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REVIEW

Ontogeny of O_2 and CO_2/H^+ chemosensitivity in adrenal chromaffin cells: role of innervation

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ABSTRACT

The adrenal medulla plays a key role in the physiological responses of developing and mature mammals by releasing catecholamines (CAT) during stress. In rodents and humans, the innervation of CAT-producing, adrenomedullary chromaffin cells (AMCs) is immature or absent during early postnatal life, when these cells possess 'direct' hypoxia- and CO_2/H^+-chemosensing mechanisms. During asphyxial stressors at birth, these mechanisms contribute to a CAT surge that is critical for adaptation to extra-uterine life. These direct chemosensing mechanisms regress postnatally, in parallel with maturation of splanchnic innervation. Here, we review the evidence that neurotransmitters released from the splanchnic nerve during innervation activate signaling cascades that ultimately cause regression of direct AMC chemosensitivity to hypoxia and hypercapnia. In particular, we consider the roles of cholinergic and opioid receptor signaling, given that splanchnic nerves release acetylcholine and opiate peptides onto their respective postsynaptic nicotinic and opioid receptors on AMCs. Recent *in vivo* and *in vitro* studies in the rat suggest that interactions involving α7 nicotinic acetylcholine receptors (nAChRs), the hypoxia inducible factor (HIF)-2α signaling pathway, protein kinases and ATP-sensitive K^+ (K_{ATP}) channels contribute to the selective suppression of hypoxic chemosensitivity. In contrast, interactions involving μ- and/or δ-opiod receptor signaling pathways contribute to the suppression of both hypoxic and hypercapnic chemosensitivity, via regulation of the expression of K_{ATP} channels and carbonic anhydrase (CA I and II), respectively. These data suggest that the ontogeny of O_2 and CO_2/H^+ chemosensitivity in chromaffin cells can be regulated by the tonic release of presynaptic neurotransmitters.

KEY WORDS: Adrenal chromaffin cells, Neonate, Hypoxia, Hypercapnia, Catecholamine secretion, Nicotine, Opioids, HIF-2α, K_{ATP} channels

Introduction

The structure of the mammalian adrenal gland was established by the middle of the 19th century with the aid of histochemical techniques that allowed visualization of the outer cortex as a distinct region from the inner medulla (Kölliker, 1854). In 1968, the term 'stimulus–secretion coupling' was coined following the discovery that calcium ions were required for catecholamine (CAT) secretion from 'chromaffin' cells in the adrenal medulla (Douglas, 1968). It is now generally recognized that in both mammalian and non-mammalian vertebrates (Perry and Capaldo, 2011), CAT release from chromaffin tissue plays a key role in the ability of the animal to adapt to environmental stressors. In the adult, activation of the sympathetic nervous system during stress initiates the 'fight or flight' response, resulting in a coordinated series of autonomic responses that include an increase in blood pressure, heart rate and cardiac contractility. In mammals, CAT release from adrenomedullary chromaffin cells (AMCs), triggered by the stimulation of preganglionic splanchnic nerves, contributes to these cardiovascular responses mainly via the release of acetylcholine (ACh) and activation of postsynaptic nicotinic ACh receptors (nAChRs). In several species including rodents and humans, splanchnic innervation of the adrenal medulla is immature or absent at birth (Seidler and Slotkin, 1985; Slotkin and Seidler, 1988). Yet, it has been known for some time that CAT release from AMCs in the neonate plays a critical role in the ability of these animals to survive stressors associated with delivery and the transition to extra-uterine life (Cheung, 1990; Lagercrantz and Slotkin, 1986). This release is vital for the modulation of cardiovascular, respiratory and metabolic responses to natural asphyxial stressors present during the birthing process, e.g. low O_2 (hypoxia) and high CO_2/H^+ (acid hypercapnia). Among these responses are the regulation of cardiac function via stimulation of α-adrenergic receptors (prior to the subsequent switch over to the adult β-adrenergic receptors), and initiation of lung respiration (Slotkin and Seidler, 1988). The latter involves the stimulation of surfactant secretion via β_2-receptors, and transformation of the physiological properties of the lung epithelium from a state where fluid is secreted to one where fluid is reabsorbed (Olver et al., 1986; Van Woudenberg et al., 2012). In the developing rat embryo, the release of CAT during hypoxic stress has been proposed to promote fetal survival by reversing hypoxia-induced bradycardia, thereby maintaining O_2 homeostasis (Ream et al., 2008). Also, in fetal sheep, CAT release during hypoxia maintains peripheral vasoconstriction and aids in the redistribution of cardiac output away from non-essential organs (e.g. gut, kidneys) and towards essential organs such as the heart, brain and adrenal glands (Giussani et al., 1993).

Seidler and Slotkin showed that in the newborn rat, acute hypoxia causes depletion of adrenal catecholamines via a 'non-neurogenic' mechanism, which is lost or suppressed postnatally along a time course that roughly parallels the maturation of the sympathetic innervation of the adrenal medulla (Seidler and Slotkin, 1985). Furthermore, they showed in denervation experiments that removal of the splanchnic innervation in mature or adult animals resulted in a gradual re-appearance of this non-neurogenic mechanism (Slotkin and Seidler, 1988). More recent studies in the rat suggest that adrenal responses to hypercapnia, acidity and hypoglycemia are also suppressed postnatally, in parallel with splanchnic innervation (Livermore et al., 2011; Livermore et al., 2012; Muñoz-Cabello et al., 2005; Rico et al., 2005). Interestingly, in mammals that are born relatively mature (e.g. sheep), the development of adrenal gland innervation occurs *in utero* (Comline and Silver, 1961). Chromaffin cells in these mammals are responsive to direct asphyxial stimuli *in utero* before the full maturation of splanchnic innervation. However, chemosensitivity of these cells is lost postnatally within 24 h of birth,

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List of abbreviations

ACh	acetylcholine
AMC	adrenomedullary chromaffin cell
CaM	Ca^{2+}/calmodulin-dependent protein kinase
CAT	catecholamine
ETC	electron transport chain
HIF	hypoxia inducible factor
K_{ATP}	ATP-sensitive K^+ channel
nAChR	nicotinic acetylcholine receptor
PKA	protein kinase A
PKC	protein kinase C
ROS	reactive oxygen species

and this parallels the complete development of splanchnic innervation (Comline and Silver, 1961; Comline and Silver, 1966).

The mechanisms by which splanchnic innervation regulates O_2 and CO_2/H^+ sensitivity of developing AMCs have remained elusive for many years. In this review, we consider recent evidence that begins to shine new light on the underlying mechanisms, particularly with regard to O_2 and CO_2 chemosensitivity. We will first review briefly current views on the signaling mechanisms by which perinatal AMCs are thought to sense acute hypoxia and hypercapnia. We will then consider evidence that supports the hypothesis that neurochemicals released tonically from the splanchnic nerve during innervation activate signaling cascades that result in the blunting of chemosensitivity. In addition to ACh, splanchnic nerve terminals release several neurochemicals including pituitary adenylate cyclase activating polypeptide, histamine and opiate peptides (Holgert et al., 1998; Kobayashi et al., 1985; Kuri et al., 2009). Our main focus will be on the potential involvement of nicotinic cholinergic and opioid receptor signaling pathways. A central theme that will emerge is that upregulation of ATP-sensitive K^+ (K_{ATP}) channels via both nAChR signaling and opioid receptor signaling pathways is a key mechanism that contributes to the blunting of hypoxia chemosensitivity in AMCs. Conversely, the available evidence suggests that activation of opioid (but not ACh) receptor signaling pathways, culminating in the downregulation of carbonic anhydrase (CA)I and II, is a major contributor to the blunting of CO_2 chemosensitivity.

Mechanisms of acute hypoxia sensing in perinatal AMCs

The mechanisms by which perinatal AMCs sense acute hypoxia were first investigated using dissociated cells from the rat adrenal gland (Mochizuki-Oda et al., 1997; Mojet et al., 1997; Thompson et al., 1997). In response to acute hypoxia, isolated neonatal rat AMCs show inhibition of outward K^+ current and membrane depolarization, leading to voltage-gated Ca^{2+} entry and CAT secretion (Mochizuki-Oda et al., 1997; Thompson et al., 1997). Exemplar traces of hypoxia-induced K^+ current inhibition and membrane depolarization in neonatal rat AMCs are shown in Fig. 1Ai,Bi and Fig. 2Ai,Bi. Acute hypoxia also induces CAT secretion when applied to fresh tissue slices of the adrenal gland (García-Fernández et al., 2007), and to superfused whole adrenal glands (Adams et al., 1996; Rico et al., 2005), from neonatal rat pups. Various K^+ channels modulate the excitability of perinatal rat and ovine AMCs and contribute to their physiological responses to hypoxia. Action potential duration and firing frequency are shaped by iberiotoxin-sensitive, large conductance Ca^{2+}-dependent (BK) and delayed rectifier-type (K_v) K^+ channels, both of which are inhibited by hypoxia (Mochizuki-Oda et al., 1997; Thompson et al.,

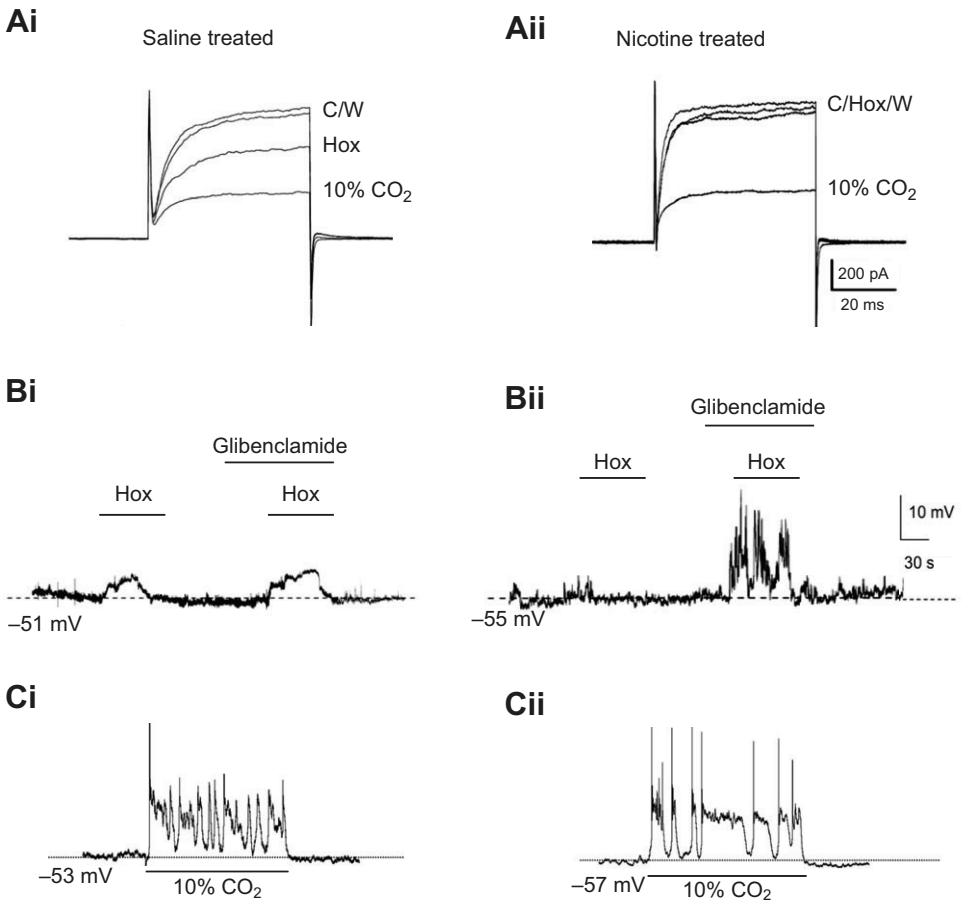

Fig. 1. Effects of hypoxia and hypercapnia on outward K^+ current and membrane potential in saline- versus nicotine-treated postnatal day 0 (P0) adrenomedullary chromaffin cells (AMCs). (Ai) In saline-treated AMCs, both hypoxia (Hox) and isohydric hypercapnia (10% CO_2, pH 7.4) cause a reversible inhibition of outward K^+ current during steps to +30 mV. (Aii) In nicotine-treated AMCs, hypoxia does not affect outward K^+ current, although hypercapnia still produces significant inhibition at +30 mV. In current-clamp recordings, both hypoxia (Bi) and isohydric hypercapnia (10% CO_2, pH 7.4; Ci) produce depolarizing excitatory responses in saline-treated AMCs. In nicotine-treated AMCs, only hypercapnia (Cii) causes excitation; hypoxia (Bii) fails to affect membrane potential. However, the hypoxic response in nicotine-treated AMCs can be rescued in the presence of glibenclamide (Bii), a K^+-sensitive (K_{ATP}) channel blocker. Note that glibenclamide on its own has no effect but it potentiates the hypoxia-induced membrane depolarization in saline-treated AMCs (Bi). C, control; W, wash (modified from Buttigieg et al., 2009).

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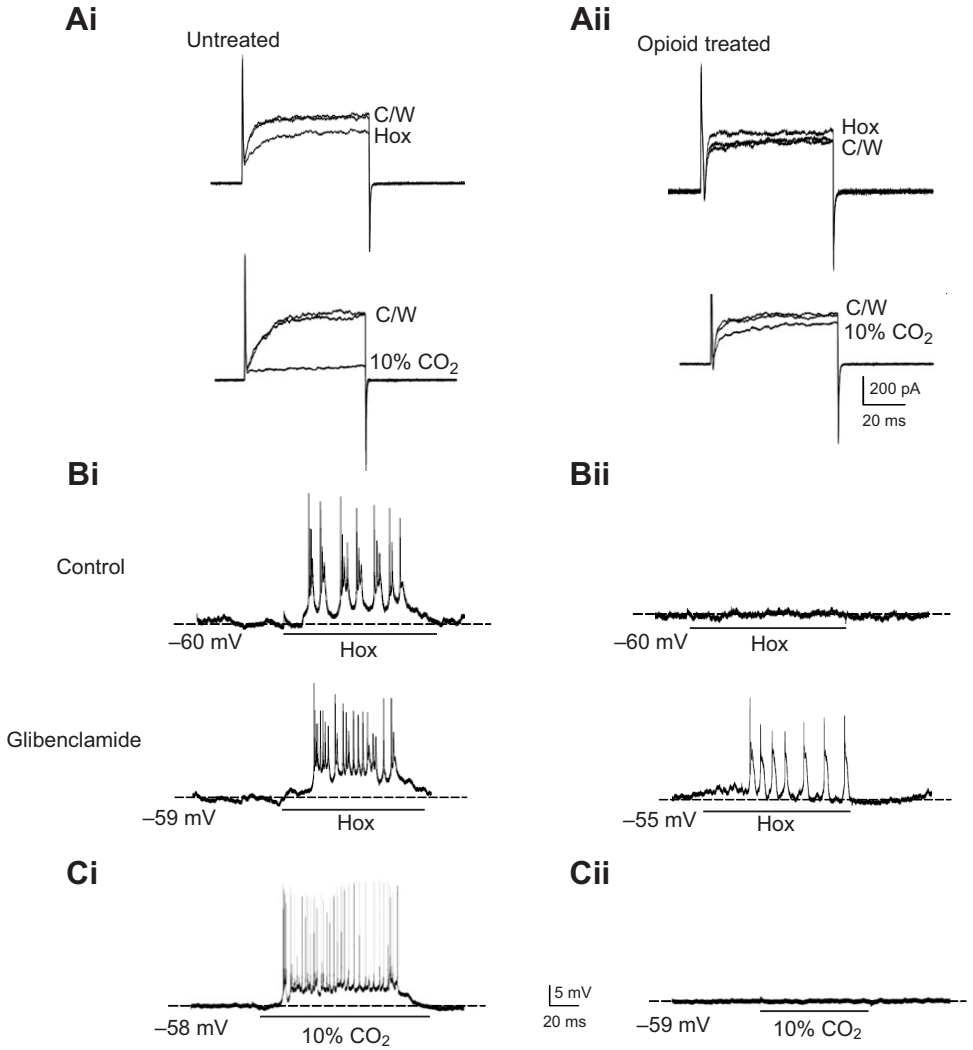

Fig. 2. Effects of chronic opioid exposure on hypoxia and hypercapnia chemosensitivity in neonatal rat AMCs. (Ai) In control (untreated) AMCs, both hypoxia (Hox; upper trace) and isohydric hypercapnia (10% CO_2, pH 7.4; lower trace) cause a reversible inhibition of outward K^+ current during steps to +30 mV. (Aii) In opioid-treated AMCs, both hypoxia (upper trace) and hypercapnia (lower trace) have little or no effect on the outward K^+ current at +30 mV. In current-clamp recordings, both hypoxia (Bi, upper trace) and hypercapnia (Ci) produce depolarizing excitatory responses in control (untreated) AMCs. In contrast, both hypoxia (Bii, upper trace) and hypercapnia (Cii) fail to affect membrane potential significantly in opioid-treated AMCs. However, the cell (Bii, upper trace) that was initially unresponsive to hypoxia fires action potentials in the presence of glibenclamide (Bii, lower trace), a K_{ATP} channel blocker. Note that glibenclamide potentiates the hypoxia-induced membrane depolarization in untreated AMCs (Bi, lower trace). C, control; W, wash (modified from Salman et al., 2013).

2002; Thompson et al., 1997; Thompson and Nurse, 1998). In addition, hypoxia inhibits apamin-sensitive, small conductance Ca^{2+}-dependent (SK) K^+ channels and this mechanism principally contributes to the depolarization (or receptor potential) of rat and ovine AMCs at the resting membrane potential (Keating et al., 2005; Keating et al., 2001; Lee et al., 2000). However, acute hypoxia is associated with a fall in intracellular ATP concentration (Varas et al., 2007) leading to the activation of glibenclamide-sensitive K_{ATP} channels, and membrane hyperpolarization in neonatal rat AMCs (Thompson and Nurse, 1998). This is thought to serve as a protective mechanism that limits membrane depolarization and Ca^{2+}-dependent CAT secretion during hypoxia. In rat embryos, expression of hypoxia-sensitive BK and K_{ATP} channels appears to be oppositely regulated during mid-to-late fetal stages (Bournaud et al., 2007). Though inhibition of K^+ channels is the main contributor to hypoxia-induced membrane depolarization and Ca^{2+}-dependent CAT secretion in rat, mouse and ovine AMCs, species differences have been noted. For example, in guinea pig AMCs, hypoxia appears to activate a non-selective cationic current, leading to membrane depolarization (Inoue et al., 1999).

Additionally, there is evidence that alterations in the expression pattern of specific Ca^{2+} channel subtypes may also contribute to the ontogeny of rat AMC responsiveness to hypoxia. In particular, low voltage-activated T-type Ca^{2+} channels ($Ca_v3.2$ subtype) were reported to be necessary for hypoxia-evoked CAT secretion in tissue slices of neonatal rat adrenal medulla (Levitsky and López-Barneo, 2009). In that study, T-type Ca^{2+} channel expression decreased with postnatal maturation, together with the loss of hypoxia sensitivity. Moreover, denervation of adult AMCs resulted in the reappearance of hypoxia sensitivity in parallel with T-type Ca^{2+} channel recruitment (Levitsky and López-Barneo, 2009). While these studies suggest that hypoxia-evoked CAT secretion in tissue slices may involve additional factors such as activation of T-type Ca^{2+} channels, the significance is uncertain, as in another study pharmacological blockade of these channels had no effect on hypoxia-evoked CAT secretion in isolated neonatal rat AMCs (Souvannakitti et al., 2010).

Though several of the downstream events during hypoxia chemotransduction in neonatal AMCs have been well characterized, the molecular identity of the 'O_2 sensor' is still unknown. The available evidence favors the mitochondrial electron transport chain (ETC), in association with a change in redox status, as playing an important role in hypoxia sensing in both rodent and ovine AMCs (Keating et al., 2005; Nurse et al., 2009). For example, various blockers of the ETC including the complex I blocker, rotenone, mimicked and occluded the effects of acute hypoxia (Keating et al., 2005; Mojet et al., 1997; Thompson et al., 2007). Further, hypoxia sensing is present in an immortalized chromaffin cell line (i.e. MAH cells) derived from fetal rat adrenal medulla (Fearon et al., 2002),

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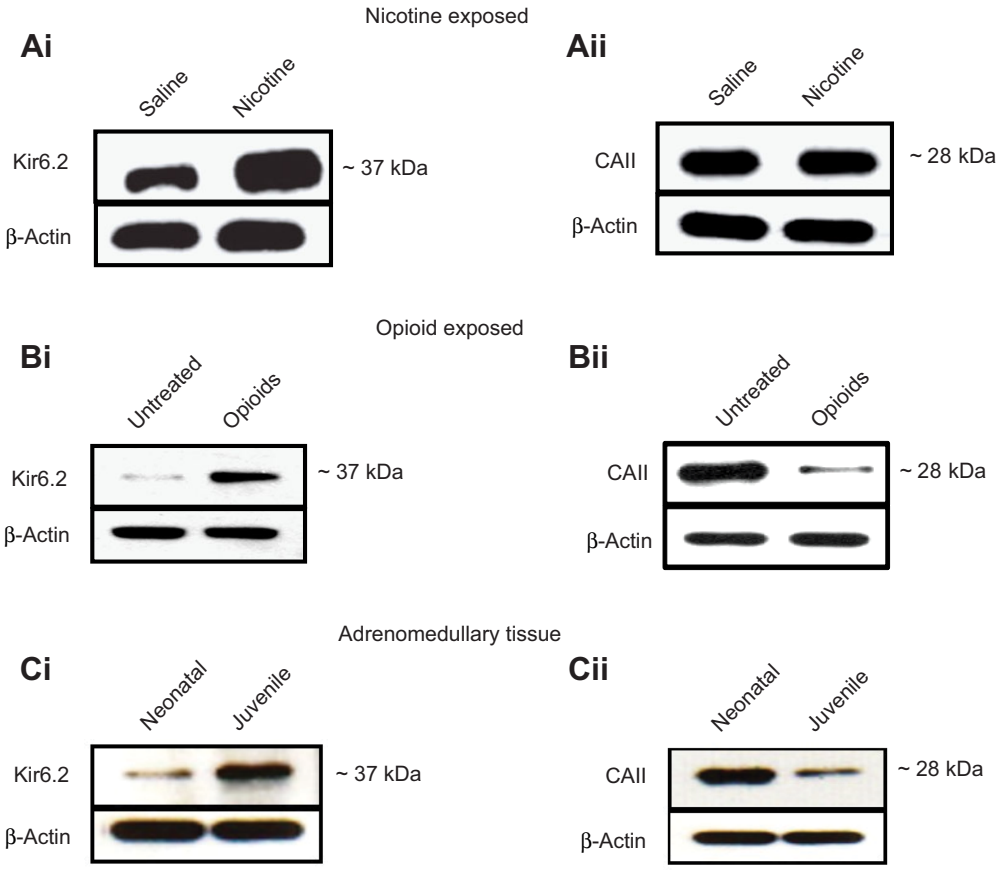

Fig. 3. K_{ATP} channel subunit Kir6.2 and carbonic anhydrase II (CAII) expression in nicotine- and opioid-exposed neonatal rat AMCs and rat adrenomedullary tissues. (Ai) Western blot analysis showing a significant upregulation of K_{ATP} channel subunit Kir6.2 in P0 AMCs from nicotine-exposed pups relative to saline-exposed pups. (Aii) Western blot analysis shows similar expression levels of CAII protein in saline- and nicotine-exposed AMCs (Buttigieg et al., 2009). (Bi) Similarly, chronic opioid exposure causes upregulation of Kir6.2 subunit expression in cultured neonatal AMCs (Salman et al., 2013). (Bii) In addition, chronic opioid exposure downregulates CAII protein expression in cultured neonatal AMCs; β-actin was used as an internal control (Salman et al., 2013). Interestingly, Kir6.2 subunit and CAII expression appear to be developmentally regulated in neonatal versus juvenile adrenomedullary enriched tissue. Western blot analysis reveals developmental upregulation in Kir6.2 subunit (Ci) and downregulation in CAII (Cii) expression in juvenile adrenomedullary tissue.

but is absent in mitochondria-deficient (ρ0) MAH cells (Buttigieg et al., 2008b). The involvement of redox pathways was suggested by the observations that reducing agents (e.g. dithiothreitol) or scavengers of reactive oxygen species (ROS; including catalase) mimicked hypoxia in inhibiting O_2-sensitive K^+ currents, whereas increasing ROS had the opposite effect (Keating et al., 2005; Thompson et al., 2007). A current working model based on these studies proposes that a decrease in H_2O_2 during acute hypoxia may modulate the gating of oxygen-sensitive K^+ channels (e.g. SK channels) in AMCs, leading to a decrease in their open probability. The resulting inhibition of these K^+ channels causes membrane depolarization, voltage-gated Ca^{2+} entry and CAT release.

Mechanisms of acute CO_2/H^+ sensing in perinatal AMCs

Compared with hypoxia sensing, there have been relatively few studies on the mechanisms and ontogeny of CO_2/H^+ sensing in AMCs. The initial study demonstrating that neonatal rat AMCs can respond directly to elevated CO_2 (hypercapnia) in a dose-dependent manner was performed on thin adrenal slices (Muñoz-Cabello et al., 2005). Using carbon fiber amperometry, these authors found that CAT secretory responses of AMCs to hypercapnia were significantly higher in neonatal compared with adult slices. These secretory responses were correlated with a fall in intracellular pH, required extracellular Ca^{2+} and were inhibited by the membrane-permeable CA inhibitor methazolamide (2 μmol l^{-1}) (Muñoz-Cabello et al., 2005). Electrophysiological studies on dissociated neonatal rat AMCs demonstrated that hypercapnia induces a depolarizing receptor potential, often leading to action potential firing, and also inhibits a voltage-dependent outward K^+ current (Buttigieg et al., 2008b; Livermore et al., 2011; Muñoz-Cabello et al., 2005). Exemplar traces of hypercapnia (10% CO_2)-induced K^+ current inhibition and membrane depolarization in neonatal rat AMCs are shown in Fig. 1Ai,Ci and Fig. 2Ai,Ci. The depolarizing receptor potential was also inhibited by methazolamide and was attributable to the activation of a resting cation conductance (Muñoz-Cabello et al., 2005). Studies on tissue slices using *in situ* hybridization revealed that only two CA isoforms, i.e. CAI and CAII, were expressed in chromaffin tissue of the neonatal rat adrenal gland; interestingly, their expression was significantly diminished or absent in the adult gland, in parallel with the diminished hypercapnia sensitivity (Muñoz-Cabello et al., 2005). As illustrated in Fig. 3Cii, western blot analysis has also revealed decreased expression of CAII protein in adrenomedullary tissue from juvenile compared with neonatal rats. Taken together, these data show that acute CO_2 sensing in neonatal AMCs involves intracellular acidification catalyzed by CAI and CAII, leading to membrane depolarization and action potential firing, voltage-gated Ca^{2+} entry, and CAT secretion. Also, the developmental loss of hypercapnia sensitivity was attributable to the downregulation of CAI and CAII as the primary events.

Role of nAChR signaling in regulating the ontogeny of hypoxia, but not hypercapnia, sensitivity in chromaffin cells

The ontogeny of hypoxia and hypercapnia sensitivity in rat AMCs, and particularly the blunting that occurs during early postnatal development, is tightly correlated with maturation of splanchnic innervation as discussed in the Introduction. Given that cholinergic innervation via the splanchnic nerve provides the major excitatory drive to AMCs, it was plausible that activation of postsynaptic nAChRs contributed to this blunting. In one attempt to test this hypothesis, AMCs were isolated from neonatal (P0) pups born to dams that were exposed to nicotine bitartrate (versus saline-treated

controls) throughout gestation, before they were tested for hypoxia and hypercapnia sensitivity (Buttigieg et al., 2008a). Because nAChRs are already expressed on the 'non-innervated' fetal AMCs (Sala et al., 2008), the aim was to stimulate prematurely these nicotinic receptors via the maternal circulation, and test whether or not there was a precocious blunting of hypoxia and hypercapnia sensitivity. Indeed, compared with age-matched saline controls, nicotine-treated P0 AMCs showed a blunted hypoxia sensitivity as revealed by: (i) the failure of acute hypoxia to inhibit voltage-gated outward K^+ current or to elicit a depolarizing receptor potential (as shown in Fig. 1Ai,ii and 1Bi,ii, respectively); (ii) the failure of acute hypoxia to elicit a significant rise in intracellular Ca^{2+}; and (iii) the failure of acute hypoxia to stimulate CAT secretion (Buttigieg et al., 2008a). Interestingly, fetal nicotine exposure had no effect on the ability of these P0 AMCs to respond appropriately to isohydric hypercapnia (10% CO_2, pH 7.4). Thus, nicotine-exposed P0 AMCs responded to isohydric hypercapnia with inhibition of voltage-gated outward K^+ current, membrane depolarization (as shown in Fig. 1Ci,ii), a rise in intracellular Ca^{2+}, and CAT secretion (Buttigieg et al., 2008a). Moreover, expression of the two main enzymes that mediate CO_2 sensitivity, i.e. CAI and CAII (Muñoz-Cabello et al., 2005), was similar in saline- and nicotine-exposed P0 AMCs when analyzed at both protein and mRNA levels (Buttigieg et al., 2008a). Though effective in blunting hypoxia sensitivity, the failure of prenatal nAChR stimulation to mimic the effects of innervation in blunting hypercapnia sensitivity suggested the involvement of other neural factors (see later).

The effects of nicotine exposure on the blunting of hypoxia sensitivity were mediated via nAChR signaling pathways. This conclusion was derived from experiments on culture models where neonatal rat AMCs and immortalized chromaffin (MAH) cells were chronically exposed to nicotine base (50 μmol l^{-1}) with or without nicotinic blockers for up to ~1 week *in vitro* (Buttigieg et al., 2009). Consistent with the results from *in vivo* nicotine exposures, hypoxia sensitivity was blunted when chromaffin cells were cultured for ~1 week in the presence of nicotine, but was retained when nicotine was omitted from the medium. These effects of nicotine could be prevented by co-incubation with the α7 nAChR antagonist α-bungarotoxin but not with a non-α7 antagonist (e.g. hexamethonium), suggesting that signaling via α7 nAChR was involved (Buttigieg et al., 2009). Despite these findings, the fate of α7 and other nAChRs expressed on chromaffin cells during chronic nicotine exposure is still unknown. This knowledge is important for a full understanding of the signaling pathways, given the well-known desensitization properties of these nAChRs following prolonged agonist exposure (Papke et al., 2009). In this regard, when chronic nicotine was applied to neuroblastoma cells expressing α7- and α3-containing receptors, there appeared to be a time-dependent loss of α7 nAChR function after ~4 days exposure, as monitored by intracellular Ca^{2+} imaging (Ridley et al., 2002).

Role of opioid receptor signaling in regulating the ontogeny of hypoxia and hypercapnia sensitivity in chromaffin cells

The failure of chronic nicotine to blunt hypercapnia sensitivity in neonatal AMCs (as discussed above) suggested other neural factors may be involved. In addition to ACh, splanchnic nerve terminals also store and release opiate peptides (Holgert et al., 1998; Kobayashi et al., 1985), and the three major subclasses of opioid receptors, i.e. μ, δ and κ, are known to be present in the adrenal medulla (Bunn et al., 1988; Keating et al., 2004; Kimura et al., 1988; Wittert et al., 1996). Also, opiate peptides are co-released with catecholamines from AMCs (Livett et al., 1981). These considerations led to an investigation of whether chronic opioids *in vitro* contribute to the blunting of AMC sensitivity to isohydric hypercapnia (Salman et al., 2013). Interestingly, exposure of primary neonatal rat AMC cultures for ~1 week to μ- or δ-opioid receptor agonists (2 μmol l^{-1}), separately or together, led to the blunting of both hypoxia and hypercapnia sensitivity as shown in Fig. 2Ai,ii, 2Bi,ii (upper traces) and 2Ci,ii. In contrast, exposure to a κ-opioid receptor agonist over the same time period was ineffective (Salman et al., 2013). The blunting effects of combined μ- and δ-opioid agonists on both chemostimuli were prevented when the agonists were co-incubated with a general opioid antagonist, naloxone (2 μmol l^{-1}), over the duration of the culture period. Western blot analysis revealed that the blunting of CO_2 sensitivity in opioid-treated AMCs was correlated with the downregulation of carbonic anhydrases, CAI and CAII (Salman et al., 2013). As discussed below, the blunting of hypoxia sensitivity was attributable to the upregulation of K_{ATP} channels, and this was the case for both chronic opioid and chronic nicotine exposures. It should be noted, however, that acute exposure of ovine AMCs to μ- and κ-opioid agonists can lead to a suppression of hypoxia sensitivity (Keating et al., 2004). These authors reported that in the case of μ-agonists, the suppression was mediated via activation of a resting O_2-sensitive, Ca^{2+}-dependent K^+ (SK) conductance, as well as inhibition of voltage-dependent Ca^{2+} channels. These mechanisms appear quite different from those involved in the effects of chronic opioids on the hypoxia sensitivity of neonatal rat AMCs, as the latter involves changes in gene expression and occurs independently of κ-opioid receptors (Salman et al., 2013).

Upregulation of K_{ATP} channel expression contributes to the blunting of hypoxia sensitivity in nicotine- and opioid-treated cells

The blunting of hypoxia sensitivity that occurs during chronic exposure of neonatal AMCs to either nicotine or opioids could occur at several levels and by a variety of mechanisms. These include: (i) downregulation of the O_2 sensor or a defective one; and (ii) alterations in the signaling cascade, including changes in expression levels of the O_2-sensitive K^+ channels, or T-type Ca^{2+} channels. As discussed earlier in this review, inhibition of SK, BK and K_v channels in neonatal AMCs contributes to the depolarization necessary for voltage-gated Ca^{2+} entry and CAT secretion during acute hypoxia. However, the magnitude of this depolarization is blunted by the hyperpolarizing action caused by the simultaneous opening of K_{ATP} channels, associated with the fall in ATP during hypoxia. Thus, the activation of K_{ATP} channels can be seen as a 'brake' on AMC secretion under these conditions. Consequently, an increase in the strength of this breaking action alone could, in principle, act as a potential mechanism for blunting hypoxia sensitivity. The available evidence suggests this is indeed the main mechanism by which chronic nicotine and chronic opioids mediate their effects on hypoxia sensing in neonatal AMCs (Buttigieg et al., 2009; Salman et al., 2013). For example, the magnitude of the K_{ATP} current density estimated from voltage-clamp studies using the K_{ATP} channel blocker glibenclamide was significantly enhanced in both nicotine-treated (Buttigieg et al., 2009) and opioid-treated (Salman et al., 2013) cells, relative to untreated controls. Also, as exemplified in Fig. 1Bii (nicotine treatment) and Fig. 2Bii (opioid treatment), individual cells that initially failed to respond to hypoxia with membrane depolarization did so when the experiment was repeated in the presence of glibenclamide. These data suggest that the O_2 sensor per se remained relatively intact, and that the apparent loss of hypoxia sensitivity could be attributed to a greater

hyperpolarizing contribution from K_{ATP} channels at the resting potential. However, the potential involvement of T-type Ca^{2+} channels in the loss of hypoxia sensitivity (Levitsky and López-Barneo, 2009) is not excluded.

Molecular studies also support the upregulation of K_{ATP} channels in neonatal AMCs after chronic nicotine and chronic opioid exposures. The K_{ATP} channel is a tetrameric complex consisting of four pore-forming Kir6.x subunits in combination with four regulatory sulfonylurea receptor subunits (Nichols, 2006). As illustrated in Fig. 3Ai and 3Bi, western blot analysis revealed that when neonatal rat AMCs were chronically exposed to nicotine and opioids *in vitro* there was a significant upregulation of Kir6.2 subunit expression (Buttigieg et al., 2009; Salman et al., 2012; Salman et al., 2013). These data are consistent with the functional evidence for increased K_{ATP} channel activity during hypoxia after both chronic treatments. The upregulation of Kir6.2 protein in nicotine-exposed (compared with saline-exposed) adrenomedullary tissue was also demonstrated in experiments where adrenal glands were isolated from newborn rat pups born to dams that were exposed chronically to nicotine throughout gestation (Buttigieg et al., 2009). In fact, *in vivo* administration of the K_{ATP} channel blocker glibenclamide to these nicotine-exposed pups prevented the loss of hypoxia tolerance seen in littermates without glibenclamide pretreatment (Buttigieg et al., 2009). These data further emphasize the critical role played by K_{ATP} channels in the ontogeny of hypoxia sensitivity in AMCs.

Is K_{ATP} channel expression correlated with the ontogeny of hypoxia sensitivity *in vivo*?

Given the proposed central role of K_{ATP} channels in regulating O_2 sensitivity and hypoxia-evoked CAT secretion, the question arises whether the ontogeny of hypoxia sensitivity in AMCs is correlated with K_{ATP} channel expression during development. Studies on fetal rat AMCs suggest there are developmental changes in the magnitude of the K_{ATP} current relative to the other O_2-sensitive K^+ currents, and these properties directly affect hypoxia-evoked CAT secretion during mid-to-late gestation (Bournaud et al., 2007). These authors reported that at fetal day (F)15 a higher level of K_{ATP} relative to other O_2-sensitive Ca^{2+}-dependent K^+ [$I_{K(Ca)}$] currents resulted in membrane hyperpolarization and blunted CAT secretion during acute hypoxia (Bournaud et al., 2007). Accordingly, at F15 the percentage of total outward current carried by glibenclamide-sensitive K_{ATP} channels $I_{K(ATP)}$ was ~50% compared with ~30% for $I_{K(Ca)}$ under normoxic conditions; however, at late fetal (F19) stages, $I_{K(ATP)}$ was reduced to ~14% of the total current, compared with ~64% for $I_{K(Ca)}$ (Bournaud et al., 2007). In that study, acute hypoxia caused an increase in action potential firing and robust CAT secretion only when applied to F19 AMCs. The failure of hypoxia to induce CAT secretion in F15 AMCs, as a result of the higher K_{ATP} channel expression, may be physiologically important in preventing premature lung fluid clearance via the action of circulating CAT on β-adrenergic receptors on pulmonary epithelial cells (Bournaud et al., 2007; Lagercrantz and Bistoletti, 1977; Olver et al., 1986).

As discussed in the Introduction, there is strong evidence that hypoxia sensitivity in AMCs declines postnatally as a consequence of splanchnic innervation *in vivo*. If the strategy discussed above of exposing perinatal AMCs to chronic nicotine or chronic opioids does indeed mimic the effects of splanchnic innervation, albeit prematurely, then this should be reflected in the expression pattern of K_{ATP} channels during normal development. As illustrated in Fig. 3Ci, western blot analysis revealed that the expression of the Kir6.2 subunit of the K_{ATP} channel (relative to β-actin) was significantly upregulated in adrenomedullary tissue dissected from adrenals glands of juvenile rats when compared with neonatal rats. This raises the possibility that innervation of AMCs per se could lead to upregulation of K_{ATP} channel expression *in vivo*, in association with the loss of hypoxia sensitivity. The result is also consistent with the idea that ACh and opiate peptides released from the splanchnic nerve during postnatal development could mediate the upregulation of K_{ATP} channels, via interaction with postsynaptic nicotinic and opioid receptors, respectively.

Molecular mechanisms by which chronic nicotine upregulates K_{ATP} channels: role of protein kinases and hypoxia inducible factor (HIF)-2α

As discussed above, interactions involving the α7 nAChR appear necessary for the nicotine-induced blunting of hypoxia sensitivity in neonatal AMCs. What are the intervening steps between the α7 nAChR interaction and the upregulation of K_{ATP} channels during chronic nicotine exposure? Though α7 nAChRs are highly Ca^{2+} permeable, the role of intracellular Ca^{2+} signaling is complicated by the observation that simply exposing neonatal AMCs to a chronic depolarizing stimulus (high K^+, 30 mmol l^{-1}), expected to stimulate voltage-gated Ca^{2+} entry, did not mimic the effects of chronic nicotine (Buttigieg et al., 2009). Nevertheless, experiments using protein kinase blockers revealed critical roles for protein kinase C (PKC) and Ca^{2+}/calmodulin-dependent (CaM) kinase, but not protein kinase A (PKA) (Buttigieg et al., 2009). These authors reported that when cultures of neonatal rat AMCs were co-incubated with nicotine plus either GF109203X (a PKC blocker) or KN-62 (a CaM kinase blocker) for ~1 week, hypoxia sensitivity was retained as measured by the inhibition of outward K^+ current. However, co-incubation with H-89, a PKA blocker, had no effect on the blunting action of nicotine exposure on hypoxia sensitivity. Confirmation that the requirement for PKC and CaM kinase function occurred upstream of K_{ATP} channel upregulation was obtained from experiments on the immortalized chromaffin cell line, i.e. MAH cells (Buttigieg et al., 2009). In these experiments, the upregulation of the Kir6.2 subunit of the K_{ATP} channel seen in MAH cells exposed to chronic nicotine for ~1 week *in vitro* was prevented during co-incubation with GF109203X or KN-62, but not H-89.

The MAH cell model revealed an additional role of the transcription factor HIF-2α in the nicotine-induced blunting of hypoxia sensitivity and the upregulation of the K_{ATP} channel subunit Kir6.2. Hypoxia-inducible factors are a family of transcription factors including HIF-1α and HIF-2α, which are key regulators of gene expression under hypoxic conditions (Semenza, 2004). The functional complex is a heterodimer consisting of an α- and a β-subunit, and though both subunits are constitutively expressed, the α-subunit is rapidly degraded under normoxic conditions (Huang et al., 1998; Wang et al., 1995). While hypoxia is a potent inducer of HIFs, several pathophysiologial conditions such as inflammation and oxidative stress can lead to HIF accumulation (Taylor, 2008). The initial indication that the HIF pathway was involved in nicotine-induced blunting of hypoxia sensitivity came from experiments on a HIF-2α-deficient (>90% knockdown) MAH cell line (shMAH) created using interference RNAi techniques (Brown et al., 2009). Both untransfected and transfected scrambled control MAH (scMAH) cells showed typical responses to hypoxia as measured by the inhibition of outward K^+ current (Buttigieg et al., 2009). Moreover, similar to primary neonatal AMCs, both of these cell lines showed a blunted hypoxia sensitivity following ~1 week exposure to chronic nicotine *in vitro*. However, when HIF-2α-deficient MAH cells were exposed to

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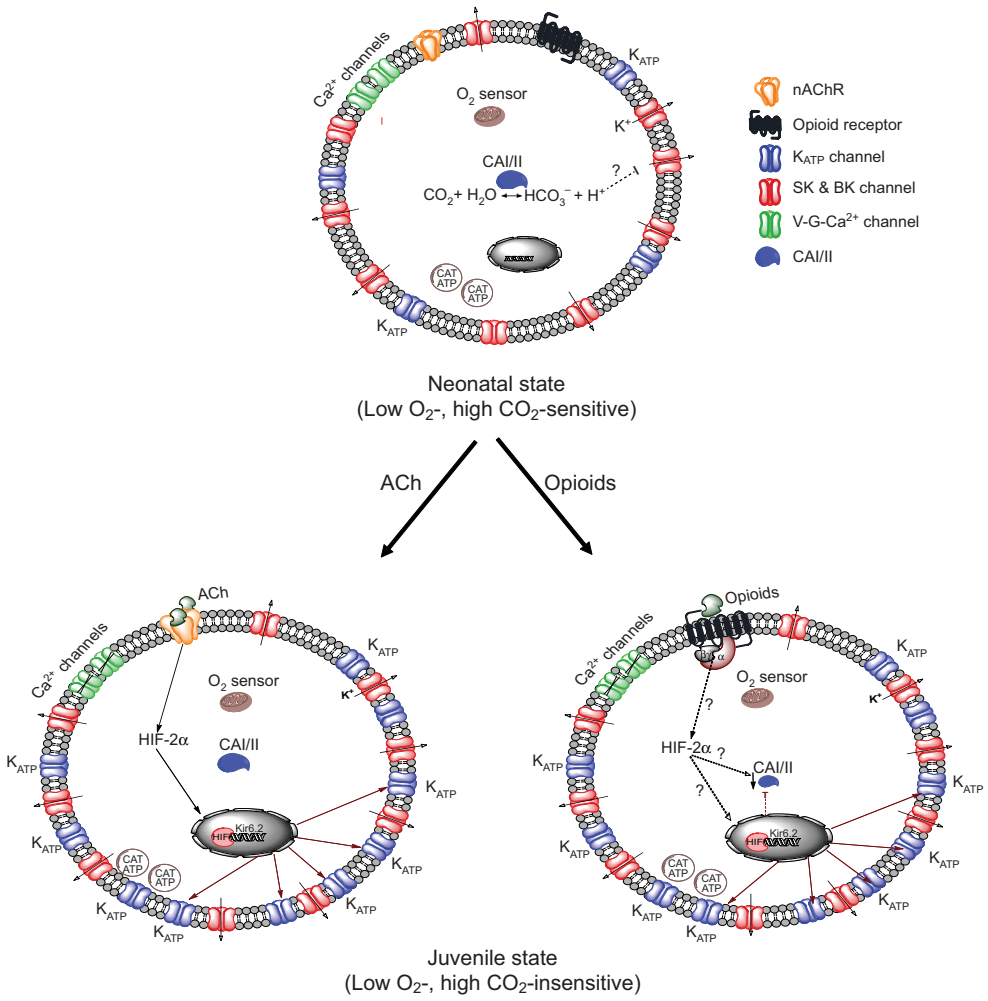

Fig. 4. Schematic representation showing the effects of stimulating nicotinic acetylcholine receptors (nAChRs) and opioid receptors on specific targets in the chemotransduction cascade in rat chromaffin cells. Chronic stimulation of the nAChRs and opioid receptors induces a signaling pathway that leads to functional hypoxia inducible factor (HIF)-dependent upregulation of the K_{ATP} channel. Activation of the K_{ATP} channel during hypoxia reduces membrane depolarization necessary for voltage-dependent Ca^{2+} entry and catecholamine (CAT) release. Unlike nAChRs, chronic stimulation of opioid receptors further downregulates CAI/II, thereby blunting hypercapnia chemosensitivity in these cells.

chronic nicotine for the same duration, hypoxia sensitivity was retained, suggesting a role for HIF-2α. More recent studies have clarified the role of HIF-2α, and particularly its involvement in the regulation of the Kir6.2 subunit in chromaffin cells. Unlike the rapid induction of HIF-2α that occurs when control MAH cells are exposed the chronic hypoxia (2% O_2) *in vitro* (Brown and Nurse, 2008), chronic nicotine exposure causes a gradual accumulation of HIF-2α. During nicotine exposure, a significant increase in HIF-2α levels in MAH cells was not detectable after 24 h, but reached near-maximum levels by 3 days and remained elevated at 7 days of exposure (Salman et al., 2012). Interestingly, western blot analyses revealed that the time course of HIF-2α accumulation paralleled that of the increased expression of the Kir6.2 subunit. This effect was not limited to Kir6.2, as another ubiquitous HIF target, i.e. vascular endothelial growth factor or VEGF, was also upregulated by chronic nicotine exposure (Salman et al., 2012). These effects of chronic nicotine on HIF-2α accumulation and Kir6.2 upregulation were prevented during co-incubation with the α7 nAChR blocker α-bungarotoxin, and were absent in HIF-2α-deficient MAH cells. Examination of the promoter region of the Kir6.2 gene revealed a hypoxia response element and, moreover, chromatin immunoprecipitation assays revealed binding of HIF-2α to this promoter region in nicotine-treated MAH cells (Salman et al., 2012). Taken together, these data suggest the linking of α7 nAChR signaling, activation of PKC and CaM kinase pathways and HIF-2α accumulation to the transcriptional upregulation of the K_{ATP} channel subunit Kir6.2 during exposure of chromaffin cells to chronic nicotine. However, the link between the α7 nAChR and HIF-2α accumulation during chronic nicotine exposure remains unclear. There is evidence that the increase in intracellular Ca^{2+} that occurs in PC12 cells exposed to chronic intermittent hypoxia leads to HIF-2α degradation via activation of Ca^{2+}-dependent proteases, i.e. calpains (Prabhakar et al., 2009). Given that chronic nicotine exposure may lead to a slow loss of α7 nAChR function, and an associated reduction in Ca^{2+} influx (Ridley et al., 2002), it is plausible that such a mechanism could contribute to the delayed HIF-2α stabilization seen in nicotine-treated MAH cells (Salman et al., 2012). Though it still remains to be determined whether PKC and CaM kinase act upstream or downstream of HIF-2α accumulation, the net result of these interactions is the blunting of hypoxia sensitivity because of the increased expression of functional K_{ATP} channels.

Concluding remarks

In this review, we have considered the evidence that neurochemicals supplied by splanchnic innervation are involved in the ontogeny of hypoxia and CO_2/H^+ chemosensitivity in mammalian AMCs. The search for such factors was triggered by the observations that CAT secretion from perinatal AMCs in response to these chemostimuli was highly correlated with the innervation status (Muñoz-Cabello et al., 2005; Nurse et al., 2009; Slotkin and Seidler, 1988). This CAT secretion is critical for the proper transition to extra-uterine life

(Lagercrantz and Slotkin, 1986). The weight of current evidence favors both cholinergic and opioid innervation of AMCs as key contributors to the suppression of chemosensitivity. In attempts to mimic the effects of cholinergic innervation, AMCs were chronically exposed to nicotine *in utero* or *in vitro*. This resulted in a blunting of hypoxia sensitivity, though high CO_2 (hypercapnia) sensitivity remained intact. Conversely, exposure of AMCs to chronic μ- and δ-opioid agonists *in vitro* resulted in the blunting of both hypoxia and hypercapnia sensitivity. It would appear that premature stimulation of nAChRs and opioid receptors on AMCs with these neurotransmitter mimetics, at earlier times than they would experience during splanchnic innervation, essentially hijacks the signaling mechanisms that mediate the normal developmental loss of chemosensitivity. A central theme that has emerged is that for both chronic nicotine and opioid exposures, the upregulation of K_{ATP} channels is a key mechanism that contributes to the blunting of hypoxia sensitivity, by favoring membrane hyperpolarization during acute hypoxia. The slow accumulation of the transcription factor HIF-2α, at least during chronic nicotine exposure, leads to the transcriptional upregulation of the K_{ATP} channel subunit Kir6.2. The effect of nicotine exposure on expression of the K_{ATP} channel subunit Kir6.2 is also dependent on the activity of PKC and CaM kinase. These kinases have previously been implicated in pathways leading to the synthesis and stabilization of HIF-1α protein, and in the transcriptional activity of HIF-1 in cells exposed to intermittent hypoxia (Semenza, 2009; Yuan et al., 2005; Yuan et al., 2008). However, it remains to be determined whether similar intervening pathways regulate HIF-2 in perinatal AMCs following nicotine exposure. Though further studies are required to clarify the opioid receptor signaling pathways, the opioid-mediated decrease in carbonic anhydrase (CAI and CAII) expression in AMCs seems to be the main contributor to the suppression of hypercapnia sensitivity. It is noteworthy, however, that chronic opioid exposure can lead to the accumulation of HIF-1 in neuroblastoma cells (Daijo et al., 2011). Thus, it is plausible that the HIF pathway may also be involved in the upregulation of K_{ATP} channels, and downregulation of CA, in AMCs following opioid exposure. Working models that summarize our current understanding of the signaling pathways by which chronic nicotine and opioids regulate chemosensitivity in developing AMCs are summarized in Fig. 4.

While the studies summarized in this review suggest possible mechanisms by which innervation may contribute to the ontogeny of hypoxia and CO_2/H^+ sensitivity in chromaffin cells, they also have clinical implications. Blunted adrenal CAT release during asphyxia contributes to the failure of arousal and elevated neonatal mortality as occurs during sudden infant death syndrome (SIDS) (Buttigieg et al., 2009; Cohen et al., 2005; Sawnani et al., 2004). Also, cigarette smoking during pregnancy has been linked to an increased risk of SIDS, likely due to the presence of nicotine (Mitchell and Milerad, 2006). Indeed, prenatal nicotine exposure is associated with an increased mortality rate when the affected offspring are exposed to a hypoxic challenge (Slotkin et al., 1995). Also, opioid medication for pain management is widespread, and opioid intake by pregnant mothers has also been linked to a higher incidence of infant mortality due mainly to SIDS (Burns et al., 2010). Thus, understanding the mechanisms by which chronic nicotine and opioid exposures lead to the blunting of chemosensitivity in AMCs is critical for the management of pathophysiological conditions associated with abnormal arousal responses in the neonate. In this regard, the observation that pre-exposure to the K_{ATP} channel blocker glibenclamide reverses the loss of hypoxia tolerance seen in rat pups born to nicotine-treated dams (Buttigieg et al., 2009) points to K_{ATP} channels as targets for therapeutic intervention in cases where abuse of these drugs is implicated.

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Competing interests
The authors declare no competing financial interests.

Author contributions
S.S. and J.B. contributed to experimental design, performed several of the molecular and electrophysiological experiments described in this review, and helped in the preparation, organization and editing of this manuscript; C.A.N. contributed to the planning, organization and design of several experiments described in this review and wrote the first draft of this manuscript.

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