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Effect of Somatostatin on Voltage-Gated Calcium Influx in Isolated Neonatal Rat Carotid Body Type I Cells

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EFFECT OF SOMATOSTATIN ON VOLTAGE-GATED CALCIUM INFLUX IN ISOLATED
NEONATAL RAT CAROTID BODY TYPE I CELLS

A thesis submitted in partial fulfillment
of the requirement for the degree of
Master of Science

By

ERIC JOSEPH DUNN
B.S., Westminster College, 2013

2015
Wright State University
I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION
BY Eric Joseph Dunn ENTITLED Effect of Somatostatin on Voltage-Gated Calcium
Influx in Isolated Neonatal Rat Carotid Body Type I Cells BE ACCEPTED IN PARTIAL
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ABSTRACT

Dunn, Eric Joseph. M.S., Department of Neuroscience, Cell Biology, and Physiology, Wright State University, 2015. Effect of Somatostatin on Voltage-Gated Calcium Influx in Isolated Neonatal Rat Carotid Body Type I Cells.

Somatostatin (SST) is a neuropeptide hormone that regulates the release of secondary hormones. Evidence suggests SST plays a neuromodulatory role due to its distribution throughout the central nervous system. Interestingly, SST has been suggested to affect the carotid body, the small peripheral chemoreceptors that regulate breathing. It has been shown that the peripheral chemoreflex sensitivity to CO₂ and hypoxia is reduced by SST in humans (Pedersen et al., 1999; Pandit et al., 2014). SST has also been found to inhibit whole cell Ca²⁺ currents recorded from adult rat carotid body type I cells (e Silva & Lewis, 1995), but the mechanism by which this occurs is unknown. This study aimed to identify the types of SST receptors (SSTR) on type I cells and confirm the mechanism by which their activation inhibits Ca²⁺ influx.

Specific antibodies were used to identify SSTR1-5 on type I cells, and results showed that SSTR1-5 were present on the membrane and cytoplasmically in type I cells. To record intracellular Ca²⁺ entry, type I cells were loaded with FURA-2 (5 μM) and depolarized in response to stimuli, including 80 mM K⁺ and hypoxia. Type I cells applied with 2, 10, and 20 minute applications of 1 μM SST had no significant inhibition on voltage-gated Ca²⁺ entry compared to controls. Also, a 2 minute application of 1 μM SST did not significantly inhibit Ca²⁺ influx in adult rat type I cells when compared to controls. These results indicate that 1 μM SST does not significantly inhibit K⁺- nor hypoxic-evoked Ca²⁺ influx in isolated type I cells from
carotid bodies. Thus the mechanism by which SST inhibits the acute ventilatory response to hypoxia and hypercapnia is not likely via inhibition of voltage-gated Ca\textsuperscript{2+} influx nor via inhibition of the chemosensory response of type I cells.

This work appeared in abstract form at the Ohio Physiological Society meeting 2014 and the Midwest Graduate Research Symposium 2015.
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INTRODUCTION
Anatomy

The carotid bodies are peripheral chemoreceptors in mammals. They are small organs located bilaterally in the neck, at the rostral end of the left and right common carotid artery bifurcation (Figure 1) (Kumar & Prabhakar, 2012). However, the exact location of the carotid body within the common carotid artery varies among species (Gonzalez et al., 1994).

The carotid body is highly vascularized organ. It contains a dense network of small blood vessels covering a large area of the organ (Gonzalez et al., 1994). Some studies have suggested that the vascularization of carotid body exceeds that of the brain by a factor of five to six (McDonald, 1981; Pallot, 1987). Blood is supplied to the carotid body via small arteries branching from the common carotid artery (Gonzalez et al., 1994). Venous drainage originates from one or a few small veins on the surface of the organ, and ends in the internal jugular vein (Gonzalez et al., 1994).

As blood crosses the carotid body, it branches and continues to the superior cervical cava (SCG). The carotid body is innervated by the SCG with the ganglioglomerular nerves (Gonzalez et al., 1994). The carotid body is also innervated by a branch of the glossopharyngeal nerve (GPN) called the carotid sinus nerve (CSN), which connects the carotid body to the brain stem and regulates respiratory control (Gonzalez et al., 1994). The CSN fibers are considered sensory fibers rather than motor fiber, since the soma of the CSN is located in the petrosal sensory ganglion and not in the brainstem (De Castro, 1928). De Castro (1928) concluded that the carotid body was a sensory organ rather than a gland, when he
found that the innervation of the carotid body parenchyma was unchanged when the SCG was removed.
Figure 1

Illustration showing the anatomical position of carotid body. The common carotid artery (CC) splits into the internal (IC) and external (EC) branches. The carotid body is located at the common carotid artery bifurcation.
Physiological Function

The carotid bodies are the body’s main peripheral chemoreceptors that modulate breathing. These organs are viewed as the primary peripheral sensor for oxygen, and maintain and restore blood gas homeostasis by quickly responding to hypoxia, hypercapnia, or acidosis (Peers et al., 2010). They have become increasingly considered as polymodal organs, as they are sensitive to other stimuli, including temperature changes and physiological osmolarity fluctuations (Peers et al., 2010).

There are two distinct cell types that make up the carotid body- type I cells and type II cells (Figure 2). In a carotid body, there are about 4 type I cells for every type II cell (McDonald, 1981; Nurse & Piskuric, 2013). Type I cells are small spherical cells (8-15 μm) that are arranged in clusters called glomoids (Nurse & Piskuric, 2013), separated by connective tissue. These cells contain neurotransmitters and synaptic vesicles, and are capable of forming electrical and chemical synapses with other type I cells as well as other neuronal fibers, including the CSN. Type I cells have a round nucleus and granular cytoplasm, though the granular appearance is absent in type II cells (Gonzalez et al., 1994). Importantly, type I cells depolarize in response to hypoxia, and transduce a chemical signal into an action potential. These chemoreceptor cells play a transducer role that underlies their presynaptic and neurosecretory function (Kumar & Prabhakar, 2012). In contrast to type I cells, type II cells are not excitable and do not synapse with the CSN. Type II cells are elongated, glia-like cells about 10 μm in diameter (Nurse &
Piskuric, 2013). They lack synaptic vesicles and are thought to play a supporting role in the carotid body (Nurse & Piskuric, 2013).
Figure 2

Picture illustrating the arrangement of cells in the carotid body. Elongated type II cells encase type I cells. Type I cells, clustered in glomoids, contain neurotransmitter and synapse with afferent chemosensory fibers such as the carotid sinus nerve. The cells of the carotid body receive a rich supply of arterial blood, shown by the capillary.
Though isolated type I cells in culture have been shown to respond to hypoxia, other transplant and regeneration studies have been performed to confirm that the type I cell is the main transducing component of the carotid body, not only in isolation (Kumar & Prabhakar, 2012). One study cryodestroyed type I cells and found the chemosensory function of the carotid body to be lost (Verna et al., 1975), suggesting that type I cells are the primary transducers of the carotid body (Kumar & Prabhakar, 2012).

**Neurotransmitter Release from Carotid Body Type I Cells**

It has been clearly demonstrated that neurotransmitters are released from type I cells under hypoxic conditions (Peers et al., 2010). When hypoxia induces neurotransmitter release, the neurotransmitter excites afferent chemosensory fibers and modulates respiratory reflexes (Peers et al., 2010). However, the multitude of autocrine and paracrine effects of neurotransmitters on the carotid body remain poorly described at this time (Gonzalez et al., 1994; Nurse, 2010; Wyatt & Peers, 1993). Nonetheless, this secretory activity is viewed as a critical component of the type I cell’s response to hypoxia (Peers et al., 2010).

The chemosensory mechanism of the type I cell that a majority of researchers agree upon is the membrane hypothesis for oxygen sensing (Figure 3). Briefly, hypoxia inhibits specific K+ channels (including BKCa channels), causing the type I cell to depolarize (Peers et al., 2010). The increase in membrane potential opens voltage-gated Ca2+ channels, and the Ca2+ influx initiates neurotransmitter release (Peers et al., 2010). Previous work has shown that dihydropyridine-sensitive L-type
Ca$^{2+}$ channels account for part of this voltage-gated Ca$^{2+}$ entry, but other voltage-gated Ca$^{2+}$ channels may also play a role since dihydropyridine receptor antagonists do not completely block Ca$^{2+}$ entry in response to high extracellular potassium (Buckler & Vaughan-Jones, 1994).

However, there are some steps in the membrane hypothesis for oxygen sensing that remain up for debate. For an example, an important, but unexplained event in this hypothesis is how exactly hypoxia causes inhibition of K$^+$ channels (Prabhakar & Peers, 2014). It is also unknown whether or not there is more than one oxygen sensor in the type I cell. Other possible oxygen sensors in the type I cell include protein complexes of the mitochondrial electron transport chain (Nurse, 2005), an extra-mitochondrial rotenone-sensitive protein (Lopez-Barneo, 2003), and a plasma membrane-associated heme oxygenase-2 (Williams et al., 2004).

The mechanisms by which type I cells respond to other stimuli, such as hypercapnia and acidosis, have also been studied. Most research indicates that hypercapnia causes a drop in intracellular pH which inhibits various acid-sensitive K$^+$ channels, such as TASK-1 and TASK-3 (Buckler & Vaughan-Jones, 1994; Buckler et al., 2000). Inhibition of K$^+$ channels causes a membrane depolarization, opens voltage-gated Ca$^{2+}$ channels, and initiates neurosecretion (Peers & Buckler, 1995). Intracellular and extracellular pH play a role in type I cells response to acidic stimuli (Buckler et al., 2000). The sensitivity of TASK-1 and TASK-3 channels to acid is due to the protonation of a histidine residue in the large extracellular loop/helical cap region (Kim et al., 2000; Morton et al., 2003; Rajan et al., 2000). However, the initial
response of type I cells to hypercapnia is dependent on the activity of carbonic anhydrase, which converts water and CO\textsubscript{2} to bicarbonate (Gonzalez et al., 1994).

The mechanism of type I cells sensing low glucose levels has been less studied. One study suggests that hypoglycemia sensing involves the inhibition of voltage-dependent (but Ca\textsuperscript{2+}-independent) K\textsuperscript{+} channels, causing a membrane depolarization and neurotransmitter release (Lopez-Barneo, 2003). The released neurotransmitter cause increase firing rates in the CSN, and then signal to the nucleus tractus solitaries (NTS) of the medulla.
Figure 3

Schematic of the membrane hypothesis for oxygen sensing. Through an unknown mechanism, hypoxia inhibits oxygen sensitive K⁺ channels, such as TASK channels or BKCa channels. The increase in cytoplasmic potassium increases the membrane potential. The increase in membrane potential activates voltage-gated Ca²⁺ channels, allowing Ca²⁺ influx. The increase in cytoplasmic Ca²⁺ concentration initiates vesicle fusion and neurotransmitter release.
Neurotransmitters of Carotid Body Type I Cells

Several biogenic amines have been identified in the type I cells of the carotid body. For an example, dopamine is abundant in the type I cell and has been well characterized (Gonzalez et al., 1994). Type I cells express tyrosine hydroxylase (TH), which is the rate-limiting enzyme in catecholamine biosynthesis (Nurse, 2005). Type I cells can be identified in immunocytochemistry experiments with TH staining. Also, the biosynthetic enzyme histamine carboxylase, which is involved in histamine synthesis, has been localized in type I cells from rat carotid bodies (Koerner et al., 2004). Although the histamine concentration in type I cells has been suggested to exceed that of dopamine by as much as 10 times, the functional role of histamine in the carotid body remains unexplained (Nurse, 2005). However, there is evidence that histamine may play an inhibitory role that opposes the excitatory effects of acetylcholine (ACh) on type I cells (Thompson et al., 2010). Moreover, type I cells also contain norepinephrine and serotonin (5-HT) (Nurse, 2005).

Many neurotransmitters are released from, and act upon the cells of the carotid body in response to stimuli. For an example, ACh, adenosine, ATP, and serotonin (5-HT) are excitatory neurotransmitters (Nurse & Piskuric, 2013). ACh has been found to act postsynaptically on CSN fibers, as these fibers are directly stimulated by ACh via nicotinic receptors (Iturriaga & Alcayaga, 2004; Fitzgerald, 2000). In addition, stimulation of nicotinic receptors on type I cells activates a cation current, causing a membrane depolarization and increase in intracellular Ca^{2+} (Wyatt & Peers, 1993; Shirata et al., 2007). Adenosine plays an excitatory role in ventilation due to its increase in chemosensory activity in the CSN (Conde et al.,
Also, purinergic (P2X) blockers were shown to inhibit hypoxic-induced postsynaptic responses, supporting ATP’s role as an excitatory neurotransmitter (Nurse, 2005; Nurse, 2010). Exogenous 5-HT has also been shown to have stimulatory effects on the CSN (Nurse & Piskuric, 2013).

Inhibitory pre- and postsynaptic neurotransmitters that act on the carotid body include GABA and dopamine (Nurse & Piskuric 2013). GABA agonists have been shown to reduce the hypoxic ventilatory response in mammals by inhibiting the CSN discharge (Kim et al., 2006; Shirahata, 2002). Dopamine receptor antagonists have been shown to increase the ventilatory response to hypoxia in rodents (Gonzalez et al., 1994; Iturriaga & Alcayaga, 2004; Shirata et al., 2007).

**Effects of Somatostatin on Type I Cells and Breathing**

SST has been localized within nerve fibers that innervate the arterial chemoreceptors of guinea pigs, but the role of this peptide in those sensory fibers has yet to be resolved (Kummer et al., 1990). Nevertheless, it has been shown that SST depresses the acute hypoxic ventilatory response, as well as sensitivity to CO₂ in conscious humans (Pedersen et al., 1999). On a scale of tens of minutes, humans infused with SST (0.5 mg·h⁻¹; 0.06 μM) were found to have reduced acute hypoxic ventilatory response by 82% and acute hypercapnic ventilatory response by 26% (Pandit et al., 2014).

Similar effects were found when the drug was intravenously injected into anesthetized rats. Rats infused with SST (100 μg·kg⁻¹; 0.95 μM) had reduced tidal volume and breathing frequency (Kaczyńska & Szereda-Prestaszewska, 2010).
In SST studies involving the breathing of humans and rats, the inhibiting mechanism of SST was hypothesized to be working at the level of the carotid body. There is evidence of SST directly affecting type I cells. SST (0.1 μM) was shown to reversibly inhibit whole cell Ca\(^{2+}\) current peaks by 24% in rat carotid body type I cells (e Silva & Lewis, 1995). Inhibition of Ca\(^{2+}\) entry may change the chemotransduction signal in type I cells, and partially account for the reductions in acute hypoxic and hypercapnic ventilatory responses in humans infused with SST.

Although studies investigating the peripheral effects of SST on breathing have been limited in number, there have been multiple studies assessing the central effects. For an example, intracisternally injected SST in rats caused apnea (Harfstrand et al., 1984). In cats, apnea occurred when SST was microinjected into a region of the medulla oblongata known as the nucleus paragigantocellularis lateralis (Yamamoto et al., 1988). Similarly in rats, microinjection of SST in the same area of the medulla oblongata induced apnea (Chen et al., 1990). However, when SST was microinjected into the rostral portion of the nucleus, the nucleus facialis, ventilatory depression occurred rather than apnea (Chen et al., 1990). These studies demonstrate the ability of SST to modulate central regulation of respiration. SST has been suggested to affect respiration at three different levels of the central respiratory network: 1) modulating the output of cranial and spinal motor neurons, 2) altering respiratory rhythmogenesis in the brainstem, and 3) regulating the chemosensory drive to the respiratory pattern generator (Llona & Eugenín, 2005).
Somatostatin

SST is a neuropeptide hormone that is distributed throughout the central and peripheral nervous systems. It is a neurotransmitter and modulates neural activity. It is naturally synthesized as an active peptide in two distinct forms: a 14-amino acid peptide chain (SST-14) and a 28-amino acid peptide chain (SST-28). SST is synthesized in various cells, such as neuroendocrine, inflammatory cells, and immune cells, in response to neurotransmitters, ions, steroid hormones, growth factors, cytokines, and nutrients (Patel, 1999). Both forms of the peptide act on multiple tissues, including the brain, gut, pituitary gland, endocrine pancreas, adrenal glands, kidneys, and immune cells (Patel & Srikant, 1997). Due to its ubiquitous nature, SST carries out a broad spectrum of biological responses upon binding to one of five SST receptor (SSTR) subtypes (Patel, 1999).

Plasma levels of SST vary among species, and even between individuals of the same species. For an example, someone who has recently eaten a meal will experience greater plasma SST levels than someone who has not. This is because SST plays a significant role in the digestive tract, as it is a potent inhibitor of many gastrointestinal hormones including gastrin, cholecystokinin, and secretin (Krejs, 1986). One study has measured normal human subjects to have fasting plasma SST concentrations of 13.3 pg•ml\(^{-1}\) (8.12 pM) on average (Saito & Saito, 1982). In addition to eating a meal, SST-secreting tumors can also alter plasma SST levels among individuals. Patients with carcinoma had plasma concentrations of SST ranging from 125 pg•ml\(^{-1}\) (80 pM) to 400 pg•ml\(^{-1}\) (244 pM), and a patient with
pheochromocytoma exhibited a SST concentration $47.0 \text{ pg}\cdot\text{ml}^{-1}$ (28.9 pM) in the plasma (Saito & Saito, 1982).

**Somatostatin Receptors**

In humans, SSTRs are encoded by five nonallelic genes located on different chromosomes (Patel & Srikant, 1997). Four of the genes contain no introns, with one exception: Splice variants of SSTR2 can give rise to SSTR2A and SSTR2B, which only differ in cytoplasmic C-tail length (Patel & Srikant, 1997). Moreover, there is a great degree of structural conservation in SSTRs among different species. Between humans and rats SSTR1 isoforms, there is 94-98% sequence identity, 93-96% for SSTR2, 88% SSTR4, and 82-83% for SSTR3 and SSTR5 (Patel & Srikant, 1997).

In order for SST to carry out its role, whether in neurotransmission, secretion, or cell proliferation, it must first bind to a family of five SSTR subtypes (SSTR1-5). SSTR1-5 have a seven transmembrane domain. Also, these receptors were identified to be G-protein coupled receptors (Yamada et al., 1992). All receptor subtypes bind to SST with great affinity, typically in the low nanomolar range (0.1-7.9 nM) (Patel & Srikant, 1994). SSTR1-4 have slightly higher affinity for SST-14, while SSTR5 has a greater affinity for SST-28 (Patel & Srikant, 1997). However, since SST-14 and SST-28 have great length and flexibility, both peptides can adopt the correct conformation allowing entry into any of the five SSTR subtype binding pockets (Patel & Srikant, 1997).

Upon binding to any of the five SSTRs, SST initiates G-protein coupled signaling cascade (Figure 4). SSTR activation is associated with decreases
intracellular cyclic AMP (cAMP) and Ca\(^{2+}\) concentrations, and stimulation of protein phosphatases (Patel & Srikant, 1997). Therefore, SSTR1-5 work via a G\(_i\)-protein coupled mechanism (Patel & Srikant, 1997).

Once activated after SST binding, the receptor then activates a pertussis toxin-sensitive GTP-binding protein, which inhibits adenylyl cyclase activity. Adenylyl cyclase causes a decrease in intracellular cAMP levels, which inhibits PKA activation and protein phosphorylation. SSTRs are also coupled to K\(^+\) channels, such as the BK\(_{Ca}\), delayed rectifier, and ATP-sensitive K\(^+\) channels (Patel et al., 1995; Reisine & Bell, 1995). SSTR activation causes K\(^+\) channels to hyperpolarize the cell membrane potential, inhibiting spontaneous action potentials. Also, this hyperpolarization causes a reduction in intracellular Ca\(^{2+}\) levels due inhibition of voltage-gated Ca\(^{2+}\) channels (Patel & Srikant, 1997). At the level of the type I cell, SST has been suggested to affect dihydropyridine-sensitive L-type Ca\(^{2+}\) channels and \(\omega\)-conotoxin GVIA-sensitive N-Type Ca\(^{2+}\) channels (e Silva & Lewis, 1995). Excitatory neurotransmitter normally depolarizes the cell membrane, which opens voltage-gated Ca\(^{2+}\) channels and induces a Ca\(^{2+}\) influx. But with no Ca\(^{2+}\) influx, vesicles will not be able to fuse with the membrane, neurotransmitter will not be released, and signaling from the excitable cell will be inhibited.

Other mechanisms have been studied to explain the inhibition of Ca\(^{2+}\) currents. For an example, SSTRs have been shown to activate protein phosphatases, such as the Ca\(^{2+}\)-dependent phosphatase calcineurin (Renström et al., 1996), protein tyrosine phosphatases (Florio et al., 1994), and serine threonine phosphatases (White et al., 1991). Serine threonine proteases dephosphorylate Ca\(^{2+}\) channel
proteins, and activate K+ channel proteins, thus hyperpolarizing the cell and inhibiting Ca2+ influx (Patel & Srikant, 1997).
Figure 4

Illustration of SST’s mechanism in modulating cell secretion. SST binding to any SSTR subtype stimulates the $G_i$ signaling pathway. Activation of a pertussis toxin-sensitive GTP-binding protein initiates three major effector pathways involving adenylyl cyclase (AC), $K^+$ channels, or protein phosphatases. The downstream effects of these three pathways include a decrease in intracellular cAMP, a decrease in intracellular Ca$^{2+}$, and an increase in calcineurin. The SST-dependent activation and increase in calcineurin, along with decreases in cAMP and Ca$^{2+}$, block exocytosis and inhibit cell secretion.
Hypothesis and Summary

The purpose of this study was to investigate the possible inhibiting mechanism of SST on the hypoxic and hypercapnic responses of type I cells from neonatal rat carotid bodies. Studies have shown that SST alters breathing, so this project was aimed at characterizing the effects of SST on voltage-gated Ca\(^{2+}\) influx in type I cells. The hypothesis of this study was that SST inhibits voltage-gated Ca\(^{2+}\) entry into isolated type I cells, and thus inhibits the acute hypoxic and hypercapnic ventilatory responses.

First, immunocytochemistry staining was performed to identify SST and SSTR1-5. Once identified, it had to be determined if SST and its receptors were functional. This was done by testing the excitability of type I cells and recording intracellular Ca\(^{2+}\) levels. The effect of SST on voltage-gated Ca\(^{2+}\) influx was measured using Ca\(^{2+}\) imaging. Voltage-gated Ca\(^{2+}\) channels were studied using a high potassium stimulus, which evokes a robust Ca\(^{2+}\) influx in type I cells. A physiological stimulus, hypoxia, was also used to test the effects of SST on the hypoxic response. Intracellular Ca\(^{2+}\) levels were measured using hypoxia to evoke voltage-gated Ca\(^{2+}\) entry into the type I cell. Since humans infused with SST also had a reduced hypercapnic ventilatory response, the effect of SST on the acid-induced depolarization of type I cells was also recorded. This was done using the perforated patch clamp technique to measure the membrane potential of type I cells.
MATERIALS AND METHODS
**Dissection and Dissociation of Neonatal Rat Carotid Body Type I Cells**

On each experimental day, three neonatal Sprague Dawley rats (12-19 days old) were placed, one at a time, in an induction chamber. In the chamber, animals were anesthetized with 4.5% isofluorane and 95.5% oxygen at a flow rate of 0.9 \( \text{L\cdotmin}^{-1} \). Once the animal was unconscious, it was transferred to a nose cone underneath a dissecting microscope. The rat in the nose cone was exposed to the same anesthetic gas and flow rate as in the induction chamber. The animals were checked to be completely unconscious by testing their withdrawal reflex using a foot and tail pinch. Once the rat had no response to the pinch, its limbs were taped down to keep it stationary, and the dissection was carried out.

Each dissection started with an incision made along the rat’s sternum to expose subcutaneous fascia. After this initial incision, a low magnification dissecting microscope (Omâno, Japan) was used for the remainder of the dissection. The fascia, salivary glands, fat tissue, and skeletal muscles lateral to the trachea were removed using fine forceps (Dumont Inox #5, Fine Science Tools), in order to view the common carotid artery. Any fat tissue and fascia still attached to the common carotid artery was carefully removed so that the bifurcation of the carotid artery was exposed. The hypoglossal nerve, which runs across the carotid artery, was cut and spread away from the bifurcation. The occipital artery, connected to the carotid artery, was then cut and proximally reflected exposing the internal carotid artery, where the carotid body is located. Using fine-tipped forceps, the carotid body was carefully removed from the connective tissue surrounding the internal carotid artery and placed directly in iced Dulbecco’s phosphate buffered saline (DPBS).
without calcium or magnesium (Sigma). While rats were still anesthetized, they were euthanized by decapitation and disposed of in accordance with Lab Animal Research requirements. The extracted carotid bodies were then examined under the Omâno microscope and any remaining excess tissue that adhered to the carotid body was removed.

Once excess tissue was removed, the carotid bodies were transferred to a separate Petri dish containing a digestive enzymatic solution (0.4 mg•ml\(^{-1}\) collagenase type I (Worthington), 0.2 mg•ml\(^{-1}\) trypsin type I (Sigma)) in DPBS with low calcium and magnesium. They were incubated at 37°C for 20 minutes to digest the connective tissue holding the organ together. The carotid bodies were then torn apart using fine forceps and incubated at 37°C for an additional 7 minutes. Next, the tissue was transferred to a 15 ml centrifuge tube where it was centrifuged at 130 g for 5 minutes. The tissue was resuspended in Ham's F-12 culture medium, containing L-glutamine and NaHCO\(_3\) (Sigma). After another 5 minute centrifugation, the digested tissue was triturated with a fire polished Pasteur pipette (Sigma). The cells were then plated onto 4 to 6 polylysine-coated coverslips and kept in Petri dishes and incubated at 37°C for 2 hours. After this incubation period, the cells were ready for experiments.

**Dissection and Dissociation of Adult Rat Carotid Body Type I Cells**

On experimental days with adult rats, two Sprague Dawley rats (150 g, 6-8 weeks old) were used. The dissection procedure was similar to that of neonatal rats with a few important differences. First, instead of using anesthesia, adult rats were
euthanized using a rising concentration of CO₂. In addition, due to the increased amount of connective, adipose, and nervous tissue in the adult rat, the carotid body was not directly taken off the bifurcation in the animal. The common carotid artery and the occipital artery were cut and removed from the animal with the bifurcation still intact. The extracted bifurcations were examined under the Omâno microscope and any excess connective and nervous tissue was removed. Once excess tissue was cleared from the bifurcation, the carotid body was carefully taken from the bifurcation. The remaining dissociation steps were identical to that of the neonatal rats, except the carotid bodies were incubated in the digestive enzymatic solution, containing 0.45 mg•ml⁻¹ collagenase type I and 0.25 mg•ml⁻¹ trypsin, for 25 minutes at 37°C.

**Immunocytochemistry**

Isolated type I cells adhered onto 22 X 22 mm square coverslips (Fisher Scientific) were fixed by immersion in methanol at -20°C for 15 minutes. The coverslips were tilted using fine forceps to remove excess solution from the top of the coverslip. Cells were then washed 3 x 5 minutes with 0.4 ml Blocking Solution (1% BSA (Sigma), 0.3% Triton-X 100 (Alfa Aesar), and phosphate buffered saline (PBS) (Sigma)). Anti-SSTR primary antibodies were used to identify the presence of SSTR1-5 on the membrane and in the cytoplasm of type I cells, while an anti-TH antibody was used to verify the cells were type I cells (Figure 5A).

The anti-SSTR1 primary antibody (ThermoFisher: PA3-206) was a rabbit polyclonal diluted in blocking solution 1:5000. Anti-SSTR2-5 primary antibodies
(Alomone Labs: ASR-006, ASR-003, ASR-004, ASR-005) were all rabbit polyclonal and diluted to 1:50 in blocking solution. The anti-TH primary antibody (Sigma) was mouse monoclonal and diluted to 1:1000 in blocking solution. Anti-SSTR and anti-TH primary antibodies were added to appropriate coverslips and incubated at 4°C overnight in a humidified chamber. Control coverslips contained no primary antibodies, only blocking solution.

After the incubation, the coverslips were washed in 0.4 ml blocking solution 2 x 10 minutes. The secondary antibodies were added to all coverslips and incubated in the dark at room temperature for 2 hours. Before the secondary antibodies were applied, they were centrifuged in blocking solution for two minutes at 1100 rpm. Rhodamine Red-X conjugated AffiniPure donkey-anti rabbit IgG secondary antibody (Jackson ImmunoResearch) was diluted to 1:200 in blocking solution and used to label SSTR1-5. Fluorescein (FITC)-conjugated AffiniPure donkey anti-mouse IgG secondary antibody (Jackson ImmunoResearch) was diluted to 1:200 in blocking solution and used to identify TH in the cytoplasm of type I cells. Coverslips were again washed in blocking solution 3 x 5 minutes. Vectashield mounting media (15 μl, Vector Laboratories) was placed on the microscope slide. The DAPI in the Vectashield stained the nucleus of the cells. After the coverslips were tilted to remove excess solution, they were placed faced down on the Vectashield. The coverslips were sealed with nail polish, placed in a slide box, and incubated in the dark at 4°C overnight. The first SSTR immunocytochemistry experiments are summarized in Figure 5B.
The DeltaVision microscope system (Applied Precision) on an inverted Olympus IX71 microscope was used to obtain images. The microscope contained an oil immersion, x63 magnification, 1.4 n.a. objective and Coolsnap HQ CCD camera (Photometrics). Each image was scaled to have a width of 15 μm. Images were set for RD TR PE 617/73 nm emission and 555/28 excitation (Percent Transmission (%T) = 50% and exposure = 0.100 s), FITC 528/38 nm emission and 490/20 excitation (%T = 32% and exposure = 0.100 s), and DAPI 457/50 nm emission and 360/40 excitation (%T = 100% and exposure = 0.050 s). As pictures were being taken, multiple Z-sections were also taken throughout the cell. Each Z-section was 0.25 μm apart, and the focal depth was 0.28 μm. All images were deconvolved with Softworx software (Applied Precision).

Due atypical perinuclear TH staining, a second SSTR immunocytochemistry experiment was performed using a new, unopened anti-TH primary antibody. The secondary antibodies were diluted 1:1000 in blocking solution in this experiment. The second set of experiments are summarized in Figure 5C.

The same procedure was also repeated for an anti-SST antibody (Sigma: SAB4502861). Anti-SST staining experiments are summarized in Figure 5D.
**Figure 5**

Staining in isolated type I cells from neonatal rats using double immunofluorescence. A) Illustration of 1° antibodies identifying SSTR, SST, and TH. The fluorescently labeled 2° antibodies bind to the 1° antibodies. SSTR and SST were stained red with Rhodamine and TH was stained green with FITC. B) Table summarizing the first set of SSTR immunocytochemistry experiments. C) Summary of second set of SSTR immunocytochemistry experiments. New anti-TH antibody was used and the 2° antibodies were diluted 1:1000. D) Summary of anti-SST immunocytochemistry experiment.
A)  

![Diagram of antibody interactions](image)

B)  

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Solutions

For the first set of Ca\(^{2+}\) imaging experiments, HEPES buffered salt solution, adjusted to pH 7.4 with NaOH at 35-37°C, was used as the extracellular solution. HEPES buffered salt solution contained the following concentrations (in mM): 140 NaCl, 4.5 KCl, 2.5 CaCl\(_2\), 1 MgCl\(_2\), 11 glucose, and 10 HEPES (NaCl and CaCl\(_2\) Fisher Scientific, all others Sigma). The high potassium stimulus (80 mM K\(^{+}\)) was HEPES buffered salt solution adjusted to pH 7.4 with KOH at 35-37°C, and contained the following concentrations (in mM): 64.5 NaCl, 80 KCl, 2.5 CaCl\(_2\), 1 MgCl\(_2\), 11 glucose, and 10 HEPES. The hypoxic stimulus was made by bubbling HEPES buffered salt solution with 100% N\(_2\) and adding 1 mM sodium dithionate (Fluka) to scavenge O\(_2\).

The next set of Ca\(^{2+}\) imaging experiments involving hypoxia used Tyrode’s solution at 35-37°C as the extracellular solution, and contained the following concentrations (in mM): 117 NaCl, 4.5 KCl, 11 glucose, and 23 NaHCO\(_3\) (Sigma). CaCl\(_2\) (2.5 mM) and MgCl\(_2\) (1 mM) was added after bubbling in 5% CO\(_2\) and 10% O\(_2\) for 15 minutes, to prevent calcium carbonate and magnesium carbonate formation. The control solution was gassed with 5% CO\(_2\) and 10% O\(_2\). The hypoxic stimulus was made by bubbling Tyrode’s solution in 95% N\(_2\) and 5% CO\(_2\) and adding 1 mM sodium dithionate to scavenge O\(_2\).

Electrophysiology experiments used the same hypoxic and control HEPES buffered salt solutions as described in the first Ca\(^{2+}\) imaging experiments, with an additional acidic stimulus. An acidic HEPES buffered salt solution was made as previously described, but it was adjusted to pH 6.9 with NaOH.
**Perforated Patch Clamp**

Isolated type I cells adhered onto 15 mm diameter round coverslips (Warner Instruments) were mounted in a perfusion chamber (0.4 ml, RC-25F, Warner Instruments) and perfused with extracellular solution at a rate of 8 ml•min⁻¹. Cells were viewed using the Nikon Eclipse TE 2000U inverted microscope. Recordings were made using the amphotericin perforated patch configuration of the whole-cell patch clamp technique (Figure 6). Data were acquired using an Axopatch 200B amplifier (Molecular Devices) controlled by Clampex 10 (Molecular Devices). Currents were sampled at 2 kHz and filtered at 1 kHz. All data were analyzed using Clampfit 10 software (Molecular Devices). All experiments were completed on an anti-vibration microscopy table (TMC) and performed in the range of 35-37°C.

Pipettes were made from 1.5 OD x 0.86 ID x 100 L mm glass capillaries (Harvard Apparatus). The ends of the glass capillaries were polished by placing over a flame from a methanol lamp. Once cooled, the pipettes were pulled using the Narishige Model PC-10 (Narishige Group). Pipette tips with a resistance of 6.5-9.5 MΩ were used in experiments. Pipettes were half-filled with 250 μg•ml⁻¹ amphotericin B (Sigma), then placed on the Ag/AgCl electrode wire. The electrode wire was mounted on a motorized micromanipulator (Sutter Instrument Company). The pipette tip was carefully lowered into the extracellular solution of the perfusion chamber using the motorized micromanipulator. Once the tip contacted the cell membrane, suction was slowly applied to form a gigaseal, allowing electrical access to the cell.
After allowing 5 minutes for amphotericin B to perforate the cell, the seal was checked. When a stable patch was obtained with a seal resistance greater than 2 GΩ, the membrane potentials of type I cells were recorded. After a stable baseline membrane potential reading, cells were exposed to the acidic HEPES buffered salt solution which caused a depolarization. Once the cell recovered to its initial baseline membrane potential, the cell was exposed to 1 μM SST (Sigma: S9129) for 2 minutes. The cell was then exposed to the acidic stimulus again in the presence of 1 μM SST.
Figure 6

Schematic showing the setup for electrophysiology experiments.
Ca\textsuperscript{2+} imaging experiments with FURA-2

Isolated type I cells adhered to 15 mm diameter round coverslips were loaded with a Ca\textsuperscript{2+}-sensitive ratiometric dye, FURA-2AM (Invitrogen), in a Petri dish at room temperature. The cells were loaded for 30 minutes in the dark to allow the dye to penetrate the cells. For Ca\textsuperscript{2+} imaging experiments using a high potassium stimulus, the loading solution contained 5 μM FURA-2AM in HEPES buffered salt solution adjusted to pH 7.4. For Ca\textsuperscript{2+} imaging experiments using a hypoxic stimulus, the loading solution contained 5 μM FURA-2AM in Ham’s F-12 culture medium and was gassed at room temperature with 10% O\textsubscript{2} and 5% CO\textsubscript{2}. FURA-2AM is a dye commonly used in Ca\textsuperscript{2+} imaging experiments and has ratiometric properties that avoid bleaching artifacts. After the loading period, the coverslips were washed twice in either pH 7.4 HEPES buffered salt solution (high potassium experiments) or Ham’s F-12 culture medium (hypoxic experiments) in the dark for 10 minutes each to ensure that the –AM groups from the dye were cleaved.

The coverslips were then mounted in a perfusion chamber and perfused with extracellular solution at a rate of 8 ml•min\textsuperscript{-1}. Cells were viewed using the Nikon Eclipse TE 2000U inverted microscope with a CFI super fluor x40 oil immersion objective. Cells were excited with 50 ms exposures to 340 nm and 380 nm light at 0.2-0.5 Hz using a Lambda 10-3 filter wheel (Sutter Instrument Company). The light traveled from the lamp source, through a neutral density filter (Chroma), and reflected from a 400 DCLP dichroic mirror (Chroma) up to the cells. The light from the high wattage lamp source (Lambda-LS xenon arc lamp, Sutter Instrument Company) first passed through neutral density filters with optical densities of 0.7,
before reaching the cells to prevent photodamage. When the light reached the cells, they emitted fluorescent light, which was brightest 510 nm. This light was then reflected through the dichroic mirror, bent 90°, and traveled through a 510 nm ± 30 nm filter. The light that passed through the 510 nm filter (Chroma) was recorded using a CoolSnap HQ2 CCD camera (Photometrics) (Figure 7). Image acquisition was controlled by Metafluor 7.1.2 software (Molecular Devices). A spherical region of interest was placed around the cells so that the software could graph the 340/380 fluorescent ratios over time.

In the first set of Ca²⁺ imaging experiments, cells were perfused with HEPES buffered salt solution. A high potassium stimulus was used to evoke an increase in voltage-gated Ca²⁺ influx in the neonatal rat isolated type I cells. The large increase in cytoplasmic Ca²⁺ levels in response to the high potassium stimulus indicated type I cells. Cells were then exposed to 1 μM SST for 2, 10 and 20 minutes, and then exposed to the high potassium stimulus again. Initially, cells were exposed to 0.1 μM, but since no inhibition was evident, the concentration for SST was increased by a factor of 10 for remaining experiments. This Ca²⁺ imaging experiments was repeated in isolated type I cells from adult rats using the high potassium stimulus. In experiments with adult rats, the effect of a 2 minute exposure of 1 μM SST was measured. The procedure was identical to the first set of Ca²⁺ experiments on neonatal rat type I cells.

The next set of Ca²⁺ imaging experiments measured the effects of a 2 minute exposure of 1 μM SST on the hypoxic response in isolated type I cells from the neonatal rat carotid body. Due to the cells instability and lack of responsiveness to a
hypoxic HEPES buffered salt solution, a hypoxic Tyrode’s solution was used. With the exception of the bathing solutions, the procedure was similar to the first set of Ca\(^{2+}\) experiments. Ca\(^{2+}\) imaging on the hypoxic response was also repeated for a longer time frame: 10 and 20 min SST exposures. The hypoxic Ca\(^{2+}\) imaging experiments with 10 and 20 minute exposures were identical to the first set of hypoxic Ca\(^{2+}\) imaging experiments.
Figure 7

Schematic showing the setup for calcium imaging experiments.
Oxygen Measurements in Solutions

$P_{O_2}$ was measured in control and hypoxic HEPES buffered salt solutions using OxyMicroDevice (World Precision Instruments, Inc.). The male fiber-optic plug was connected to the ST-plug of the OxyMicroDevice. The spring of the male fiber-optic plug of the microsensor was inserted into the groove of the female fiber-optic plug. A safety nut was carefully turned to ensure the fiber-optic plug was locked into the OxyMicroDevice. The other end of the fiber-optic cable contained the syringe and microsensor. The oxygen-sensitive needle-type microsensor was housed in a 0.4 x 40 mm syringe needle mounted to a 1 ml plastic syringe.

The protective plastic cap was carefully removed to expose the syringe needle. The syringe needle was placed above the perfusing HEPES buffered salt solution, with the glass fiber retracted. Then, the glass fiber with the microsensor tip was completely extended from the syringe needle by carefully pressing the syringe plunger so that the tip was submerged in the solution. The microsensor is based on luminescence quenching caused by collisions between luminescent dye molecules in the excited state and molecular oxygen. In the presence of oxygen, a collision occurs where energy is transferred from the excited indicator molecule (on microsensor) to oxygen. Oxygen therefore changes from its ground state into an excited state. This makes the indicator molecule unable to emit luminescence, and its luminescence signal decreases. In the absence of oxygen, the excited indicator molecule does not collide with oxygen. Since no collisions occur, the excited indicator molecule remains in the excited state, emits luminescent light, and has a
measurable luminescence signal that is greater than the signal when oxygen is present.

After the oxygen level was recorded in the control HEPES buffered salt solution, the solution was changed to the hypoxic solution and the oxygen level was recorded (Figure 8). When finished, the glass fiber with the microsensor tip was rinsed with distilled water to remove any sample residues. The microsensor tip was then retracted into the protective housing and the plastic cap was placed back on the syringe.
Figure 8

Measuring $P_O_2$ levels in control and hypoxic solutions using OxyMicroDevice. The initial $P_O_2$ in control solution is 140 torr. When the control solution is switched to the hypoxic solution (100% $N_2$), the $P_O_2$ descended from 140 torr to 20 torr in a minute, and it took another minute for the $P_O_2$ to reach under 10 torr. When switched back to the control solution, the $P_O_2$ recovered to 140 torr in a minute.
Statistics

Data are presented as mean ± standard error of the mean. Differences between individual means were determined by an unpaired Student’s t test, and statistical significance was indicated by values of p<0.05.
RESULTS
**Immunocytochemistry**

**Staining for SSTRs in Isolated Type I Cells**

SSTR1-5 were qualitatively identified on isolated type I cells from the carotid bodies of neonatal rats using double immunofluorescence. Five distinct rabbit anti-rat primary antibodies were used to characterize all five SSTR. Receptors were stained red with the secondary antibody containing Rhodamine. TH, which indicates type I cells, was stained green with the secondary antibody containing FITC. DAPI stained the nucleus blue. In control experiments, the cells were not exposed to primary antibodies, only secondary antibodies.

Staining results show SSTR1-5 are all present cytoplasmically within the type I cell, with SSTR1 also appearing on the cell membrane (Figure 9). The control image, containing no primary antibodies, shows only the nucleus from DAPI staining. This indicates that there was no non-target binding of the secondary antibodies. All images show green staining of TH, marking the cells as type I cells. However, the TH staining appears abnormally perinuclear. TH staining is typically more obvious in the cytoplasm, because anti-TH antibodies should be present in cytoplasmic vesicles containing dopamine. The first set of immunocytochemistry experiments used an old anti-TH antibody, which might have accounted for the perinuclear staining.

The staining procedure was repeated for a second set of experiments. To account for the abnormal TH staining in the previous experiment, a new primary anti-TH antibody was used, and the dilutions for all secondary antibodies was 1:1000 instead of 1:200. The images show that SSTR1-5 are all present in the
cytoplasm (Figure 10). There is some TH staining in the cytoplasm, but it is predominantly perinuclear again. Because the sources of the primary and secondary antibodies were the same as the first experiment, no control image is shown in this figure; refer to Figure 9 for a control image.

Staining for SST in Isolated Type I Cells

Type I cells were also stained for SST using a rabbit anti-rat primary antibody. SST red was stained red with Rhodamine, TH was stained green with FITC, and the nucleus was stained blue with DAPI. The image shows that SST is present throughout the cytoplasm and membrane in type I cells (Figure 11).
Figure 9

SSTR staining in isolated neonatal rat carotid body type I cells using double immunofluorescence. SSTR1-5 are all present in the cytoplasm in type I cells. SSTR1 staining also suggests that the receptor may be present on the membrane. SSTRs are stained red with Rhodamine. TH, indicating a type I cell, is stained green with FITC. The nucleus of the cells are stained blue with DAPI. Scale bar is 10 microns.
SSTR1  SSTR2  SSTR3  SSTR4  SSTR5  Control

Red: SSTR
Green: Tyrosine Hydroxylase
Blue: Nucleus
Figure 10

Repeated SSTR staining in isolated neonatal rat carotid body type I cells using double immunofluorescence. SSTR1-5 are all present cytoplasmically in type I cells. SSTRs are stained red with Rhodamine. TH, indicating a type I cell, is stained green with FITC. The nucleus of the cells are stained blue with DAPI. Secondary antibodies were diluted 1:1000. Scale bar is 10 microns.
Figure 11

SST staining in isolated neonatal rat carotid body type I cells using double immunofluorescence. SST is present in the cytoplasm and membrane of type I cells.

The secondary antibodies used were the same as those used when staining for SSTRs, refer to Figure 9 for control staining. Scale bar is 10 microns.
Calcium Imaging

High K⁺ Stimulus in Neonatal Rat Carotid Body Type I Cells

The effect of SST on voltage-gated Ca²⁺ influx was measured using Ca²⁺ imaging with FURA-2. A high potassium stimulus was used to open voltage-gated Ca²⁺ channels, evoking an increase in intracellular Ca²⁺ levels. The high potassium stimulus was used to indicate healthy type I cells. Cells that did not respond to the high potassium stimulus were not used for recordings, because this meant the cell was either damaged or not a type I cell.

Cells that had a robust response to high potassium were quickly placed in control HEPES buffered salt solution and recovered to baseline Ca²⁺ levels. On average, cells had an initial baseline Ca²⁺ 340/380 ratio of 0.578 (n=18). Once baseline levels were reached cells recovered for an additional 2 minutes, and then exposed to the high potassium stimulus again (Figure 12A). In experiments with SST, once baseline Ca²⁺ levels were reached, the cells recovered for 2 minutes in HEPES buffered salt solution containing 1 μM SST (Figure 12B). After the 2 minute recovery, cells were exposed to the high potassium stimulus in the presence of 1 μM SST.

The first Ca²⁺ peak was compared to the smaller second Ca²⁺ peak, and this difference was measured as the percent inhibition (Figure 12C). FURA-2 fluorescence ratios show no significant Ca²⁺ inhibition for type I cells exposed to 1 μM SST for 2 minutes compared to controls (p=0.53). Control cells had an average inhibition of 9.3 ± 2.9% inhibition (n=8). Cells exposed to 1 μM SST had an average inhibition of 7.1 ± 2.1% inhibition (n=10).
Figure 12

Effect of 2 minute exposure of 1 μM SST on K⁺ evoke Ca²⁺ currents in isolated type I cells from the carotid body of neonatal rats using Ca²⁺ imaging. A) Control with 2 minute recovery in HEPES buffered salt solution. B) 2 minute recovery in 1 μM SST and HEPES buffered salt solution. C) Histogram showing the percent inhibition of K⁺ evoked Ca²⁺ influx for type I cells (p>0.05, error bars are standard error of the mean).
This experiment was repeated with longer SST exposures to test if the mechanism of 1 μM SST took longer than 2 minutes to have an effect on the type I cell. The cells used in the experiments with 10 and 20 minute exposures of 1 μM SST had an average initial baseline Ca\textsuperscript{2+} 340/380 ratio of 0.578 (n=22). In control experiments, cells were initially exposed to high potassium, opening voltage-gated Ca\textsuperscript{2+} channels and thus causing a Ca\textsuperscript{2+} influx. The cells then recovered to the baseline Ca\textsuperscript{2+} level. 10 minutes after baseline Ca\textsuperscript{2+} levels were reached, the cells were exposed to high potassium, and then again after 20 minutes (Figure 13A). In experiments with SST, cells were exposed to 1 μM SST during the 10 and 20 minute recoveries, and exposed to 1 μM SST in the presence of the high potassium stimulus (Figure 13B).

The 10 and 20 minute Ca\textsuperscript{2+} peaks were separately compared with the initial potassium induced Ca\textsuperscript{2+} peak (Figure 13C). FURA-2 fluorescence ratios show no significant Ca\textsuperscript{2+} inhibition for type I cells exposed to 1 μM SST when compared to controls, for both 10 minutes (p=0.22) and 20 minutes (p=0.10). Control cells had an average inhibition of 12.2 ± 1.5% after 10 minutes (n=12), while cells exposed to 1 μM SST had an average inhibition of 15.5 ± 2.2% after 10 minutes (n=10). After 20 minutes, control cells had an average inhibition of 22.6 ± 2.1%, and cells exposed to 1 μM SST had an average inhibition of 28.6 ± 2.9%.
Figure 13

Effect of 10 and 20 minute exposures of 1 μM SST on K⁺ evoked Ca²⁺ influx in isolated type I cells from the carotid body of neonatal rats using Ca²⁺ imaging. A) Control with 10 and 20 minute recovery in HEPES buffered salt solution. B) 10 and 20 minute recovery in 1 μM SST and HEPES buffered salt solution. C) Histogram showing the percent inhibition of K⁺ evoked Ca²⁺ influx for type I cells (p>0.05, error bars are standard error of the mean).
**Hypoxic Stimulus in Neonatal Rat Carotid Body Type I Cells**

To test whether SST has an effect on the hypoxic response in type I cells, Ca\(^{2+}\) imaging was repeated using a hypoxic stimulus. Type I cells were exposed to a 2 minute application of 1 μM SST and the change in intracellular Ca\(^{2+}\) levels were recorded using FURA-2. The cells were bathed in Tyrode’s solution for all hypoxic experiments. Initially, cells were exposed to a hypoxic Tyrode’s solution, and if hypoxia evoked an intracellular Ca\(^{2+}\) increase, then the cell was a type I cell. Only cells that responded to hypoxia were used for this experiment.

Cells were used for recording if they had a robust response to hypoxia during a 2 minute exposure. The cells used in this hypoxic Ca\(^{2+}\) imaging experiment had an average initial baseline Ca\(^{2+}\) 340/380 ratio of 0.56 (n=32). During the first hypoxic stimulus in control experiments, cells had an average fluorescence ratio increase of 0.53 ± 0.05 (n=15). After the initial stimulus, the cells recovered in Tyrode’s solution to allow cytoplasmic Ca\(^{2+}\) levels to return to baseline. After 5 minutes from the point when baseline was reached, the cells were exposed to the hypoxic stimulus again for 2 minutes (Figure 14A). During the second hypoxic stimulus in control experiments, cells had an average fluorescence ratio increase of 0.50 ± 0.04.

In experiments with 1 μM SST, the average fluorescence ratio increase during the initial hypoxic stimulus was 0.77 ± 0.06 (n=16). Once baseline Ca\(^{2+}\) were reached from the 2 minute stimulus, the cells recovered in Tyrode’s solution for 3 minutes. After 3 minutes, the cells were exposed to Tyrode’s solution containing 1 μM SST for 2 minutes, and then the hypoxia solution containing 1 μM SST for an
additional 2 minutes (Figure 14B). During the second hypoxic exposure, in the presence of 1 μM SST, cells had an average fluorescence ratio increase of 0.70 ± 0.06.

The average fluorescence ratio of the first hypoxic response was compared to the average fluorescence ratio of the second hypoxic response. This difference was measured as percent inhibition (Figure 14C). FURA-2 fluorescence ratios show no significant Ca^{2+} inhibition for type I cells exposed to 1 μM SST for 2 minutes compared to controls (p=0.64). Control cells had an average inhibition of 14.2 ± 2.7% (n=15). Cells exposed to 1 μM SST for 2 minutes had an average inhibition of 12.5 ± 2.2% (n=16).
Figure 14

Effect of 2 minute application of 1 μM SST on the hypoxic response of isolated type I cells from the carotid body of neonatal rats using Ca^{2+} imaging. A) Control with 5 minute recovery in Tyrode's solution. B) 3 minute recovery in Tyrode's solution, then 2 minute exposure to Tyrode's solution containing 1 μM SST. C) Histogram showing the percent inhibition of hypoxic evoked Ca^{2+} influx in type I cells (p>0.05, error bars are standard error of the mean).
To test the effects of longer exposures of SST on the hypoxic response, $\text{Ca}^{2+}$ was repeated with 10 and 20 minute exposures of 1 $\mu$M SST using a hypoxic stimulus. To identify type I cells, the cells were initially exposed to a hypoxic Tyrode’s solution. Only the cells that had responded to hypoxia were used for this experiment.

The cells used in this hypoxic $\text{Ca}^{2+}$ imaging experiment had an average initial baseline $\text{Ca}^{2+}$ 340/380 ratio of 0.49 ($n=23$). During the first hypoxic stimulus in control experiments, cells had an average fluorescence ratio increase of $0.72 \pm 0.07$ ($n=8$). Once baseline $\text{Ca}^{2+}$ levels were reached from the initial stimulus, the cells recovered in Tyrode’s solution for 10 minutes (Figure 15A). At the 10 minute mark, the cells were exposed to the hypoxic stimulus again for 2 minutes. Cells had an average fluorescence ratio increase of $0.67 \pm 0.06$ during the hypoxic stimulus at 10 minutes. After 20 minutes from the $\text{Ca}^{2+}$ baseline recovery point from the initial hypoxic stimulus, the cells were exposed to hypoxia for 2 minutes a final time. During the third hypoxic exposure, at 20 minutes, cells had an average fluorescence increase of $0.56 \pm 0.05$.

In experiments with 1 $\mu$M SST, the average fluorescence ratio increase during the initial hypoxic stimulus was $0.84 \pm 0.04$. Once baseline $\text{Ca}^{2+}$ were reached from the initial 2 minute stimulus, the cells recovered in Tyrode’s solution with 1 $\mu$M SST for 10 minutes (Figure 15B). After 10 minutes, the cells were exposed to a hypoxic Tyrode’s solution containing 1 $\mu$M SST for 2 minutes. Cells had an average fluorescence ratio increase of $0.77 \pm 0.04$ ($n=15$) during the hypoxic stimulus at 10 minutes. A second hypoxic Tyrode’s solution with 1 $\mu$M SST was applied to the cells
20 minutes after the initial hypoxic response. Cells had an average fluorescence ratio increase of $0.75 \pm 0.05$ (n=12) during the hypoxic stimulus at 20 minutes.

The average fluorescence ratio of the first hypoxic response was compared to the average fluorescence ratio of the hypoxic responses after 10 minutes, and after 20 minutes. This difference was measured as percent inhibition (Figure 15C). FURA-2 fluorescence ratios show no significant Ca$^{2+}$ inhibition for type I cells exposed to 1 μM SST for 10 minutes compared to controls (p=0.77). Also, Ca$^{2+}$ entry was not significantly inhibited for type I cells exposed to 1 μM SST for 20 minutes compared to controls. After 20 minutes, control cells actually had a greater percent inhibition than those cells exposed to 1 μM SST, but the difference between the two groups is not statistically significant (p=0.19). After 10 and 20 minutes, control cells had an average inhibition of 7.1% ± 3.3%, and 20.5% ± 4.1%, respectively (n=8). Cells exposed to 1 μM SST had an average inhibition of 8.1% ± 1.7% (n=15) after 10 minutes, and 14.0% ± 2.8% (n=12) after 20 minutes.
Figure 15

Effect of 10 and 20 minute exposures of 1 μM SST on the hypoxic response of isolated type I cells from neonatal rat carotid bodies using Ca\(^{2+}\) imaging. A) Control with 10 and 20 minute recovery in Tyrode’s solution. B) 10 and 20 minute recovery in 1 μM SST and Tyrode’s solution. C) Histogram showing the percent inhibition of hypoxic evoked Ca\(^{2+}\) influx for type I cells (p>0.05, error bars are standard error of the mean).
Electrophysiology

Perforated Patch Clamp

The effects of SST on the acid-induced depolarization in type I cells was tested by exposing the cells to 1 μM SST for 2 minutes. The membrane potentials of type I cells was recorded using the amphotericin perforated-patch configuration of the whole-cell patch clamp technique (Burlon et al., 2009). Type I cells had an average resting membrane potential of -42.16 mV ± 2.1 mV, (n=9). In control experiments, acidic HEPES buffered salt solution was applied to type I cells, and the change in membrane potential was recorded (Figure 16A). Once the depolarized cell returned to baseline membrane potential, the cell recovered in control HEPES buffered salt solution for 3 minutes. After the recovery period, the cell was exposed to 1 μM SST for 2 minutes and exposed to acidic HEPES in the presence of 1 μM SST (Figure 16B). After the cell recovered again for 5 minutes in HEPES buffered salt solution, the acidic stimulus was applied again and the membrane depolarization was recorded (Figure 16C).

The cells’ average depolarization was compared in the presence of acid and no SST, acid and 1 μM SST, and acid after SST exposure (recovery) (Figure 17). The average depolarization of a type I cell in the presence of acid was 9.7 ± 0.8 mV. The average depolarization after a 2 minute exposure of 1 μM SST decreased to 8.2 ± 1.3 mV. However, this decrease in acid-induced depolarization in cells exposed to 1 μM SST is not significant when compared to controls (p=0.29). The average recovery depolarization was 8.9 ± 1.7 mV, and is not significantly different when compared to
the depolarization in acid only (p=0.57) nor acid plus SST (p=0.72). Therefore, 1 μM SST did not significantly inhibit the acid response of type I cells.
Figure 16

Effect of 2 minute application of 1 μM SST on acid-induced depolarization of type I cells using perforated patch clamp. Type I cell membrane potential in A) acid only, B) 1 μM SST and acid, and C) acid after SST exposure.
Figure 17

Histogram comparing average depolarization in acid only, acid plus 1 μM SST, and acid after SST exposure. Acid caused type I cells to depolarize 9.7 ± 0.8 mV on average, while acid with 1 μM SST caused an average depolarization of 8.2 ± 1.3 mV. The reduction in acid-induced depolarization of 1 μM SST is not significant (n=8, p > 0.05, error bars are standard error of the mean).
Calcium Imaging in Adult Rats

High K⁺ Stimulus in Adult Rat Carotid Body Type I Cells

To test whether SST acts upon the carotid body in an age-dependent manner, Ca²⁺ imaging experiments were repeated with 2 minute applications of 1 μM SST in adult rat type I cells. The effect of SST on voltage-gated Ca²⁺ influx in isolated type I cells from adult rat carotid bodies was measured using Ca²⁺ imaging with FURA-2.

In adult rat Ca²⁺ imaging experiments, a high potassium stimulus was used to evoke voltage-gated Ca²⁺ influx. On average, cells had an initial baseline Ca²⁺ 340/380 ratio of 0.53 ± 0.02 (n=19). In control experiments, cells exposed to the initial high potassium stimulus had an average fluorescence ratio increase of 0.96 ± 0.10 (n=11). Once baseline levels were reached from the initial high potassium stimulus, cells recovered for an additional 2 minutes before being exposed to the high potassium stimulus again (Figure 18A). Cells had an average fluorescence ratio increase of 0.87 ± 0.09 during the high potassium stimulus at 2 minutes.

In experiments with SST, cells exposed to the initial high potassium stimulus had an average fluorescence ratio increase of 0.79 ± 0.06 (n=8). Once baseline Ca²⁺ levels were reached, the cells recovered for 2 minutes in HEPES buffered salt solution containing 1 μM SST (Figure 18B). After the 2 minute recovery, cells were exposed to the high potassium solution in the presence of 1 μM SST. Cells exposed to the high potassium stimulus and 1 μM SST at the 2 minute mark had an average fluorescence ratio increase of 0.68 ± 0.06.

The first Ca²⁺ peak was compared to the second Ca²⁺ peak, and this difference was measured as the percent inhibition (Figure 18C). FURA-2 fluorescence ratios
show control cells had an average inhibition of 10.5 ± 1.9% inhibition (n=11). Cells exposed to 1 μM SST had an average inhibition of 15.0 ± 1.7% inhibition (n=8). However, the larger percent inhibition for cells exposed to 1 μM SST is not significantly different from controls (p=0.11). Therefore, exposure of 1 μM SST for 2 minutes does not significantly inhibit voltage-gated Ca^{2+} influx in isolated type I cells from adult rat carotid bodies.
Figure 18

Effect of 2 minute exposure of 1 μM SST on isolated type I cells from the carotid body of adult rats using Ca\textsuperscript{2+} imaging. A) Control with 2 minute recovery in HEPES buffered salt solution. B) 2 minute recovery in 1 μM SST and HEPES buffered salt solution. C) Histogram showing the percent inhibition of K\textsuperscript{+} evoked Ca\textsuperscript{2+} influx for type I cells (p>0.05, error bars are standard error of the mean).
DISCUSSION
Summary of Results

These experiments have identified the presence of SST and SSTR1-5 in isolated type I cells from neonatal rat carotid bodies. However, when the functionality of these receptors were tested with 1 μM SST, no effects were evident. The data presented in this study show that SST does not significantly inhibit voltage-gated Ca$^{2+}$ influx in the type I cells of both neonatal and adult rats, nor the acid-induced depolarization in type I cells from neonatal rats.

Previous studies have shown that when humans were infused with SST, their ventilatory responses to hypoxia were reduced (Pedersen et al., 1999; Pandit et al., 2014). SST was hypothesized to be working at the level of the carotid body, and e Silva and Lewis (1995) have shown 0.1 μM SST to inhibit whole cell Ca$^{2+}$ current in rat carotid body type I cells. However, there are some important differences in the experimental setup between the current work and the work of e Silva and Lewis (1995). First, e Silva and Lewis (1995) did not mention how long they applied SST on the type I cells before recording Ca$^{2+}$ currents. Another key difference is that e Silva and Lewis (1995) recorded currents at room temperature (22-26°C), while all recordings in this report were done at 35-37°C. Finally, in e Silva and Lewis (1995), they applied the 0.1 μM SST solution through a micropipette near the recording cell (Figure 19A). This technique differs from the setup in present study because temperature cannot be accounted for when using a micropipette to apply the drug. If a colder solution is being applied to a type I cell, then the temperature, rather than the drug, may account for a significant reduction in Ca$^{2+}$ currents. In the present
study, the cells were perfused with a solution with a concentration of 1 μM SST at 35-37°C (Figure 19B).
Figure 19

Schematic illustrating the different setups for Ca\(^{2+}\) recordings in isolated type I cells.

A) The setup of e Silva and Lewis (1995). SST was not diluted in the extracellular solution being perfused, but rather, diluted in a separate solution in a micropipette. The temperature of the SST solution in the micropipette could not be controlled, and this solution was applied directly onto type I cells. The cells were perfused with an extracellular solution and Ca\(^{2+}\) currents from type I cells were recorded at 22-26°C.

B) The setup of the present work. SST was diluted in the perfusing extracellular solution, therefore the SST concentration was constant in the perfusion chamber. The temperature for both control and experimental solutions were regulated at 35-37°C.
A

Extracellular Solution

Glass Pipette

[0.1 µM SST]
Temp = ?

Type I Cell

22-26 °C

B

Temperature Regulator

Extracellular Solution [1 µM SST]

[1 µM SST]

Type I Cell

35-37 °C
In response to chemostimuli, the carotid body releases neurotransmitter due to opening of voltage-gated Ca\(^{2+}\) channels and Ca\(^{2+}\) influx. To test whether or not SST has an inhibitory effect on voltage-gated Ca\(^{2+}\) entry, 2, 10, and 20 minute exposures of 1 μM SST were applied to isolated type I cells and intracellular Ca\(^{2+}\) levels were recorded. A 2 minute exposure was conducted to test if SST was working on the type I cell on a scale of minutes. Longer exposures of 10 and 20 minutes were also tested because Pandit et al. (2014) found SST to reduce the acute hypoxic and hypercapnic ventilatory response in humans that were infused with SST after undergoing a 15 minute period of resting breathing. In addition, Perry and Sandle (2009) found SST to inhibit apical BK Channels in surface colonocytes in the human colon after a 5 minute period, but maximal inhibition occurred after 25 minutes.

1 μM SST was chosen for all experiments in the present work. Initially, 0.1 μM was used in experiments, but this concentration was increased 10-fold when no significant effects on Ca\(^{2+}\) influx were evident. Work presented in this thesis has shown that SST is present in the type I cell, which suggests that the type I cell is capable of releasing SST. Despite resting SST plasma levels occurring in the picomolar range, the concentration of SST in the synapse between a type I cell and afferent chemosensory fibers may be much greater than the picomolar range. It is widely accepted that synaptically released ACh reaches millimolar concentration at the neuromuscular junction (Scimemi & Beato 2009). This became evident when Kuffler and Yoshikami (1975) calculated the peak concentration of ACh in the neuromuscular synapse of the snake to be 0.3 mM. A study later calculated the frog
and mouse neuromuscular junctions to have peak ACh cleft concentrations of 1 mM (Matthews-Bellinger & Salpeter, 1978). Therefore, this study used a concentration of SST much greater than what has been observed in the plasma.

When SST was shown not to have a significant inhibition on voltage-gated $\text{Ca}^{2+}$ entry, the hypoxic response of type I cells was tested. Similarly, 2, 10, and 20 minute exposures of SST had no significant effect on the hypoxic response of type I cells.

In e Silva and Lewis (1995), they saw SST reduce $\text{Ca}^{2+}$ currents in adult rats (150-350 g). To test if SST affected voltage-gated $\text{Ca}^{2+}$ entry in an age-dependent mechanism, $\text{Ca}^{2+}$ imaging experiments were repeated and SST was applied to adult rat type I cells. However, again, there was no significant inhibition in $\text{Ca}^{2+}$ when the cells were exposed to a high potassium stimulus after a 2 minute SST exposure.

Finally, humans infused with SST also experienced a reduced ventilatory response to hypercapnia (Pandit et al., 2014). In the present study, the effects of 1 $\mu$M SST were tested on the acid-induced depolarization of type I cells, and again, SST had no significant inhibition.

The data reported reject the hypothesis that SST is inhibiting the acute hypoxic and hypercapnic ventilatory response at the level of voltage-gated $\text{Ca}^{2+}$ entry in type I cells. But SST may still have peripheral effects in response to chemostimuli, despite these negative results. SST may have direct effects on vesicle fusion machinery, inhibiting neurotransmitter release in type I cells. SST has been shown to inhibit the fusion process between secretion vesicles and the plasma membrane in the anterior pituitaries of rats (Draznin et al., 1986). If SST directly
inhibits vesicle fusion in type I cells, then neurotransmitter release will be inhibited, and the firing rate in the CSN will be decreased, ultimately reducing the hypoxic ventilatory response. In addition, SST may act post-synaptically on the CSN. If SSTRs are expressed post-synaptically on the CSN, then it could be possible that SST directly reduces the firing rate of the CSN, and thus decreases the acute ventilatory response to hypoxia.

**Future Experiments**

Since SST had no effect on type I cells in all of the experiments reported, one future experiment will be a positive control to test whether or not SSTRs are functional. The experiment could test if SST is altering cAMP levels, using imaging and a cAMP-sensitive dye. All SSTR subtypes are G coupled and efficiently inhibit adenyl cyclase (Patel, 1999), so it would be predicted that SST reduces cAMP fluorescence ratios. Jacobs et al. (2010) conducted a similar study, monitoring intracellular cAMP levels using fluorescence resonance energy transfer (FRET). Transgenic mice with ubiquitous expression of highly sensitive FRET-based cAMP biosensor exchange proteins were used to measure cAMP levels in pituitary cells (Jacobs et al., 2010). However, the FRET-based cAMP biosensor exchange protein technique would not be useful in this project because genetically encoded protein cannot be transfected into rat primary tissue such as the type I cell. If this technique were to be used, all previous experiments would have to be repeated using mouse type I cells. Many problems can arise when using type I cells isolated from the
mouse carotid body, which include difficult dissections, very low cell yield, and unresponsive or damaged type I cells.

Moreover, there are reports of neurotransmitters attenuating Ca\textsuperscript{2+} signaling via muscarinic ACh receptor activation. One study has shown that histamine, acting via autoinhibitory H3 receptors, inhibited Ca\textsuperscript{2+} signaling initiated by the activation of muscarinic receptors in rat type I cells (Thompson et al., 2010). Similarly, another possible future study would address whether SST interacts with excitatory muscarinic Ca\textsuperscript{2+} signaling in isolated rat type I cells, using Ca\textsuperscript{2+} imaging. Considering SSTR\textsubscript{1-5} are G\textsubscript{i}-coupled proteins, SST would be expected to oppose the stimulatory effects of muscarinic receptors and thus attenuate Ca\textsuperscript{2+} signaling in the type I cell.

First, activation of muscarinic ACh receptors are known to inhibit TASK channels (Czirják et al., 2001; Inoue et al., 2008). SSTRs decrease PKA via G\textsubscript{i}-protein coupled receptors, and a PKA-dependent pathway that inhibits TASK channels in rat carotid body type I cells has been characterized (Xu et al., 2007). Therefore, the decrease in PKA caused by SSTRs could activate TASK channels, opposing the effects of muscarinic receptors (Figure 20A). Second, SSTR4 has been shown to inhibit voltage-gated L-type Ca\textsuperscript{2+} currents via G\textsubscript{\beta\gamma} signaling (Farrell et al., 2014), and this could oppose voltage-gated Ca\textsuperscript{2+} influx initiated by muscarinic receptors (Thompson et al., 2010) (Figure 20B). Third, muscarinic receptors have been shown to directly activate G\textsubscript{s}-proteins (Michal et al., 2007), and thus any excitatory signaling could be opposed by simultaneous activation of inhibitory G\textsubscript{i}-coupled SSTRs (Figure 20C).
Potential pathways by which SSTR activation might inhibit muscarinic receptor induced Ca\(^{2+}\) signaling. A) Muscarinic receptor (M) activation depolarizes the type I cell by inhibiting TASK channels and opening voltage-gated Ca\(^{2+}\) channels. SSTR activation inhibits PKA, which leads to an activation of TASK channels, and thus opposes the Ca\(^{2+}\) influx and depolarization induced by muscarinic receptor activation. B) SSTR activation causes G\(\beta\gamma\) subunits to inhibit voltage-gated Ca\(^{2+}\) channels and Ca\(^{2+}\) influx. C) Muscarinic receptors may elevate cAMP levels by a G\(s\)-coupled mechanism. Activation of SSTRs would directly oppose this action with G\(i\)-coupled inhibition of adenylyl cyclase and thus reduce cAMP levels.
Although any one of these three pathways could potentially cause SST to inhibit Ca\textsuperscript{2+} signaling initiated from muscarinic receptor activation, the most plausible mechanism is the G\textsubscript{i}-protein of SSTR counteracting the G\textsubscript{s}-protein of the muscarinic receptor, and causing a decrease in intracellular cAMP levels. This is because the experiments in this work have already demonstrated that SST has no significant effect on the hypoxic response in type I cells, which involve TASK-channels. These experiments have also shown that SST has no significant effect on voltage-gated Ca\textsuperscript{2+} influx. However, Ca\textsuperscript{2+} imaging experiments with muscarinic receptor stimulation would only show if SSTRs are functional. To test if SSTRs are working via a G\textsubscript{i}-protein in the type I cell, Ca\textsuperscript{2+} imaging experiments must be repeated with the cells incubating in the presence of the G\textsubscript{i}-protein inhibitor, pertussis toxin, for 3 hours. This would test if SSTRs work in a pertussis toxin-sensitive G\textsubscript{i}-protein coupled mechanism that inhibits excitatory signaling mediated by G\textsubscript{s}-coupled muscarinic receptors. With the G\textsubscript{i}-protein inhibited, it can be predicted that the inhibitory effects of SST would be attenuated in the presence of pertussis toxin, and muscarinic receptor excitation would cause an increase in intracellular Ca\textsuperscript{2+} levels.

Another possible experiment to pursue would be to measure neurotransmitter release from the carotid body type I cells in the presence of SST. Draznin et al. (1986) have shown SST to inhibit vesicle fusion in rat anterior pituitaries. Neurotransmitter release can be measured in the type I cell using amperometry. Neurotransmitters that are released from the cell stimulate a postsynaptic carbon fiber electrode and are recorded. Carpenter et al. (2000) used
amperometric recordings to show that hypoxia evokes catecholamine release from isolated type I cells in the presence of high extracellular potassium. However, this is a difficult technique and measuring neurotransmitter release in isolated type I cells is challenging.

One more future experiment for this project would test whether or not SST has a post-synaptic effect on the CSN, and this can be tested using whole nerve recordings. By measuring the membrane potential and firing rate of the CSN, it would be anticipated that SST would reduce the firing activity in response to hypoxia. Reduced action potential firing would be expected in the presence of SST due to the Gi-proteins of SSTRs. But this experiment wouldn’t directly show if SST was inhibiting at the level of the type I cell or at the level of the CSN. Immunocytochemistry would also have to be done on the CSN to confirm the presence of SSTRs.

**Conclusion**

Humans infused with SST were shown to have reduced acute ventilatory responses to hypoxia and hypercapnia. Previous work has suggested SST to inhibit Ca\(^{2+}\) currents in the carotid body, which could affect type I cell neurotransmitter release, CSN firing, and reduced signaling to the brainstem, ultimately altering breathing reflexes. In contrast, the results of this work show SST does not significantly affect voltage-gated Ca\(^{2+}\) influx, the hypoxic response, nor the acid response of type I cells. This data suggests that the mechanism by which SST inhibits the acute hypoxic and hypercapnic ventilatory response is not coupled to
voltage-gated Ca\(^{2+}\) influx nor the chemosensory response of type I cells. SST is likely affecting breathing at a different level, and further work will examine the effects of SST at the levels of the CSN and muscarinic receptors in the type I cell. These future studies need to be completed to gain a better understanding of SST’s role in the carotid body.


