

ELUCIDATING MECHANISMS OF NEUROGENIC INFLAMMATION IN THE MOUSE
OLFACTORY EPITHELIUM

By

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ABSTRACT

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Inflammation in the nervous system is linked to neurological disorders and neurodegenerative disease, and inflammation can be initiated by exposure to pollutants. The olfactory epithelium (OE) is directly exposed to the environment and prone to damage; however, the mechanisms of inflammation in the OE are not well understood. As a precursor to understanding chronic inflammation, I study modulation of acute inflammation in the mouse OE. Neurogenic inflammation is a form of acute inflammation caused by the release of pro-inflammatory peptides from nerves. Neurogenic inflammation can be initiated via local depolarization or axonal reflexes rather than cellular damage or infiltration of pathogens as in classical inflammation. Here, I tested the hypothesis that irritants initiate neurogenic inflammation in the mouse OE through activation of the trigeminal nerve and neuropeptide release. The pattern of immunoreactivity of irritant-sensing channels transient receptor potential -vanilloid 1 (TRPV1) and -ankyrin 1 (TRPA1) in OE tissue suggests a role in activation of the trigeminal fiber and secondary chemosensory cells (i.e., microvillous cells) in the OE. The pro-inflammatory neuropeptide substance P was released from tissue upon exposure to TRPV1 and TRPA1 agonists capsaicin and cinnamaldehyde, causing plasma extravasation, a hallmark of inflammation. Substance P treatment of OE tissue induced the release of cytokines tumor necrosis factor- α , interleukin-6, and monocyte chemoattractant protein-1, presumably from macrophages, a major source of

inflammatory mediators. The receptor for substance P, neurokinin-1, was found throughout the OE, including on macrophages and non-neuronal cells within the OE, implicating these cells in neurogenic inflammation of the OE. Macrophages were identified in the OE and found at increased levels in degenerating tissue and after exogenous application of substance P. This study identifies the trigeminal nerve, TRP channels, and macrophages as key factors in the initiation of the inflammatory response, i.e., the release of pro-inflammatory cytokines, and suggests that environmental irritation may initiate acute inflammation in the OE. Although acute inflammation can be beneficial in recovery from damage, it is important to understand the underlying mechanisms involved, as progression to chronic inflammation can lead to disease states.

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For Matt and McCarthy- let's blow this popsicle stand

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LIST OF ABBREVIATIONS

2-aminoethoxydiphenyl borate	2-APB
3-isobutyl-1-methylxanthine	IBMX
Adenosine triphosphate	ATP
Adenylyl cyclase	AC
Alzheimer's disease	AD
Bulbectomy	OBX
Calcitonin gene-related peptide	CGRP
Copy deoxyribonucleic acid	cDNA
Cyclic adenosine monophosphate	cAMP
Cyclic nucleotide-gated	CNG
Deoxyribonucleic acid	DNA
Diacylglycerol	DAG
Enzyme-linked immunosorbant assay	ELISA
Globose basal cell	GBC
Green fluorescent protein	GFP
G protein coupled receptor	GPCR
Glycogen triphosphate	GTP
Guanosine 5'-[β thio]diphosphate trilithium salt	GDP β s
Horizontal basal cell	HBC
Huntington's disease	HD
Immunohistochemistry	IHC
Immunoreactivity	IR
Inositol 4,5-triphosphate	IP3
Inositol 4,5-triphosphate receptor type 3	IP3R3
Interferon gamma	IFN- γ

Interleukin	IL
Leukemia inhibitory factor	LIF
Lipopolysaccharide	LPS
Luteinizing hormone-release hormone	LHRH
Macrophage inflammatory protein	MIP
Microvillous	MV
Monocyte chemotactic protein-1	MCP-1
Myeloid dendritic cell	MDC
Neurokinin-1	NK-1R
Neuropeptide Y	NPY
Olfactory epithelium	OE
Olfactory marker protein	OMP
Olfactory sensory neuron	OSN
Paraformaldehyde	PFA
Parkinson's disease	PD
Particulate matter	PM
Phosphodiesterase	PDE
Phospholipase C	PLC
Phosphatidylinositol 4,5 diphosphate	PIP2
Plasmacytoid dendritic cell	PDC
Polymerase chain reaction	PCR
Protein kinase C	PKC
Residual oil fly ash	ROFA
Ribonucleic acid	RNA
Solitary chemosensory cells	SCC
Substance P	sub P
Toll-like receptor	TLR

Transient receptor potential ankyrin member 1	TRPA1
Transient receptor potential melastatin member 5	TRPM5
Transient receptor potential vanilloid member 1	TRPV1
Tumor necrosis factor	TNF
Uridine-5'-triphosphate	UTP

Chapter 1: Literature Review

Introduction

The olfactory system is a popular model system for studying neurogenesis. Neurons of the peripheral olfactory epithelium (OE) organ continuously turn over, both in response to damage and under physiological conditions. The brain region to which these cells connect, the olfactory bulb, too, has proven capable of adult neurogenesis (Luskin, 1993; Byrd and Brunjes, 2001). There is much we can learn from this system about the interplay between the peripheral and the central nervous systems and its effect on development and plasticity. The olfactory system is also becoming a useful model in studying neurodegenerative diseases. An impaired sense of smell is one of the earliest symptoms of Alzheimer's (AD), Parkinson's (PD), and Huntington's (HD) diseases. Olfactory dysfunction has been called one of the most important non-motor symptoms of PD and is generally more severe in PD than in other Parkinsonian syndromes. For this reason, olfactory function tests are being used as a tool in differentiating between these related syndromes (Ponsen et al., 2010).

It is not currently understood how a neurodegenerative disease with cognitive decline as a primary symptom (AD), and others with gross motor abnormalities (PD and HD), have olfactory dysfunction in common, but inflammation may be a factor. Neurodegenerative diseases in general exhibit chronic activation of the immune system (Amor et al., 2013). There are many possible causes of this, but especially pertinent to human health and olfactory dysfunction is the initiation of inflammation due to exposure to pollutants (Block and Calderón-Garcidueñas, 2009), which has negative effects in

children, adults, and the elderly (Perera et al., 2008, Suglia et al., 2008, Calderón-Garcidueñas et al., 2011; Chen and Schwartz, 2009, Fonken et al., 2011; and (Ranft et al., 2009), respectively). Mechanisms of inflammation and its role in regeneration is an understudied area. By studying the OE of the mouse, we can learn more about how inflammation can be initiated by environmental factors, and how inflammation is modulated in a tissue that is capable of neurogenesis.

The olfactory system

General structure and function

The nasal cavities are openings through which air is filtered, humidified, warmed, and sensed, before traveling to the pharynx and lungs. The nasal cavities are lined by the nasal mucosa, which is made up of four different types of epithelia, the anterior portion consisting of squamous, cuboidal, and respiratory epithelia, and the dorsal posterior region containing the OE (Harkema et al., 2006). Thus, the nasal cavity houses the sense organs for the sensation of smell, or olfaction. In most terrestrial vertebrates, though not humans, the system also includes the vomeronasal organ and the accessory olfactory bulb, which are auxiliary olfactory organs specializing in pheromone detection and processing, respectively. Beneath the nasal mucosa is the lamina propria, an underlying layer made up of connective tissue, blood vessels, and glands (Farbman, 1992).

Olfaction arises from the activity of the olfactory system, which consists of a peripheral receptive portion, the OE, and a central processing portion called the olfactory bulb (Figure 1.1). In vertebrates, the OE is housed within the nasal cavity, and

the olfactory bulb is a projection of the brain that sits atop the nasal cavity. The nasal epithelium is covered in mucus that is secreted from Bowman's glands. These glands originate in the lamina propria and have ducts that extend through the tissue and release serous product onto the surface. The mucus secretion protects the epithelium from damage and infectious agents, and also provides protection from extreme temperatures and drying.

When volatile odorants enter the nasal cavities, they dissolve across the mucosal surface and bind to a G protein linked odorant receptor found in OSNs. Odorant binding causes the release of GTP-coupled $G\alpha(\text{olf})$ (Jones and Reed, 1989), which then stimulates adenylyl cyclase III to produce cyclic adenosine monophosphate (Pace et al., 1985). The increased level of cAMP causes the opening of cyclic nucleotide-gated channels, which allows the influx of cations. Action potentials travel down the axons and terminate in the olfactory bulb. Olfactory sensory neurons (OSNs) extend axons from the OE to the olfactory bulb, and from the bulb, signals travel to olfactory cortical areas of the brain (Figure 1.1). Thus, olfactory signals from the periphery bypass the thalamus, distinguishing olfaction from other senses.

The olfactory bulb is organized in diffuse layers: the olfactory nerve layer, the glomerular layer, the mitral cell layer, and the granule cell layer (Pinching and Powell, 1971; Byrd and Brunjes, 1995). The olfactory nerve layer consists mainly of the axons of OSNs which criss-cross in this layer as they locate the correct glomerulus to target in the glomerular layer. Glomeruli are spherical regions of neuropil made up of synapses between OSNs and juxtglomerular cells, which are bulbar interneurons, and mitral cells, which are projection neurons. Input to the olfactory bulb is organized such that

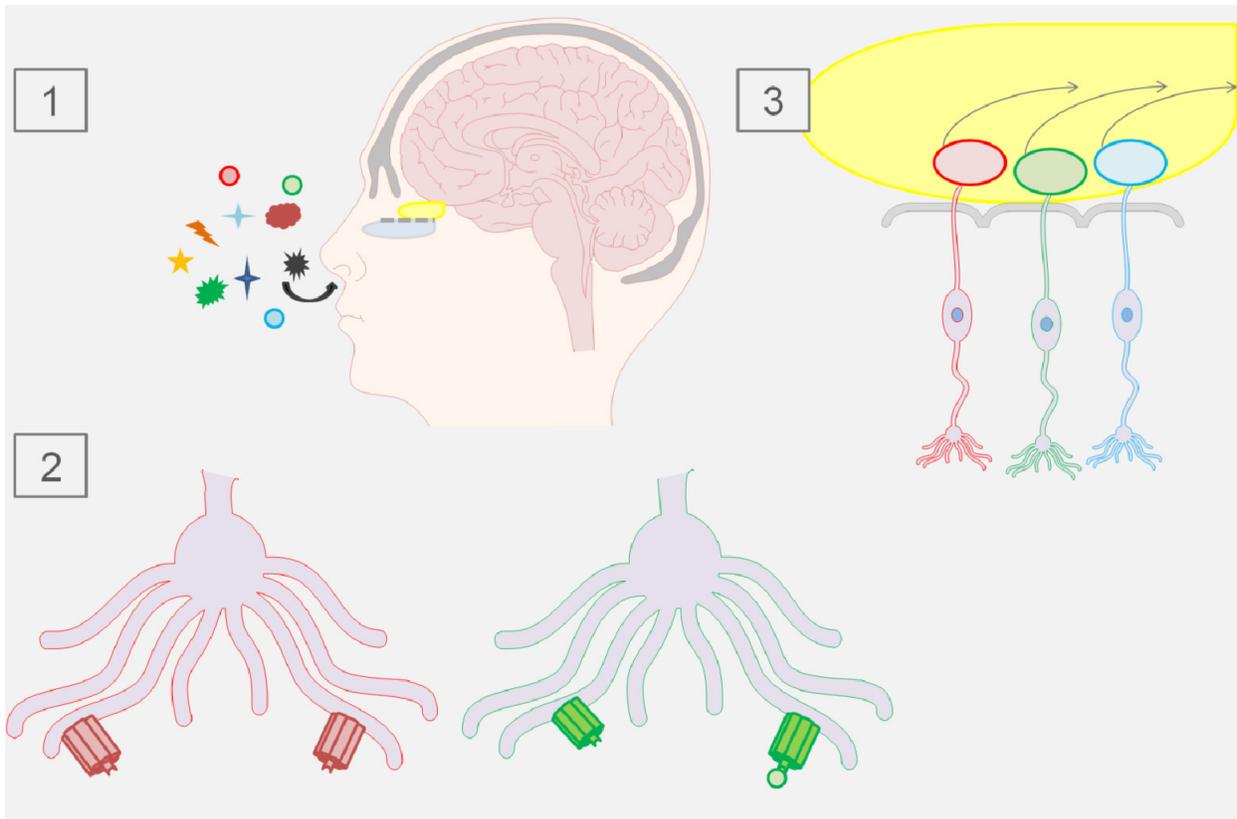


Figure 1.1. Model of the structures of olfaction. (1) The olfactory epithelium is housed within the nasal cavity, a small distance from the olfactory bulb of the central nervous system, which is situated on the underside of the brain. Air containing odorants is breathed into the nasal passages. (2) Odorants make contact with odorant receptors contained on the cilia of OSNs. (3) Signals are transmitted along the axons of OSNs, which exit the olfactory bulb, pass through perforations that separate the OE from the olfactory bulb, and terminate on glomeruli. Olfactory information can be transmitted to other regions of the brain for further processing and integration (Axel, 2005).

each glomerulus receives input only from OSNs expressing the same odorant receptor, creating a kind of odorant topography in the olfactory bulb. For further processing and integration, mitral cells carry information out of the olfactory bulb to the main olfactory cortical areas, which includes the anterior olfactory nucleus, olfactory tubercle, posterior piriform cortex, amygdala, entorhinal cortex, as well as parts of the hippocampus and hypothalamus (Axel, 2005).

Cell types of the olfactory epithelium

The OE is a pseudostratified tissue. The apical layer consists of the cell bodies of non-neuronal cells, i.e., sustentacular and microvillous cells. The basement membrane lies atop the lamina propria and consists of basal progenitor cells. Between the apical cells and the basal cells are the OSNs (Figure 1.2).

The major cell type of the OE is the OSN (alternatively called olfactory receptor neuron), which makes up about 70-80% of the cells in the OE (Farbman, 1992). The cell bodies of mature OSNs are situated in the middle of the epithelium. An OSN is considered mature when it expresses olfactory marker protein (OMP), a dendritic knob, and an axon that targets the olfactory bulb (Schwob et al., 1994). A population of immature neurons lies beneath the layer of mature OSNs, which can be identified by the expression of growth associated protein-43, as well as lack of OMP and dendritic knob. These cells are bipolar; cilia project apically from the cell body into the nasal cavity where they can make contact with odorants, and axons project away from the cell body, through the lamina propria and perforations of the cribriform plate, and terminate on glomeruli of the olfactory bulb (Axel, 2005). The cilia of mature OSNs contain odorant

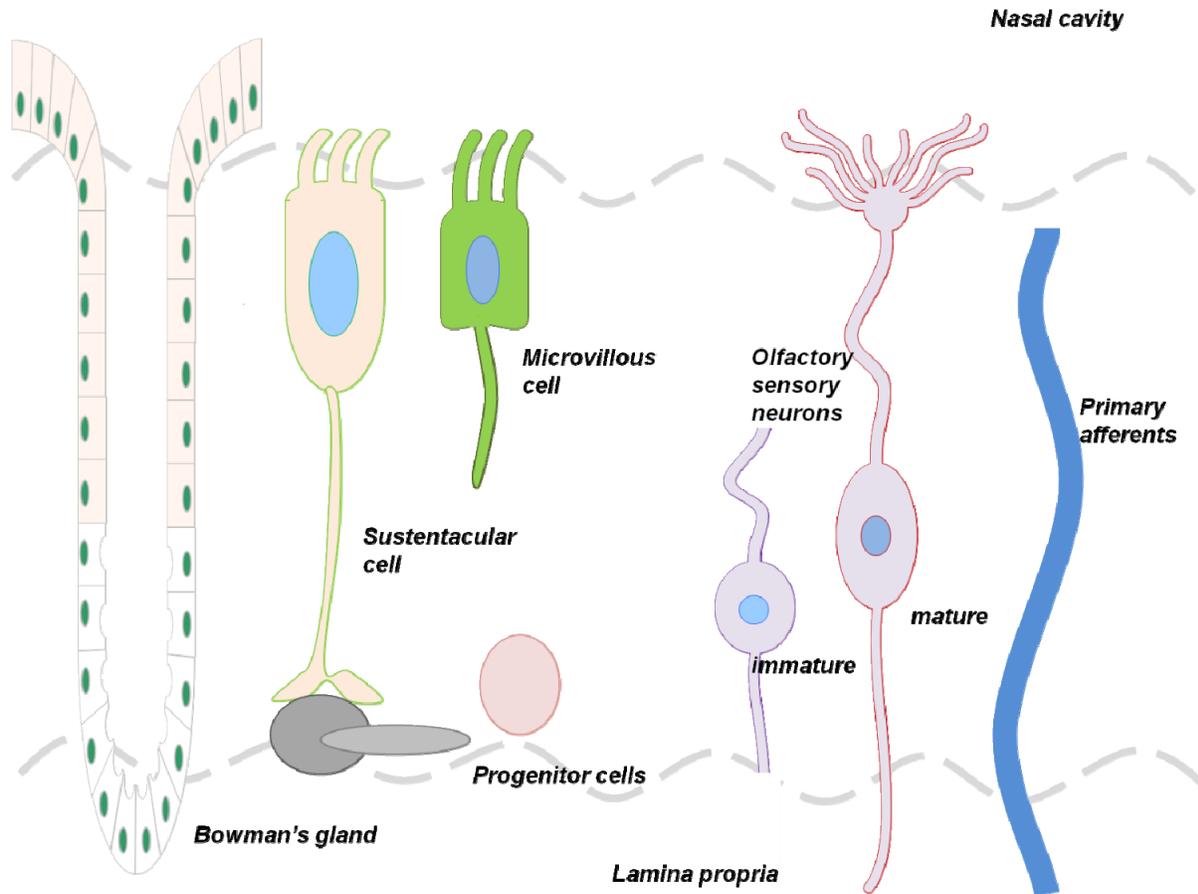


Figure 1.2. Main structures of the OE. The cell types of the OE are the OSNs, non-neuronal sustentacular and microvillous cells, and progenitor cells. Bowman's glands are located throughout the tissue and provide mucus secretions to the mucosal surface. The tissue is innervated by the peptidergic trigeminal nerve. Sustentacular cells span the length of the OE, with cell bodies in the apical portion of the tissue, and endfeet that terminate above the progenitor cells on the basement membrane. Microvillous cells are also located in the apical layer. The cell bodies of the mature OSNs are in the mid-section of the tissue, below the layer of non-neuronal cell bodies. The mature OSN has an extension ending in a dendritic knob with cilia that protrude into the mucosa surface and nasal cavity. Microvilli from sustentacular and microvillous cells also extend into the nasal cavity. The axon of the mature OSN exits into the lamina propria and targets

Figure 1.2 (cont'd) the olfactory bulb. Cell bodies of immature OSNs sit below the layer of mature OSNs. The OE itself is not vascularized, but the lamina propria contains many blood vessels, as well as connective tissue, glands, and bundles of axons.

receptor molecules and each OSN contains only one type of odorant receptor. Humans express approximately 350 odorant receptor genes, while the mouse expresses approximately 1,000. While each neuron only expresses one type of receptor, that receptor may be responsive to more than one type of odorant. Likewise, different odorants may involve the activation of a combination of receptors (Buck and Axel, 1991).

OE tissue is regularly damaged due to contact with pollutants and pathogens from the environment, but loss of the sense of smell is not a regular occurrence. Upon damage to mature OSNs, immature OSNs can be activated to quickly mature by extending a dendritic knob apically and an axon to the olfactory bulb to maintain the circuitry and functionality of the tissue (Schwob, 2002; Krolewski et al., 2012). The population of immature neurons and the regenerative ability of the OE are due to the presence of two types of progenitor cells in the epithelium: the globose basal cells (GBCs) and the horizontal basal cells (HBCs). HBCs and GBCs are found atop the basement membrane (Graziadei and Graziadei, 1979; Mackay-Sim and Kittel, 1991; Levey et al., 1991; Murdoch and Roskams, 2007), with the HBCs sitting lower in the basal membrane than GBCs (Levey et al., 1991). These progenitor cells are activated to develop into both OSNs and non-neuronal cell types, the sustentacular and microvillous cells. Notch signaling regulates progenitor cell fate (Carson et al., 2006) in the OE (Schwartz et al., 2007). When Notch signaling is inhibited, GBCs proliferate and differentiate into neurons (Nelson et al., 2007). Approximately 37% of the total cell population consists of immature neurons and basal cells (Mackay-Sim and Kittel, 1990).

The sustentacular cells span the length of the OE, with the nuclei found in the apical portion of the OE above the OSN layer. Sustentacular endfeet terminate on the basement membrane. This cell type plays an important role in the OE because it functions both as epithelial and glial-like support cells. As epithelial-like cells, sustentacular cells are involved in secretion (Menco and Morrison, 2003) and endocytosis (Bannister and Dodson, 1992). As glial-like cells, sustentacular cells physically and chemically insulate OSNs (Breipohl et al., 1974), phagocytose cellular debris (Suzuki et al., 1996), and regulate the ionic environment of the tissue (Breipohl et al., 1974; Rafols and Getchell, 1983; Getchell, 1986). Sustentacular cells protect the nasal organs by metabolizing foreign compounds through the actions of cytochrome P450 (Dahl, 1988), glutathione-S-transferase mu 2, and carbonyl reductase2 (Beites et al., 2005). Thus, sustentacular cells expand the function of the nasal cavity beyond odorant detection; they also protect the sensitive tissues of the lower airways and lungs from damaging toxicants.

The olfactory microvillous cell is a heterogenous class of cells, as there appears to be a variety of subtypes that have differing roles in the OE. They are so named because they contain fingerlike projections atop the cell body, similar to sensory cells around the body. The cell bodies of microvillous cells are located in the apical portion of the tissue, at or just below the level of sustentacular cell bodies. Some microvillous cells are primary sensory neurons; they respond to odorants and have projecting axons, but unlike mature OSNs, they do not express OMP (Moran et al., 1982; Liman et al., 1999). However, most microvillous cells of the OE in humans and rodents lack a projecting axon and are not primary sensory neurons (Carr et al., 1991; Braun and

Zimmermann, 1998). These non-primary sensory microvillous cells respond to numerous irritants and at high concentrations also respond to classical odorants, classifying these cells as secondary sensory cells. The different subtypes of microvillous cells can be differentiated based upon the expression of certain proteins and morphology. There appear to be two morphologically different TRPM5 expressing microvillous cells (Lin et al., 2008; Hansen and Finger, 2008), which are differentiated based mainly on their size and shape. Another subtype of microvillous cell in the rodent OE does not express TRPM5, but is still considered a secondary chemosensory cell because it expresses phospholipase C beta-2 (PLC β 2), transient receptor potential type C6 (TRPC6), and inositol trisphosphate receptor type 3 (IP3R3) (Elsaesser et al., 2005), and the ectonucleotidase CD73 that catalyzes 5-AMP to adenosine (Pfister et al., 2012 p.73). As these cells do not have axons that exit the OE but function as secondary chemosensory cells, they likely have a role in modulating the OE and its cell types, though the mechanisms of this have not been fully elucidated.

Disorders of the olfactory system

Although the OE is capable of regeneration to maintain functionality, olfactory disorders do exist. It was once thought that loss of smell was a conductive disorder. Conductive disorder of the olfactory system is characterized by diminished access to olfactory cells, such as due to obstruction or build up of mucus, and decreased airflow through the nasal passages, such as due to nasal polyps. It is now known that sensorineural disorders may also cause olfactory deficits. Sensorineural disorders of the olfactory system can be caused by damage to the olfactory bulb or pathways.

There are no lesion-specific tests to determine whether the olfactory dysfunction is due to conductive or sensorineural reasons.

Olfactory dysfunction or anosmia (loss of the sense of smell) can occur in response to allergic and non-allergic sinusitis, head trauma, acute viral assaults, and unknown causes, and can precede neurodegenerative diseases. Inflammatory changes in sinusitis were believed to occur only in the respiratory mucosa, sparing the OE, but studies have demonstrated that olfactory function is impaired in mice suffering from allergic rhinitis (Ozaki et al., 2010). Likewise, biopsies taken from human patients undergoing nasal surgery for chronic rhinosinusitis demonstrated changes in morphology in nasal tissue including the OE, indicating inflammation (Kern, 2000).

There is also an age-related decline in olfactory function. In humans, fifty percent of the population above 65 years old suffers from olfaction dysfunction (Stevens and Cain, 1987; Murphy, 2002). Age-related olfactory dysfunction has been described in rodents (Enwere et al., 2004), as well. OE impairment with age can be caused by both extrinsic and intrinsic mechanisms, such as cumulative damage from ambient particulate matter and pathogens, or a reduction in trophic factor activity and accumulation of mutations, respectively. The increased expression of pro-apoptotic procaspase-3 and bax in aging rats suggests that there are changes in expression of genes in the OE that can make the tissue more susceptible to apoptosis (Robinson et al., 2002). A gradual thinning of the OE due to decreased neuron and sustentacular cell populations leads to reduced sensitivity. This reduction is due to the combination of decreased proliferation of olfactory progenitor cells and increased cell death (Naessen, 1971; Nakashima et al., 1984; Loo et al., 1996; Weiler and Farbman, 1997; Kondo et

al., 2010). The timeline of neurogenesis, from birth of the cell to maturation, remains similar throughout life (Kondo et al., 2010).

The olfactory system as a model of study

A characteristic of the OE that makes it a popular model of study is its ability to repopulate dying and injured cells, especially neurons. The olfactory system is a convenient model to study, as the structures of the olfactory system are easily accessible, and are thus more easily manipulated and studied than regions of the central nervous system. Neurogenesis in the OE is morphologically and functionally similar to neurogenic areas of the central nervous system, which are primarily in the dentate gyrus and the subventricular zone. These neurogenic areas share stages of neurogenesis: proliferation of progenitor cells, differentiation of daughter cells, migration of newborn cells into the appropriate layer, and maturation of cells such that they become integrated into the circuitry of the system. The cycle of neuronal progenitor cells- proliferation, differentiation into neurons, and death, begins in embryonic development of animals and continues through adulthood (Graziadei and Graziadei, 1979a).

In the mature OE, neurogenesis occurs to a small extent to replace old OSNs. The lifespan of the OSN can vary from 3 months in mice kept in normal laboratory settings (Mackay-Sim and Kittel, 1991), to 12 months in a pure-air setting (Hinds et al., 1984). In response to significant damage, be it chemical, infectious, or traumatic in nature, the rate of neurogenesis accelerates in the OE (Schultz, 1960; Oley et al., 1975; Graziadei and Graziadei, 1979b; Costanzo and Graziadei, 1983). The rate of

physiological OE turnover has been traced using tritiated thymidine ($^3\text{H-TdR}$) in mice. One day after administration, labeled cells cluster at the basal membrane, indicating various basal cells were in the S-phase at the time of exposure. By 9 days, labeling moves midline, and by 20 days, label is present near the epithelial surface (Graziadei and Graziadei, 1979). It has been a matter of debate as to which basal cell gives rise to the different cell types of the OE. In an autoradiographic study of mouse OE following bullectomy, both GBCs and HBCs pick up the radioactive label $^3\text{H-TdR}$, but only labeled GBCs travel apicalward and populate the receptor cell layer (Levey et al., 1991). This suggests that GBCs are the progenitors in the OE that can be induced to divide after cell death of mature cells within the tissue, and their progeny can migrate to the area populated by mature neurons to replace lost cells. It is now thought that HBCs are in a quiescent state until gross injury initiates proliferation, in which case, they can differentiate into GBCs. As mentioned previously, a population of immature neurons exists in the OE. Dying or damaged neurons are not necessarily directly replaced by a newly divided cell, but are more often replaced by an immature neuron while new dividing cells are generated. After axonal lesion of the garfish olfactory nerve, a small but rapid phase of regeneration reconstitutes 3-5% of the original population of nerves. 12 days after axonal lesion, the main phase of regeneration reconstitutes 50-70% of the original population of axons at a rate 7X slower than the rapid phase. The rapid phase of fibers comes from the population of immature neurons growing at the time of injury that are capable of quickly maturing. Thus, division of basal cells gives rise to immature neurons that replace dying OSNs. This process ensures continual functioning of the OE (Cancalon and Elam, 1980; Cancalon, 1987).

Chemicals are routinely employed to damage the OE in order to study recovery. Intranasal irrigation of rodents with zinc sulfate causes degeneration of the epithelium and axons, followed by widespread recovery of the OE (Burd, 1993; Herzog and Otto, 1999). Detergent aspiration (Nadi et al., 1981; Baker et al., 1983; Cummings et al., 2000) and methyl bromide inhalation (Schwob et al., 1999) have been used in rodents, as well as in fish (Cancalon, 1983; Iqbal and Byrd-Jacobs, 2010), with similar results. While these studies are informative, using potent chemicals to destroy neuroepithelium may not be a pertinent model for human health. The effect of chronic low-level damage from exposure to harmful agents in the environment is under-studied, representing an important gap in our knowledge. Due to its contact with the environment, the OE represents an ideal model for understanding the effects of environmental irritants on a neuronal tissue.

Common chemical sense

Odorant sensation and processing is not the only function of the olfactory system. The detection of chemical irritants, called the common chemical sense, is a form of defense exhibited by the nasal cavity. In response to exposure to irritants, the respiratory system has developed a number of protective reflexes to aid in clearing out harmful agents: sneezing, coughing, and apnea.

The common chemical sense in the OE is primarily signaled to the brain by trigeminal fibers (Silver et al., 1985; Hunter and Dey, 1998). The trigeminal nerve, or cranial nerve V, is a polymodal nerve. It has a motor function involved in mastication and proprioception, but the trigeminal nerve is primarily considered a somatosensory

nerve because the sensations of touch, temperature, and pain are transmitted along this nerve. Trigeminal fibers also carry chemosensory information, along both a-delta myelinated and non-myelinated C fibers. To humans, the chemosensory qualities transmitted by the trigeminal fiber are often described as “pungent, tickling, warm, cool, burning, stinging, sharp,” (Doty et al., 1978). Because these sensations are elicited in response to irritants, the function of these qualities is thought to warn the organism of dangerous stimuli in order to avoid further exposure (Stone et al., 1968). In addition to triggering avoidance behavior, trigeminal responses initiate a number of physiological responses, including secretion of mucus, cerebral arousal, increased vascular permeability, and sneezing, as well as changes in pattern of respiration, circulation to the nasal mucosa, and heart rate (Farbman, 1992).

Trigeminal fibers in the nasal mucosa

The nasal mucosa is innervated by two branches of the trigeminal nerve, which originate from cells of the trigeminal ganglion located near the pons. The ophthalmic division of the trigeminal nerve branches off into the anterior ethmoid nerve, which innervates the anterior region of the nasal mucosa. The maxillary division of the trigeminal nerve gives rise to the nasopalatine nerve that innervates the posterior region of the mucosa (Farbman, 1992).

It was once thought that trigeminal fibers did not innervate the OE. In a study of murine OE, capsaicin-sensitive fibers that are immunoreactive to substance-P are noted in the lamina propria and surrounding Bowman’s glands, but not in the main OE (Papka and Matulionis, 1983), but more recent studies have provided evidence for trigeminal

innervation of the OE. Trigeminal fibers are often identified by their immunoreactivity toward calcitonin gene-related peptide (CGRP) and substance P (sub P), which are neuropeptides contained within the fibers. Of structures that label for trigeminal neuropeptides in the rat nasal epithelium, approximately 82% are immunoreactive toward sub P and 35% for CGRP (Hunter and Dey, 1998). Likewise, the frog OE is richly innervated by sub P- immunoreactive fibers. Transection of the ophthalmic branch of the trigeminal nerve causes denervation of the sub P- immunoreactive fibers in the OE, confirming that they are trigeminal fibers. These fibers are prevalent in the lamina propria, especially around blood vessels and Bowman's glands, but also extend very near the apical surface of the OE (Bouvet et al., 1987). Thus, since earlier reports, there has been ample evidence of trigeminal innervation of the nasal mucosa in both the respiratory epithelium and the OE.

TRP channels

Responses to irritants from primary sensory nerves are mediated through the activity of irritant-sensing channels, such as the Transient Receptor Potential (TRP) family of channels. TRP channels control information processing of nociceptors, a subset of neurons that transmit signals of pain when activated (Bautista et al., 2006; Salas et al., 2009). The members of the TRP channel family have six transmembrane segments and a pore loop that allows permeability to cations. Beyond a basic structural similarity, TRP family members vary greatly in terms of selectivity and activation mechanisms. The family is divided into two groups. Those with the closest homology to the drosophila TRP, where TRP channels were first discovered, are called classical

TRP, or TRPC. Group 1 consists of the classical channels: TRPC, TRPV, TRPM, TRPA, and TRPN. Group 2 contains TRPP and TRPML.

The activity of TRPV1 is commonly described in response to vanilloids, and most notably, capsaicin, but TRPV1 is modulated by many things, including heat (43°C and higher), anandamide, camphor, piperine, allicin, ethanol, nicotine, proinflammatory cytokines, protons, and PIP₂. The highest expression of this channel is found in the trigeminal ganglion, dorsal root ganglia, nociceptors, urinary bladder, and testes (Mandadi and Roufogalis, 2008). TRPA1 often co-localizes with TRPV1 (Gerhold and Bautista, 2009), and was originally thought to be a cold transducer, but is now considered a sensor of inflammatory state. TRPA1 is sensitive to many irritants found in plants, including isothiocyanate, which is found in mustard oil and garlic, tetrahydrocannabinol, which is a cannabinoid derived from a plant, and cinnamaldehyde, found in cinnamon (Gerhold and Bautista, 2009). Many TRPA1 activators are found in the environment, such as acrolein, which is present in cigarette smoke, tear gas, vehicle exhaust, and can be produced by metabolism of chemotherapy medications (Bautista et al., 2006), α -terpineol, amyl acetate, benzaldehyde, toluene α,β -unsaturated aldehydes (Andr  et al., 2008), reactive oxygen species, alkenyl aldehydes, and 15d-PGJ₂-eoxyl-12,14 prostaglandin (Andersson et al., 2008).

Inflammation

When the health of a tissue is compromised, immune defenses are initiated. There are two major classes of defenses, innate immunity and adaptive immunity. Innate immunity involves external defenses such as the epithelium and secretion of

mucus and internal defenses such as the activity of phagocytic cells. Phagocytic cells recognize foreign cells through the expression of membrane molecules, such as lipopolysaccharides on gram negative bacteria and peptidoglycans on gram positive bacteria. The receptors for these molecules are known as toll-like receptors, and are found on phagocytic cells like macrophages and dendritic cells. These phagocytic cells abate the spread of infections or damaging toxins by engulfing and destroying the harmful agents with digestive enzymes contained in lysosomes. The adaptive immune defense is elicited in response to specific threats.

Hallmark symptoms of inflammation include redness, swelling, increased temperature, and pain. Acute inflammation is the initial response to damage and is a non-specific response of clearing dead tissue and allowing access of immune cells. Once the damaged tissue is removed, there can be restitution, replacement of damaged tissue that is organized in the same way as before the damage. However, sometimes cells cannot be replaced, or too much of the structure may be destroyed, and fibrous replacement occurs instead, resulting in scar tissue. If the damaging agent persists in the tissue, chronic inflammation can occur, causing continual tissue damage, immune responses, and fibrous repair. Chronic inflammation often leads to loss of function of that tissue, such as, in olfaction, olfactory dysfunction or anosmia. The activities of immune cells are paramount to the form and regulation of inflammation and recovery that occurs in the damaged tissue.

Inflammatory mediators

The local release of neuropeptide and dilation of blood vessels allows for the recruitment of inflammatory mediators, namely, immune cells, and the factors used by immune cells for communicating and activating pathways involved in inflammation and recovery, i.e., cytokines. There are a number of different types of immune cells with differing modes of activation.

Both the innate and adaptive immune responses can be involved in the events of inflammation, and the type of cell involved determines much of the outcome of the inflammatory response. The 'type' of inflammation often refers to the characteristic activity of TH1 cells or classically activated macrophages that promote inflammation, extracellular matrix destruction, and apoptosis, or TH2 cells and alternatively activated macrophages, which promote extracellular matrix reconstruction, proliferation, and angiogenesis. The roles of these cell types and other immune cells in inflammation are discussed below.

B cells

B cells are cells of the adaptive immune system. These lymphocytes secrete antibodies that identify specific antigens of foreign molecules to target the cell for destruction. For example, when a macrophage digests a cell, it will often present the antigen from the digested cell on its own surface membrane, which can then be recognized by B cells (Mauri and Bosma, 2012). B cells are known to respond to viruses infecting the OE (Tan and Stevenson, 2014) and systemic infection produced by

intravenous lipopolysaccharide exposure results in elevated levels of B cells in the olfactory bulb (Doursout et al., 2013).

T and Helper T cells

T cells are another cell type of the adaptive immune response. When a T cell encounters an antigen-presenting cell, the T cell becomes activated, and will then track and destroy similarly infected cells. Proliferating helper T (TH) cells develop into two major subtypes of effector T cells, TH1 and TH2 cells. TH1 cells provide defenses against intracellular bacteria and protozoa. TH1 cells are activated by IL-12 and IL-2 and can kill bacteria through IFN- γ - mediated production of nitric oxide free radicals or through the activation of their main effector cells, macrophages, which T cells can also activate with the productions and release of IFN- γ . TH2 cells provide defenses against extracellular parasites including parasitic worms. TH2s are activated by IL-4 and can produce and release the cytokines IL-4, IL-5, IL-9, IL-10 and IL-13, which in turn can activate a variety of effector cells, including eosinophils, basophils, mast cell, B cells, and T cells (Spellberg and Edwards, 2001).

Dendritic cells

Dendritic cells are motile cells with stellar morphology characterized primarily by their ability to migrate from non-lymphoid to lymphoid organs and stimulate T lymphocytes. They are antigen-presenting cells derived in bone marrow from a precursor common to the both the dendritic cell and monocyte. Dendritic cells are classified into two major divisions, tissue cells and peripheral blood cells. Tissue

dendritic cells are concentrated in tissues that are more readily infiltrated by microorganisms, such as skin, intestinal mucosa, and airways. Peripheral blood contains two major subsets of dendritic cells, the plasmacytoid dendritic cell (PDC), and the myeloid dendritic cell (MDC). Antigens are digested by dendritic cells into shorter polypeptides which can be moved to cell surfaces and associated with histocompatibility antigens, allowing for the activation of T lymphocytes. Oftentimes, dendritic cells will secrete chemokines and migrate toward lymphoid organs in order to interact with the appropriate T lymphocytes (Schraml and Reis e Sousa, 2015). Dendritic cells are the primary cell type in identifying and defending against viruses (Hartmann et al., 2006). When defending against viruses, PDCs will produce, in large quantities, IFN- α , a potent anti-microbial cytokine. Dendritic cells are the only cell type capable of activating naïve T lymphocytes (Iwasaki, 2007).

Dendritic cells have been identified in the nasal mucosa of humans, in both healthy and diseased tissues. Mucosal dendritic cells are found in the basolateral space, separated from inhaled substances by the epithelial tight-junction barrier (Schraml and Reis e Sousa, 2015). Dendritic cells can sample the content of airway lumen without disruption of the epithelium barrier through expression of tight-junction proteins claudin-1, claudin-7, and zonula-2 (Hammad and Lambrecht, 2008). Both PDCs and MDCs were identified in human nasal mucosa and are found in equal levels in healthy noses. Interestingly, both types of dendritic cells are found in decreased numbers in tissue from allergy sufferers, with PDCs being the most decreased. Patients treating their allergies with glucocorticoid steroids had further reduced levels of dendritic

cells. However, in patients with non-allergic acute rhinitis (probably due to infection) levels of dendritic cells had greatly increased (Hartmann et al., 2006).

Innate lymphoid cells

Similar to dendritic cells, innate lymphoid cells (ILC) integrate functions of the adaptive and innate immune responses. This classification of cells includes both cytotoxic and non-cytotoxic cells. These cells do not express cell lineage markers nor do they respond in an antigen-specific manner but can be identified due their classic lymphoid morphology. The cytotoxic class of cells is made up of the natural killer cells. The non-cytotoxic subset is divided into three classes, ILC1, ILC2, ILC3, based on the effector cytokines expressed, analogous to the subsets of T cells. All three non-cytotoxic subsets and the natural killer cells arise from a common lymphoid progenitor cell. ILCs are numerous in epithelia that are barriers to the environment, especially those prone to viral and bacterial infection. ILC1s characteristically release TNF and IFN- γ in response to tumors as well as virus infections. ILC2s produce IL-4, -5, -9, and -13, primarily in response to parasitic worms and allergic reactions. ILC3s release IL-17A and IL -22 and are found primarily in mucosal tissues, and are essential to the development of lymphoid tissues (Artis and Spits, 2015).

Neutrophils

Neutrophils, also known as polymorphonuclear leukocytes, are the most prominent immune cell present up to 24 hours after an inflammatory injury, especially in bacterial infections and infarctions. Neutrophil level is often used in clinical diagnosis of

inflammation. The earliest responders are neutrophils released from bone marrow into the blood. In an inflamed state, vessel walls express adhesion molecules and integrins, allowing neutrophils to adhere to epithelium and roll along vessel walls. Neutrophils then aggregate, become ameboid, and enter damaged tissue, following the concentration gradient of chemotactic factors to the site of damage. Neutrophils engulf bacteria and pathogens when foreign material is bound to a membrane receptor on the surface of the neutrophil. The foreign material is ingested through the activity of proteases contained in cytoplasmic granules and through the generation of free radicals. Neutrophils also produce arachidonic acid which initiates the production of prostaglandins, a dilation factor also involved in the sensation of pain at injured sites. These cells have a short lifespan, so their numbers must be regularly replaced. Most die in the inflamed area, though some may be removed through the lymphatic system. When these cells die, they contribute to the structural breakdown of the area common to inflamed sites and can also lead to necrotic tissue if present in high numbers.

Neutrophils have been shown to be early responders to nasal damage as well. In mouse nasal tissue treated with black mold toxin satratoxin G, neutrophilic rhinitis was established after 24 hours, with increases in cytokines interleukin (IL)-6, IL-1, and macrophage inflammatory protein (MIP)-2 (Islam et al., 2006). In mice treated with Poly (I:C) to simulate viral infection, neutrophil infiltration was measured starting at 8 hours and present at much higher number by 3 days, but were absent from the epithelium by 9 days (Kanaya et al., 2014).

Mast Cells

Mast cells are resident to tissues throughout the body: in blood vessels and nerves and in tissues that interact with the outside environment. Mast cells have a role in innate immunity, they are involved in host defense mechanisms against parasitic infestations, as well as immunomodulation of the immune system, tissue repair, and angiogenesis. Mast cells are activated by an interaction between antigens and IgE receptors, but can also be activated by neuropeptides, complement compounds, basic compounds (such as compound 48/40) and opiates. After activation, mast cells secrete granule-associated mediators and generate lipid-derived substances. Mast cell activation may also be followed by the synthesis and release of chemokines and cytokines. Within minutes of mast cell activation, degranulation of the mast cell causes the release of histamine, leukotrienes, cytokines, and proteases, which leads to the immediate reactions. The exact outcome of the immediate reaction depends on the tissue in which it takes place, but can include increased permeability of blood vessels due to dilation, or bronchconstriction via smooth muscle contraction. The late-phase reaction occurs hours later, with the secretion of cytokines and chemokines, especially IL-4 and IL-13, which brings more immune mediators into the area (Metcalfe et al., 1997).

In the nasal mucosa, mast cells are integral to the pathogenesis of allergic rhinitis. Mast cells in the respiratory mucosa release histamine in response to trigeminal activation (Saunders et al., 2014). A recent study demonstrated that the subset of mast cells present in the OE during allergic rhinitis are the same as the subset present in the respiratory mucosa (Li et al., 2014).

Macrophages

Macrophages are resident to tissues throughout the body, and arise from monocytes that originate in bone marrow and circulate in the blood, during which time they lack phagocytic capacity. Once a macrophage enters an inflamed site, it can become either classically or alternatively activated. Classical activation results in a phenotype similar to TH1 cells, which promotes inflammation through destruction of the extracellular matrix through the production of matrix metalloproteinases, and cell death through the process of apoptosis. Factors that can push a macrophage toward the classical activation phenotype include IFN- γ , bacterial lipoproteins, and cytokines such as tumor necrosis factor (TNF)- α , IL-1, or IL-12. The alternatively activated macrophage promotes a phenotype similar to TH2 cells, and initiates angiogenesis, proliferation of cells, and extracellular matrix reconstruction. Factors that can initiate the alternative activated profile of a macrophage include transforming growth factor- β , IL-4,-10,-14, and glucocorticoids (Duffield, 2003). Activation of macrophages is through the activity of IFN- γ and presence of microbe or bacterial product such as LPS (Mosser and Edwards, 2008).

Macrophages are typically not the first immune cell to the site of inflammation, but they are able to survive longer than neutrophils, and thus can be in the damaged tissue for prolonged times. The secretion of growth factors and cytokines from macrophages are essential steps in the inflammatory response, but also mediate recovery of tissue. Macrophages also produce nitric oxide, a powerful smooth muscle relaxant and vessel dilator.

Macrophages are present in the OE after damage. Bulbectomy is a method used to cause the retroactive degeneration of OSNs, and approximately 16 hours post-bulbectomy, macrophages enter the OE (Getchell et al., 2002). A simulation of viral damage to the OE using Poly (I:C) indicated that while neutrophils have a transient presence in the OE after injury, which starts around 8 hours and is absent after 9 days, macrophages infiltrate around the same time as neutrophils, albeit in smaller numbers, and then persist in the tissue to at least 24 days (Kanaya et al., 2014). Thus, macrophages may be present during both acute and chronic inflammation, but likely in larger numbers in the latter. The effect the macrophage has in the tissue is largely determined by the environment encountered, determining a classical or alternative activation and which effectors are subsequently released.

Cytokines

Cytokines are soluble polypeptides produced by lymphocytes and monocytes. Cytokines have a diverse array of functions. In general, cytokines function as growth and differentiation factors, alarm signals for the inflammatory response, chemotactic factors for leukocytes, and modulators of immune cell functions. IL-1 α , IL-1 β , and IL-6 are all endogenous pyrogens, which induce fever by stimulating prostaglandins and the hypothalamus. IL-1 α , IL-1/3, IL-6, and leukemia inhibitory factor (LIF) all stimulate increased acute-phase protein production by hepatocytes, which act to destroy or inhibit microbes. Others are pro-coagulant, which may function to trap pathogens in blood clots. IL-4 and IL- β share many macrophage suppressive activities and support the propagation of certain TH lymphocytes.

TNF- α is a cytokine associated with infection or injury in many cell-types (Sandborn and Hanauer, 1999; Kollias et al., 1999; Feldmann and Maini, 1999) and is present in sinonasal inflammatory disease. However, not much is known about the role of TNF- α in chronic sinusitis in humans. Lane and colleagues developed a genetic mouse model of chronic human rhinosinusitis in which sustentacular cells are made to produce TNF- α , and this production can be turned on and off. Long-term production of the cytokine decreased the responsiveness of the tissue to odorants before death of OSNs and gross changes in tissue morphology. Once production of the cytokine was halted, morphological changes were reversed and neuroregeneration was initiated, demonstrating that inflammatory cytokines and inflammation can cause damage to tissue and hinder neurogenesis until inflammation is abated. Mature OSNs were preferentially affected by TNF- α expression, while sustentacular and basal cells remained largely unaffected. The authors of the study noted a marked increase in the submucosal space due to the infiltration of immune cells, and they suggest that OSNs may have been affected due to damage specific to their axon bundles by incoming immune cells into the lamina propria, triggering apoptosis (Lane et al., 2010). This study demonstrated an important role for TNF- α in olfactory dysfunction of chronic inflammatory conditions of the OE.

Conversely, there is evidence that cytokines may also be neuroprotective. For example, increased expression of the mRNA of cytokine LIF occurs before the onset of progenitor cell proliferation (Bauer et al., 2003), and there is a corresponding increase in LIF and IL-6 receptors by GBCs (Nan et al., 2001). The parallel timelines for cytokine expression and basal cell proliferation suggests that cytokines such as LIF and IL-6

have a role in neurogenesis. Macrophage depletion, which would decrease the levels of cytokines, also corresponds to decreased neurogenesis and recovery following injury (Borders et al., 2007a, 2007b). Cytokines such as monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein -1 α (MIP-1 α) are chemoattractants for macrophages. MCP-1 and MIP-1 α mRNA reaches peak levels 3-5 days post bulbectomy (Getchell et al., 2002). To test the hypothesis that reducing the number of macrophages after injury reduces the number of proliferative GBCs, MIP-1 α ^{-/-} mice were bulbectomized to initiate apoptosis and recovery. Neurogenesis and remodeling was decreased in the knockout mouse, but macrophage recruitment and, subsequently, recovery from injury, were restored with exogenous application of MIP-1 α (Kwong et al., 2004).

Neurogenic inflammation

One form of inflammation can be initiated by activation of nerves and subsequent release of inflammatory neuropeptides. This inflammation, coined neurogenic, is caused by the activation of primary sensory afferents and release of neuropeptides, rather than infiltration of foreign agents or tissue damage directly. Neurogenic inflammation causes symptoms typical of inflammation, vasodilation and fluid extravasation, as well as changes in cellular excitability (Richardson and Vasko, 2002). Trigeminal fibers transmit information of pain and irritation to the central nervous system, but they also have a local effect; sub P and CGRP, released from trigeminal fibers, initiate neurogenic inflammation (Black, 2002; Chiu et al., 2012). The release of neuropeptide causes dilation of nearby blood vessels. This allows for increased blood

flow to the damaged site and immune cell infiltration. Nearby mast cells are also activated, and, in turn release histamine, causing an increase in the dilation of nearby blood vessels.

Particulate matter

A major factor that can enter the airways and initiate neurogenic inflammation is particulate matter (PM). The airways are the entry points of pollutants into our bodies, and approximately 44.3 million Americans (14%) live in a county with unhealthy levels of small PM (American Lung Association, 2014). PM in ambient air is categorized into coarse particles (10 μm aerodynamic diameter in at least one dimension, or PM₁₀) and fine particles (PM_{2.5}), which is also commonly subdivided into ultrafine or nanosized particles (PM_{0.1}). The smaller the particle, the greater the potential to cause damage, as smaller particles can penetrate deep into the airways of the respiratory tract and into the lungs and alveoli (Valavanidis et al., 2008). Cross-sectional studies found a correlation between PM pollution and mortality (Dockery et al., 1993; Krewski et al., 2009). Exposure routes for manganese are studied more often than other inhalants because of its role in neurotoxicity, especially in relation to development of neurodegenerative diseases. In an occluded nostril model, the majority of manganese found in the olfactory bulb was delivered via the olfactory route following acute inhalation exposure (Brenneman et al., 2000). The rat trigeminal nerve may also deliver manganese from the nasal cavity to the brain (Lewis et al., 2005). Olfactory transport rapidly delivers manganese to the olfactory bulb and tract (within 8–48 hr), but not to more distant brain areas. Conversely, inhaled tungsten (Radcliffe et al., 2009) and iron

(as the sulfate salt) are poorly transported from the nasal cavity (Rao et al., 2003).

Thus, there are limitations in which PM can access the rest of the body through the OE. Regardless, some PM may still be initiating neurogenic inflammation in the OE, which could have more wide-reaching effects depending on which immune cells and mediators become involved.

Inflammatory neuropeptides

In the 1930s, a substance was discovered in the brain and gut of the rabbit that caused a fall in blood pressure and contraction of the ileum. This factor was given the name sub P. It was later determined to be an undecapeptide, a peptide consisting of 11 amino acid residues, in the family of tachykinins. First described in amphibians and invertebrates, tachykinins are peptides that are primarily involved in the processes of pain and inflammation (Andrews and Rudd, 2004). These peptides are largely referred to as neuropeptides due to their origins in neuronal cells. Sub P is widely distributed throughout the central and peripheral nervous systems and stored in synaptosomes of unmyelinated sensory nerve endings. However, tachykinins, sub P included, are found more extensively than this. Sub P is produced by inflammatory cells such as macrophages, eosinophils, lymphocytes, and dendritic cells (Weinstock et al., 1988; Bost et al., 1992; Killingsworth et al., 1997; Joos et al., 2000), and also may function as a chemoattractant of monocytes (Saban et al., 1997; Schratzberger et al., 1997)(Saban et al., 1997).

There are three types of tachykinin receptors, neurokinin-1 receptor (NK-1R), NK-2R, and NK-3R. Each receptor is preferentially activated by a specific tachykinin.

The NK-1R is activated preferentially by sub P, the NK-2R by NKA, and the NK- 3R by NKB. However, at high enough concentrations, any tachykinin can activate any tachykinin receptor (Nakanishi, 1991). Tachykinin receptors are part of the G protein coupled receptor (GPCR) superfamily (Hershey and Krause, 1990). These receptors are glycoproteins that consist of seven α -helical transmembrane segments, an extracellular amino- terminus and an intracellular carboxyl tail. The second and third membrane-spanning domains are involved in ligand binding. The third transmembrane loop interacts with G proteins. The receptor can be desensitized when serine and threonine residues of the carboxyl terminal are phosphorylated. Cells can be desensitized to sub P signaling through a mechanism of receptor internalization upon binding of sub P to NK-1R. Agonist stimulation of the NK-1R in many tissue and cell types initiates intercellular signaling mechanisms through the release of calcium from internal sources.

Summary of dissertation experiments

Inflammation in the peripheral and central nervous system is increasingly being coupled to neurological disorders and neurodegenerative diseases. For instance, asthma and rhinitis, forms of inflammation in the lungs and nose, are linked to dementia in humans. Environmental air pollutants negatively affect cognitive brain functions such as learning and memory and cause cell death and neurodegeneration. Markers of inflammation are observed in the brain following exposure to ambient particulate matter, indicating that components of pollution cause neuroinflammation. Inflammation can be detrimental, as it hinders neuroregeneration in the adult CNS, and was also shown to

inhibit proliferation and regeneration in the OE by Lane and colleagues, described above. This may be due to the acute activation of monocytes and subsequent production of pro-inflammatory cytokines, however, the exact mechanism is not known. To elucidate mechanisms of neuroregeneration that occur subsequent to or simultaneous with inflammation, I studied inflammation in the mouse OE, a highly regenerative tissue. Furthermore, I studied ways in which irritants from the environment can initiate neurogenic inflammation in the OE, to better understand how the environment can affect the OE, as well as to understand the extent to which inflammation can occur in the OE. I hypothesized that irritants from the environment activate trigeminal nerve fibers in the OE, causing a local release of sub P which then initiates an increase in macrophages and their cytokines in the tissue. If neurogenic inflammation occurs in the OE on a regular basis, inflammation may be an important factor in maintaining a highly regenerative environment. The information from my studies adds to studies in which pathogens or lesions are used to investigate inflammation and recovery.

Chapter two is a description of experiments testing the hypothesis that the mouse OE contains irritant-sensing channels that respond to noxious agents in the environment and activate free nerve endings in the OE to initiate neurogenic inflammation. Thus, the expression and cellular location of TRP channels and trigeminal fibers and function of these receptors is investigated. Activation of irritant-sensing channels can cause the local release of neuropeptide. Studies were carried out to measure the release of sub P and subsequent plasma extravasation in the OE in response to activation of irritant-sensing channels.

The sensitivity of trigeminal fibers can be expanded by innervating secondary chemosensory cells. In Chapter three, I investigate the signal transduction mechanisms of one such secondary chemosensory cell in the OE- the IP3R3-expressing microvillous cell subtype. Mechanisms involved in responses to odorants and purines are investigated using live cell calcium imaging. A role in irritant signaling is also investigated through immunohistochemical studies.

The effects of the release of neuropeptide into tissue can be widespread. The role of local sub P release in the mouse OE is investigated in Chapter four. OE tissue was treated with sub P to investigate the role of this neuropeptide in the recruitment of macrophages and the release of cytokines. The expression and function of sub P receptor NK-1R was examined using immunohistochemistry and live cell calcium imaging to investigate other downstream targets of neurogenic inflammation.

Overall, the studies of this dissertation identify the trigeminal nerve, TRP channels, and macrophages as key factors in the neurogenic inflammatory response in the mouse OE (Figure 1.3). The presence of irritant sensing channels on trigeminal fibers and secondary chemosensory cells suggests that environmental irritation can initiate acute inflammation in the OE. The sub P-induced increase in macrophages and cytokines suggests that these inflammatory mediators are involved in not only chronic inflammation, but also acute neurogenic inflammation. Although acute inflammation can be beneficial in recovery from damage, it is important to understand the underlying mechanisms involved, as progression to chronic inflammation can lead to disease states. Therefore, these studies contribute to the understanding of the effects of environmental pollutants on our health.

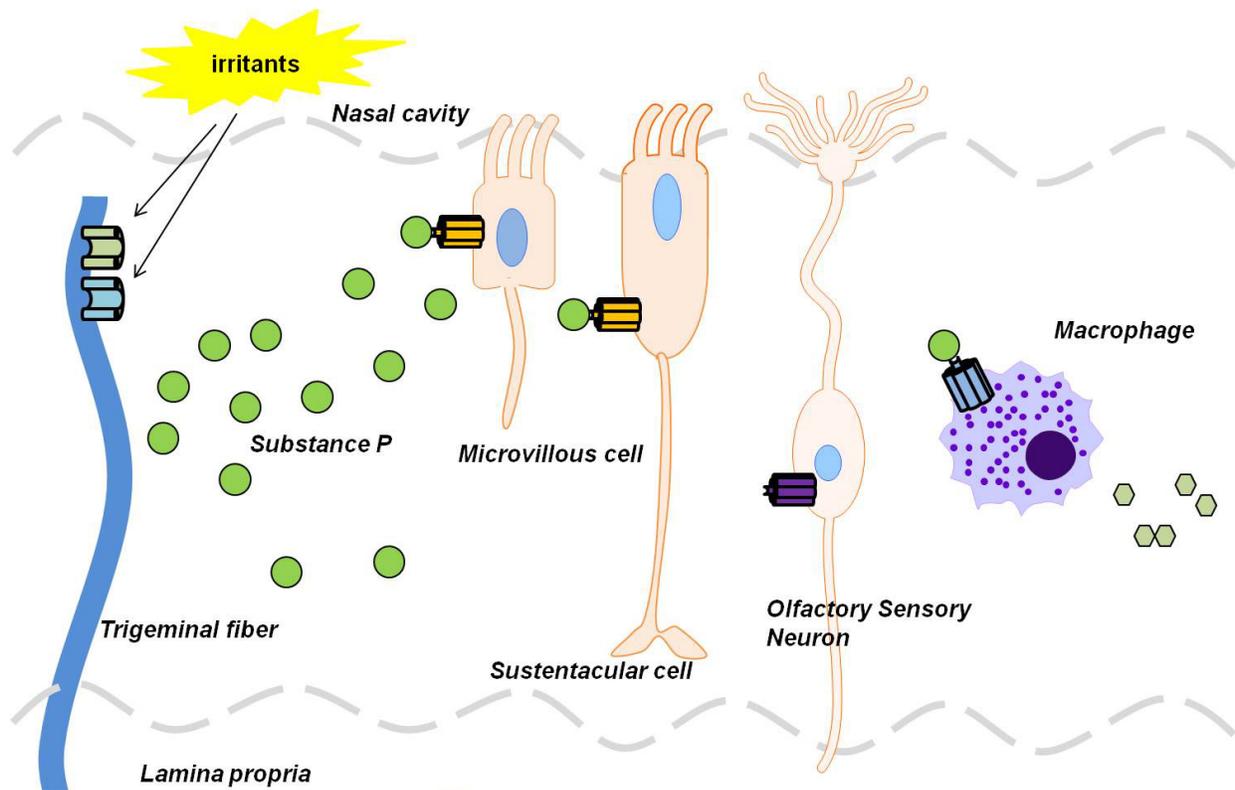


Figure 1.3. Model of initiation of neurogenic inflammation in the mouse OE.

Irritants from the environment enter the nasal cavity and descend into the OE and activate TRP channels on trigeminal fibers. This activation of TRP channels by irritants initiates the release of sub P from the trigeminal fiber. Sub P instigates an increase in intracellular calcium in non-neuronal microvillous cells and the release of inflammatory mediators, called cytokines, from macrophages.

Chapter 2: TRPA1 and TRPV1 in irritant initiated neurogenic inflammation of the mouse olfactory epithelium

Introduction

The olfactory epithelium (OE) is open to the environment and the pollutants therein, and thus it is in contact with factors that can cause damage. The nasal cavity maintains many defenses against toxins. Specifically, in the OE, sustentacular cells contain high levels of enzymes, such as cytochrome p450, that metabolize toxins. The OE is also found in the rostral-most aspect of the nasal cavity, so air is filtered through the respiratory epithelium before encountering the sensitive tissue of the OE. Large particles from the air become trapped in hair and mucus, while many reactive gases are absorbed (Harkema et al., 2006). However, damaging and irritating agents in the air can still access and compromise the OE.

Rhinosinusitis, a form of inflammation often initiated in response to bacteria, virus, or allergens, is a common affliction of the upper airways and OE (Kern, 2000). Rather than by direct tissue damage or the infiltration of pathogens into the tissue, the neurogenic form of inflammation is initiated by the stimulation of sensory nerves (Lundblad et al., 1983; Baraniuk, 2001; Lacroix and Landis, 2008). As the OE contains primary sensory nerve fibers, i.e. trigeminal innervation (Bouvet et al., 1987; Finger et al., 1990), and is in constant contact with the outside environment, I expect that neurogenic inflammation is a form of inflammation that is readily initiated in the OE.

Upon activation of sensory afferents, neuropeptides are released directly from nerve terminals into the local environment. This release is through the activity of irritant

sensing channels such as transient receptor potential channel type Vanniloid 1 (TRPV1) or ankyrin 1 (TRPA1). Capsaicin is the prototypical agonist of TRPV1, and in concentrations that do not cause axonal damage initiates the release of neuropeptide from terminals without axonal conductance (Németh et al., 2003). Capsaicin activation of TRPV1 causes cation influx into the sensory terminal, creating a concentration adequate in causing the release of neuropeptide. One downstream effect of this capsaicin initiated release of neuropeptide is blood vessel dilation and the activation of plasma extravasation, a common symptom of acute inflammation. Blood vessel dilation increases the flow of blood to the inflamed area, causing the warmth and redness associated with inflammation, and the accompanying plasma extravasation that is especially characteristic of neurogenic inflammation increases the access of immune cells and liquid exudation from vessels, leading to edema also typical of inflammation.

The OE is innervated by primary afferents originating from the trigeminal ganglion and trigeminal nerve, or cranial nerve V. These innervating fibers are peptidergic, and are often identified due to immunoreactivity toward neuropeptides substance P (sub P) or CGRP (Hunter and Dey, 1998). TRPV1 and TRPA1 often co-localize, and TRPV1 is often found expressed on primary afferents such as trigeminal nerve fibers (Mandadi and Roufogalis, 2008). Thus, TRP channels expressed on trigeminal fibers can be a means for the initiation of neuropeptide release into the OE, in other words, their activation can initiate neurogenic inflammation.

The goal of this study is to determine the components present in the mouse OE that initiate an inflammatory response to irritants. I localized immunoreactivity to irritant-sensing channels TRPA1 and TRPV1 to trigeminal nerve fibers, as expected, but I also

identified secondary chemosensory cells that either express irritant sensing channels or are contacted by trigeminal fibers. Additionally, I used live cell calcium recordings to demonstrate that TRPA1 and TRPV1 are responsive to irritants. I also demonstrated that the activation of TRPA1 and TRPV1 initiates the efferent release of inflammatory peptide sub P in mouse OE tissue which leads to plasma extravasation from local blood vessels, a classic symptom of inflammation. Thus, I demonstrate the components of the system by which irritants from the environment can initiate inflammation within the OE.

Methods and Materials

Animals

Swiss Webster (CFW) mice were used in all experiments. Adult male mice (6-8 week) were used in immunohistochemistry, plasma extravasation, and polymerase chain reaction studies. Neonatal mice of both sexes (P0-4) were used in calcium imaging, culture media collection for enzyme-linked immunosorbant assay, and nitrocellulose membrane staining studies. In immunohistochemical studies, a transgenic animal in the C57 BL/6 background was used to compare immunoreactivity to green fluorescence protein (GFP)-expressing microvillous cells. This transgenic mouse line, the inositol triphosphate receptor 3 (IP3R3)-tauGFP mouse (kindly provided by Dr. Diego Restrepo, University of Colorado Denver, Aurora, CO), has the first exon of the *Itpr3* gene replaced by the coding region for a fusion protein of tau and GFP (Hegg et al., 2010). This GFP expression identifies a particular microvillous cell type in the mouse OE, and will henceforth be referred to as IP3R3-MV.

Mice were given food and water *ad libitum*. Animal rooms were kept at 21–24°C and 40–60% relative humidity with a 12-h light/dark cycle. All procedures were conducted in laboratory animals as approved by Michigan State University Institutional Animal Care and Use Committee.

Immunohistochemistry (IHC)

Anesthetized adult mice (65 mg/kg ketamine+5 mg/kg xylazine, intraperitoneally) were transcardially perfused with 0.1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA), then decapitated and post-fixed in 4% PFA overnight. Heads were decalcified for 5 days with ethylenediaminetetraacetic acid (0.5M, pH=8), cryoprotected overnight in 30% sucrose solution, frozen, and then sectioned coronally (20 µm), from level 3 at the level of the second palatal ridge of the mouse nasal cavity, with a HM525 cryostat (Microm International, Waldorf, Germany) while embedded in TissueTek Optimal Cutting Temperature compound (Sakura Finetek USA, Torrance, CA). Tissue was collected onto positive-charged slides, and stored at -20 °C until use, at which time they are rehydrated with PBS. Tissue is permeabilized using 0.3% Triton X-100 and blocked in normal goat serum (10%) for 1 hour. Primary antibodies (Table 2.1) were incubated on the tissue overnight followed by cy3-conjugated anti-rabbit immunoglobulin (1:200, Jackson Immunoresearch Lab, West Grove, PA, USA). TRPM5 immunoreactivity was assessed using a Tyramide signaling amplification 546 kit (Invitrogen, Eugene, OR). Slides were mounted with Vectamount with or without nuclei staining DAPI included in medium (Vector Labs, Burlingame, CA). Immunoreactivity was visualized on an Olympus FV1000 Confocal laser scanning microscope (Olympus

America Inc., Center Valley, PA, USA) with cy3 excited at 543nm. Antibody specificity was examined by omitting the primary antibody or secondary antibody. No immunoreactivity was observed in any of the controls (data not shown).

Antibody	Target	Concentration	Manufacturer
Rabbit α -TRPV1	Irritant-sensing channel TRPV1	1: 2000	Abcam; Cambridge, England
Rabbit α -TRPA1	Irritant-sensing channel TRPA1	1: 1000	Abcam; Cambridge, England
Rabbit α -CGRP	Sensory nerve fibers, including Trigeminal fibers	1: 500	Peninsula; San Carlos, CA
Rabbit α -TRPM5	Subset of OE microvillous cell	1: 100	Alomone; Jerusalem, Israel

Table 2.1: Antibodies used in immunohistochemical studies

Reverse transcriptase polymerase chain reaction (PCR) of TRPA1 and TRPV1

Turbinates were dissected from adult C57BL/6 and CFW strain mice (n=4/strain). The animals were anesthetized with a ketamine/xylazine injection of 0.05 mL and then decapitated. The skin and jaw were removed, the skull hemisected, and OE tissues were rapidly dissected and stored at -80°C until RNA isolation was performed. On the day of enzyme immunoassay performance, the OE tissues were homogenized by sonication in Tris buffer [10 mM Tris-HCl (pH 7.6) containing 100 mM NaCl, 1 mM EDTA, 2 M activated Na_3VO_4 , 50 mM NaF, and a protease inhibitor cocktail (1:1000, Sigma-Aldrich, St. Louis, MO)]. The concentration of protein in the homogenate was measured using a bicinchoninic acid protein assay kit (Pierce, Rockford, IL). TRIzol reagent (Life Technologies) was added to turbinate tissue, then the mixture was sonicated and added to autoclaved tubes containing 100 μL of chloroform, centrifuged for 10min at 12000 RPM (4°C). The clear phase of the remaining supernatant was

mixed with 125 μL of isopropanol to precipitate the RNA, then centrifuged for 8 min at 12000 RPM and 4°C. The supernatant was aspirated and the pellets washed with 75% ethanol, then centrifuged for 5 min at 12000 RPM and 4°C. The pellets were allowed to air dry. 60 μL of diethylpyrocarbonate (DEPC) water was added, immediately followed by incubation in a 60°C water bath for 10 minutes. Following the manufacturer's protocol (High-Capacity cDNA Reverse Transcription Kit constructed by Applied Biosystems), RNA samples were diluted with DEPC water such that samples contained 1000 ng per μL of RNA. Reaction mixture containing random primers and reverse transcriptase was added for a total volume of 20 μL per sample. As control, an equal volume of RNA of each sample was treated according to the same protocol with addition of water instead of the reverse transcriptase. Samples were processed in a thermocycler. Reaction products were then used for PCR amplification of mouse TRPV1 and TRPA1 receptors by the HotStar Taq Polymerase Kit (Qiagen) according to the manufacturer's recommendations, in a final volume of 20 μL . After an initial activation step at 95°C for 15 min, cDNAs were amplified with custom primers. cDNA were subjected to 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, extension at 72°C for 1 min, followed by a final extension at 72°C for 10 mins. cDNA were separated by size in a 1.5% agarose gel containing ethidium bromide using 100V for 1 hour alongside a DNA ladder. Products were visualized with UV light.

mRNA	Primer	Sequence	Product size	Reference
TRPV1	forward	GCTGCTAACGGGGACTTCTT	285	Taylor-Clark et al., 2008
	reverse	CTTCAGTGTGGGGTGGAGTT		
TRPA1	forward	ATATGCAGTGGCAATGTGGA	175	Taylor-Clark et al., 2008
	reverse	CTGAGGCCAAAAGCCAGTAG		
Actin	forward	GTGGGAATGGGTCAGAAGA	303	N/A
	reverse	GAGGGCATACAGGGACAGCA		

Table 2.2. RT-PCR primers

Western blotting

Anesthetized (4% isoflurane) adult male (6-8 weeks old) C57BL/6 mice were decapitated and heads were hemisected and OE tissues dissected and stored at -80°C. Tissue was processed following the protocol described previously (Jia et al., 2009). Briefly, tissues were homogenized by sonication in Tris buffer. Homogenates (30 µg/lane) were resolved on 12.5% gels and then transferred to nitrocellulose membranes. Membranes were incubated overnight at 4 °C with primary antibodies (Table 1) made in blocking buffer (0.2% g/LI-Block, Millipore, Bedford, MA, USA). After washing, the membranes were incubated with horseradish peroxidase-labeled secondary antibody (Jackson Laboratory, West Grove, PA, USA). Immunoreactive proteins were detected by incubating nitrocellulose membrane with an enhanced chemiluminescence reagent (Amersham Biosciences, Piscataway, NJ, USA) and the nitrocellulose membrane was then exposed to Kodak X-ray film, which was then developed.

Confocal live cell calcium imaging

Neonatal mice were decapitated and their skin and lower jaw were removed. The head was embedded in a block made of carrot and mounted onto a vibratome. Tissue was kept in ice-cold Ringer's solution while sectioned into 400- μ m slices. Ringer's solution contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM HEPES, 10 mM glucose, and 500 μ M Probenecid (pH 7.4, 290–320 mOsm). Slices were incubated with 50 μ g/mL calcium indicator dye X-Rhod 1 AM (Molecular Probes) made in Ringer's solution with 0.04% pluronic F-127 (Invitrogen, Calsbad, CA) and 0.4% DMSO for 60 min at room temperature before imaging.

Tissue slices were placed in a laminar flow chamber (Warner Instruments, Hamden, CT, USA) and perfused continuously with Ringer's solution at a flow rate of 1.5–2.0 mL/min. Test solutions were applied using bath exchange and a small-volume loop injector (200 μ L). Our perfusion system exchanges the bath in *ca.* 7–10 s and traces were not corrected for this delay. Following application of bioactive compound, 5 minutes of Ringer's alone was perfused over the slice. Using a FV1000 confocal microscope (Olympus), images were collected over 2 minutes at 0.2–1 Hz. A helium–neon laser excited X-Rhod-1 AM at 543 nm. Fluorescence emissions were filtered at 560–660 nm. The fluorometric signals obtained were expressed as relative fluorescence (F) change, $\Delta F/F_0 = (F - F_0)/F_0$, where F_0 is the baseline fluorescence level (mean F of first 5 frames). Data were collected from a minimum of three slices for each experiment.

Nitrocellulose membrane staining for sub P

Slices of OE from neonatal CFW mice were cultured on nitrocellulose membrane (0.45 μm ; GE Osmonics, Minnetonka, MN) placed in a 35 mm petri dish with cinnamaldehyde (1 μM) or Ringer's solution for 1 hour. Slices were then removed, and membranes were put into a chamber for vapor fixation at 75°C for 2 hours. The membrane was incubated for 1 hour with blocking reagent (5% Tween 20 and 5% carnation lowfat milk in PBS) to block non-specific staining, then anti-Sub P (1:1000; AbdSerotec) at 4°C overnight. Sub P immunoreactivity (IR) was visualized using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and Vector VIP (Pierce, Rockford IL), with equal staining times.

Enzyme-linked immunosorbant assay (ELISA) measurement of sub P

OE slices (400 μm) were collected as described previously from neonatal CFW mice (both sexes). For each treatment group 3–4 slices were used from one animal, with each treatment group containing at least 3 animals. Slices were placed on a cell culture plate insert (Millicell-CM PICMORG50, Milipore Corp., Billerica, MA) and incubated at 37°C with 5% CO₂ in neurobasal media with 0.02 g/L B-27 supplement, 0.01 g/L penicillin/streptomycin and 0.01 g/L L-glutamine (Invitrogen, Carlsbad, CA). Conditioned media were collected 1 hr after capsaicin treatment. Levels of sub P in the media were assayed using an ELISA kit (Peninsula Laboratories, San Carlos, CA) according to manufacturer's instructions. All samples were run in duplicate, averaged, and reported as mean \pm SEM of Sub P/ μg protein (Jia and Hegg, 2010).

Plasma extravasation

Mice were anesthetized using 4% isoflurane. The capsaicin treatment group received 2 μ M capsaicin (25 μ L) applied dropwise to the right naris (n=4 adult male CFW mice). The antagonist plus capsaicin group (n=3 adult male CFW mice) first received 10 μ M capsazepine (25 μ L) to the right naris and ten minutes later received capsaicin. The cinnamaldehyde treatment group received 100 μ M cinnamaldehyde (25 μ L) applied dropwise to the right naris (n=3 adult male CFW mice). The antagonist plus cinnamaldehyde group (n=3 adult male CFW mice) first received 100 μ M HC-030031 (25 μ L) to the right naris and ten minutes later received cinnamaldehyde. Five minutes after nasal stimulation, mice were placed on a warming pad and Alexafluor 555 conjugated to albumin (25mg/kg in saline) was injected into the tail vein. Five minutes following tail vein injection, heads were removed and fixed in 4% PFA overnight. Following fixation, heads were bisected in the midsagittal plane and the nasal septum removed to expose turbinates. Pictures were taken and analyzed using an Inverted Nikon Fluorescent microscope (Nikon TE2000-U Fluorescence Microscope) with an excitation of 555/emission 565 nm for Alexafluor 555 dye. Fluorescence intensity was measured using ImageJ software (NIH). Images were converted to 16-bit and the turbinates were highlighted. The equation used for the corrected integrated density (integrated density- area of selected cell X mean fluorescence of background readings) took into consideration the area of the selected cell and normalized the fluorescence of the cell using the fluorescence of the background.

Results

TRPV1 and TRPA1 irritant sensing channels are present in the mouse OE

Though trigeminal fibers are known to innervate the OE, expression of receptors that activate the fibers are not well described in the mouse OE. To confirm the presence of these irritant sensing channels in the mouse OE, turbinate tissue from adult mice was probed for the expression of mRNA to TRPA1 and TRPV1 (Figure 2.1A). TRPA1 band expression was found at the expected weight (175 bp, lane 1) and absent from the control (lane 2). TRPV1 band was expressed at the expected weight (285 bp,), but further optimization is needed because a band was also observed in the control (not shown). Using western blotting analysis (Figure 2.1B), TRPV1 and TRPA1 protein was detected from OE tissue, further verifying the presence of both irritant-sensing channels in the mouse OE.

TRPV1 and TRPA1 channels are found on putative nerve fibers and multiple cell types in the mouse OE

I sought to determine the location of irritant sensing channels in the mouse OE using IHC. I found that TRPV1-IR was punctate and present throughout the OE, including adjacent to IP3R3-MVs and on OSNs (Figure 2.2, A-C). TRPA1-IR was observed throughout the epithelium in fiber-like structures that spanned the height of the epithelium (Figure 2.2, D). These fibers co-labeled with CGRP (Figure 2.2, F), a neuropeptide contained in trigeminal nerve fibers, indicating TRPA1 expression on trigeminal nerve fibers in the OE. TRPA1-IR was not present in the IP3R3-MV subtype, but was present in another microvillous cell sub-type (Figure 2.2, G and H). Double-labeling for TRPM5 and TRPA1 demonstrated instances of co-labeling, indicating a

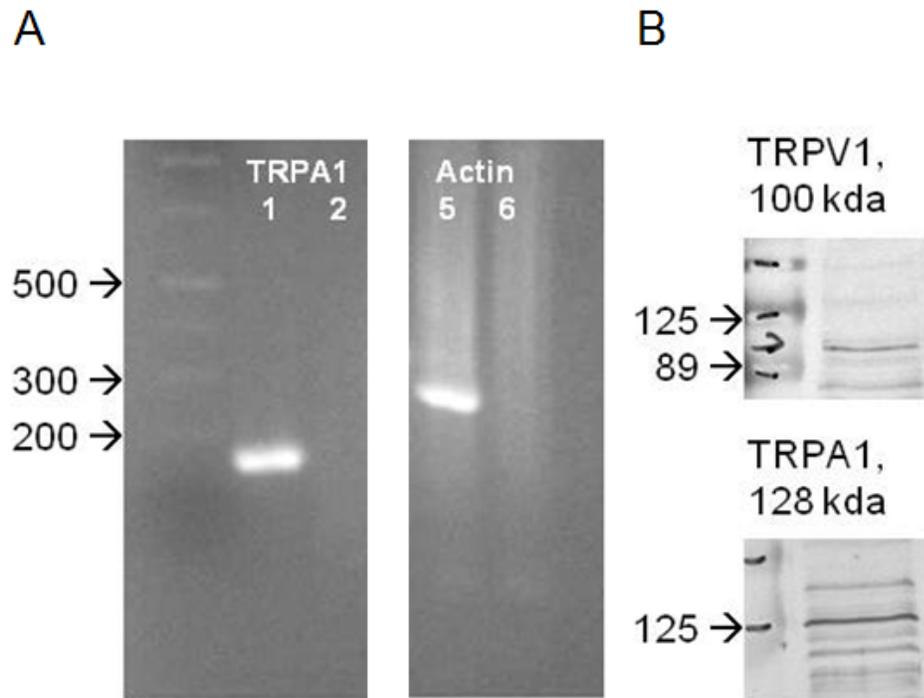


Figure 2.1. mRNA and protein expression of TRPA1 and TRPV1 in the mouse OE.

(A) Electrophoresis gel of PCR products testing for the gene sequences of TPRA1 and TRPV1 in mouse OE. This gel is representative of all samples (n=4) of the C57 and CFW strains. Both strains showed expression of TRPA1. RT-PCR indicated expression of TRPA1 mRNA at 175bp (lane 1) and not in the cDNA negative lane (lane 2). Positive control actin was evident in the cDNA positive lane (lane 5) and absent from the cDNA negative lane (lane 6). Arrows indicate control marker sizes of 500, 300, and 200 bp. (B) Representative blots of western blotting performed on adult mouse OE tissue to probe for TRVP1 and TRPA1 protein expression. Both TRPV1 (100 kda) and TRPA1 (125) expression was present in all samples (n=3). Arrows indicate molecular weights of the protein ladder that was run alongside samples.

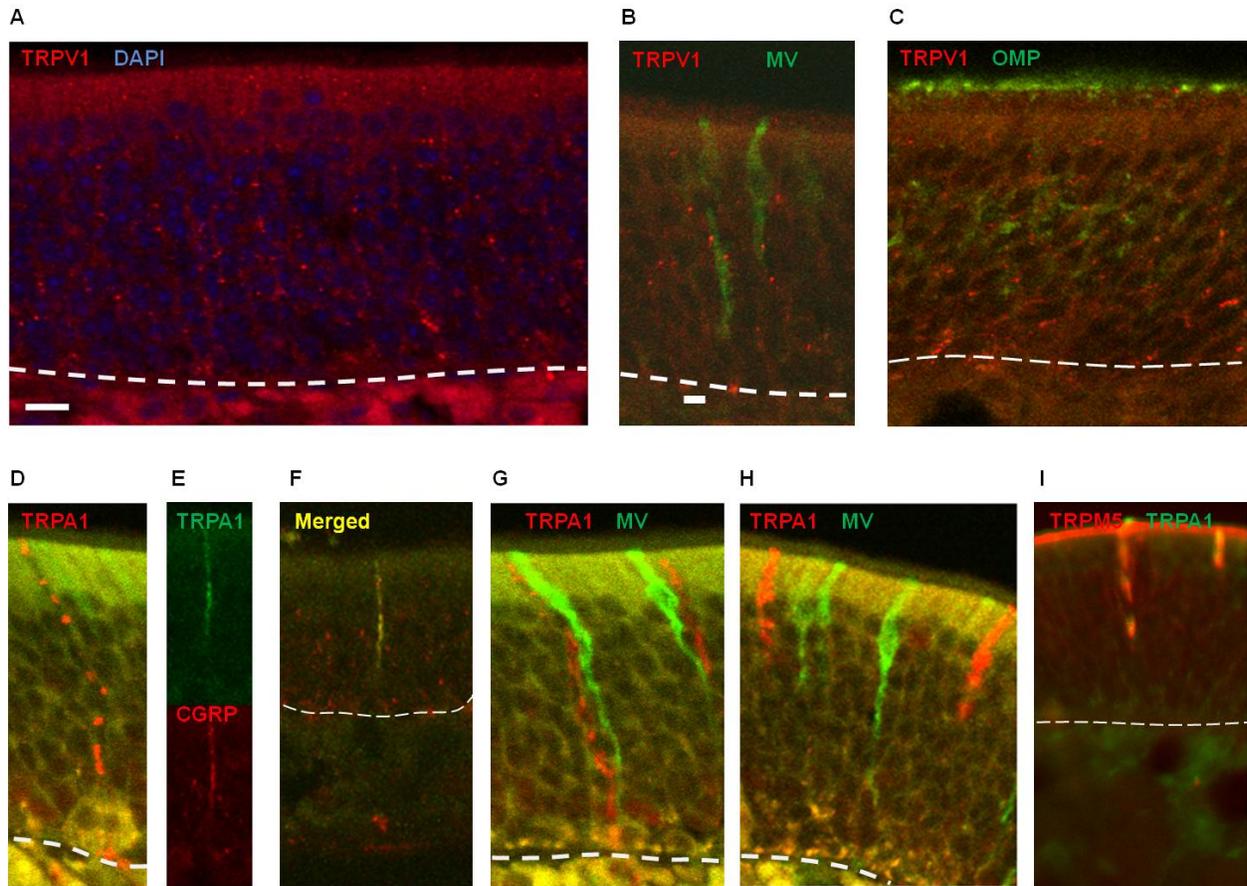


Figure 2.2. Irritant-sensing channels TRPV1 and TRPA1 are expressed in mouse OE. (A) TRPV1-IR (red) was found throughout the OE. Labeling was punctate (red) and (B) often found adjacent to IP3R3-MV cells (green). (C) and neurons (OMP, green). (D) TRPA1 (red) labeled fibers at intervals throughout the epithelium, which spanned the OE and crossed into lamina propria (dotted white line). (E) TRPA1-IR (green, E1), when co-labeled with a marker for trigeminal fibers, CGRP (red), was found to co-localize in the same structures (F). (G) These fiber-like projections with TRPA1-IR occasionally come into contact with GFP-expressing IP3R3-MVs (green). (H) TRPA1-IR (red) was occasionally expressed in a cell with microvillous-cell like morphology, but did not co-localize with IP3R3-MV cells (green) (I) TRPA1-IR also co-labeled for a marker for a subtype of olfactory microvillous cell, TRPM5 (merged image of TRPA1 in

Figure 2.2 (cont'd) green and TRPM5 in red) (G) Scale bar= 10 μ m in B, C, and E.

Dotted lines indicate lamina propria.

population of microvillous cell that has been previously described to express the transduction component TRPM5 (henceforth TRPM5-MV; Figure 2.2 I). Thus, irritant-sensing channels TRPV1 and TRPA1 may be activating trigeminal fibers using both direct and indirect means. TRPA1 co-localization with CGRP indicated a direct activation, whereas TRPV1-IR adjacent to IP3R3-MV indicated that the fiber could be activated in response to the IP3R3-MV responding to stimulants that would not otherwise activate the trigeminal fiber directly.

TRP channel activation causes changes in intracellular calcium across OE cell types

I postulated that trigeminal fibers of the OE would be stimulated by irritants through TRPA1 and TRPV1 activity but my IHC studies indicated that TRPA1 and TRPV1 are expressed on more structures than just the trigeminal fiber. To determine that the TRP channels were not only present but also functional on these other structures, live-cell calcium imaging experiments were conducted using TRPV1 and TRPA1 agonists. TRPV1 agonist capsaicin (100 nM, 1 μ M, and 10 μ M) and TRPA1 agonist cinnamaldehyde (100 μ M and 1 mM) increased calcium in multiple cells types of the OE (Table 2.3). At the lowest concentration of capsaicin (100 nM), the average $\Delta F/F_0$ was 26.2 ± 7.8 , and all the responses noted were from cells identified as OSNs (5/5), and this identification was based on their morphology and location in the OE. At the higher concentrations (1 and 10 μ M) responses were seen in both neuronal and non-neuronal cell types, though the majority of responses were still from OSNs. Additionally, at both higher capsaicin concentrations, the average $\Delta F/F_0$ from OSNs was greater than that of the non-neuronal responses (49.1 ± 28.3 versus 6.11 ± 0.0 and

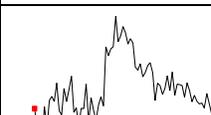
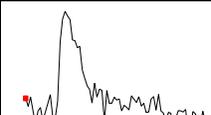
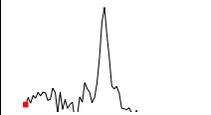
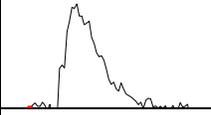
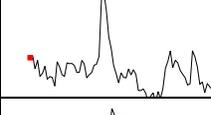
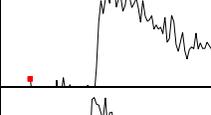
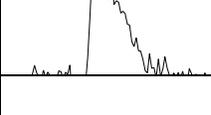
Agonist	Concentration	Responding cell type	Number of responses	Number of slices	Average $\Delta F/F_0$	Representative trace
capsaicin	100 nM	sensory neurons	5/5	1	26.2 ± 7.8	
capsaicin	1 μM	sensory neurons	11/12	2	49.1 ± 28.3	
capsaicin	1 μM	non-neuronal	1/12	2	6.1 ± 0.0	
capsaicin	10 μM	sensory neurons	11/15	2	71.5 ± 35.9	
capsaicin	10 μM	non-neuronal	4/15	2	11.2 ± 4.8	
cinnamaldehyde	100 μM	sensory neurons	13/13	3	51.5 ± 28.0	
cinnamaldehyde	1 mM	sensory neurons	19/32	3	26.7 ± 5.6	
cinnamaldehyde	1 mM	non-neuronal	13/32	3	72.0 ± 27.3	

Table 2.3. TRP channel activation causes changes in intracellular calcium across OE cell types. Slices of neonatal OE were loaded with X-Rhod-AM1 and increases in intracellular calcium in response to agonists were measured using a confocal microscope and imaging software. TRPV1 agonist capsaicin (100 nM, 1 μM, 10 μM) evoked increases in calcium from OSNs (5 of 5 responding cells from 1 slice; 11 of 12 responding cells from 2 slices; 11 of 15 responding cells from 2 slices, respectively). Higher concentrations (1 μM and 10 μM) elicited responses from non-neuronal cells

Table 2.3 (cont'd) (1 of 12 from 2 slices and 4 of 14 responding cells from 2 slices, respectively). TRPA1 agonist cinnamaldehyde (100 μ M, 1 μ M) evoked increases in calcium from OSNs (13 of 13 responding cells from 3 slices and 19 of 32 responding cells from 3 slices). Higher concentrations (1 mM) elicited responses from non-neuronal cells (13 of 32 responding cells from 3 slices).

71.5±35.9 versus 11.2±4.8, respectively). At the lowest concentration of cinnamaldehyde (100 µM), the average $\Delta F/F_0$ was 51.5±28.0, and all the responses noted were from cells identified as OSNs (13/13). At the higher concentration (1 mM) responses were seen in both neuronal (19/32) and non-neuronal cell types (13/32) and the average $\Delta F/F_0$ from OSNs was greater than that of the non-neuronal responses (49.1±28.3 versus 6.1±0.0 and 71.5±35.9 versus 11.2±4.8, respectively). Collectively, these data support the expression of functional irritant sensing TRPA1 and TRPV1 channels in the OE.

Sub P is released from tissue exposed to irritants

Sub P is used in signaling pain, but it also has an efferent effect- it can be released locally from trigeminal fibers. To demonstrate that TRPV1 and TRPA1 agonists capsaicin and cinnamaldehyde cause the efferent release of sub P in the OE, I placed OE slices on nitrocellulose membranes in the presence of different treatments to evoke the release of sub P, and then processed the nitrocellulose for sub P-IR. When the slices were exposed to cinnamaldehyde (1 µM), the density of immunostaining for sub P was greater than the immunostaining in control treatments (Figure 2.3B). When primary antibody or secondary antibody was omitted, sub P-IR was absent (not shown). Sub P release was not exclusive to a particular region but was found in every slice from rostral to caudal. This indicates that even though trigeminal innervations may be denser in the respiratory tissue of the rostral nasal cavity, there is still ample innervation of the caudal olfactory portions to have detectable sub P release from trigeminal fibers.

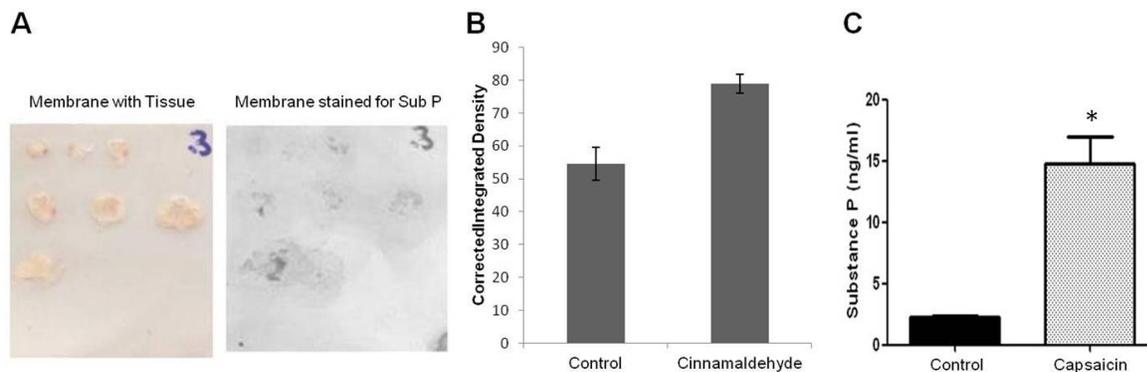


Figure 2.3. Substance P is released from neonatal olfactory epithelial slices exposed to irritants. (A) Nitrocellulose membranes were cultured with tissue exposed to cinnamaldehyde (varying concentrations) or PBS. After fixation, nitrocellulose membranes were stained for sub P. (B) Intensity of labeling is greater in cinnamaldehyde exposed tissue compared to control. (C) Exposure of OE slices to capsaicin evoked a significantly greater release of sub P than control (saline), detected by ELISA analysis of media containing slices (n=3 animals, 3 slices/treatment). *P<0.05 vs. control.

To determine the extent to which the TRPV1 irritant induces sub P release in the OE, I exposed neonatal mice OE to capsaicin (for exposure to olfactotoxicant satratoxin G, see supplemental data). Sub P release was quantified using ELISA. TRPV1 agonist capsaicin (100 μ M) significantly ($p < 0.05$) increased sub P release from OE tissue slices of neonatal CFW mouse compared to control (8.3 ± 3.3 and 2.3 ± 0.1 ng/ml) (Fig. 2.3 C).

In these studies, I show that sub P is released from the OE of mice upon TRPA1 and TRPV1 stimulation. This indicates that these channels, whether they are found on trigeminal fiber themselves, or on cells that trigeminal fibers innervate, can cause the release of neuropeptide content from sensory fibers within the olfactory tissue.

Importantly, this result indicates that not just cellular damage or infiltration of allergens or xenobiotics activates inflammation, but irritants can activate inflammation in the OE.

TRPV1 and TRPA1 agonists cause plasma extravasation in the mouse nasal cavity

One of the main symptoms of inflammation is plasma extravasation. Capsaicin, the agonist for TRPV1-mediated peptide release, can cause plasma extravasation in the mouse nasal cavity by initiating the release of sub P from sensory fibers (Saunders et al., 2014). The effect of plasma extravasation in the nasal cavity was shown to be via the release of sub P from sensory fibers by denervating the tissue and then treating with capsaicin, which resulted in no leakage of the nearby vessels (Saunders et al., 2014). In our studies, the leakage of albumin measured as relative fluorescence was significantly higher in capsaicin and cinnamaldehyde treated OEs ($p < 0.05$), compared to vehicle control (Figure 2.4 A-C). Additionally, I demonstrate that this increase in

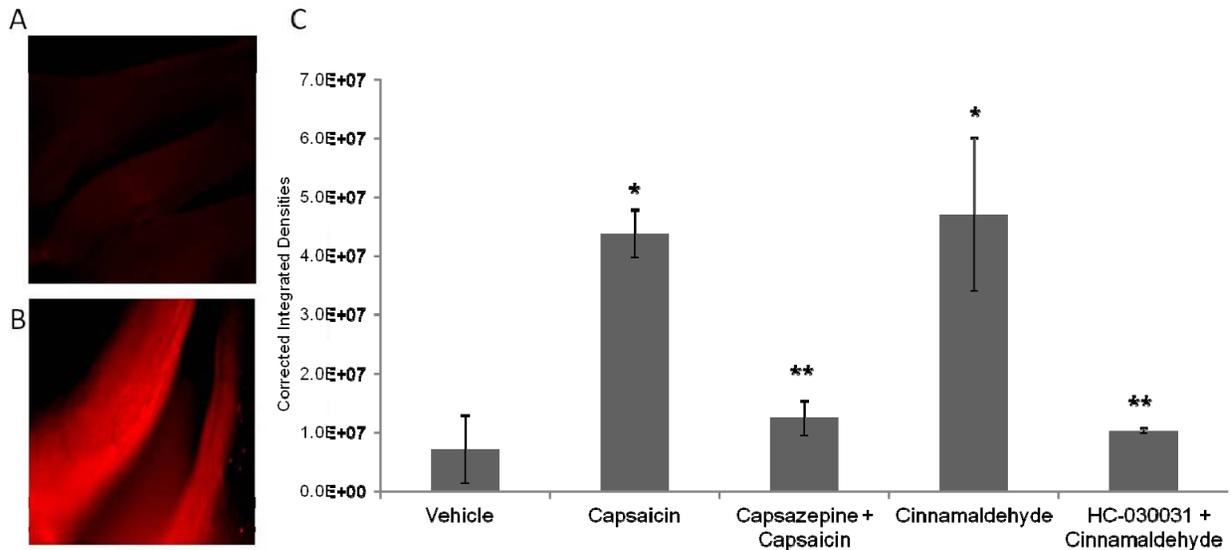


Figure 2.4. Activation of irritant-sensing TRP channels activates a pro-inflammatory pathway and initiates plasma extravasation. To identify areas of plasma leakage, adult mice aspirated saline vehicle, capsaicin (2 μ M), or cinnamaldehyde (100 μ M) into right nasal cavity. Some mice were pre-treated with TRPV1 antagonist capsazepine (10 μ M) or TRPA1 antagonist HC-030031 (100 μ M) 10 minutes before above agonists. 5 minutes after capsaicin or cinnamaldehyde aspiration, Alexaflour-555 conjugated albumin was administered via tail vein injection. Fluorescence images of whole mounts of hemisected nasal cavity treated with (A) vehicle or (B) agonist were converted to 16-bit black and white images and then quantified using ImageJ software. (C) Aspiration of capsaicin or cinnamaldehyde caused a significant increase in plasma extravasation compared to saline control (*, $p < 0.05$). Treatment with the TRPV1 antagonist, capsazepine (10 μ M) or TRPA1 antagonist, HC-030031 (100 μ M), before aspiration of capsaicin or cinnamaldehyde (respectively), significantly reduced subsequent plasma extravasation (**, $p < 0.05$ compared to agonist) to levels similar to control (saline).

fluorescence and thereby, inflammation, is initiated through the activity of TRPV1 and TRPA1, as pretreatment with the TRPV1 antagonist capsazepine and TRPA1 antagonist HC-030031 does not result in an increase in fluorescence compared to control, and was significantly lower than fluorescence levels of the agonist only groups ($p < 0.05$).

Discussion

The studies of this chapter suggest that the activation of trigeminal fibers in the mouse OE can occur both directly, through expression of irritant sensing channels TRPA1 and TRPV1, and indirectly, through the innervation of microvillous secondary chemosensory cells. I demonstrated that TRPA1 and TRPV1 are found more extensively than only on trigeminal fibers. TRPA1 is also found on TRPM5-MVs and TRPV1 on OSNs, suggesting that these cell types may have a role in modulation of olfactory responses to irritants, or conversely, that irritants can modulate responses to odorants (Schaefer et al., 2002). The activation of these channels leads to the release of inflammatory neuropeptide sub P, as shown through the use of TRPA1 agonist cinnamaldehyde and TRPV1 agonist capsaicin, and downstream effects of inflammation are initiated, such as plasma extravasation (Figure 2.5).

Localization of TRP channels

The IHC studies of this chapter suggest neurogenic inflammation can be initiated by a number of mechanisms and is not restricted to direct activation of trigeminal fibers. I demonstrated TRPA1 and TRPV1 labeling on secondary chemosensory non- neuronal

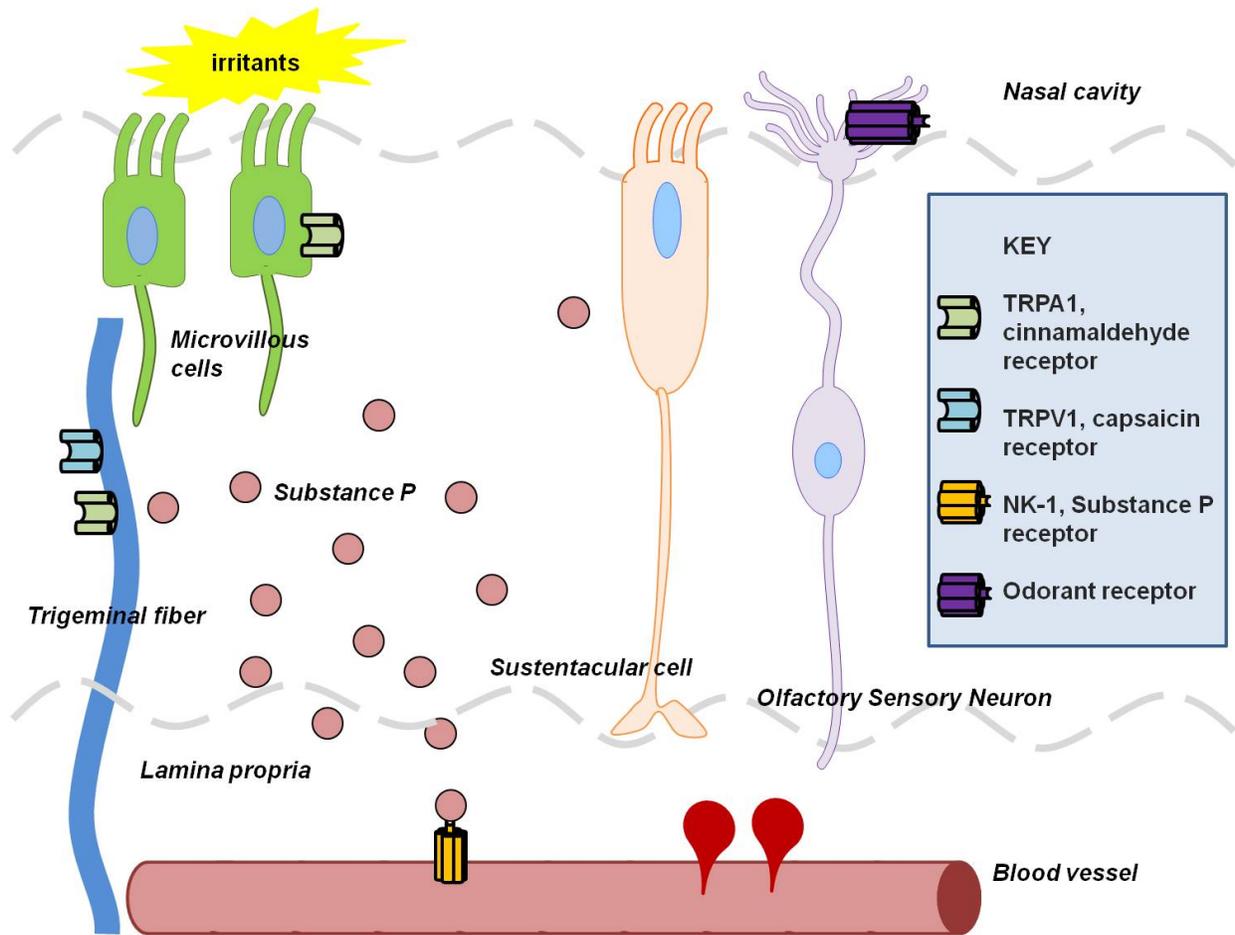


Figure 2.5. Secondary chemosensory cells, trigeminal fibers, and TRP channels in initiating neurogenic inflammation in the mouse OE. TRPA1 and TRPV1 are found on trigeminal fibers and microvillous cells and their activation can cause substance P release, leading to plasma extravasation.

cells such as TRPM5-MVs, and on putative nerve fibers contacting secondary chemosensory cells, such as the IP3R3-MV. Trigeminal fibers always stop short of the line of tight junctions along the apical surface of the tissue, preventing direct contact with some environmental irritants. Thus, innervation of secondary chemosensory cells can extend the activation of trigeminal fibers beyond lipophilic compounds that can descend into the epithelium and contact trigeminal fibers. This relationship between trigeminal fibers and secondary chemosensory cells has been described for SCCs in the respiratory epithelium, which also form synaptic contacts with trigeminal nerve fibers and contribute to nasal trigeminal sensitivity (Finger et al., 2003; Gulbransen et al., 2008b). TRPM5-MVs are responsive to bitter compounds, ATP, and acetylcholine (Gulbransen et al., 2008a). IP3R3-MVs have also been shown to be responsive to ATP. Additionally, compounds that stimulate olfactory responses from OSNs and do not traditionally activate TRP channels can become irritants at high concentrations, such as amyl acetate at a concentration 4000 higher than concentrations that elicit an odorant response (Tucker, 1971).

Our finding of TRPV1 and TRPA1 protein expression in the OE is supported by a recent study in which immunoreactivity toward several TRP channels was described in mouse OE (Nakashimo et al., 2010). This previous study described TRP-IR in relation to OSNs, with no analysis of immunoreactivity in non-neuronal cells or innervations, structures which have important roles in sensing the olfactory environment. Therefore, my study adds to the understanding of what cell types may be involved in trigeminal activation and olfactory-trigeminal interactions. Though I do not currently understand the function of this activity, I have shown that multiple cell types are responsive to

TRPA1 and TRPV1 agonists capsaicin and cinnamaldehyde. It is interesting that mRNA toward TRPA1 and TRPV1 was found within OE tissue, as the cell bodies of trigeminal fibers are found outside of the tissue, in the trigeminal ganglia. Some of this mRNA is likely due to cell types found to express TRPA1 and TRPV1 protein. However, mRNA can also be localized outside of the cell body (Mohr et al., 2001), as in the creation of dendritic spines involved in long term potentiation (Doyle and Kiebler, 2011).

Release of inflammatory neuropeptide

I demonstrated that application of TRPV1 agonist capsaicin and TRPA1 agonist cinnamaldehyde results in release of sub P. Other studies have demonstrated the initiation of neurogenic inflammation mediated through sub P innervation of the OE (Saunders et al., 2014), but to my knowledge, there has not been direct demonstration of sub P release from the mouse OE as demonstrated here. Though it is expected that capsaicin treatment would cause a release of sub P from peptidergic sensory fibers, it was important to demonstrate the initiation of neurogenic inflammation through a TRPA1 mechanism as well. Andre and colleagues demonstrated that capsaicin-sensitive sensory neurons were activated by α,β -unsaturated aldehydes, found in cigarette smoke, and caused neurogenic inflammation in the form of bronchi constriction and elevated intracellular calcium levels in guinea pig airway (Andrè et al., 2008). In their study, inflammation was not decreased with use of TRPV1 antagonist, capsazepine, but was decreased with TRPA1 antagonist glutathione, suggesting tissue differences in activation of inflammation. My studies showed that TRPA1 and TRPV1 classical agonists cinnamaldehyde and capsaicin, respectively, cause changes in

intracellular calcium, sub P release, and plasma extravasation; it would be interesting to follow up on these studies using other irritants. For example, our lab has worked with environmental irritants satratoxin G and nickel sulfate. I have shown that there are olfactory deficits in mice upon exposure to these toxicants (Figure S1 in Appendix), and I have also measured sub P release from neonatal OE slices upon satratoxin G exposure (Figure S2 in Appendix). Whether these toxicants and particulate matter cause changes in intracellular calcium, sub P release, and plasma extravasation also and whether the effects are through TRPA1 or TRPV1 activity should be investigated in the future.

Neurogenic initiation of inflammation

Plasma extravasation is a symptom of inflammation that causes the classical symptoms of inflammation, redness, warmth, and swelling. Sub P was shown to be a major initiator of plasma extravasation (Saunders et al., 2014). However, there are other sources of sub P, such as immune cells, and thus, activation of immune cells could cause the release of sub P and subsequent plasma extravasation. However, only low levels of immune cells are resident to the OE (discussed in chapter 4). Sub P was measured within an hour of irritant application, and therefore not within the normal timeframe of immune cell infiltration. Histamine release from local mast cells is also linked to plasma extravasation. In these studies, I decreased plasma extravasation with the use of TRPV1 and TRPA1 antagonists, and thus I demonstrated the neurogenic aspect of inflammatory activation, i.e., release of sub P from trigeminal fibers.

Limitations of study

Innervation of the OE

In this current study, I focused specifically on trigeminal fibers because they have a known role in inflammation and pain transduction, but I cannot rule out a possible role for the terminal nerve in neurogenic inflammation of the OE. The terminal nerve is found in all vertebrate animals save for cartilaginous fish, and is located around blood vessels and mucosal glands. Fibers enter the vomeronasal epithelium, but it is unclear whether they enter the main OE. Terminal nerve fibers can be differentiated from other innervations of the nasal cavity due to the presence of luteinizing hormone-release hormone (LHRH), as trigeminal, olfactory, and vomeronasal nerves are not immunoreactive toward LHRH. Some terminal fibers are immunoreactive toward acetylcholinesterase, and these rarely co-localize with LHRH fibers. There is no immunohistochemical evidence of acetylcholinesterase or LHRH immunoreactive fibers in the OE. However, placing a tracer in the OE leads to back labeling of terminal nerve ganglion cells (reviewed in (Farbman, 1992)). Thus, terminal nerve innervation of the OE cannot be ruled out.

Calcium imaging experiments and receptor functionality

In live-cell calcium imaging experiments, increases in intracellular calcium were measured in both neuronal and non-neuronal cells to both capsaicin and cinnamaldehyde. Response to capsaicin in OSNs is supported by our IHC, which indicated extensive TRPV1 labeling, including the neuronal layer of the OE. However, our IHC study did not indicate TRPA1 expression on OSNs. Cinnamaldehyde activated

non-neuronal cells at higher concentrations. Thus, there may be concentration dependent activation of odorant receptors and TRP channels. Furthermore, there were occasional increases in latency in responses to both cinnamaldehyde and capsaicin. I hypothesize that some of the responses to cinnamaldehyde and capsaicin noted at later time points may have actually been responses to sub P, which would have been released subsequent to activation of trigeminal fibers by capsaicin and/or cinnamaldehyde. To test this, I first determined that cinnamaldehyde was causing changes in intracellular calcium in some cells through the activation of TRPA1 by pre-treating tissue with the TRPA1 antagonist HC-030031. This resulted in the abolishment of responses, confirming that there are responses to cinnamaldehyde due to the activation of TRPA1 in the mouse OE (Figure S3 in Appendix). Next, I would need to pre-treat tissue with the NK-1R antagonist, L-732-138, and then apply cinnamaldehyde, to see if the latent responses are removed, which would indicate that the latent responses were indeed due to sub P release and activation of cells with NK-1.

Other neuropeptides in the olfactory mucosa

In this study, I have chosen to focus on sub P because of its proven role in inflammation, especially through the initiation of plasma extravasation (Saunders et al., 2014). Trigeminal fibers contain both CGRP and sub P and are likely co-transmitted (Stjärne et al., 1989; Maggi, 1995), though there appear to be more sub P-IR fibers in the OE (Hunter and Dey, 1998). Additionally, neuropeptide content can change depending on inflammatory state (Donnerer et al., 1992; Sikora et al., 2003). The olfactory system also contains vasoactive intestinal peptide (Miller et al., 2014) and

neurokinin-A, and future studies should elucidate the role these peptides may very well also have in neurogenic inflammation.

Conclusion

Environmental irritants enter the nasal cavity and can contact the OE, and through activation of trigeminal fibers, can potentially activate acute inflammation in the OE. This has distinct implications for human health, especially for residents of areas stricken with high levels of pollution or even allergens. Inflammation needs to be tightly regulated in order to not turn into chronic inflammation, and in the OE, must be abated to allow for regeneration of neurons (Lane et al., 2010; Sultan et al., 2011).

Furthermore, compromised nasal tissue allows easier bacterial access to the olfactory bulb, an aspect of the central nervous system (Herbert et al., 2012). While the OE can come into contact with damaging or irritating agents on a regular basis, olfactory dysfunction is not a common occurrence, and regenerative capabilities are typically maintained. This study provides information on how inflammation can be initiated by environmental factors. Future studies are needed in order to understand the regulation of inflammation in this interesting neuroepithelium. By learning how acute inflammation is initiated in the mouse OE, we may be able to understand what is changed or disrupted in chronic inflammation, which can protect us from the adverse effects of chronic inflammation, or prevent the occurrence of chronic inflammation altogether.

Chapter 3- Elucidating signal transduction pathways involved in secondary chemosensation in IP3R3-containing microvillous cells

Introduction

The sensation of smell arises from the detection of odorants by olfactory sensory neurons (OSNs) of the olfactory epithelium (OE), but this tissue is also capable of detecting irritants. Irritant detection in the nasal cavity is primarily signaled to the brain by trigeminal fibers that innervate the OE (Silver et al., 1986; Hunter and Dey, 1998). The sensitivity of trigeminal fibers can be expanded by cells with a chemosensory function, as seen in the relationship between trigeminal fibers and solitary chemosensory cells (SCCs) in the respiratory epithelium (Gulbransen et al., 2008a). We recently described a microvillous cell present in the OE distinguishable due to its expression of the type 3 receptor for inositol 1,4,5 triphosphate (IP3R3), which henceforth will be referred to as IP3R3-MV. This cell responds to purine adenosine triphosphate (ATP), neurokinin substance P (sub P), and a mixture of odorants. The IP3R3-MV is also occasionally innervated by trigeminal fibers (Hegg et al., 2010). Thus, a chemosensory function for this cell type is suggested.

The responses to ATP in the IP3R3-MV are mediated by the ionotropic purinergic receptor P2X3, as expression of this receptor by IP3R3-MV was previously shown (Hegg et al., 2010). Responses to odorants were also reported. In OSNs, odorant binding causes the release of GTP-coupled G α (olf) (Jones and Reed, 1989), which then stimulates adenylyl cyclase III (ACIII) to produce cyclic adenosine monophosphate (cAMP) (Pace et al., 1985). The increased level of cAMP causes the opening of cyclic

nucleotide-gated (CNG) channels, which allows the influx of cations (Figure 3.1). However, the IP3R3-MV does not express the canonical odorant transduction components $G\alpha(\text{olf})$, CNGA2, or ACIII, nor does it express olfactory marker protein (OMP) (Hegg et al., 2010). There are other microvillous cells present in the OE that express a variety of signal transduction components, including transient receptor potential cation channel subfamily M member 5 (TRPM5) (Lin et al., 2008; Hansen and Finger, 2008), phospholipase C beta-2 (PLC β 2), and TRP C6 (Elsaesser et al., 2005). Thus, it is possible that the IP3R3-MV carries out signal transduction components that are outside of the canonical odorant transmission mechanisms.

In this chapter, I utilize pharmacological blockers of specific factors in signal transduction (Figure 3.1 and 3.2). I utilized GDP β s to block the activity of GPCRs. GDP β s is a non-hydrolyzable analogue of GDP that competitively inhibits the binding of guanine nucleotides to G proteins. G protein coupled receptors remain inactive until agonist binding. In this inactive state, the α subunit and the $\beta\gamma$ complex are associated. Upon agonist binding to an extracellular domain, the receptor undergoes a conformational change that catalyzes the exchange of GDP for GTP on the α subunit. GTP-bound $G\alpha$ subunit and the $\beta\gamma$ complex then dissociate and activate downstream effectors. When the agonist dissociates and GTP is hydrolyzed back to GDP, the $G\alpha$ subunit and $\beta\gamma$ complex associate once again, which terminates the signaling and brings the receptor back to the inactive state. Thus, with a non-hydrolyzable form of GDP, ie GDP β s, GDP cannot be exchanged for GTP, and the receptor stays in the inactivated state even in the presence of an agonist. One downstream target of GPCR activation is AC. Forskolin is a pharmacological agent that activates AC, and so the

application of forskolin would increase intracellular levels of cAMP. After the conversion of ATP to cAMP, cAMP is converted to AMP by phosphodiesterase (PDE). 3-isobutyl-1-methylxanthine (IBMX) is an inhibitor of PDE, and thus its use maintains levels of cAMP inside cells. Another downstream target of GPCRs is PLC. The enzyme PLC then cleaves the phosphate group from phosphatidylinositol 4,5 diphosphate (PIP₂), creating IP₃ and diacylglycerol (DAG). To block the involvement of internal stores of calcium gated by IP₃, I used 2-APB. 2-APB is rapidly cell permeant. The exact mechanism by which 2-APB antagonizes IP₃R3-calcium release is under debate. There is evidence 2-APB blocks calcium release from internal stores, but there is also evidence that it can inhibit store-operated channels without the involvement of IP₃R3, as 2-APB has been found to be effective at blocking cation entry in drosophila photoreceptors, a cell type that does not express IP₃R3s (Bootman et al., 2002).

As mentioned previously, the IP₃R3-MV is occasionally contacted by trigeminal fibers, and the IP₃R3-MV has been shown to be responsive to sub P, a neuropeptide found in trigeminal nerve fibers in the OE. This suggests that the IP₃R3-MV plays a role in irritant or pain transduction. Trigeminal fibers express irritant-sensing channels to respond to irritants in the environment, such as transient receptor potential-Vanniloid-1 (TRPV1) and TRP-ankyrin-1 (A1) (Silver et al., 2006; Kim et al., 2010). Trigeminal fibers have been found to contact secondary chemosensory cells, such as the SCC of the respiratory epithelium of the nasal cavity, which is thought to aid in expanding the range of molecules to which the trigeminal fiber responds (Gulbransen et al., 2008b, 2008a).

Currently, a role as a secondary chemosensory cell is suggested for the IP3R3-MV, but it is not known what signal transduction elements are utilized in these responses. In this study, I set out to determine signal transduction pathways of the IP3R3-MV in odorant and purinergic signaling. This study utilizes a transgenic mouse in which the expression of green fluorescent protein (GFP) is driven by the promoter for IP3R3, allowing for identification of IP3R3-MV. Using pharmacological agents to manipulate various signal transduction pathways, I demonstrated that purinergic responses occur not only through the ionotropic P2X3 receptor, but also via metabotropic P2Y receptors. Responses to odorants were verified to not involve ACIII activation, but do appear to be G protein activated. The expression of a receptor for sub P, NK-1, was also present on IP3R3-MV, and occasional contact with structures containing irritant-sensing channels TRPA1 and TRPV1 suggest a role for the IP3R3-MV in irritant signaling.

Materials and Methods

Animals

Throughout this study, IP3R3^{tm1(tauGFP)} transgenic mice in which GFP is expressed in cells containing IP3R3 were used (Hegg et al., 2010). Briefly, the first exon of the *Itpr3* gene was replaced by the coding region for a fusion protein of tau and GFP (tauGFP), three polyadenylation sites (3xpA), and a floxed neomycin gene. IP3R3^{tm1(tauGFP)} mice were then crossed with actin-CRE mice (in C57BL/6 background), and then all subsequent crosses were with C57BL/6 mice. Expression is bi-allelic such that IP3R3-heterozygous (IP3R3⁺/IP3R3⁻ tauGFP⁺) mice express both IP3R3 and GFP,

and the IP3R3-knockout (IP3R3⁻ tauGFP⁺/IP3R3⁻ tauGFP⁺) mice express GFP but have non-functional IP3R3.

Mice of both sexes, postnatal 0–5 days of age, were used in physiological experiments. Tissue from adult mice aged 6-8 weeks were used in immunohistochemical studies. All animals were bred and housed in the animal facilities of Michigan State University and procedures were in compliance with Michigan State University's Institutional Animal Care and Use Committee.

Polymerase Chain Reaction (PCR)

Presence of GFP, IP3R3, and Cre were determined with PCR using Red-Extract-N-Amp following manufacturer's instructions (Sigma-Aldrich). Tissue was digested in Extraction solution, Tissue preparation solution, and Neutralization buffer from PCR kit. Digested tissue was added to primer oligonucleotides (Invitrogen, Carlsbad, CA, USA) and PCR Reaction mix from kit, with samples for each primer run separately. The mixture is then placed in a thermocycler. The thermocycling program consisted of stages of initial denaturation at 94°C for 3 minutes followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 68°C for 1 minute, extension at 72°C for 2 minutes. Final extension lasts for 10 minutes at 72°C and then samples are cooled to 4°C. mRNA products were separated by size in a 1.5% agarose gel containing ethidium bromide using 100V for 1 hour alongside a DNA ladder. Products were visualized with UV light.

mRNA	Primer	Sequence	Product size (bp)
IP3R3	5'	GGTGAGTGAGCCTAGGGCAAAGAGA	1142
	3'	TCTTCTCCAAGCATCCTCCAGGC	
GFP	5'	TTCAAGGACGACGGCAACT	417
	3'	ACTTGTACAGCTCGTCCATGC	
Cre	5'	GCTGGTTAGCACCGCAGGTGTAGAG	421
	3'	CGCCATCTTCCAGCAGGCGCACC	

Table 3.1. Primers used in genotyping of transgenic mice

Confocal live cell calcium imaging

Neonatal mice were decapitated and their skin and lower jaw were removed. The head was embedded in a block made of carrot and mounted onto a vibratome. Tissue was kept in ice-cold Ringer's solution while sectioned into 400 μ m slices. Ringer's solution contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 10 mM HEPES, 10 mM glucose, and 500 μ M Probenecid (pH 7.4, 290–320 mOsm). Slices were incubated with 50 μ g/mL calcium indicator dye X-Rhod 1 AM (Molecular Probes) made in Ringer's solution with 0.00005% pluronic F-127 (Invitrogen) and 0.0001% DMSO for 60 min at room temperature.

Tissue slices were placed in a laminar flow chamber (Warner Instruments, Hamden, CT, USA) and perfused continuously with Ringer's solution at a flow rate of 1.5–2.0 mL/min. Test solutions were applied using bath exchange and a small-volume loop injector (200 μ L). Our perfusion system exchanges the bath in *ca.* 7–10 s and traces were not corrected for this delay. Using a FV1000 confocal microscope

(Olympus America Inc., Center Valley, PA, USA), images were collected for data analysis. A krypton–argon ion laser excited GFP at 488 nm and a helium–neon laser excited X-Rhod-1 AM at 543 nm. Fluorescence emissions were filtered at 505–525 and 560–660 nm, respectively. Along with a 0.80 NA water objective, IP3R3 MV cells were visualized and changes in calcium levels measured simultaneously. However, to prevent cross talk of fluorescence emission, the 488 laser line was turned off after capturing the second frame.

To determine the response profiles of IP3R3-MV cells to bioactive compounds, an odorant mixture (10 μ m each of cineole, octanol, heptanol, isoamyl acetate and r-carvone; Sigma Aldrich, St. Louis, MO), ATP (5 mM in Ringer's solution, Sigma Aldrich, St. Louis, MO), sub P (500 nM in Ringer's solution, Sigma Aldrich, St. Louis, MO), the adenylyl cyclase activator Forskolin (2 mM in Ringer's solution, Sigma Aldrich, St. Louis, MO), or the PDE inhibitor 3-isobutyl-1-methylxanthine (IBMX; 100 mM in Ringer's solution, Sigma Aldrich, St. Louis, MO) were superfused individually with the loop injector, and fluorescence was recorded. The number of applications performed on any slice was limited to five due to the observation of calcium rundown and photobleaching following more than five applications. Following application of bioactive compound, five minutes of Ringer's alone was perfused over the slice, and then one of four antagonists was applied. The non-hydrolyzable G protein blocker guanosine 5'-[β thio]diphosphate trilithium salt (GDP β S, 100 mM; Sigma Aldrich, St. Louis, MO) was incubated with the tissue slice for 20 minutes without solution flowing. The IP3 receptor inhibitor, 2-aminoethoxydiphenyl borate (2-APB; 100 μ M, Cayman Chemical, Ann Arbor, MI) made in Ringer's solution was continuously superfused over the slice for 10 minutes. Finally,

five minutes of Ringer's only perfusion was performed before application of a bioactive compound to measure recovery of calcium transients. To be included in the data set, the peak amplitude of the recovery calcium transient had to be at least 75% of the initial transient amplitude. All data were normalized to the initial calcium transient. Paired Student's t-tests were used to determine significant differences ($p < 0.05$) between the antagonist peak amplitude and the recovery of agonist peak amplitude. This was not done in the case of GDP β s treatment, as its non-hydrolyzable nature does not allow for a recovery measurement. The fluorometric signals obtained were expressed as relative fluorescence (F) change, $\Delta F/F = (F - F_0)/F_0$, where F_0 is the baseline fluorescence level (mean F of first 5 frames). Data were collected from a minimum of three slices for each experiment.

Immunohistochemistry (IHC)

Anesthetized mice (65 mg/kg ketamine+5 mg/kg xylazine, intraperitoneally) were transcardially perfused with 0.1 M PBS followed by 4% PFA, then decapitated and post-fixed in 4% PFA overnight. Heads were decalcified for 5 days with EDTA (0.5M, pH=8), cryoprotected overnight in 30% sucrose solution, frozen, and then sectioned with a HM525 cryostat (Microm International, Waldorf, Germany) while embedded in TissueTek Optimal Cutting Temperature compound (Sakura Finetek USA, Torrance, CA). Slices were collected onto positive-charged slides and rehydrated with PBS, permeabilized with 0.02-0.03% Triton X-100 and blocked with 1% normal goat serum. The sections were incubated with primary antibody overnight, followed by cy3 or TRITC-conjugated anti-rabbit immunoglobulin (1:200, Jackson Immunoresearch Lab, West

Grove, PA, USA). Slides were mounted with Vectamount (Vector Labs, Burlingame, CA). Immunoreactivity was visualized on an Olympus FV1000 Confocal laser scanning microscope (Olympus America Inc., Center Valley, PA, USA) with Cy3 excited at 543nm and TRITC at 532 nm. Antibody specificity was examined by omitting the primary antibody or secondary antibody. No immunoreactivity was observed in any of the controls (data not shown).

Antibody	Target	Concentration	Manufacturer
Rabbit α -P2Y2	Metabotropic purinergic receptor	1:200	Alomone; Jerusalem, Israel
Rabbit α -NK-1	Sub P receptor neurokinin 1	1:1000	Millipore; Billerica, MA
Rabbit α -TRPV1	Irritant-sensing channel TRPV1	1: 2000	Abcam; Cambridge, England
Rabbit α -TRPA1	Irritant-sensing channel TRPA1	1: 1000	Abcam; Cambridge, England

Table 3.2. Antibodies used in immunohistochemical studies

Results

The IP3R3-MV exhibits increases in intracellular calcium in response to odorants, ATP, and sub P, yet it does so without the canonical odorant signaling machinery, ACIII, $G\alpha(olf)$, and OMP (Hegg et al., 2010). This study investigates the signaling mechanisms of the IP3R3-MV by utilizing pharmacological agents directed to various signaling pathways in the IP3R3^{tm1(tauGFP)} mouse (Figure 3.1 and 3.2). I also utilized IHC to confirm the presence of purinergic and neurokinin receptors.

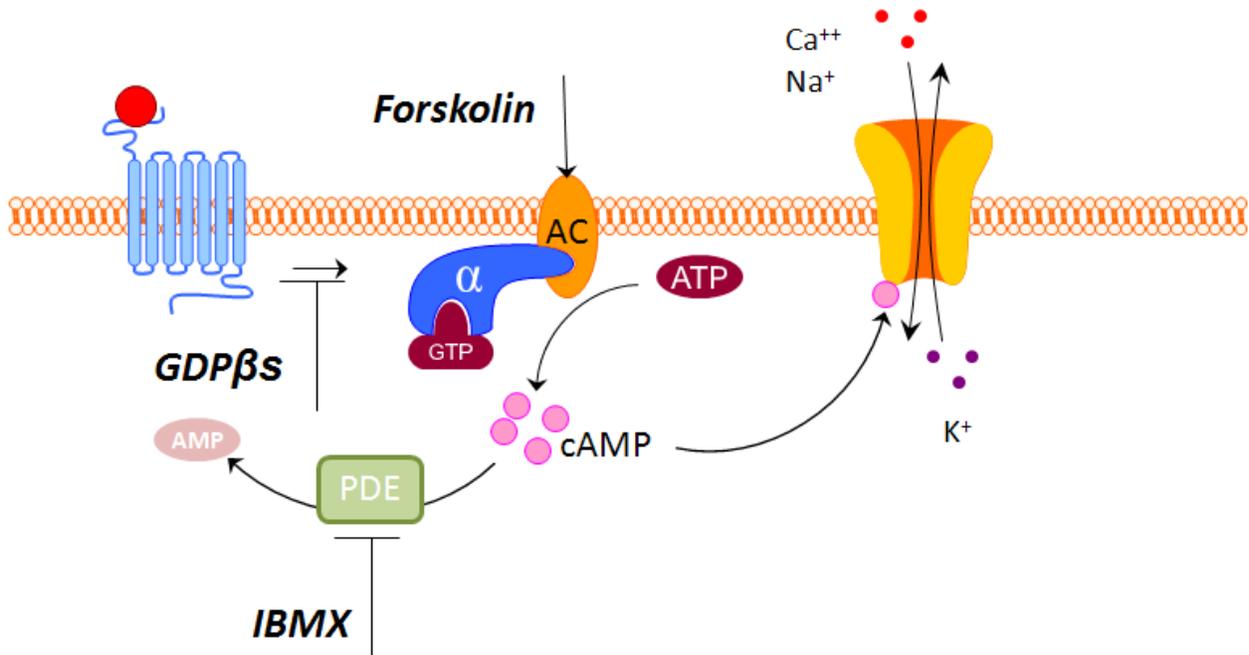


Figure 3.1. cAMP pathway with pharmacological manipulations. Odorant binding to a GPCR causes the release of GTP-coupled G α (olf), which then stimulates ACIII to produce cAMP. The increased level of cAMP causes the opening of CNG channels, which allows the influx of cations. GDP β s is a non-hydrolyzable competitive analog of GDP used to block G protein couple receptors. Forskolin is an activator of ACIII, the activation of which would cause an increase in cAMP. IBMX blocks the conversion of cAMP to AMP, the use of which would keep cAMP available to active CNG channels.

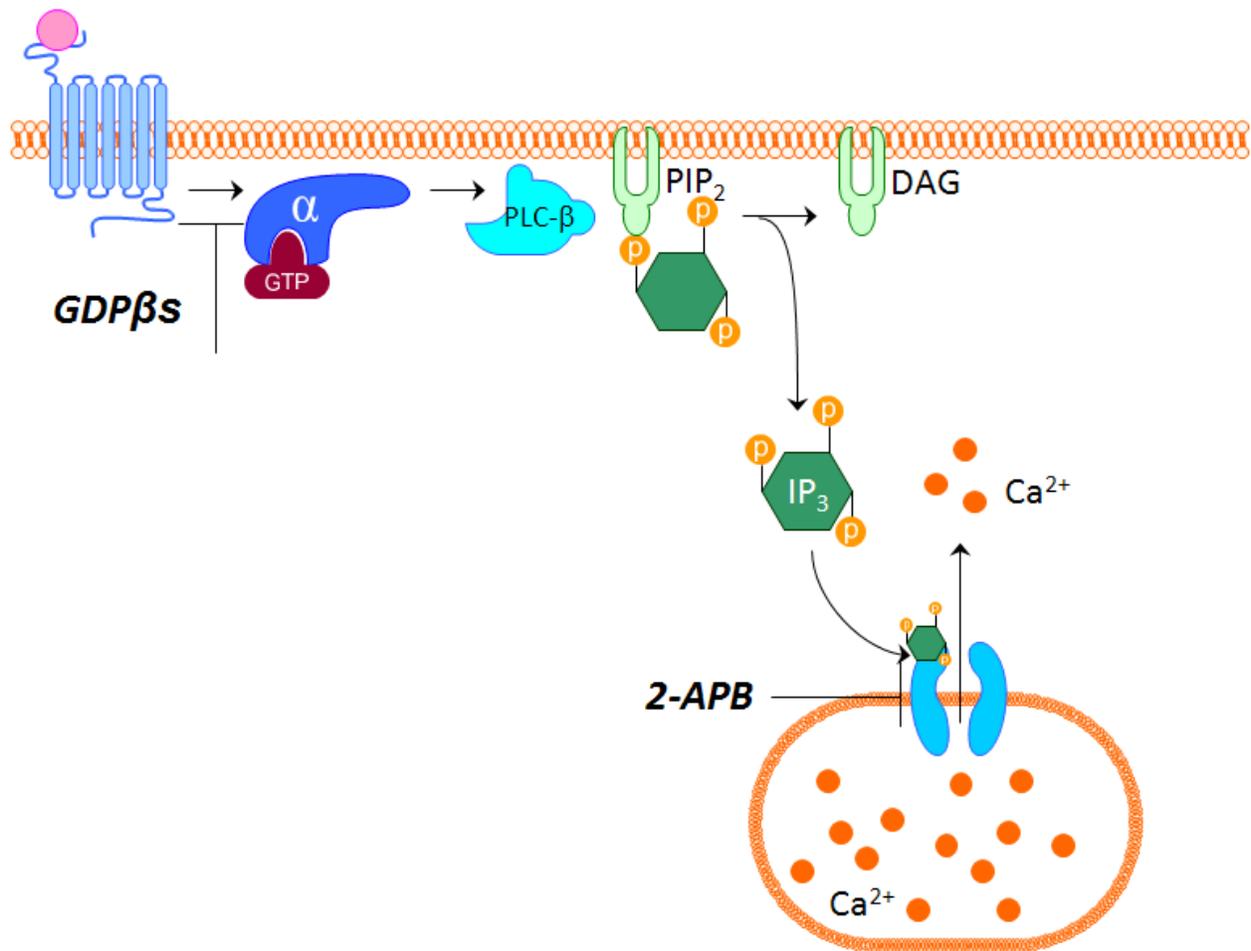


Figure 3.2. IP₃ pathway with pharmacological manipulations. Ligand binding to a GPCR causes the release of GTP-coupled G α (q/11), which then stimulates PLC β to cleave PIP₂, forming DAG and IP₃. Receptors gated by IP₃ are activated on intracellular stores of calcium, allowing the release of calcium into the intracellular space. **GDP β s** is a non-hydrolyzable competitive analog of GDP used to block G protein couple receptors. **2-APB** is an antagonist of IP₃R₃, the use of which blocks the release of internally stored calcium.

Mechanisms of odorant signaling in IP3R3-MV cells

Forskolin (F) increases the activity of ACIII, thereby increasing cAMP levels. IBMX inhibits PDE activity, thereby inhibiting the breakdown of cAMP and causing its accumulation (Figure 3.1). Because ACIII and PDE are not present in the IP3R3-MV, the application of F/IBMX was not expected to elicit changes in this microvillous cell. As expected, simultaneous application of these drugs to OE slices evoked significantly larger increases in intracellular calcium in OSNs compared to IP3R3-MV ($p < 0.05$) (Figure 3.3 A, B). Responses to the odorants mixture also caused significantly larger increases in intracellular calcium measurements in the IP3R3-MV than the FIBMX mixture ($p < 0.05$) (Figure 3.3C). Binding of odorants to G protein-coupled receptors (GPCR) on OSNs is the initial step in odorant signaling activity. To determine whether GPCRs mediate responses to odorants in the IP3R3-MV, the non-selective G protein inhibitor GDP β s was used. Responses to odorant mixture were reduced by 32%, a significant reduction ($p < 0.05$), in the presence of GDP β s in 16 of 21 IP3R3-MV cells that were previously responsive to odorants (Fig.3.3D). Note that in 5 out of 21 total cells, there was no effect of GDP β s on peak amplitude of the odorant response, but they could not be excluded from the analysis based on Grubb's outlier test.

Mechanisms of purinergic signaling in IP3R3-MV cells

We previously demonstrated that IP3R3-MV cells respond to ATP with increases in intracellular calcium, and these transients are significantly decreased, but not abolished, in calcium free solution. Furthermore, most IP3R3-MVs express the ionotropic P2X3 receptor (Hegg et al., 2010), which when activated would allow

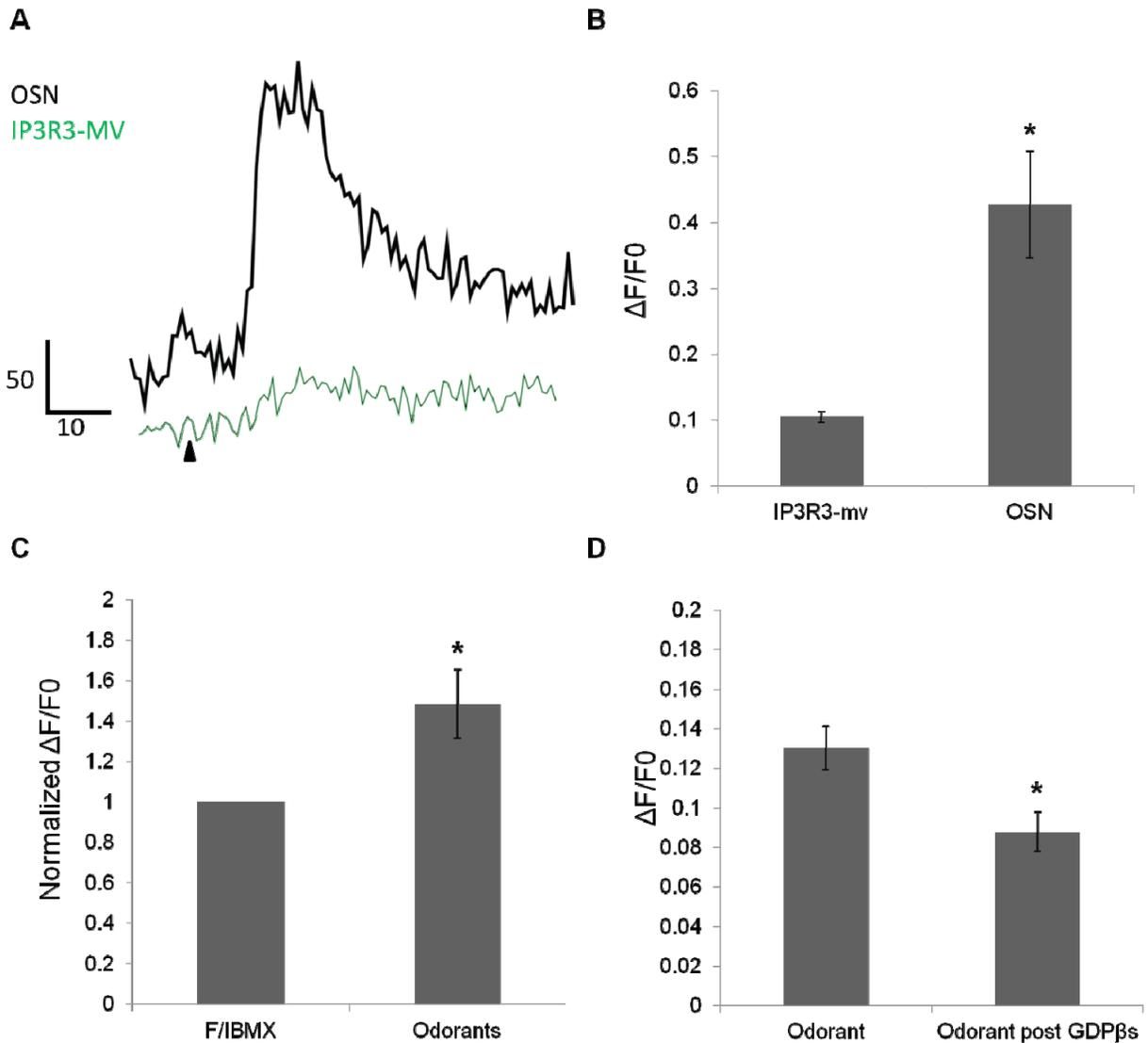


Figure 3.3. IP3R3-MV responses to odorants are not mediated by a cAMP pathway but involve GPCRs. (A) Representative Ca^{2+} traces of responses to Forskolin and IBMX mixture (F/IBMX). \blacktriangle indicates application of F/IBMX. (B) Average peak amplitude of intracellular Ca^{2+} increases in IP3R3-MV cells (n=44 cells) and OSNs (n=14 cells) in response to F/IBMX. (C) Exposure to odorant mixture elicited significantly larger responses from IP3R3-MVs compared to F/IBMX solution (n=21). (D) GDPβs treatment decreased the odorant-induced Ca^{2+} transients in most IP3R3-MV cells that were responsive to odorants (16 of 21). * $p < 0.05$, Student's t-test.

entrance of cations into the cell. To determine whether the increase in calcium upon ATP stimulation in the IP3R3-MV also involves IP3 activation of intracellular calcium stores, slices were incubated with the IP3 receptor antagonist, 2-APB (Figure 3.2). The ATP-evoked response was decreased 35% by 2-APB, a significant reduction ($p < 0.05$) from the initial and recovery calcium measurements (Figure 3.4A). The release of calcium from internal stores suggests the activity of metabotropic ATP receptors. Thus, GDP β s was applied to block metabotropic receptor G protein activation, and caused a decrease in ATP-induced calcium transients in 13 of 19 IP3R3-MV, a significant reduction of 48% ($p < 0.05$) from initial, pre-GDP β s application (Figure 3.4B). Uridine 5'-triphosphate (UTP) is an agonist for metabotropic purinergic receptors. Its application resulted in robust increases in intracellular calcium (Figure 3.5A). These responses were significantly ($p < 0.05$) reduced with the application of GDP β s (Figure 3.5B).

Irritant signaling

Trigeminal fibers are known to release ATP and sub P, and the IP3R3-MV responds with increases in intracellular calcium to both of these factors. Immunoreactivity toward P2Y2, a metabotropic purine receptor, is found mostly in the apical layer of the OE where the cell bodies of IP3R3-MVs and sustentacular cells are found (Figure 3.6A). Immunoreactivity toward TRPV1 and TRPA1 indicated structures in close apposition to the IP3R3, most likely trigeminal fibers, though expression of TRPV1 could be on the IP3R3-MV itself (Figure 3.6 B and C). Immunoreactivity toward NK-1, the primary receptor for sub P, was also found on the IP3R3-MV (Figure 3.6D).

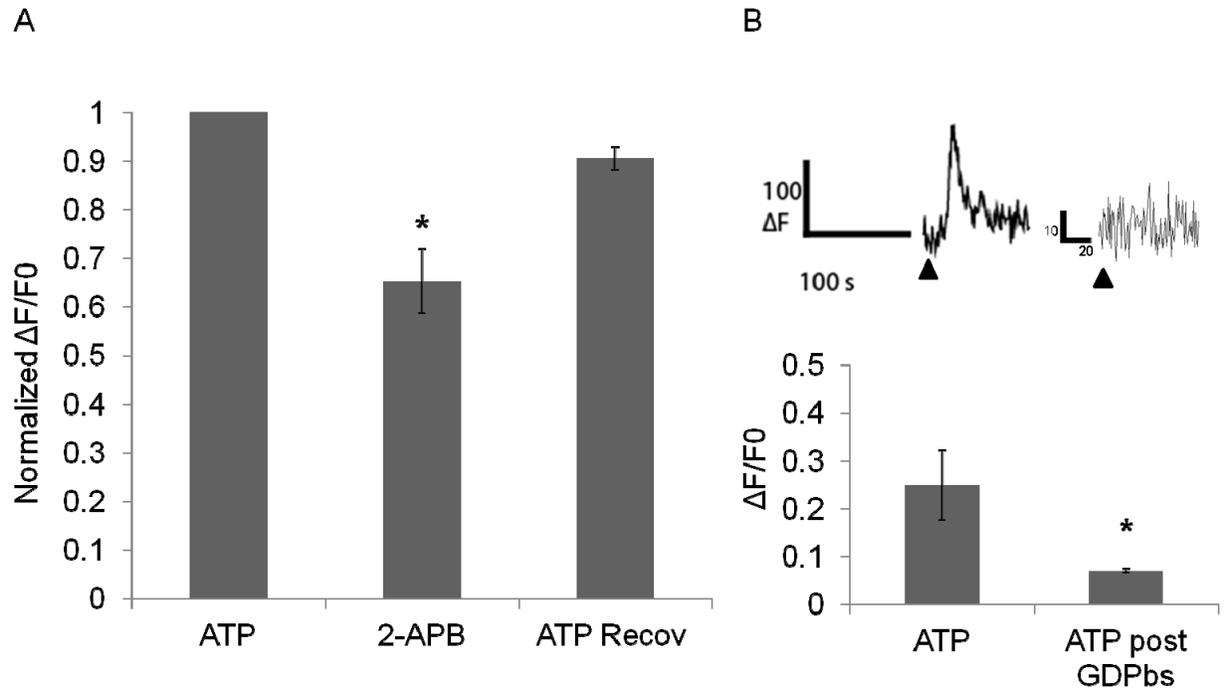


Figure 3.4. Increases in intracellular calcium from IP3R3-MVs involve internal calcium sources and G protein-coupled receptors. (A) Application of ATP and IP3 receptor blocker 2-APB to OE slices. 2-APB (100 μ M) application resulted in a 35% decrease in ATP-induced calcium transients (n=14 cells, 5 slices) in IP3R3-MV cells. Washout of 2-APB and application of ATP (ATP recovery) resulted in calcium levels similar to initial responses to ATP without the presence of 2-APB. Note that to be included in the data set, the peak amplitude of the ATP recovery calcium transient had to be at least 75% of the initial transient amplitude. All data were normalized to the initial calcium transient. (B) GDP β s pretreatment led to a $48 \pm 0\%$ decreased in ATP-evoked calcium transients in 13 of 19 IP3R3-MV cells of neonatal mice (n= 3 slices). Representative traces from IP3R3-MV cells in response to ATP before and after GDP β s incubation are presented above graph. * $p < 0.05$, Student's t-test.

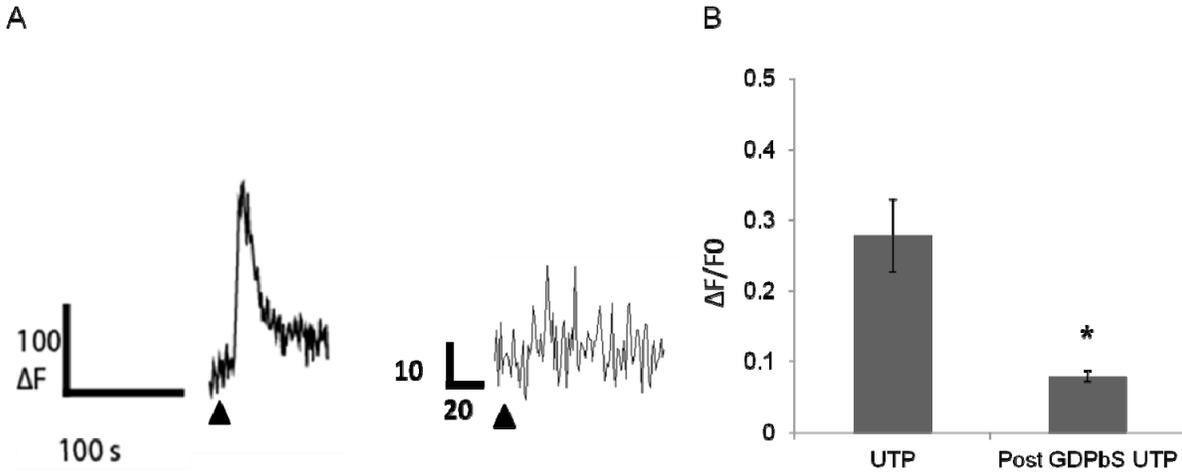


Figure 3.5. Application of purinergics induced robust calcium transients in IP3R3-MVs. (A) Representative calcium traces from IP3R3-MV cells in response to UTP before and after GDPβs incubation (n= 34 cells, 4 slices). (B) Calcium fluorescence responses from IP3R3-MV cells upon treatment with UTP alone, or UTP with GDPβs incubation. GDPβs pretreatment UTP-evoked calcium transients by $48 \pm 0\%$ (n=19 cells, 3 slices). * p<0.05, Student's t-test.

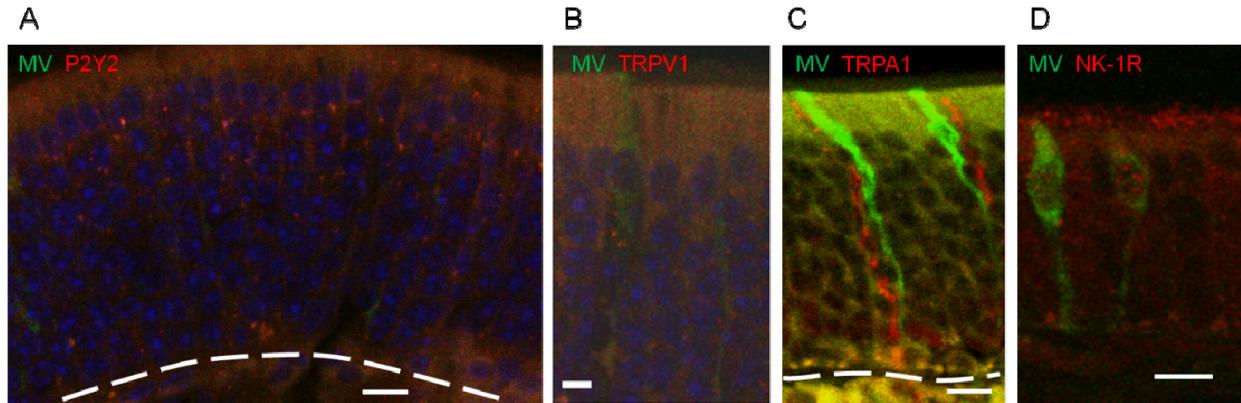


Figure 3.6. Immunohistochemical evidence of NK1, TRPA1, and TRPV1 receptors in relation to the IP3R3-MV. (A) Immunoreactivity (IR) toward metabotropic purinergic receptor P2Y2 indicated expression on cell bodies of the apical layer. (B and C) TRPV1 and TRPA1 expression was near IP3R3-MVs. It is unclear whether TRPV1 co-localizes with the IP3R3-MV, but it appears that TRPA1 does not. (D) NK-1R IR was also found throughout the epithelium, spanning all layers, including on IP3R3-MV cell bodies as well as microvilli and cilia of the mucosal surface. Dotted line indicates division between basement membrane of OE and lamina propria.

Discussion

Odorant signaling

A response to odorants may be unusual in a cell type that lacks the major olfactory transduction components, but there is evidence of odorant signaling outside of the cAMP pathway. In mice in which the A2 subunit of CNG is disrupted, several odorants still produce responsiveness in the OE (Lin et al., 2004). A subset of OSN that does not express cAMP signaling components instead utilizes a cGMP signaling pathway (Meyer et al., 2000). In our experiments, the responses to F/IBMX in IP3R3-MV cells were significantly smaller than calcium transients produced by OSNs, lending further support for the lack of ACIII-mediated odorant transduction. The small responses to the odorant mixture were lowered after incubation with GDP β s, indicating that, like odorant transduction in OSNs, GPCRs are involved. Although G α (olf) is not expressed in the IP3R3-MV, G α q/11 is present within the IP3R3-MV. The downstream effect of G α q/11 is commonly PLC. Thus, the activation of G α q/11 could cause increase in intracellular calcium in IP3R3-MVs through the production of IP3 and the release of calcium from internal stores.

Purinergic signaling

In the olfactory system, trigeminal afferents innervating the OE may be a source of ATP released from synaptic vesicles (Finger et al., 1990; Getchell and Getchell, 1992), or via plasma membrane nucleotide transport proteins (Roman et al., 1997). Furthermore, ATP is also released from ischemic, stressed, and injured cells (Kilgour et al., 2000). Thus, the responses to ATP by the IP3R3-MV may be part of a pain and

damage signaling system in the OE (Sawynok and Sweeney, 1989; Tominaga et al., 2001). The IP3R3-MV cell is known to contain the ionotropic purinergic receptor, P2X3 (Hegg et al., 2010). Thus, increases in intracellular calcium in response to ATP were considered to be due to the opening of cationic channels in the cell membrane. The reduction of ATP-induced calcium transients with use of the IP3 receptor blocker 2-APB indicated that the increase in calcium levels is due, at least in part, to intracellular stores of calcium. Surprisingly, the GPCR blocker, GDP β s, also lowered ATP-induced calcium transients. Therefore, the reduction produced in these two experiments suggested the presence of an additional purinergic receptor, one that is metabotropic and gates intracellular stores of calcium. This was further supported by our finding in live cell calcium imaging experiments that UTP, an agonist for metabotropic P2Y receptors, elicited robust responses from IP3R3-MVs, which were subsequently decreased with the addition of GDP β s. Thus, there appear to be both metabotropic and ionotropic receptors to purinergics in the IP3R3-MV, which was also supported by IHC studies. The presence of both ionotropic and metabotropic purinergic receptors was also described in rat submandibular ductal cells, where the ionotropic receptor is coupled to the secretion of a serine protease, while the function of the metabotropic receptor in this cell type is unknown (Amsallem et al., 1996). Further studies would need to be completed to ascertain whether the IP3R3-MV utilizes these two modes of purinergic signaling in a similar vein, one for secretion of a bioactive factor, the other with another purpose. Indeed, in another study from our lab, ATP was shown to initiate the release of proliferative factor neuropeptide Y (NPY) from the IP3R3-MV (Jia and Hegg, 2010).

IP3R3-MV role in irritation signaling

In the experiments described herein, the IP3R3-MV responded to a mixture of odorants that was applied at a relatively high concentration. Perhaps this is enough to activate possible irritant pathways. However, irritant-sensing channels TRPA1 and TRPV1 were not expressed on the cell itself, nor were responses to TRPA1 agonist cinnamaldehyde observed (not shown). There may be other mechanisms by which the IP3R3-MV senses irritants, but at this time it seems more likely that this cell type does not sense irritants, but is instead a part of downstream signaling of pain or irritation. IP3R3-MV cells are occasionally innervated by trigeminal fibers (Hegg et al., 2010), supported by my studies that indicate structures containing TRPV1 and TRPA1 occasionally contacting the cell, as well as an earlier study indicating IP3R3-MV contact with sub P expressing structures (Hegg et al., 2010). Thus, it is suggested that the IP3R3-MV may be involved in modulating activity of innervating trigeminal fibers. SCCs of the respiratory epithelium communicate with innervating trigeminal fibers with the release of acetyl choline (Saunders et al., 2014). A similar mechanism is not yet known for the IP3R3-MV. ChAT, the enzyme that catalyzes acetyl choline, is absent in the IP3R3-MV, though we do know that the cell type can release NPY in response to ATP (Kanekar et al., 2009; Hegg et al., 2010). Sustentacular cells of the OE communicate with one another through gap junctions, and connexins are found linking multiple cell types in the OE, except OSNs (Rash et al., 2005). Thus, it is possible that microvillous cells express gap junctions and can communicate with trigeminal fibers and sustentacular cells through electrical synapses.

How the activities of multiple receptors coordinate with each other in the IP3R3-MV is not currently understood. NK-1R activation could lead to phosphorylation of TRPV1, if this channel is indeed expressed on the IP3R3-MV (Figure 3.7). TRPV1 is sensitized by phosphorylation, and desensitized by dephosphorylation (Por et al., 2013), and repeated or prolonged application of capsaicin also causes TRPV1 desensitization. ATP is known to increase TRPV1 activity and pretreatment with ATP reverses TRPV1 desensitization. P2Y2 reverses the desensitization caused by capsaicin by activating PKC (Wang et al., 2010). Activation of PKC causes PIP2 to be cleaved, forming DAG and IP3. However, PIP2 itself can also modulate TRP channels, including TRPV1. Upon calcium entry following maximal activation of TRPV1 using pharmacological agents, PI4P and PIP2 are broken down, which causes TRPV1 desensitization. Activation of nearby bradykinin receptors also leads to a decrease in PIP2 and TRPV1 sensitization, while decreases in both PI4P and PIP2 cause TRPV1 inhibition (Lukacs et al., 2013). On the other hand, activation of P2Y2 channels can also lead to the inhibition of P2X3 channels (Gerevich et al., 2007), which become desensitized after prolonged ATP exposure. Thus, expression of multiple channels with varying activation mechanisms (ie ionotropic versus metabotropic) can be a system for regulating the activation, sensitization, and desensitization of channels.

Trigeminal fibers contain sub P, a neuropeptide involved in signaling pain centrally. Sub P is also released peripherally when inflammation is initiated, causing a multitude of effects, including neuronal stimulation, protein extravasation, and vasodilation (Maggi, 1995). This study demonstrated that the IP3R3-MV responds to sub P, mediated through its expression of NK-1R. The role for responses to sub P is

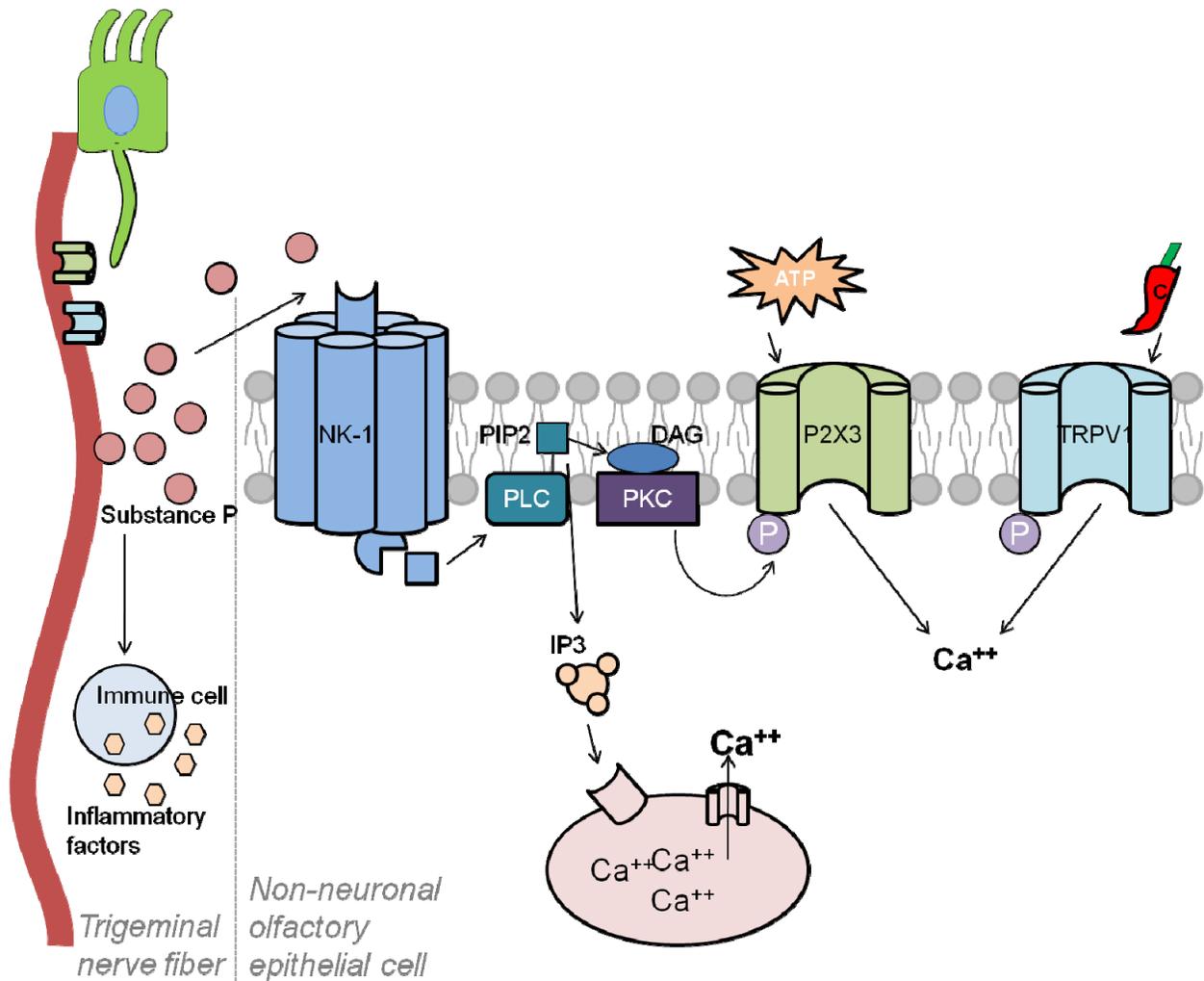


Figure 3.7. Possible mechanism of potentiation of TRP responses to irritants by NK-1R. If the IP3R3-MV does indeed express both NK-1R and TRP channels, release of sub P from contacting trigeminal fibers can bind to NK-1R. Activation of NK-1R would initiate the phosphorylation of nearby TRP channels through the activity of PLC and PKC. Phosphorylation of TRPV1 is known to potentiate responses to capsaicin.

currently unknown, but just as ATP release is a precursor to the release of NPY from IP3R3-MVs, perhaps a response to sub P initiates the release of a bioactive compound in order to communicate with nearby cells. Future studies should investigate the function of the IP3R3-MV response to sub P.

Limitations of study

GDPβs was used to demonstrate that a GPCR is involved in signal transduction in response to UTP and odorants. Gαq/11 is present in IP3R3-MVs. Gαq/11 leads to the activation of PLC-β, which hydrolyzes PIP2 into IP3. IP3 can then act on IP3 receptors on internal stores of calcium, causing the release of calcium into the cell. The role of Gαq/11 in the response to odorants seen in the IP3R3-MV could be confirmed with the use of a selective Gαq/11 inhibitor (Takasaki et al., 2004). 2-APB, which is an antagonist of IP3R3, should also be used in future experiments to determine whether activation of Gαq/11 causes changes in intracellular calcium through the PLC/IP3 pathway typical of Gαq/11. Interestingly, a study of mGluR5 channels coupled to Gαq/11 demonstrated that the classical activation of PLC and subsequent IP3 production did not occur in neurons of the brain, and instead lead to a pathway that regulated transcription of activator protein-1 (Yang et al., 2006). Thus, there are possible unexpected downstream effects of metabotropic receptor activation.

Pharmacological agents tend to have unintended actions, and likewise, there are typical a number of different agents for the same target. Heparin and Xestospongin are inhibitors of IP3R3. However, heparin also uncouples G protein signaling and activates ryanodine receptors, thus an inactivation of IP3R3 is muddled by release of calcium

gated by ryanodine receptors. Xestospongine is another IP3R3 antagonist, but has pitfalls of its own, including its higher cost and slow action (Ehrlich et al., 1994). 2-APB was used in this study to block IP3R3, but 2-APB is also known to block TRP channels (Bootman et al., 2002).

At this time, a specific blocker of PLC was not applied to determine the role of PLC in IP3R3-MV signal transduction. U73122 should be utilized in future experiments to confirm its role in generating IP3 and activation release of intracellular calcium, and the role of this transduction pathway in purinergic, odorant, and neurokinin signaling (Figure 3.7). Likewise, in addition to the use of F/IBMX, SQ22536 could be used to determine the role of ACIII in the IP3R3-MV. SQ22536 is a direct inhibitor of AC. As ACIII is not expressed in the IP3R3-MV, no effect of SQ 22536 is expected (Figure 3.8).

The immunohistochemical localization of TRPV1 did not clearly demonstrate whether the channel is expressed on the IP3R3-MV or on trigeminal fibers making contact with the cells. TRPA1 appeared to be on trigeminal fibers near the cell, and not on the cell proper, and live cell calcium imaging supported this conclusion, as responses to TRPA1 agonist cinnamaldehyde were not observed. Future studies should examine whether the IP3R3-MV is responsive to capsaicin in order to clarify the expression of this channel.

Conclusion

I have elucidated that GPCRs and intracellular calcium are a part of the signal transduction pathway for responses to purines, and GPCRs, but not the cAMP pathway, are needed for response to odorants in the IP3R3-MV. A role for this cell in irritant

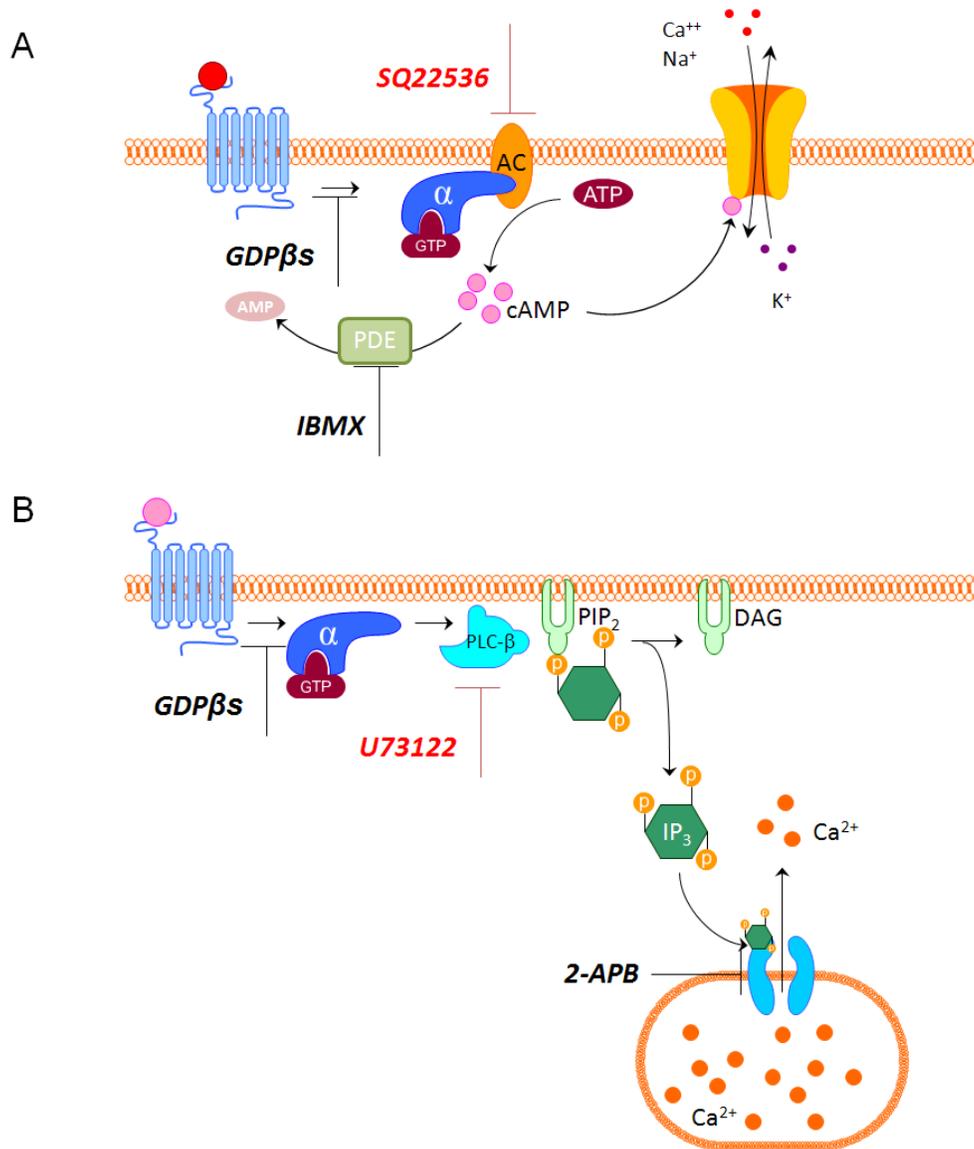


Figure 3.8. Model of pharmacological agents to be utilized for future experiments.

(A) In addition to the use of GDPβs and F/IBMX, SQ22536 could be used to determine the role of ACIII in the IP3R3-MV. SQ22536 is a direct inhibitor of AC. As ACIII is not expressed in the IP3R3-MV, no effect of SQ 22536 is expected. (B) Internal stores of calcium gated by IP3R3 can be pharmacologically blocked with the use of 2-APB.

U73122 should be utilized in future experiments to block the activity of PLC, an enzyme that creates IP3 and DAG from PIP2.

sensation remains unclear, but certainly a relationship with the trigeminal system is possible. Future studies are needed to address the function of these responses. For example, in the SCC, activation of gustducin and TRPM5, signaling factors common to bitter tastant transduction, cause the release of acetylcholine. The release of acetylcholine can then activate metabotropic acetylcholine receptors on nearby trigeminal nerve fibers (Saunders et al., 2014). The IP3R3-MV does not appear to utilize acetylcholine, but this cell is the primary source of NPY in the OE. NPY is a neuroproliferative factor, and we have shown that the release of NPY is initiated by ATP (Kanekar et al., 2009). Future studies should be done to determine the function of responses to sub P. NPY could be released in response to substance P, or another currently unknown factor could be released to modulate nearby cells or trigeminal fibers.

Chapter 4- Investigating the role of substance P in macrophage recruitment and activation

Introduction

The olfactory epithelium (OE) is a tissue that comes into contact with damaging agents from the environment on a regular basis, and thus, inflammation is a common occurrence in the tissue. In chapter 2, I demonstrated the presence of components of the neurogenic inflammatory response in the mouse OE. Specifically, I demonstrated that transient receptor potential –vanniloid-1 and ankyrin-1 (TRPV1 and TRPA1) channels are present and functional in the OE, and that their activation causes the local release of neuropeptide substance P (sub P) and plasma extravasation.

There is bountiful evidence of sub P's role in the inflammatory cascade. Sub P initiates the increase of immune cells to sites of injury by causing dilation of local vessels. In addition to increasing access of immune cells through dilation of blood vessels, sub P can also directly stimulate the chemotaxis of lymphocytes, monocytes, neutrophils, and fibroblasts (Haines et al., 1993; Kahler et al., 1993; Schratzberger et al., 1997). The inflammatory effects of many toxins and irritants are mediated through the activity of sub P and its receptor neurokinin-1 (NK-1R). For example, in NK-1R knockout mice, cigarette smoke-induced increases in macrophages and dendritic cells are absent (De Swert et al., 2009) and cytokine levels are reduced in NK-1R knockout mice exposed to parasites (Garza et al., 2008).

Cytokines are small proteins used in chemical messaging between cells and are major mediators of the inflammatory cascade. In the central nervous system, cytokines

regulate apoptosis and cell proliferation, synaptic plasticity, neural transmission, and Ca^{2+} signaling. It is unclear as to what the source of cytokines is in the mouse OE. Neutrophils can release cytokines and are first to the scene in inflammation (Harkema studies). Macrophages are another major source of cytokines, especially of tumor necrosis factor (TNF)- α . Interleukin (IL)-6 is expressed by olfactory ensheathing cells and microglial cells of the olfactory bulb (Herbert et al., 2012). A residential source of cytokines is not well described for the OE.

Bulbectomy is often used to study the infiltration and activation of macrophages in the mouse OE. In this study, I examine whether sub P can initiate an increase in macrophages and cytokines in the mouse OE. I also examine the expression of the sub P receptor NK-1R in order to understand what other downstream effects sub P release may have. I demonstrate that NK-1Rs are found throughout the mouse OE, on multiple cell types, including macrophages, within the tissue. Furthermore, I show that macrophage content in the mouse OE can be increased upon exogenous application of sub P, which corresponds with an increase in cytokines, including TNF- α .

Methods and Materials

Animals

Swiss Webster (CFW) mice were used in all experiments. Adult male mice (6-8 week) were used in immunohistochemistry and enzyme-linked immunosorbant assay studies. Neonatal mice of both sexes (P0-4) were used in culture media collection for cytometric bead array and calcium imaging studies. In immunohistochemical studies, a transgenic animal in the C57 BL/6 background was used to compare immunoreactivity

to green fluorescent protein (GFP)-expressing microvillous cells. This transgenic mouse, the inositol triphosphate receptor 3 (IP3R3)-tauGFP mice (kindly provided by Dr. Diego Restrepo, University of Colorado Denver, Aurora, CO) has the first exon of the *Itpr3* gene replaced by the coding region for a fusion protein of tau and GFP, which allows for identification of the IP3R3 expressing microvillous cell (IP3R3-MV) in the OE (Hegg et al., 2010).

Mice were kept in rooms at 21–24°C with 40–60% relative humidity with a 12-h light/dark cycle and given food and water *ad libitum*. All procedures conducted in laboratory animals as approved by Michigan State University Institutional Animal Care and Use Committee.

Western Blotting

Anesthetized adult male (6-8 weeks old) C57BL/6 mice were decapitated. The olfactory epithelia were immediately dissected and stored at -80 °C. The olfactory tissue was processed following the protocol described previously (Jia et al., 2009). Briefly, tissues were homogenized by sonication in Tris buffer. Homogenates (30 µg/lane) were resolved on 12.5% gels and transferred to nitrocellulose membranes. Membranes were left overnight at 4 °C after incubation with primary antibodies (rabbit anti-NK1), made in blocking buffer (0.2% g/LI-Block, Millipore, Bedford, MA, USA). After washing, the membranes were incubated with horseradish peroxidase-labeled secondary antibody (Jackson Laboratory, West Grove, PA, USA). Immunoreactive proteins were detected with a chemiluminescence reagent (ECL, Amersham

Biosciences, Piscataway, NJ, USA) and then exposed to Kodak X-ray film which was then developed.

Immunohistochemistry (IHC)

Anesthetized adult mice (65 mg/kg ketamine+5 mg/kg xylazine, intraperitoneally) were transcardially perfused with 0.1 M phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA), then decapitated and post-fixed in 4% PFA overnight. Heads were decalcified for 5 days with ethylenediaminetetraacetic acid (0.5M, pH=8), cryoprotected overnight in 30% sucrose solution, frozen, and then sectioned coronally (20 μ m), from level 3 at the level of the second palatal ridge of the mouse nasal cavity, with a HM525 cryostat (Microm International, Waldorf, Germany) while embedded in TissueTek Optimal Cutting Temperature compound (Sakura Finetek USA, Torrance, CA). Tissue was collected onto positive-charged slides, and stored at -20 deg C until use. Or, 4% PFA was used in cell culture fixation for cells grown on cover slides and used for IHC studies. Tissue or cells are rehydrated with PBS and permeabilized with 0.3% Triton X-100 and incubated with blocking reagent normal donkey serum (10%) to decrease non-specific binding. Tissue was incubated with primary antibody (rat anti-CD-68; rabbit anti-NK-1) overnight at 4°C followed by washing with PBS and incubation with secondary antibody conjugated to a fluorophore (TRITC or FITC at 1:200; Jackson Laboratories) for two hours at room temperature.

Antibody	Target	Concentration	Manufacturer
Rat α -CD68	Macrophage	1: 1000	AbD Serotec; Kidlington, UK
Rabbit α -CD-163	Macrophage	1: 1000	Epitomic; Burlingame, CA
Rat α -F4/80	Mature macrophage	1: 1000	AbD Serotec; Kidlington, UK
Rabbit α -Iba1	Microglia	1:1000	Wako; Osaka, Japan
Rabbit α -NK-1R	Sub P receptor	1:500	Millipore; Billerica, MA

Table 4.1. Antibodies used in immunohistochemical studies

Bulbectomy

Mice were anesthetized using 4% isoflurane to initially anesthetize, followed by 2% isoflurane to maintain anesthesia. The scalp is then incised and a hole is drilled through bone that is directly above the right olfactory bulb. The olfactory bulb was aspirated using a small diameter glass pipette, the hole packed with gel foam, and the skin closed with surgical staples. The contralateral bulb is left intact. One day later, tissue was collected and processed for immunohistochemistry.

RAW 264.7 cell culture

The murine cell line of macrophages, RAW 264.7 (gift from the Pestka lab, Michigan State University), were maintained at 37°C and 5% CO₂ in a humidified incubator in Dulbecco's Modified Eagles Medium supplemented with 10% (volume/volume) fetal bovine serum (Gibco, Gaithersburg, MD), 100 units/mL penicillin, and 100 μ g/mL streptomycin (Gibco, Gaithersburg, MD).

Sub P aspiration

Adult male CFW mice were anesthetized using 4% isoflurane. 25 μ L of a solution of sub P (100 μ M) was applied dropwise to right naris and breathed in. Lavage and tissue were collected 1 day after aspiration.

Plasma extravasation

Mice were anesthetized using 4% isoflurane. The sub P treatment group received 100 μ M sub P (25 μ L) applied dropwise to the right naris (n=3 adult male CFW mice). Five minutes after nasal stimulation, mice were placed on a warming pad and Alexafluor 555 conjugated to albumin (25mg/kg in saline) was injected into the tail vein. Five minutes following tail vein injection, heads were removed and fixed in 4% PFA overnight. Following fixation, heads were bisected in the midsagittal plane and the nasal septum removed to expose turbinates. Pictures were taken and analyzed using an Inverted Nikon Fluorescent microscope (Nikon TE2000-U Fluorescence Microscope) with an excitation of 555/emission 565nm for Alexafluor 555 dye. Fluorescence intensity was measured using ImageJ software (National Institute of Health). Images were converted to 16-bit and the turbinates were highlighted. The equation used for the corrected integrated density (integrated density- area of selected cell X mean fluorescence of background readings) took into consideration the area of the selected cell and normalized the fluorescence of the cell using the fluorescence of the background.

Cytometric Bead Array

OE slices (400 μm) were collected from neonatal CFW mice (both sexes). For each treatment group, 3–4 slices were attained from each animal, which each treatment group containing 4 animals. Slices were placed on a cell culture plate insert (Millicell-CM PICMORG50, Milipore Corp., Billerica, MA) with sub P (20 ng/ml) or satratoxin G (60 mg/ml) and incubated at 37°C with 5% CO_2 in neurobasal media with 0.02 g/L B-27 supplement, 0.01 g/L penicillin/streptomycin and 0.01 g/L L-glutamine (Invitrogen, Carlsbad, CA). Conditioned media were collected 1 and 24 hours after treatment.

The concentrations of Interleukin-6 (IL-6), Interleukin-10 (IL-10), Monocyte Chemoattractant Protein-1 (MCP-1), Tumor Necrosis Factor-alpha (TNF- α), Interferon- γ (IFN- γ), Interleukin 12p70 (IL-12p70), and Interleukin-12 (IL-12) in nasal lavage fluid were determined using Cytometric Bead Array (CBA) Mouse Inflammation Kit (BD Biosciences, San Diego, CA, USA). Measurements were carried out according to manufacturer's instructions using a FACSCalibur Flow Cytometry System and BD CBA Analysis software (BD Biosciences, San Jose, CA, USA). Cytokine concentrations are expressed as pg/ml.

Enzyme-linked immunosorbant assay (ELISA) for TNF measurement

1 day after sub P treatment, nasal lavages were collected from mice immediately after death, with 300 μL of saline applied retrogradely through the nasopharyngeal meatus using a 20-gauge cannula and a 1 mL syringe. Samples were stored in -80°C until further analyzed. Levels of TNF- α in nasal lavage fluid were assayed using a mouse OptEIA TNF ELISA kit (BD Biosciences, San Jose, CA) according to

manufacturer's instructions. All samples ($n= 3-5$ animals per group) were run in duplicate.

Confocal live cell calcium imaging

Neonatal mice were decapitated and their skin and lower jaw were removed. The head was embedded in a block made of carrot and mounted onto a vibratome. Tissue was kept in ice-cold Ringer's solution while sectioned into 400- μm slices. Ringer's solution contained 140 mM NaCl, 5 mM KCl, 1 mM MgCl_2 , 2 mM CaCl_2 , 10 mM HEPES, 10 mM glucose, and 500 μM Probenecid (pH 7.4, 290–320 mOsm). Slices were incubated with 50 $\mu\text{g}/\text{mL}$ calcium indicator dye X-Rhod 1 AM (Molecular Probes) made in Ringer's solution with 0.04% pluronic F-127 (Invitrogen, Calsbad, CA) and 0.4% DMSO for 60 min at room temperature before imaging.

Tissue slices were placed in a laminar flow chamber (Warner Instruments, Hamden, CT, USA) and perfused continuously with Ringer's solution at a flow rate of 1.5–2.0 mL/min. Test solutions were applied using bath exchange and a small-volume loop injector (200 μL). Our perfusion system exchanges the bath in *ca.* 7–10 s and traces were not corrected for this delay. Following application of bioactive compound, 5 minutes of Ringer's alone was perfused over the slice. Using a FV1000 confocal microscope (Olympus), images were collected over 2 minutes at 0.2–1 Hz. A helium–neon laser excited X-Rhod-1 AM at 543 nm. Fluorescence emissions were filtered at 560–660 nm. The fluorometric signals obtained were expressed as relative fluorescence (F) change, $\Delta F/ F_0 = (F - F_0)/F_0$, where F_0 is the baseline fluorescence

level (mean F of first 5 frames). Data were collected from a minimum of three slices for each experiment.

Results

Immunoreactivity toward CD-68 indicates macrophage presence in the mouse OE

Once inflammation is initiated in a tissue, it is common for immune cells to be present and active. There are a number of immune cell and macrophage markers available. Immunoreactivity to macrophage marker was first characterized in murine macrophage RAW 264.7 cells, with CD-68 allowing the most labeling (Figure 4.1). CD-68 was then characterized in OE tissue from unilateral bulbectomized mice.

Bulbectomy is removal of the olfactory bulb, which is the target of OSN axons in the central nervous system. Without the olfactory bulb, retrograde degeneration of OSNs occurs. Bulbectomy is a common way in which to increase and therefore study the presence of macrophages in the OE (Getchell et al., 2002). Macrophages were not evident in the intact bulb (Figure 4.2B), but were plentiful in the area of the removed bulb 1 day after bulbectomy (Figure 4.2A). In the ipsilateral OE of bulbectomized mice (OBX side), CD-68+ cells were large and round (Figure 4.2C), which is typical of macrophage morphology (Getchell et al., 2006; Borders et al., 2007). CD-68+ cells were apparent in tissue pertaining to the OE of the intact bulb (intact side), but most of these cells were in the lamina propria and not in the OE proper. Counting CD68+ profiles in the OE, excluding the lamina propria, found macrophages in significantly higher numbers ($p < 0.05$) in the OE of the OBX side compared to the OE of the intact

Antibody	Target	IR
CD-163	Surface glycoprotein of macrophages that functions as Acute-phase regulator receptor	No
CD-68	Glycoprotein of macrophages that binds to lipoproteins	Yes
F4/80	Transmembrane surface protein of mature macrophage	No
Iba1	Calcium binding molecule that is upregulated in activated microglia	No

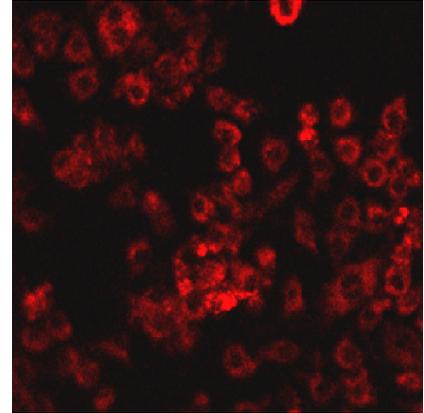


Table 4.2. Antibodies used in immunohistochemical labeling of macrophages.

There are a variety of different antibodies for labeling macrophages. Using the same methods, CD-163, F4/80, and Iba1 did not label RAW 264.7 cells, whereas CD-68 resulted in positive immunoreactivity in RAW 264.7 cell culture.

Figure 4.1. Immunohistochemical labeling of RAW macrophages. CD-68 was used to label macrophages of the RAW 264.7 cell line.

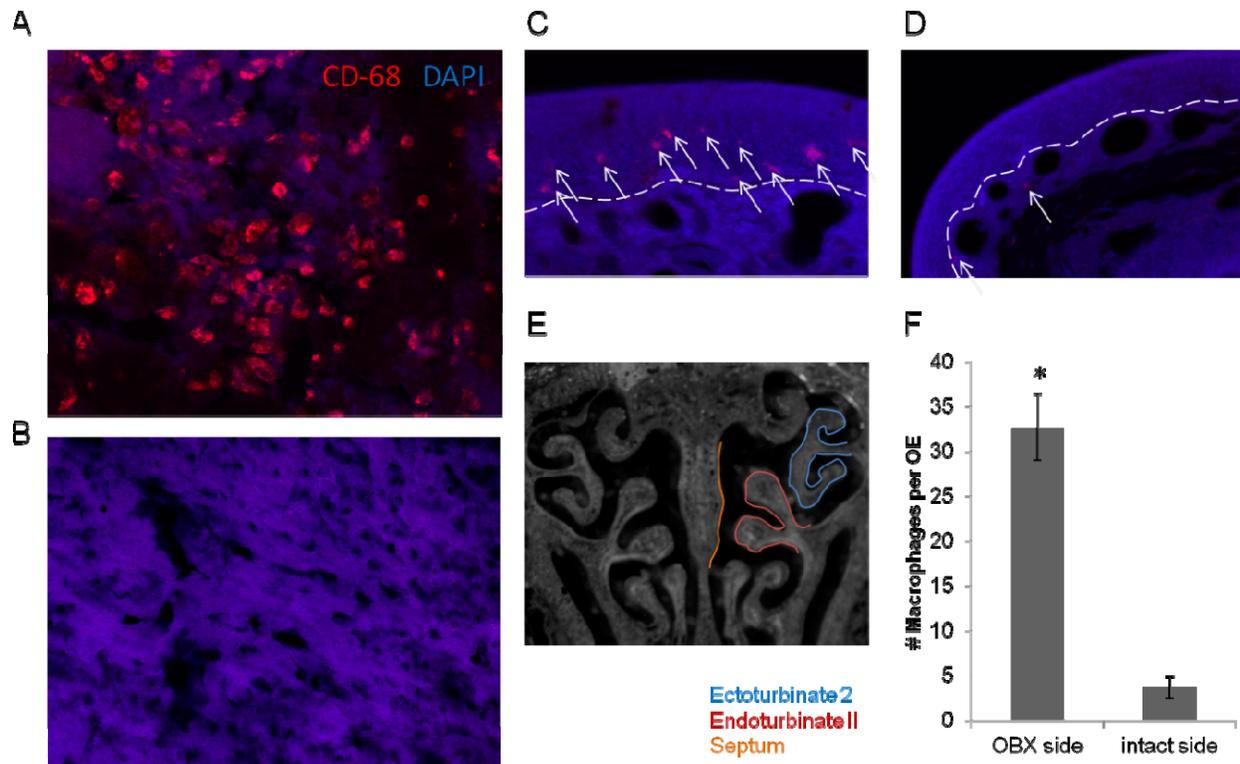


Figure 4.2. Labeling of macrophages in intact and bulbectomized tissue. (A)

Bulbectomy causes retrograde degeneration of OSNs and has been used to recruit macrophages into the OE, labeled with CD-68(red). (B) Macrophages were more apparent in the intact contralateral bulb.

(C) In the OE ipsilateral to bulbectomy, CD-68 (red, arrows) labeling was present. (D) Low levels of CD-68 positive macrophages were found in the OE without damage, and were more likely found in the lamina propria (red,

arrows). (E) CD-68+ profiles were counted from ectoturbinate 2, endoturbinate II, and septum.

(F) Counting the macrophages in the OE indicated that bulbectomy greatly increased the number of macrophages in the side ipsilateral to the removed bulb, compared to the OE contralateral to the removed bulb, ipsilateral to the intact side. * $P < 0.05$ Student's t-test.

side (Figure 4.2E). This indicated that CD-68+ positive immune cells are plentiful in injured tissue but very low levels of macrophages are present in undamaged tissue.

The role of sub P in recruitment and cytokine release into the OE

In order for sub P to have a direct effect on macrophages, NK-1R would need to be expressed on the cell type. CD-68+ cells in the OE of bulbectomized mice, as well as RAW 264.7 cells, exhibited immunoreactivity to NK-1R (Figure 4.3). A role for sub P in recruitment of macrophages into the OE was investigated by investigating immunoreactivity toward CD-68 in tissue from adult mice that had aspirated sub P solution (100 μ M in saline) (Figure 4.4A). There was a significant increase in immunoreactivity toward CD-68 in tissue treated with sub P compared to saline control ($p < 0.05$). This suggests increases in macrophages in the OE can be initiated by sub P, which is released from trigeminal fibers that innervate the tissue. Pre-treatment of animals with NK-1R blocker L732-138 followed by administration of sub P significantly decreased the number of macrophages in the tissue ($p < 0.05$), confirming that this increase in macrophages is mediated through the release of neuropeptide sub P and its receptor, NK-1. Application of this concentration of sub P also initiated plasma extravasation, which was seen to be significantly higher ($p < 0.05$) in the mouse OE compared to control (Figure 4.4B). This supports the idea that one mechanism by which sub P increases the macrophage content in the OE is by initiating plasma extravasation, which increases blood flow and the permeability of nearby blood vessels.

Sub P has also been shown to have a role in the expression and release of cytokines from immune cells. To investigate whether sub P can cause the release of

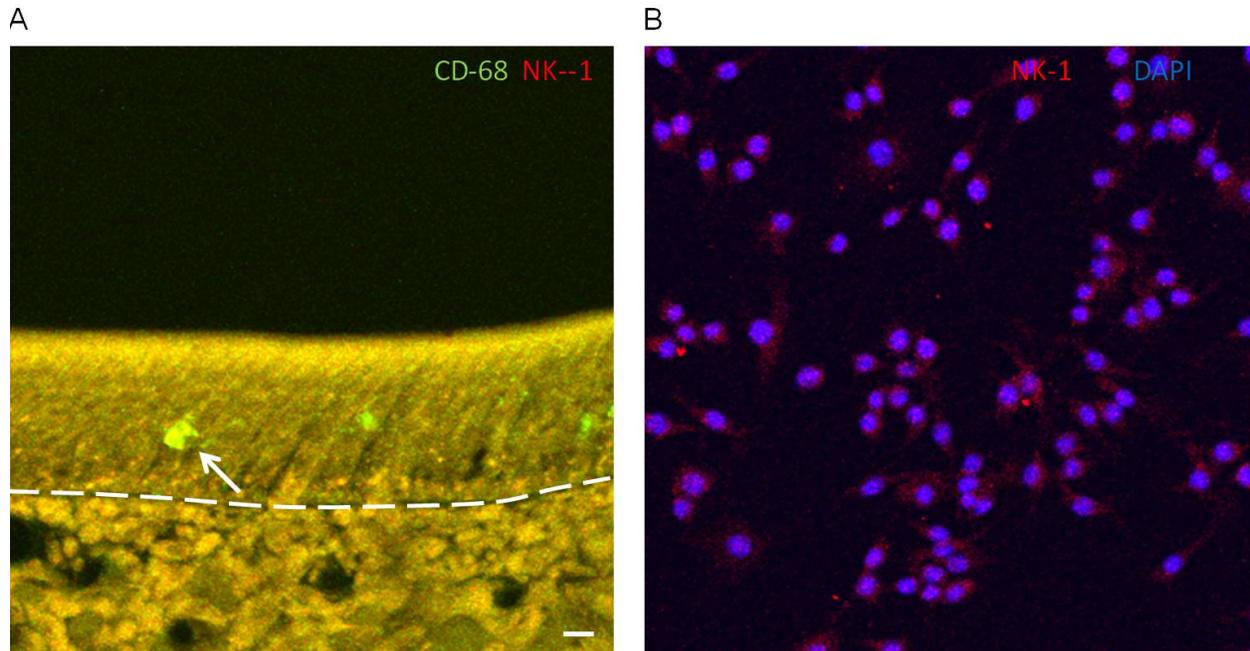


Figure 4.3. NK-1R immunoreactivity in the mouse OE indicates co-labeling in CD-68+ macrophages. (A) In OE tissue from bulbectomized mice, macrophages, indicated by CD-68 (green), co-label (yellow; indicated by arrow) for the marker for the sub P receptor NK-1R (red). (B) Raw 264.7 macrophages also label for NK-1R. All cells in the cell culture were positive for NK-1R (red), as determined by DAPI (blue) labeling of cell nuclei. Scalebar represents 5 μ M, dotted line indicated basement membrane.

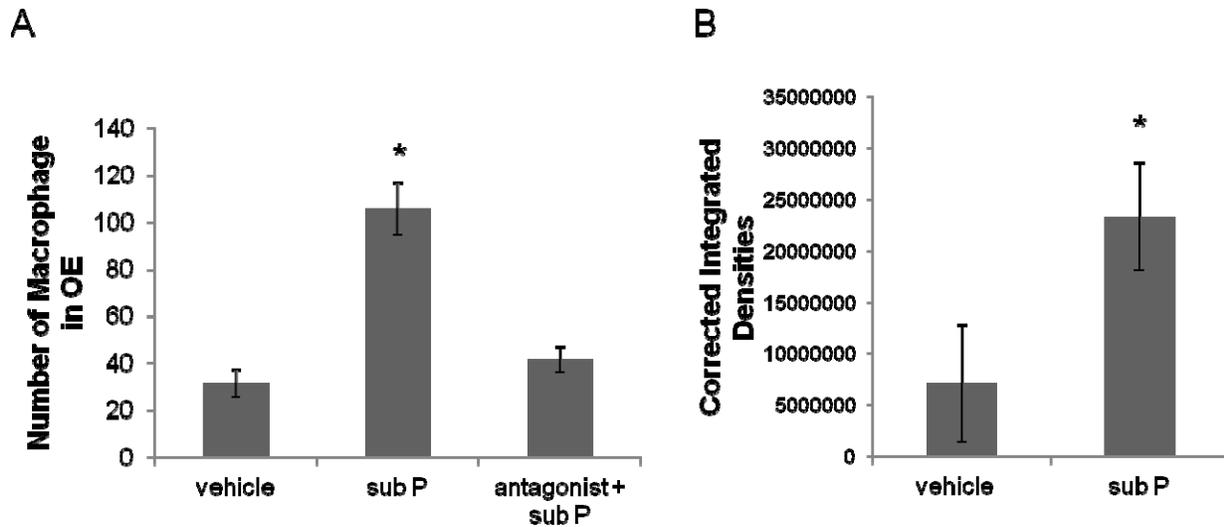


Figure 4.4. Nasal administration of sub P initiates an increase in macrophages within the mouse OE. (A) The number of CD68+ profiles in the mouse OE after sub P (100 μ M) aspiration was significantly higher than in control (saline aspiration). This increase was blocked with the use of L732-138, an antagonist of NK-1R, to levels similar to control (vehicle). (B) Application of sub P (100 μ M) caused a significant increase in plasma extravasation compared to control (vehicle), suggesting a mechanism by which macrophages are increased in the mouse OE. * $P < 0.05$ Student's t-test.

cytokines from OE tissue, and what cytokines are subsequently released, slices of neonatal mouse OE were incubated with sub P for 1 or 24 hours. Another group of slices were incubated with satratoxin G as a positive control, as this toxin has been found to cause the release of cytokines into lavage from mouse nasal tissue (Islam et al., 2006). Incubation of slices for 1 hour with sub P initiated small increases in cytokines TNF- α , MCP-1, and IL-6, with no appreciable levels of IL-12p70, IFN- γ , or IL-10. Larger increases were elicited after 24 hours incubation with sub P for TNF- α , MCP-1, and IL-6, with little to no release of IL-12p70, IFN- γ , or IL-10. Similar increases were seen with the positive control satratoxin G (Table 4.3). In this setup, cells could be releasing cytokines due to cell death. Thus, lavages were also collected 1 day after adult mice aspirated sub P (100 μ M) or saline (control). Also, to demonstrate the specific role of sub P, the antagonist for NK-1, L-732-138 (10 mM), was also used. One group was pre-treated with L-732-138 followed by aspiration of sub P, and another group received pretreatment with L-732-138 followed by aspiration of saline. None of the above groups had measurable release of TNF- α (data not shown).

NK-1R labeling and calcium imaging studies identify various responding cell types

Macrophages are just one possible cell type that may be responding to the release of sub P. I carried out preliminary IHC studies to locate receptors for sub P, and have demonstrated immunoreactivity toward NK-1R throughout the OE, including in apical portions where dendritic processes and microvilli extend (Figure 4.5). The expression of functional NK-1R on sustentacular cells is further supported by my calcium imaging studies. Our lab has also previously shown that the IP3R3-MV exhibits

Pg/ml ± SEM	TNF-α	MCP-1	IL-6	IFN	IL-10	IL-12p70
SG, 1 hr (n=3)	0 ± 0	0 ± 0	4.97 ± 2.86	0 ± 0	0 ± 0	0 ± 0
SG, 24 hr (n=3)	37.59 ± 4.23	1192.02 ± 190.49	663.18 ± 62.97	0 ± 0	2.3 ± 2.3	0 ± 0
SubP, 1 hr (n=3)	4.88 ± 0.35	20.083 ± 4.53	274.14 ± 44.53	0 ± 0	0 ± 0	0 ± 0
SubP, 24 hr (n=3)	116.50 ± 12.40	14449.93 ± 2722.05	5952.09 ± 786.0	1.27 ± 0.19	11.31 ± 2.44	0 ± 0

Table 4.3. Environmental irritant satratoxin G and substance P induce release of cytokines from neonatal mouse OE slices. Slices taken from CFW mice (n= 3 slices/animal, 3 animals/treatment) were incubated for either 1 or 24 hours with either satratoxin G (SG; positive control; 60 mg/ml), or substance P (20 ng/ml). Collected media was analyzed for cytokine levels using Cytometric bead array mouse inflammation kit (BD Biosciences, San Diego, CA). Measurements were carried out using flow cytometry and FCAP array software (SoftFlo Inc, St. Louis Park, MN) as per manufacturer instructions.

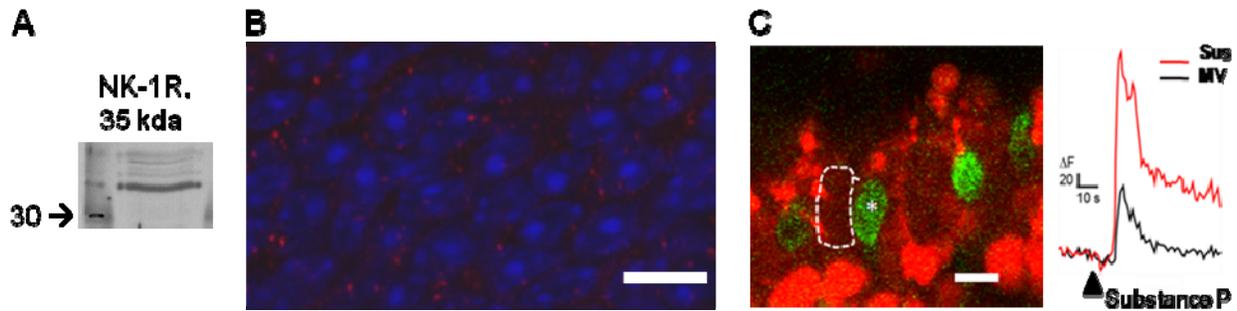


Figure 4.5. Western blotting, IHC, and confocal live cell calcium imaging studies demonstrate the presence of NK-1R within the OE. (A) Adult mouse OE tissue processed for western blotting analysis indicated expression of NK-1R protein at the expected weight of 35 kda. Arrow indicates weight of molecular weight indicator ladder, which was run alongside OE sample. (B) NK-1R IR (red) was punctate and found around cells (DAPI, blue) throughout the OE (C) Left, X-Rhod AM-1 -loaded OE slice containing IP3R3+ microvillous cell (green) and sustentacular cells (outlined). Right, Timecourse of Ca²⁺ transients in response to substance P (500 nM) from a putative sustentacular cell (red trace) and an IP3R3-mv cell (black trace). Changes in intracellular calcium are measured as percent changes in fluorescence (ΔF). Black triangle indicates time of sub P superfusion. Scale bar = 5 μ m (B), 10 μ m (C).

increases in intracellular calcium in response to sub P, and the studies of Chapter 3 demonstrate immunoreactivity to NK-1R on the IP3R3-MV.

Discussion

In this study, I investigated the role of the release of sub P in the mouse OE (Figure 4.6). I demonstrated that sub P initiates an increase in the macrophage and cytokine content in mouse OE. By additionally investigating the localization and activity of receptors to sub P, I identified cell types that may be involved in neurogenic inflammation, including macrophages and sustentacular cells.

Role of sub P in macrophage recruitment

The current study demonstrates the presence of macrophages in healthy and degenerating tissue, but at significantly higher numbers in the latter. This indicates that the majority of CD-68+ macrophages are recruited into the tissue upon damage or arise from cellular divisions of residential cells, and that residential macrophages are not present in significant numbers. Bulbectomy is commonly used to cause the infiltration and activation of macrophages. In this study, aspiration of sub P also caused an increase of macrophages into the OE. These CD-68+ macrophages co-labeled for NK-1R and human and rat macrophages also express NK-1R (Marriott and Bost, 2001; Simeonidis et al., 2003). This lends support to a mechanism of activation of macrophage activity through sub P. Activation of NK-1R is also an initiator of plasma extravasation (Saunders et al., 2014), which allows increased access of the OE to immune cells. During extravasation from blood vessels, leukocytes undergo tethering,

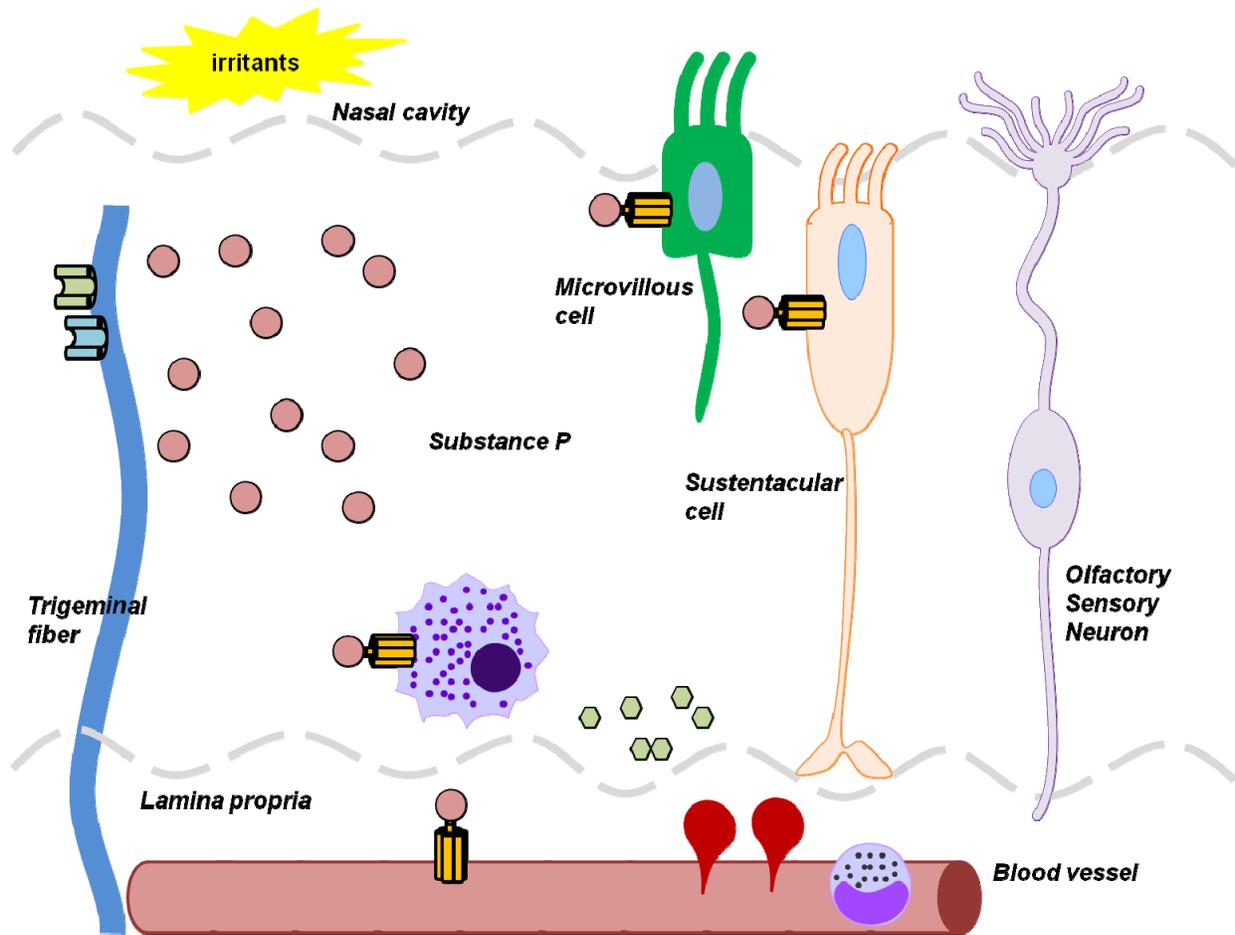


Figure 4.6. A model of the effects of sub P release in the mouse OE. Sub P is released from trigeminal fibers and initiates plasma extravasation, which allows increased access of macrophages into the OE. Sub P also initiates an increase in cytokines from slices of neonatal OE. Receptors for sub P are found throughout the OE, and calcium imaging experiments demonstrated responses to sub P from IP3R3-MVs and sustentacular cells.

rolling, adhesion, and then diapedesis through junctions in the blood vessels. Specific interactions between integrins and adhesion molecules as well as contact with chemoattractant molecules are required to allow for diapedesis (Schenkel et al., 2004). Sub P initiates the release of factors that are chemoattractant to immune cells. Macrophage inflammatory protein-2 (MIP-2) and MCP-1 are two chemokines synthesized in response to sub P (Ramnath and Bhatia, 2006; Sun et al., 2007, 2008). MCP-1 and MIP-1 α mRNA reaches peak levels 3-5 days post bullectomy, which also corresponds to when CD-68+ macrophages are found at peak levels (Getchell et al., 2002). Thus, the increase of macrophages in the OE upon exogenous sub P administration is likely caused by a combination of sub P-initiated release of chemoattractant factors from resident macrophages and plasma extravasation allowing increased access of recruited macrophages.

In my study, cytokine levels were not increased 1 hour after sub P treatment, but were significantly raised after 24 hours. The delay in cytokine release could correspond to time needed for immune cell infiltration. The lack of macrophages in uninjured tissue and the marked increase 24 hours after sub P treatment supports this hypothesis. Importantly, one of the cytokines that was increased was MCP-1, a known chemoattractant of macrophages. There is support for the sub P-initiated release of cytokines. For example, the increase in mRNA expression and secretion of IL-8 is initiated through sub P's activation of NF- κ B (Lieb et al., 1997), whereas the sub P-induced expression of cytokine IL-6 is independent of NF κ B activation, and is instead mediated through the signal transduction component p38 mitogen-activated protein kinase (p38 MAPK) (Fiebich et al., 2000). The bacterial lipopolysaccharides (LPS)

cause the release of IL-1 from monocytes, and when complemented with sub P, IL-1 production is quadrupled (Martin et al., 1993). With sub P priming, lower levels of LPS can induce IL-6 mRNA expression from monocytes than when LPS is presented alone, and sub P can elicit IL-6 production by astrocytes (Lieb et al., 1997). The lack of measurement of TNF- α release into lavages from adult mice that aspirated sub P could indicate that while macrophages numbers may be increased through the activity of sub P, sub P may not be an adequate signal for production and release of cytokines into a tissue that is otherwise uninjured. It is also possible that different time points would have yielded measurement of TNF release, thus warranting future studies.

IHC, western blotting, and live cell calcium imaging studies indicated that NK-1R is found throughout the OE, including non-neuronal sustentacular and microvillous cells (Figure 4.5). At this time, the function of the responses to sub P from these cells is unknown, but sustentacular and microvillous cells play important roles in maintaining the health of the OE. While the role of these particular cells in neurogenic inflammation is currently unclear, the role of NK-1R in inflammation is well documented. The initiation of inflammation in the intestines is mediated through the activity of NK-1R, as demonstrated by a study in which mice genetically deficient in NK-1R had decreased epithelial damage and TNF- α levels induced by toxin A compared to mice with normal NK-1R expression (Castagliuolo et al., 1998). However, in the same knockout model, TNF- α levels were increased in the lungs after immuno-challenge, suggesting that the role of sub P and NK-1R differs between systems (Bozic et al., 1996). Furthermore, expression of NK-1R can change. After injury to the optic nerve, NK-1R is expressed by the reactive astrocytes of the glial scar, but is not otherwise expressed by the optic

nerve (Mantyh et al., 1989). Thus, it is important to understand the role of sub P and NK-1R in the mouse OE if we are to fully understand neurogenic inflammation of this tissue. Future studies are needed to elucidate the role of sustentacular and microvillous cells in sub P-mediated mechanisms.

The cytokine cocktail

The cytokines that were significantly increased 24 hours after sub P treatment in this study, TNF- α , MCP-1, and IL-6, have also been shown to be increased by other toxins and pathogens. For example, the mycotoxin satratoxin G causes the infiltration of macrophages and increases mRNA expression for cytokines TNF- α , IL-1 α , IL-1 β , and IL-6 in the OE (Islam et al., 2006). Exposure to roridin-A (another mycotoxin) also increases levels of TNF- α , MCP-1, and IL-6 cytokines, measured in nasal lavages from mice (Corps et al., 2010). TNF- α is a cytokine associated with infection or injury in many cell-types (Feldmann and Maini, 1999; Kollias et al., 1999; Sandborn and Hanauer, 1999), and is present in sinonasal inflammatory disease. MCP-1 has been shown to be a chemoattractant of macrophages, including into the OE (Getchell et al., 2002). IL-6 is expressed in OE after bullectomy (Nan et al., 2001). However, immune cells contain multiple cytokines that are co-released, and the role of a cytokine can differ from one environment to another (reviewed in (Borsini et al., 2015)). Thus, it is important to identify which cytokines are present in the OE at the same time, as they may have a synergistic effect. More studies are needed to understand the role of releasing TNF- α , IL-6, and MCP-1 together in the mouse OE.

Limitations of study

CD-68 was used throughout this study to label macrophages in the OE, however several other macrophage labels were used but not found to reliably label macrophages. This could be because the methodology used for CD-68 IHC did not have favorable conditions for the other markers. Likewise, fixation can often cause differences in antibody labeling and paraformaldehyde (4%) may not be the ideal fixative for some antibodies. Iba1 and F4/80 are considered markers of activated and mature macrophages. While it could be the case that macrophages that enter the OE due to sub P initiated dilation and chemotaxis have not encountered a signal in the tissue to become fully activated, and thus only labeled for CD-68, it seems unlikely that this would be true for the OE tissue of bulbectomized mice, as OSNs would be actively degenerating at the time. The number of macrophages in the OE could also have been increased due to cellular division (Evans et al., 1973; Amano et al., 2014). Cell lineage markers could be used to determine if macrophages were recently undergoing cell division, but it would be difficult to ascertain the source of the dividing cells, as cell lineage marker BrdU, which would label dividing and subsequent daughter cells, is toxic and not typically applied directly to the nasal passages, and intraperitoneal application of BrdU would label macrophages originating from blood and bone monocytes as well as those cells that divided locally.

In previous measurements of TNF- α that were not reported in this chapter, all samples, including saline controls, yielded high levels of TNF- α from lavages as well as media collected from plates of treated RAW 264.7 macrophages. The high levels of TNF- α in saline samples was concerning, and so great care was taken to use sterilized

saline in future replications, which resulted in the experiment described in the results that yielded saline samples with no measurable TNF- α levels, but also no TNF- α measured from sub P treated samples. Thus, future studies are warranted for determining whether sub P is not capable of initiating release of TNF- α from infiltrating macrophages, or if the experimental design needs to be altered, such as the timing of lavage collection, in order to accurately measure TNF- α from nasal lavage.

In this study, I focused on the macrophage as a possible source of cytokines in the mouse OE. However, there are likely many sources for cytokines. Studies have shown that epithelial cells are an important source of cytokines in the airways. Cytokines released by airway epithelial cells are chemotactic for T-cells and eosinophils, which are the typical immune cells present in allergic responses (Renauld, 2001). However, it seems unlikely that sustentacular cells would release cytokines after such a delay. That is not to say that the sustentacular cells of the OE do not release cytokines, but only that they may require a different set of circumstances in order to do so, and may not release cytokines in response to local sub P release. Given the anatomy of the nasal cavities and the portions of OE slices used for the cytokine release experiments, the nasopharynx were likely also included in the tissue slices. The nasopharynx would be a source for other cytokine expressing cells, especially lymphoid cells (Kiyono and Fukuyama, 2004). T lymphocytes are a source of cytokines: IL-2, IFN- γ , TNF- β from TH1 lymphocytes, and IL-4, IL-5, IL-13 from TH2. T lymphocytes are typically activated in response to allergic responses, but the cytokines they express were not looked for in this study, so while unlikely, the role of T lymphocytes as a cytokine source in neurogenic (sub P-initiated) inflammation cannot be ruled out at this

time. Neutrophils are immune cells that respond to damage before macrophages and have been shown to increase cytokine levels in the nasal mucosa, as well. Thus, neutrophils likely also contributed to the expression of cytokines after sub P treatment. Future studies would need to be performed to determine the relative contribution of each type of immune cell. Studies in which either neutrophils or macrophages are depleted (with Ly6g monoclonal antibody or clodronate, respectively) and then cytokine release in response to sub P is measured could provide clarification.

Conclusions

Inflammation can be initiated a number of ways. Well studied is the activation of toll-like receptors in response to LPS, and specific to the nasal mucosa, olfactotoxicants such as satratoxin G, bulbectomy, and TNF over-production have been illustrative of aspects of inflammation that occur in the OE. For example, we know that neutrophils are early responders to damage caused by satratoxin G. Bulbectomy has been used to initiate degeneration and regeneration so we can study the role of factors such as macrophages, LIF, and MIP-1 α . The construction of a transgenic model for TNF overproduction demonstrated the hindrance of neuroregeneration by this cytokine. Neurogenic aspects are less well-understood. Here I demonstrate that macrophages are increased in the mouse OE through the actions of the inflammatory neuropeptide, sub P. In Chapter 2, I demonstrated the presence of components of the neurogenic inflammatory response in the mouse OE. Activation of TRPV1 and TRPA1 causes the local release of neuropeptide sub P and plasma extravasation. The increase in macrophages seen here could be a product of plasma extravasation as well as release

of factors that attract macrophages. Understanding the role of sustentacular cells and microvillous cells and their responses to sub P may also be informative in understanding the process of neurogenic inflammation in the mouse OE. Future work is warranted to determine what signals may be released in the OE to initiate the expression and release of cytokines such as TNF- α , and if these signals are present during neurogenic inflammation.

CHAPTER 5- Discussion

Significance and contributions

Inflammation in the nervous system is linked to neurological disorders and neurodegenerative disease, and can be initiated by exposure to pollutants. The olfactory epithelium (OE) is directly exposed to the environment, and as such, exposed to irritants, but the process of inflammation has not been well described in this tissue. Understanding inflammation in the OE is especially important because it is a neuroepithelium that is readily capable of neurogenesis. Therefore acute inflammation occurs in the tissue subsequent to damage or irritation, but must be resolved in order to allow for regeneration. Thus, as a precursor to understanding chronic inflammation, and to better understand the environment in which neurogenesis occurs in the mouse OE, I investigated the initiation of acute neurogenic inflammation in the mouse OE.

The studies of this dissertation identify the trigeminal nerve, transient receptor potential (TRP) channels, substance P (sub P) and non-neuronal cells as key factors in the initiation of neurogenic inflammation. My studies also reveal that neurogenic inflammation in the mouse OE involves the activity of macrophages and cytokines (Figure 5.1). In this chapter, I will discuss the implications of these results, focusing on what we can learn from the location and types of irritant sensing channels found to be expressed and functional in the mouse OE, and the implications of the involvement of mediators that are generally considered part of the chronic inflammatory response- macrophages and cytokines. Additional hypotheses and future implications of this work will be discussed, including the role of neurogenic inflammation in the genesis of chronic inflammation in the mouse OE, the role of chronic inflammation of the olfactory system

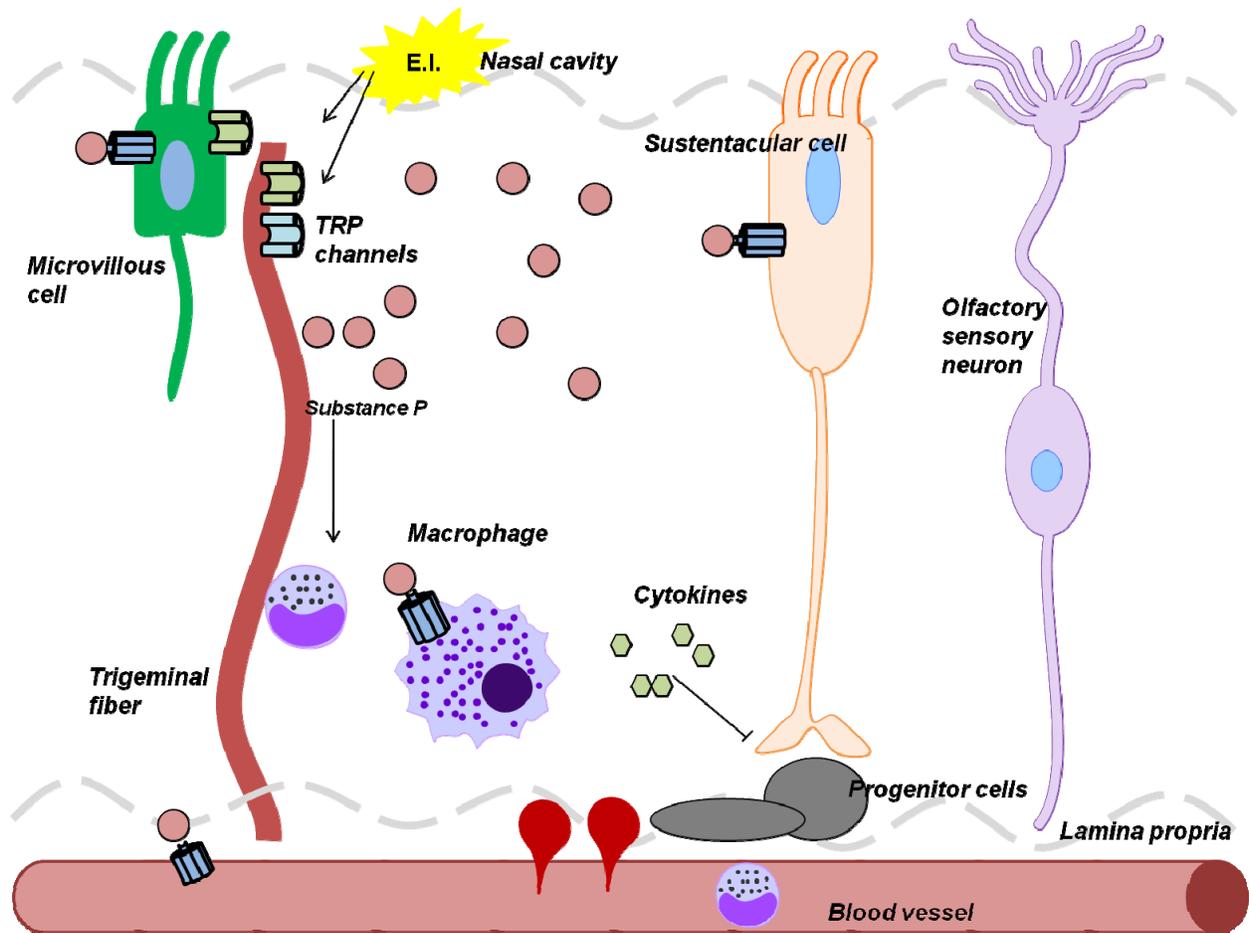


Figure 5.1. Summary of experiments and findings. In the experiments of this dissertation, I tested the hypothesis that irritants initiate neurogenic inflammation in the OE through activation of the trigeminal nerve and neuropeptide release. The pattern of immunoreactivity of irritant-sensing channels TRPV1 and - TRPA1 in OE tissue suggests their role in activation of the trigeminal fiber and secondary chemosensory cells (i.e., microvillous cells) in the OE. The pro-inflammatory neuropeptide sub P was released from tissue upon exposure to TRPV1 and TRPA1 agonists capsaicin and cinnamaldehyde, causing plasma extravasation, a hallmark of inflammation. Sub P treatment of OE tissue induced plasma extravasation and the increase in macrophages within the OE. Cytokines TNF- α , IL-6, and MCP-1 were measured from OE tissue

Figure 5.1 (cont'd) treated with sub P. The receptor for sub P, neurokinin-1 was found throughout the OE, including on macrophages and microvillous cells within the OE, implicating these cells in neurogenic inflammation of the OE.

in the pathogenesis of disease in the central nervous system, and possible mechanisms of modulation.

Initiation of neurogenic inflammation in the mouse OE

The studies of Chapter 2 point to different ways neurogenic inflammation can be initiated: through the activation of irritant-sensing channels TRP-Ankyrin1 (A1) and TRP-Vanilloid1 (V1) on trigeminal fibers and through trigeminal fiber innervation of secondary chemosensory cells that employ various other mechanisms of activation. My immunohistochemical studies of TRPA1 and TRPV1 indicated that their expression was not limited to trigeminal fibers. TRPA1 immunoreactivity co-localized with TRP-subfamily Melastatin member 5 (M5)-expressing microvillous cells (TRPM5-MV). TRPM5 is known in the gustatory system as a channel with sweet, amino acid, and bitter sensing capabilities. TRPM5 signaling has also been found in brush cells of the respiratory and gastrointestinal tracts, and thus, may be a more common component of mammalian sensory transduction with an important role in allowing tissues to sample aspects of their environment, rather than solely part of the taste system (Kaske et al., 2007). In the nasal cavity, TRPM5-MVs respond to odorous irritants such as ethyl propionate and valeric acid (Lin et al., 2008). TRPM5-expressing OSNs are also purported to allow for the transduction of pheromone responses in the OE (López et al., 2014).

Trigeminal fibers are known to occasionally innervate IP3R3-MV (Hegg et al., 2010). However, the role of IP3R3-MV as a secondary chemosensory cell is not fully understood, and thus we do not fully understand the role of this cell in the OE or how to

target its activities. For example, we know that solitary chemosensory cells express TRPM5, which is a monovalent-selective cation channel gated by intracellular calcium levels. When a stimulant, such as a sweet tastant, binds to a G protein coupled receptor, the $\beta\gamma$ subunit dissociates and creates diacylglycerol (DAG) and inositol triphosphate (IP3) from phosphatidylinositol biphosphate (PIP2). IP3 activates the release of calcium from IP3-Receptor3 gated intracellular stores. This increase in intracellular calcium then activates the TRPM5 channel and allows the entrance of Na^+ (Liu and Liman, 2003). The signaling mechanisms by which the IP3R3-MV transducts information about diverse stimulants was previously unknown. Chapter 3 describes experiments investigating IP3R3-MV responses to ATP, a major alarmin or danger-associated molecular patten released upon cellular injury, and indicated involvement of both ionotropic and metabotropic purinergic receptors. My immunohistochemical (IHC) studies of TRPA1 and TRPV1 expression suggested that IP3R3-MVs are closely associated with structures with both channels, likely trigeminal fibers, but no apparent responses to TRPA1 agonist cinnamaldehyde were exhibited by the IP3R3-MV. Regardless, there appear to be several mechanisms of initiating neurogenic inflammation even when looking only at TRPV1 and TRPA1 location. Having multiple mechanisms can expand the range of irritants to which the trigeminal fiber and the OE itself can respond. All of these mechanisms may not necessarily lead to the initiation of neurogenic inflammation, and so the activation of these different cell types should be further studied in order to understand their role in the OE.

To assess the function of TRPV1 and TRPA1 receptors in the mouse OE, the prototypical TRPV1 and TRPA1 agonists were used in live-cell calcium imaging studies;

capsaicin was used to activate TRPV1 and cinnamaldehyde to activate TRPA1. It is through these calcium-imaging studies that we can begin to determine the characteristics of responses to TRPA1 and TRPV1 activators. I have described which cells do and do not respond to cinnamaldehyde and capsaicin and the importance of concentration in differentially activating the different cell types. It is important to note, however, that a much wider range of molecules likely activate these receptors. A recent study indicates that lipopolysaccharides (LPS), a by-product of gram-negative bacteria, can activate TRPA1 and initiate a neurogenic inflammatory response, though to a lesser degree than through the activity of toll-like receptors (TLRs) on immune cells, considered the canonical mechanism of LPS activation of inflammation. The researchers found that the shape of Lipid A was an important determinant of whether LPS could activate TRPA1 (Meseguer et al., 2014).

TRPV1 can be activated by components of particulate matter (PM). Residual oil fly ash (ROFA) is a common PM pollutant. Treatment of airway cells with ROFA can lead to common signs of biological activity typical to inflammation, such as increased interleukin (IL)-8, IL-6, tumor necrosis factor- α (TNF- α), and increased intracellular calcium. The release of these factors of inflammation was blocked with use of the TRPV1 antagonist capsazepine, suggesting that inflammation is initiated by ROFA through activation of capsaicin-sensitive fibers such as trigeminal fibers. ROFAs have a negative charge, but become surrounded by a protionic micro-environment, and it is in this way that ROFA is expected to activate TRPV1, as TRPV1 is known to be activated by acid (Veronesi and Oortgiesen, 2001).

These studies involving non-classical irritants demonstrate how unexpected agents can initiate neurogenic inflammation, and that the activation of TRP receptors may occur more expansively than previously described. My studies showed that TRPA1 and TRPV1 classical agonists cinnamaldehyde and capsaicin, respectively, cause changes in intracellular calcium, sub P release, and plasma extravasation; it would be interesting to follow up on these studies using other irritants and particulate matter to investigate whether changes in intracellular calcium, subP release, and plasma extravasation also occur in response to these noxious agents and whether the effects are through TRPA1 or TRPV1 activity.

The role of particulate matter in inflammation and disease

In understanding the initiation of neurogenic inflammation in the OE, PM from the environment is of particular interest because it enters our body through airway passages on a regular basis, including the nose. Adverse health effects have been linked to exposure to PM, including in the central nervous system. In the 1980s in Germany, high ambient pollution levels were connected to increased mortality and morbidity, which led to a number of studies, in Germany and elsewhere, investigating disease and mortality caused by pollution (Spix et al., 1993). A study from 1995-1996 in Boston, MA, USA, showed that nitrogen dioxide and PM_{2.5} were associated with life-threatening arrhythmia, and that PM_{2.5} concentrations were higher in the hours and days before onset of myocardial infarction in a large group of patients (Peters et al., 2001). Thanks to heightened awareness in recent history, air quality has improved in most industrialized nations. While PM above PM_{2.5} is limited and well controlled,

ultrafine PM remains an area of concern because ambient pollution is made up more and more of particles of size, distribution, and composition that have not been thoroughly studied, and thus the mechanisms of toxicity of ultrafine PM still need to be understood. Indoor air quality is also of great concern given that many people spend the majority of their day indoors. Smoking, cooking, candle or incense burning, cleaning product use, and mold can all contribute to poor indoor air quality and adverse health effects.

Exposure to PM increases systemic inflammation, which can directly affect the brain and central nervous system. This was demonstrated in a study in which mice were exposed to concentrated ambient particles from a polluted area composed of metals, nitrates, sulfates, and elemental and organic carbons for two weeks. Exposure to PM significantly increased the expression of NF κ B in mouse brain fractions, a transcription factor that promotes the expression of proinflammatory factors such as nitric oxide synthase, complement factors, IL-1 α , and TNF- α (Campbell et al., 2005). Increased black carbon, a marker for traffic pollution, predicted decreased verbal and non-verbal intelligence scores in children from Boston, MA from 1986-2001 (Suglia et al., 2008). The majority of olfactory bulbs of autopsy subjects from Mexico City, an area of high levels of pollution, exhibited immunoreactivity to beta amyloid proteins in neurons, glia, or blood vessels. Patients living in Mexico City, compared to control groups, score lower on the University of Pennsylvania Smell Identification Test olfactory test (Calderón-Garcidueñas et al., 2010). A study of healthy children and young adults of Mexico City that died suddenly revealed that exposure to air pollution causes neuroinflammation, disruption of the blood-brain barrier, altered innate immune

responses in the brain, and accumulation of A β 42 and α -synuclein starting in childhood (Calderón-Garcidueñas et al., 2008).

As described above, exposure to PM can cause increases in markers of neurodegenerative disease, and this pathology often begins in the olfactory system. Olfactory dysfunction is a common precursor to many neurodegenerative diseases, including Alzheimer's disease (AD) and Parkinson's disease (PD) (Albers et al., 2006; Haehner et al., 2011), and alterations in olfaction is being used as an early biomarker for neurodegenerative disease diagnosis and disease progression (Attems et al., 2014). Approximately 90% of early PD patients experience olfactory dysfunction, and this symptom can precede the onset of motor symptoms. The changes in olfaction in neurodegenerative diseases involve the OE, olfactory tract, primary olfactory cortices, and their secondary targets (Doty, 1989). Olfactory impairment is also prevalent in patients with multiple sclerosis, likely due in part to reduced proliferation of neural stem cells, as the subventricular zone of mice with multiple sclerosis-like pathology of the forebrain have decreased neuroblast generation, which appears to cause impaired long-term olfactory memory (Tepavčević et al., 2011). Asthma, a form of chronic inflammation of the airways, is linked to a modest increase in AD and dementia and to a shorter lifespan expectancy in AD patients (Eriksson et al., 2008). Allergy appears to enhance phosphorylation of tau protein, a protein associated with AD (Sarlus et al., 2012). Accumulation of ultrafine PM has been identified in the postmortem olfactory bulb and OE (Calderon-Garciduenas et al., 2011; Calderon-Garciduenas et al., 2010; Calderon-Garciduenas et al., 2012), and PD and AD -like neuropathology in human olfactory bulb can be initiated by toxicants, such as those found in diesel exhaust

(Calderon-Garciduenas et al., 2004; Levesque et al., 2011). Exposure to manganese has been especially implicated in the etiology of idiopathic PD. Manganese can travel to the olfactory bulb, striatum, cortex, and cerebellum carried along by nanoparticles along the olfactory tracts. Welders, workers involved in battery production, ferro-alloy production, and processing of ore are particularly vulnerable to manganese exposure, and motor abnormalities are prevalent amongst a group of workers at a ferro-alloy plant that were studied, along with children and the elderly living in the area around the plant (Zoni et al., 2012).

While scientists are beginning to appreciate a link between environmental factors and neurodegenerative diseases (Pan-Montojo and Reichmann, 2014), much remains to be studied in understanding the mechanisms involved in linking the two. For example, the olfactory system in PD and the substantia nigra, the area of the brain most classically affected in PD, are differentially affected. In studying the expression of α -synucleopathy as a marker of disease, it was found that dopaminergic cells are largely unaltered in the olfactory bulb, whereas in the substantia nigra, they are overwhelmingly targeted by α -synucleopathy. However, glutamatergic cells and cells expressing calcium-binding protein and sub P cells are the most vulnerable along the olfactory pathway (Ubeda-Bañon et al., 2010). Understanding the different mechanisms involved is the key to treating inflammation and subsequent disease.

Inflammation and recovery

Complex cellular and molecular interactions within the central nervous system are initiated upon spinal cord and brain injuries. These processes are initiated in an

attempt to repair the initial tissue damage. GeneChip[®] microarray technology has demonstrated that there is an upregulation of transcription and inflammation genes shortly following spinal cord injury and a downregulation of structural proteins and neurotransmission proteins in the same timespan. At later timepoints, growth factors, axonal guidance factors, and extracellular matrix genes are upregulated. This indicates that the tissue is making attempts to repair itself. However, there is also an increase in stress genes and proteases, and down-regulation of mRNAs associated with the cytoskeleton and synapses, which suggests that the tissue is struggling to maintain function and survive (Bareyre and Schwab, 2003).

Recently, there have been a number of studies targeting neuroinflammation in order to treat neurodegenerative disease. But in many clinical trials, reducing inflammation was actually found to have adverse effects on the disease. Despite positive results in animal studies in which progesterone was found to decrease inflammation, the treatment of patients with traumatic brain injury with progesterone in a phase 3 randomized clinical trial was found to have no effect, or slightly harmful effects, compared to placebo (Skolnick et al., 2014). Likewise, the treatment of amyotrophic lateral sclerosis with the antibiotic minocycline to decrease inflammation was found to have an adverse effect on patients, as well (Gordon et al., 2007). Without fully understanding the role of inflammation in these conditions, we will not fully understand which aspects need to be targeted. Furthermore, these interventions were in patients who already had clinical diagnoses of diseases, and thus the inflammation occurring may be trying to alleviate, albeit ineffectively, the dysfunction in the system, and thus generally trying to decrease inflammation may be impeding the body's intrinsic attempt

to control the condition. Thus, it may not be a matter of inflammation being a harmful process that needs to be stopped, but rather, inflammation may have a role in the recovery that we need to better understand. A better understanding of all the factors involved in inflammation will provide a fuller appreciation of which factors cause an adverse versus a protective outcome.

Macrophages and cytokines such as TNF- α are often considered part of the chronic inflammatory response due to the latency of their actions and their connection to cell death and proteinopathies. TNF- α initiates cell death in the OE through the activation of caspases 1, 2, and 3 (Suzuki and Farbman, 2000). AD patients have an increased degenerative potential in brain tissue compared to age-matched controls due to increased activation of TNF- α receptor associated death domain and caspase-3 pathways (Zhao et al., 2003). Cytokines TNF- α , IL-1b and IL-6 stimulate protein kinases involved in tau hyper-phosphorylation in AD (Morales et al., 2010). Adverse effects of TNF- α are also linked to chronic inflammatory conditions of the nose, such as chronic rhinosinusitis. A model of chronic rhinosinusitis was created by genetically targeting sustentacular cells and inducing the cell type to produce TNF- α , allowing for the production of TNF- α in a spatially and temporally controlled manner. In this model, a 20-fold increase in the level of TNF- α after 7 days corresponds with a large infiltration of immune cells into the tissue. Fourteen days later, while the epithelium appears structurally normal, olfactory sensation and transduction is significantly impaired. By forty-two days, the OE is depleted of neurons and electrical responses. Remarkably, with cessation of TNF- α production, the epithelium recovers its structure and electrical

responses after two weeks (Lane et al., 2010). This study showed us the potent effect of just one cytokine expressed chronically in the OE.

Bulbectomy initiates apoptosis of OSNs, infiltration of macrophages, and proliferation of olfactory progenitor cells, but when researchers depleted macrophages using clodronate capsules, they found a significant decrease in the thickness of the OE, number of OSNs, and number of proliferating cells, compared to control tissue. This suggested that macrophages support neuronal survival and proliferation (Borders et al., 2007a). A follow-up study identified no less than 4,000 genes differentially up-regulated in tissue with macrophage activity after injury versus macrophage-depleted tissue after injury (Borders et al., 2007b). There can be many factors at work here, but macrophages are a major source of cytokines, and some of these cytokines may be necessary for regeneration and recovery of neurons after injury. For example, the cytokine leukemia inhibitory factor (LIF) is important in injury-induced neurogenesis, as its expression increases after injury and exogenous LIF increases basal cell proliferation. Furthermore, LIF knockout models do not exhibit injury-induced proliferation (Bauer et al., 2003). Evidence even exists for a pro-regenerative role for TNF- α , a cytokine that is almost universally regarded as a pro-inflammatory mediator. TNF- α knockout mice with brain injury performed better on rotorod and balance beam tests than injured WT mice during the acute phase of the injury. However, wildtype mice recovered from injury faster than knockout mice, and knockout mice also had more cortical tissue loss than wildtype (Scherbel et al., 1999). TNF may even protect neurons against neurodegeneration, as pre-treatment of hippocampal cell cultures with TNF- α

and TNF- β lessened β -amyloid-induced neuronal degeneration and iron toxicity (Barger et al., 1995).

Studying inflammation to understand regeneration

We can learn more about the role of inflammation in regeneration by studying animals and tissues in which regeneration readily occurs in response to injury. For example, a study of neurogenesis following injury of the zebrafish brain identified the role of inflammatory factors in the activation of proliferative glial cells. The brain of the zebrafish is very plastic. Even after traumatic brain injury to the telencephalon, tissue architecture can be restored and lost neurons can be replenished primarily by the activity of radial glial cells. To determine whether inflammation is needed to initiate the proliferative response of radial glial cells, inflammation was produced in the zebrafish telencephalon without tissue injury by injecting yeast product zymosan A. This was followed by increased markers for proliferation and then increased proliferation of radial glial cells. The investigators then used a molecular transcriptome to screen for genes that were upregulated in radial glial cells after injury and found a gene of interest, CysLT1, a receptor for inflammatory factors known as leukotrienes. Leukotriene C4 is a ligand for CysLT1 and its injection into zebrafish brain caused significantly elevated proliferation, up to 21 days after administration. This study indicated the role of inflammatory factors leukotrienes in signaling injury and initiating proliferation of progenitor cells upon traumatic brain injury (Kyritsis et al., 2012).

The role of non-neuronal cells in regeneration

Immunohistochemical studies presented in Chapter 2 demonstrate apposition of TRPV1 immunoreactivity to the IP3R3-MV, and suggested innervation of this cell type by trigeminal fibers. Occasional contact of the IP3R3-MV with trigeminal fibers labeled with antibody to sub P was described previously by our lab (Hegg et al., 2010). This previous work suggested a function of the IP3R3-MV as a secondary chemosensory cell due to its innervation by trigeminal fibers and its responsiveness to bioactive compounds such as purinergics and neurokinins, much like the solitary chemosensory cell of the respiratory epithelium of the nasal cavity (Lin et al., 2008). Experiments from Chapter 3 demonstrated that the IP3R3-MV is at least not responsive to the TRPA1 agonist cinnamaldehyde and does not express irritant-sensing channels TRPA1 or TRPV1, but could be involved in modulating pain and irritant signaling, as the IP3R3-MV does express the receptor to sub P, NK-1R, and is contacted by trigeminal fibers. The IP3R3-MV is known to release neurotrophic factor Neuropeptide Y (NPY) in response to the injury signal ATP (Kanekar et al., 2009); NPY is not the only factor regulated by ATP in the OE. Our lab has also demonstrated that ATP can upregulate fibroblast growth factor-2 and transforming growth factor- α (Jia et al., 2010). It would be interesting to know if NPY is released under other conditions, such as in response to irritation.

In many systems, NPY has been shown to have a role in modulating inflammation (Malva et al., 2012). NPY has been found to modify neurogenic inflammation through the inhibition of inflammatory neuropeptide release through inhibition of voltage-gated calcium channels. In a study of guinea pig atria, NPY inhibited neuropeptide release in response to electrical stimulation of nerves but had no

effect on neuropeptide release stimulated by capsaicin treatment, suggesting that NPY inhibits voltage-sensitive calcium channels of sensory fibers (Giuliani et al., 1989). NPY was also found to inhibit the release of sub P in response to electrical stimulation of the cat tibial nerve (Duggan et al., 1991). Plasma extravasation caused by stimulation of excitatory non-adrenergic non-cholinergic nerve fibers of the airways is inhibited by NPY, which is thought to be by inhibiting sub P release because NPY did not have an inhibitory effect on plasma extravasation caused by exogenous sub P (Takahashi et al., 1993). Furthermore, NPY could decrease inflammation by effecting release of cytokines from monocytes, as seen with the dose-dependent application of NPY causing an inhibition of IL-6 from macrophages of the spleen (Straub et al., 2000a, 2000b). NPY also appears to regulate the change of microglia to phagocytic behavior triggered by cytokine IL-1 β (Ferreira et al., 2011). It is also possible that NPY in the OE could utilize a mechanism similar to that employed by other neuropeptides that have a modulatory effect on inflammation. For example, vasoactive intestinal peptide and pituitary adenylate cyclase-activating peptide have been found to inhibit the productions of inflammatory mediators from activated microglia. This inhibitory function appears to take place through a specific receptor, VPAC1, which causes an increase in cAMP. The increase in cAMP inhibits the nuclear translocation of p65, subsequently down-regulating NF κ B binding to the promoters of inflammatory mediators such as cytokines TNF- α , IL-1 β , and IL-6 (Delgado et al., 2003).

Future hypotheses

Sub P is released into the epithelium upon irritation of the nasal cavity from trigeminal fibers, initiating plasma extravasation, infiltration of macrophages, and cytokine release, which obstructs neuroregeneration. Damage to the OE also stimulates the release of ATP. Previous research in our lab demonstrated that IP3R3 regulates the release of NPY from IP3R3-MV cells (Jia et al., 2013), and that this release is initiated by ATP (Kanekar et al., 2009). Utilizing IP3R3-knockout mice, changes in cellular composition, proliferation, and olfactory-mediated behaviors were identified. The IP3R3- knockout mouse has impaired NPY release. Beginning at the age of 2 months and lasting throughout adulthood, basal cell and immature neuron cell populations are decreased in IP3R3-knockout mice compared to wildtype mice. There is also decreased neuronal differentiation in the IP3R3-knockout mouse, which corresponds to the decrease in basal cell numbers. In addition to deficits in physiological turnover, the IP3R3- knockout mouse has a limited capacity to proliferate after injury to the OE (Jia et al., 2013). Thus, changes in NPY expression and release correspond with alterations to the morphology of the OE as well as the ability of the OE to proliferate and recover after injury. As we age, we experience olfactory dysfunction. In the aged- IP3R3-knockout mouse, a decrease in mature OSN numbers and proliferation levels is seen, as well as compromised olfactory-mediated behaviors (Jia and Hegg, 2015). Studies from our lab utilizing the IP3R3- knockout mouse have associated a decrease in the proliferative factor NPY with altered OE morphology, decreased proliferative ability, and deficits in olfaction.

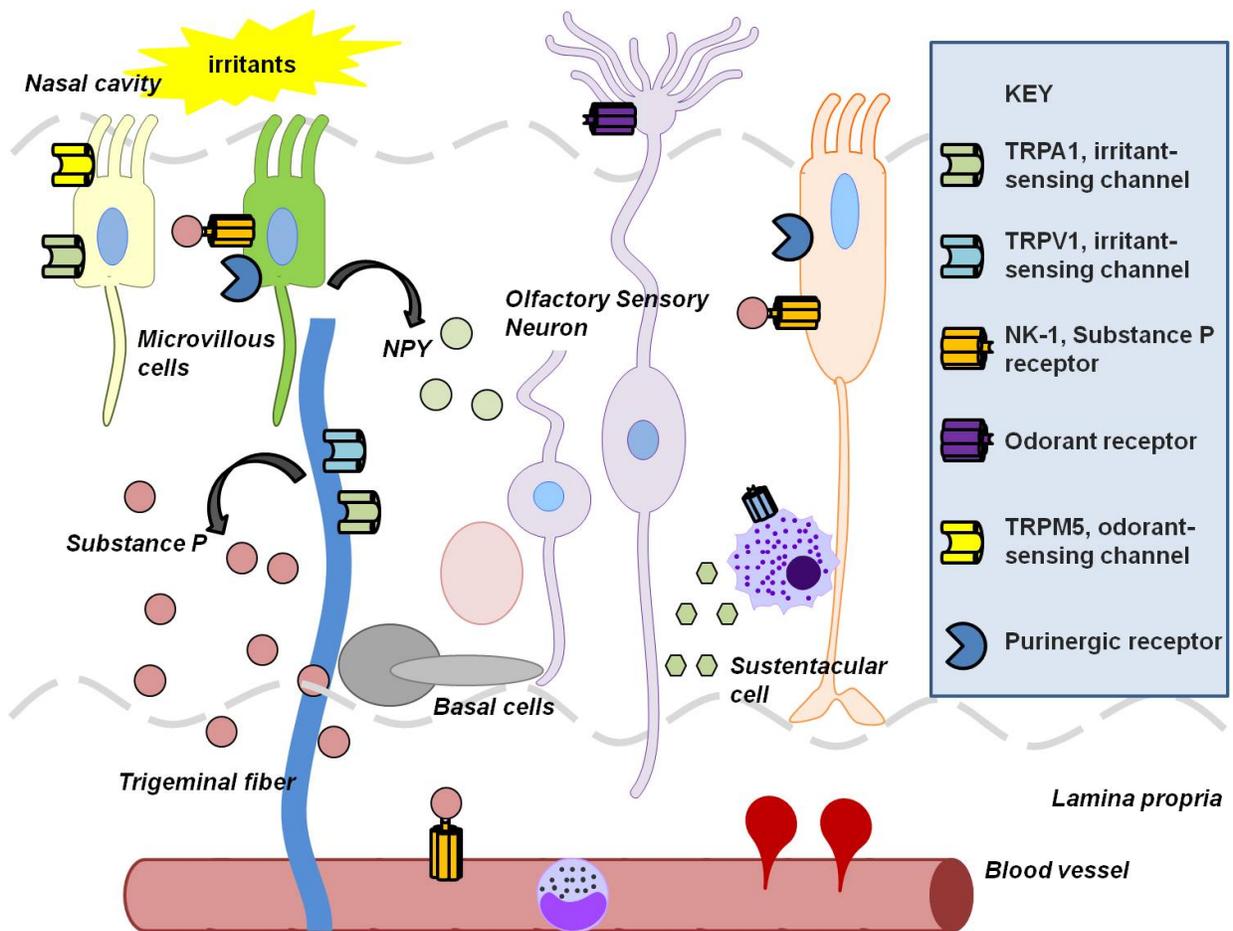


Figure 5.2. Model of future hypothesis. Environmental irritants activate TRP channels on trigeminal fibers and microvillous cells within the OE. Activation of TRP channels on trigeminal fibers leads to the release of neuropeptide sub P from the nerve fibers into the local environment. Sub P initiates mechanisms of inflammation, including plasma extravasation, macrophage infiltration, and cytokine release. NPY is a neuroproliferative factor expressed in IP3R3-MV cells of the OE. The release of NPY is initiated by ATP and causes an increase in proliferating cells in the OE. NPY is also known to inhibit the release of sub P from nerves innervating the airways.

Another mechanism by which the OE may maintain the balance between inflammation and regeneration is through the cannabinoid system, which was recently described to have a role in regeneration of the mouse OE by our lab (submitted papers, 2015). Cannabinoids, through the activity of their receptors CB1 and CB2, have also been shown to have a role in immunomodulation. CB1 is primarily expressed in the central nervous system, whereas CB2 is primarily expressed in the periphery, including T cells and macrophages (Buckley et al., 2000). Expression of CB2 on macrophages depends on the state of the cell. CB2 is undetectable in residential peritoneal macrophages, but macrophages primed by IFN- γ or thioglycolate express high levels of CB2. Not all inflammation activators caused this up-regulation in receptor expression, and LPS priming did not cause a similar increase (Carlisle et al., 2002). The non-psychoactive component of cannabinoids, called cannabidiol, causes an increase in IL-12, a decrease in expression of IL-10, and also causes a decrease in chemotaxis of macrophages (Sacerdote et al., 2005). Cannabinoids also cause a decrease in the release of nitric oxide upon microglial cell activation (Waksman et al., 1999). An interaction between the cannabinoid system and trigeminal innervation has been described. Cannabinoids cause the release of sub P, and CB1 receptor antagonist AM-251 inhibited the internalization of NK-1R, which occurs upon sub P binding (Zhang et al., 2010). The synthetic cannabinoid WIN also initiates the release of neuropeptide from trigeminal ganglion, but not through TRPV1 (Price et al., 2004). It would be interesting to further my current studies and investigate the relationship between cannabinoid signaling and neurogenic inflammation in the mouse OE.

I hypothesize that exposure to environmental irritants initiates both inflammatory and regenerative mechanisms within the OE via the release of neuropeptides sub P and NPY, respectively (Figure 5.2). Inflammatory mediators inhibit neuroregeneration, as seen in the overexpression of TNF studies from Lane and colleagues. NPY aids in proliferation of the OE, and abates neurogenic inflammation, by either inhibiting the release of sub P, or inhibiting the action of sub P on effector cells like macrophages.

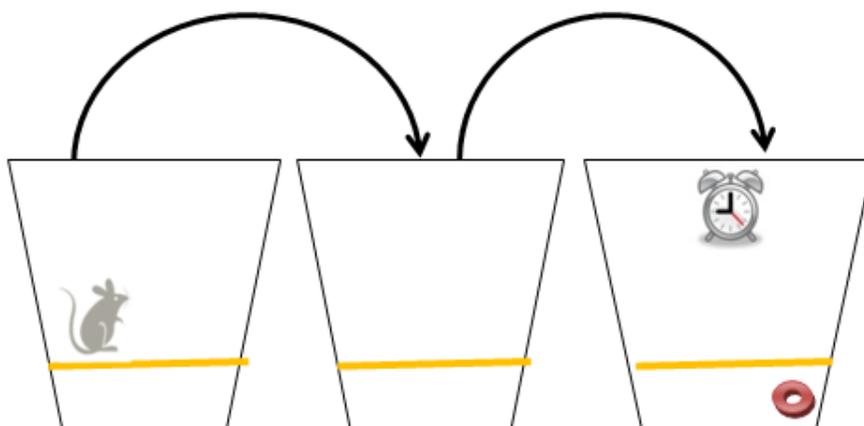
Final conclusions

Earlier studies, including from our lab, have identified neurotrophic factors that aid in the recovery and neuroproliferation of the mouse OE, especially in response to injury. What is less described is the role of inflammation. The studies in this dissertation identify TRP channels, trigeminal fibers, non-neuronal cells, and macrophages as factors involved in neurogenic inflammation of the mouse OE. Understanding inflammation in the OE is important because it is a neuroepithelium that is readily capable of neurogenesis. Focusing on neurogenic inflammation adds to our knowledge of how the environment can affect our health, which is especially important in considering the OE, as this is a tissue that is open to the environment and exposed to PM, irritants, and pollution. Acute inflammation occurs in tissue subsequent to damage or irritation, but must be resolved in order to allow for regeneration. Thus, as a precursor to understanding chronic inflammation, and to better understand the environment in which neurogenesis occurs in the mouse OE, I have investigated the initiation of acute neurogenic inflammation in the mouse OE. Although acute inflammation can be beneficial in recovery from damage, it is important to understand underlying mechanisms involved, as progression to chronic inflammation can lead to

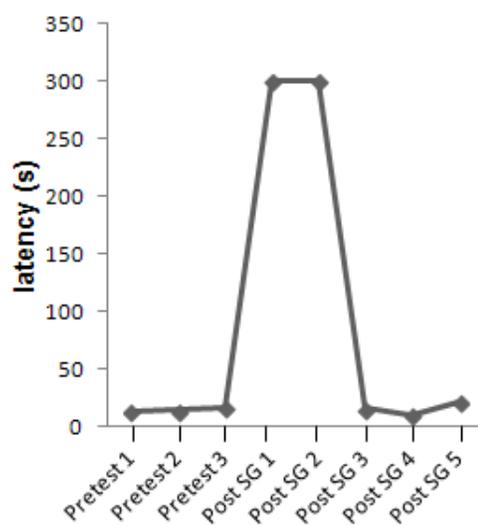
disease states. Therefore, these studies contribute to the understanding of the effects of environmental pollutants on our health and suggest future studies to add to our current understanding.

APPENDIX

A



B



C

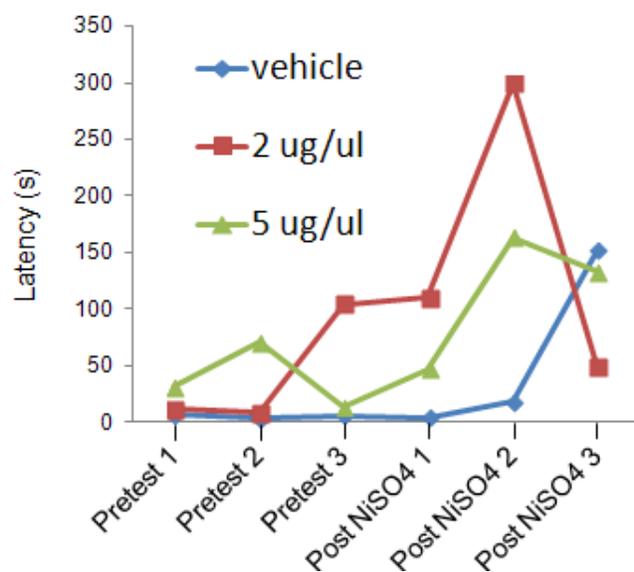


Figure S1: Buried food test was used to assess olfaction in mice exposed to environmental toxicants satratoxin G and nickel sulfate. (A) Mice were found to have significantly increased latencies in uncovering buried food 2 and 4 days following exposure to satratoxin G. Decreased latency by the seventh day post-exposure suggests that numbers of mature OSNs have recovered. (B) Increases in latency to uncovering buried food were not as profound with nickel sulfate exposure, but likely exist, as well.

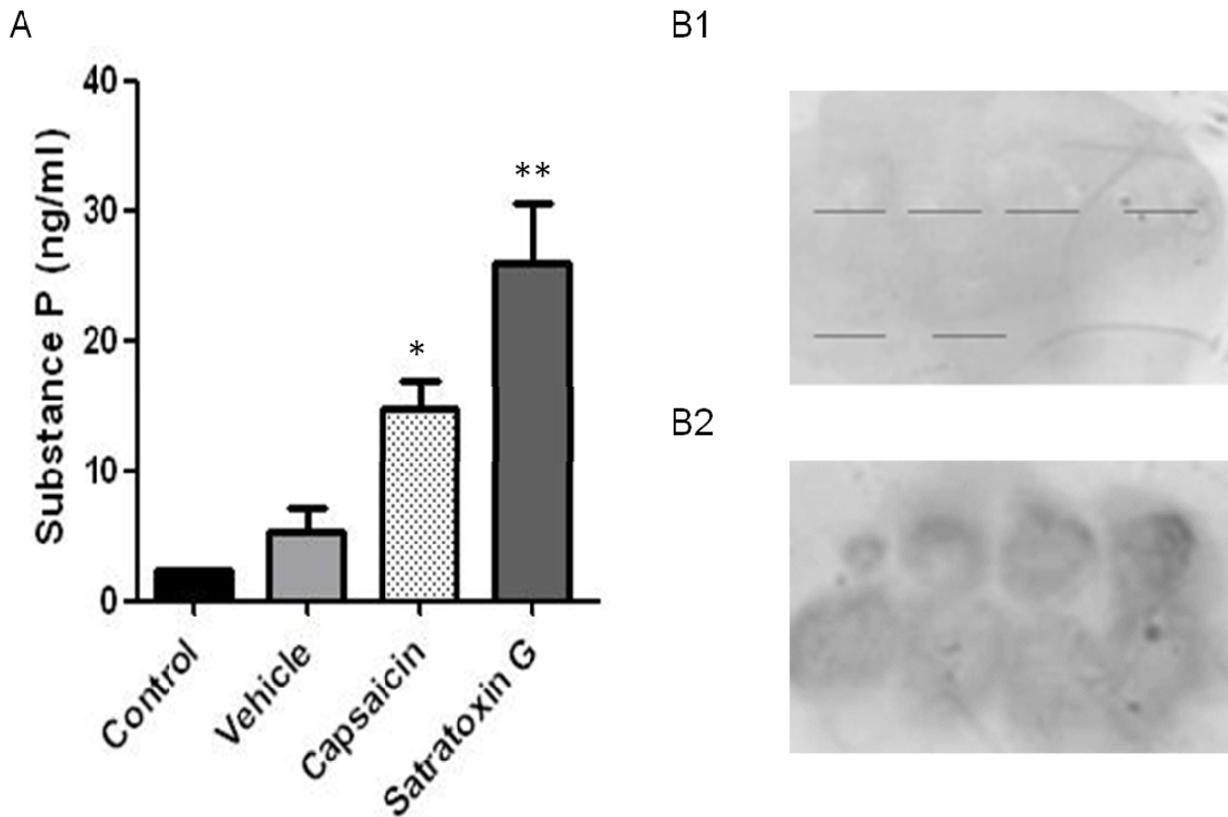


Figure S2: Satratoxin G and nickel sulfate induce sub P release from neonatal OE slices. (A) Capsaicin and satratoxin G significantly increase sub P released from OE slices. OE slices (n= 3 animals, 3 slices/treatment) were incubated in media in the presence of 100 ul of Ringer's solution (control), ethanol (vehicle), capsaicin (100 uM), or satratoxin G (60 ug/ml). $P < 0.05$ vs *control or **vehicle (one-way ANOVA followed by Bonferonni post-hoc test). (B) The release of sub P by nickel sulfate could not be measured using an ELISA due to the interference of metals with the sub P ELISA kit. Instead, nitrocellulose membrane labeling after exposure of slices to nickel sulfate was used. OE slices cultured on nitrocellulose membrane with (B1) nickel sulfate (5 ug/ul) or (B2) Ringer's solution for 1h. Placement of slices is indicated by underlining. Immunoreactivity for sub P indicated that sub P was release from all slices, not just in anterior portions.

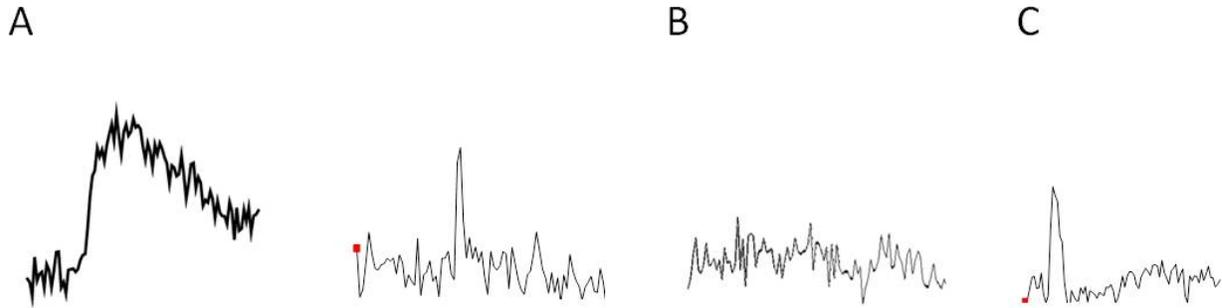


Figure S3: Responses to cinnamaldehyde are mediated through the activation of TRPA1. (A) Representative traces of responses to cinnamaldehyde. The second trace is an example of an increased latency in a response. (B) After treatment with TRPA1 antagonist HC-030031, responses to cinnamaldehyde are blocked. (C) After TRPA1 antagonist HC-030031 is washed out, responses to cinnamaldehyde return (n=1).

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