MAH cells, as well as adrenal tissues isolated from *in utero* nicotine-exposed rat pups were used to understand the role of HIF-2 α in the nicotine-induced upregulation of K_{ATP} channel expression. Western blot and quantitative RT-PCR (QPCR) analyses were conducted to investigate the effects of HIF-2 α deficiency on K_{ATP} channel expression. Furthermore, chromatin immunoprecipitation (ChIP) assays were performed to test the binding of HIF-2 α to the promoter region of the gene encoding the K_{ATP} channel subunit (Kir6.2). Finally, *in utero* nicotine-exposed adrenal tissues were used to confirm the predictions made *in vitro* using the immortalized MAH cell line.

Chapter 3 is a collaborative study that examines the role of chronic stimulation of opioid receptors in mediating the suppression of the direct hypoxic and hypercapnic sensitivity in neonatal rat AMCs. In these studies, primary neonatal rat AMCs were exposed to chronic opioids for 1 week in culture. Initial electrophysiological experiments monitoring hypoxia- and hypercapnia-induced inhibition of outward K⁺ current and membrane depolarization were

conducted by Dr. J. Buttigieg. Electrophysiological experiments involving the use of glibenclamide to test the functional up-regulation of the K_{ATP} channel expression following chronic opioids were conducted in collaboration with Dr. M. Zhang. I performed western blot analyses using primary neonatal AMCs to examine the effects of chronic opioids on the expression of a number of markers involved in hypoxia and hypercapnia chemosensitivity (i.e. K_{ATP} channel subunit, Kir6.2, and carbonic anhydrase enzymes). Finally, I performed fluorescence immunocytochemistry to visualize the expression of μ - and δ -opioid receptors in neonatal rat AMCs.

In Chapter 4, I examined the molecular mechanisms involved in the effects of chronic opioids on the expression of K_{ATP} channel subunit, Kir6.2, and carbonic anhydrase II enzyme in MAH cells. Techniques used in this chapter include western blot, quantitative RT-PCR analysis, and ChIP analysis using wild type and HIF-2 α -deficient MAH cells. In addition, western blot analysis was applied to adrenal tissues isolated from pups exposed to chronic morphine *in utero*.

Finally, Chapter 5 contains a general discussion, in addition to a model that integrates all the findings of this thesis.

**CHAPTER 2: CHRONIC NICOTINE INDUCES HYPOXIA INDUCIBLE FACTOR-2 α
IN PERINATAL RAT ADRENAL CHROMAFFIN CELLS: ROLE IN
TRANSCRIPTIONAL UPREGULATION OF K_{ATP} CHANNEL SUBUNIT Kir6.2**

This study has previously been published and reprinted with permission:
Shaima Salman, Stephen T. Brown and Colin A. Nurse (2012).
Am J Physiol Cell Physiol **302**: C1531–1538.

I performed all the experiments, analysis and preparation of the manuscript. Stephen Brown generated and kindly provided the HIF-2 α -deficient cell line and was involved in data interpretation. Alison Holloway generously provided assistance with animal experiments.

ABSTRACT

Fetal exposure to nicotine causes impaired adrenal catecholamine secretion and increased neonatal mortality during acute hypoxic challenges. Both effects are attributable to upregulation of K_{ATP} channels and can be rescued by pre-treatment with the blocker, glibenclamide. Though use of *in vitro* models of primary and immortalized, fetal-derived rat adrenomedullary chromaffin cells (i.e. MAH cells) demonstrated the requirements for $\alpha 7$ nAChR stimulation and the transcription factor, HIF-2 α , the latter's role was unclear. Using western blots, we show that chronic nicotine causes a progressive, time-dependent induction of HIF-2 α in MAH cells that parallels the upregulation of K_{ATP} channel subunit, Kir6.2. Moreover, a common HIF target, VEGF mRNA, was also upregulated after chronic nicotine. All the above effects were prevented during co-incubation with α -bungarotoxin (100 nM), a specific $\alpha 7$ nAChR blocker, and were absent in HIF-2 α -deficient MAH cells. CHIP assays demonstrated binding of HIF-2 α to a putative HRE in Kir6.2 gene promoter. Specificity of this signalling pathway was validated in adrenal glands from pups born to dams exposed to nicotine throughout gestation. Whereas medullary tissue showed upregulation of both HIF-2 α and Kir6.2, cortical tissue which normally expresses K_{ATP} channels but lacks $\alpha 7$ nAChR, showed no changes in either HIF-2 α or Kir6.2. This study has uncovered a signalling pathway whereby a non-hypoxic stimulus (nicotine) promotes HIF-2 α -mediated transcriptional upregulation of a novel target, Kir6.2 subunit. The data suggest that the HIF pathway may be involved in K_{ATP} channel- mediated neuroprotection during brain ischemia, and in the effects of chronic nicotine on ubiquitous brain $\alpha 7$ nAChR.

INTRODUCTION

The proper transition of the neonate to extrauterine life depends critically on catecholamine (CAT) secretion from adrenomedullary chromaffin cells (AMC), triggered by asphyxial stressors associated with birth, e.g. hypoxia (Seidler & Slotkin, 1985; Lagercrantz & Slotkin, 1986). We recently demonstrated that this direct response to hypoxia is blunted in AMCs derived from the offspring of rat dams exposed to nicotine throughout gestation, providing a potential link between cigarette smoke and impaired arousal as occurs in Sudden Infant Death Syndrome (Buttigieg *et al.*, 2008). The underlying mechanisms were attributable to the upregulation and subsequent opening of K_{ATP} channels (Kir6.2) during acute hypoxia, thereby favoring membrane hyperpolarization and reduced excitability (Buttigieg *et al.*, 2009). Indeed, in the latter study pre-treatment with the K_{ATP} channel blocker glibenclamide reversed the increased neonatal mortality seen in nicotine-exposed pups subjected to acute hypoxic challenges. Complementary studies using isolated neonatal rat AMCs or a fetal-derived immortalized chromaffin cell line (MAH cells), combined with *in vitro* exposures to 50 μ M nicotine lasting 1 week, demonstrated that the nicotine-induced K_{ATP} channel upregulation required activation of α -bungarotoxin-sensitive $\alpha 7$ nicotinic ACh receptors (nAChR) (Buttigieg *et al.*, 2009). Moreover, there was an obligatory requirement for the transcription factor, hypoxia inducible factor (HIF)-2 α , because the above effects of nicotine were absent in MAH cells deficient in HIF-2 α (Buttigieg *et al.*, 2009). In these studies, however, the mechanisms underlying the role of HIF-2 α were unclear, as there were no obvious changes in HIF-2 α levels in MAH cells after 24hr exposure to nicotine, in contrast to its robust induction after exposure to hypoxia (2% O₂) for a similar period (Brown & Nurse, 2008; Buttigieg *et al.*, 2009). Understanding these mechanisms is of broad interest given the importance of K_{ATP} channel function in neuroprotection and neurovascular remodelling following oxygen/glucose deprivation during brain ischemia and stroke (Ballanyi, 2004; Sun & Hu, 2010), and in the physiology of pancreatic beta cells which becomes dysregulated after fetal nicotine exposure (Bruin *et al.*, 2008).

In the present study, we re-visited the role of HIF-2 α in K_{ATP} channel regulation after noting a potential hypoxia response element (HRE) or HIF binding site in the promoter region of Kir6.2 gene that encodes one of two dissimilar subunits of the K_{ATP} channel. Each of four channel pore-forming Kir6.2 subunits combines with a regulatory subunit consisting of the sulphonylurea receptor (SUR) to form a functional tetrameric complex (Nichols, 2006). We hypothesized that nicotine exposure may cause a time-dependent induction of HIF-2 α , which in turn could lead to transcriptional upregulation of Kir6.2 subunit and consequently, increased functional K_{ATP} channel expression. Indeed, we found that both HIF-2 α and Kir6.2 protein displayed a time-dependent, parallel, and progressive upregulation in wild type and scrambled control MAH cells exposed to chronic nicotine over 1 week. Notably, this upregulation of HIF-2 α and Kir6.2 was absent in a stable HIF-2 α -deficient MAH cell line (>90% knockdown) generated using interference RNAi techniques (Brown *et al.*, 2009), and chromatin immunoprecipitation (ChIP) assays demonstrated binding of HIF- 2 α to the promoter region of the Kir6.2 gene. To validate the specificity of the signalling pathway we used an *in vivo* model of the rat adrenal gland subjected to chronic nicotine exposure during fetal development. In this model, we took advantage of the fact that the adrenal cortex expresses ATP-dependent K⁺ channels (Xu & Enyeart, 2001) but, in contrast to the medulla, lacks expression of α -bungarotoxin-sensitive α 7 nicotinic AChR (Criado *et al.*, 1997; Mousavi *et al.*, 2001). Interestingly, this *in vivo* model supported the main conclusions of the present study.

MATERIALS AND METHODS

Cell culture: The *v-myc* immortalized adrenal chromaffin (MAH) cells were incubated in a humidified atmosphere of 95% air: 5% CO₂ at 37°C and grown in L-15/CO₂ medium containing 0.6 % glucose, 1% penicillin/streptomycin, 10% fetal bovine serum and 5 µM dexamethasone as previously described (Fearon *et al.*, 2002). Cells were plated on poly-D-lysine- and laminin-coated 35 mm culture dishes, fed every 1–2 days, and routinely passaged every 3-4 days. HIF-2a knockdown (and scrambled control) MAH cells were generated using short hairpin RNAi (shRNA) interference techniques and propagated as a stable cell line as previously described (Brown & Nurse, 2008).

Preparation of adrenal tissues from nicotine- vs saline-treated pups: Experiments were approved by the the Animal Research and Ethics Board at McMaster University, and performed in accordance with the guidelines of the Canadian Council for Animal Care. The procedures for nicotine vs saline injections into pregnant Wistar rats were identical to those described in detail in our previous studies (Buttigieg *et al.* 2008). Experimental animals were kept at constant temperature (22°C) and lighting (12 h light/dark) with free access to water and food. Female Wistar rats were randomly treated with saline (vehicle) or nicotine bitartrate (1 mg/kg body weight/d) subcutaneously (s.c.) once a day for two weeks before mating and then throughout the gestation period as previously described (Holloway *et al.*, 2005; Buttigieg *et al.*, 2008). Upon delivery, pups were removed and both adrenal glands from each animal were isolated and kept in sterile medium. In the same medium, most of the outer cortex was trimmed and isolated from the central medullary tissue. The enriched cortical and medullary fractions were kept separate for molecular analysis.

Chromatin Immunoprecipitation (ChIP) Assay: ChIP assay was performed using a standard protocol (Millipore) as previously reported (Brown *et al.*, 2009). MAH cells were plated on 100 mm dishes and treated with nicotine base (50 µM) for 7 days at a confluency of 75%. This dose of nicotine was previously shown to be subsaturating for nicotinic receptors on primary rat chromaffin cells (EC₅₀ ~25 µM) (Buttigieg *et al.*, 2008);

this dose also produced a similar upregulation of K_{ATP} channel expression in MAH cells following *in vitro* exposure and in primary neonatal chromaffin cells following both *in vitro* and *in utero* exposure to nicotine (Buttigieg *et al.*, 2009). Histones were cross-linked to DNA using 1% formaldehyde for 10 min at 37°C. Immunoprecipitation was performed using antibodies against HIF-2 α or control IgG. PCR analysis was utilized to detect HIF-2 α binding using primers specific for putative HIF responsive element (HRE) on Kir6.2 promoter sequence (forward: 5` CTG GAA GGA AGC CAG TCT TG 3`; and reverse: 5` CCT TCT TGT CCC CCT TTC TC 3`), and negative primers downstream from the promoter region (forward: 5` GCA TAA ATG TTT CCC ACT CC 3`; and reverse: 5` TAA CTG AAG AAG GGC AGG AA 3`). Primers were designed using Gene Fisher (Giegerich *et al.*, 1996). Thermal cycling conditions included Platinum *Taq* DNA polymerase activation at 95°C for 2 min, 35 cycles of denaturing at 95°C for 30 s, and annealing and extension at 60°C for 30 s.

Western Immunoblot analysis: Nuclear and cytoplasmic extracts obtained from adrenal tissues and MAH cells were loaded and resolved on SDS-PAGE gel and transferred to PVDC membranes as previously described (Brown & Nurse, 2008). Membranes were incubated with either primary Kir6.2 rabbit polyclonal antibody (1:1000 dilution; Alomone Labs Ltd.), HIF-2 α rabbit polyclonal antibody (1:1000 dilution; Novus Biologicals, Littleton, CO, USA), SUR1 rabbit polyclonal antibody (1:5000 dilution; Millipore, Canada), TATA-binding protein rabbit polyclonal antibody (1:2000 dilution; Santa Cruz, CA, USA), or primary β -actin rabbit monoclonal antibody (1:10,000 dilution) at 4°C overnight.

Quantitative Real Time-PCR (Q RT-PCR): Quantitative RT-PCR analysis was performed using the Stratagene (Mx3000p) detection system using Absolute QPCR SYBR Green Mix. Kir6.2 primers (forward: 5' ACA AGA ACA TCC GAG AGC A 3' and reverse: 5' CTG CAC GAT CAG AAT AAG GA 3') and VEGF primers (forward: 5' AATGATGAAGCCCTGGAGTG 3' and reverse: 5' AATGCTTTCTCCGCTCTGAA 3' (Huang *et al.*, 2010), were designed using Gene Fisher (Giegerich *et al.*,

1996) and specificity was confirmed using BLAST. Thermal cycling conditions included Platinum *Taq* DNA polymerase activation at 95°C for 2 min, 40 cycles of denaturing at 95°C for 3 s, annealing and extension at 60°C for 30 s, followed by routine melting curve analysis. Samples with no template were used as a negative control. Data were compared using the arithmetic equation $2^{-\Delta\Delta CT}$ (Livak & Schmittgen, 2001) and mRNA levels were normalized to Lamin A/C expression (primers: forward: 5'-GCAGTACAAGAAGGAGCTA and reverse: 5' CAGCAATTCCTGGTACTCA 3') as an endogenous reference (Brown *et al.*, 2009), and expressed as transcript fold change relative to mRNA from untreated control MAH cells.

Drugs: All drugs were purchased from Sigma-Aldrich and applied fresh to the medium every 2 days.

Statistical analysis: Data from experiments were expressed as means \pm SEM and statistical analyses were carried out using one-way ANOVA with Dunnett post-hoc multiple comparison. Differences were considered significant if $P < 0.05$.

RESULTS

Effects of chronic nicotine on Kir6.2 subunit expression in control and HIF-2 α -deficient MAH cells

Consistent with previous reports from this laboratory (Buttigieg *et al.*, 2009), exposure of control, wild type (w.t.) MAH cells to chronic nicotine (50 μ M) for 7 days caused a significant upregulation of K_{ATP} channel Kir6.2 subunit at the protein level (Fig. 1A). Moreover, this effect was abolished during continuous co-incubation with α -bungarotoxin (α Btx; 100 nM), but not hexamethonium (Hex; 100 μ M) (Fig. 1A), confirming the involvement of homomeric α 7 (but not α 3-containing) neuronal nAChR (Buttigieg *et al.*, 2009). It is known that multiple α and β subunits of neuronal nAChR including α 3, α 5, α 7, β 2, β 4 are expressed in neonatal rat chromaffin cells (Buttigieg *et al.*, 2009; Souvannakitti *et al.*, 2010) and MAH cells (our unpublished microarray and PCR data). Importantly, this nicotine-induced upregulation of Kir6.2 was dependent on HIF-2 α function because it was absent in HIF-2 α -deficient (shHIF2 α ; >90% knockdown) MAH cells regardless of incubation conditions (Fig. 1B).

Our microarray data on MAH cells also revealed a significant up-regulation in Kir6.2 transcript after exposure to chronic hypoxia (2% O₂; 24 hr), a condition known to cause robust induction of HIF-2 α in these cells (Brown and Nurse 2008). This raised the question whether or not chronic nicotine exposure caused induction of HIF-2 α in MAH cells as a necessary and sufficient condition for the upregulation of Kir6.2. Because short-term (24 hr) exposures of MAH cells to chronic nicotine failed to cause any significant HIF-2 α accumulation in our previous study (Buttigieg *et al.*, 2009), we next investigated whether longer-term exposures were required.

Time-dependent effects of chronic nicotine on HIF-2 α accumulation and expression pattern of Kir6.2 and SUR1 subunits of the K_{ATP} channel in MAH cells: Role of α 7 nAChR

To determine whether or not HIF-2 α accumulation in nicotine-treated MAH cells is a time-dependent phenomenon, western blot analysis was used to probe for HIF-2 α at 24 hr, 3 days and 7 days of treatment. As illustrated in Fig. 2A (lower panel), there was a progressive increase in HIF-2 α accumulation that was significant at 3 and 7 days (but *not* at 24 hrs) of exposure, with HIF-2 α levels being significantly greater at 7 days compared to 3 days (Fig. 2A; histogram).

We next reasoned that if HIF-2 α induction was indeed causally related to the nicotine-induced upregulation of Kir6.2, then the kinetics of Kir6.2 expression should follow *pari passu* that of HIF-2 α accumulation. This was indeed the case as illustrated in Fig. 2B (upper panel) where Kir6.2 protein levels also increased progressively with exposure time and, similar to HIF-2 α accumulation, was also significant at 3 and 7 days (but *not* at 24 hrs) (Fig. 2B; D). Interestingly, the stimulatory effect of chronic nicotine was specific for the Kir6.2 subunit of the K_{ATP} channel because expression of the regulatory SUR1 subunit remained constant (Fig. 2C; D).

We also confirmed that, as for Kir6.2 upregulation, the nicotine-induced HIF-2 α accumulation at 7 days exposure was dependent on homomeric α 7 nAChR because it was prevented during co-incubation with the α 7 nAChR blocker, α -bungarotoxin (100 nM), but not hexamethonium (100 μ M) (Fig. 3A). Moreover, as illustrated in Fig. 3B, HIF-2 α accumulation was markedly suppressed in nicotine-treated HIF-2 α -deficient (shHIF2 α) MAH cells as expected, though it was robust in nicotine-treated scrambled control (ScCont) cells (Fig. 3B). Similarly, exposure to shorter durations of chronic hypoxia (2% O₂; 24hr) caused a robust induction of HIF-2 α in control (wtMAH, Fig. 3A; ScCont MAH), but not in HIF-2 α -deficient (shHIF2 α MAH), MAH cells (Fig. 3B).

Chronic nicotine upregulates both Kir6.2 and VEGF mRNA in MAH cells

The observed HIF-2 α -dependent upregulation of Kir6.2 protein in MAH cell after chronic exposure to nicotine raised the possibility that this could occur at the transcriptional level. To test this possibility we probed for Kir6.2 mRNA expression after a 7-day exposure of MAH cells to chronic nicotine. As illustrated in Fig. 4A, chronic nicotine caused ~3 fold increase in Kir6.2 mRNA expression (relative to lamin) as assessed by quantitative real-time PCR analysis. Moreover, this increased expression was prevented during co-incubation with the $\alpha 7$ nAChR blocker, α -bungarotoxin (100 nM), but not hexamethonium (100 μ M) (Fig. 4A). These data are consistent with the idea that the effects of chronic nicotine are mediated via HIF-2 α -dependent transcriptional upregulation Kir6.2 mRNA, leading to increased expression of K_{ATP} channels at the protein level.

The posit that chronic nicotine exposure causes HIF-2 α induction in MAH cells implies that the transcripts for other well-characterized HIF targets may also be concomitantly upregulated. Our microarray analysis on nicotine-treated MAH cells indicated upregulation of vascular endothelial growth factor (VEGF), a well known HIF-induced gene. To confirm these data and assess whether the nicotine-induced HIF-2 α accumulation was associated with an increase in VEGF mRNA, we used quantitative real-time PCR analysis following a 7-day exposure of MAH cells to chronic nicotine. Indeed, chronic nicotine and as expected chronic hypoxia (2% O₂, 24 hr) caused a significant increase in VEGF mRNA in control MAH cells; this effect was absent in HIF-2 α -deficient (shHIF2 α) MAH cells (Fig. 4B). Moreover, co-incubation of nicotine with α -bungarotoxin (α Btx; 100 nM), but not with hexamethonium (100 μ M), prevented the increase in VEGF mRNA expression (Fig. 4B), confirming that the effect was mediated via $\alpha 7$ nAChR. Taken together, these results demonstrate that chronic nicotinic activation of $\alpha 7$ nAChR in MAH cells leads to the accumulation of HIF-2 α which in turn mediates upregulation of Kir6.2 mRNA and other common HIF targets such as VEGF mRNA.

ChIP assay reveals binding of HIF-2 α to the promoter region of the Kir6.2 gene

Given that the nicotine-induced upregulation of Kir6.2 mRNA in MAH cells was HIF-2 α dependent (Fig. 4A), it was of interest to determine whether or not Kir6.2 gene expression is directly regulated by HIF-2 α . We therefore analyzed the promoter region of the Kir6.2 gene and found a potential hypoxia response element (HRE) or HIF-binding site (Fig. 5A). This site contains the HIF core sequence 5'-GCGTG-3' spanning nucleotides -1087 to -1083 and the HIF ancillary site 5'-CACAG-3' spanning nucleotides -1065 to -1061. The region containing both elements is considered the HRE and seems to be conserved in both the rat and mouse sequence (Wenger et al., 2005) (Fig. 5A, upper). It is similar in structure to the HRE element found in the promoter of adenosine A2a receptor (A2aR) gene that was recently shown to be regulated by HIF-2 α in MAH cells (Brown *et al.*, 2011). In order to determine whether or not HIF-2 α binds to this region of the Kir6.2 promoter, we performed a chromatin immunoprecipitation (ChIP) assay. Indeed, the ChIP assay on chromatin isolated from nicotine-treated wild type (wt MAH) and scrambled control (ScCont MAH) cells revealed a prominent band of 288bp that was conspicuously absent in HIF-2 α -deficient (shHIF2 α MAH) cells (Fig. 5B). In control experiments, no bands were detectable in control IgG immunoprecipitates nor in samples that included a pair of primers (negative primer) designed to amplify potential immunocomplexes, should they form downstream of the promoter region. These negative primers failed to amplify DNA suggesting that there was specific binding of HIF-2 α to an upstream site in the promoter region. These findings strongly suggest that HIF-2 α binds to the distal promoter region that contains a putative HRE and show that HIF-2 α binding to Kir6.2 can be stimulated by a non-hypoxic stimulus, i.e. nicotine.

Effects on chronic nicotine in utero on expression of Kir6.2 and HIF-2 α in adrenal medulla vs cortex

It was of interest to determine whether or not the model proposed above for the chronic effects of nicotine on the MAH cell line could be validated *in vivo*. To address this, we used an *in vivo* model where maternal administration of nicotine was previously shown to cause impaired O₂ sensing in chromaffin cells of the

affected offspring, attributable to upregulation of K_{ATP} channels (Buttigieg *et al.*, 2008; Buttigieg *et al.*, 2009). Previous studies have demonstrated that K_{ATP} channels are expressed in both the adrenal cortex and medulla (Xu & Enyeart, 2001; Buttigieg *et al.*, 2009), whereas α -bungarotoxin-sensitive $\alpha 7$ nicotinic AChRs are expressed only in the medulla (Criado *et al.*, 1997; Mousavi *et al.*, 2001). This presented a unique opportunity to test the specificity of the proposed pathway by comparing expression patterns in the adrenal cortex versus medulla of nicotine-exposed pups. As illustrated in Fig. 6, medullary tissue from nicotine-treated pups showed the expected upregulation of both HIF-2 α and Kir6.2 protein relative to saline-treated controls. By contrast, there was no difference in Kir6.2 expression (nor HIF-2 α) in the adrenal cortex between nicotine- and saline-treated pups. These data confirm that a signalling pathway involving stimulation of $\alpha 7$ nicotinic AChRs is required for the nicotine-induced upregulation of Kir6.2, via HIF-2 α .

DISCUSSION

The novel findings of this study are that chronic nicotine exposure leads to a slow, progressive accumulation of HIF-2 α in perinatal chromaffin cells via the selective activation of $\alpha 7$ nAChR, and subsequent transcriptional upregulation of ATP-sensitive K⁺ channel subunit, Kir6.2. Though these studies were greatly aided by use a control and HIF-2 α -deficient immortalized chromaffin (MAH) cell lines, importantly, the main conclusion of the study was validated in an *in vivo* model. In particular, western blot analyses of adrenal gland tissues taken from newborn pups exposed to chronic nicotine *in utero* revealed that upregulation of HIF-2 α and Kir6.2 expression also occurred, however it was confined to the medullary, but *not* cortical, region. Thus, even though K_{ATP} channels are normally expressed in both the adrenal medulla and cortex (Xu & Enyeart, 2001; Buttigieg *et al.*, 2009), our failure to observe its upregulation in the cortex is easily explained by the restricted expression of $\alpha 7$ nAChRs to the medulla (Criado *et al.*, 1997; Mousavi *et al.*, 2001). In addition to providing an additional control using an *in vivo* model, these data further emphasize the specificity of the signalling pathway leading to K_{ATP} channel upregulation after chronic nicotine.

Our previous studies demonstrated that chronic nicotine *in utero* and *in vitro* blunts the acute hypoxic sensitivity of neonatal adrenal chromaffin cells via upregulation of K_{ATP} channels, and involved CaMkinase and PKC signalling pathways (Buttigieg *et al.*, 2008; Buttigieg *et al.*, 2009). Moreover, we demonstrated that *in vivo* administration of the K_{ATP} channel blocker glibenclamide was able to reverse the increased neonatal mortality seen in nicotine-exposed pups subjected to acute hypoxic challenges (Buttigieg *et al.*, 2009). The present study helps to provide a mechanistic understanding at the molecular, cellular, and whole animal level of the adverse effects of nicotine exposure in mediating the loss of hypoxia tolerance in the neonate (Slotkin *et al.*, 1995). Thus, the ability of the K_{ATP} channel blocker glibenclamide to reverse the increased neonatal mortality seen in nicotine-exposed pups subjected to acute hypoxic challenges is explained by the slow accumulation of HIF-2 α induced by nicotine acting via $\alpha 7$ nAChR, leading to the transcriptional and functional

upregulation of K_{ATP} channels. This in turn causes membrane hyperpolarization and decreased catecholamine secretion during hypoxic challenges.

HIF stabilization and accumulation appears to be a multistep pathway that involves a number of post-translational modifications. Because $\alpha 7$ nAChRs are highly Ca^{2+} -permeable, it is likely that extracellular Ca^{2+} entry through these receptor channels initiates the signalling cascade, leading to activation of PKC and CaM kinase and ultimately HIF-2 α stabilization (Buttigieg *et al.*, 2009). Whether or not the pathway is similar to the one proposed for the role of Ca^{2+} , PKC and CaM kinase in promoting increased synthesis, stabilization, and transcriptional activity of HIF-1 α in PC 12 cells (see Fig. 12; (Yuan *et al.*, 2008)), remains to be determined. In this regard, there is precedence for the idea that nicotine exposure can also lead to HIF-1 α accumulation, as reported in a previous study on human small cell lung cancers (Zhang *et al.*, 2007). In the latter study, the action of nicotine was also mediated via $\alpha 7$ nAChR and resulted in increased VEGF expression, as observed in the present study. Other proposed signalling pathways such as the phosphatidylinositol 3-kinase (PI3K)/Akt pathway, involved in HIF-1 α stabilization (Mottet *et al.*, 2003), may potentially play a role in HIF-2 α stabilization in MAH cells, however, this possibility remains to be tested. While the status of HIF-1 α protein in our study on HIF-2 α -deficient MAH cells remains uncertain, our microarray data suggests that HIF-1 α mRNA is relatively unchanged in these mutant cells (our unpublished data). In general, HIF-2 α appears to be important in cells of the sympathoadrenal lineage and is the predominant and best-studied HIF in MAH cells as it is stabilized during exposure to either chronic hypoxia, prolyl hydroxylase inhibitors, or iron chelators (Brown & Nurse, 2008; Brown *et al.*, 2009). Irrespective of any potential role of HIF-1 α in the present study, our demonstration that HIF-2 α binds directly to the promoter region of the Kir6.2 gene in MAH cells strongly suggests that HIF-2 α is the key regulator of K_{ATP} channel expression and therefore the mediator of the nicotine-induced loss of hypoxia tolerance in neonatal adrenal chromaffin cells (Slotkin *et al.*, 1995; Buttigieg *et al.*, 2009). It is noteworthy that there does appear to be a

role for HIF-1 α in MAH cells exposed to chronic intermittent hypoxia, where several potential HIF-1 targets appear to be regulated (Brown *et al.*, 2010).

CLINICAL SIGNIFICANCE

The demonstration that K_{ATP} channel expression is under transcriptional control by HIF-2 α is of general interest given the importance of these channels in neuroprotection during brain ischemia (Jiang & Haddad, 1997; Ballanyi, 2004), stroke prevention (Sun & Hu, 2010), and as protectants against metabolic stress (Minami *et al.*, 2004). For example, during oxygen/ glucose deprivation central neurons respond to the fall in ATP by opening K_{ATP} channels as a protective mechanism that results in the suppression of membrane excitability and consequently, a reduction in energy demand (Jiang & Haddad, 1997; Ballanyi, 2004). Such low O₂ ischemic conditions are likely to induce the ‘master regulator’ HIF transcription factor(s) (Semenza, 2004, 2009), and potentially upregulate K_{ATP} channel expression (as demonstrated herein) as part of a global positive feedback mechanism to further enhance neuroprotection. K_{ATP} channels also play a central role in diabetes and particularly, in the hyperglycemic response of pancreatic beta cells which also express nicotinic receptors, including α 7 nAChR (Yoshikawa *et al.*, 2005). Indeed, maternal nicotine use during pregnancy has been reported to result in mitochondrial dysfunction in pancreatic beta cells attributable to oxidative stress (Bruin *et al.*, 2008), raising the possibility that the HIF pathway was activated under these conditions (Yuan *et al.*, 2008). Finally, the widespread distribution α 7 nAChR in brain adds fuel to the idea that chronic nicotine exposure, e.g. from cigarette smoke, may cause HIF stabilization in several regions of central nervous system. Such stabilization could lead to adaptive responses (e.g. neuroprotection) (Bencherif, 2009) on the one hand, or maladaptive responses (e.g. disease conditions) on the other, depending on cell type and the particular pattern of HIF expression (Semenza, 2009).

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Table 1: List of primers used for Q RT-PCR and ChIP analyses

TABLE 1

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Accession Numbr
<u>Q RT-PCR</u> Kir6.2 VEGF Lamin	ACA AGA ACA TCC GAG AGC A AATGATGAAGCCCTGGAGTG GCAGTACAAGAAGGAGCTA	CTG CAC GAT CAG AAT AAG GA AATGCTTTCTCCGCTCTGAA CAGCAATTCCTGGTACTCA	NM_031358 NM_001110333 NM_001002016
<u>ChIP</u> Kir6.2 (experimental) Kir6.2 (negative)	CTG GAA GGA AGC CAG TCT TG GCA TAA ATG TTT CCC ACT CC	CCT TCT TGT CCC CCT TTC TC TAA CTG AAG AAG GGC AGG AA	NM_031358 NM_031358

Figure 1. Effects of chronic nicotine and nicotinic antagonists on Kir6.2 protein expression in MAH cells. A) Western blot analysis of Kir6.2 expression (top) in wild-type MAH cells (wtMAH) exposed to either nicotine (Nic; 50 μ M), nicotine plus α -bungarotoxin (α Btx; 100 nM), or nicotine plus hexamethonium (Hex; 100 μ M), for 7 days; densitometric analysis (bottom) of relative Kir6.2 protein expression relative to β -actin (n =3). B) Comparable western blot analysis of Kir6.2 expression showing lack of nicotine-induced Kir6.2 upregulation in HIF-2 α -deficient (shHIF2 α) MAH cells. Results were normalized to β -actin and represented as mean \pm SEM relative to control (Anova; *, P < 0.05). Values are represented as mean \pm SEM of three independent experiments.

FIGURE 1

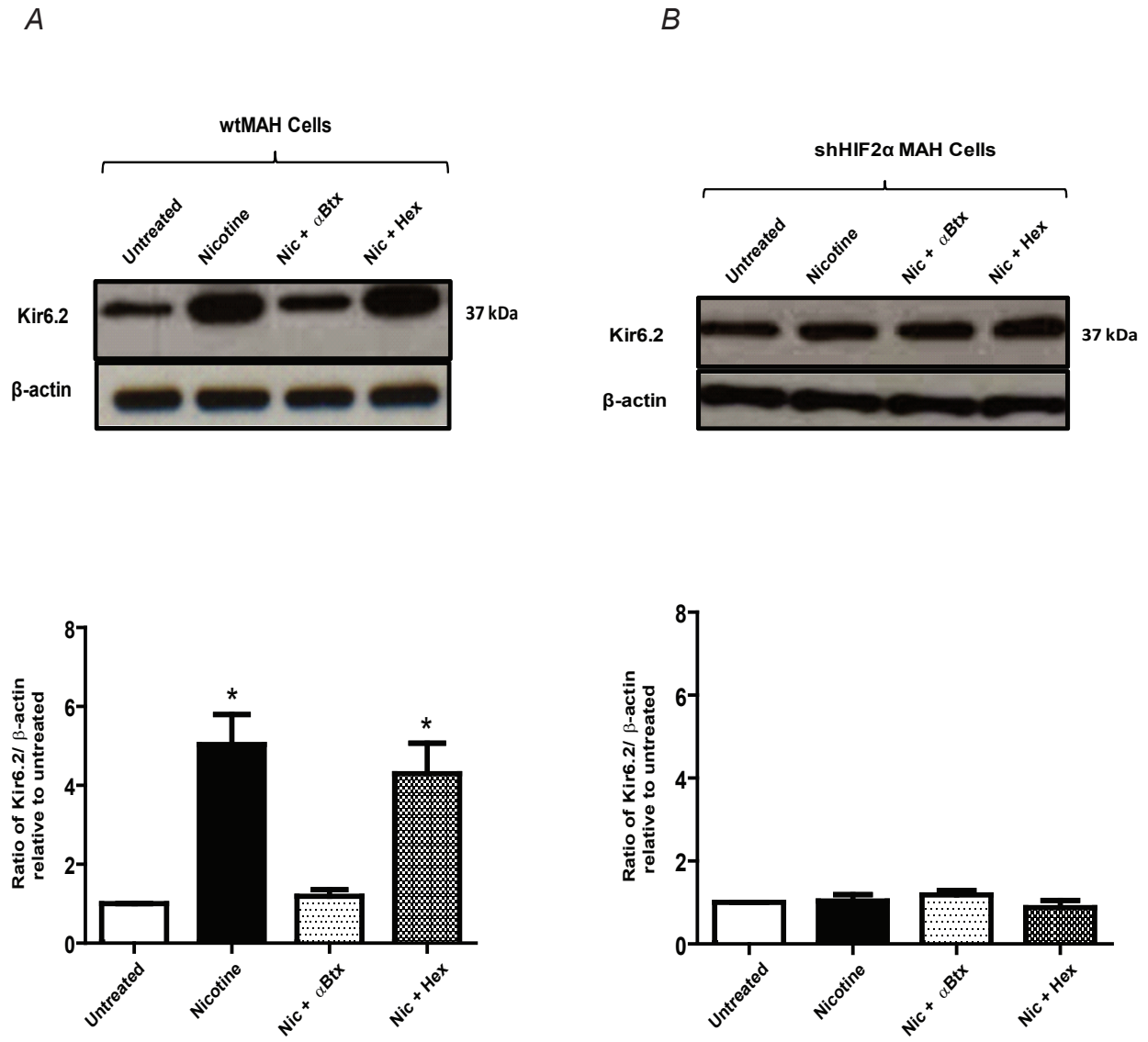


Figure 2. Time course of the effects of chronic nicotine on Kir6.2, SUR1, and HIF-2 α expression in MAH cells. Time-dependent HIF-2 α (A), Kir6.2 (B), and SUR1 (C) protein expression in MAH cells exposed to nicotine (Nic; 50 μ M) for 24 hr, 3 days, and 7 days in culture. Densitometric quantitation (bottom) of expression data normalized to loading control. i.e. β -actin (for cytoplasmic extracts in the case of Kir6.2 and SUR 1) and TATA-Binding Protein (TBP) (for nuclear extracts in the case of HIF-2 α). Values are represented as mean \pm SEM of three independent experiments.

FIGURE 2

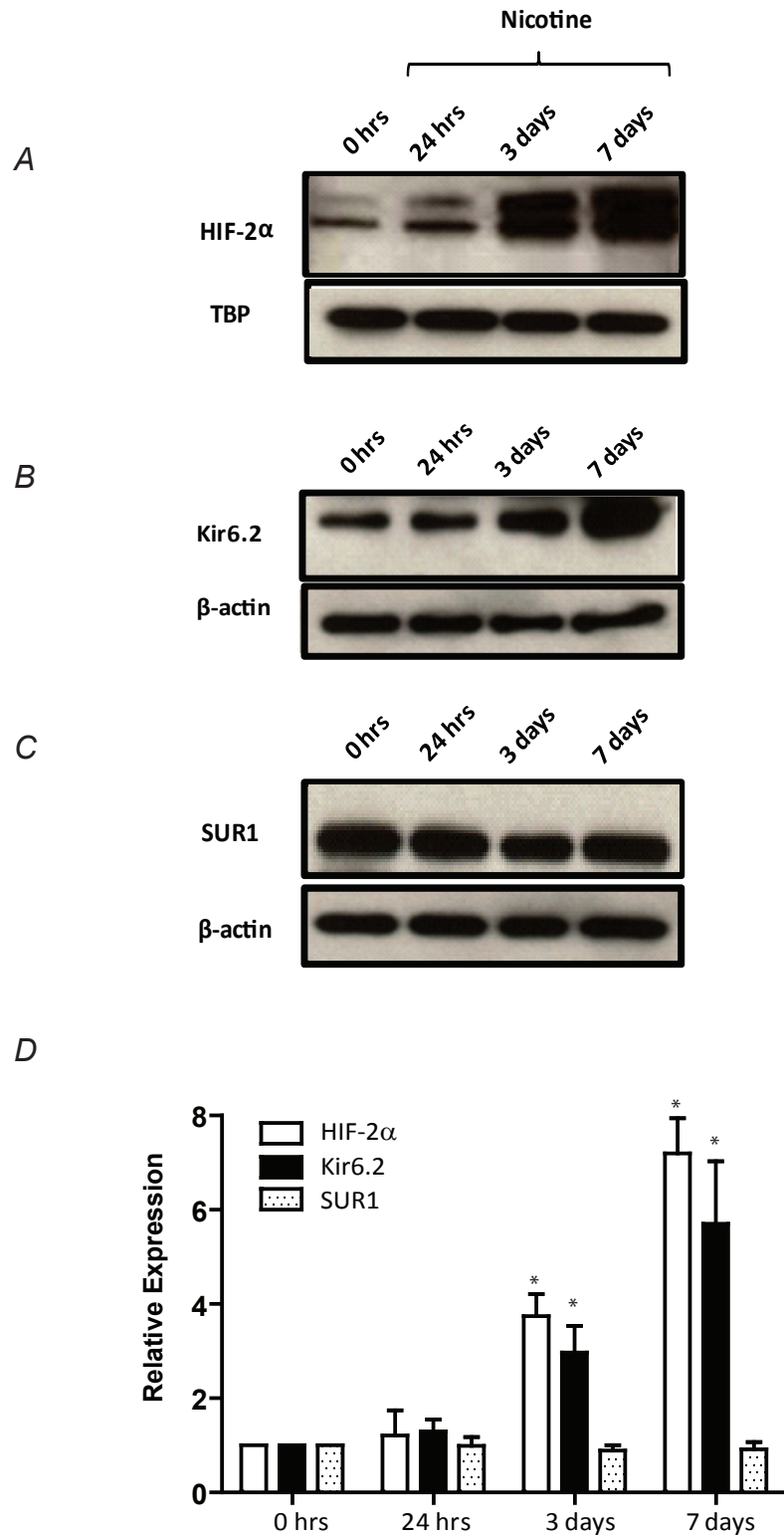


Figure 3. HIF-2 α accumulation in MAH cells exposed to nicotine, nicotine plus nicotinic blockers, or hypoxia. A) Western blot analysis of HIF-2 α accumulation (top) in wtMAH cells exposed to either nicotine (Nic; 50 μ M), nicotine plus α -bungarotoxin (α Btx; 100 nM), nicotine plus hexamethonium (Hex; 100 μ M) for 7 days, or to chronic hypoxia (Hox; 2% O₂, 24 hrs). B) Western blot analysis of HIF-2 α accumulation in HIF-2 α -deficient (shHIF2 α) and scrambled control (ScCont) MAH cells exposed to 50 μ M nicotine for 7 days. Densitometric analysis of relative HIF-2 α accumulation is shown in the lower histograms (n= 3 independent experiments). Results were normalized to TATA-Binding Protein (TBP) and represented as mean \pm SEM relative to control (Anova; *, P < 0.05).

FIGURE 3

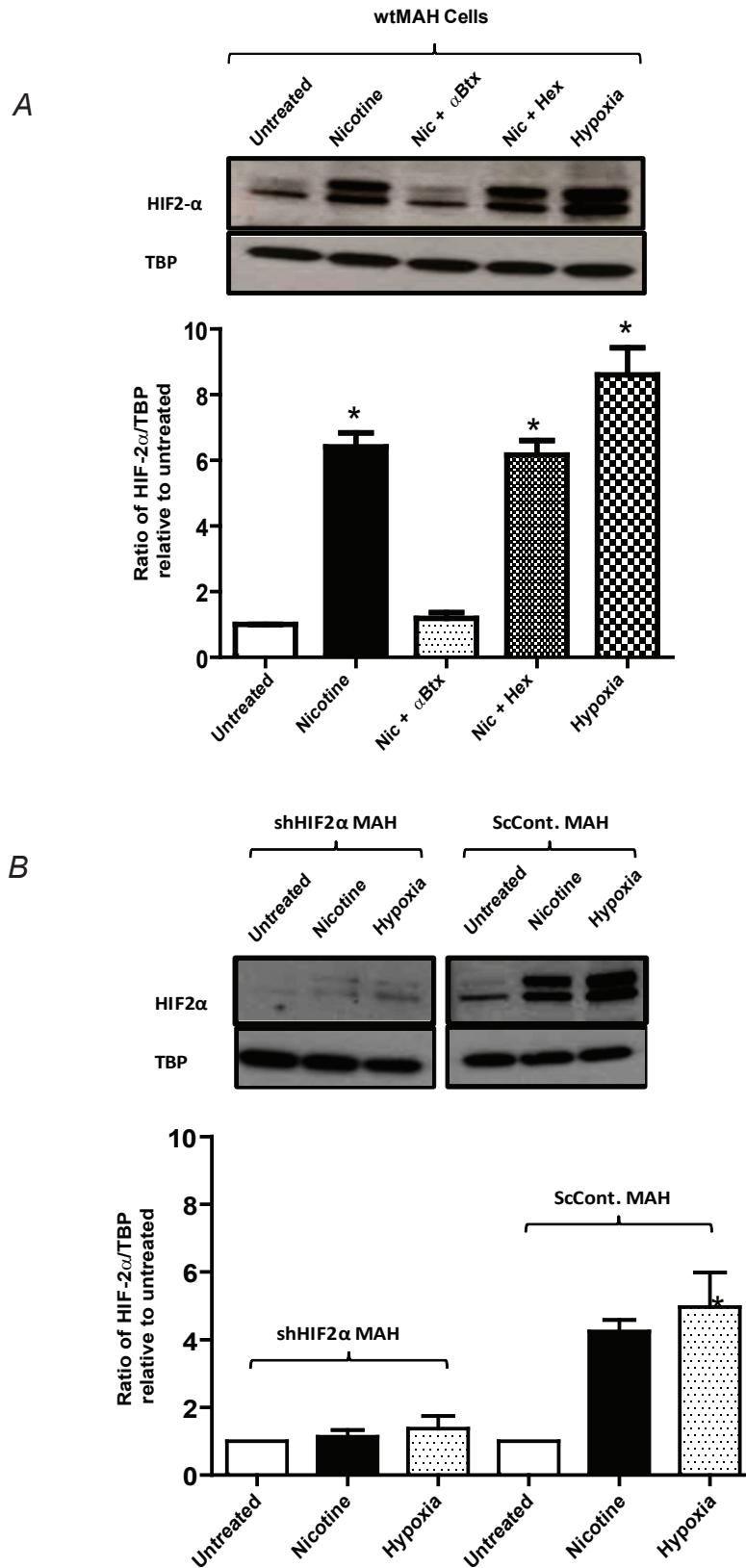
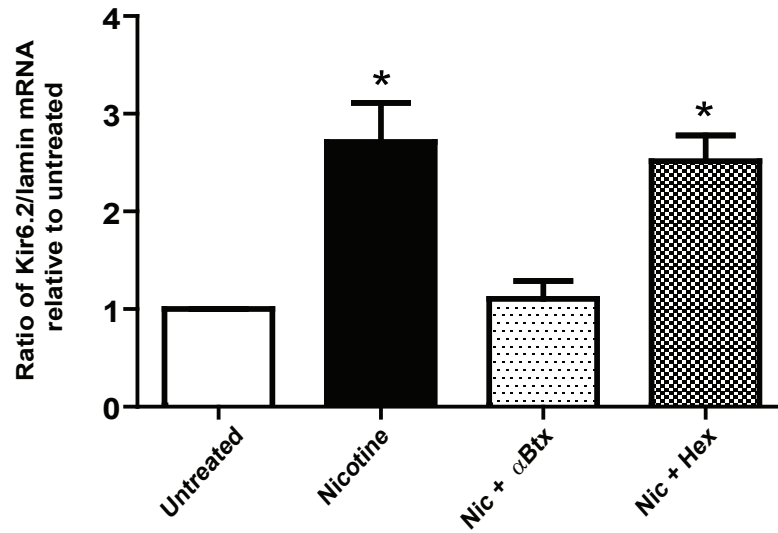


Figure 4. Effects of chronic nicotine \pm nicotinic blockers on Kir6.2 and VEGF mRNA expression in MAH cells. A) Quantitative RT-PCR analysis of Kir6.2 mRNA levels in wild-type MAH cells (wtMAH) exposed to either nicotine (Nic; 50 μ M), nicotine plus α -bungarotoxin (α Btx; 100 nM), or nicotine plus hexamethonium (Hex; 100 μ M), for 7 days in culture. Collected data were normalized to Lamin A/C and calibrated to control (untreated MAH cells) and plotted as relative fold change of mean \pm SEM (n = 3; Anova; *, P < 0.05). B) Similar analysis for VEGF mRNA expression in control and HIF-2 α -deficient (shHIF2 α) MAH cells exposed to either nicotine, nicotine plus α -bungarotoxin (α Btx; 100 nM), nicotine plus hexamethonium (Hex; 100 μ M) for 7 days, or chronic hypoxia (Hox; 2% O₂, 24 hrs). Histogram summarizes quantitative RT-PCR data for VEGF mRNA expression normalized to Lamin A/C and represented as relative to control. Data are presented as mean \pm SEM (* P < 0.05) for three independent experiments; note VEGF mRNA was not upregulated in HIF-2 α -deficient cells.

FIGURE 4

A



B

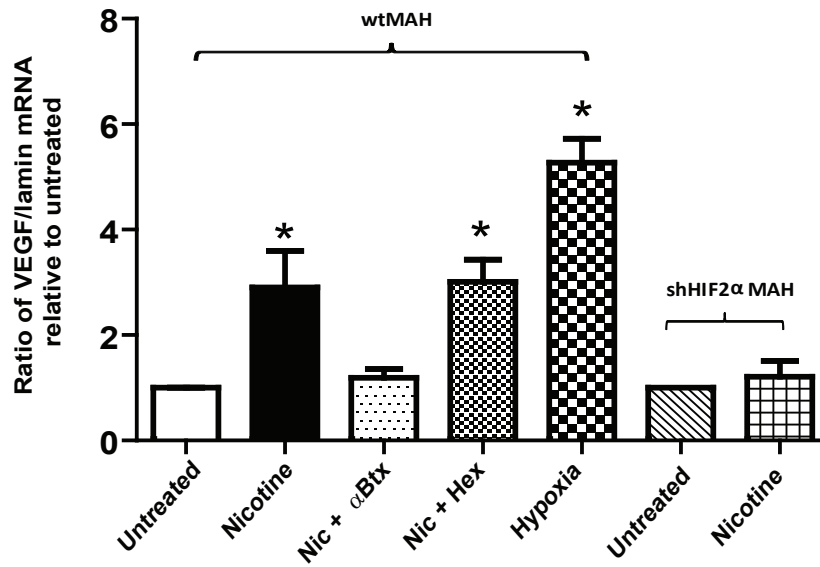
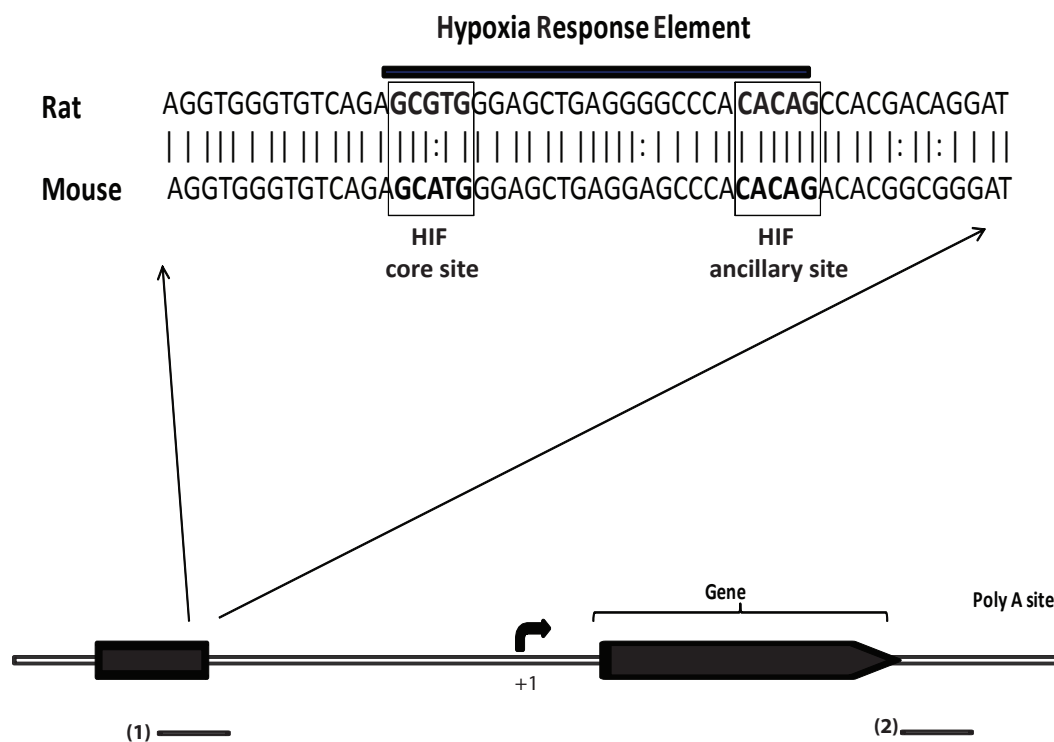


Figure 5. Hypoxia Response Element in Kir6.2 gene and chromatin immunoprecipitation (ChIP) assay. A) Putative HRE within promoter region of Kir6.2 gene. B) Lysates from untreated control (Untr) and nicotine (Nic) -treated wild type (wt), shHIF2 α , and ScCont MAH cells were subjected to ChIP assay using a HIF-2 α polyclonal antibody. PCR analysis was performed using a primer pair designed to span the putative HRE (experimental primer) or downstream exon (negative primer). Controls include a ChIP performed using non-specific IgG monoclonal antibody (IgG) and a starting material control (Input). The gel shown is representative of three independent experiments.

FIGURE 5

A



B

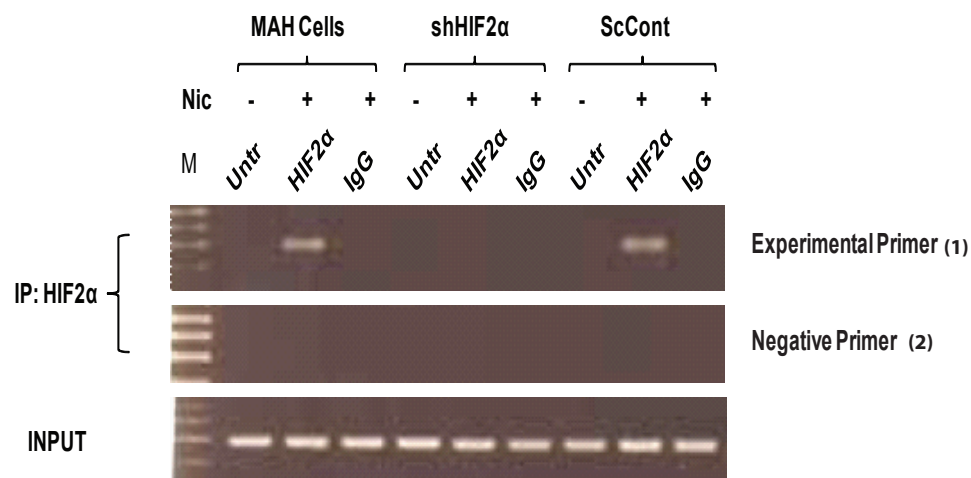
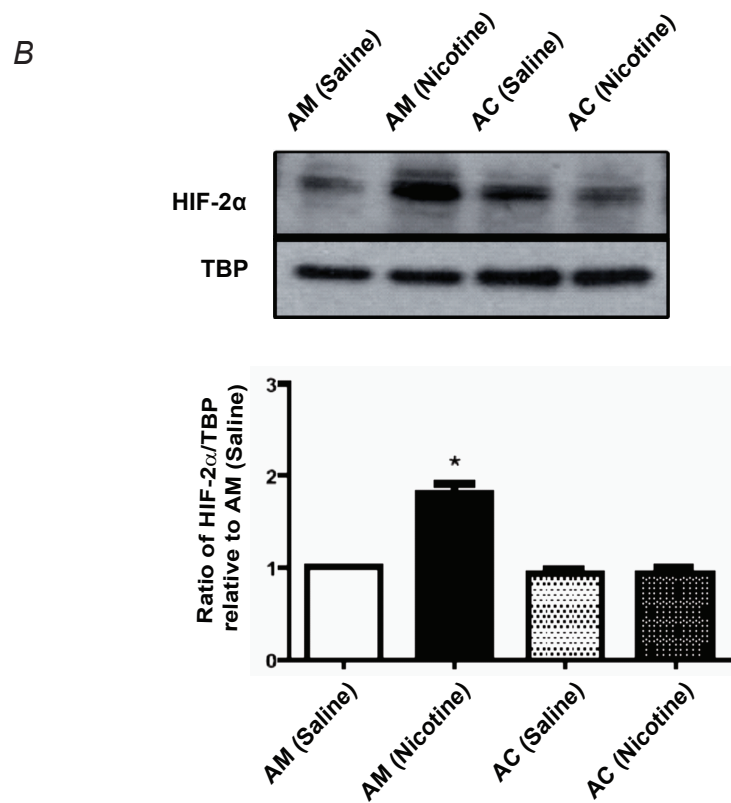
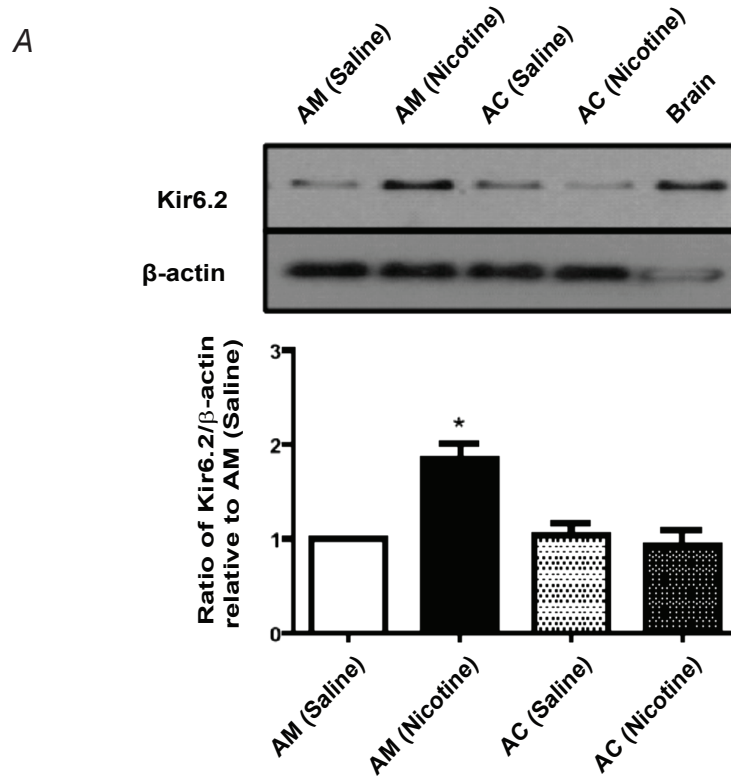


Figure 6. Effects of *in utero* chronic nicotine exposure on the K_{ATP} channel subunit (Kir6.2) and HIF-2 α expression in neonatal adrenal gland tissues. Western blot analyses of K_{ATP} channel subunit, Kir6.2, expression (A) and HIF-2 α expression (B) in adrenal medulla (AM) and adrenal cortex (AC) of saline- and nicotine-exposed pups. Note increased expression of Kir6.2 subunit in AM but not AC of nicotine exposed pups. Densitometric quantitation (*bottom*) of relative expression data normalized to loading control, i.e., β -actin (in the case of Kir6.2) or TBP (in the case of HIF-2 α) and plotted as means \pm SE compared with control [AM (saline)] of 3 independent experiments (ANOVA; **P* 0.05).

FIGURE 6



CHAPTER 3: CHRONIC EXPOSURE OF NEONATAL RAT ADRENOMEDULLARY CHROMAFFIN CELLS TO OPIOIDS *IN VITRO* BLUNTS BOTH HYPOXIA AND HYPERCAPNIA CHEMOSENSITIVITY

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Shaima Salman*, Josef Buttigieg*, Min Zhang, and Colin A. Nurse (2013).
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I performed the molecular and immunocytochemical experiments and assisted with some of the electrophysiology data acquisition and analysis. Josef Buttigieg performed the initial electrophysiology experiments. Min Zhang was involved in data acquisition for some of the electrophysiology experiments. *Both authors contributed equally to this study.

Abstract

At birth, rat adrenomedullary chromaffin cells (AMCs) respond directly to asphyxial stressors such as hypoxia and hypercapnia by triggering catecholamine secretion, which is critical for proper transition to extrauterine life. These non-neurogenic responses are suppressed postnatally in parallel with the development of splanchnic innervation, and reappear following denervation of the adult adrenal gland. To test whether neural factors released from the splanchnic nerve may regulate AMC chemosensitivity, we previously showed that nicotinic agonists *in utero* and *in vitro* suppressed hypoxia, but *not* hypercapnia, sensitivity. Here, we considered the potential role of opiate peptides which are also released from the splanchnic nerve and act via postsynaptic μ -, δ -, κ - opioid receptors. Treatment of neonatal rat AMC cultures for ~ 1week with μ - and/or δ - (but not κ) opioid agonists (2 μ M) led to a marked suppression of both hypoxia and hypercapnia sensitivity, as measured by K^+ current inhibition and membrane depolarization; co-incubation with naloxone prevented the effects of combined opioids. The suppression of hypoxia sensitivity was attributable to upregulation of K_{ATP} current density and the K_{ATP} channel subunit Kir6.2, and was reversed by the K_{ATP} channel blocker, glibenclamide. By contrast, suppression of hypercapnia sensitivity was associated with down-regulation of two key mediators of CO_2 sensing, i.e. carbonic anhydrase (CA) I and II. Collectively, these studies point to a novel role for opioid receptor signalling in the developmental regulation of chromaffin cell chemosensitivity, and suggest that prenatal exposure to opioid drugs could lead to impaired arousal responses in the neonate.

Introduction

Catecholamine (CAT) secretion from adrenomedullary chromaffin cells (AMCs) is a crucial physiological response during the transition of the fetus to extrauterine life. Early in development of several mammalian species, and prior to innervation of the adrenal medulla by the splanchnic nerve, neonatal AMCs release CAT in response to asphyxial stressors including hypoxia (low PO₂) and hypercapnia (high PCO₂) experienced during the birthing process (Lagercrantz & Slotkin, 1986; Thompson *et al.*, 1997; Munoz-Cabello *et al.*, 2005; Nurse *et al.*, 2009; Livermore *et al.*, 2011). CAT secretion from AMCs is required for the regulation of cardiac conduction and in the preparation of the lungs for air breathing (Lagercrantz & Bistoletti, 1977; Jones, 1980; Seidler & Slotkin, 1985; Lagercrantz & Slotkin, 1986). In general, these non-neurogenic responses to asphyxial stimuli are suppressed postnatally along a time course that parallels the development of splanchnic innervation (Seidler & Slotkin, 1985, 1986; Cheung, 1990; Thompson *et al.*, 1997; Garcia-Fernandez *et al.*, 2007; Nurse *et al.*, 2009). Hence, juvenile AMCs show markedly reduced sensitivity to asphyxial stimuli (Slotkin & Seidler, 1988; Thompson *et al.*, 1997; Munoz-Cabello *et al.*, 2005; Rico, 2005; Garcia-Fernandez *et al.*, 2007), and, moreover, adrenal denervation in the adult results in the restoration of direct hypoxia chemosensitivity (Levitsky & Lopez-Barneo, 2009).

Taken together, the above data are consistent with the hypothesis that factors released from splanchnic nerve terminals following innervation may contribute to the suppression of asphyxial sensitivity in developing AMCs, via their action at postsynaptic receptors. In one recent test of this hypothesis, we focused on the well-known preganglionic cholinergic innervation of adrenal chromaffin cells. Indeed, we found that chronic exposure to nicotinic ACh receptor agonists (i.e. nicotine) *in utero* and *in vitro* resulted in a selective blunting of hypoxia sensitivity in neonatal

AMCs (Buttigieg *et al.*, 2007; Buttigieg *et al.*, 2008). However, the sensitivity to isohydric hypercapnia (10% CO₂; pH =7.4) remained intact in these preparations (Buttigieg *et al.*, 2007), suggesting that other neural factors released from the splanchnic nerve may also be involved. In addition to ACh, splanchnic nerve terminals release a number of neurotransmitters or neuromodulators including opiate peptides, pituitary adenylate cyclase activating peptide (PACAP), and histamine that act on postsynaptic receptors expressed on AMCs (Kobayashi *et al.*, 1985; Holgert *et al.*, 1998; Kuri *et al.*, 2009). Therefore, any one or more of these agents could also be involved, and particularly in the developmental regulation of hypercapnia sensitivity in AMC.

In the present study, we examined the potential role of opioid receptor signalling in the developmental regulation of chemosensitivity in AMC, which are known to express μ -, δ - , κ - opioid receptors (Kimura *et al.*, 1988; Wittert *et al.*, 1996; Keating *et al.*, 2004). To facilitate these studies we used an *in vitro* model of dissociated neonatal rat AMC exposed for ~1 week to selective opioid agonists, either singly or in combination, and in the presence or absence of the general opioid receptor blocker, naloxone. To characterize AMC sensitivity to hypoxia and isohydric hypercapnia, we used perforated-patch whole -cell recording to monitor whole-cell K⁺ currents and membrane potential (Thompson *et al.*, 1997; Buttigieg *et al.*, 2007; Buttigieg *et al.*, 2008). Interestingly, we found that exposure to μ - and δ - opioid agonists led to a suppression of both hypercapnia and hypoxia sensitivity in AMC, and these effects were prevented during co-incubation of naloxone. To probe at potential underlying molecular mechanisms, we further investigated components in the signal transduction pathway previously known to regulate hypoxia and hypercapnia sensitivity in these cells, i.e. the expression of ATP-sensitive K⁺

channels (Buttigieg *et al.*, 2008), and carbonic anhydrase (CA) isoforms I and II (Munoz-Cabello *et al.*, 2005), respectively.

MATERIALS AND METHODS

Ethical Approval: All procedures for animal handling and tissue dissections were carried out according to the guidelines of the Canadian Council on Animal Care (CCAC) and institutional guidelines. The authors have read, and the experiments comply with, the policies and regulations of *The Journal of Physiology* given by (Drummond, 2009).

Cell culture: Soon after birth, neonatal rat pups [postnatal day 0 P0] (Wistar, Charles River, Quebec, Canada), were first rendered unconscious by a blow to the back of the head and then killed immediately by decapitation. Adrenal glands were excised bilaterally and most of the surrounding cortex was trimmed away and discarded. Cultures of primary adrenomedullary chromaffin cells (AMCs) were prepared using a combination of enzymatic and mechanical dissociation of the medullary-rich tissue as previously described (Thompson *et al.*, 1997; Buttigieg *et al.*, 2008). Dissociated cells were plated on 35 mm culture dishes coated with Matrigel (Collaborative Research, Bedford, MA, USA) and grown in DMEM-F12 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and various additives as described previously (Thompson *et al.*, 1997). Cultures were incubated in a humidified atmosphere of 95% air and 5% CO₂ at 37°C for ~1 week in the presence or absence of μ -, δ -, and/or κ - opioid agonists (2 μ M; see below); the general opioid receptor blocker naloxone (2 μ M) was present in some experiments. The medium was changed every 2 days and fresh drugs were added at that time in the case of opioid-treated cultures.

Electrophysiology: The nystatin perforated-patch clamp technique was used for electrophysiological studies on whole-cell currents and membrane potentials in isolated AMCs as previously described (Thompson & Nurse, 1998; Thompson, 2002; Buttigieg *et al.*, 2007; Thompson *et al.*, 2007; Buttigieg *et al.*, 2008). The pipette solution consisted of (mM): K

gluconate, 115; KCl, 25; NaCl, 5; CaCl₂, 1; Hepes, 10 at pH 7.2, and nystatin (300-450 µg ml⁻¹). Experiments were carried out at 37°C in HCO₃⁻-buffered extracellular solution containing (mM): NaCl, 115; KCl, 5; CaCl₂, 2; MgCl₂, 2; glucose, 10; and NaHCO₃, 24, bubbled with 5% CO₂ at pH 7.4. Hypoxic solutions (PO₂ ~15 mmHg) were generated by bubbling N₂ gas; in the case of hypercapnic solutions (10% CO₂) the bicarbonate concentration was increased to 48 mM (equimolar substitution with NaCl) so as to maintain the pH at 7.4. Tetrodotoxin (TTX; 0.5 µM) was present in some experiments. The extracellular solution was applied to the cells by gravity flow or via a rapid perfusion system (Thompson *et al.*, 1997; Zhang & Nurse, 2004). In some experiments, cells were held at a holding potential of -60 mV and currents recorded during voltage steps to +70 mV; in other experiments the voltage was ramped from -120 to +80 mV over a period of 500 msec at a frequency of 0.1 Hz, followed by voltage steps to +30 and +60 mV from a holding potential of -60 mV. Membrane potentials were monitored in current clamp mode (I=0). Results are presented as mean ± SEM. Current density (pA/pF), obtained by dividing the whole-cell current (pA) by cell capacitance (pF), and membrane potentials (mV) were compared using paired or independent (Mann-Whitney) Student's t tests with significance set at $P < 0.05$. (Microcal Origin version 7.5).

Western Immunoblot analysis: Cells were homogenized and lysed in Buffer A solution containing: 10 mM HEPES pH 7.6, 10 mM KCl, 0.1 mM EDTA pH 8, 0.1 mM EGTA pH8, 1 mM DTT and protein inhibitors (Complete Mini, Roche, Laval, QC, Canada), 1 mM PMSF, 5 mg/mL Aprotinin, 5 mg/mL Leupeptin). Protein samples were boiled at 95-100°C for 5 minutes. 10µg of protein samples, measured using the Bradford assay (1:5 dilution reagent and 1 mg/mL BSA), were loaded and resolved on 10% SDS-PAGE gel and transferred onto PVDF membranes. Membranes were then washed and incubated with either primary rabbit polyclonal

antibody against Kir6.2 (1:1000 dilution; Alomone Labs Ltd.), rabbit polyclonal anti-human carbonic anhydrase I antibody (1:1000 dilution; abcam), sheep polyclonal anti-human carbonic anhydrase II antibody (1:1000 dilution; AbD Serotec), or primary rabbit monoclonal [β -actin antibody (1:10,000 dilution; Millipore) (loading control) at 4°C overnight. Membranes were then washed in phosphate-buffered saline (PBS) and incubated in a goat anti-rabbit horseradish peroxidase (HRP)-linked secondary antibody (1:10,000 dilution; Jackson Labs, Bar Harbor, ME, USA) for 1 hr at room temperature. Immunoreactions were visualized using ECL and exposed to XAR-film.

Immunofluorescence: Cultures of neonatal rat AMCs were grown on glass cover slips attached to the underside of central wells of modified Nunc 35 mm dishes (Buttigieg *et al.*, 2008). Medium was aspirated and cells were gently rinsed with pre-warmed PBS at pH 7.2, and quickly fixed in 2 ml of ice cold 4% paraformaldehyde in PBS for 1 hr at 4°C. Cells were then washed 3 times with PBS for 3 min and incubated with 100 μ l of primary antibodies (rabbit polyclonal anti- μ -opioid receptor; rabbit polyclonal anti- δ -opioid receptor; Alomone labs, Jerusalem) diluted in 1% BSA/PBS overnight. Samples were washed the next day with PBS 3 times (10 min each) and incubated with FITC-conjugated secondary antibody (1:50; Jackson ImmunoResearch) for 1 hr at room temperature in the dark. Cells were then washed three times with PBS (10 min each) and covered with Vectashield to avoid photobleaching. Pre-adsorption controls were performed by incubating primary antibody overnight at 4°C in the presence of 3x excess antigen (1 μ g of antibody incubated with 3 μ g of antigen). Cells were visualized and imaged using a Zeiss inverted microscope (IM 35).

Drugs: All drugs including TTX, were purchased from Sigma-Aldrich. Fresh opioid drugs were applied in medium every 2 days at a concentration of 2 μ M. The following opioid drugs were

used for chronic treatments: μ -opioid agonist DALDA ([D-Arg², Ly⁴]-dermorphin-(1-4)-amide); δ -opioid agonist DPDPE ([D-Pen², 5, P-Cl-Phe⁴]-enkephalin); κ -opioid agonist U-62066 ((\pm)-(5 α ,7 α ,8 β)-3,4-Dichloro-N-methyl-N-[7-(1-pyrrolidinyl)-1-oxaspiro[4.5]dec-8-yl] benzeneacetamidemesylate salt). Naloxone hydrochloride dehydrate (2 μ M) was used as a general opioid antagonist.

Statistical analysis: Data are expressed as means \pm SEM and statistical analyses were carried out using non-parametric test (Mann-Whitney U) and ANOVA as appropriate. Differences were considered significant if $P < 0.05$.

RESULTS

For studies on opioid-treated cells, we incubated AMC cultures with the selective μ -, δ - , κ - opioid receptor agonists, i.e. DALDA, DPDPE, and U-62066 respectively, separately or together at a concentration of 2 μ M for ~1 week. This concentration is similar to that used in previous studies (Keating *et al.*, 2004) and is likely to be in the physiological range as the opiate concentration near single chromaffin cells has been estimated to be in the micromolar range (Castanas *et al.*, 1985a, 1985b). Also, a concentration of 2 μ M is more than 100 times greater than the binding affinity for these agonists at their respective receptors (Cheng *et al.*, 1992; Schlosser *et al.*, 1995; Keating *et al.*, 2004). Under phase contrast microscopy, the appearance of neonatal rat AMC cultures grown for ~1 week in the presence of opioid receptor agonists, was indistinguishable from that of control sister cultures (not shown). Similarly, in electrophysiological studies the mean resting potential of control untreated cells was (-60.7 ± 1.2 mV; $n = 12$), a value not significantly different from that of opioid-treated cells (-64.4 ± 3.5 mV; $n = 10$; $p < 0.05$), after 1 week in culture.

Electrophysiological studies on the effects of chronic opioid exposure on hypoxic sensitivity in neonatal rat AMCs

In late-fetal and neonatal rat AMCs, hypoxia inhibits a variety of O₂-sensitive K⁺ channels (SK, BK, and K_v channels), leading to or facilitating membrane depolarization, voltage-gated Ca²⁺ entry and catecholamine (CAT) release (Mochizuki-Oda *et al.*, 1997; Thompson *et al.*, 1997; Thompson & Nurse, 1998; Bournaud *et al.*, 2007; Garcia-Fernandez *et al.*, 2007; Nurse *et al.*, 2009). In the first experimental series we used perforated-patch, whole-cell recording to investigate the effects of μ -, δ - , κ - opioid agonists (DALDA, DPDPE, and U-62066; 2 μ M),

present individually or in combination, on hypoxic sensitivity of AMC cultured for 7 days. In control cultures, approximately 81% ($n = 9/11$) of AMCs showed the expected reversible inhibition of outward K^+ current at positive potentials under voltage clamp (Fig. 1A; upper traces and I-V plot), and membrane depolarization under current clamp (Fig. 1A; lower trace), during acute hypoxia ($PO_2 \sim 15$ mmHg); the mean depolarization during hypoxia was 4.5 ± 0.80 mV ($n = 10$). In contrast, AMCs from sister cultures chronically exposed for 7 days to combined μ -, δ -, and κ -opioid agonists ($2 \mu\text{M}$) showed a marked suppression of hypoxic sensitivity, reflected by the relative lack of effect of hypoxia on outward K^+ current during depolarizing voltage steps (Fig. 1B; upper traces, and I-V plot). In these experiments only $\sim 18\%$ ($n = 2/11$) opioid-treated cells tested appeared to show a detectable hypoxia-evoked inhibition of K^+ current at positive potentials. Also, as exemplified in Fig. 1B (lower trace), in current clamp studies hypoxia had no detectable effect on membrane potential of opioid-treated cells ($n = 12$). The blunting effects of combined agonist exposure were mediated via the opioid receptor signalling pathway because they could be prevented by co-incubation with the general opioid receptor antagonist, naloxone ($2 \mu\text{M}$), over the entire treatment period (Fig. 1C); naloxone had no significant effect on whole cell currents or membrane potential when present alone ($n = 12$).

We next investigated whether the blunting of hypoxic sensitivity in opioid-treated AMCs was linked to specific subtypes of opioid receptors. Following chronic exposure to either μ - (DALDA; $2 \mu\text{M}$) or δ - (DPDPE; $2 \mu\text{M}$) opioid agonist alone, hypoxic sensitivity in neonatal AMCs was almost completely abolished as indicated by the relative lack of effect of acute hypoxia on whole-cell K^+ currents and membrane potential (Fig. 1D,E). These blunting effects, however, were not observed when neonatal AMCs were chronically exposed to the κ -opioid agonist (U-62066; $2 \mu\text{M}$) alone (Fig.1F). These findings suggest that chronic exposure to μ -

and/or δ -, but not κ -, opioid receptor agonists renders neonatal AMCs relatively insensitive to acute hypoxia.

Role of ATP-sensitive K⁺ (K_{ATP}) channels in mediating the blunting effects of chronic opioids on hypoxic sensitivity in neonatal AMCs

The blunting effect of chronic opioids on hypoxic sensitivity could occur at the level of the O₂ sensor or at downstream steps in the signal transduction pathway. We previously showed that hypoxic sensitivity in neonatal AMCs is also blunted following exposure to chronic nicotine, and this was attributable to up-regulation of K_{ATP} channel expression (Buttigieg *et al.*, 2007; Buttigieg *et al.*, 2008). These K_{ATP} channels open during hypoxia favoring membrane hyperpolarization which counteracts the depolarizing effects of hypoxia on membrane potential, arising from inhibition of other K⁺ (e.g. SK, BK, and K_V) channels (Buttigieg *et al.*, 2008; Nurse *et al.*, 2009). To test whether or not increased K_{ATP} channel activity plays a similar role in opioid-treated cells, we added the K_{ATP} channel blocker, glibenclamide (Glib; 50 μ M) to the recording solution. Consistent with our previous studies (Thompson & Nurse, 1998; Buttigieg *et al.*, 2008), the hypoxic suppression of outward K⁺ current in control (untreated) neonatal AMCs after 7 days *in vitro* was augmented in the presence of glibenclamide as exemplified in Fig. 2A (upper traces). Current density versus voltage plot for a group of 6 cells showing the enhancing effects of glibenclamide on the hypoxic inhibition of outward K⁺ current at positive potentials is shown in Fig. 2A (lower). Interestingly, in the case of opioid-treated cells cultured for 7 days, addition of glibenclamide to the recording solution resulted in the unmasking of hypoxic sensitivity in cells that initially failed to show hypoxic inhibition of outward K⁺ current (e.g. Fig. 2B, upper traces). Pooled data from a group of 7 opioid-treated cells, exposed sequentially to

hypoxia, glibenclamide, hypoxia plus glibenclamide, and wash are shown in the lower I-V plot (Fig. 2B; lower); note the significant enhancing effect of glibenclamide on the hypoxic inhibition of outward current at more positive potentials. These data are consistent with the hypothesis that the O₂-sensor itself is functionally intact in opioid-treated AMCs and that the blunting effect of chronic opioids on hypoxia sensitivity is attributable to increased K_{ATP} channel activity.

To address whether the increased K_{ATP} channel activity after chronic opioids is effective in blunting the hypoxia-induced receptor potential in neonatal AMCs, we tested the effects of glibenclamide under current clamp conditions. As exemplified in Figs. 2C1 and C2 respectively, control (untreated) cells that initially responded to hypoxia with bursts of action potentials appeared to show increased hypoxia-induced spike activity superimposed on a more depolarized membrane potential after glibenclamide (cf. Figs. 2Ca, Cb; n = 10) (see also, (Buttigieg *et al.*, 2008). On the other hand, many opioid-treated cells that initially failed to show hypoxia-induced membrane depolarization responded with positive shifts in membrane potential when glibenclamide was present in the bathing solution. Among these opioid-treated cells we identified several categories: (i) cells which showed no obvious response to hypoxia initially but responded to hypoxia plus glibenclamide with a burst of action potentials (Fig. 2Da,Db; n = 5); (ii) cells which showed no obvious response to hypoxia initially but responded to hypoxia plus glibenclamide with a subthreshold membrane depolarization (Fig. 2Ea,Eb; mean depolarization = 5.8 ± 1.06 mV, n= 7); and (iii) cells which even showed a slight hyperpolarization to hypoxia initially but responded to hypoxia plus glibenclamide with a positive shift in membrane potential (Fig. 2Fa,Fb; n = 3). These categories likely reflect cell groups with different expression levels of K_{ATP} channels relative to the other O₂-sensitive K⁺ channels that are inhibited by hypoxia.

Chronic opioids upregulate expression of Kir6.2 subunit of the K_{ATP} channel

One possible explanation for the enhancement of hypoxic inhibition of K⁺ current and the decreased hypoxia-sensitivity in opioid-treated AMCs exposed to glibenclamide (see Fig. 2) is an increased expression of functional K_{ATP} channels. To obtain a quantitative estimate of the increased contribution of K_{ATP} channel currents in control vs opioid-treated cells we calculated the glibenclamide-sensitive difference current density under hypoxia, using data similar to those in Figs. 2A,B. The resulting estimate of I_{K_{ATP}} current density (pA/pF) was plotted against voltage (mV) over the voltage range -120 to +80 mV as shown in Fig. 3A (n =10). As expected, the I_{K_{ATP}} currents reversed near the K⁺ equilibrium potential (~ -84 mV) and was significantly larger in opioid-treated cells; this was especially noticeable at positive membrane potentials as the driving force on K⁺ increased. For a voltage step to +30 mV (from a holding potential of -60 mV) the glibenclamide-sensitive difference current density increased from 11.5 ± 1.85 (n =11) pA/pF in control to 21.6 ± 3.03 pA/pF in opioid-treated cells (n = 15; p<0.05; Fig. 3B). To address whether this increase was attributable to increased expression of K_{ATP} channels at the protein level, we used western blot analysis on extracts from 7 day-old control and opioid-treated AMC cultures. As indicated in Fig. 3C, the expression level of the channel pore-forming subunit Kir6.2 (relative to β-actin) increased ~7 fold following opioid exposure. These data suggest that chronic opioids cause an increased expression of K_{ATP} channel proteins in neonatal AMCs during 1 week of exposure.

Chronic opioid exposure blunts CO₂ chemosensitivity in neonatal AMCs

Sensitivity of neonatal rat AMCs to hypercapnia (10% CO₂) also decreases postnatally in parallel with splanchnic innervation, and is mediated by inhibition of outward K⁺ current, membrane

depolarization, voltage-gated Ca^{2+} entry and catecholamine secretion (Munoz-Cabello *et al.*, 2005; Buttigieg *et al.*, 2007; Buttigieg *et al.*, 2008) To address whether or not hypercapnic sensitivity is also regulated by chronic opioid exposure we monitored membrane currents and membrane potential in AMCs exposed high CO_2 (10% CO_2) under isohydric conditions (pH = 7.4). In control conditions, isohydric hypercapnia caused inhibition of outward K^+ current under voltage clamp (Fig. 4A; upper trace), and induced a depolarizing receptor potential under current clamp (Fig. 4A; lower trace) in several neonatal AMCs cultured for 7 days. For this experimental series, pooled data from a group of cells (n =11) examined under voltage clamp are summarized in the I-V plot in Fig. 4A, where isohydric hypercapnia caused a significant suppression of outward K^+ current at positive potentials. By contrast, exposure to combined μ -, δ - , κ - opioid agonists (DALDA, DPDPE, and U-62066; 2 μM) for 7 days resulted in a marked reduction or blunting of CO_2 sensitivity as illustrated in the exemplar traces in Fig. 4B (upper and lower trace), and in the I- V plot of Fig. 4B (middle). Moreover, continuous co-incubation of these combined agonists with the general opioid receptor blocker, naloxone (2 μM), resulted in the retention of CO_2 sensitivity (Fig. 4C). As was the case for opioid-mediated blunting of hypoxic sensitivity, the blunting of CO_2 sensitivity involved mainly μ - and δ - opioid receptors. For example, as illustrated in Fig. 4 D-F, incubation for 7 days with either the μ - or δ - opioid receptor agonist alone resulted in a marked suppression of CO_2 sensitivity (Figs. 4D,E), whereas incubation with the κ - opioid agonist alone was ineffective (Fig. 4F).

Whereas inhibition of outward K^+ current is thought to contribute to CO_2 sensitivity of neonatal AMC via prolongation of the action potential duration, the origin of the receptor potential or initial depolarization at rest is attributable to activation of a cation conductance (Munoz-Cabello *et al.*, 2005). To examine whether this component of the CO_2 sensing pathway

was also impaired in opioid-treated cells we first monitored the receptor potential in control versus opioid-treated cells exposed briefly to isohydric hypercapnia (10% CO₂ ; pH =7.4). As exemplified in Figs. 4A,B (lower traces) and Fig. (5Ba,Bb), whereas hypercapnia readily induced a subthreshold membrane depolarization (mean = 5.5 ± 0.25 mV; n = 4) or action potential firing (n = 12) in the majority (~80%) of control cells, opioid-treated cells were largely (Fig. 4B, lower trace; Fig. 5Ca), though not exclusively (Fig. 5Cb), unresponsive (>80% non-responsive cells). Quantification of the relative proportions of responsive versus non-responsive cells in the two conditions is summarized in Fig. 5D for this experimental series. The blunting effect of opioids on hypercapnia sensitivity was prevented during continuous co-incubation with 2 μ M naloxone (Fig. 4C; lower trace; n= 12) and, as in the case of hypoxia (see above), involved mainly μ - and δ - (but not κ -) opioid receptors (Figs. 4D-F).

We also compared changes in holding current in control versus opioid-treated cells during a switch from normocapnic (5% CO₂) to hypercapnic (10% CO₂) solutions at constant pH. As indicated in Fig. 5Aa, and consistent with a previous report (Munoz-Cabello *et al.*, 2005), under voltage clamp there was a significant inward shift in the holding current (at -60 mV) when control AMCs were exposed to hypercapnia, and the effect was readily reversible. By contrast, under similar conditions hypercapnia failed to induce a significant shift in the holding current in opioid-treated cells, consistent with the current clamp data showing suppression or loss of hypercapnia-induced membrane depolarization at the resting potential (Fig. 4B,E, lower traces; Fig. 5Ca).

Chronic opioid exposure reduces expression of carbonic anhydrase isoforms I and II in neonatal AMCs

Tests for the presence of 7 different carbonic anhydrase (CA) isoforms using RT-PCR and *in situ* hybridization revealed that only CAI and CAII mRNA were expressed in the neonatal rat adrenal gland and, interestingly, expression of both isoforms was virtually absent (>1000 fold lower) in the adult gland (Munoz-Cabello *et al.*, 2005). Moreover, in electrophysiological studies the hypercapnic response of neonatal AMC was markedly inhibited by the CA inhibitor, methazolamide, leading these authors to conclude that the CO₂ sensitivity of these cells was at least partially dependent on high expression levels of the CO₂-hydrating enzymes CAI and CAII (Munoz-Cabello *et al.*, 2005). These considerations led us to examine whether or not the opioid-induced suppression of CO₂ sensitivity in cultured neonatal AMCs was associated with reduced expression of CAI and CAII at the protein level. Indeed, using western blot analysis with β -actin as a control, we found that expression of both CAI and CAII proteins were significantly reduced in opioid-treated neonatal AMCs compared to control cells after 7 days *in vitro*. Exemplar gels of CAI and CAII expression patterns for control versus opioid-treated cells and summary data from triplicate experiments are illustrated in Figs. 6A,B.

Immunocytochemical detection of μ - and δ -opioid receptors on cultured neonatal rat chromaffin cells

Based on gene expression studies using RT-PCR analysis, the three major opioid receptors, μ , δ and κ , have been identified in *adult* rat adrenal gland (Wittert *et al.*, 1996). To corroborate the above electrophysiological findings demonstrating key roles for μ - and δ -opioid receptor signalling in regulation of O₂ and CO₂ sensing in *neonatal* AMC we used fluorescence immunocytochemistry. As shown in Fig. 7, both μ - (MOR) and δ - (DOR) opioid receptor immunostaining was localized selectively to chromaffin cell clusters in dissociated neonatal

AMC cultures. These data suggest that the blunting effects of chronic opioid exposure on chemosensing were mediated via direct interactions with μ - or δ - opioid receptors on neonatal AMC.

DISCUSSION

In this study we demonstrate for the first time that chronic exposure of neonatal rat AMCs to μ - and/or δ -opioid receptor agonists for ~ 1 week *in vitro* leads to a naloxone-sensitive suppression of direct hypoxic and hypercapnic chemosensitivity. These results are of interest because they suggest a general mechanism that could account for the developmental loss or suppression of direct O₂ and CO₂ sensitivity in AMC that normally occurs during preganglionic splanchnic innervation (Slotkin & Seidler, 1988; Thompson *et al.*, 1997; Munoz-Cabello *et al.*, 2005). In particular, because the amount of opiate peptides (i.e. enkephalin) increases in the developing rat preganglionic splanchnic nerve in parallel with the suppression of direct hypoxic chemosensitivity (Holgert *et al.*, 1998; Nurse *et al.*, 2009), the possibility is raised that activation of postsynaptic receptors on AMC by released opiate peptides is involved. Indeed, the involvement of opiate peptides in suppressing the non-neurogenic response of adrenal medulla to hypoxia has been previously considered, however, these experiments involved *acute* exposures to opioid agonists. For example, Keating *et al.* (2004) reported that hypoxia-induced catecholamine secretion from ovine AMC was inhibited in the presence of μ - and κ - opioid agonists and this was attributable to opioid-mediated enhancement of a K⁺ conductance (SK) and/or inhibition of voltage-gated Ca²⁺ channels. By contrast, similar studies in the rat failed to show any effect of opioid agonists on hypoxia-induced catecholamine secretion from neonatal AMC (Rico, 2005). While the difference between these two studies may be species related, it should be noted that in the present study opioid agonist exposure lasted for ~ 1 week *in vitro* and, importantly, opioid agonists were *not* present in the bathing solution when chemosensitivity was tested. Chronic exposure of neonatal AMC to opioids is more likely to mimic the developmental effects of splanchnic innervation given that the latter matures gradually over the first 1-2

postnatal weeks in the rat (Seidler & Slotkin, 1985). Moreover, our finding that both hypoxic and hypercapnic sensitivity are suppressed in neonatal AMC after chronic opioid exposure *in vitro* closely mimics the *in vivo* developmental pattern seen in these cells, when examined acutely before and after splanchnic innervation (Slotkin & Seidler, 1988; Thompson *et al.*, 1997; Munoz-Cabello *et al.*, 2005). It is noteworthy that the splanchnic nerve also supplies an important nicotinic cholinergic innervation to the adrenal gland; however, in our previous studies chronic exposure of neonatal AMC to nicotine *in utero* and *in vitro* led to a selective blunting of hypoxic, but *not* hypercapnic, sensitivity (Buttigieg *et al.*, 2007; Buttigieg *et al.*, 2008). It therefore appears that the opioid receptor signalling pathway contributes to the developmental regulation of both hypoxic and hypercapnic sensitivity in AMC, whereas the nicotinic receptor pathway contributes only to the regulation of hypoxic sensitivity.

Mechanisms underlying opioid-mediated suppression of hypoxic chemosensitivity

Hypoxic sensitivity of neonatal AMC involves regulation of various subtypes of K⁺ channels, leading to membrane depolarization, voltage-gated Ca²⁺ entry, and catecholamine secretion (reviewed in Nurse *et al.* 2009). Both small (SK) and large (BK) conductance K⁺, as well as voltage dependent K⁺ (K_V), channels are inhibited by hypoxia leading to or facilitating membrane depolarization. This depolarizing action, however, is blunted by the simultaneous activation of ATP-sensitive K⁺ (K_{ATP}) channels, which favors membrane hyperpolarization during hypoxia (Thompson & Nurse, 1998; Bournaud *et al.*, 2007). After chronic exposure to μ - and/or δ - (but not κ -) opioid agonists, at concentrations (2 μ M) greatly exceeding their K_d values but comparable to estimated opiate extracellular concentrations near AMC *in vivo* (Castanas *et al.*, 1985a, 1985b; Keating *et al.*, 2004), the normal hypoxia-induced inhibition of outward K⁺

currents at positive potentials was markedly reduced or eliminated. Moreover, the hypoxia-induced membrane depolarization or receptor potential, due mainly to inhibition of SK channels at the resting potential (Lee *et al.*, 2000; Keating *et al.*, 2005; Bournaud *et al.*, 2007), was also significantly suppressed. Dramatically, however, hypoxia-sensitivity as determined by both assays could be restored in these opioid-treated cells if the K_{ATP} channel blocker glibenclamide was present in the recording extracellular solution. In fact, some opioid-treated cells that were initially silent when exposed to acute hypoxia generated a burst of action potentials when the same stimulus was applied to the *same* cell in the presence of glibenclamide. These data suggest that upregulation of K_{ATP} channel function, which would favor membrane hyperpolarization during acute hypoxia, is a major contributor to the blunting of hypoxic sensitivity in these cells. Supportive evidence for this mechanism was obtained from electrophysiological estimates of K_{ATP} current density, determined from the glibenclamide-sensitive difference current during hypoxia, and by western blot analysis of the expression of the K_{ATP} channel subunit, Kir6.2, in control versus opioid-treated cells. Taken together, these data are reminiscent of a similar role played by K_{ATP} channel upregulation in the blunting of hypoxia sensitivity in perinatal AMC following exposure to chronic nicotine *in utero* and *in vitro* (Buttigieg *et al.*, 2008; Salman *et al.*, 2012). However, further experiments are required to elucidate the intracellular signalling pathway that leads to K_{ATP} channel upregulation during opioid exposure. It should also be noted that we cannot presently exclude the possibility that other potential targets in the O_2 chemotransduction pathway may also be modified by chronic opioid exposure. These include subtle changes in expression levels of the other O_2 -sensitive K^+ channels (i.e. SK, BK and K_v), or of T-type Ca^{2+} channels which have been reported to play a key role in the hypoxic sensitivity of neonatal AMC (Levitsky & Lopez-Barneo, 2009).

Mechanisms underlying opioid-mediated suppression of CO₂ chemosensitivity

An especially novel aspect of our study was the demonstration that chronic opioids also blunted the sensitivity of neonatal AMC to high CO₂ (hypercapnia), thereby mimicking the developmental loss or reduction in hypercapnic sensitivity following splanchnic innervation (Munoz-Cabello *et al.*, 2005). To our knowledge, this evidence is the first to suggest how innervation of AMC could lead to loss of CO₂ sensitivity. A previous study highlighted several aspects associated with hypercapnic sensitivity in neonatal rat AMC (Munoz-Cabello *et al.*, 2005). These include: (i) high CO₂-mediated activation of a resting cationic conductance leading to membrane depolarization or the receptor potential; (ii) high CO₂-mediated inhibition of voltage-dependent K⁺ channels, leading to prolongation of action potential duration and increased catecholamine secretion; and (iii) blockade of high CO₂-mediated responses by the membrane-permeable carbonic anhydrase (CA) inhibitor, methazolamide (Munoz-Cabello *et al.*, 2005). In the present study, chronic exposure to μ - and/or δ - opioid agonists markedly reduced or abolished not only the high CO₂-mediated receptor potential, but also inhibition of voltage-dependent outward K⁺ current. Moreover, because CAI and CAII are the main CA isoforms expressed in neonatal rat AMC, and both isoforms are downregulated during splanchnic innervation (Munoz-Cabello *et al.*, 2005), we compared their expression patterns in control versus opioid-treated AMC using western blot analysis. Indeed, we found that both CAI and CAII proteins were significantly downregulated in neonatal AMC exposed to chronic opioids *in vitro*. The intracellular signalling cascade leading to reduced CAI and CAII expression in opioid-treated cells remains to be elucidated. Nevertheless, these data suggest a molecular mechanism

by which opioid innervation can lead to suppression of CO₂ sensitivity in AMC during postnatal development.

CLINICAL SIGNIFICANCE

Our main finding that chronic opioid exposure can impair the ability of neonatal AMC to sense asphyxial stimuli such as hypoxia and hypercapnia has important clinical implications. The deleterious consequence is that the catecholamine surge that normally occurs in response to asphyxial stressors at birth would become seriously impaired, thereby compromising the proper transition of the neonate to extra-uterine life (Seidler & Slotkin, 1985; Slotkin & Seidler, 1988). This catecholamine release is critical for maintenance of cardiac conduction and for transformation of the lung into an air-breathing epithelium (Slotkin & Seidler, 1988). Though the use of opioid medication is widespread for pain management, opioid drugs (e.g. heroin) are major contributors to drug abuse and fatalities. In the adult, these fatalities often result from the adverse side effects of opioids, particularly the marked respiratory depression that becomes exacerbated during sleep (Walker & Farney, 2009). To date, brain sites involved in respiratory control (e.g. the preBötzing complex) have received the most attention for the targets of opioid-mediated respiratory depression in the adult (Morin-Surun *et al.*, 1992; Montandon *et al.*, 2011). In the neonate, however, hypoxic activation of catecholaminergic pathways, especially via the adrenal medulla, stimulates breathing and arousal responses that are critical for survival (Sawnani *et al.*, 2004; Cohen *et al.*, 2005). Thus, blunted adrenal catecholaminergic responses during perinatal asphyxia contributes to the failure of arousal and elevated perinatal mortality as occurs during Sudden Infant Death Syndrome (SIDS) (Sawnani *et al.*, 2004; Cohen *et al.*, 2005; Buttigieg *et al.*, 2008). In this regard, opioid intake by mothers during pregnancy has been linked

to an increased incidence of infant mortality due mainly to SIDS (Burns *et al.*, 2010). It is possible that the mechanisms underlying opioid-mediated suppression of O₂ and CO₂ sensing in neonatal chromaffin cells, as uncovered in the present study, are broadly applicable to other chemosensitive sites involved in respiratory control. If so, they could help clarify the relationship between opiate abuse during pregnancy and abnormal arousal responses associated with perinatal disorders that result in infant morbidity, e.g. SIDS.

ACKNOWLEDGEMENTS

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Figure 1: Effects of chronic exposure of neonatal rat adrenomedullary chromaffin cells (AMCs) to opioid agonists for ~1 week *in vitro* on hypoxia sensitivity. A. In control (untreated) AMCs cultured for ~1 week, hypoxia ($PO_2 \sim 15\text{mmHg}$) typically causes inhibition of outward K^+ current (top trace) and membrane depolarization (bottom trace); current density (pA/pF) versus voltage (I-V) plot (middle) shows significant inhibition of outward current ($p < 0.05$) at potentials positive to +10 mV. These responses are markedly reduced or abolished in neonatal AMCs chronically exposed for ~1 week to either a combination of μ -, δ -, and κ -opioid agonists (2 μM) (B), or to either μ - or δ -opioid agonists (2 μM) alone (D and E respectively). These blunting effects of chronic opioids on the hypoxia-induced responses are prevented during continuous co-incubation with the general opioid receptor antagonist, naloxone (2 μM ; Fig. 1C). Also, chronic exposure to the κ -opioid agonist (2 μM) alone was ineffective (F). Data are presented as mean \pm SEM ($n = 11$). TTX was present in the extracellular solution. C = control; Hox = hypoxia; W = wash.

FIGURE 1

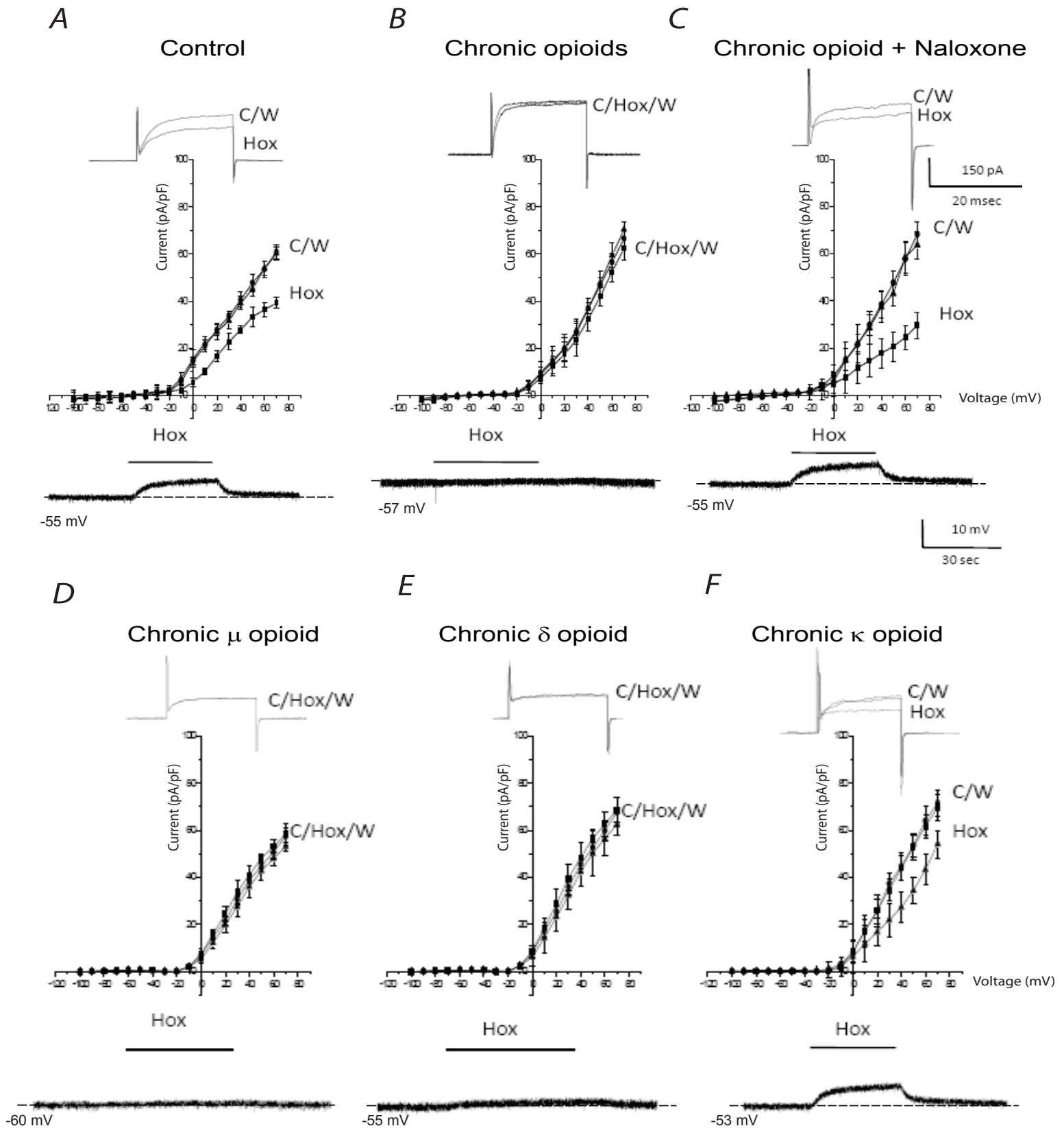


Figure 2: Effects of glibenclamide on outward K⁺ current and membrane potential during hypoxia in control vs opioid-treated neonatal AMCs. In control (untreated) AMCs, glibenclamide (50 μ M) enhances the hypoxia-induced inhibition of outward K⁺ current as shown in upper sample traces (steps to +30 mV) and lower current density vs voltage (I-V) plot (Aa); significant difference ($P < 0.05$) between current density in hypoxia (Hox) and hypoxia plus glibenclamide (Hox + Glib) at potentials $\geq +30$ mV. By contrast, opioid-treated cells failed to respond to hypoxia alone, however, when combined with glibenclamide, there was a pronounced inhibition of outward current at positive potentials ($> +20$ mV), as shown in upper traces and lower I-V plot (Ab). Data are presented as mean \pm SEM. ($P < 0.05$). Under current clamp, the hypoxia-induced membrane depolarization and increased excitability (Ba) was potentiated when glibenclamide was applied to the *same* control cell (Bb) ($n = 11$). By contrast, membrane depolarization was absent or weak when hypoxia was applied to opioid-treated cells (Ca, Da), and even a weak hyperpolarization occurred in a few cases (Ea). However, when these *same* cells were exposed to hypoxia in the presence of glibenclamide, there was a marked positive or depolarizing shift in membrane potential as shown in Cb, Db and Eb respectively; note the cell Ca that was initially quiescent during hypoxia actually fired action potentials when hypoxia was combined with glibenclamide (Cb). The 'n' values for each type of response is shown on right. C = control; Hox = hypoxia; W = wash; Glib = glibenclamide.

FIGURE 2

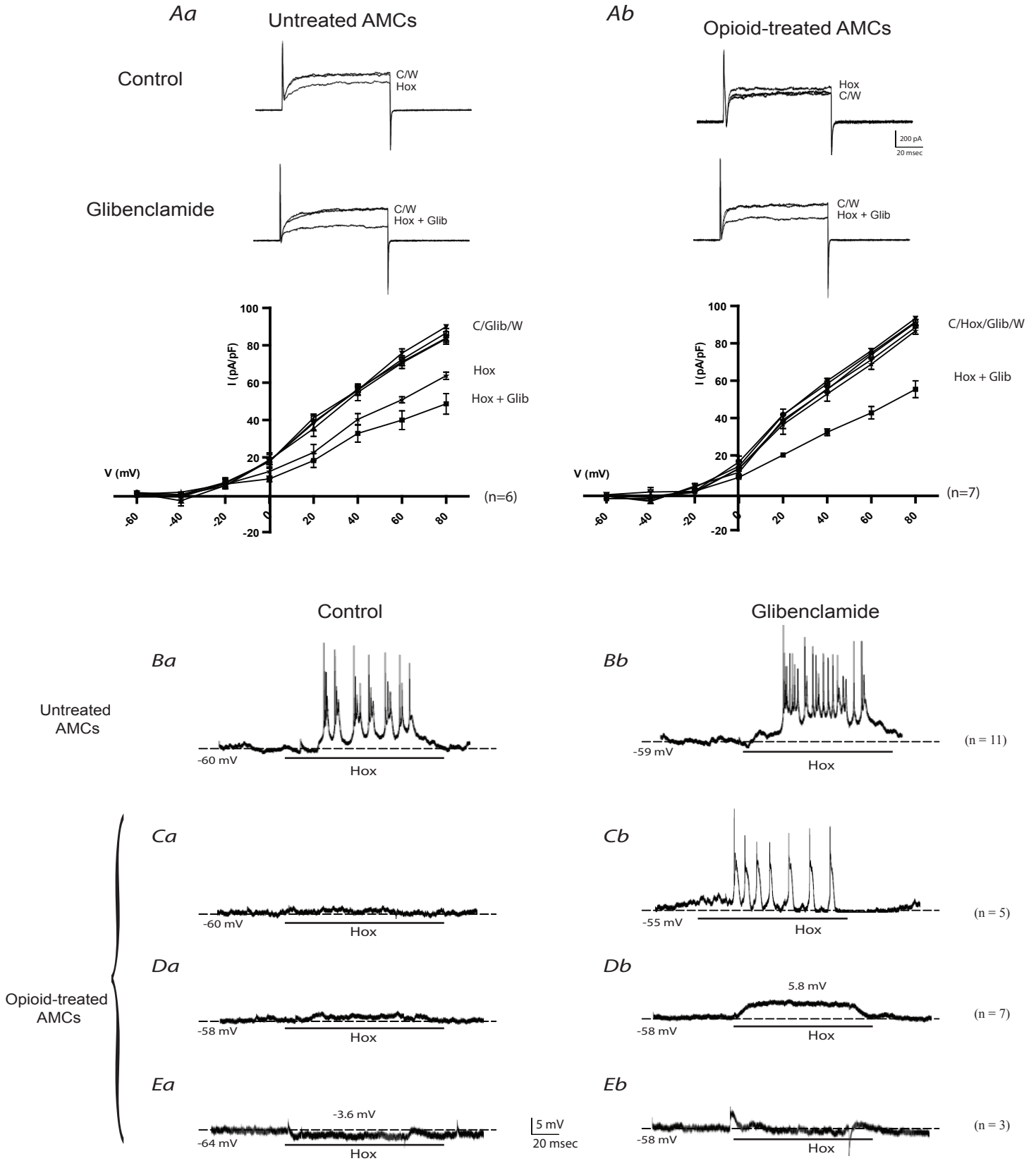
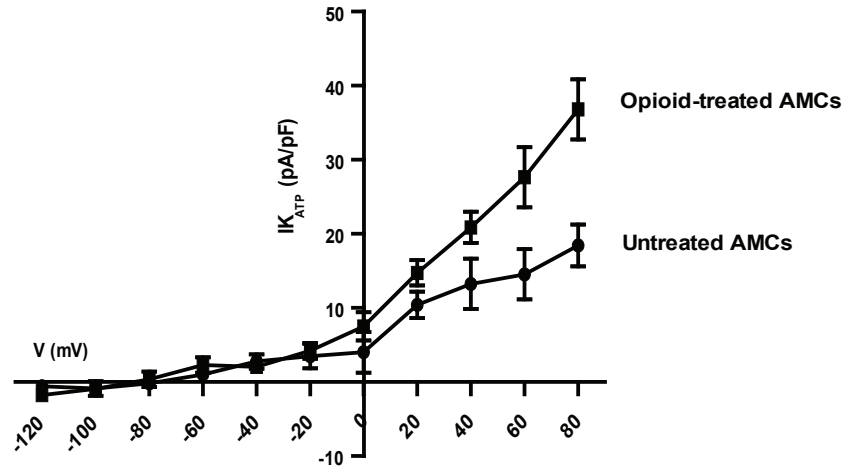


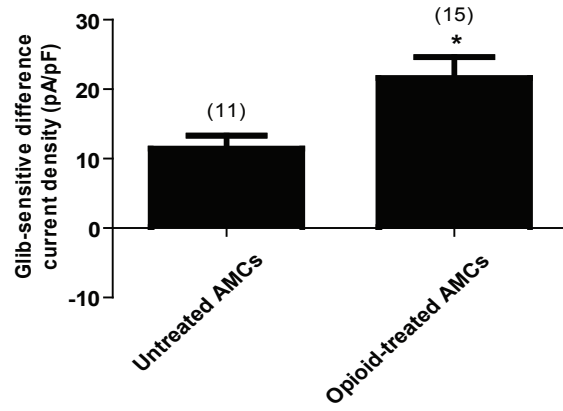
Figure 3: Upregulation of functional K_{ATP} channels and Kir6.2 subunit in opioid-treated neonatal rat AMCs. The glibenclamide-sensitive difference current density ($I_{K_{ATP}}$ (pA/pF)) in untreated versus opioid-treated AMCs is plotted against voltage in the I-V plot (A), and during steps to +30 mV (B). Note the significant increase in K_{ATP} current density in opioid-treated relative to control untreated AMCs ($P < 0.05$ in B). (C) Western blot analysis of K_{ATP} channel subunit, Kir6.2, in untreated AMCs and in AMCs cultured with combined μ -, δ -, and κ -opioid agonists (2 μ M) for 7 days. Note increased expression of Kir6.2 during chronic opioid exposure; β -actin was used as an internal control. Values are represented as mean \pm SEM of three independent experiments ($*P < 0.05$).

FIGURE 3

A



B



C

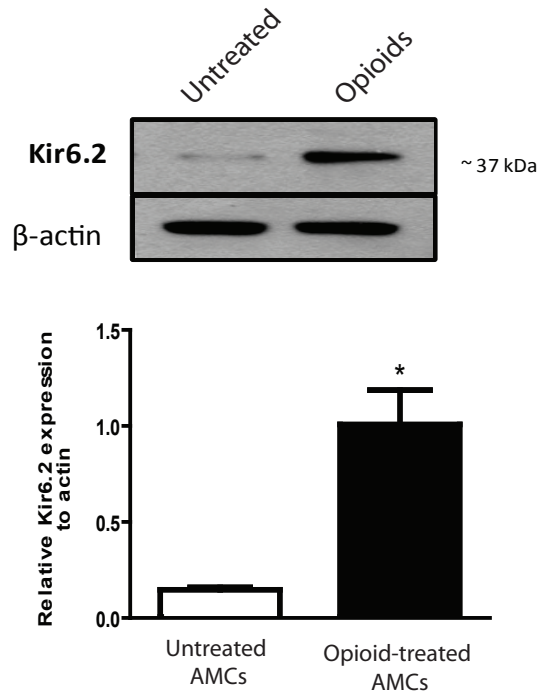


Figure 4: Effects of chronic opioid exposure on CO₂ sensitivity in neonatal rat AMCs. In control neonatal AMCs cultured for ~1 week, isohydric hypercapnia (10% CO₂; pH 7.4) typically causes inhibition of outward K⁺ current (top trace) and membrane depolarization (bottom trace); current density (pA/pF) versus voltage (I-V) plot (middle) shows significant inhibition of outward current ($p < 0.05$) at positive potentials (A). These responses are markedly reduced or abolished in neonatal AMCs chronically exposed for ~1 week to either a combination of μ -, δ -, and κ -opioid agonists (2 μ M) (B), or to either μ - or δ -opioid agonists (2 μ M) alone (D and E respectively). These blunting effects of chronic opioids on the CO₂-induced responses are prevented during continuous co-incubation with the general opioid receptor antagonist, naloxone (2 μ M) (C). Also, chronic exposure to the κ -opioid agonist (2 μ M) alone was ineffective (F). Data are presented as mean \pm SEM ($n = 11$). TTX was present in the extracellular solution. C = control; W = wash.

FIGURE 4

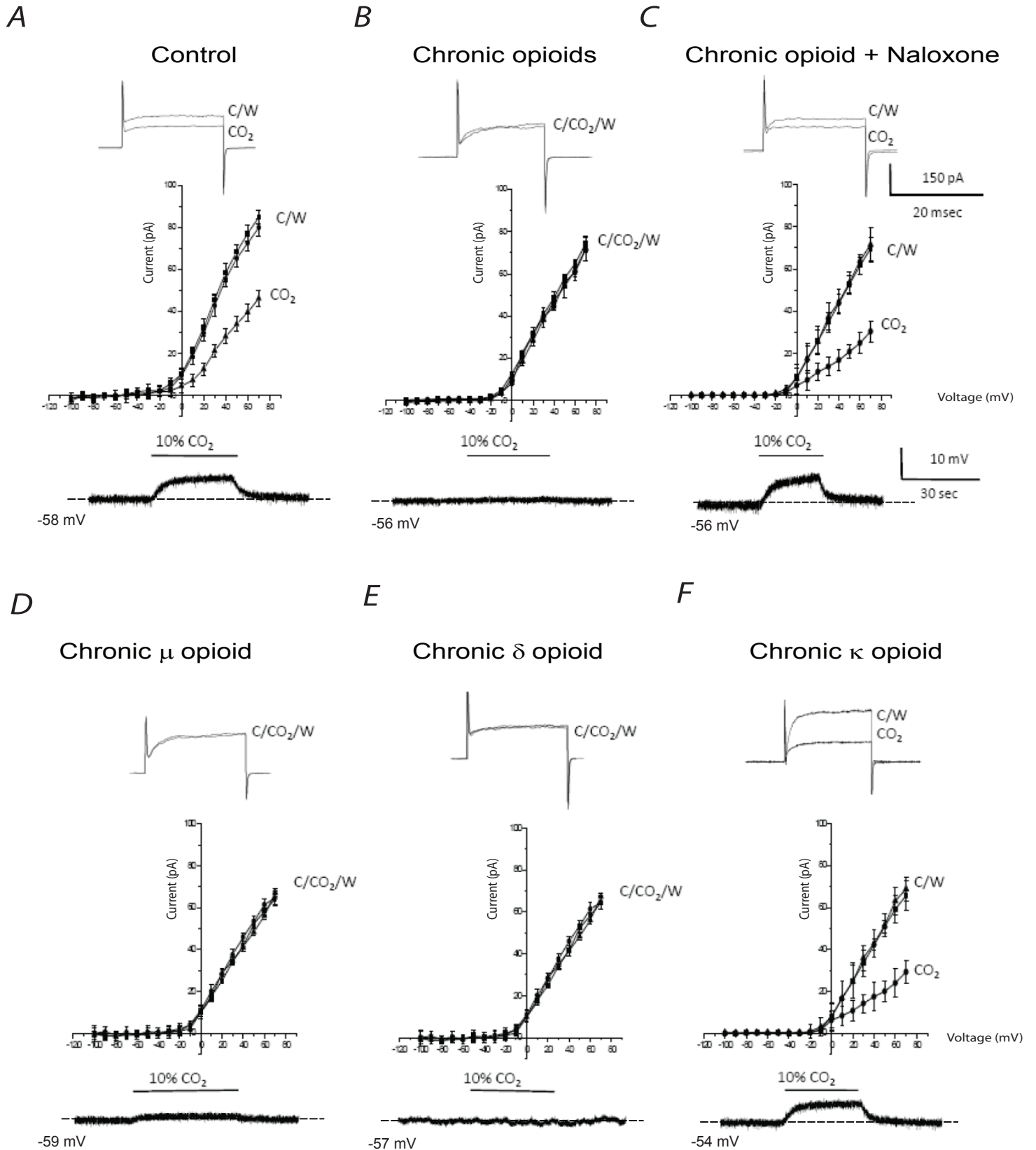


Figure 5: Comparative estimates of the blunting effects of chronic opioids on CO₂ -mediated responses in neonatal AMCs. Comparison of the CO₂-induced changes in holding current at -60 mV in control (*Aa*) versus opioid-treated (*Ab*) cells; note the significant (* $p < 0.05$) inward shift in holding current normally seen in control cells during high CO₂ is abolished in opioid-treated cells. The variability in CO₂-induced changes in membrane potential is shown for control cells (*Ba, Bb*) versus opioid-treated cells (*Ca, Cb*); data of response frequency for each condition is summarized in *D*. Note that CO₂-induced action potential firing or spikes (*Ba*) or subthreshold depolarizations (*Bb*) occur frequently in untreated (control) cells (*D*), but is rare in opioid-treated cells (*Cb, D*). Also, the majority of opioid-treated cells fail to show either CO₂-induced membrane depolarization or spikes, i.e. are non-responsive (*Ca, D*), in contrast to untreated cells (*D*).

FIGURE 5

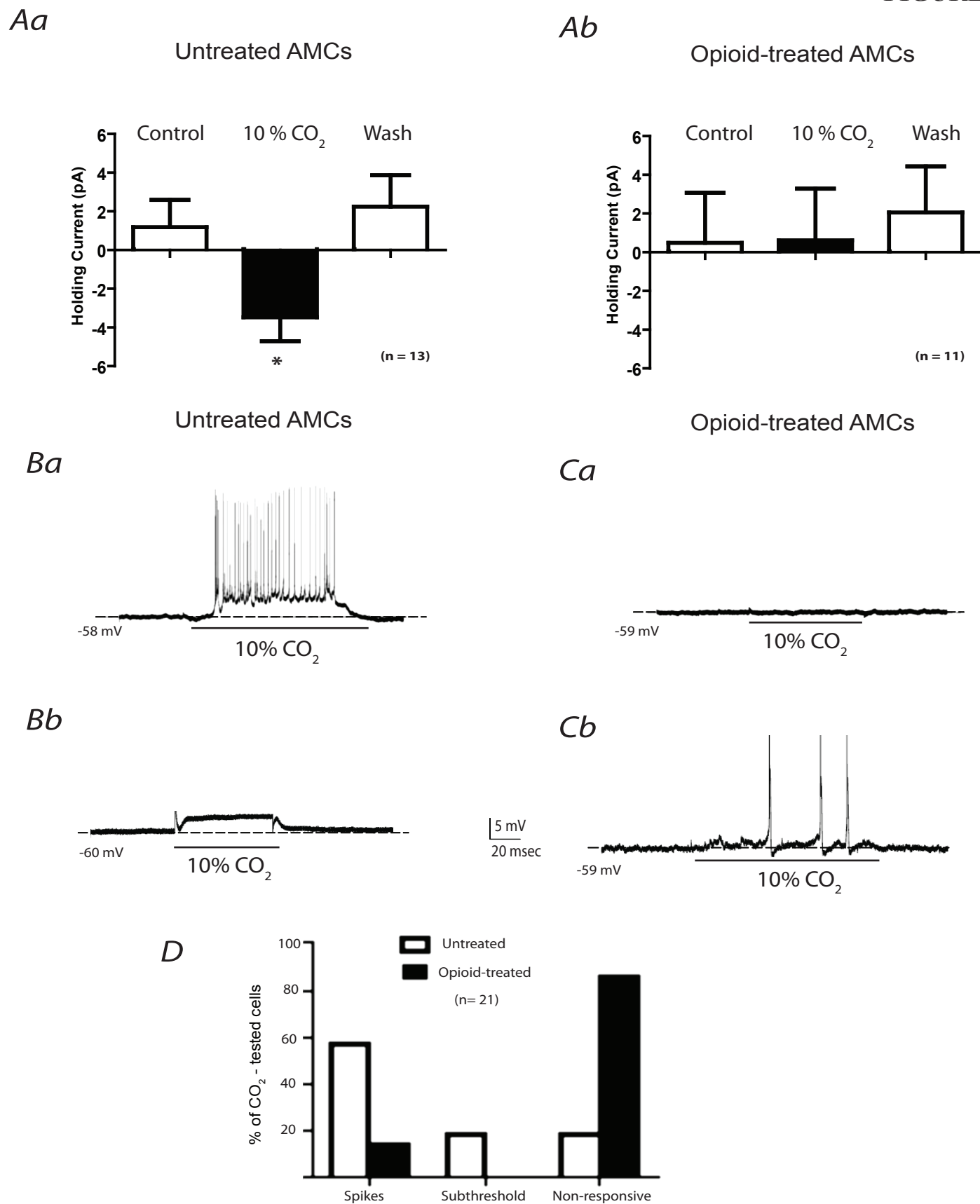


Figure 6: Effects of chronic opioid exposure on carbonic anhydrase enzyme expression in neonatal rat AMCs. (A) Western blot analysis showing expression of carbonic anhydrase (CA) isoforms CAI and CAII in 7 day-old cultures of control untreated AMCs versus AMCs treated with combined μ -, δ - and κ -opioid agonists ($2\mu\text{M}$); β -actin was used as an internal control. Note the downregulation in CAI and CAII expression in neonatal AMCs following chronic opioid exposure. Values are represented as mean \pm SEM of three independent experiments ($*P < 0.05$).

FIGURE 6

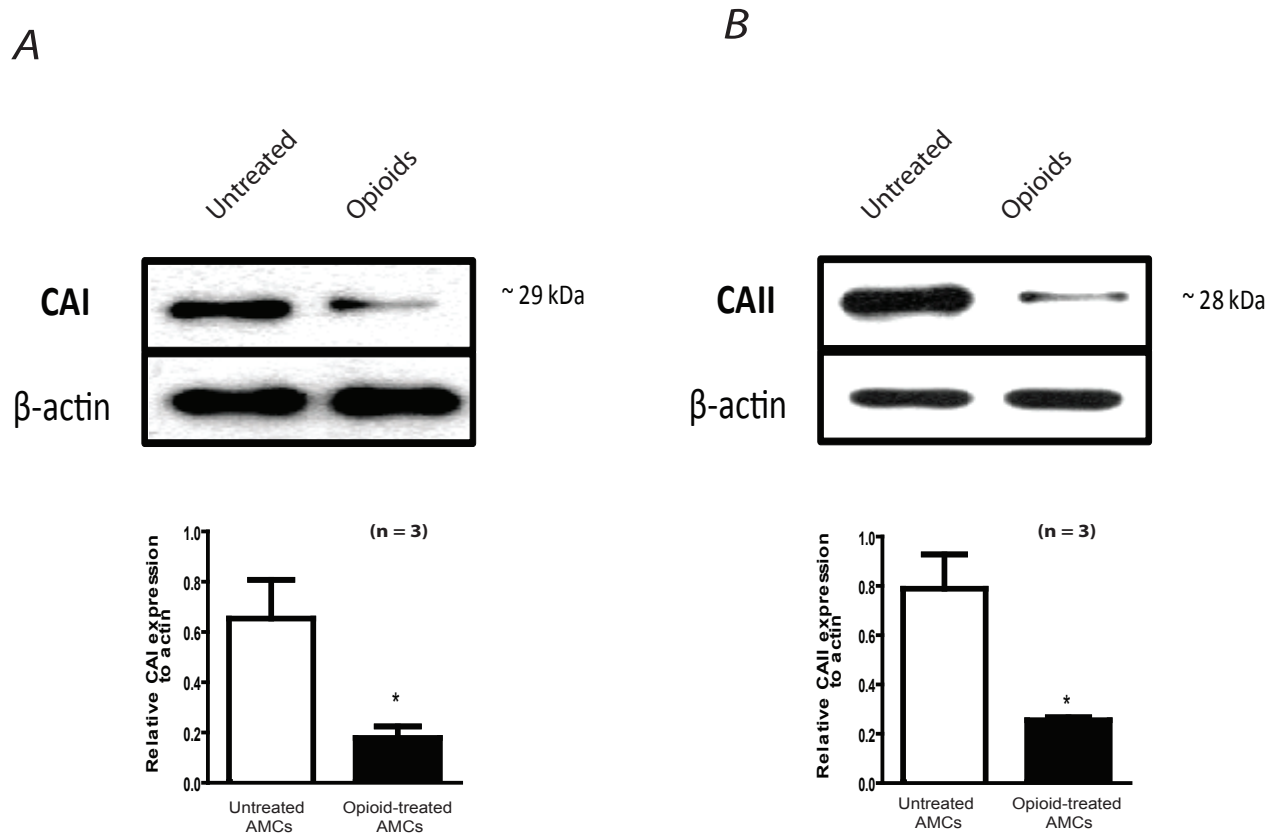
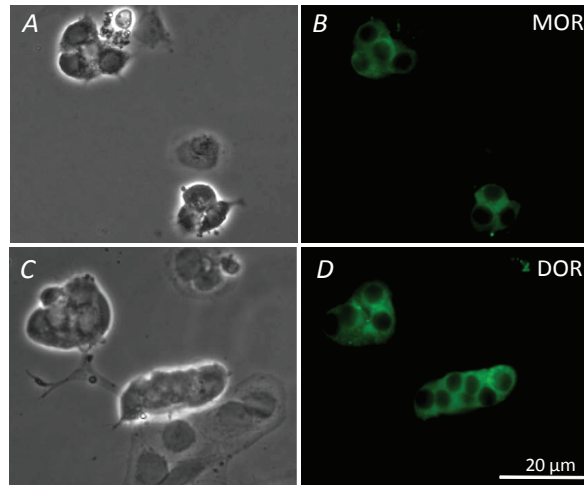
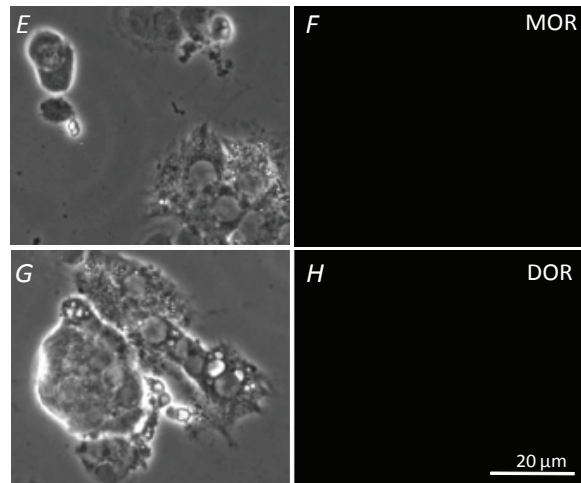


Figure 7: Immunofluorescence staining of neonatal rat AMC cultures for μ - and δ -opioid receptor expression. Corresponding phase and fluorescence (FITC) images of cultures showing positive immunostaining of AMCs for μ -opioid receptor (MOR) (A and B respectively), and for δ -opioid receptor (DOR) (C and D respectively). Pre-absorption controls with excess antigen (see Methods) confirming staining specificity for each antibody are shown in E-H. The data are representative of three independent experiments for each antibody staining.

FIGURE 7



Pre-adsorption control



CHAPTER 4: REGULATION OF K_{ATP} CHANNEL SUBUNIT Kir6.2 AND CARBONIC ANHYDRASE I & II IN PERINATAL RAT ADRENAL CHROMAFFIN CELLS EXPOSED TO CHRONIC OPIOIDS: ROLE OF PKA AND HYPOXIA INDUCIBLE FACTOR (HIF)-2 α

Authors: **Shaima Salman**, Alison Holloway, and Colin A. Nurse

I performed all the experiments, analysis and preparation of the manuscript for potential submission. Alison Holloway provided assistance with animal experiments, data collection and analysis.

ABSTRACT

At birth, asphyxial stressors such as hypoxia and hypercapnia are important physiological stimuli for adrenal catecholamine release that is critical for the proper transition of neonate to extra-uterine life. We recently showed that chronic opioids blunt chemosensitivity of neonatal rat adrenomedullary chromaffin cells (AMCs) to hypoxia and hypercapnia. This blunting was attributable to increased K_{ATP} channel and decreased carbonic anhydrase (CA) I, II expression respectively, and involved μ - and δ -opioid receptor signalling pathways. To address underlying molecular mechanisms, we first exposed an O_2 - and CO_2 -sensitive, immortalized rat chromaffin cell line (MAH cells) to combined μ - (DALDA) and δ - (DPDPE) opioid agonists (2 μ M) for ~7 days. Western blot and QPCR analysis revealed that chronic opioids increased K_{ATP} channel subunit, Kir6.2, and decreased CAII expression; both effects were blocked by naloxone and were absent in hypoxia inducible factor (HIF)-2 α -deficient MAH cells. Chronic opioids also stimulated HIF-2 α accumulation along a time-course similar to Kir6.2. Chromatin immunoprecipitation assays on opioid-treated cells revealed the binding of HIF-2 α to a hypoxia response element in the promoter region of the Kir6.2 gene. The opioid-induced regulation of Kir6.2 and CAII were dependent on PKA, but not PKC or CaM kinase, activity. Interestingly, a similar pattern of HIF-2 α , Kir6.2, and CAII regulation (including the downregulation of CAI) was replicated in chromaffin tissue obtained from rat pups, born to dams exposed to morphine throughout gestation. Collectively, these data reveal novel mechanisms by which chronic opioids blunt asphyxial chemosensitivity in AMCs, thereby contributing to abnormal arousal responses in the offspring of opiate-addicted mothers.

INTRODUCTION

Catecholamine (CAT) secretion from adrenomedullary chromaffin cells (AMCs) in response to asphyxial stressors at birth is a crucial physiological response during the transition of the fetus to extra-uterine life (Lagercrantz & Slotkin, 1986; Slotkin & Seidler, 1988). In particular, CAT secretion in the neonate aids in metabolic regulation, cardiac conduction, and in the transformation of the lungs into an air-breathing epithelium (Lagercrantz & Slotkin, 1986). This ‘non-neurogenic’ response is triggered by the direct action of stressors such as low O₂ (hypoxia) and high CO₂/H⁺ (acid hypercapnia) on AMCs, leading to membrane depolarization, voltage-gated Ca²⁺ entry, and CAT secretion (Mochizuki-Oda *et al.*, 1997; Thompson *et al.*, 1997; Munoz-Cabello *et al.*, 2005; Nurse *et al.*, 2009). These direct chemosensing mechanisms are suppressed postnatally, in parallel with the development of splanchnic innervation (after the first postnatal week in the rat), and return following denervation of adult AMCs (Seidler & Slotkin, 1985, 1986; Thompson *et al.*, 1997; Munoz-Cabello *et al.*, 2005; Levitsky & Lopez-Barneo, 2009; Nurse *et al.*, 2009). Thus, it is plausible that neurochemicals released from the splanchnic nerve during innervation activate signalling cascades that lead to the suppression of hypoxia and hypercapnia chemosensitivity. In recent tests of this hypothesis, we considered the potential involvement of nicotinic cholinergic and opioid receptor signalling pathways, given that ACh and opiate peptides are among the presynaptic neurochemicals released from the splanchnic nerve (Kobayashi *et al.*, 1985; Holgert *et al.*, 1995; Holgert *et al.*, 1998). Interestingly, we found that whereas exposure of neonatal rat AMCs to chronic nicotine suppressed only hypoxia chemosensitivity (Buttigieg *et al.*, 2008a), exposure to μ - and/or δ -opioid agonists led to the suppression of both hypoxia and hypercapnia sensitivity (Salman *et al.*, 2013). In both instances, the suppression of hypoxia sensitivity was attributable to the increased expression of functional

ATP-sensitive K^+ (K_{ATP}) channels, which induce membrane hyperpolarization and decreased excitability during hypoxia (Buttigieg *et al.*, 2009; Salman *et al.*, 2013). On the other hand, the suppression of hypercapnia sensitivity in opioid-treated AMCs was associated with decreased expression of the CO_2 hydrating enzymes, carbonic anhydrase (CA) I & II (Salman *et al.*, 2013).

In the present study, we were interested in the intracellular signalling mechanisms leading to the blunting of O_2 and CO_2 chemosensitivity in opioid-treated chromaffin cells, and therefore focussed on factors regulating K_{ATP} channel and CA expression. As a first step, we considered the potential role of the transcription factor, hypoxia inducible factor (HIF)-2 α , because: (i) HIF-2 α stabilization in nicotine-treated AMCs mediated the transcriptional upregulation of K_{ATP} channel subunit Kir6.2 (Salman *et al.*, 2012) (Chapter 2); and (ii) the HIF pathway has previously been implicated in the signalling cascade activated in neuroblastoma cells during chronic opioid exposure (Daijo *et al.*, 2011). To facilitate these studies we used a fetal-derived, immortalized rat chromaffin cell line, i.e. MAH cells, which are sensitive to both hypoxia and hypercapnia (Fearon *et al.*, 2002; Buttigieg *et al.*, 2008b), and express Kir6.2 and CAII mRNA and protein (Piskuric *et al.*, 2008; Buttigieg *et al.*, 2009; Salman *et al.*, 2012). Moreover, the availability of a stable HIF-2 α -deficient (>90% knockdown) MAH cell line (shMAH) allowed us to investigate the role of HIF-2 α in the opioid signalling cascade. Because protein kinases (e.g. PKC and CaM kinase) were implicated in the upregulation of Kir6.2 in nicotine-treated chromaffin cells (Buttigieg *et al.*, 2009), we also investigated their potential involvement in opioid receptor signalling in MAH cells using pharmacological inhibitors. Finally, to validate the predictions of this *in vitro* model, we used a physiologically-relevant *in vivo* model where pregnant dams received daily injections of morphine just prior to and throughout gestation. The

expression patterns of HIF-2 α , Kir6.2, and CAI &II were then compared in chromaffin tissues from the adrenal glands of neonates born to morphine- vs. saline- exposed dams.

MATERIALS AND METHODS

Cell culture: the *v-myc* immortalized chromaffin (MAH) cells, HIF-2 α -deficient MAH cells (shHIF-2 α) and scrambled control (ScCont) MAH cells (Brown *et al.*, 2009) were grown in L-15/CO₂ medium containing 0.6 % glucose, 1% penicillin/streptomycin, 10% fetal bovine serum and 5 μ M dexamethasone, as previously described (Fearon *et al.*, 2002). A stable HIF-2 α -deficient MAH cell line (shMAH), generated using interference RNAi techniques (Brown *et al.*, 2009), was used in some experiments and grown under similar conditions. All cultures were incubated in a humidified atmosphere of 95% air, 5% CO₂ at 37°C for varying periods up to ~7 days *in vitro*. Cells were fed every 1–2 days and routinely passaged every 3-4 days when cell density reached sub-confluency. When passaging cells, medium was removed and cells were detached using 0.25% trypsin–EDTA. Suspended cells were pelleted by centrifugation and the pellet was re-suspended in pre-warmed medium. Cells were then plated on 35 mm culture dishes coated with poly-D-lysine and laminin.

Adrenal Gland Tissues: Adrenal tissues were obtained from neonatal rat pups following maternal exposures to morphine or saline (control) solutions. Saline (vehicle) or morphine sulphate (Sigma Aldrich, St Louis, MO) dissolved in saline was administered to adult female rats (Wistar, Charles River, Quebec, Canada) 1 week before pregnancy, during pregnancy, and up to 4 days postpartum as described below:

Day 1-3 pre-pregnancy: 5 mg/kg/day sc, once daily;

Day 4-7 pre-pregnancy: 10 mg/kg/day, sc, once daily;

Gestational day 1-postpartum day 4: 10 mg/kg/day, sc, once daily.

All procedures for animal handling and tissue dissections were carried out according to the guidelines of the Canadian Council on Animal Care (CCAC) and institutional guidelines. To

minimize side effects, the dose administered was near that required to produce analgesia in rats (i.e. 2-5mg/kg sc every 2-4 hr). Female rats were weighed twice daily, and monitored for the first 15 min after injection and then again at 60 min post-injection. For each female, litter size, litter weight, sex of littermates, birth weight, and the number of stillbirths were recorded. Live birth index ($[\# \text{ of live offspring} / \# \text{ of offspring delivered}] * 100$) and sex ratio ($\# \text{ of male offspring} / \# \text{ of female offspring}$), were calculated (Table 1). The 10th percentile value for birth weight of the control group was obtained (Mean - 2*SD). Rat pups with birth weights less than the 10th percentile were labelled small for gestational age (SGA). Following delivery, the pups were isolated and weighed before tissue collection. Soon after birth, neonatal rat pups [postnatal day 0 P0], were first rendered unconscious by a blow to the back of the head and then killed immediately by decapitation. Adrenal glands were excised bilaterally and most of the surrounding *adrenal cortex* (AC) was trimmed and isolated from the central *adrenal medulla* (AM) for separate molecular analysis of the two tissues (Salman *et al.*, 2012).

Immunofluorescence: MAH cells were grown on modified Nunc 35mm dishes with central wells to which glass coverslips were attached as previously described (Buttigieg *et al.*, 2008a). Immunofluorescence staining was performed as outlined in our previous study (Salman *et al.*, 2013). Briefly, medium was removed and cells were washed with pre-warmed PBS, pH 7.2, and fixed with ice cold 4% paraformaldehyde in PBS for 1 hr at 4°C. Cells were then washed with PBS and incubated with 100 μ l of primary antibodies (rabbit polyclonal anti- μ -opioid receptor; rabbit polyclonal anti- δ -opioid receptor; Alomone) diluted in 1% BSA/PBS overnight. For pre-adsorption controls, primary antibodies were incubated in the presence of 3x excess antigen overnight at 4°C. Following incubation with primary antibodies, samples were washed the next day with PBS and incubated with FITC-conjugated secondary antibody (1:50; Jackson

ImmunoResearch Laboratories, Inc., West Grove, PA, USA) in the dark for 1 h at RT. Samples were then washed with PBS and covered with Vectashield to prevent photobleaching.

Fluorescence visualization and images were obtained using a Zeiss inverted microscope (IM 35).

Quantitative Real-Time PCR (QPCR): QPCR analysis was performed using the Stratagene (Mx3000p) detection system and ABsolute QPCR SYBR Green Mix. Primers were designed using Gene Fisher and specificity was confirmed using BLAST. Thermal cycling conditions included, Platinum *Taq* DNA polymerase activation at 95°C for 2 min, 40 cycles of denaturing at 95°C for 3 s, and annealing and extension at 60°C for 30 s, followed by routine melting curve analysis. Samples with no template were used as a negative control. Data were compared quantitatively using the arithmetic equation $2^{-\Delta\Delta CT}$ (Livak & Schmittgen, 2001); mRNA levels were normalized to Lamin A/C expression and expressed as transcript fold change relative to mRNA from untreated control MAH cells. Each experiment was repeated 3-4 times. Primers used were as follows: Kir6.2 subunit, forward: 5' ACA AGA ACA TCC GAG AGC A 3', reverse: 5' CTG CAC GAT CAG AAT AAG GA 3' (Accession No. NM_008428); CAII (Carbonic anhydrase II), forward: 5' CCG ACA GTC CCC TGT GGA 3', reverse: 5' GCG GAG TGG TCA GAG AGC CA 3' (Accession No. NM_010602); VEGF, forward: 5' AATGATGAAGCCCTGGAGTG 3', reverse: 5' AATGCTTTCTCCGCTCTGAA 3' (Huang *et al.*, 2010); Lamin, forward: 5' GCAGTACAAGAAGGAGCTA 3', reverse: 5' CAGCAATTCCTGGTACTCA 3' (Accession No. NM_008084).

Chromatin Immunoprecipitation (ChIP) Assay: ChIP assay was performed using a standard protocol provided by ChIP assay kit (Millipore) as previously described (Brown *et al.*, 2009; Salman *et al.*, 2012). Briefly, MAH cells were plated on 100-mm dishes (Corning Incorporated) at a confluency of $\sim 0.75 \times 10^6$ cells/dish and treated with opioid agonists for 7 days in culture.

Following chronic treatments, the cultures were treated with 1% formaldehyde for 10 min at 37°C in order to cross-link histones to DNA. Medium was removed and cells were washed with ice-cold phosphate buffered saline (PBS, pH 7.4) containing protease inhibitors, scraped and pelleted at 2000 rpm for 4 min at 4°C. The pellet was then resuspended in 200µl SDS lysis buffer. Cross-linked DNA was sheared, and pelleted at 13,000 rpm for 10 min at 4°C. The lysate supernatant was diluted 10x using the ChIP dilution buffer and a small portion (1%) was kept for DNA quantification and used as an input control. For immunoprecipitation, lysates were pre-cleared by the addition of Protein A Agarose/Salmon Sperm DNA (50% slurry) and maintained under constant agitation for 30 min at 4°C. Samples were incubated with rabbit polyclonal antibody against HIF-2α (Novus Biologicals) overnight at 4°C with constant rotation. For negative controls, the antibody was omitted from the samples. Following immunoprecipitation, Agarose beads were pelleted, washed, and the DNA was eluted and reversed cross-linked by adding 5 M NaCl and heating at 65°C for 4 hr. DNA was recovered by phenol/chloroform extraction and ethanol precipitation. PCR analysis was utilized to detect HIF-2α binding using primers specific for a putative hypoxia responsive element (HRE) present in KCNJ11 (Kir6.2 subunit) sequence (forward: 5` CGG ACT CTC AGA GCA GTG TA 3`; and reverse 5` GCA GAC TCT GAC AGT GCC TTT 3).

Western Immunoblot Analysis: MAH cell cultures and adrenal gland tissues were lysed so as to obtain cytoplasmic and nuclear fractions as previously described (Brown & Nurse, 2008). Briefly, MAH cells were lysed in buffer A (10 mM HEPES pH 7.6, 10 mM KCl, 0.1 mM EDTA pH 8, 0.1 mM EGTA pH 8, 1 mM DTT, and protease inhibitors (Complete Mini; Roche, Laval, Quebec, Canada)) then incubated on ice for 15 min with intermittent vortexing. A final concentration of 0.6% NP40 was added and samples were vortexed for 1 min then centrifuged at

13,000 rpm for 30s. The supernatant (cytoplasmic-extract) was collected and the pellet was resuspended in buffer C (20 mM HEPES pH 7.6, 0.4 M NaCl, 1 mM EDTA pH 8, 1 mM EGTA pH 8, 1 mM DTT, 5% glycerol, and protease inhibitors) and frozen at -80°C over night. Lysate was then thawed and centrifuged at 13,000 rpm for 5 min and the resulting supernatant (nuclear-extract) was collected to determine total protein concentration using Bradford assay (1:5 dilution reagent and 1 mg/mL BSA). 5µg of cytoplasmic-extracts and 2.5µg of nuclear-extracts were loaded and resolved on a 10% and a 4-15 % gradient SDS-PAGE gel respectively and transferred onto PVDC membranes. Membranes were then washed and incubated with either primary rabbit polyclonal antibody against Kir6.2 (1:1000 dilution; Alomone Labs Ltd. Jerusalem, Israel), rabbit polyclonal anti-human carbonic anhydrase I antibody (1:1000 dilution; Abcam Inc., Cambridge, MA, USA), sheep polyclonal anti-human carbonic anhydrase II antibody (1:1000 dilution; AbD Serotec, Kidlington, UK), HIF-1 α or HIF-2 α rabbit polyclonal antibody (1:1,000 dilution; Novus Biologicals, Littleton, CO), primary rabbit monoclonal β -actin antibody as loading control for cytoplasmic extracts (1:10,000 dilution; Millipore, Billerica, MA, USA), or primary rabbit polyclonal TATA-binding protein (TBP) antibody as a loading control for nuclear extracts (1:25,000 dilution; Santa Cruz, CA) at 4°C overnight. Membranes were then washed in PBS, and incubated in goat anti-rabbit horseradish peroxidase (HRP)-linked secondary antibody (1:10,000 dilution; Jackson Labs, Bar Harbor, ME, USA) for 1 hr at room temperature. Immunoreaction products were visualized using enhanced chemiluminescence (ECL) and images were obtained after exposure to XAR-film.

Luciferin-Luciferase Chemiluminescence detection of ATP release from MAH cells: ATP release from MAH cells was measured using a Labsystem Luminoskantm luminometer as previously described (Buttigieg & Nurse, 2004). Briefly, cell medium was removed and replaced

with 900 μ l of extracellular solution containing in mM: NaCl, 135; KCl, 5; CaCl₂, 2; MgCl₂, 2; glucose, 10; and Hepes, 10, at pH 7.4. For hypoxic stimulation, an equal volume of the same solution was bubbled with N₂ gas (PO₂ ~15–20mmHg). A 200 μ l luciferin–luciferase solution, prepared from the ATP determination kit (Molecular probes # A22066), was added and the culture dish was placed in the luminometer. Chemiluminescence readings were expressed as relative light units (RLU) at intervals of 4s for a period of 3 min. Data were analyzed using Labsystems Ascent Software.

Drugs: All drugs were purchased from Sigma-Aldrich (St Louis, MO, USA). In cell culture studies, fresh drugs were added to the growth medium every 2 days. For chronic opioid treatments, a combination of the μ -opioid agonist DALDA ([D-Arg², Lys⁴]-Dermorphin (1-4) amide) and the δ -opioid agonist DPDPE ([D-Pen², 5, P-Cl-Phe⁴]-enkephalin) was used at a concentration of 2 μ M each. In some experiments, naloxone hydrochloride dehydrate (2 μ M) was used as a general opioid antagonist and added at the same time as the agonists. Protein kinase inhibitors used in this study include: 2 μ M of H-89 (PKA inhibitor), 2 μ M of GF109203X (PKC inhibitor), and 3 μ M of KN-62 (CaMK inhibitor).

Statistical Analysis: Molecular data were normalized to loading control and results were expressed as mean \pm SEM, and statistical analyses were made using ANOVA and non-parametric Mann-Whitney *U*-test using GraphPad Prism, version 4.0. For pregnancy outcomes, statistical analyses were performed using student t test (using the SigmaStat, v.2.03; SPSS, Chicago, Ill package) by comparing the morphine injected group to the control group. Fisher's exact test ($\alpha = 0.05$) was used when categorical variables were compared. Littermates were used as an experimental unit and values were presented as mean \pm SEM. * indicates $p < 0.05$.

Diagrams: Diagrams were made using BioDraw Ultra 12.0.

RESULTS

In the *in vitro* experiments reported below, all opioid exposures were performed on immortalized chromaffin (MAH) cell cultures that were incubated with combined μ - and δ - opioid agonists, i.e. DALDA and DPDPE, respectively, at a concentration of 2 μ M each. The duration of the exposure period varied from 24 hr to 7 days as indicated in the text. Similar to primary neonatal rat adrenomedullary chromaffin cells (AMCs) (Salman *et al.*, 2013), MAH cells express both μ - and δ - opioid receptors as exemplified in the immunocytochemical experiments shown in Fig. 1 (n =3). In addition, microarray data from our laboratory suggest that MAH cells express μ - and δ -opioid, but not κ -opioid, receptors (data not shown). Thus, MAH cells represent a simple surrogate model to study signalling pathways activated by μ - and δ -opioid receptors in chromaffin cells.

Effects of chronic opioid exposure on K_{ATP} channel expression in MAH cells

In our recent study, chronic exposure of primary neonatal rat AMCs to combined μ - (DALDA) and δ - (DPDPE) opioid agonists (2 μ M) for ~7 days *in vitro* caused an increased expression of the K_{ATP} channel subunit, Kir6.2, as determined by western blot analysis (Salman *et al.*, 2013). Using both quantitative real-time PCR (QPCR) and western blotting, we first investigated whether upregulation of Kir6.2 subunit also occurs in MAH cells exposed for ~7days to medium containing combined opioids. As illustrated in Fig. 2A, the transcript level of Kir6.2 subunit was significantly increased (>2 fold) in opioid-treated MAH cells compared to untreated controls. Moreover, this enhanced Kir6.2 expression was mediated via opioid receptor signalling pathways because it was prevented in MAH cells exposed to combined opioids plus the general opioid receptor blocker, naloxone (2 μ M; Fig. 2A). Western blot analysis also confirmed the upregulation of Kir6.2 expression at the protein level in opioid-treated MAH cells, and its

prevention during co-incubation with naloxone (Fig. 2B). These data are consistent with those we previously reported for primary neonatal AMCs (Salman *et al.*, 2013), and suggest that the MAH cell line is a suitable model for probing opioid-mediated signalling pathways in rat chromaffin cells.

HIF-2 α stabilization is required for opioid-induced upregulation of K_{ATP} channel subunit (Kir6.2) expression in MAH cells

We previously demonstrated that exposure of both perinatal rat AMCs and immortalized MAH cells to chronic nicotine causes stabilization of the transcription factor, HIF-2 α , which in turn led to the transcriptional upregulation of Kir6.2 subunit and K_{ATP} channel expression (Buttigieg *et al.*, 2009; Salman *et al.*, 2012). In other cell types, e.g. neuroblastoma cells, stimulation of μ - and δ - opioid receptors can lead to HIF activation under non-hypoxic conditions (Daijo *et al.*, 2011). It was therefore of interest to investigate whether or not the opioid-induced upregulation of Kir6.2 subunit expression in MAH cells was HIF-2 α -dependent. To test this, we used western blots to compare Kir6.2 expression in a HIF-2 α -deficient (>90% knockdown) MAH cell line (shHIF-2 α MAH; (Brown *et al.*, 2009)) following exposure to chronic opioids. As shown in Figs. 3A,B, the opioid-induced upregulation of Kir6.2 subunit relative to β -actin was present in non-transfected control (w.t. MAH) and transfected scrambled control (ScCont MAH) cells, but was absent in HIF-2 α -deficient (shHIF-2 α MAH) cells. These results demonstrate that HIF-2 α is required for the opioid-mediated upregulation of Kir6.2 subunit in MAH cells.

We next investigated whether chronic opioid exposure could stabilize HIF-2 α in nuclear extracts of w.t. MAH cells. As illustrated in Fig. 4A, there was a robust accumulation of HIF-2 α

in MAH cells exposed to chronic opioids for ~7 days *in vitro*, and this effect was abolished during co-incubation with the opioid receptor blocker, naloxone (2 μ M).

Time-dependent effects of chronic opioids on HIF-2 α accumulation and Kir6.2 subunit expression in MAH cells

In a previous study, we found that chronic nicotine exposure caused a parallel, progressive, and time-dependent accumulation of HIF-2 α and Kir6.2 subunit expression in MAH cells (Salman *et al.*, 2012). To test whether a similar pattern occurs during opioid exposure, we monitored HIF-2 α and Kir6.2 subunit expression in opioid-treated MAH cells at 0 hr, 24hr, 3 days and 7 days in nuclear and cytoplasmic extracts, respectively. As illustrated in Figs. 4B, C, western blot analysis revealed a slow progressive increase in HIF-2 α accumulation that occurred in parallel with the increase in Kir6.2 subunit over the 7 day treatment period. Both increases were significant at exposure periods of 3 and 7 days, but not at 24 hr (Fig. 4C); by contrast, exposure of MAH cells to chronic hypoxia (2% O₂) normally results in a robust HIF-2 α accumulation at 24 hr (data not shown) (Brown & Nurse, 2008; Salman *et al.*, 2012). This expression pattern of HIF-2 α and Kir6.2 subunit is reminiscent of that seen in MAH cells during exposure to chronic nicotine over a similar time period (Salman *et al.*, 2012).

HIF-2 α binds to the promoter region of Kir6.2 gene in opioid-treated cells

The promoter region of the Kir6.2 gene contains a putative hypoxia response element (HRE), where the HIF core site (GCGTG) spans nucleotides -1087 to -1083 and the HIF ancillary site (CACAG) spans nucleotides -1065 to -1061 (Fig. 3B; upper). Using a chromatin immunoprecipitation (ChIP) assay, we previously demonstrated that HIF-2 α bound to the

promoter region of Kir6.2 gene, following its stabilization in MAH cells exposed to chronic nicotine (Salman *et al.*, 2012). To test whether opioid receptor signalling promotes binding of HIF-2 α to the promoter region of Kir6.2 gene, we performed ChIP assays on control and HIF-2 α -deficient MAH cells exposed to chronic opioids for ~7 days. Indeed, we found that under these conditions HIF-2 α bound to the Kir6.2 promoter region in w.t. and scrambled control (ScCont) MAH cells, but not in HIF-2 α -deficient (shHIF-2 α) MAH cells, as illustrated in Fig. 3B (lower). These data complement those demonstrating that HIF-2 α deficiency prevents upregulation of Kir6.2 in opioid-treated MAH cells (Fig. 3A), and imply that the mechanism of HIF-2 α action most likely involves increased expression of Kir6.2 at the transcriptional level.

To confirm transcriptional activity of HIF-2 α in opioid-treated MAH cells, we measured mRNA expression levels of a common HIF-2 α target gene, vascular endothelial growth factor (VEGF), using quantitative real-time PCR (QPCR) analysis. These experiments demonstrated that VEGF mRNA was significantly increased in opioid-treated MAH cells (Fig. 3C).

Chronic opioid exposure downregulates the expression of carbonic anhydrase II in MAH cells: Role of HIF-2 α

Sensitivity of neonatal rat AMCs to high CO₂ (hypercapnia) is dependent on the activity of the CO₂ hydrating enzymes, carbonic anhydrase (CA) I and II (Munoz-Cabello *et al.*, 2005). We previously showed that exposure of primary neonatal rat AMC to chronic opioids blunts hypercapnia sensitivity, in association with the downregulation of CAI and CAII (Salman *et al.*, 2013). Though MAH cells express only CAII, they show CO₂ sensitivity similar to primary neonatal AMC (Buttigieg *et al.*, 2008b). This suggests MAH cells may serve as a suitable model for investigating opioid-mediated blunting of CO₂ sensing in chromaffin cells. Indeed, exposure

of MAH cells to chronic opioids for ~7 days resulted in a significant reduction in CAII transcript as determined by QPCR; further, this reduction was prevented during continuous co-incubation with the opioid receptor blocker, naloxone (2 μ M; Fig. 5A). Western blot analysis revealed that chronic opioids also caused a naloxone-sensitive downregulation of CAII at the protein level, as illustrated in Fig. 5B.

Because chronic opioids stabilize HIF-2 α in MAH cells (Fig. 4), we wondered whether or not the opioid-mediated downregulation of CAII expression was HIF-2 α -dependent. To test this, we used western blot analysis to compare CAII expression in HIF-2 α -deficient (shHIF-2 α) MAH cells following exposure to chronic opioids for ~7days. As shown in Fig. 6A, the opioid-mediated downregulation of CAII relative to β -actin was present in non-transfected control (w.t. MAH) and transfected scrambled control (ScCont MAH) cells, but was absent in HIF-2 α -deficient (shHIF-2 α MAH) cells. These results demonstrate that HIF-2 α is required for the opioid-mediated downregulation of CAII in MAH cells.

HIF-mediated repression of several genes such as α -fetoprotein and LIFR under hypoxia may occur via hypoxia response elements (HREs) present on the antisense strand (Narravula & Colgan, 2001; Mazure *et al.*, 2002; Jeong *et al.*, 2007). We therefore examined sites within the CAII proximal promoter, ~1.7 kb upstream from the transcription initiation site ATG, for potential HREs. Interestingly, this analysis revealed a putative HRE element on the antisense strand suggesting possible binding of HIF transcription factors (Fig. 6B). While this suggests a potential mechanism by which HIF-2 α stabilization may lead to the downregulation of CAII in MAH cells, further studies are required to validate this point.

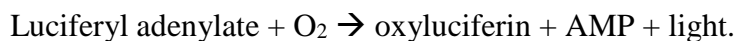
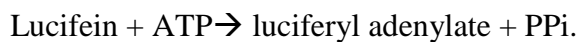
Central role of protein kinase A (PKA) in mediating the downstream effects of opioid receptor signalling in MAH cells

Various protein kinases are known to be activated during opioid receptor signalling (Feng *et al.*, 2011). We therefore used pharmacological inhibitors to probe for the potential involvement of three common kinases, i.e. PKC, CaMK, and PKA, in mediating the opposing effects of chronic opioid exposure on Kir6.2 and CAII expression in MAH cells. As illustrated in Fig. 7A, western blot analysis revealed that inhibition of PKA by H-89 (2 μ M) prevented the opioid-induced upregulation of Kir6.2 evident in MAH cells following chronic opioid exposure. By contrast, inhibition of PKC and CaMK by GF109203X (2 μ M) and KN-62 (3 μ M) respectively, had no significant effect on the upregulation of Kir6.2 protein in comparable experiments (Fig. 7A). Likewise PKA, but not PKC or CaMK, inhibition prevented the opioid-induced downregulation of CAII evident in MAH cells following chronic opioid exposure (Fig. 7B). These data suggest a central role of PKA in the opioid receptor signalling pathway, leading to the regulation of key proteins involved in the blunting of O₂ and CO₂ chemosensitivity in chromaffin cells.

Given that both PKA activity (Figs. 7A,B) and HIF-2 α accumulation (Figs. 3,5,6), are necessary for the regulation of Kir6.2 and CAII in opioid-treated MAH cells, the question arises whether HIF-2 α accumulation is dependent on PKA activity. To address this, we investigated whether the opioid-induced HIF-2 α accumulation could occur in the presence of PKA inhibition. As illustrated in Fig. 7C, inhibition of PKA by H-89 had no significant effect on the opioid-induced HIF-2 α accumulation in MAH cells. Furthermore, inhibition of PKC by KN-62 and CaMK by GF109203X were similarly ineffective (Fig. 7C). Taken together, these data suggest that the regulation of Kir6.2 and CAII in opioid-treated MAH cells requires the independent actions of both PKA activity and HIF-2 α accumulation.

Chronic opioids blunt hypoxia sensing in MAH cells as monitored by ATP release

Catecholamines are co-stored with ATP in chromaffin granules and both agents are co-released during stimulus-evoked secretion (Castillo *et al.*, 1992). To test whether or not chronic opioids blunt hypoxia-evoked secretion in MAH cells, ATP release was monitored in control vs. opioid-treated cells using luciferase chemiluminescence (Buttigieg & Nurse, 2004). In this assay, ATP acts as a catalyst in the oxidation of luciferin by luciferase:



These experiments revealed that exposure of control (untreated) MAH cells to hypoxia (PO_2 ~15–20 mmHg) caused a significant increase in extracellular ATP levels (Fig. 8). By contrast, when the same stimulus was applied to opioid-treated MAH cells, extracellular ATP levels remained unchanged (Fig. 8). Further, this blunting effect of chronic opioids on hypoxia sensing was prevented during co-incubation with 2 μM naloxone (Fig. 8). These data suggest that chronic opioids blunt hypoxia-evoked secretion in MAH cells via opioid-receptor signalling pathways. This effect is most likely due to the upregulation of K_{ATP} channels in MAH cells (thereby favoring membrane hyperpolarization during acute hypoxia), as recently demonstrated for opioid-treated neonatal AMC (Salman *et al.*, 2013).

Effects of chronic maternal morphine exposure on Kir6.2, CAI, CAII, and HIF-2 α expression in the adrenal glands of affected neonates

To validate the main conclusions from our *in vitro* model in a physiologically-relevant *in vivo* system, we administered morphine (or saline) to pregnant Wistar rats and compared the

expression pattern of Kir6.2, CAI & II, and HIF-2 α in adrenal glands of the offspring. For these experiments, female rats (200-250 g) were randomly assigned to receive daily injections of morphine sulphate or saline (vehicle) for 1 week prior to pregnancy, during pregnancy, and up to 4 days postpartum (as outlined in Materials and Methods). Whole adrenal glands were dissected from the offspring soon after birth, and the outer cortical tissue was trimmed with a scalpel so as to obtain separate 'enriched' tissue fractions from the adrenal medulla (AM) and adrenal cortex (AC). As illustrated in Fig. 9A-C, western blot analysis revealed there was a significant increase in expression of Kir6.2 and HIF-2 α , and a decrease in expression of CAI and CAII, in the medullary-rich regions of glands taken from morphine-exposed pups relative to saline-exposed controls. Interestingly, this change in expression pattern for Kir6.2, HIF-2 α , and CAII was similar to that seen in opioid-treated MAH cells *in vitro*. On the other hand, the changes in expression pattern seen in the medullary region were not replicated in cortical-rich regions of glands taken from morphine-exposed pups. Notably, CAI and CAII expression was robust in the medullary-rich regions of control (saline-exposed) glands, but was almost absent from cortical-rich regions, suggesting minimal cross-contamination occurred during our attempts to separate cortical from medullary tissue. These data suggest that in this *in vivo* model, the morphine-induced changes in expression pattern were confined to the medullary chromaffin cells, which are the predominant cell type expressing opioid receptors in the adrenal gland (Salman *et al.*, 2013).

DISCUSSION

In a recent study we reported that chronic exposure of neonatal rat adrenomedullary chromaffin cells (AMCs) to μ - and δ -opioid receptor agonists *in vitro* blunts hypoxia and hypercapnia chemosensitivity via upregulation of K_{ATP} channels and downregulation of carbonic anhydrase (CA) I and CAII, respectively (Salman *et al.*, 2013). The goals of the present study were to obtain a more complete understanding of the underlying molecular mechanisms and signalling pathways involved. Using an immortalized, fetal-derived rat chromaffin (MAH) cell line as a model, we found that chronic opioid exposure led to increased expression of Kir6.2 subunit of the K_{ATP} channel, and decreased expression of CAII, the only one of the CA isoforms expressed in MAH cells. Both effects were mediated via PKA, and were dependent on the transcription factor, HIF-2 α , because they were abrogated in HIF-2 α -deficient MAH cells. Strong support for this schema was obtained from an *in vivo* model where adrenal tissue from pups born to morphine-exposed dams was probed for Kir6.2, CAI, CAII, and HIF-2 α expression. Interestingly, compared to saline-treated controls, adrenal tissue from morphine-exposed pups showed increased expression of Kir6.2 and HIF-2 α , and decreased expression of both CAI and CAII.

Role of HIF-2 α in opioid-induced regulation of Kir6.2 and CAII expression

One key factor involved in gene regulation in MAH cells following exposure to chronic μ - and δ -opioids was the transcription factor, HIF-2 α . Whereas the opioid-induced regulation of Kir6.2 and CAII was observed in wild type and transfected scrambled control MAH cells, it was absent in HIF-2 α -deficient MAH cells. Moreover, following opioid exposure there was a gradual increase in HIF-2 α accumulation in MAH cells that occurred along a time course similar to the

increase in Kir6.2 expression. The importance of HIF-2 α in this signalling pathway was further demonstrated using a ChIP assay, which revealed the direct binding of HIF-2 α to the promoter region of the Kir6.2 gene in opioid-treated MAH cells. These data suggest a molecular explanation for the increased expression of Kir6.2 subunit and increased K_{ATP} current density previously reported in primary neonatal rat adrenomedullary chromaffin cells (AMCs) exposed to chronic opioids *in vitro* (Salman *et al.*, 2012). In the latter study, the increased expression of K_{ATP} channels accounted largely for the opioid-mediated blunting of hypoxia sensitivity, which could be restored by simply adding the K_{ATP} channel blocker, glibenclamide, to the extracellular solution. Thus, the ability of chronic opioid exposure to blunt hypoxia sensing in chromaffin cells appears to be dependent on the induction of HIF-2 α , leading to the transcriptional upregulation of Kir6.2 and increased expression of functional K_{ATP} channels. The opening of these K_{ATP} channels in response to a fall in ATP concentration during hypoxia favors membrane hyperpolarization, and therefore a decreased sensitivity to the hypoxic stimulus.

Evidence that a non-hypoxic stimulus such as opioid exposure could lead to HIF induction has been obtained in a previous study on human SH-SY5Y neuroblastoma cells (Daijo *et al.*, 2011). These authors reported that chronic exposure of SH-SY5Y cells to μ - and δ -opioid receptor agonists led to the induction of HIF-1 α protein and an increase in HIF-1 target gene expression. While these data demonstrated a link between HIF and the biological effects of chronic opioid exposure, there are notable differences when compared with the present study. In particular, HIF-2 α protein (and not HIF-1 α) was the major HIF involved in the regulation of Kir6.2 and CAII in MAH cells and, moreover, its induction was not apparent at 24 hr exposure. By contrast, in SH-SY5Y cells the induction of HIF-1 α protein was apparent as early as 8 hr and peaked at 24 hr exposure (Daijo *et al.*, 2011). Though the fate of HIF-1 α in MAH cells is unknown in the present

study, it is noteworthy that in the adrenal tissue of morphine-exposed pups, we found that HIF-2 α was elevated (relative to saline-exposed controls) whereas HIF-1 α remained unchanged. Thus, it still remains plausible that at earlier time points after opioid exposure other target genes, specific to HIF-1, may be regulated in chromaffin cells, however, this idea remains to be tested. Alternatively, the opioid-induced regulation of HIFs may occur via the same opioid receptors but in a cell-type specific manner, depending on the intracellular signalling pathways. In this regard, chronic exposure of different cells to another non-hypoxic stimulus may lead to the differential regulation of HIF-1 α and HIF-2 α . For example, activation of nicotinic acetylcholine (ACh) receptor signalling pathways by exposure to chronic nicotine led to HIF-1 α accumulation in human small cell lung cancers (Zhang *et al.*, 2007). On the other hand, a similar exposure led to HIF-2 α accumulation in MAH cells (Salman *et al.*, 2012), though there was increased expression of the general HIF target gene, VEGF, in both studies.

A particularly novel aspect of the present study was the demonstration that the opioid-induced downregulation of CAII seen in control MAH cells was absent in HIF-2 α - deficient MAH cells. Functional CA activity is critical for the ability of neonatal AMCs to sense elevated CO₂ (hypercapnia), and CAII is one of only two carbonic anhydrases (CAs) expressed in these cells (Munoz-Cabello *et al.* 2005). To our knowledge this is the first demonstration that CAII expression can be regulated by HIF, and particularly by HIF-2 α . In this regard, HIF-1 α has been reported to act as a transcriptional repressor of a number of genes such as α -fetoprotein and LIFR under hypoxia, by direct binding to a reverse HRE (rHREs) located on the antisense strand (Narravula & Colgan, 2001; Mazure *et al.*, 2002; Jeong *et al.*, 2007). Interestingly, a search for a HRE in the promoter region of CAII gene revealed a putative rHRE (~1.7 kb upstream from the transcription initiation site) located on the antisense strand (Fig. 6B). This raises the attractive

possibility that the CAII gene may act as a novel transcriptional target of HIF-2 α , however, this requires further validation. It is also noteworthy that the tumor-associated CAs, i.e. CAIX and CAXII, represent a class of HIF-1 dependent genes that are strongly induced in tumor cell lines under hypoxia (Wykoff *et al.*, 2000); in that study a HRE was also identified on the antisense strand of CAIX. In addition, a recent study identified a large number of genes that were repressed by HIF-1 α in arterial endothelial cells under hypoxia though no specific pattern of HRE was identified (Manalo *et al.*, 2005). Therefore, further studies are required to clarify the mechanisms underlying the HIF-2 α dependent downregulation of the CAII gene as observed in the present study.

Role of protein kinase A in opioid-induced regulation of Kir6.2 subunit and CAII expression

Using inhibitors of three common protein kinases, we found that PKA (but not PKC or CaM kinase) activity was necessary for the opioid-induced regulation of Kir6.2 subunit and CAII expression in MAH cells. Because HIF-2 α induction was also necessary for this regulation, the question arose whether the requirement for PKA activation occurred upstream or downstream of HIF-2 α induction. Our results showed that PKA inhibition did not significantly affect HIF-2 α accumulation in opioid-treated MAH cells, suggesting that PKA activation was required downstream of (or in parallel with) HIF-2 α accumulation (Fig. 10). It remains to be determined whether PKA activation during opioid-induced signalling is involved in the transcriptional or posttranslational modification of Kir6.2 subunit and CAII expression. In a recent report, activation of μ - and δ -opioid receptors in SH-SY5Y neuroblastoma cells led to HIF-1 α accumulation via the PI3K/Akt/mTOR signalling pathway (Daijo *et al.*, 2011). It is tempting to speculate that at least part of the opioid receptor signalling in MAH cells is mediated via

activation of this pathway, given the evidence that HIF-2 α expression may also be regulated via a distinct mTOR complex (mTORC2) and Akt isoform (Akt2), as shown in renal carcinoma cells (Toschi *et al.*, 2008). However, further experiments are required to test this idea.

Effects of chronic morphine in utero on expression of Kir6.2, CAII and HIF-2 α in adrenal medulla vs cortex of affected neonates

It was of interest to test the predictions of the *in vitro* opioid-treated MAH cell model in a physiologically-relevant *in vivo* system. To this end, both medullary and cortical tissues from the adrenal glands of rat pups exposed to chronic morphine *in utero* were examined for the expression pattern of Kir6.2, CAI & II, and HIF-2 α . The rat adrenal gland expresses all three types of opioid receptors and these are restricted to the chromaffin cells of the medulla (Wittert *et al.*, 1996; Salman *et al.*, 2013). Interestingly, even though Kir6.2 is expressed in both adrenal cortex and medulla (Xu & Enyeart, 2001; Buttigieg *et al.*, 2009), there was a selective upregulation of Kir6.2 protein in the medullary region of morphine-exposed pups compared to saline-exposed controls. This is the expected result if an intact opioid receptor signalling pathway is required for Kir6.2 upregulation, and argues against a non-specific action of morphine following injections *in vivo*. Moreover, even though the MAH cell model allowed us to test only for the regulation of CAII following opioid exposure, morphine-exposed pups showed downregulation of both CAI and CAII expression. Not surprisingly, this downregulation was again confined to the medullary tissue, the only region of the gland where the two carbonic anhydrases (CAs) are known to be expressed (Munoz-Cabello *et al.*, 2005).

Similar to Kir6.2 subunit expression, we detected HIF-2 α protein in both medullary and cortical 'enriched' tissues of the adrenal gland. However, chronic morphine exposure led to a

selective accumulation of HIF-2 α in the medullary tissue only. This result, not only validated our *in vitro* MAH cell model, but once again emphasized the requirement for an intact opioid receptor signalling pathway for HIF-2 α induction, given that the cortical cells do not express μ - and δ -opioid receptors (Salman *et al.*, 2013). Additionally, the effects of chronic morphine on HIF accumulation were found to be restricted to HIF-2 α , for though HIF-1 α was expressed in the medulla its levels were not significantly affected. Generally, the importance of HIF-2 α function is thought to be tissue specific, and HIF-2 α appears to play a major role in cells of the sympathoadrenal lineage (Bishop *et al.*, 2008; Brown & Nurse, 2008; Brown *et al.*, 2009). Although the two transcription factors are paralogs, they seem to play distinct target-specific roles even when expressed in the same tissue. In fact, in studies using partially deficient HIF-1 α and HIF-2 α mice, a mutual antagonism between HIF-1 α and HIF-2 α was found to regulate intracellular redox status and hypoxia sensitivity of carotid body glomus cells and adrenomedullary chromaffin cells (Yuan *et al.*, 2013). Using the MAH cell model, we demonstrated that chronic opioids led to HIF-2 α accumulation, and that HIF-2 α deficiency prevented the opioid-induced upregulation of Kir6.2 and downregulation of CAII (Fig. 10). These observations suggest that the increased HIF-2 α accumulation seen in morphine-exposed neonatal medullary tissue likely led to the transcriptional upregulation of Kir6.2 and downregulation of CAII (and probably CAI as well) in that tissue.

CLINICAL SIGNIFICANCE

While our studies contributed to a general understanding of the role of opioid receptor signalling in the developmental regulation of hypoxia and hypercapnia sensitivity in chromaffin cells, they also have clinical significance. Prenatal exposure of pregnant mothers to opiates (e.g. heroin and

related illicit drugs) has been linked to higher rates of stillbirths and sudden infant death syndrome (SIDS) that are often characterized by impaired arousal during asphyxial challenges (Kato *et al.*, 2003; Burns *et al.*, 2010). Indeed, in the present study we noted that chronic morphine exposure *in utero* was associated with decreased litter size, and an increase in the percentage of dams delivering stillborns (Table 1), as reported in previous studies (Zagon & McLaughlin, 1977). In addition, infants born small for gestational age (SGA) have been proposed to have higher risk of becoming victims to SIDS (Getahun *et al.*, 2004). In this regard, morphine exposure *in utero* also resulted in a significantly higher proportion of offspring that were growth restricted (See proportion of SGA pups in Table 1). A recent study reports that use of opiates by pregnant mothers has climbed and that the number of neonates born addicted to painkiller medication (e.g. opioid pain relievers) has tripled in the past decades due to the rise in use and abuse of prescription pain medications (Patrick *et al.*, 2012). Because adrenal catecholamine release in response to perinatal asphyxia is important for proper arousal, the adverse effects of chronic opioid exposure on the chemosensing properties of chromaffin cells may well contribute to the increased susceptibility of drug-exposed neonates to SIDS. If so, further studies on these chromaffin cells should aid in the development of therapeutic strategies that could benefit the offspring of women who use opiates during pregnancy, whether for pain management or in cases of drug abuse.

ACKNOWLEDGMENT

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was supported by grants from the Natural Sciences and Engineering Research Council (NSERC) of Canada, and the Canadian Institutes of Health Research (CIHR).

Table 1: Effects of chronic morphine exposure on pregnancy outcomes

TABLE 1

Pregnancy outcomes	Saline (N=10)	Morphine (N=17)	p-value
Litter size (N)	13.0 ± 0.7	8.5 ± 0.9	0.004*
Litter weight (g)	79.4 ± 5.0	48.5 ± 4.9	<0.001*
Birth weight (g)	6.3 ± 0.1	6.0 ± 0.1	0.123
Proportion of SGA pups (%)	13.1	39.2	<0.0001*
Live birth index (%)	100	84.6 ± 6.0	0.025*
Proportion of dams delivering stillborns (%)	0	53	0.0088*
Sex ratio (M/F)	1.1 ± 0.1	2.1 ± 0.4	0.114

Abbreviations: SGA, Small for Gestational Age; F, female; M, male. * indicates $p < 0.05$.

Figure 1: Immunofluorescence staining of μ - and δ -opioid receptors in MAH cells.

MAH cell cultures were immunostained with antibodies against μ - and δ -opioid receptors, visualized with FITC-conjugated secondary antibodies. Corresponding phase contrast (upper left) and fluorescence (upper right) images of MAH cells, immunostained for μ -opioid (A,B) and δ -opioid (C,D) receptors. In control experiments for μ -opioid (lower; E,F) and δ -opioid (lower, G,H) receptor, the primary antibody was pre-incubated with excess antigen before application to the cells, followed by FITC-conjugated secondary antibody.

FIGURE 1

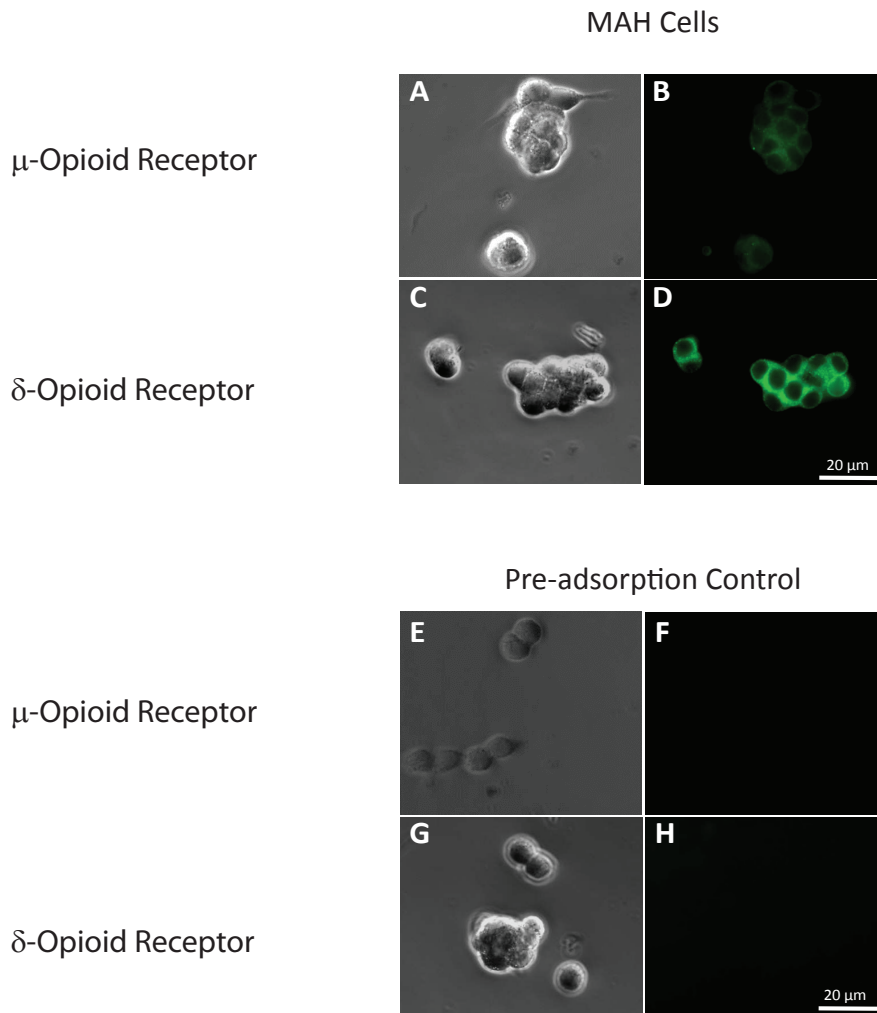
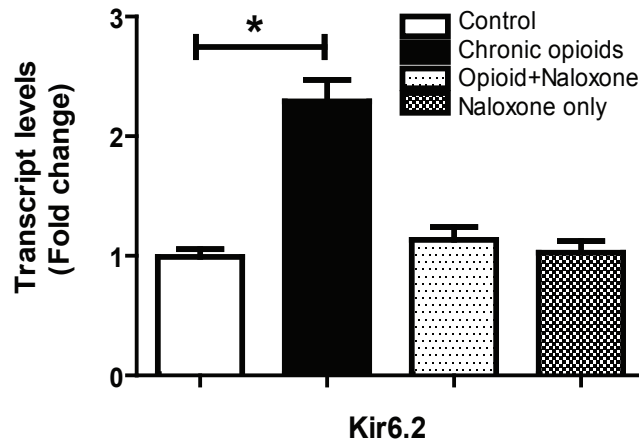


Figure 2: Quantitative RT-PCR (QPCR) and western blot analyses of K_{ATP} channel subunit Kir6.2 expression in MAH cells. (A) QPCR analysis of Kir6.2 mRNA in control MAH cells, in MAH cells cultured with combined μ - and δ - opioid agonists (2 μ M) \pm naloxone (2 μ M), and in MAH cells cultured with naloxone, for 7 days. Note significant upregulation of Kir6.2 transcript in opioid-treated cells ($*P < 0.05$; $n=4$ for each group); one-way ANOVA was used for multiple comparisons within groups. (B) Western blot analysis showing increased expression of Kir6.2 protein in opioid-treated MAH cells, and its prevention in presence naloxone; β -actin was used as an internal control.

FIGURE 2

A



B

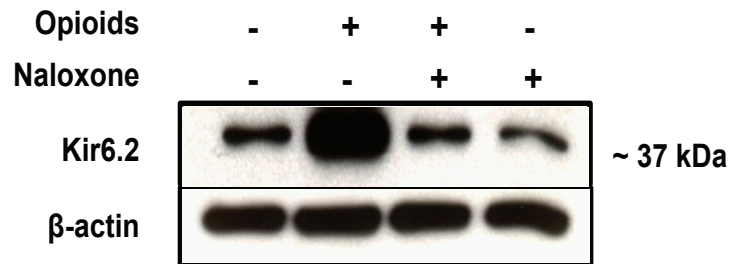
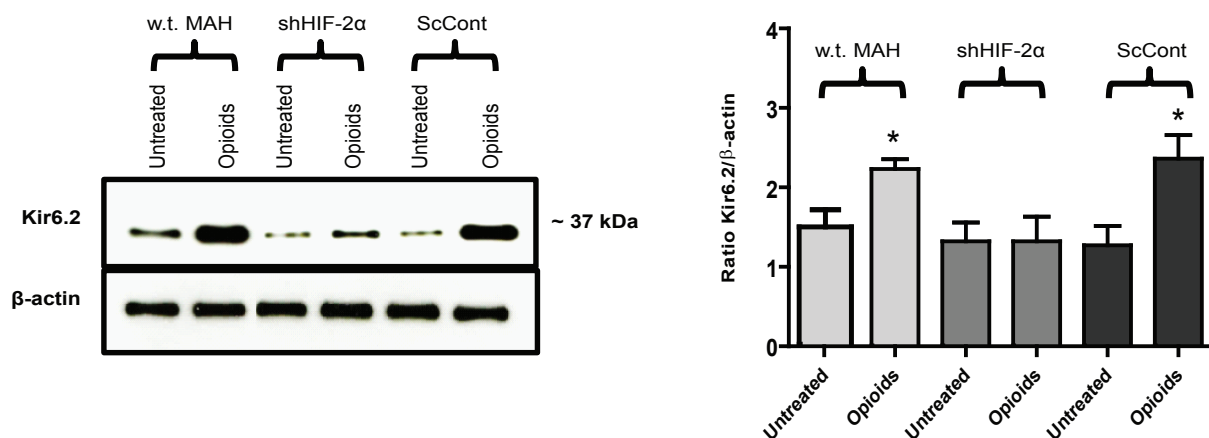


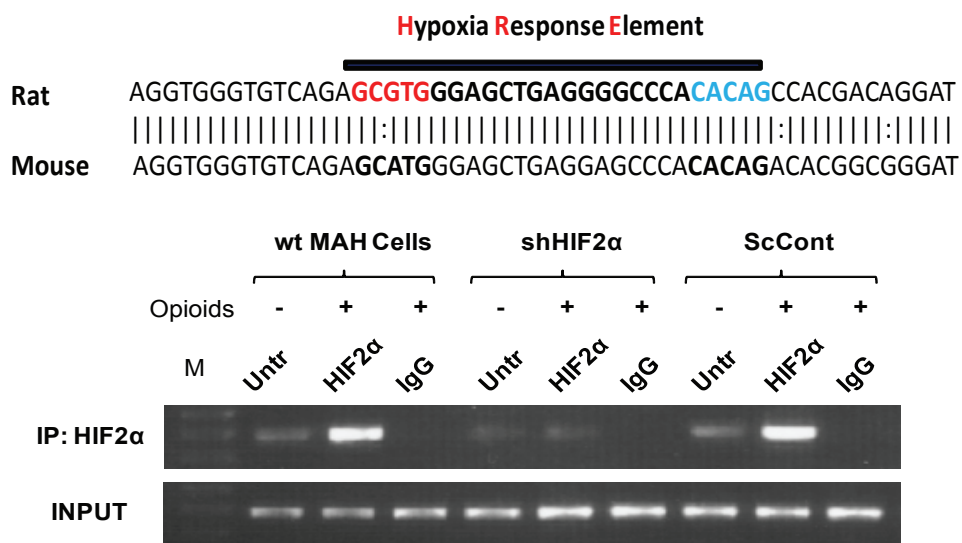
Figure 3: Effects of chronic opioid exposure on Kir6.2 subunit expression in HIF-2 α -deficient MAH cells. Western blot analyses of Kir6.2 subunit expression in w.t., HIF-2 α -deficient (shHIF-2 α) and scrambled control (ScCont) MAH cells cultured with combined opioid agonists (2 μ M) for 7 days (A; left) (n=3; Anova; * P < 0.05). Note absence of Kir6.2 subunit upregulation in HIF-2 α -deficient (shHIF-2 α) MAH cells. Densitometric analysis of changes in Kir6.2 subunit expression normalized β -actin (A; right). (B) Chromatin immunoprecipitation (ChIP) assay demonstrating binding of HIF-2 α to the promoter region of Kir6.2 gene in opioid-treated MAH cells. Note hypoxia response element (HRE) within the promoter region of rat and mouse Kir6.2 gene (upper); the HIF core site (GCGTG) spans nucleotides -1087 to -1083 and HIF ancillary site (CACAG) spans nucleotides -1065 to -1061. Lysates from untreated control (Untr) and opioid-treated wild type (w.t.), shHIF2 α , and ScCont MAH cells were subjected to ChIP assay using a HIF-2 α polyclonal antibody (lower). PCR analysis was performed using a primer pair designed to span the putative HRE upstream of the promoter region. Technical controls include a ChIP performed using non-specific IgG monoclonal antibody (IgG) and a starting material control (Input). (C) VEGF mRNA expression as determined by Q-PCR analysis in control (untreated) and opioid-treated MAH cells (n=3). Data are expressed as means \pm SEM (n = 3; Mann-Whitney U -test; * P < 0.05).

FIGURE 3

A



B



C

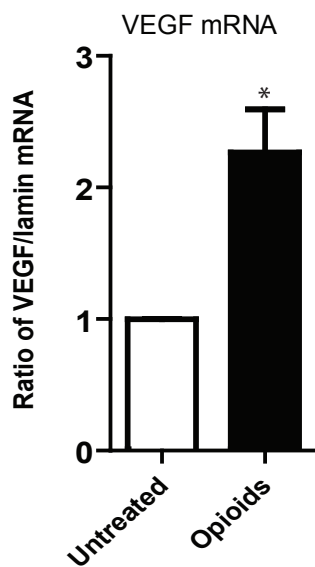


Figure 4: Effects of chronic opioid exposure on Kir6.2 and HIF-2 α expression in MAH cells.

(A) Western blot analysis of HIF-2 α accumulation in MAH cells cultured with combined μ - and δ - opioid agonists (2 μ M) \pm naloxone (2 μ M), or with naloxone only (2 μ M), for 7 days. (B) Time-dependent HIF-2 α and Kir6.2 protein expression in MAH cells exposed to combined opioids (2 μ M) for 24 hr, 3 days, and 7 days in culture. β -actin was used as loading control for cytoplasmic extracts in the case of Kir6.2, and TATA-Binding Protein (TBP) for nuclear extracts in the case of HIF-2 α . Results show progressive increase in Kir6.2 expression that parallels HIF-2 α accumulation following chronic opioids; for both Kir6.2 and HIF-2 α the increase is significant at 3 and 7 days (B,C). (C) Densitometric analysis demonstrating the fold induction in Kir6.2 subunit expression and HIF-2 α accumulation normalized to loading control. Data are expressed as means \pm SEM (n = 3; Anova; * P < 0.05).

FIGURE 4

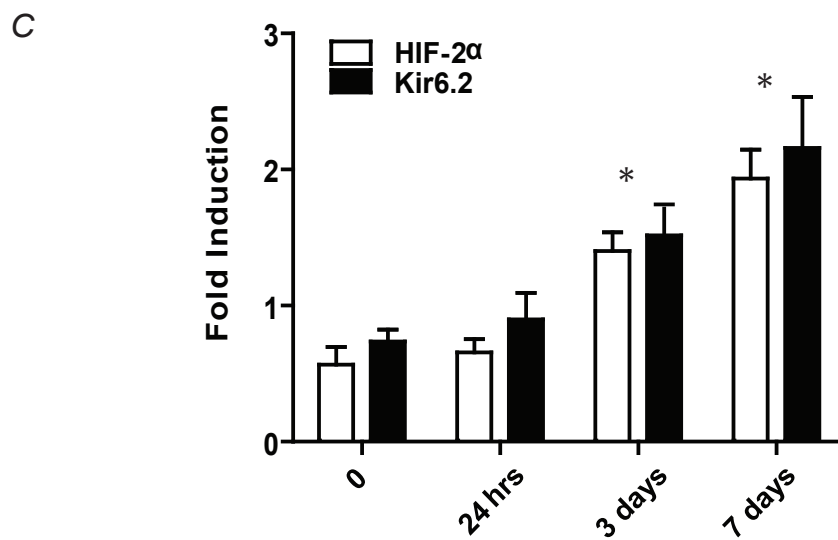
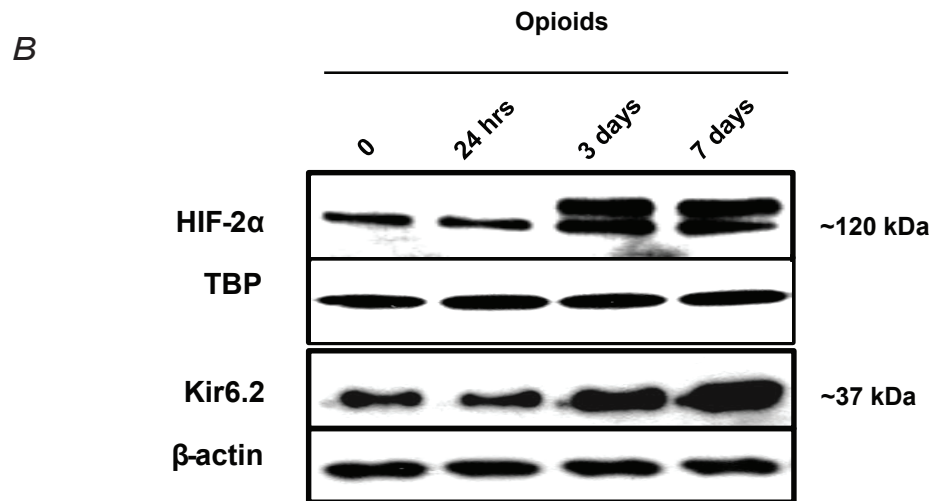
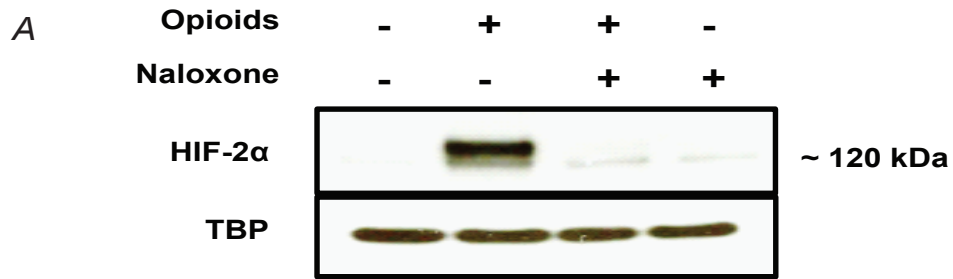
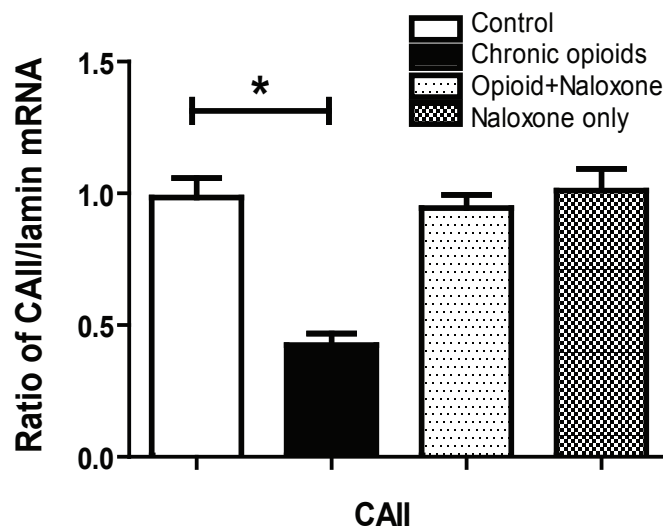


Figure 5: Effects of chronic opioid exposure on carbonic anhydrase II (CAII) expression in MAH cells. (A) mRNA expression analysis of CAII in control MAH cells, in MAH cells cultured with combined μ - and δ - opioid agonists (2 μ M) \pm naloxone (2 μ M), and in MAH cells cultured with naloxone (2 μ M) only, for 7 days. $*P < 0.05$, $n=3$ for each group. One-way ANOVA was used for multiple comparisons within groups. (B) Western blot analysis showing expression of CAII protein in MAH cells grown under similar conditions. β -actin was used as an internal control. Note naloxone-sensitive increase in CAII expression in MAH cells following chronic opioids in A and B.

FIGURE 5

A



B

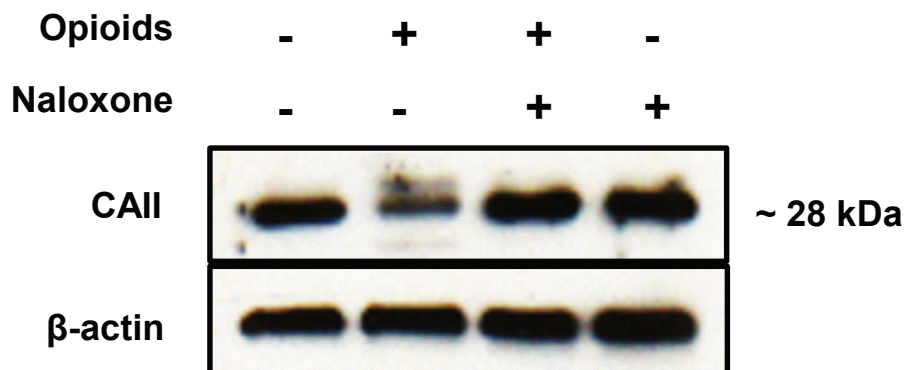


Figure 6: Effects of HIF-2 α -deficiency on expression of carbonic anhydrase II (CAII) expression in MAH cells. (A, upper) Western blots of CAII protein expression in control MAH cells, HIF-2 α -deficient (shHIF2 α) MAH cells, and scrambled control (ScCont) MAH cells, cultured with or without combined opioid agonists (2 μ M) for 7 days. Lower histogram (A) shows densitometric analysis of CAII protein expression normalized to β -actin under the various conditions indicated. Note the opioid-mediated downregulation of CAII is absent in HIF-2 α - deficient MAH cells. Data are expressed as means \pm SEM (n = 3; Anova; * P < 0.05). (B) Putative hypoxia response element (HRE) within the promoter region of CAII gene. The HIF core site (ACGTG) spans nucleotides -1774 to -1769 and HIF ancillary site (CACGT) spans nucleotides -1793 to -1788. Note the putative HRE is located on the antisense strand.

Figure 7: Effects of protein kinase inhibitors on expression of Kir6.2, CAII and HIF-2 α in MAH cells following chronic opioid exposure. (A) Western blot analysis showing the effects of protein kinase inhibition on Kir6.2 expression in opioid-treated MAH cells. Inhibitors of protein kinase A (H-89) (2 μ M), but not inhibitors of protein kinase C (GF109203X) (2 μ M) or CaM kinase (KN-62) (3 μ M), prevented the upregulation of Kir6.2 in opioid-treated cells. Densitometric analysis of Kir6.2 subunit expression normalized to β -actin is shown in histogram (right). A similar protocol was used to study the effects of protein kinase inhibitors on CAII expression relative to β -actin (B), and HIF-2 α accumulation relative to TBP (C), in opioid-treated MAH cells. Note the PKA inhibitor (H-89) prevented the opioid-induced downregulation of CAII expression, but not HIF-2 α accumulation, in MAH cells. Data are expressed as means \pm SEM (n = 3; Anova; * P < 0.05).

FIGURE 7

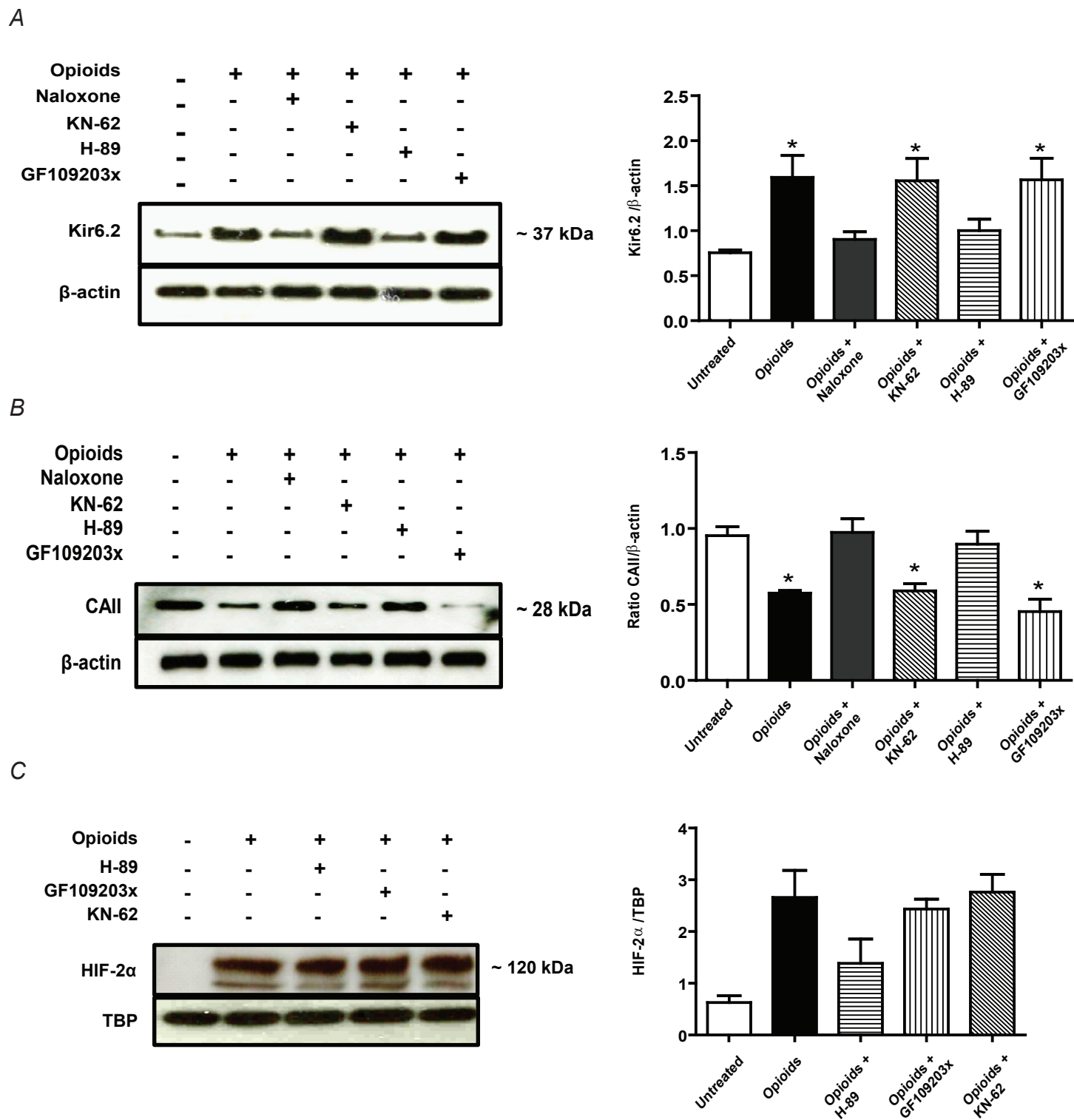


Figure 8: Effects of chronic opioids on hypoxia-evoked ATP release from MAH cells.

Bioluminescence recordings expressed as relative light units (RLU) were normalized to control (Normoxia). Chemostimulation with hypoxia ($PO_2 \sim 15\text{--}20$ mmHg) caused a significant (Anova; $*P < 0.01$) increase in extracellular ATP levels in control MAH cells (left), and the effect was reversible after a return to a normoxic solution (recovery). Following exposure of MAH cells to chronic opioids for ~ 1 week *in vitro*, the effect of hypoxia on ATP release was blunted; co-incubation of opioids with naloxone prevented this blunting effect. Inset (right) shows a plot of normalized RLU values (\pm SEM) versus ATP concentration.

FIGURE 8

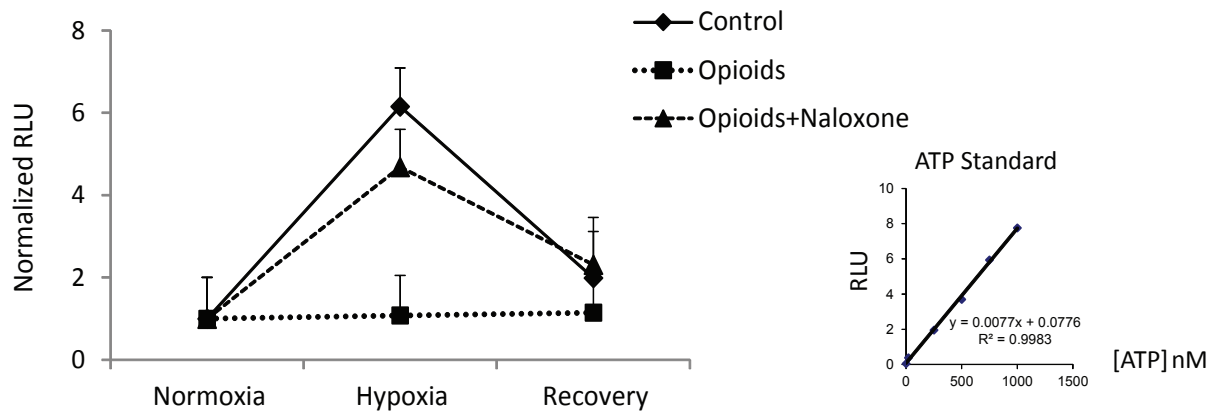


Figure 9: Effects of maternal morphine injections on the expression of K_{ATP} channel subunit (Kir6.2), carbonic anhydrase (CAI & II) enzymes and hypoxia inducible factors (HIFs) in adrenal gland tissues of affected neonates. Western blot analyses of K_{ATP} channel subunit, Kir6.2 (A), CAI & II (B), and HIF-1 α & HIF-2 α (C) expression in adrenal medulla (AM) and adrenal cortex (AC) of saline- and morphine-exposed rat pups. Note the increased Kir6.2 subunit and reduced CAI and CAII expression (relative to β -actin) in AM, but not AC, of morphine-exposed pups. Also, note the selective increase in HIF-2 α , but not HIF-1 α , accumulation (relative to TBP) in AM of morphine-exposed pups as shown in C. Data are expressed as means \pm SEM (n = 3; Anova; * $P < 0.05$).

FIGURE 9

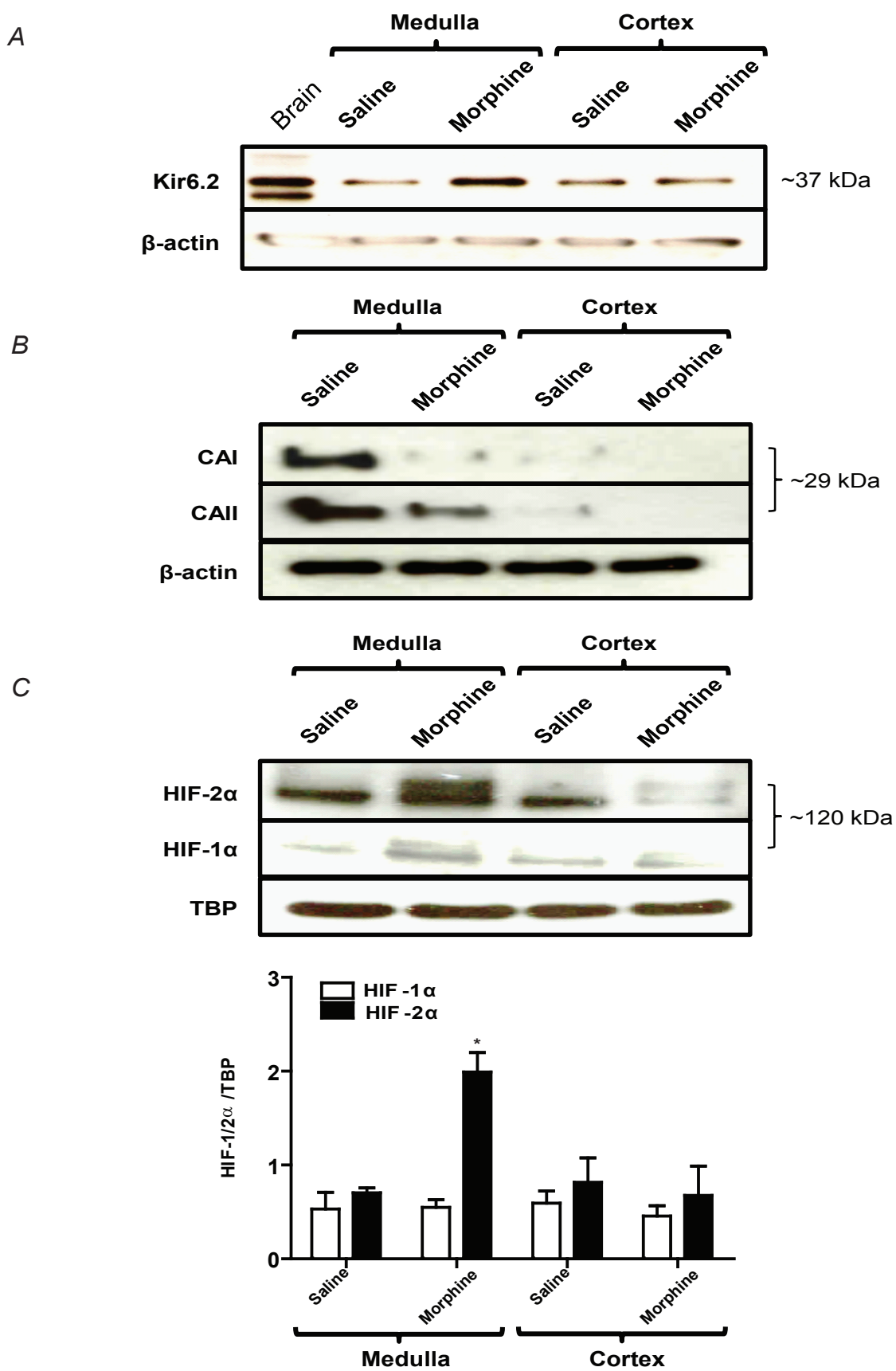
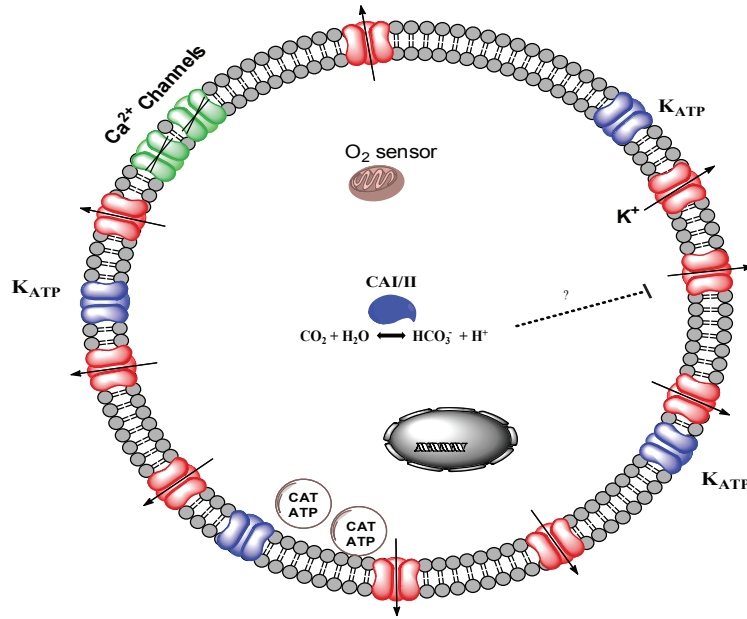
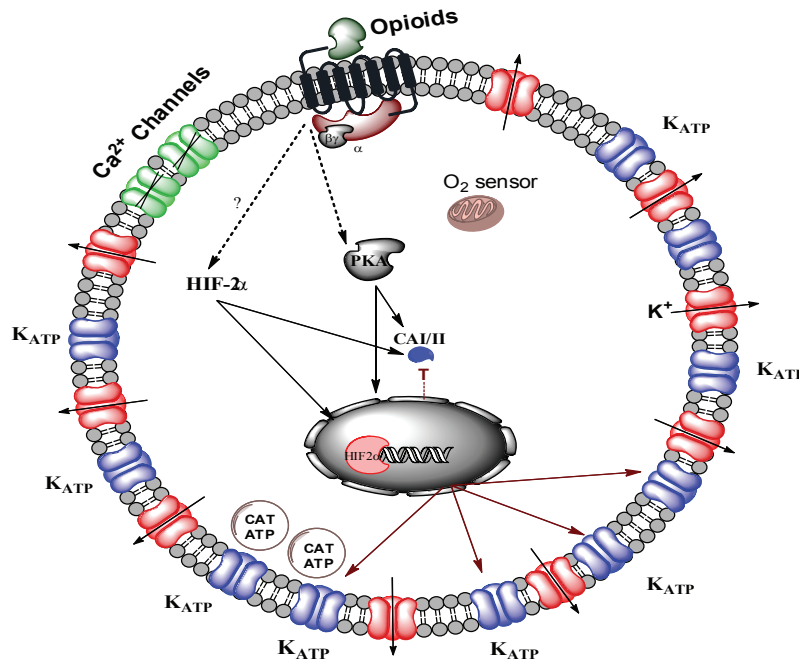


Figure 10: Proposed signalling pathway for chronic opioid-induced regulation of hypoxia and hypercapnia sensitivity in neonatal chromaffin cells. In neonatal (untreated) chromaffin cells, hypoxia causes inhibition of Ca^{2+} -dependent (SK, BK) K^+ and K_v currents, and simultaneous activation K_{ATP} current, resulting in membrane depolarization, voltage-gated Ca^{2+} entry and CAT release. The depolarization in response to hypercapnia is dependent on carbonic anhydrase (CA) I and/or II activity. Opioid-treated cells on the other hand are no longer sensitive to hypoxia or hypercapnia due to the opioid-induced upregulation of K_{ATP} channels and downregulation of CA I & II, respectively. The regulation of K_{ATP} and (at least) CAII expression is dependent on PKA activation and HIF-2 α accumulation.






FIGURE 10



Neonatal AMCs
(Low O₂ - high CO₂ -sensitive)



Opioid-treated AMCs
(Low O₂ - high CO₂ -insensitive)

-  Opioid receptor
-  K_{ATP} Channel
-  SK, BK & K_v Channels
-  V-G-Ca²⁺ Channel
-  Carbonic Anhydrase I/II (CAI/II)

CHAPTER 5: GENERAL DISCUSSION

This thesis addressed the cellular and molecular mechanisms involved in the ontogeny of low O₂ (hypoxia) and high CO₂ (hypercapnia) chemosensitivity of rat adrenomedullary chromaffin cells (AMCs), and particularly the role of nicotinic and opioid receptor signalling. These asphyxial stressors are experienced by the newborn during the stages of labor and birth, and occur as a result of uterine contractions (Lagercrantz & Slotkin, 1986). Sensitivity of AMCs to these stressors is important for the neonate as it triggers catecholamine (CAT) secretion, which is a critical event for a number of physiological adaptations that ensure proper transition to extrauterine life. These adaptations include maintenance of cardiac contraction and preparation of lungs for air breathing. In addition, soon after birth, the neonate experiences apneic episodes (resulting from intermittent cessation of breathing) during which CAT release from AMCs in response to asphyxial stressors is essential for maintaining homeostasis (Lagercrantz & Bistoletti, 1977; Seidler & Slotkin, 1985; Lagercrantz & Slotkin, 1986; Slotkin & Seidler, 1988; Stokowski, 2005; Hillman *et al.*, 2012). However, these ‘direct’ responses to asphyxial stimuli in AMCs are lost postnatally following the development of splanchnic innervation of the adrenal medulla. Thus, in juvenile and adult animals, CAT secretion from the adrenal gland during stress is ‘neurogenic’ and is mediated principally via cholinergic transmission at nicotinic synapses between splanchnic nerve terminals and AMCs (Lagercrantz & Bistoletti, 1977; Lagercrantz & Slotkin, 1986; Seidler & Slotkin, 1986; Slotkin & Seidler, 1988; Munoz-Cabello *et al.*, 2005; Rico *et al.*, 2005). Denervation of the adrenal gland results in a gradual return of the ‘direct’ hypoxic chemosensitivity (Slotkin & Seidler, 1988; Levitsky & Lopez-Barneo, 2009), suggesting that the ontogeny of O₂ sensitivity in AMCs is under neural control. Moreover, responses of AMCs to additional stressors such as hypoglycemia also

appear to be suppressed postnatally following splanchnic innervation (Livermore *et al.*, 2011; Livermore *et al.*, 2012). Therefore, it was hypothesized that maturation of splanchnic innervation plays a role in the developmental regulation of chromaffin cell responses to stressors such as hypoxia, hypercapnia, and hypoglycemia. However, the underlying mechanisms were poorly understood when this thesis began.

This thesis has made novel contributions towards our understanding of the cellular and molecular mechanisms involved in the regulation of the direct O₂ and CO₂ chemosensitivity in developing AMCs. In addition to identifying key contributions from nicotinic ACh and opioid receptor signalling pathways, this thesis also uncovered novel roles of the transcription factor HIF-2 α , in the regulation of chemosensitivity. A combination of both *in vitro* and *in vivo* models were employed during my studies. For most of the cellular and functional studies, primary AMCs were isolated from neonatal rat pups and exposed to nicotinic ACh or opioid receptor agonists (\pm antagonists) in culture for ~7 days. For studies of the underlying molecular and intracellular signalling mechanisms, use of the immortalized fetal rat adrenomedullary chromaffin (MAH) cell line proved to be advantageous. This line shares similar chemosensitive properties to primary neonatal AMCs and, additionally, both HIF-2 α -deficient (>90% knockdown) and scrambled control MAH cell lines were readily available in our laboratory (Brown *et al.*, 2009).

The *in vivo* models used in this thesis involved isolation of neonatal adrenal glands from rat pups exposed to either chronic nicotine or morphine *in utero* (Fearon *et al.*, 2002; Buttigieg *et al.*, 2008b). Chapter 2 examined the role of the transcription factor, HIF-2 α , in the nicotine-induced upregulation of K_{ATP} channel expression in chromaffin cells using both *in vivo* and *in vitro* models. Chapter 3 provided evidence for the contribution of chronic stimulation of postsynaptic μ - and δ -opioid receptors to the suppression of the direct O₂ and CO₂ chemosensitivity in primary neonatal

chromaffin cells using an *in vitro* model. Chapter 4 identified molecular mechanisms involved in the chronic opioid-induced suppression of the hypoxic and hypercapnic sensitivity in chromaffin cells and the underlining role of HIF-2 α in the signalling pathway using both *in vivo* and *in vitro* models. Collectively, the findings in the three data chapters of this thesis showed: (i) a common HIF-2 α -dependent upregulation of K_{ATP} channel expression as the main contributor to the nicotine- and opioid-induced blunting of hypoxic sensitivity; and (ii) a HIF-2 α -dependent downregulation of carbonic anhydrase (CA) I and II as the main contributor to the opioid-induced blunting of hypercapnic sensitivity.

Role of postsynaptic opioid receptor stimulation in regulating the direct O₂ and CO₂/H⁺ chemosensitivity in neonatal rat AMCs

As noted earlier, chemosensitivity in neonatal AMCs is lost postnatally following maturation of splanchnic innervation, and returns following denervation of adult AMCs. This led to the hypothesis that tonic release of neurochemicals from the splanchnic nerve, stimulated postsynaptic receptors on chromaffin cells, leading to the suppression of direct asphyxial chemosensitivity. In addition to the neurotransmitter acetylcholine (ACh), splanchnic nerve terminals release a few other chemicals including pituitary adenylate cyclase activating polypeptide (PACAP) (Kuri *et al.*, 2009), histamine (Holgert *et al.*, 1998), vasoactive intestinal peptide (VIP) (Yoshikawa *et al.*, 1990) and opiate peptides (Kobayashi *et al.*, 1985). Buttigieg *et al.*, (2009) tested the effects of chronically stimulating the nicotinic ACh receptors (nAChRs) on the direct O₂ and CO₂/H⁺ chemosensitivity in neonatal rat AMCs; they found that chronic nicotine, *in vitro* and *in utero*, selectively blunted the direct O₂ chemosensitivity, but not the CO₂ chemosensitivity (Buttigieg *et al.*, 2008a). This suggested that other factors may account for the postnatal loss of CO₂

chemosensitivity in the developing AMCs. Among these are opioid peptides that are present in splanchnic nerve terminals (Holgert *et al.*, 1998), and they are known to activate opioid receptors on AMCs (Bunn *et al.*, 1988; Kimura *et al.*, 1988; Wittert *et al.*, 1996; Keating *et al.*, 2004). This hypothesis was tested in Chapter 3 of this thesis, where it was shown that chronic treatment of neonatal rat AMCs with μ - and/or δ -opioid agonists (2 μ M) *in vitro* suppressed both hypoxia and hypercapnia chemosensitivity. In contrast, treatment with the κ -opioid agonist had no effect on hypoxia or hypercapnia sensitivity in neonatal rat AMCs. The blunting effects of chronic opioid exposure on hypoxia and hypercapnia chemosensitivity were prevented during co-incubation with the general opioid receptor antagonist, naloxone (2 μ M), demonstrating specificity of signalling pathway through opioid receptors.

The above data contrast with a previous study showing that *acute* exposure of neonatal rat adrenal medulla to opioid peptides did not prevent hypoxia-induced CAT secretion (Rico *et al.*, 2005). In the present study, however, chronic exposures (~1 week) to opioid agonists were used as this is more likely to mimic the time-dependent effects of splanchnic innervation that occurs gradually over the first 1-2 postnatal weeks (Seidler & Slotkin, 1985, 1986). In addition, opioid agonists were omitted from the bathing solution when the electrophysiological experiments in Chapter 3 were performed to test for chemosensitivity. Therefore, our chronic experiments are likely to be a more realistic model for mimicking the effects of splanchnic innervation. In summary, these experiments support the hypothesis and provide a general model to explain the developmental loss of direct O₂ and CO₂ chemosensitivity in AMCs following splanchnic innervation.

Fundamental mechanisms involved in nicotine- and opioid-induced suppression of the direct O₂ chemosensitivity in chromaffin cells

Responsiveness to hypoxia in chromaffin cells involves inhibition of O₂-sensitive K⁺ channels (e.g. SK channels) which is the primary event initiating membrane depolarization. Membrane depolarization signals intracellular Ca²⁺ increase through voltage-gated Ca²⁺ channels and CAT secretion. At the same time, mitochondrial inhibition results in a fall in intracellular ATP levels and activation of ATP-sensitive K⁺ (K_{ATP}) channels. Activation of these channels is thought to act as a 'brake' that modulates cell excitability and limits CAT secretion (Nurse *et al.*, 2009). As mentioned earlier, both chronic nicotine and chronic opioids contribute to the blunting of hypoxic chemosensitivity in neonatal rat AMCs (Buttigieg *et al.*, 2008a; Salman *et al.*, 2013). This blunting effect can occur via a variety of molecular mechanisms including: modulation of the oxygen sensor, modulation in the expression of O₂-sensitive K⁺ (i.e. SK, BK, K_v and K_{ATP} channel) and/or voltage-gated Ca²⁺ (e.g. T-type) channels involved in regulating hypoxic sensitivity. During hypoxia, the depolarization induced by K⁺ channel (i.e. SK, BK and K_v) inhibition predominates over the hyperpolarization induced by K_{ATP} channel activation, leading to a net depolarization and CAT secretion. If this balance is disturbed (e.g. due to relative changes in channel expression), it would accordingly modulate the effects of hypoxia on CAT secretion. For example, if the effect of K_{ATP} channel-induced hyperpolarization predominates over the depolarization induced K⁺ channel inhibition, this would result in a net hyperpolarization that ultimately suppresses hypoxic sensitivity. This mechanism has been previously proposed to explain the chronic nicotine-induced blunting of hypoxia chemosensitivity in neonatal AMCs (Buttigieg *et al.*, 2009). Similarly, chronic opioid exposure causes an increase in the magnitude of the K_{ATP} current density, leading to a blunting of hypoxia sensitivity in neonatal AMCs (shown in Chapter 3). The fact that responses to

hypoxia were rescued in opioid-treated AMCs by blocking K_{ATP} channel activation with glibenclamide suggests that the 'defect' is predominantly downstream of the oxygen sensor, which appears to be functionally intact. These observations, however, do not rule out a potential contribution from the T-type Ca^{2+} channel, which has been reported to be critically involved in the hypoxia-induced CAT secretion from neonatal rat adrenal slices (Levitsky & Lopez-Barneo, 2009).

The molecular studies in Chapter 3 demonstrated an increased expression of the K_{ATP} channel subunit, Kir6.2, in opioid-treated neonatal AMCs relative to untreated controls. These data supported the electrophysiological studies showing an increased K_{ATP} current density in these opioid-treated cells (Chapter 3; NOTE chapter 4 deals with MAH cell exposures).

The *in vitro* studies in Chapters 2 and 3 demonstrated that the upregulation of the K_{ATP} channel subunit, Kir6.2, is a common molecular mechanism for blunting hypoxia sensitivity in chromaffin cells exposed chronically to either nicotine or opioids. The validity of this conclusion was further confirmed with the use of *in vivo* models where adrenomedullary tissue or primary neonatal AMCs were obtained from rat pups chronically exposed throughout gestation to either nicotine or morphine *in utero*. Interestingly, western blot analysis showed an increased K_{ATP} channel subunit (Kir.6.2) expression in medullary (but not cortical) tissues excised from adrenal glands of both nicotine- and opioid-exposed pups, when compared to saline-treated controls (Chapters 2 and 4). Moreover, it was previously demonstrated that pre-treatment of nicotine-treated pups with the K_{ATP} channel blocker, glibenclamide, reduced their mortality rate following exposure to a hypoxic challenge (Buttigieg *et al.*, 2009). These observations are significant since they highlight the important role of K_{ATP} channel regulation in the nicotine- and opioid-induced blunting of hypoxic chemosensitivity in neonatal AMCs.

Signalling mechanisms leading to K_{ATP} channel upregulation in nicotine- and opioid-treated AMCs: Role of the transcription factor HIF-2 α and protein kinase activation

Simulation of either nAChRs (Buttigieg *et al.*, 2009) or opioid receptors (Chapter 3) activates signalling cascades that ultimately lead to upregulation of K_{ATP} channel expression in neonatal rat AMCs. In Chapter 4, the underlying molecular mechanisms involved in these signalling cascades were explored using an immortalized, fetal-derived rat chromaffin (MAH) cell line as a model. One important key regulator in both nicotine and opioid signalling pathways was the transcription factor, hypoxia-inducible factor (HIF)-2 α . HIFs are a family of transcription factor complexes consisting of an α subunit (e.g. HIF-1 α or HIF-2 α) and a β subunit (HIF-1 β) that are involved in regulating oxygen homeostasis (Semenza, 2009). Historically, they have been known to be induced by hypoxic stimuli, however, it has recently been appreciated that they can also be induced by ‘non-hypoxic’ stimuli such as vasoactive hormones (Richard *et al.*, 2000), thermal and mechanical stress (Kim *et al.*, 2002), and inflammation (Taylor, 2008).

The involvement of HIF-2 α in the signalling cascade leading to K_{ATP} channel upregulation during nicotine exposure was first demonstrated using a HIF-2 α -deficient MAH cell line (shMAH) (Brown *et al.*, 2009; Buttigieg *et al.*, 2009). In these studies, though chronic nicotine was found to be effective in blunting hypoxia sensitivity in wild type and scrambled control MAH cells, it was ineffective in HIF-2 α -deficient MAH cells (Buttigieg *et al.*, 2009). Molecular studies in Chapter 2 revealed that chronic nicotine induced a gradual, progressive increase in HIF-2 α accumulation that occurred in parallel with the increase in K_{ATP} channel subunit, Kir6.2, in MAH cells. These effects of nicotine were sensitive to the $\alpha 7$ nicotinic acetylcholine receptor ($\alpha 7$ nAChR) blocker, α -bungarotoxin, suggesting the involvement of $\alpha 7$ nAChRs in the signalling pathway. Moreover,

the effect was specific to the Kir6.2 subunit of the K_{ATP} channel, as the SUR1 subunit expression was unaffected. Further investigation using a ChIP assay revealed the direct binding of HIF-2 α to a hypoxia response element (HRE) located in the promoter region of the gene encoding the K_{ATP} channel subunit, Kir6.2 (Figure 1).

Interestingly, the use of inhibitors of general kinases revealed the requirement of PKC and CaMK activation for nicotine-induced upregulation of Kir6.2 subunit (Buttigieg *et al.*, 2009). Collectively, these data suggested the nicotine-induced signalling cascade involved $\alpha 7$ nAChR, kinase activation (PKC and CaMK), and HIF-2 α -dependent transcriptional upregulation of the K_{ATP} channel subunit, Kir6.2 (Chapter 2; (Salman *et al.*, 2012). The relationship between $\alpha 7$ nAChR, HIF-2 α , and protein kinase activation in this pathway is currently unknown. However, we speculate that changes in intracellular Ca^{2+} homeostasis via $\alpha 7$ nAChR are involved. In PC 12 cells, intermittent hypoxia is known to activate Ca^{2+} -dependent protease pathways that lead to HIF-2 α degradation (Prabhakar *et al.*, 2009). The status of $\alpha 7$ nAChRs during the chronic experiments described in Chapter 2 is uncertain, however, desensitization of nAChRs is generally a common feature of chronic nicotine exposure (Albuquerque *et al.*, 2009). It is possible that chronic nicotine induced a loss of $\alpha 7$ nAChR function, leading to the progressive increase in HIF-2 α accumulation in chromaffin cells, as suggested for HIF-1 α in a previous study on nicotine-treated SH-SY5Y neuroblastoma cells (Ridley *et al.*, 2002). However, it remains to be tested whether PKC and CaMK involvement occurs upstream or downstream of HIF-2 α accumulation in this signalling pathway.

Similar to chronic nicotine, exposure of perinatal AMCs to chronic μ - and δ -opioid led to a slow progressive accumulation of HIF-2 α that paralleled the increase in Kir6.2 expression. Moreover, ChIP analysis in Chapter 4 demonstrated the direct binding of HIF-2 α to the HRE located in

promoter region of Kir6.2 gene in opioid-treated MAH cells. This study suggested that HIF-2 α -dependent upregulation of K_{ATP} channel expression could explain the blunting effects of chronic opioids on hypoxia chemosensitivity in primary rat neonatal AMCs, as described in Chapter 3 (see Figure 1). The idea that chronic opioid exposure could induce HIF accumulation was previously proposed in human SH-SY5Y neuroblastoma cells (Daijo *et al.*, 2011). In that study, exposure to μ - and δ -opioid agonists led to an increase in HIF-1 α accumulation that was apparent after 8 hours. In the present study however, exposure to μ - and δ -opioid agonists triggered accumulation of a different HIF isoform, i.e. HIF-2 α , and the effect was significant only after 3 days of exposure (Chapter 4). Although the fate of HIF-1 α in opioid-treated MAH cells is unknown, it is noteworthy that HIF-2 α , but not HIF-1 α , was upregulated in adrenomedullary tissue of morphine-exposed rat pups (see later). Therefore, it is possible that the effects of opioids are cell-type specific, depending on the particular factors and second messengers expressed in those cells.

Similarly, exposure to chronic nicotine (a non-hypoxic stimulus) led to HIF-2 α accumulation in chromaffin cells (Chapter 2), whereas the same stimulus led to HIF-1 α accumulation in human small cell lung cancers (Zhang *et al.*, 2007). It is noteworthy that the effects of HIF-2 α accumulation in the nicotine- and opioid-induced signalling pathways were not limited to the upregulation of Kir6.2 subunit only. Western blot analysis revealed that another common HIF target, i.e. vascular endothelial growth factor (VEGF), was also upregulated in both nicotine- and opioid-treated MAH cells (Chapters 2 and 4), further confirming the increase in transcriptional activity of HIF-2 α under these conditions.

The use of protein kinase inhibitors demonstrated that PKA activation (but not PKC or CaMK) was required for the opioid-induced Kir6.2 upregulation in MAH cells. These experiments identified another signalling component in this pathway. Further investigations showed that PKA

inhibition had no effect on HIF-2 α accumulation, suggesting that PKA activity in this signalling pathway is required downstream of (or in parallel to) HIF-2 α induction (Figure 1). A recent study showed that HIF-1 α is induced by μ - and δ -opioid receptor activation in SH-SY5Y neuroblastoma cells via activation of PI3K/Akt/mTOR signalling pathway (Daijo *et al.*, 2011). It is possible that opioids activate a similar signalling pathway (e.g. distinct mTOR complex (mTORC2) and Akt isoform (Akt2) specific for HIF-2 α (Toschi *et al.*, 2008)) leading to HIF-2 α accumulation in MAH cells. This possibility, however, remains to be tested.

Molecular mechanisms involved in opioid-induced suppression of the direct CO₂ chemosensitivity in chromaffin cells

As discussed earlier, chronic opioid exposure blunts both hypoxia and hypercapnia sensitivity in perinatal AMCs, thereby mimicking the effects of innervation. Hypercapnia sensitivity is mediated by activation of a resting cationic conductance that triggers membrane depolarization, as well as the inhibition of voltage-gated K⁺ channels that contributes to prolongation of the action potential and increased CAT secretion (Munoz-Cabello *et al.*, 2005). These responses are prevented in the presence of the carbonic anhydrase (CA) inhibitor, methazolamide (Munoz-Cabello *et al.*, 2005). Neonatal rat AMCs express only two of the seven carbonic anhydrase (CA) isoforms, i.e. CA I and II (Munoz-Cabello *et al.*, 2005; Buttigieg *et al.*, 2008a). Chronic exposure of primary neonatal rat AMCs to μ - and δ -opioids blunted the hypercapnia-induced receptor potential and inhibition of outward K⁺ current (Chapter 3). Consistent with the postnatal downregulation of CAI and II expression in the rat adrenal medulla following splanchnic innervation (Munoz-Cabello *et al.*, 2005), molecular analyses in Chapter 3 showed a marked downregulation in CAI and II expression in opioid-treated neonatal AMCs. Interestingly, medullary tissues from adrenal glands isolated

from *in utero* morphine-exposed pups showed decreased expression of CAI and CAII relative to saline-treated controls (see below). It is noteworthy that the expression of both CAI and CAII was not affected in primary neonatal AMCs isolated from nicotine-exposed pups (Buttigieg et al., 2008a). This is consistent with the idea that, in contrast to opioid innervation, nicotinic cholinergic innervation is not involved in the regulation of CO₂ sensitivity in postnatal rat AMCs.

Molecular mechanisms leading to carbonic anhydrase (CA) II downregulation in opioid-treated chromaffin cells: Role of transcription factor HIF-2 α and protein kinase activation

An interesting and novel finding in this thesis was the requirement of HIF-2 α for the opioid-induced downregulation of CAII as demonstrated by the use of HIF-2 α -deficient MAH cells. HIF has recently been recognized to function as both a transcriptional activator and repressor (Manalo *et al.*, 2005). Some genes such as α -fetoprotein and LIFR are known to be repressed by the direct binding HIF-1 α to a reverse HRE (rHREs) that is located on the antisense strand (Narravula & Colgan, 2001; Mazure *et al.*, 2002; Jeong *et al.*, 2007). Likewise, a search for (a) rHRE(s) in CAII promoter region identified a putative rHRE (~1.7 kb upstream from the translation initiation site) on the antisense strand suggesting a mechanism by which HIF-2 α may downregulate CAII expression in opioid-treated MAH cells. However, this possibility needs to be validated in future experiments (e.g. ChIP analysis). Despite evidence suggesting HIF binding to a rHRE may serve as a repressive mechanism, a recent study identified a large number of genes that are repressed by HIF-1 α in situations where no specific HRE pattern was identified (Manalo *et al.*, 2005). Therefore, the presence of rHRE on the antisense strand may not necessarily be a requirement for HIF-repression mechanisms. On the other hand, tumor-associated CAs, i.e. CAIX and CAXII, are known to be induced by HIF-1 α during hypoxia, though a HRE has been identified on the antisense

strand of CAIX gene promoter (Wykoff *et al.*, 2000). In the case of opioid-treated MAH cells, it is also plausible that HIF-2 α may regulate another factor that ultimately leads to the repression of CAII expression. Therefore, while data in this thesis provide novel evidence for the HIF-2 α -dependent downregulation of CAII in opioid-treated chromaffin cells, further experiments are required to understand the underlying mechanisms.

Additionally, and as previously discussed for the opioid-induced upregulation of Kir6.2, PKA activation was required for the opioid-induced downregulation of CAII in MAH cells (Chapter 4; see also Figure 1). Overall, these studies identified key elements in the μ - and δ - opioid receptor signalling pathway that are involved in the regulation of CAII expression in AMCs.

Effects of *in utero* exposure to chronic nicotine and morphine on the expression of Kir6.2, CAII and HIF-2 α in the neonatal adrenal medulla vs cortex

In Chapters 2 and 4, I tested the predictions made *in vitro* (using primary cell cultures of neonatal AMCs or immortalized MAH cells) using an *in vivo* model that was more physiologically relevant. Adrenomedullary tissues isolated from rat pups exposed to either nicotine or morphine *in utero* showed an increase in Kir6.2 expression relative to (control) saline-treated pups. By contrast, cortical tissues, which also normally express Kir6.2 subunit (Xu & Enyeart, 2001; Buttigieg *et al.*, 2009), showed no change in Kir6.2 expression after morphine exposure (Chapter 4). These data, however, match perfectly the known distribution of the respective nicotinic and opioid receptors in the adrenal gland. For example, α -bungarotoxin-sensitive- $\alpha 7$ nAChRs (Criado *et al.*, 1997; Mousavi *et al.*, 2001), as well as μ - and δ -opioid receptors (Wittert *et al.*, 1996) are expressed on chromaffin cells of the adrenal medulla, whereas the cortex lacks expression of these receptors. Thus, the restricted expression of these receptors can explain the selective nicotine- and opioid-

induced upregulation of Kir6.2 expression in the medulla (versus the cortex), and further points to specificity in the actions of nicotine and opioid agonists during these studies.

In addition, use of the *in vivo* model allowed the investigation of the effects of morphine exposure on the expression of the two CO₂ markers, CAI and CAII. Examination of adrenal gland tissues from morphine-exposed pups revealed the downregulation of both CAI and CAII protein, and this effect was also confined to the adrenal medullary tissue where the two isoforms have been shown to be expressed (Munoz-Cabello *et al.*, 2005).

Finally, HIF-2 α accumulation in this *in vivo* model was also tested. Both *in utero* nicotine and morphine exposures led to an increase in HIF-2 α accumulation in the medullary region of the adrenal glands of affected pups. Interestingly, and similar to the Kir6.2 expression pattern, this effect was specific to the medulla only, even though HIF-2 α expression was found in both the medullary and cortical tissues. These results validated the predictions made *in vitro* using the MAH cell line and primary AMC cell cultures, and emphasized the requirement for a functional receptor-mediated signalling pathway, thereby ruling out non-specific actions of the drugs. Moreover, the effect of morphine was restricted to HIF-2 α accumulation only, as HIF-1 α expression was not significantly affected in morphine-exposed tissues. Unlike the ubiquitous expression of HIF-1 α , HIF-2 α expression is more tissue specific and in general, it has been implicated in the functions of cells derived from the sympathoadrenal lineage (Bishop *et al.*, 2008; Brown & Nurse, 2008; Brown *et al.*, 2009). This confirms that although the two transcription factors are paralogs, they may activate distinct signalling pathways that are cell type -specific even when both are expressed in the same cells/tissues. In this regard, a mutual antagonism has been recently reported between HIF-1 α and HIF-2 α that controls the cellular redox status and regulates O₂ sensitivity in the carotid body and adrenal medulla (Yuan *et al.*, 2013). Collectively, it is possible that the morphine-

induced increase in HIF-2 α accumulation seen in the *in vivo* studies leads to the upregulation of Kir6.2 subunit and the downregulation in CAI and CAIIs, as predicted by the *in vitro* MAH cell.

CLINICAL IMPLICATIONS OF THIS THESIS

1. Maternal health and its implication in Sudden Infant Death Syndrome

Sudden Infant Death Syndrome (SIDS) is the third leading cause of postnatal death in North America, and is characterized by an unexplained sudden death of an infant with no previous medical history (Kung *et al.*, 2008). Research has shown that SIDS-affected infants display a failure in activating natural respiratory and arousal reflexes (Kato *et al.*, 2003), which can protect the infant from prolonged fatal apneic events (van der Hal *et al.*, 1985). Respiratory and arousal reflexes are mediated by input from chemoreceptors localized in the central nervous system and periphery (carotid body and adrenal gland in neonate) (Feldman *et al.*, 2003). The underlying causes for a blunted reflex activation in SIDS victims are largely unknown but have been significantly correlated to prenatal exposure to either nicotine or opiates, via maternal cigarette smoking or drug use respectively. Importantly, this thesis suggests potential mechanisms by which exposure to these drugs might lead to abnormal adrenal responses in the newborn, and consequently, impaired arousal reflexes during apneic episodes, and uncovered novel molecular and physiological effects of both nicotine and opiate exposure.

1.1. Prenatal Nicotine Exposure

Maternal cigarette smoking during pregnancy has been linked to poor pregnancy outcome and a higher rate of Sudden Infant Death Syndrome (SIDS) (Slotkin *et al.*, 1995; Sawnani *et al.*, 2004; Cohen *et al.*, 2005; Rogers, 2008; Buttigieg *et al.*, 2009). Nicotine is the most pharmacologically

active component of cigarette smoke (Dani & Harris, 2005) and is known to readily cross the fetal circulation (Law *et al.*, 2003). Nicotine readily crosses the placenta and binds to nicotinic acetylcholine receptors (nAChRs) (Law *et al.*, 2003), which are expressed in the adrenal gland, brain, and brainstem regions involved in cardiorespiratory and arousal control (Kinney *et al.*, 1993; Mousavi *et al.*, 2001; Falk *et al.*, 2005). Many studies have reported abnormalities in arousal reflexes in nicotine-exposed infants (Chang *et al.*, 2003; Richardson *et al.*, 2009), as well as abnormalities in cholinergic and serotonergic transmission in these brain centres (Falk *et al.*, 2005; Lavezzi *et al.*, 2005). Central chemoreceptors in the retrotrapezoid nucleus (Guyenet *et al.*, 2008) and preBötzing complex (Montandon *et al.*, 2011) are primarily sensitive to changes in arterial PCO₂ and pH (Solomon *et al.*, 2000; Mulkey *et al.*, 2004). Together, these studies suggest that the brainstem is likely a key target of nicotine exposure in SIDS infants. However, nicotinic AChRs are also present in peripheral chemoreceptors which represent other potential targets of circulating nicotine. For instance, nicotinic nAChRs have been identified in peripheral carotid body chemoreceptors (Wyatt & Peers, 1993; Obeso *et al.*, 1997), and it has been proposed that the inhibitory effect of nicotine-induced dopamine release from these receptors attenuates cardiorespiratory reflexes to hypoxia (Hafstrom *et al.*, 2002; Hafstrom *et al.*, 2005; Gauda *et al.*, 2009). However, the carotid body chemoreceptors are relatively insensitive to hypoxia at birth (Donnelly, 2000). By contrast, this period coincides with the time when chromaffin cells of the adrenal medulla investigated in this thesis are most sensitive to hypoxia (and hypercapnia), and therefore likely to play a more dominant role in arousal reflexes in the neonate. As discussed in the Introduction, catecholamine secretion from adrenal chromaffin cells in response to asphyxial stressors at birth is essential for the proper establishment of cardiovascular and respiratory adaptations to air-breathing life (Seidler & Slotkin, 1985; Slotkin & Seidler, 1988). Key findings

uncovered in this thesis and previous studies from our laboratory demonstrated impaired ability of neonatal chromaffin cells to sense hypoxic stimuli after chronic nicotine exposure (Buttigieg *et al.*, 2009). Importantly, in the latter study there was increased mortality in nicotine-exposed (vs saline-exposed) pups when subjected to a 45 min bout of hypoxia; moreover, this loss of hypoxia tolerance could be prevented by pre-injecting nicotine-exposed pups with the K_{ATP} channel blocker, glibenclamide (Buttigieg *et al.*, 2009). This thesis uncovered the link between nicotine exposure and the K_{ATP} channel, by demonstrating the key role of $\alpha 7$ nicotinic nAChRs and subsequent accumulation of HIF-2 α , leading to the transcriptional upregulation of Kir6.2 subunit of the K_{ATP} channel. Thus, the peripheral action of nicotine on adrenal chromaffin cells *in utero* may also contribute to abnormal arousal reflexes and the pathophysiology of SIDS.

1.2 . Prenatal Opioid Exposure

Similar to nicotine, opiates readily cross into the fetal circulation (Farid *et al.*, 2008). The use of opiates during pregnancy whether for drug abuse (e.g. heroin) or replacement therapy (e.g. methadone and naltrexone) has been linked to a number of adverse fetal and neonatal effects (Kandall & Gaines, 1991; Burns *et al.*, 2010). Methadone, for example, is the most commonly used substance for replacement therapy to treat heroin addiction in USA, UK and Australia (Burns *et al.*, 2007). Similar to morphine, it binds selectively to the μ -opioid receptor to produce morphine-like euphoric effects (Volpe *et al.*, 2011). However, human and animal studies link the use of methadone during pregnancy to a number of negative pregnancy outcomes associated with increased neonatal and fetal mortality and the incidence of SIDS (Slotkin *et al.*, 1976; Kashiwagi *et al.*, 2005; Burns *et al.*, 2010), in addition to respiratory and heart rate depression (Wouldes *et al.*, 2004; Jansson *et al.*, 2005). These observations are reminiscent of our *in vivo* study showing

that chronic morphine exposure *in utero* was associated with decreased litter size and higher rate of dams delivering stillborns. The surviving offspring would likely have been more prone to neonatal mortality if exposed to asphyxial stressors, as demonstrated for their nicotine-exposed counterparts (Buttigieg *et al.*, 2009). Therefore, the present thesis raises the possibility that peripheral action of opiates in the blunting of O₂- and CO₂- chemosensitivity in neonatal AMCs may contribute to the higher incidence and the pathophysiology of SIDS in infants born to opioid-dependent mothers.

Naltrexone has emerged recently as an alternative replacement therapy for heroin- addicted pregnant mothers by acting as a general, non-selective opioid antagonist (Hulse *et al.*, 2001). Small blood concentrations of naltrexone have been shown to effectively block higher concentrations of opiates, thereby minimizing the effects on the fetus. Recent human studies suggest it may have positive therapeutic potential for heroin-addicted mothers as it lacks the detrimental effects on the neonate (Hulse *et al.*, 2001; Hulse & O'Neil, 2002; Hulse & O'Neill, 2002). In conclusion, our studies showing the negative effects of opioid exposure *in utero* on the chemosensitivity of neonatal adrenal chromaffin cells offer an explanation for the harmful effects of opiate use during pregnancy. Moreover, they provide mechanistic insight into the signalling pathways by which opioid exposure might lead to perinatal morbidities and loss of hypoxia tolerance in the newborn. As was the case for nicotine exposures, the transcription factor HIF-2 α and the regulation of K_{ATP} channel play central roles in chromaffin cell responses to chronic opioids. It is therefore likely that the K_{ATP} channel blocker glibenclamide may have protective effects for SIDS victims born to opioid-addicted mothers, by rescuing chromaffin cell responses to hypoxia. However, the adverse effects of chronic opioids on CO₂ sensitivity of chromaffin cells are not alleviated by this

treatment, though drugs that specifically target HIF-2 α might have therapeutic potential in this regard.

FUTURE DIRECTIONS

Findings in chapter 2 demonstrated that nicotine-induced Kir6.2 subunit upregulation was dependent on the accumulation of the transcription factor, HIF-2 α . A previous study from our laboratory (Buttigieg *et al.*, 2009) showed that the effects of nicotine on hypoxia chemosensitivity was prevented when inhibiting PKC and CaMK, indicating their involvement in the nicotine-induced signalling pathway. We speculate that changes in intracellular Ca²⁺ are involved in this signalling pathway resulting in K_{ATP} channel upregulation, however further studies are required to elucidate the link between $\alpha 7$ nAChR and HIF-2 α accumulation. Also, additional studies are needed to clarify whether PKC and CaMK act upstream or downstream of HIF-2 α in this signalling pathway.

Chapters 3 and 4 of this thesis investigated the role of opioid receptor stimulation in regulating the direct O₂ and CO₂/H⁺ chemosensitivity in neonatal AMCs. Chapter 3 showed that exposure of neonatal AMCs to chronic opioids led to suppression of both O₂ and CO₂/H⁺ chemosensitivity. However, splanchnic nerve terminals release a few other chemicals including pituitary adenylate cyclase activating polypeptide (PACAP) (Kuri *et al.*, 2009), vasoactive intestinal peptide (VIP) (Yoshikawa *et al.*, 1990) and histamine (Holgert *et al.*, 1998). The release of these chemicals may also contribute to regulation of asphyxial chemosensitivity in chromaffin cells. Future experiments are required to address whether or not these chemicals also contribute to the postnatal regulation of direct chemosensitivity in chromaffin cells.

The focus of Chapter 2 in this thesis was to investigate cellular and molecular mechanisms involved in the opioid-induced blunting of O₂ and CO₂ chemosensitivity in chromaffin cells.

However, chromaffin cells are also known to be sensitive to other asphyxial stressors such as acidity (low pH) and hypoglycemia (low glucose) that are also suppressed postnatally following innervation (Munoz-Cabello *et al.*, 2005; Rico *et al.*, 2005; Livermore *et al.*, 2011; Livermore *et al.*, 2012). It is currently unknown whether chronic opioid or nicotine exposure can regulate sensitivity to low pH or hypoglycemia. Furthermore, a number of ion channels have been proposed to be involved in acid sensitivity and shown to be modulated by extracellular or intracellular pH. In carotid body glomus cells, these channels include Ca^{2+} -activated K^+ channels (Peers & Green, 1991), background tandem-p-domain, acid-sensitive K^+ (TASK) channels (Buckler *et al.*, 2000), and the acid-sensing ion channels (ASICs) (Tan *et al.*, 2007). The canonical transient receptor potential (TRPC) channel family has been implicated in hypoglycemia sensitivity in the carotid body (Garcia-Fernandez *et al.*, 2007). Therefore, expansion of these studies with experiments similar to those described in Chapter 3 and 4 can help answer such interesting questions, and would shed more light on the postnatal regulation of asphyxial sensitivity in the developing AMCs.

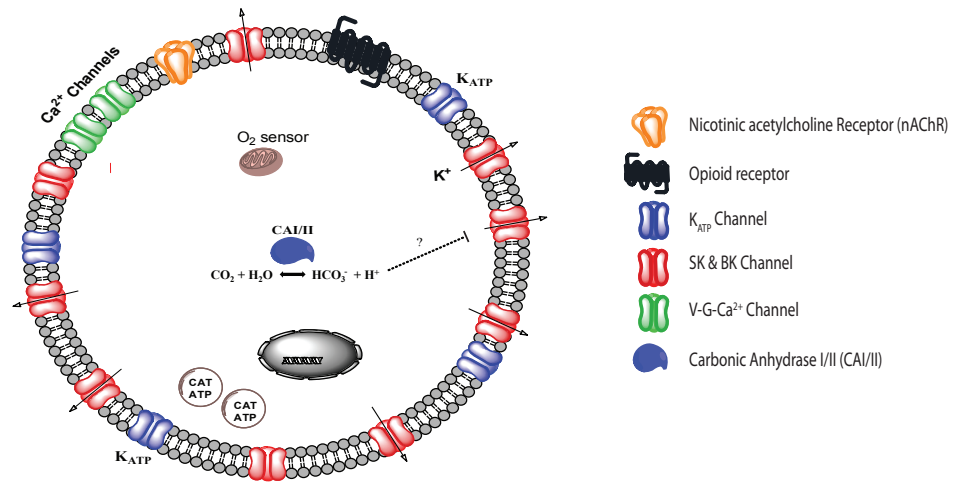
Furthermore, neonatal rat AMCs express low voltage T-type Ca^{2+} channels which have been proposed to play a role in regulating hypoxia sensitivity (Levitsky & Lopez-Barneo, 2009). Their expression decreases postnatally following splanchnic maturation (Levitsky & Lopez-Barneo, 2009). It is possible that chronic exposure to opioids (or other neurochemicals released from splanchnic nerve terminals) activates a signalling pathway that regulates T-type Ca^{2+} channel expression. Additional studies should address whether opioid peptides or other neurochemicals play a role in regulating T-type Ca^{2+} channel expression in the developing rat AMCs.

Finally, a recent study demonstrated HIF-1 α induction in SH-SY5Y neuroblastoma cells via μ - and δ -opioid receptor activation of the PI3K/Akt/mTOR signalling pathway (Daijo *et al.*, 2011). Data in Chapter 4 revealed that inhibition of PKA, PKC and CaMK did not significantly affect

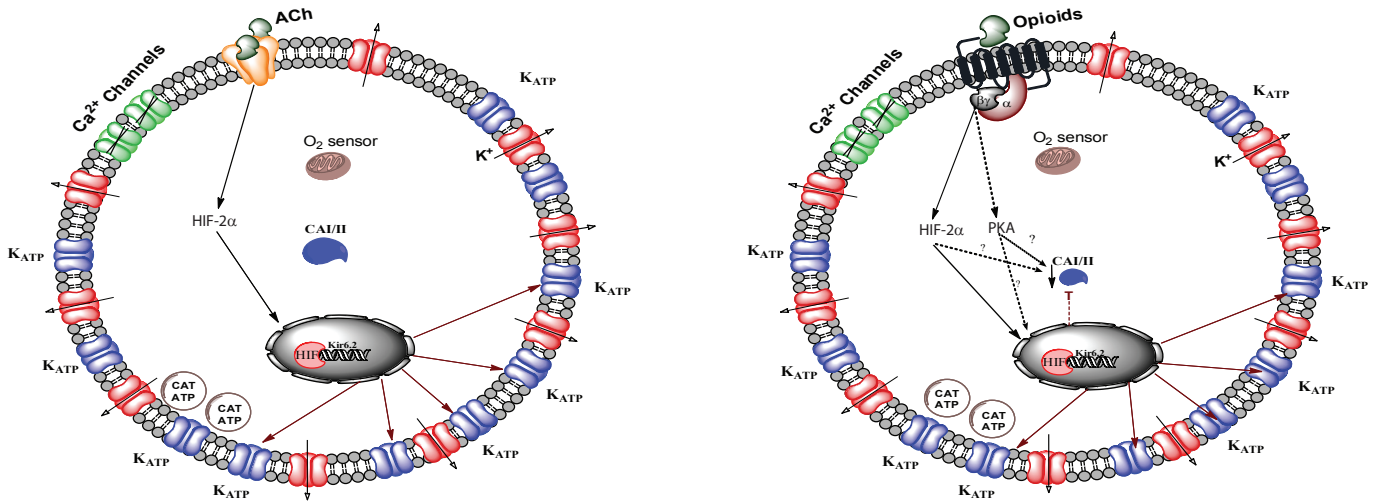
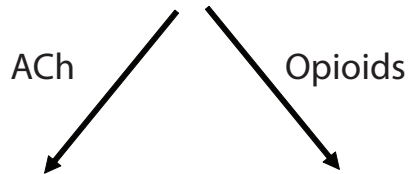
HIF-2 α accumulation. Therefore, it would be of interest to test the potential involvement of PI3K signalling pathway in the effects of chronic opioids on HIF-2 α accumulation demonstrated in this thesis using specific inhibitors (e.g. LY294002). Furthermore, while data in Chapter 4 suggest the dependence of opioid-induced regulation of CAII on HIF-2 α accumulation, the exact mechanisms are unknown. It is possible that HIF-2 α associates directly with CAII to repress its expression. It is also possible that HIF-2 α regulates a cellular messenger that mediates the opioid-induced repression of CAII expression. Therefore, further promoter and expression analysis studies (e.g. ChIP assay) are required to reveal the link between HIF-2 α and CAII expression.

Figure 1. Schematic representation showing the effects of stimulating nicotinic acetylcholine receptors (nAChRs) and opioid receptors on specific targets in the chemotransduction cascade in rat neonatal adrenomedullary chromaffin cells (AMCs). Chronic stimulation of the nAChRs and opioid receptors leads to suppression of the hypoxic chemosensitivity via mechanisms of HIF-2 α -dependent K_{ATP} channel upregulation. Activation of K_{ATP} channels during hypoxia induces membrane hyperpolarization that predominates membrane depolarization induced by inhibition of O₂-sensitive K⁺ channels (i.e. SK, BK, and K_v). This effect blunts the hypoxic response and CAT secretion. Unlike nAChRs, opioid receptor stimulation further blunts the hypercapnic response in neonatal AMCs, which correlates with the downregulation of carbonic anhydrase I and II (CAI/II). The downregulation of CA enzyme expression reduces the rate of CO₂ hydration and the decrease in intracellular pH, which is proposed as a potential mechanism to induce K⁺ channel inhibition leading to voltage-dependent Ca²⁺ entry and CAT secretion (Munoz-Cabello *et al.*, 2005).

FIGURE 1



**“Neonatal” State
(Low O₂ - high CO₂ -sensitive)**



**“Juvenile” State
(Low O₂ - high CO₂ -insensitive)**

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