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Targeting Fatty Acid-Activated Pathways in the Somatosensory System

Tian Yu
Utah State University

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TARGETING FATTY ACID-ACTIVATED PATHWAYS IN THE SOMATOSENSORY SYSTEM

by

Tian Yu

A dissertation submitted in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY in

Biology

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Logan, Utah

2010
ABSTRACT

The Role of Fatty Acids-Activated Receptors in Fat Perception

by

Tian Yu, Doctor of Philosophy
Utah State University, 2010

Major Professor: Dr. Timothy A. Gilbertson
Department: Biology

Given the dramatic rise in obesity and the diseases linked with it, it is becoming increasingly important to understand the mechanisms that underlie the body’s ability to recognize fat. The chemoreception of dietary fat in the oral cavity has largely been attributed to activation of the somatosensory system that relays the textural properties of fat. However, the ability of fatty acids, which are believed to represent the proximate stimulus for fat taste, to activate trigeminal ganglionic neurons has remained unexplored. In general, my research has provided the first evidence of fatty acids activating the somatosensory system by increasing the intracellular calcium concentration and generating receptor potentials. Other experiments were focused on identifying fatty acids-responsive pathways in acute isolated trigeminal neurons involved with fat somatosensory perception. My results revealed that fatty acids-activated pathways involved the release of intracellular calcium stores in subpopulations of trigeminal neurons. By using pseudorabies virus as a “live-cell” tracer, a subpopulation of lingual-
innervated trigeminal neurons was labeled. Quantitative real-time polymerase chain reaction on individual neurons showed several transient receptor potential channel markers were expressed in these labeled neurons, which indicated the identification of lingual-innervated neurons was successful. This technique helped resolve the problem of trigeminal neurons being a mixed population of cells, and confirmed the role of the release of intracellular calcium stores in fatty acid-activated pathways. Using patch clamp recording, I discovered that the linoleic acid activated signaling pathway involved the activation of G protein and phospholipase C. I further began to characterize the downstream conductance that is activated by linoleic acid in rat trigeminal neurons. Using perforated patch clamp recording, I have recorded linoleic acid-induced currents that exhibited many of the properties of transient receptor potential-like channels, suggesting that this “cellular sensor” is probably playing an important role in the somatosensory perception of fat. Taken together, this dissertation research has revealed the ability of fatty acids to act as effective tactile stimuli and identified several elements of the fatty acids-activated signaling pathway involved in the somatosensory perception of fat.

(142 pages)
ACKNOWLEDGMENTS

I have lived in the U.S. for almost five years. Everybody said I have a big improvement in my English skills, but I still encountered times that I was “stuck” in front of people. My face was turning pink, my lips were moving but I couldn’t finish even one sentence. I know this was my sign of being angry, nervous, emotional, or thankful. Yes, I am sticking here again now, thousands of words in my mouth, but don’t know where to start.

This dissertation is dedicated to the many people who continually supported and helped me.

I would like to acknowledge my major professor, Dr. Timothy A. Gilbertson, for his tremendous support and guidance in not only my graduate study, but building me up into a strong person. I came to USU from China five years ago without any experience of leaving my family for more than a month and half. From my first day in USU, Tim claimed himself as my “scientific father,” who would love to help me in both science and life. He indeed gave me encouragements that nobody has provided me as a professor or teacher in my life so far. It was he who told me again and again when I hit my low periods in experiments that science was a “creative art” that requires innovation and persistence, but the latter was always the most important quality. It was he who told me “Tian, you will be successful in future” so affirmatively even when I started to doubt myself. It was he who asked me to fight with my weakness because “every time you fight and succeed, you are stronger than before” when I first served as a teaching assistant and had to deal with 90 students. And, I could never forget that it was he who told me to
“follow my heart” and make my own decisions, no matter what decisions were, he would do his best to help me. Undoubtedly, I could never complete this doctoral program without his assistance, and his influence on me will last for a lifetime.

I would also like to express my deepest appreciation to all my other committee members. Dr. David York had been really supportive when I was seeking for future postdoctoral positions. Dr. Joseph Li and Dr. Daryll DeWald were the ones who kept encouraging me on my research and even pushing me to communicate with other scientists around the world. Dr. Anhong Zhou was the one who introduced me to Utah State and watched each and every step of my graduate study carefully. Once a while, we got together and had long discussions covering almost all parts about academic life. He tried really hard to lead me to the right track of being a successful scientist.

In addition, my sincere gratitude is going to Dr. MieJung Park. She gave me enormous assistance on my virus project. Without her help, I would not even have the courage to start this new project in my last year of graduate study which was in an absolutely new area to me. Thanks a lot, MieJung.

Also, I would like to thank the whole Gilbertson Lab. If Tim is my “scientific father,” Dr. Dane Hansen would be my “lab mother.” She was the one who helped me on a daily basis. Every time I had questions or problems on almost anything, my first reaction (or conditioned reflex) would be asking “Dane! Do you know…/Where is…/How to…/Can you help me on…?” I never had a negative answer from Dane in my five years in the Gilbertson Lab. I cannot imagine how I could survive without Dane’s help. Bhavik was my true “lab buddy.” We sat next to each other for four years, and he
was the one I spent most of time talking with in the lab. In the beginning, I was looking for his help on almost every problem in experiments. As I have grown up to be a more skilled scientist, we started to solve the problems together. We went through so many difficult times side by side that I would never forget the days and nights we spent together in the lab. Of course, Cherry and Han are the best fellow students in the world. You made the lab environment so friendly and relaxed, and that is exactly the reason I can spend so much time in the lab or even made the lab as my “home.” Arian, Hala, Doug, Spencer, and Ben gave me endless support on my experiments. Chad and Bemate are two previous undergraduate students in the lab that I would like to give special thanks to. They did their best to cheer me up and taught me how to enjoy life in Logan.

I am thankful to many friends I met in the Biology Department and CIB: Dr. Kamal Rashid, Sitaram, Kum, Hyoung, Yuho, Ninglin, Zhang Chun, Huo Junling, Shi bi, Yang Dawei, Jin Jiming, and all others. We spent such a good time together.

约莫也是同样的六月，五年前我睡在华中科技大学韵苑21栋525的床上幻想著未来的留学生活。似乎梦都还没来得及做醒，就又到毕业时节（飞光飞光，劝尔一杯酒！）。我对留学生活的幻想大概从来 Logan 的第一天起就被击得粉碎，也从来不曾想到之后要面对的，是无边无际的实验和寂寞。现在回首五年间的点滴，汇成这用中文写成的致谢外一篇，是为了更精确的表达我心中最真挚的感谢。

Jerpin, 胡静和 Michelle 是我在 Logan 最先结识的朋友。他们帮我度过了在 Logan 最困难的时光，并且给了我最初的异乡生活里最无与伦比的温暖。后来又加上 Ivy, Rachel 和许晗，我最珍贵的室友们。我和你们一起把 Logan 小镇能玩儿的
东西几乎穷尽。从摘樱桃李子到山谷里裹着毯子看星星，到万圣节走玉米迷宫，再到周末的火锅 party 和整人游戏，当然还有无数次的倾心交谈。是这些让我最后终于适应了小镇生活，最后甚至开始发自内心的享受。也因为这些，给我的 PhD 生涯里增添了不少色彩，变成人生最值得回忆的一段时光之一。

我在 Logan 也终于有机会结识许许多多背景不同的朋友，他们让我看到了学术之外更宽广的天空。吴滟是我遇到的第一个专业艺术人士，他教我用相机记录 Logan 山谷天堂一般的秋色，让我接触到一种从未想过的观察生活的方式。我想我不会成为一个摄影家，但是会努力成为一个敏锐的生活观察家。华子龙是我认识的真正享受弹钢琴的人。跟你偷偷溜进学生宿舍的地下室或者晚上十点半的艺术系琴房听你弹琴，不论那天实验是成功还是失败，都一定是我最佳的心情愉悦剂。齐旭和 Skyler 是我最后的室友兼房东。跟你一般大，却用实际行动教会我责任心，让我看到真实的家庭生活。跟你们共度的一年时光，虽然要不停的和低体温作战，却也是我在 Logan 最放松最温暖的时光。张匀律是一个几乎在任何时候都能让我马上开口大笑的小妹妹。她的开心与乐观让我 Logan 生活的最后一年充满了欢乐。

还有张志尧，谢谢你给我进行社会学的“社普”。跟你的交谈使我开始意识到自己思维的单一性，而多元化思维也正应该是一个科学家要努力做到的。当然还要谢谢你给我看你的“谢志”，启发我认认真真的来写这论文的最后几页字。

还有 Drew, 洪国钟, 汪欣, 徐妍妍, 赖超群, 梁屹, 武勇, 钞海洋, 常冉, 韩乙丁, 王琦, 李嫒, 刘朔, 邹薇, 初媛, 等等等等。谢谢你们，跟你们共度的每一天时光都清晰的刻在脑海里，没有你们，我不会成为现在快乐的我。
另外，我的 PhD 生活里不能不提到的是不能经常见面的老朋友们：王温静，徐亮，何倩，管乐，高莹，杨扬，李璇，刘丹丹，韩小飞，熊悦，沈黎平，王展。虽然我们仅仅依靠电话和网络联系，我们的友谊却从来没有因此而冲淡过。你们给过我的精神和物质支持（比如我回国请我吃的大餐，遥远的电脑技术支持，万能的图书馆账号…），支撑着我完成洋插队的学业，从“老鱼”成功进化为“灭绝师太老鱼”，却仍然没有磨灭当初的理想和激情。

最后是我的家人。我的爸爸妈妈大概是这个世界上能够想象到的最完美的父母了。他们教我建立了恰当的价值观和评判标准，然后给我充分的自由来做我自己的决定。不论我如何选择，他们都尽最大的可能给我支持和鼓励，能够生在这样的家庭，是我上辈子的造化。我也要感谢我的爷爷奶奶外公外婆。爷爷虽然去世，但他一辈子的严谨治学也是我一辈子的榜样。奶奶对文字的敏感遗传给了我，我用这份敏感写自己的文字，也体会到她在写给我的诗里对我的期待。外公外婆的故事一直都像是传说一般。外公和我是隔了近七十年的同行，他的刚毅，坚强和不服输，是我在脆弱的时候鼓励自己的良药，因为我身上流着四分之一如此坚强刚毅的血液。还有我的两个叔叔和两个舅舅。你们对我的关怀对我的好，我在心头铭记。老妹刘思余，再坚持两年，你一定会度过高考的难关！最后是刘敬哥哥。从我二十一岁那年起，你就成为了我的“人生导师”。我做所有重大决定都要先问过你的意见才安心。没有你的指引和鼓励，我一定不是现在敢闯敢做的我。

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<tr>
<td>2-APB</td>
<td>2-aminophenyl borane</td>
</tr>
<tr>
<td>5-HT\textsubscript{1B}</td>
<td>5-hydroxytryptamine receptor 1B</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>BSL2</td>
<td>Biosafety level 2</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+}</td>
<td>Calcium ions</td>
</tr>
<tr>
<td>[Ca\textsuperscript{2+}]_i</td>
<td>Intracellular calcium concentration</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
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<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>CD36</td>
<td>Cluster of differentiation 36</td>
</tr>
<tr>
<td>CGRP</td>
<td>Calcitonin-gene related peptide</td>
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<tr>
<td>CNS</td>
<td>Central nervous system</td>
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<td>CPE</td>
<td>Complete cytopathic effect</td>
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<tr>
<td>CRAC</td>
<td>Calcium released activated calcium channel</td>
</tr>
<tr>
<td>DAG</td>
<td>Diacylglycerol</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
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<td>DRGs</td>
<td>Dorsal root ganglionic neurons</td>
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<td>DRK</td>
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<td>FAs</td>
<td>Fatty acids</td>
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<td>FFAs</td>
<td>Free fatty acids</td>
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<tr>
<td>FGF21</td>
<td>Fibroblast growth factor 21</td>
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<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
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GFP   Green fluorescent protein
GLP-1  Glucagon-like peptide-1
GPCR  G protein-coupled receptors
HPI   Hours post inoculation
IB4   Isolectin B4
IP$_3$ Inositol 1,4,5-trisphosphate
IP$_3$R Inositol 1,4,5-trisphosphate receptor
K$^+$ Potassium ions
KCNA Shaker family of delayed rectifying potassium channels
KCNB Shab family of delayed rectifying potassium channels
KCNC Shaw family of delayed rectifying potassium channels
KCNK 2P domain and background potassium channels
LA Linoleic acid
LEPR Leptin receptor gene
MA Myristic acid
Na$^+$ Sodium ions
NGF Nerve growth factor
NKCC1 Na$^+$K$^+$2Cl$^-$ cotransporter 1
OA Oleic acid
P2X$_3$ P2X Purinoceptor 3
PFU Plaque forming units
PIP$_2$ Phosphatidylinositol 4,5-bisphosphate
<table>
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<tr>
<th>Acronym</th>
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<td>PK15</td>
<td>Porcine kidney 15</td>
</tr>
<tr>
<td>PLC</td>
<td>Phospholipase C</td>
</tr>
<tr>
<td>PLCβ2</td>
<td>Phospholipase C β2 isoform</td>
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<tr>
<td>PNS</td>
<td>Peripheral nervous system</td>
</tr>
<tr>
<td>PrV</td>
<td>Pseudorabies virus</td>
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<tr>
<td>PUFAs</td>
<td>Polyunsaturated fatty acids</td>
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<tr>
<td>qRT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
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<td>RFP</td>
<td>Red fluorescent protein</td>
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<td>ROCE</td>
<td>Receptor-operated calcium entry</td>
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<td>SERCA</td>
<td>Sarco/endoplasmic reticulum calcium ATPase</td>
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<td>Trigeminal ganglionic neurons</td>
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<td>TNF-α</td>
<td>Tumor necrosis factor-alpha</td>
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<td>TPPO</td>
<td>Triphenylphosphine oxide</td>
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<td>Trk-A</td>
<td>TRK1-transforming tyrosine kinase protein</td>
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<td>TRCs</td>
<td>Taste receptor cells</td>
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<td>TRP</td>
<td>Transient receptor potential</td>
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CHAPTER 1
INTRODUCTION

Rationale for the Proposed Research

Obesity is becoming a major cause of many health problems in developed countries and a growing one in the developing world. There are as many as 30 diseases related to the obese state, including cardiovascular disease, diabetes, end-stage renal disease and even some forms of cancer. While obesity is clearly a disease that has multiple etiologies, there are compelling data indicating a link between the growth in the obese population and the corresponding increment of dietary fat intake (1, 2).

In our diets, energy comes from three primary sources – fat, carbohydrate and protein. Of these, fat is the most concentrated energy source, providing nine calories per gram compared with four calories per gram from either carbohydrate or protein. Ingested fat is predominantly in the form of triglycerides, which is an ester form of glycerol and three fatty acids, although there are significant amounts of mono- and di-glycerides, free fatty acids and glycerol in fat-containing food. The hydrolysis of fat (i.e. the breakdown into free fatty acids and glycerol) occurs primarily in the intestine via the action of lipases with presence of bile secretions. The by-products of fat digestion can move to the blood vessels via the lymphatic thoracic duct for energy use, or be rebuilt into triglycerides and lipoprotein.

Despite the obvious link between the fat intake and the obesity, there has been comparably little attention paid to the sensory cues provided by dietary fats. Over the past decade, however, the idea that fats might provide salient cues to the gustatory system and
act as a “taste of fat” was introduced to the field. While the “taste of fat” remains controversial, the textual cues of dietary fat or triglyceride are generally accepted. In some cases, textural properties appear to be sufficient to identify fat content. Oiliness rating of stimuli was reported when samples were rated with or without the blocking of additional olfactory and visual cues (3). However, more commonly, subjective ratings by sensory panels reveal predominant textual attributes, but also odor and taste dimensions (4). Thus, it is more likely that fats play multiple roles in foods and lack a unitary sensory property.

On the other hand, there are reports suggesting that the fatty acid concentrations in edible oil generally range between 0.01%-0.1% (5). Meanwhile, lingual lipase activity could generate an additional 0-12 µMoles fatty acids/min per l (6). Although fatty acids’ role in the taste system attracts more attention, and is under intense study, fatty acid activity in the somatosensory system remains largely unexplored. Sensory neurons isolated from somatosensory system have been utilized as the model neurons to study the molecular and cellular based signaling pathways excited by somatosensory stimuli. Whether fatty acids could be the analog stimulation to sensory neurons would be the obvious question. Indeed, electrophysiological studies from our lab and others (7) confirmed the efficacy of free fatty acids as effective tactile stimuli. My dissertation study was therefore focused on fatty acids’ action on isolated trigeminal ganglionic neurons, and the identification of the detailed players that involved in the fatty acid-sensitive signaling pathway.
Excess intake of dietary fat is linked with obesity (1), which has become a worldwide epidemic, especially evident in Western countries. For decades, intense research has been carried out to determine the underlying cause(s) of obesity. The most promising early data in this area was the identification of genes that were apparently responsible for some forms of obesity. Among the most consequential discoveries were the $\text{Lept}^{\text{Lep}}$ gene and its product leptin and the leptin receptor gene (LEPR) (8, 9). Because leptin is an adipocyte-derived cytokine whose administration reverses obesity caused by leptin deficiency, it was initially viewed as an adipocyte-derived signal that functioned primarily to prevent obesity. Now, however, researchers have generally agreed that leptin is a signal that conveys the level of fat storage to the brain, relaying information as whether the body is starving or full. The focus on leptin further lead to the discovery of a series of adipocyte hormones related to obesity in central and enteric nervous systems. For example, adiponectin, visfatin, tumor necrosis factor-α (TNF-α) and fibroblast growth factor 21 (FGF21) are some regulators of glucose homeostasis (10-12).

In recent years, there has been an intense focus on the obesity regulators acting in the central nervous system (CNS), yet comparatively little emphasis has been placed on the fat-derived signals in the PNS. It has become increasingly clear that fat itself and its by-products, particularly fatty acids, also act as peripheral stimuli to a variety of fat-responsive tissues (13, 14). For example, in skeletal, smooth and cardiac muscle and some other fat-responsive tissues (pancreas, liver, intestine), fatty acids appear to be acting as primary messengers. In these tissues, fatty acids’ effects could be either indirect,
involving intracellular effects of arachidonic acid or other fatty acids as the substrate (15), or direct, where fatty acids interact with a variety of voltage-activated ion channel (16) and receptor (17) signaling pathways. Specifically in the oral cavity, dietary fat could be a signal conveying information for the impending change of body physiology in response to fat intake as well. There is compelling evidence that oral administration of lipids increases the protein content of pancreatobiliary juice in esophagectomized rats (13). Taken together, our laboratory and others have found that fat could be a peripheral signal that activates the taste system as well. The ultimate goal is to determine if this putative “taste and texture of fat” plays a role in the obesity epidemic.

**Tactile Information of Fat Is Perceived Through the Somatosensory System**

For a long time in the chemosensory field, fat was thought to have virtually no taste and its most salient cue was its texture (18-20), with its sensory attributes given descriptors like “mouth feel,” “oiliness,” “creaminess,” or “slipperiness.” The perception of the texture of fat is perceived largely through activation of the somatosensory system and specifically through activation of trigeminal nerve fibers that originate from the trigeminal ganglia and send projections into the oral cavity. These polymodal chemosensory cells respond to a variety of stimuli (21-23). However, very few studies have attempted to explore their responsiveness to the components of dietary fats and fewer have attempted to look at the cellular and molecular mechanisms for fat perception.

Thus, the general perception remains that fat is perceived largely through activation of the somatosensory system. Somatic sensations are of four modalities: tactile, thermal, pain, and proprioception. The tactile sensations are usually regarded as those of touch,
pressure, vibration, itch, and tickle. The dorsal root is responsible for the somatic sensations of the body. It conducts nerve impulses from sensory receptors in the skin, muscles, and internal organs into the CNS. In the head and neck, the trigeminal nerve serves as the corresponding nerve of dorsal root. There are three cranial nerves that innervate the face area, cranial nerve V, VII and IX. Cranial nerve V is also known as the trigeminal nerve, while cranial nerves VII and IX are referred to as the facial and glossopharyngeal nerves, respectively. The facial and glossopharyngeal nerves are mixed cranial nerves (conveying both afferent and efferent information) that are responsible for much of taste perception. The facial nerve has its sensory axons extend from the taste buds of the anterior two-thirds of the tongue through the geniculate ganglion, and end in the pons. The chorda tympani branch of facial nerve is primarily sensitive to taste quality and intensity of chemical stimuli (24). However, they can also be activated by thermal and mechanical stimuli (25, 26). The glossopharyngeal nerve carries taste information from the posterior tongue. The trigeminal nerve is also mixed and the largest of the cranial nerves. This nerve emerges from two roots on the ventrolateral surface of the pons. The large sensory root has a swelling called the trigeminal ganglion, which is located in a fossa on the inner surface of the petrous portion of the temporal bone. The ganglion contains cell bodies of most of the primary sensory neurons. As indicated by its name, the trigeminal nerve has three branches: ophthalmic, maxillary, and mandibular. The ophthalmic nerve, the smallest branch, enters the orbit via the superior orbital fissure. The maxillary nerve is intermediate in size between the ophthalmic and mandibular nerves and enters the foramen rotundum. The mandibular nerve, the largest branch, exits
through the foramen ovale. This mandibular nerve is the one that contains somatosensory axons from the anterior two-thirds of the tongue, and some other areas on the face. It is sensitive to primarily tactile aspects of stimuli, only strong chemical stimuli could elicit a response from this nerve (27, 28). The tactile nature of the lingual nerve, usually regarded as thermal and nociceptive sensitivity, was also supported by clinical evidence of anesthesia of the anterior tongue following impairment of the trigeminal nerve (29). The input from facial nerve and lingual nerve converges on individual solitary neurons which will allow interactions between sensory modalities, e.g., taste and texture (touch) (30).

**The Classification of Trigeminal Ganglionic Neurons (TGNs)**

As stated above, trying to study this complicated system is obviously challenging. One of the most productive ways has been to study the characteristics of isolated trigeminal ganglionic neurons (TGNs). In general, most of the studies carried out in somatosensory neurons were focused on their role in pain perception, like trigeminal neuralgia, not on the other sensations. If one isolates TGNs, it is commonly accepted that the different cell body sizes represent functionally different cell types. Thus, in our goal to characterize the ability of these cells to respond to fatty acids, it will be helpful if we have some information of the functional classification of different types of TGNs.

The most accepted classification of TGNs in this respect is to divide them into the mechanoreceptors and the nociceptors according to their function. Mechanoreceptors are sensory receptors that respond primarily to mechanical pressure or distortion. When a mechanoreceptor receives a stimulus it begins to fire impulses or action potentials at an elevated frequency. Nociceptors, however, are sensory receptors that send signals that
cause the perception of pain in response to potential damaging stimulus. There are three major classes of nociceptors—thermal, mechanical, and polymodal. Specifically, thermal nociceptors are activated by extreme temperatures. They usually have small-diameter, thinly myelinated A_δ fibers that conduct signals at about 5-30 m/s. Mechanical nociceptors are activated by intensive pressure applied to the skin, and also have thinly myelinated A_δ fibers conducting at 5-30 m/s. Polymodal nociceptors are activated by high-intensity mechanical, chemical, or thermal (both hot and cold) stimuli. These nociceptors have small-diameter, unmyelinated C fibers that conduct more slowly, less than 1.0 m/s velocity (31).

The most widely used classification of TGNs tends to separate them based upon their soma diameter and the use of specific markers for labeling. The studies of structural differences of TGNs were closely related to the dorsal root ganglionic neurons (DRGs). Mature primary sensory ganglia neurons have been classified in three main types (A, B, C) based on their sizes and the distribution of their organelles (32). Type A neurons are large neurons (40-75 μm in diameter), type B compose of medium neurons (20-50 μm in diameter), whereas type C neurons are the smallest ganglion cells with a diameter of less than 20 μm (32), however, it should be noted that these classification strategies vary considerably in the literature. Type A and B neurons have myelinated fibers and are mainly mechanoreceptive, whereas type C neurons give rise to largely unmyelinated fibers and are mainly nociceptive (33).

There are several markers used to classify TGNs as well. *Griffonia simplicifolia* isolectin B4 (IB4)-fluorescein isothiocyanate (FITC) binds to a portion of small diameter
DRGs (soma diameter $\leq 26\mu m$) (34) and TGNs (35), which were thought to be nociceptors. These IB4-positive non-peptidergic neurons and IB4-negative peptidergic neurons were reported to have some functional differences. Neuropeptides such as calcitonin-gene related peptide (CGRP), substance P and nerve growth factor (NGF) receptor Trk-A were expressed in the IB4-negative neurons, while P2X purinoceptor 3 (P2X$_3$) receptor was expressed in IB4-positive neurons (36-38). 5-hydroxytryptamine receptor 1B (5-HT$_{1B}$) mRNA was found expressed in medium size TGNs (soma diameter 25-37 $\mu m$), which was virtually absent from IB4-labelled cells. In the neuropeptide coexpression experiments, people found that 5-HT$_{1B}$ cells were immunoreactive for Trk-A and CGRP in medium-size Trk-A cells, while substance P cells did not shown 5-HT$_{1B}$ expression (33). In another report, Na$^+$/K$^+$/2Cl$^-$ cotransporter 1 (NKCC1) mRNA is predominately expressed by small and medium diameter DRGs and TGNs. Specifically in trigeminal ganglia, NKCC1 mRNA-expressing neurons frequently colocalized with peripherin, CGRP, or transient receptor potential vanilloid 1 (TRPV1) immunoreactivity and N52 antibody (39).

Taken together, it is clear that texture perception is conducted by the trigeminal nerve. Cells from the trigeminal ganglia contain mixed cell types which could be distinguished by their soma diameters and a series of markers, but none of these methods currently provides an unequivocal means of identifying subtypes of TGNs that innervate the oral cavity and, importantly, are those that respond to fatty acids. Thus, part of the dissertation research was spent attempting to develop methods utilizing pseudorabies
virus as a tracer (see below) to identify subsets of rat TGNs that respond to the textural properties of fats in the oral cavity.

**Pseudorabies Virus as the “Live-Cell” Tracing Tool**

Pseudorabies virus (PrV) is a swine herpes virus of the *Alphaherpesvirinae* subfamily, which cause Aujeszky’s disease in pigs. Although swine are the reservoir for the virus, it has a broader host range which includes nearly all mammals except for the higher primates and humans and other vertebrate species (40). PrV naturally infects the oronasopharyngeal route. After primary infection of the epithelial cells, the viral particles invade nerve endings innervating the mucosae, traveled back to the neuron bodies and further ascend toward the CNS, resulting in a non-suppurative meningoencephalitis (41, 42).

The host range of PrV made it a useful tool in a wide range of animal models but remains safe for laboratory workers. PrV contains DNA rather than RNA, which brings the convenience for manipulating the genome. It is also neurotropic virus by nature: it invades neurons and produces infectious progeny that cross synapses to infect other neurons in the same circuit. That is, PrV spreads faithfully among functionally connected neurons (synapses), rather than simple releases virus particles to extracellular space. These features make PrV a good tool as a neural circuit tracer. Indeed, carefully designed studies of PrV transport have confirmed its reliability. First, the circuit pathway developed by PrV infection resembles the results from the nonviral tracers. Second, infection does not spread interaxonally to non-synaptically connected, physically adjacent circuitry. Third, PrV can only work with intact neuronal circuit. Last, PrV spreading is
not dependent upon a certain kind of neurotransmitter or specific anatomy (41). Thus, PrV holds promise as a good candidate of identifying the oral cavity-innervated TGNs.

Actually, PrV has been used as a circuit tracer for about 20 years. Among many virulent strains, the attenuated strain PrV Bartha, is the most widely used PrV for neuronal tracing. It spreads only from post- to pre-synaptic cells in the circuit (retrograde spread). PrV-Bartha was first exploited for defining CNS circuits that modulate the autonomic and somatic peripheral outflow (43). Later studies utilized the genetic recombinant technique to insert reporter genes like β-galactosidase or green- or red-fluorescent protein (GFP, RFP) genes to determine the connections of the CNS to many peripheral organs and tissues. Some of the examples includes: tongue (44), nose (45), adipose tissues (46-49), muscle (50), heart (51), and stomach (52). In a recent report, a recombinant PrV-Kaplan strain with GFP gene was used to successfully trace the pathway to the trigeminal and brainstem neurons (53), although the wild-type Kaplan strain was thought to have a powerful inflammatory response that usually killed the animal within 2-3 days. However, the careful study of post-inoculation-time-window enables the researchers to utilize the anterograde and retrograde abilities of the Kaplan strain, and obtain the high animal survival rate at the same time. More importantly, both PrV-Bartha and PrV-Kaplan infected neurons retain relatively normal electrophysiological properties for some time after infection (45, 54-57). This feature gives us possibilities of not only tracing the circuit, but also studying the functions of the traced neurons.
Putative Fatty Acid-Responsive Proteins

**G Protein-Coupled Receptors (GPCRs).** Over the past few years, several GPCRs considered orphans were identified and found to be activated by a variety of fatty acids. These GPCRs include GPR40, GPR41, GPR43, GPR84 and GPR120 (Table 1.1). Among these, GPR40 has been the most completely characterized. GPR40 is thought to be a receptor of long chain fatty acids (carbon chain length > 12), which appears expressed relatively specifically in pancreatic islets across species. It couples with $G_{q/11}$, resulting in elevation of intracellular calcium and regulator of protein kinase A and C activities, consistent with the interpretation that the receptor may play a role in insulin secretion (17). Interestingly, a connection between GPR40 activation and delayed rectifying potassium (DRK) channel inhibition (see below) has been proposed to involve cyclic adenosine monophosphate (cAMP) and protein kinase A (58).

GPR41 and GPR43’s ligands are short chain fatty acids (carbon chain length < 6) (59, 60). These two are closely related receptors that are activated by propionate and other short chain carboxylic acids (60). Another member of this family is GPR42 appears to be a non-functional gene duplication of GPR41. GPR41 has highest expression level in adipose tissue where it has been linked with the release of leptin (61). GPR43 has been shown to be present in lymphatic tissues and gastrointestinal tract and play a role in differentiation of leukocyte progenitors in hematopoietic cells (62). Both GPR41 and GPR43 couple to inositol 1,4,5-trisphosphate (IP$_3$) formation via $G_{i/o}$ (GPR41 & GPR43) or $G_{q}$ (GPR43 only).
More recently, GPR84 has been identified as a receptor for medium chain fatty acids (63). GPR84 couples primarily to a pertussis toxin-sensitive $G_i/G_o$ pathway and induces a cAMP increase after application of medium chain fatty acids. GPR84 functions as a specific receptor for medium chain fatty acids of C9 to C14, with C10-C12 being the most potent. Expression analysis revealed significant induction of GPR84 in monocytes/macrophages upon lipopolysaccharide stimulation, suggesting that medium chain fatty acids may regulate inflammatory responses through activation of GPR84 (63).

GPR120 is an additional fatty acid receptor that has to be characterized in enteroendocrine cells of the small intestine. These cells, including a corresponding cell line, STC-1, share many characteristics of the mammalian taste receptor cells (TRCs) (64). STC-1 cells express sweet (65) and bitter receptor families as well as gustducin (66, 67). Interestingly, they also express GPR120, a GPCR that is activated by long chain fatty acids (68). Activation of GPR120 results in release of an enteroendocrine hormone, glucagon-like peptide-1 (GLP-1), as well as inhibits apoptosis induced by serum deprivation through $G_q$ pathway (68, 69).

CD36. Cluster of differentiation 36 (CD36) is a glycoprotein that was identified first in human platelets (70, 71). It was first thought to be a receptor for thrombospondin and collagen. However, this molecule is now considered a transmembrane protein that binds lipids including long chain fatty acids (72, 73). One of the roles proposed for CD36 is in the transport fatty acids across plasma membranes from the extracellular to the intracellular side (74), though this role has not been firmly established. CD36 is broadly expressed and could be functioned in cellular lipid transfer, as a scavenger receptor, and
in cell adhesion (75). It plays a role in cellular triglyceride synthesis, lipid storage in adipose tissue, and fatty acid oxidation in muscle. One report in 1997 came out revealing that CD36 is expressed in the epithelial layer of circumvallate papillae of the tongue (76). Recently, Laugerette et al. provided compelling evidence to implicate CD36 as playing an important role in oral fat perception and even in triggering neural mechanisms that lead to bile secretion (77). In the oral cavity, expression of CD36 is restricted to the lingual gustatory papillae, particularly the circumvallate papillae, which are located on the back side of the tongue (77). The Laugerette group further examined the physiological responses of both wild type and CD36 knockout mice. Wild type mice showed obvious preference for a solution containing linoleic acid or a solid diet containing linoleic acid. CD36 knockout mice, however, did not exhibit such preference (77).

**Delayed Rectifying Potassium (DRK) Channels.** Since the early 1990’s, fatty acids have been known to affect ion channels. Arachidonic acid has been shown to activate several types of potassium channels (78-80), whereas some polyunsaturated fatty acids (PUFA) could block some other potassium channel classes (81). Fatty acids also have been reported to affect sodium channels (82) and calcium channels (83, 84) as well. More recently, a specific transient receptor potential (TRP) family channel that could be activated by arachidonic acid has been identified (85). These results suggest that fatty acids may be important regulators of signal transduction in a variety of systems.

As described above, DRK channels have been implicated in fatty acid transduction in the taste system. PUFAs can lead to TRC activation through an inhibition of DRK
channels. Subsequent research has shown that the predominant fatty acid sensitive DRK channel in taste cells is the *Shaker* *K*$_{V}$.1.5 (KCNA5) channel, which is known to be highly sensitive to PUFAs (81, 86). Heterologous expression studies from our laboratory have suggested that all members of the KCNA channel family are very sensitive to PUFAs, KCNB channels are moderately sensitive and KCNC channels are insensitive to fatty acids (unpublished data). More interestingly, comparison of DRK expression in obesity-prone and obesity-resistant rat models has concluded that the ratio of fatty acid-sensitive DRK channels to fatty acid-insensitive DRK channels might be an important to contributor to overall peripheral fatty acids sensitivity and influence the strength of the resulting chemosensory response to fat (87). Furthermore, it seems that there is a correlation between activity in peripheral potassium channels and feeding behavior. High fat diets and/or obesity reduce the responsiveness of peripheral DRK channels to fatty acids. In addition, a number of studies have implicated potassium channels’ roles directly or indirectly in food intake. As an example, antagonists of potassium channels lead to a decrease in food intake, while agonists exert the opposite effect (88). Thus, DRK channels are important players or possibly act as receptors in peripheral gustatory system.

**TRP Channels as Cellular Sensors**

The TRP channels are now known to constitute important components of sensory systems. They form a large and functionally versatile family of ion channels that have distinct activation mechanisms and biophysical properties throughout a diverse series of organisms. For example, yeast use a TRP channel to perceive and respond to osmolarity change (89, 90); *C.elegans* use TRP channels to detect and avoid noxious chemicals (91);
Male mice use a pheromone-sensing TRP channel to sense their female partners (92). Humans use TRP channels to appreciate sweet, bitter and umami tastes (93), as well as to discriminate among warmth, heat and cold (94-97). In general, the mammalian TRP superfamily is divided into six subfamilies based on the sequence homology, which includes TRPC (canonical, TRPC1-7), TRPM (melastatin, TRPM1-8), TRPV (vanilloid, TRPV1-6), TRPA (ankyrin, TRPA1), TRPP (polycystin) and TRPML (mucolipin) families (98). The mammalian TRP family tree is shown in Fig. 1.2.

The TRP channel subunit consists of six putative transmembrane spanning domains (S1-S6) with a pore forming loop between S5 and S6. The two termini (-NH$_2$ and –COOH) of the polypeptide are both located intracellularly (99). All TRP channels are known to be cation-selective. Most functionally characterized TRP channels are permeable to calcium ions except for TRPM4 and TRPM5, which are permeable to monovalent cations.

Although knowledge on these channels accumulated rapidly, the physiological roles of most channels are still obscure. Here I will focus on introducing the four TRP channels—TRPV1, TRPA1, TRPM8 and TRPM5, which are involved in either somatosensation in primary sensory neurons or taste transduction in TRCs.

TRPV channels are activated by heating in heterologous expression systems and sensory neurons (95-97, 100). Specifically, TRPV1 was generally known as the ‘hot’ pepper derived vanilloid compound capsaicin receptor, but it is also activated by heat, decreased pH and some other agonists as well (101-104). In sensory neurons like DRGs or TGNs, TRPV1 expression predominates in a subset of neurons with small diameters
unmyelinated C-fiber nociceptors (105). TRPA1 is distinguished from other TRP channels by the presence of ~14 ankyrin repeats in its N terminus. Although TRPA1 was described as a cold-sensitive nonselective cation channel (106), further studies reveal that it is a ligand-gated channel activated by pungent agents like mustard oil and garlic (107-110). Like TRPV1, TRPA1 is also expressed in a subset of unmyelinated C-fiber nociceptors, which are thought to be involved in the pain pathway in primary sensory neurons (103, 106, 109, 111).

Two members of the TRPM subfamily are involved in chemical senses. TRPM8 has been most convincingly described as an environmental cold- and menthol-activated nonselective cation channel. It activates unmyelinated C-fibers as well as the myelinated Aδ fibers (94). TRPM5 is found expressed in taste tissues and played important roles in sweet, bitter, umami signaling transduction on taste receptor cells. Taste preference test with TRPM5-/- mice showed diminished sensitivity in perception of these tastants (93). Different than TRPV1, TRPA1 or TRPM8, which have ligands directly binding to the channels, TRPM5 is activated by intracellular Ca^{2+} increases following the generation of IP_{3} and diacylglycerol (DAG) via phospholipase C (PLC), which is initiated by tastants binding to taste receptors on the membrane (93). These taste receptors consist of the sweet and umami receptor (T1Rs) and bitter receptor (T2Rs) families and are G protein-coupled receptors (GPCRs) that couple to the activation of PLCβ2 (93).

A Model Linking GPCRs, CD36 and Fatty Acid-Sensitive DRK Channels

As described above, there are a number of proteins that have been implicated as putative receptors for fatty acids. While it is entirely plausible that these putative
receptors (fatty acid-activated GPCRs, CD36 and fatty acid-sensitive DRK channels) could function independently in the transduction of fatty acids in the somatosensory and taste systems, I favor the hypothesis that these proteins are functionally linked. Fig. 1.3 illustrates our model of the putative fatty acids receptors responding to fatty acids and initiating a signaling transduction cascade. Fatty acids, either transported by CD36 to the GPCRs rich regions or directly bound to GPCRs activates a G protein that stimulates production of PLC, production of the second messenger IP₃ and release of calcium from intracellular stores. The release of calcium stores may lead to activation of a member of calcium released activated calcium channel (CRAC) family and influx of calcium that depolarizes the cell (i.e. produces a receptor potential). This depolarization increases the open probability of DRK channels which in this open state are blocked directly by fatty acids further depolarizing the cell leading to the opening of voltage-gated calcium channels. Experiments in this dissertation were testing this specific hypothesis in TGNs. (Fig. 1.3)

Dissertation Outline

This dissertation research explores the mechanisms underlying dietary fat evoked somatosensation in oral cavity. In general, my research is focused on identifying dietary fat responsive pathways in sensory neurons which would reflect somatosensation in the oral cavity (i.e. TGNs). To better study the fatty acid-sensitive signaling pathway in oral-innervated polymodal TGNs, I have made use of the neural circuit tracer PrV to label a subpopulation of lingual-innervated TGNs. Furthermore, I studied the mechanisms that underlie fatty acids stimulation specifically in those PrV-labeled TGNs. In Chapter 2,
using adult Sprague-Dawley rat model, my research is focused on using ratiometric calcium imaging and patch clamp recording as the functional output to make the first attempt to explore fatty acids’ effect on TGNs. More specifically, I took pharmacological and ion substitution approaches to identify specific proteins involved in fatty acids transduction pathway in TGNs. These results demonstrated for the first time that fatty acids are capable of activating TGNs. To explore this pathway in TGNs from the oral cavity, in Chapter 3, I describe results from experiments where I (1) developed a technique to identify a subpopulation of neonatal TGNs that innervate the oral cavity and (2) showed that these cells respond in a qualitatively similar fashion to fatty acids as the larger population of adult TGNs studied earlier.

My data in Chapter 2 and Chapter 3 implied that a TRP-like channel was likely involved downstream in the fatty acids transduction pathway. In Chapter 4, I followed up on this observation and turned to recording and characterizing TRP-like currents initiated by linoleic acid (LA) in TGNs. In this research, I made the first attempt to use perforated whole-cell patch clamp recording to record the TRP-like current from the acutely isolated TGNs and attempted to elucidate the properties of this LA-induced current.

While I faced a number of technical and methodological challenges in the course of this dissertation research and I have not been able to completely determine the transduction pathway for fatty acids in the trigeminal system, a number of important outcomes have emerged from this project. First, I have shown that one of the components in dietary fat, specifically fatty acids, is able to produce a depolarizing receptor potential and activate a rise in intracellular calcium in TGNs consistent with fatty acids being a
somatosensory stimulus (Chapter 2). Second, using fluorescent PrV constructs I have
developed approaches to identify a subset of TGNs which innervate the oral cavity and
show conclusively that these cells are also activated by fatty acids (Chapter 3). Third, I
have made significant initial progress to identify the components of the transduction
pathway for fatty acids in TGNs (Chapters 2-4). Like in other chemosensory cells
including TRCs and enteroendocrine cells, the pathway appears to be initiated by binding
of fatty acids to fatty acids-activated GPCRs (a progress that might positively be
influenced by the fatty acids binding protein, CD36), activation of PLC, generation of the
second messengers IP₃ and DAG, and the release of calcium from intracellular stores.
The calcium rise then apparently leads to activation of a cation permeable TRP-like ion
channel. While I have identified a number of TRP-like channels in TGNs that may play a
role in the fatty acids pathway, the lack of specific pharmacological tools currently have
precluded me from being able to identify a specific ion channel in this pathway. Given
the progress made, I anticipate that future research will be able to more completely
elucidate this pathway.

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   products using free-choice profiling and genetic responsivity to 6-n-


Table 1.1: Summary of fatty acid-sensitive membrane proteins that may play a role in fatty acids detection in oral cavity. CCK, cholecystokinin; GLP-1, glucagon-like peptide-1.

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Figure 1.1: Diagram of the branches of the trigeminal nerve that innervate the oral, nasal, and ocular cavities. The chemosensitive structures innervated by each trigeminal branch are indicated in parentheses. (112)
Figure 1.2: Mammalian TRP family tree. The mammalian TRP channels comprise six related protein families with sequence identity as low as 20%. (99)
Figure 1.3: A model linking GPCRs, CD36 and fatty acid-sensitive DRK channels. Hypothesized transduction pathway activated by fatty acids acting at GPCRs, CD36 and DRKs in chemosensory cells. FA, Fatty Acids; R, GPCRs; CRAC, calcium released activated calcium channels; VGCC: voltage gated calcium channels.
CHAPTER 2

FATTY ACIDS ACTIVATE TRIGEMINAL GANGLIONIC NEURONS IN ADULT SPRAGUE-DAWLEY RATS

Abstract

Understanding the mechanisms that contribute to the sensory recognition of dietary fat has become increasingly important given the epidemic of obesity which is partially driven by high dietary fat intake. While the textural properties of dietary fat (e.g. triglycerides) have been well documented to occur via the activation of trigeminal ganglionic neurons (TGNs) in the somatosensory system, the ability of fatty acids (FAs) to activate TGNs remained unexplored. To investigate the ability of FAs to activate the somatosensory system, I have used fura-2 based calcium imaging and patch clamp recording as the functional readout to explore the ability of FAs to activate TGNs from adult Sprague-Dawley rats. FAs (1-100 µM) elicit robust changes in intracellular calcium concentration ([Ca^{2+}]_i) in approximately two-thirds of TGNs in a concentration-dependent fashion. In general, responses to monounsaturated or saturated FAs occurred in neurons that also responded to polyunsaturated FAs. Moreover, cells exhibit FA-responses that are less dependent of extracellular sodium (Na^+) but are either dependent or independent of extracellular calcium (Ca^{2+}) possibly indicating multiple functional cell types. Store depletion by thapsigargin significantly reduces but does not abolish the FA-induced Ca^{2+} responses. I also measured FA-induced membrane depolarization in TGNs by patch clamp recording. Linoleic acid (LA) elicits membrane depolarization in TGNs with a time course similar to that seen for the rise in [Ca^{2+}]. Inhibition of G protein
activation with GDP-β-S and inhibition of phospholipase C activity with U73122 blocked 77% and 58% of LA-induced depolarization of TGNs, respectively, which suggests the involvement of G protein/PLC pathways and is consistent with the activation of upstream FAs sensitive G protein-coupled receptor (GPCRs) in the FAs signaling pathway in TGNs.

Introduction

One role of the peripheral somatosensory system in the oral cavity is to enable the recognition of the temperature, pungency and the textural properties of foods. Cellular and molecular approaches have yielded information regarding the molecular underpinning of these processes. Several membrane ion channels have been discovered as the receptors of capsaicin (from hot chili) (1), menthol (2, 3) and pungent agents (4, 5) such as mustard oil and garlic. These unconventional sensations, along with the more conventional ones like taste and olfaction, contribute to the ability of enjoying the palatability of foods while rejecting those compounds that are potentially dangerous.

The ability to sense fat either in terms of its texture or taste appears to be correlated with dietary fat preference and, ultimately, obesity. Human studies suggest that individuals with a high body mass index (BMI) have lower sensitivity to oral FAs compared to those with lower BMIs (6). Animal behavioral studies indicate a clear preference to FAs compare to mineral oil (textural) control (7), consistent with the notion that FAs have a taste quality. The conditioned taste aversion test showed strong aversions to LA at the concentration of 100 µM (8). The cellular/molecular mechanisms underlying the taste of FAs remain to be elucidated. The textual cues associated with dietary fat (e.g.
triglycerides) are generally accepted, though the concentrations that produce this somatosensory experience have not been well established empirically. Recent reports suggest that the concentrations of free fatty acids (FFAs) in edible oil generally range between 0.01%-0.1% (9). Lingual lipase activity, on the other hand, could generate additional 0-12 µmol fatty acids/min per litter (6), which helps increase the FFAs to a detectable level in the oral cavity. Therefore, based upon the concentrations found in fat-containing foods and/or generated by the activity of lingual lipases, FAs have the potential of being effective sensory stimuli in the oral cavity. Indeed, research had indicated these concentrations of FAs were able to activate taste receptor cells (TRCs) by inhibiting delayed rectifying potassium channels (DRKs) (10). However, whether FAs are capable of activating the somatosensory system directly like capsaicin and menthol is unknown.

The peripheral nervous system (PNS) within oral cavity contains three cranial nerves. Cranial nerves VII and IX (referred to as the facial and glossopharyngeal nerves, respectively) are responsible for the taste perception, while cranial nerve V (referred to as trigeminal nerve) is the one that transmits somatosensory information. Generally speaking, noxious stimuli are detected by nociceptors that have small to medium diameter perikarya that further correspond to unmyelinated C and lightly myelinated Aδ nerve fibers. Innocuous stimuli, such as light touch, are detected by large diameter neurons that correspond to the more heavily myelinated Aα or Aβ fibers (referred to as mechanoreceptors). Based on molecular markers and pharmacological characteristics, there are about 50% of sensory neurons that respond to capsaicin (transient receptor
potential vanilloid 1 (TRPV1) positive) (4), 50% of capsaicin sensitive cells respond to mustard oil (mainly allyl isothiocyanate) (TRPV1 and transient receptor potential ankyrin 1 (TRPA1) positive), the other 50% respond to sanshool (2P domain and background potassium channels (KCNK) positive), which by itself activates 52% of sensory neurons (5). Menthol activates a separate subpopulation (~20%) that does not respond to capsaicin or mustard oil (transient receptor potential melastatin 8 (TRPM8) positive only) (5). Most of these neurons are small diameter myelinated neurons that are not sensitive to stretch, except for some TRPV1 and sanshool sensitive neurons that are stretch sensitive also, and fall into the class of non-peptidergic C-fiber nociceptors (11).

The primary targets for studies on somatosensation occur in sensory neurons from the dorsal root ganglia and trigeminal ganglia. The trigeminal nerve is a mixed nerve containing both afferent and efferent nerve fibers with broad innervation patterns. TGNs are considered polymodal neurons that consist of a heterogeneous population of cells. How to identify and functionally separate these neurons has been a major confound in the research of the physiology of sensory neurons. The most widely used approach is to divide sensory neurons by their sizes, i.e. small and medium neurons (<26 µm diameter) are classified as nociceptors, while larger neurons (>28 µm diameter) are believed to represent the population of mechanoreceptors (11, 12).

Although progress has been made in the cellular and molecular basis for the transduction of spicy and pungent compounds in somatosensory system, the ability of TGNs to respond to the components contained in fat and the mechanisms underlying the ability of FAs to activate sensory neurons remains elusive. Here, I take the multiple
approaches to elucidate the molecular and cellular basis of the FA-induced signaling pathway in TGNs. I have shown that FAs activate a subset of TGNs by increasing the 

$[Ca^{2+}]$, and depolarizing the membrane. Moreover, I found that FAs caused the release of intracellular $Ca^{2+}$ store in this subset of neurons, a process that contributes to the development of the FA-induced receptor potential. Preliminary data implicated the primary receptor for FAs appears to be one or more of the recently identified FA-activated GPCRs. Several FA-sensitive GPCRs were found expressed in the trigeminal ganglia, and I provided the first evidences that the G protein/phospholipase C (PLC) pathway is involved in FAs signaling. Thus, I conclude that FAs are effective stimuli for somatosensory neurons, which contribute to our perception of dietary fat.

**Materials and Methods**

**Acute isolation of trigeminal ganglionic neurons.** All surgical and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee, Utah State University. TGNs were isolated according to methods modified from Simon and colleagues (13, 14). Four-to 8-week-old male Sprague-Dawley rats were deeply anaesthetized by 50 mg/kg i.p. injection of sodium pentobarbital. The paired trigeminal ganglia were then harvested and diced into small pieces into Hank’s balanced salt solution (HBSS), followed by a 45-minute incubation with 2.5 mg/ml collagenase (Type XI-S) at 37°C in HBSS also and a 10-minute incubation at 37°C in 100 µg/ml DNase I in culture medium (DMEM/F-12 1:1 medium supplemented with 10% FBS). The neurons were centrifuged and resuspended in culture medium by gentle trituration with a flame-polished Pasteur pipette. Subsequently, neurons were plated onto poly-D-
lysine (0.1 mg/ml)-coated glass coverslips (12-15 mm in diameter) and cultured at 37°C in a water-saturated atmosphere with 5% CO₂. For electrophysiology, neurons with soma diameters ranging between 18 to 25 µm were used within 8 hours after plating on coverslips. For calcium imaging, neurons were cultured 36-48 hours in culture medium before use. These cell based assays were handled in the room temperature unless stated.

**Calcium imaging.** TGNs were loaded with fura-2/AM (5 µM; Molecular Probes, Eugene, OR) for 1 hour in Tyrode’s saline solution with 10% pluronic acid at 37°C in the dark. Neurons were then rinsed and placed in culture medium for 30 minutes to allow de-esterification of the acetoxyethyl ester group from fura-2. The coverslips were mounted into an imaging chamber (RC-25F and RC-26Z, Warner Instruments, Hamden, CT), placed on an inverted microscope (Nikon, Eclipse TS100, Japan) and perfused continuously with Tyrode’s solution. Neurons were illuminated with a 100-watt xenon lamp and excitation wavelengths (340/380 nm) were delivered by a monochromator (Bentham FSM150, Intracellular Imaging Inc., Cincinnati, OH) at a rate of 20 ratios per minute. Fluorescence was measured by a CCD camera (pixelFly, Cooke, MI) coupled to a microscope and controlled by imaging software (Incyt Im2™, Intracellular Imaging). The ratio of fluorescence (340/380 nm) was directly converted to Ca²⁺ concentrations using a standard curve generated for the imaging system using a fura-2 calcium imaging calibration kit (Molecular Probes, Eugene, OR). FAs were applied extracellularly with a bath perfusion system at a flow rate of 4 ml/min permitting complete exchange of the extracellular solution in less than 20 seconds.
Electrophysiological recording and analysis. Whole-cell patch clamp recording was used to measure membrane potential (current clamp mode) in TGNs with an Axopatch-200B amplifier (Molecular Devices, Sunnyvale, CA). Patch pipettes were fabricated from borosilicate glass on a Flaming-Brown micropipette puller (model P-97; Sutter Instrument, Novato, CA) and subsequently fire-polished on a microforge (model MF-9, Narishige, Japan) to a resistance of 2-8 MΩ. Commands were delivered and data were recorded using pCLAMP software (v. 10, Molecular Devices, Sunnyvale, CA) interfaced to an Axopatch-200B amplifier with a DigiData 1322A analog-to-digital board. Data were filtered online at 1 kHz and sampled at 2-4 kHz. The membrane potentials (V_M) of TGNs were recorded continuously before, during and after bath application of LA using the current clamp mode of the amplifier while holding the cells at its zero current level (at rest).

Solutions. Standard extracellular saline solution (Tyrode’s) contained (in mM): 140 NaCl, 5 KCl, 1 CaCl_2, 1 MgCl_2, 10 HEPES, 10 glucose, and 10 Na pyruvate; pH 7.40 adjusted with NaOH; 310 mOsm. Sodium-free saline (Na\(^+\) free Tyrode’s) contained (in mM): 140 N-methyl-D-glucamine (NMDG), 5 KCl, 1 CaCl_2, 1 MgCl_2, 10 HEPES, 10 glucose; pH 7.40 adjusted with HCl; and 310 mOsm (adjusted with NMDG). Calcium free saline (Ca\(^{2+}\) free Tyrode’s) contained (in mM): 140 NaCl, 5 KCl, 1 EGTA, 1 MgCl_2, 10 HEPES, 10 glucose, and 10 Na pyruvate; pH 7.40 adjusted with NaOH; 310 mOsm. The potassium-based intracellular solution was used for measurement of membrane potential contained (in mM): 140 K gluconate, 1 CaCl_2, 2 MgCl_2, 10 HEPES, 11 EGTA, 3 ATP, and 0.5 GTP; pH 7.2 adjusted with KOH; 310 mOsM. Thapsigargin (the SERCA
inhibitor on endoplasmic reticulum), U-73122 (an inhibitor of PLC) and U-73343 (the inactive analog of U-73122) were purchased from Sigma (St. Louis, MO). GDP-β-S, an inhibitor of G protein activation was obtained from EMD Biosciences (La Jolla, CA). The linoleic acid, oleic acid (OA) and docosahexaenoic acid (DHA) stocks were made in 100% ethanol and stored under nitrogen to prevent oxidation. The myristic acid (MA) stock was made in distilled water. The caproic acid was dissolved in Tyrode’s directly. The working solutions were made from stock solutions immediately prior to use.

**Quantitative real time-PCR (qRT-PCR).** For RNA Isolation, the paired trigeminal ganglia were either stored at 20°C in RNAlater (Ambion, Austin, TX) or placed in TRI Reagent (MRC, Cincinnati, OH) for immediate extraction of RNA. Extraction was done according to manufacturer’s protocol. For PCR, first strand cDNA was synthesized using the iScript cDNA synthesis kit from Bio-Rad (Hercules, CA). To get a final volume of 20 µl, 50 ng trigeminal RNA was used for the reaction. DNA contamination was evaluated by setting up a reaction where the reverse transcriptase was omitted. After first-strand synthesis, 2 µl cDNA was added to a PCR reaction mix. The HotMaster Taq DNA polymerase kit (5 Prime, Gaithesburg, MD) was used for the PCR reaction (final concentration: 1X reaction buffer, 5.0 mM Mg^{2+}, 200 μM dNTPs, 300-900 nM forward and reverse primers, and 1.25 U/µl HotMaster Taq). Primer sequences for GPR40, 43, 120 and CD36 are shown in Table 2.1. For GPR84 detection, a commercially available TaqMan® Gene Expression Assay (ABI, Foster City CA) was used.
**Statistical analysis.** The significant effects of all the treatments were determined by a two-tailed Student’s t-test (α=0.05) compared with their controls as described in the text. Data are presented as mean ± SEM, unless otherwise indicated.

**Results**

**LA depolarizes and elicits a rise in [Ca^{2+}]_{i} in TGNs.** In the present study I have used cell-based assays (ratiometric calcium imaging and patch clamp recording) to explore the ability of FAs to activate a signaling cascade in isolated rat TGNs. As the first approach, I used the prototypical FA stimulus, LA (1-100 µM), to elicit the [Ca^{2+}]_{i} changes in isolated TGNs. As shown in Fig. 2.1A, 30 µM LA elicited a robust and reversible increase in [Ca^{2+}]_{i} in a subset of rat TGNs. The inset in Fig. 2.1 shows the concentration-response function for the ability of LA to elicit significant [Ca^{2+}]_{i} rise where the EC_{50} for the response is approximately 17.5 µM. In subsequent experiments exploring the detailed mechanism of this FA-induced signaling cascade, I have focused on using 30 µM LA, a concentration that produced a significant, but not maximal, response in TGNs. On average, approximately 65% of TGNs (n = 332) responded to LA with an average increase in [Ca^{2+}]_{i} of 272.8 ± 9.4 nM. Neurons that showed less than a 100 nM change in [Ca^{2+}]_{i} were counted as non-responders and cells that did not return to near baseline Ca^{2+} levels after LA application were excluded from subsequent analyses.

To determine if LA was capable of depolarizing rat TGNs, patch clamp recording was performed in the whole cell current clamp configuration. I tested 9 neurons for LA-induced depolarization, which have an average resting (zero current) potential of -62.7 ± 3.3 mV. LA elicited a large depolarizing response averaging 61.5 ± 6.4 mV in 7 neurons.
2 out of 9 neurons did not respond to the stimulus. Inherent in these LA-induced responses were delays of about 2 min for both the imaging and electrophysiological assays (Fig. 2.1A and Fig. 2.1B, respectively), though these two parameters were not simultaneously measured in individual neurons in the present study. This significant delay in time could not be solely attributed to perfusion delays from the bath application system because the delay was not seen with activation of TGNs by other pungent stimuli (data not shown). Moreover, of the FAs examined (see Table 2.1), another polyunsaturated FAs DHA showed a similar consistent delay as LA, while the monounsaturated and saturated FAs had a more variable response time, but once initiated, generated a much more rapid \([\text{Ca}^{2+}]\) rise.

**Other classes of FAs activate TGNs.** To determine the specificity of FA responses in rat TGNs, I performed a series of experiments using several FAs varying in carbon length, position and number of double bonds (Table 2.2). Fig. 2.2 A-D shows representative \(\text{Ca}^{2+}\) responses of various bath applied FAs (30 µM). One of the diet-rich monounsaturated FAs, oleic acid (OA, C18:1) elicited rapid, reversible rise in \([\text{Ca}^{2+}]\), which often showed biphasic characteristics \((n=19/83)\). Another medium chain saturated FA, myristic acid (MA, C14:0), showed a response type similar to OA \((n=39/151)\). It consistently caused a rapid and sometimes biphasic rise in \([\text{Ca}^{2+}]\) in a subset of TGNs as well. DHA (C22:6), another polyunsaturated omega-3 FA, elicited the \([\text{Ca}^{2+}]\) changes in a similar manner with LA. However, caproic acid (C6:0), a short chain saturated FA, failed to elicit a rise in \([\text{Ca}^{2+}]\) in TGNs \((n=72)\). Additional differences between MA and
OA responses and those generated by LA (Fig. 2.1A) and DHA were that responses to the former FAs adapted, while those to LA and DHA did not.

To determine the specificity of the FAs responsiveness on individual TGNs, I applied several different classes of FAs (LA, OA and MA) to the same individual cells. As stated above, about 65% TGNs responded to LA, which comprised the biggest responding population to FAs. OA and MA could activate TGNs separately in some cases, however, most neurons responded to OA and MA were also LA-responsive. Therefore, I counted the number of neurons that were LA and OA and/or MA sensitive. As shown in Fig. 2.2E, 23% (n=83) of the LA-sensitive neurons were also OA-sensitive, while 26% (n=151) of LA-sensitive neurons were MA-sensitive. Moreover, a small proportion of neurons (4%; n=58) responded to all three FAs.

LA-induced [Ca\(^{2+}\)]\(_i\) increases depend to varying degrees on extracellular Ca\(^{2+}\) but not on extracellular Na\(^+\). Since LA reliably elicited [Ca\(^{2+}\)]\(_i\) changes and membrane depolarization in rat TGNs, I next conducted a series of imaging experiments to determine the dependence of the LA-induced [Ca\(^{2+}\)]\(_i\) increases on extracellular Na\(^+\) and Ca\(^{2+}\) and the relative contribution of intracellular Ca\(^{2+}\) stores to this process. To determine if extracellular Na\(^+\) influx was contributing to [Ca\(^{2+}\)]\(_i\) increase, I conducted calcium imaging experiments using a saline solution whereby I substituted for Na\(^+\) with N-methyl-D-glucamine (NMDG). Half of the experiments were conducted using Na\(^+\)-free solution first followed by a regular Tyrode’s solution, while the rest were conducted in the reverse order. As shown in Fig. 2.3A and 2.3B, Na\(^+\) substitution did cause a small but significant decrease in the magnitude of the LA-induced rise in [Ca\(^{2+}\)]\(_i\). There were no
obvious order effects in these experiments. Further, to avoid the response amplitude
differences caused by cell to cell variation, I normalized each cell’s LA-induced \([\text{Ca}^{2+}]_i\)
increase in Na\(^+\) free solution with the LA-induced \([\text{Ca}^{2+}]_i\) increase in regular Tyrode’s
solution. The resulting percentages were used as the functional output to further compare
across groups. Fig. 2.3C showed the comparison of the normalized percentages between
regular Tyrode’s (100%) and Na\(^+\) free Tyrode’s (92.0 ± 2.9 %; n=138; P=0.006, paired
Student’s t-test). The deduction by Na\(^+\) substitution was only 8%, which indicated that it
is probably not physiologically relevant.

In contrast to extracellular Na\(^+\) substitution, removal of extracellular Ca\(^{2+}\) lead to
significant reduction in LA-induced \([\text{Ca}^{2+}]_i\) increase in over 70% of neurons examined
(Fig. 2.3D, 2.3E), which indicated a role for extracellular Ca\(^{2+}\) entry in these responses.
However, the dependence of the LA-induced \([\text{Ca}^{2+}]_i\) rise upon extracellular Ca\(^{2+}\) was
highly variable among cells (Fig. 2.3D-F). Using the same normalization method, the
percentages of LA-induced \([\text{Ca}^{2+}]_i\) increase in Ca\(^{2+}\) free Tyrode’s over the LA-induced
\([\text{Ca}^{2+}]_i\) increase in regular Tyrode’s were used as the functional output. According to
these normalized percentages, I divided TGNs into two classes—the ones that have the
normalized percentages less than 50% (mean percentage 24.1 ± 1.6%; n=114), and the
ones that have the normalized percentages greater than 50% (mean percentage 72.9 ±
2.5%; n=48). These data revealed that about 70% (n=162) of the cells tested in this
manner showed greater than a 50% reduction in the magnitude of the LA-induced \([\text{Ca}^{2+}]_i\),
increase upon removal of extracellular Ca\(^{2+}\). Nevertheless, although performed in the
same condition, there were 30% (n=162) of neurons that showed little dependence of extracellular Ca$^{2+}$.

**Thapsigargin treatment blocked LA-induced [Ca$^{2+}$]$_i$ increases in a subset of TGNs.** Data shown in Fig. 2.3 indicated extracellular Ca$^{2+}$ played important role in LA-induced TGNs responses, but it also became apparent that Ca$^{2+}$ release from intracellular stores was contributing to the overall changes in [Ca$^{2+}$]$_i$ during LA application in many of the neurons examined. To determine the role of intracellular Ca$^{2+}$ release, TGNs were pretreated for up to 1.5 hour with thapsigargin (1 µM), a non-competitive SERCA (sarco/endoplasmic reticulum Ca$^{2+}$ ATPase) inhibitor, which leads to the depletion of intracellular Ca$^{2+}$ stores. Most TGNs treated with thapsigargin showed an approximately 2.5-3 fold increase in basal/resting Ca$^{2+}$ levels, which might indicate the activation of store operated Ca$^{2+}$ entry (SOCE) during store depletion (15-17). Application of LA elicited a rapid decrease in basal Ca$^{2+}$ concentration because of its ability to inhibit SOCE (16) followed by its characteristic slow increase due to LA stimulation (i.e. Fig. 2.1, 2.3). After the depletion of Ca$^{2+}$ stores, TGNs were still capable of responding to LA, though there appeared to be two classes of neurons again—more than 68% of neurons (n=119) contained predominantly thapsigargin-sensitive stores (TG+), characterized by a complete inhibition of LA-induced responses in Ca$^{2+}$-free extracellular solution (Fig. 2.4B and 2.4C) (from 193.8 ± 14.6 nM to 4.7 ± 1.1 nM; n=81), while the remainder of cells (32%; n=119) responded robustly to LA and these responses were not significantly inhibited by extracellular Ca$^{2+}$ removal (Fig. 2.4A and 2.4C) and were broadly classified as thapsigargin-insensitive (TG-) (from 350.3 ± 28.1 nM to 305.4 ± 30.0 nM; n=38).
Interestingly, the magnitude of LA-induced $[\text{Ca}^{2+}]_i$ increase in TG- neurons (350.3 ± 28.1 nM) was significantly greater than TG+ neurons (193.8 ± 14.6 nM) (Fig. 2.4C, P<0.001), which might be another hint of the differences of thapsigargin sensitivity between the two classes of TGNs.

**Activation of G protein and phospholipase C pathways in LA-induced depolarization.** GPR120 is a long chain polyunsaturated FAs receptor, which has been shown to play an instrumental role in the FA-induced $[\text{Ca}^{2+}]_i$ rise in enteroendocrine cell line STC-1 cells (18-20). GPR40 is another long chain polyunsaturated FAs receptor that proved to play a role in FA-induced insulin secretion in pancreatic β cells (20, 21). In this dissertation research, I checked this group of FA-sensitive GPCRs in trigeminal ganglia with quantitative real time PCR (qRT-PCR). As shown in Fig. 2.5F, these GPCRs were all expressed in trigeminal ganglia, although the expression level differed dramatically. In order to confirm the role of G protein in the signaling pathway, I examined the effect of blocking G protein activation with the reversible blocker GDP-β-S (1 mM) on LA-induced depolarization. In current clamp recording, GDP-β-S was included in the intracellular solution and membrane potential was recorded 2-3 minutes after achieving the whole cell configuration. LA-induced depolarization was significantly reduced in presence of GDP-β-S (14.3 ± 4.9 mV; n=4; p<0.001; Fig. 2.5A, 2.5D, 2.5E) compared to control (61.5 ± 6.4 mV; n=7; p<0.001; Fig. 2.5A, 2.5E) TGNs. Next, I determined the involvement of PLC in LA signaling in TGNs. Depolarization in response to LA is significantly reduced in presence of the PLC blocker U73122 (3 μM) (25.8 ± 4.6 mV;
n=8; Fig. 2.5B, 2.5E) compared to the treatment with the inactive analog U73343 (3 µM) (61.3 ± 2.6 mV; n=7; p<0.005; Fig. 2.5C, 2.5E).

Discussion

The prevailing view of how fats are detected in oral cavity is attributed largely to their textural properties (22, 23). Inherent in this viewpoint is the assumption that fats or their chemical constituents are capable of activating the somatosensory system. However, direct evidence about the ability of fats to stimulate somatosensory system is lacking and the mechanisms by which they do so remain to be elucidated. Dietary fat, presumably in the form of triglycerides contains a significant amount of FAs like linoleic and oleic acids (24, 25). The amount of FAs would further increase by hydrolysis during heating (26). Our results demonstrated that FAs could stimulate a [Ca²⁺]i increase as well as directly depolarize TGNs, which provided the first direct evidence that FAs may act as somatosensory stimuli at concentrations found in vivo.

I further attempted to unravel the LA-initiated signaling cascade that leads to the [Ca²⁺]i rise. Careful studies of Na⁺ substitution revealed that LA-induced [Ca²⁺]i increase was reduced about as low as 8% across all the neurons I tested. This result indicated that the extracellular Na⁺ did involve in the general FA-sensitive signaling, but the small amount of reduction suggested its role not crucial. Meanwhile, I performed the parallel study of substituting for extracellular Ca²⁺. Interestingly, the Ca²⁺ dependence of responses to LA varied across neurons. Over 65% TGNs showed more than 50% reduction in the magnitude of LA-induced Ca²⁺ responses, while the rest (~35%) appeared independent of extracellular Ca²⁺. Although this variability precludes me from
making any definite conclusions about the role of extracellular Ca\(^{2+}\) in LA-induced responses, it did lead to the interpretation that there may be different classes of neurons in respect to their ability in responding to FAs. Furthermore, these results indicated that there may be differences in the relative involvement of intracellular Ca\(^{2+}\) stores in the LA signaling pathway. I took the use of the non-competitive SERCA inhibitor thapsigargin in this study. Interestingly, thapsigargin blocked the LA-induced [Ca\(^{2+}\)]\(_i\) rise in 68% of TGNs, which was consistent with the percentage of neurons that were dependent of extracellular Ca\(^{2+}\). On the other hand, the neurons that were independent of extracellular Ca\(^{2+}\) seemed to have the thapsigargin-insensitive stores. The underlying physiological function differences between these two groups are still unclear.

There are several receptor candidates of FAs that have been identified recently. These include, a group of FA-sensitive GPCRs and the FA translocase, CD36 (18, 27-30). Long chain FAs receptor GPR40, for example, has been reported to amplify glucose-stimulated insulin secretion in pancreatic β cells (20, 21). GPR120, another long chain unsaturated FAs receptor, was thought to regulate the GLP-1 secretion in enteroendocrine cell line STC-1 (18). In this dissertation, I first showed the mRNA expression of FA-sensitive GPCRs in trigeminal ganglia. Further, I checked whether G protein/PLC pathway was involved in the signaling cascade. The results from pharmacological studies using G protein blocker GDP-β-S and general PLC blocker U73122 yielded results consistent with the interpretation that the G protein/PLC pathway was involved in the FAs (i.e. LA) signaling pathways in TGNs. Nevertheless, direct evidence implicating the
involvement of specific GPCRs as FAs receptors in somatosensory neurons will require additional studies.

TGNs responded to a series of FAs, which varied in carbon chain length, and the number and position of double bonds. I observed significant delays in FA-evoked physiological changes in [Ca\(^{2+}\)], and in membrane depolarization, which was particularly evident for polyunsaturated FAs. While the nature of this delayed response is not clear, there could be several explanations. First, the LA-induced [Ca\(^{2+}\)] increase after store depletion indicates that SOCE might be involved. It was reported that some potential components in the SOCE pathways might exhibit physiologically relevant, slow responses (31, 32). Secondly, FA-induced activation of sensory neurons might involve other membrane proteins, like CD36 (30, 33). The exact mechanisms of FAs’ binding to GPCRs and the involvement, if any, with the FAs transporter CD36 is unclear (34). A rate limiting process might exist to slow the physiological response seen in these experiments. Thirdly, I tried other FAs which include OA, MA, and DHA. The [Ca\(^{2+}\)] increases to OA (long chain monounsaturated FA) and MA (medium chain saturated FA) were faster than LA and DHA, which suggested that they might activate different receptors (28, 35). The details of the cellular/molecular explanations of the delay remain to be studied.

A significant confounding variable in studying isolated TGNs lies in the fact of the mixed population of polymodal neurons. The trigeminal nerve contains both efferent and afferent nerves, not to mention its innervations to the face, nasal and neck areas. Indeed, I observed the variations among TGNs in the extracellular Ca\(^{2+}\) substitution and
thapsigargin treatment experiments, consistent with there being multiple populations of cell types that are capable of responding to FAs. However, the underlying physiological explanations of these variations remained unanswered. To this end, however, I will partially answer this question in Chapter 3, by using pseudorabies virus constructs as a “live-cell tracer,” to identify that subpopulation of neurons that innervates the oral cavity.

My studies of FAs activating somatosensory system provide new insights into the dietary fat detection in oral cavity. The somatosensory system apparently recognizes not only the triglyceride form of fat but also shows strong cellular activation by FAs, the proximate stimulus for fat “taste.” Future studies on identified subpopulations of TGNs may better refine the transduction pathways including identifying the FA-activated TRP channels, their relationship with intracellular Ca\(^{2+}\) store, and direct evidence of GPCRs involvement in the FA-transduction pathway.

References


Table 2.1: The primer sequences for GPR40, 43, 120 and CD36.

<table>
<thead>
<tr>
<th>Target</th>
<th>GenBank Accession No.</th>
<th>Sense primer/Antisense primer/Probe</th>
<th>Corresponding nucleotide sequence</th>
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<td>GPR40</td>
<td>NM_153304</td>
<td>5'-TTCTTTCTGCCCTTGGTTAT-3'</td>
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<td></td>
<td></td>
<td>5'-GCCCTGAGCTTCGTTTG-3'</td>
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</tr>
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<td>5'-ACTGCTTTCTGCTATGTGGGTGCTCTC-3'</td>
<td>596-622</td>
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<tr>
<td>GPR43</td>
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<td>5'-GCGGGCATCAGCAGTAA-3'</td>
<td>301-318</td>
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<td>5'-CCACCTGCTCGGAGTTAGT-3'</td>
<td>451-470</td>
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<td>5'-TGCTTTCGCCGTCAGTACAAGCTATC-3'</td>
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<td>GPR120</td>
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<td>5'-CTCTGAGGCTCTCGAGATA-3'</td>
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<td>738-764</td>
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<td>CD36</td>
<td>AF072411</td>
<td>5'-GTGTGCTGGACATTTGG-3'</td>
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<td>5'-CTATGCTTCAGGTTGCTTGGG-3'</td>
<td>1108-1127</td>
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<td>5'-TCAAGCCCTCGATAGGTTGCTGAGCATCA-3'</td>
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Table 2.2: The structural information of fatty acids used in the present study.

<table>
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<th>Compounds</th>
<th>Structural Nomenclature</th>
<th>Bond Positions</th>
<th>Response in TGNs</th>
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<tr>
<td>Caproic acid</td>
<td>C6:0</td>
<td></td>
<td>no response</td>
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<tr>
<td>Myristic acid</td>
<td>C14:0</td>
<td></td>
<td>Yes (26%, n=151)</td>
</tr>
<tr>
<td>Oleic acid</td>
<td>C18:1, n-9</td>
<td>Δ9</td>
<td>Yes (23%, n=83)</td>
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<tr>
<td>Linoleic acid</td>
<td>C18:2, n-6</td>
<td>Δ9, 12</td>
<td>Yes (65%, n=332)</td>
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<tr>
<td>Arachidonic acid</td>
<td>C20:4, n-6</td>
<td>Δ5, 8, 11, 14</td>
<td>Yes (36%, n=22)</td>
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<tr>
<td>Docosahexaenoic acid</td>
<td>C22:6, n-3</td>
<td>Δ4, 7, 10, 13, 16, 19</td>
<td>Yes (59%, n=22)</td>
</tr>
</tbody>
</table>
Figure 2.1: Linoleic acid elicits a rise in $[\text{Ca}^{2+}]_i$ and depolarize TGNs. Bath application of LA (30 µM) induces increase in intracellular calcium concentration (A) and depolarization (B) in acute isolated TGNs. Inset, the concentration-response function for LA-activated $[\text{Ca}^{2+}]_i$ in TGNs.
Figure 2.2: Other fatty acids activate TGNs. The long chain monounsaturated fatty acid, oleic acid (30 µM) (A) and the medium chain saturated fatty acid myristic acid (30 µM) (B) activate rapid, biphasic and adapting [Ca^{2+}]_{i} increases in TGNs. Another long chain polyunsaturated fatty acid docosahexaenoic acid (DHA, 30 µM) (C) elicits [Ca^{2+}]_{i} increases in a similar time course as LA (Fig. 2.1A). The short chain saturated fatty acid caproic acid (30 µM) (D) failed to activate TGNs. (E) shows the relative proportions of cell responses. In adult rat TGNs, 65% (n=332) of TGNs respond to LA, 23% (n=83) and 26% (n=151) of LA-responded cells respond to oleic acid and myristic acid, respectively.
Figure 2.3: Ionic dependence of the LA-induced \([\text{Ca}^{2+}]\) increase. LA (30 µM) induced similar \([\text{Ca}^{2+}]\) in Tyrode’s and Na⁺ free Tyrode’s solutions (A) even in the reverse order (B). (C) Comparison of LA-induced \([\text{Ca}^{2+}]\) increase in regular Tyrode’s solution and Na⁺ free Tyrode’s solution. The response amplitude in Na⁺ free Tyrode’s is significantly less than Tyrode’s solution (C). (D-F) LA-induced \([\text{Ca}^{2+}]\) in Tyrode’s, Na⁺ free Tyrode’s and Ca²⁺ free Tyrode’s solutions. The \([\text{Ca}^{2+}]\) increase could be dependent (D), moderately dependent (E) or independent of extracellular Ca²⁺ in subsets of TGNs.
Figure 2.4: Thapsigargin treatment blocked the LA-induced \([\text{Ca}^{2+}]_i\) increase in a subset of TGNs. Thapsigargin (TG) pretreated TGNs respond to LA (30 µM) in two different ways in TGNs. (A) shows a representative response of TGNs that have TG-insensitive stores, (B) shows a representative response of TGNs that have TG-sensitive stores. (C) Comparison of LA-induced \([\text{Ca}^{2+}]_i\) changes ± SEM in Tyrode’s and \(\text{Ca}^{2+}\) free Tyrode’s solutions in TG-insensitive TGNs and TG-sensitive TGNs. LA-induced \([\text{Ca}^{2+}]_i\) changes in Tyrode’s solution were significantly different between TG-insensitive TGNs and TG-sensitive TGNs. In TG-sensitive neurons, LA-induced \([\text{Ca}^{2+}]_i\) changes in \(\text{Ca}^{2+}\) free Tyrode’s was significantly smaller than in Tyrode’s solution. *, P<0.001
Figure 2.5: Current clamp studies in TGNs held at their zero current level show that depolarization in response to LA is greatly reduced upon blocking activity of G protein and phospholipase C (PLC). LA-induced depolarization in control (A), U73122 (3 µM) treated (B) and in U33433 (3 µM, the inactive analog of U73122) treated (C) GDP-β-S (1 mM) treated (D), TGNs. (E) Mean responses ± SEM of 30 µM LA in control conditions, GDP-β-S treatment, U73122 & U73343 treatments in TGNs. (F) Relative expression of fatty acids sensitive proteins: CD36, GPR40, GPR43, GRP120 and GPR84. *, P<0.001.
CHAPTER 3

PSEUDORABIES VIRUS NERVE TRACING REVEALS LINGUAL TRIGEMINAL GANGLIONIC NEURONS ARE ACTIVATED BY FATTY ACIDS

Abstract

Fatty acids (FAs) are capable of activating a subset of neurons isolated from the trigeminal ganglia in adult rats, consistent with the ability of these compounds to act as somatosensory stimuli (Chapter 2). These experiments, like the majority of research done on the cellular neurophysiology of trigeminal ganglionic neurons (TGNs), are confounded by the fact that neurons from the trigeminal ganglia are a mixed population of cells of both afferent and efferent origins which innervate multiple sites within the region of the head. In this study, I adopted and refined the technique of using pseudorabies virus (PrV) as a trigeminal nerve tracer. The goal of this series of experiments was to validate in a population of TGNs that innervate the tongue, my earlier findings showing that FAs could cause a rise in intracellular calcium concentration ([Ca$^{2+}$]) in TGNs and lead to membrane depolarization. PrV-KaΔgGfp injected to the tongue successfully labeled a population of lingual-innervated TGNs. Quantitative real-time PCR on individual neurons showed several transient receptor potential (TRP) channel markers were expressed, as expected, in PrV-labeled TGNs. Of these, TRPM8, which is normally expressed in only 17% of TGNs (1), was widely expressed in PrV-labeled TGNs. Several FAs were able to activate PrV-labeled TGNs, as well as PrV-unlabeled ones, which indicated that PrV was only capable of labeling a subpopulation of linoleic acid (LA)-sensitive TGNs. Ion substitution experiments conducted using calcium
imaging revealed that the LA-induced $\left[\text{Ca}^{2+}\right]_i$ increase in PrV-labeled TGNs was not dependent on extracellular $\text{Ca}^{2+}$ influx, but on the intracellular $\text{Ca}^{2+}$ stores release. Thapsigargin treatment further confirmed the role of intracellular $\text{Ca}^{2+}$ stores in the LA signaling in lingual TGNs.

Introduction

Primary sensory neurons of trigeminal nerve in the peripheral nervous system (PNS) are providing most of the somatosensory information in the head. They mediate mechanical, thermal, and chemosensory information from many tissues, including the meninges, the cornea and conjunctiva of the eyes, the facial skin, and the mucous membranes of the oral and nasal cavities. Therefore, single TGNs mediate sensations from selective areas of the head and is specialized for different qualities of somatosensory information (2). However, once the ganglia are taken out for experimentation, the information of “where these neurons come from” and “what are their functions” is lost. Conventional strategies of identification of neurons by either soma diameter sizes or pharmacological markers are not comprehensive and may lead to misidentification of the function and origin of individual TGNs.

Pseudorabies virus (PrV) is a swine herpesvirus of the *Alphaherpesvirinae* subfamily, which cause Aujeszky’s disease in pigs. It is a neurotropic virus by nature which invades neurons and produces infectious progeny that cross synapses to infect other neurons in the same circuit. That is, PrV spreads faithfully among the functionally connected neurons (synapses), rather than simply releasing virus particles into the extracellular environment (3). These features make PrV a good tool as a neural circuit
tracer. Recently, several studies have shown that PrV-Bartha and PrV-Kaplan strains could be utilized to identify TGNs that innervate the mucous membranes of the nasal cavity or the epithelial cells of the facial skin (4, 5). The attenuated strain PrV Bartha, is the most widely used tracing PrV. It spreads only in the retrograde direction. The wild-type PrV-Kaplan strain, on the other hand, is capable of spreading in both anterograde and retrograde directions (3). However, for tracing primary TGNs from the terminal organs, no trans-synaptic passage of viral particles is needed. Therefore, the data obtained with PrV-Kaplan infection would be comparable with data from PrV-Bartha strain infection, although the post-inoculation-time-window is different (5). For the PrV-Bartha strain, about 40 hours post inoculation (hpi) would be needed to observe a robust PrV-mediated expression of marker proteins in the TGNs. Longer hpi would cause the secondary infection, leading to potential unspecific labeling (4). For the PrV-Kaplan strain, the expression of the marker protein GFP was found expressed as early as 12 hpi. The nonspecific spread was observed after 24 hpi (5). Furthermore, evidence has shown that careful electrophysiology and calcium imaging studies after the PrV-Bartha and PrV-Kaplan infections revealed no detectable influences of PrV infection on many physiological properties including the resting membrane potentials, threshold for activation of voltage-gated sodium channels and amplitude of the inward current initiated by TRP channel agonists menthol and capsaicin (for TRPM8 and TRPV1, respectively) (4, 5).

PrV has been injected into the tongue successfully by Card, et. al as early as 1990 (6). A later study had successfully identified the neural circuits that innervate the tongue
muscles using PrV-Bartha strain (7). In my present study, I used the PrV-Kaplan strain with GFP as the reporter gene (PrV-KaΔgGgfp) to trace the tongue-innervated population of TGNs. Using this population of lingual TGNs (i.e. PrV-labeled), I validated my earlier findings conducted in the entire TGNs population (Chapter 2) which showed that FAs were capable of activating these cells, consistent with the ability of FAs to activate the somatosensory system. My results have refined these data to demonstrate unequivocally the ability of FAs to activate lingual TGNs.

Materials and Methods

PrV recovery procedure. The DNA construct of PrV-Kaplan derived recombinant PrV-KaΔgGgfp was a generous gift from Prof. Mettenleiter at Friedrich-Loeffler-Institut, Bundesforschungsinstitut für Tiergesundheit, Germany. For virus recovery, the DNA construct was transfected into porcine kidney (PK15) cells (ATCC, Manassas, VA) using SuperFect Transfection Reagent (Qiagen, Valencia, CA). Virus was collected for infection of fresh PK15 cells to obtain a higher titer when the complete cytopathic effect (CPE) was shown. The virus was further propogated on monolayer of PK15 cells and finally titered with 1% methocel and 1X DMEM semisolid medium mixture supplemented with 2% FBS and penicillin/streptomycin (100 U/ml). The virus stocks with the titer of 10⁷ to 10⁸ plaque forming units (PFU) per ml were aliquoted into 100 µl stocks and frozen at -80 °C for future use. The stocks remain viable for at least 1 year without loss of infectivity. At the times of infection, stocks were removed from the freezer and kept on ice until use.
**PrV stock titer.** A 1% methocel, 1X DMEM semi-solid medium was prepared by mixing 2% methocel and 2X DMEM media. The virus sample was thawed and frozen three times followed by a 10 seconds sonication with 1 second pulses at 40% amplitude. A 10 fold serial dilution of virus stock was made to obtain the low concentration virus. To determine the titer, the virus dilutions were plated on PK15 monolayer with the 1% methocel, 1X DMEM semi-solid medium. This semi-solid medium facilitates the formation of plaques by physically blocking the diffusion of the virus. The plaques could be visible after 2-3 days of culture by staining the cells with 0.5% methylene blue in 70% methanol.

**PrV injection procedure.** The study was conducted using P0-P7 neonate Sprague-Dawley rats. The animals were maintained on a 12-hour: 12-hour light cycle with water and food available *ad libitum*. All animal experimental procedures were reviewed and approved by the Laboratory Animal Research Center, Utah State University. The virus-infected animals were kept separate, and biosafety level 2 (BSL2) procedures were used.

For tongue injection, neonate rats were anesthetized with hypothermia. Each neonate rat was put in a latex glove finger to avoid freeze damage to the skin. The latex wrapped animals were immersed into ice-water mixture for 3.5 to 4 minutes. The anesthetized neonate rats were placed on their backs and a blunt tip forceps were used to gain access to the tongue. Ten µl of viral suspension (~$10^8$ PFU/ml) was injected to the tongue with a pre-sterilized Hamilton syringe (25 µl) with 26s gauge and a sharp tip needle. The virus was injected from the back of the tongue proceeding in an anterior
direction (i.e. toward the tip) while withdrawing the needle. The entire injection procedure took less than 30 seconds to complete. The animals were recovered from anesthesia under an incandescent lamp, and transferred to their mother.

**Acute isolation of trigeminal ganglionic neurons.** All surgical and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee, Utah State University. TGNs were isolated according to methods modified from Simon and colleagues (8, 9). Pseudorabies virus injected Sprague-Dawley P0-P7 neonate rats were deeply anaesthetized by hypothermia (see above). The paired trigeminal ganglia were then harvested and diced into small pieces into Hank’s balanced salt solution (HBSS), followed by 45-minute incubation with 2.5 mg/ml collagenase (Type XI-S) at 37°C in HBSS and a 10-minute incubation at 37°C in 100 µg/ml DNase I in culture medium (DMEM/F-12 1:1 medium supplemented with 10% FBS and 400 µg/ml Foscarnet (Alfa Aesar, Ward Hill, MA) and 100 µg/ml penicillin/streptomycin). The neurons were centrifuged and resuspended in culture medium by gentle trituration with a flame-polished Pasteur pipette. Subsequently, neurons were plated onto poly-D-lysine (0.1 mg/ml)-coated glass coverslips (15 mm in diameter) and cultured at 37°C in a water-saturated atmosphere with 5% CO₂. For collection of individual neurons for quantitative real-time PCR (qRT-PCR), Foscarnet was excluded from the culture medium. Either fluorescent large (soma diameter larger than 20 µm), fluorescent small (soma diameter smaller than 20 µm) neurons or non-fluorescent neurons were chosen for further studies. Neurons were cultured 1-2 hours after isolation for individual neurons collection for qRT-PCR, or 24-36 hours for calcium imaging.
**Calcium imaging.** TGNs were loaded with fura-2/AM (5 µM; Molecular Probes, Eugene, OR) for 1 hour in Tyrode’s saline solution with 10% pluronic acid at 37 °C in the dark. Cells were then rinsed and placed in culture medium for 30 minutes to allow the de-esterification of the acetoxymethyl ester group from fura-2. The coverslips were mounted into an imaging chamber (RC-25F and RC-26Z, Warner Instruments, Hamden, CT), placed on an inverted microscope (Nikon, Eclipse TS100, Japan) and perfused continuously with Tyrode’s. Neurons were illuminated with a 100-watt xenon lamp and excitation wavelengths (340/380 nm) were delivered by a monochromator (Bentham FSM150, Intracellular Imaging Inc., Cincinnati, OH) at a rate of 20 ratios per minute. A 470 nm light source coupled with a corresponding filter was used for detecting green fluorescent protein expression. Fura-2 fluorescence was measured by a CCD camera (pixelFly, Cooke, MI) coupled to a microscope and controlled by imaging software (Incyt Im2™, Intracellular Imaging). The ratio of fluorescence (340/380 nm) was directly converted to calcium concentrations using a standard curve generated for the imaging system using a Fura-2 calcium imaging calibration kit (Molecular Probes, Eugene, OR). FAs were applied extracellularly with a bath perfusion system at a flow rate of 4 ml/min permitting complete exchange of the extracellular solution in less than 20 seconds.

**Solutions.** Standard extracellular saline solution (Tyrode’s) contained (in mM): 140 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, 10 glucose, and 10 Na pyruvate; pH 7.40 adjusted with NaOH; 310 mOsm. Sodium-free saline (Na⁺ free Tyrode’s) contained (in mM): 280 mannitol, 5 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, 10 glucose; pH 7.40 adjusted with TrisOH; 310 mOsm (adjusted with mannitol). Calcium free saline (Ca²⁺
free Tyrode’s) contained (in mM): 140 NaCl, 5 KCl, 1 EGTA, 1 MgCl₂, 10 HEPES, 10 glucose, and 10 Na pyruvate; pH 7.40 adjusted with NaOH; 310 mOsm. Thapsigargin (a non-competitive SERCA (sarco/endoplasmic reticulum Ca²⁺ ATPase) inhibitor), and all the FAs were purchased from Sigma (St. Louis, MO). The LA and oleic acid (OA) stocks were made in 100% ethanol and stored under nitrogen. The myristic acid (MA) stock was made in distilled water. The working solutions were made from stock solutions immediately prior to use.

**Quantitative real-time PCR (qRT-PCR).** For qRT-PCR analysis on limited numbers of identified TGNs were collected under an inverted microscopy using borosilicate hematocrit capillary pipettes. The capillary pipettes were pulled and fire-polished to a bore slightly smaller that the size of the cell to guard against collection of cell debris. Target cells were sucked into unfilled collection pipettes, which were controlled by a micromanipulator. After capture, the tip of the pipette was broken into a 0.5 ml microfuge tube containing 50 µl RLT buffer (supplemented with B-mercaptoethanol) from the RNeasy Micro Kit (Qiagen) and kept on ice. Total RNA isolated using the RNeasy Micro kit and MessageBOOSTER kit was used for amplification. In this study, we collected PrV-labeled medium to large cells (soma diameter >20 µm), PrV-labeled small cells (soma diameter < 20 µm) or non-fluorescent mixed cells to assay for TRPM8 or TRPA1 expression. The HotMaster Taq DNA polymerase kit was used for the PCR reaction, and 1 µl cDNA per reaction was amplified using a 2-step PCR (6-second denaturation, 60-second annealing and extension). A TaqMan (ABI, Foster City, CA) detection system was used to detect the specific targets
and a housekeeping gene GAPDH, for comparison of expression levels. The primers were from either TaqMan Gene Expression Assays or designed by ourselves.

**Statistical analysis.** The significant effects of all the treatments were determined by a two-tailed Student’s t-test (α=0.05) compared with their controls as described in the text. Data are presented as mean ± SEM, unless otherwise indicated.

**Results**

**PrV injected into the tongue successfully labeled a population of neurons in the trigeminal ganglia.** In the present study, I extended the use of PrV-KaΔgGgfp to identify the population of TGNs that innervate the tongue. In order to obtain the best infectious efficiency, I injected PrV into neonate rats instead of adult rats, which were used in the previous studies of FAs responsiveness in TGNs. Following the procedures of Rothermel et al. that showed inoculations of PrV-KaΔgGgfp to nasal cavity and skin took about 12 hpi to reach the trigeminal ganglia. A 12-24 hpi time-window was initially chosen to allow the broadest possible PrV infection, but the non-specific infection would still be restricted (5). Foscarnet (400 µg/ml) was supplemented to the regular culture medium for TGNs to inhibit the non-specific viral spread (4). Therefore, I injected PrV-KaΔgGgfp into the tongue muscle in the vicinity of the lingual nerve without any lateral restrictions in order to obtain the largest labeled neuron population in the trigeminal ganglia. As shown in Fig. 3.1, 10% of all TGNs were expressing GFP, which indicated their lingual origin. Different soma diameters (i.e. functional classes of cells) of acutely isolated TGNs showed the expression of the reporter protein, although the smaller cells with soma diameter less than ~20 µm were much more commonly observed.
To validate that PrV-labeled TGNs included a subpopulation of sensory neurons that were responsible for chemosensory somatosensation, I checked the expression of TRPM8 and TRPA1 mRNA in individual PrV-labeled neurons using quantitative real-time polymerase chain reaction (qRT-PCR). However, I failed to find a consistent relationship between neuron soma size and TRP channel expression in these PrV-labeled TGNs. As indicated in Table 3.1, TRPM8 was consistently found in both PrV-labeled medium to large (soma diameter more than 20 µm) and small (soma diameter less than 20 µm) neurons (n=9/13). In 2 out of 5 assays conducted with medium to large neurons, TRPA1 was found. In small neurons, however, TRPA1 was not found in any assays (n=6), but 5 out of 7 assays still showed TRPM8 expression. The non-labeled TGNs showed both TRPA1 and TRPM8 expression (data not shown). I also performed calcium imaging experiments to further verify the TRP channels expression. Both menthol and mustard oil could activate portions of the PrV-labeled TGNs, although menthol was more responsive in PrV-labeled TGNs, which was consistent with my molecular data (data not shown). Taken together, the ‘live-cell’ tracing tool PrV-KaΔgGfp was successfully adopted and refined to my study for identifying a population of lingual-innervated, somatosensory TGNs.

**FAs activate a subpopulation of PrV-labeled, lingual-innervated TGNs.** The objective of developing the PrV labeling technique was to validate in lingual TGNs that FAs could activate TGNs (Chapter 2). As shown in Fig. 3.2A and B, LA, OA and MA (each at 30 µM) activated neonatal, PrV-labeled (i.e. lingual) TGNs in a similar manner to the original total population of adult TGNs (Chapter 2). The time course of LA, OA
and MA-induced $[\text{Ca}^{2+}]_{i}$ increases were comparable with my previous data—polyunsaturated FAs activated TGNs in a slower, gradual manner; while responses to the monounsaturated and saturated FAs have a more rapid onset and show adaptation.

However, the percentages of cells responding to each FA slightly increased in PrV-labeled TGNs, compared to the previous data generated in adult TGNs (Fig. 3.2C). There were 67%, 41%, and 31% of TGNs responding to LA, OA, and MA, respectively, as compared to 65%, 23%, and 26% in the pooled data from adult rats. I further checked whether there was a difference in the amplitude of LA-induced $[\text{Ca}^{2+}]_{i}$ increase between adult TGNs and neonatal TGNs (Fig. 3.2D, first two bars). In PrV-labeled TGNs, the LA-induced $[\text{Ca}^{2+}]_{i}$ increase was 197.3 ± 4.7 nM ($n=221$), compared to 253.4 ± 9.3 nM ($n=241$) in the TGNs isolated from adult rats. There was a significant difference ($P<0.005$) between these two groups, mainly attributable to the differences between adult rat TGNs and neonatal TGNs. On the other hand, the amplitudes of PrV-labeled TGNs (193.2 ± 7.2 nM; $n=104$) and PrV-unlabeled TGNs (200.9 ± 6.1 nM; $n=117$) were similar enough that no obvious differences could be observed (Fig. 3.2D; $P=0.41$). These data suggested that PrV was not able to isolate the whole population of LA-sensitive TGNs, but capable of labeling a subpopulation of LA-sensitive TGNs.

**LA-induced $[\text{Ca}^{2+}]_{i}$ increase in PrV-labeled TGNs was not dependent on extracellular sodium, but could be dependent on intracellular calcium release.** According to the features of PrV-labeled TGNs stated above, I performed a parallel series of experiments on PrV-labeled TGNs to check the ionic dependence of LA-induced $[\text{Ca}^{2+}]_{i}$ increase as I did previously (Chapter 2). To determine if extracellular sodium
(Na\(^+\)) and calcium (Ca\(^{2+}\)) influx was contributing to [Ca\(^{2+}\)], increase on PrV-labeled TGNs, I conducted calcium imaging experiments with saline substituting mannitol for extracellular Na\(^+\) for (Na\(^+\) free Tyrode’s) or EGTA for extracellular Ca\(^{2+}\) (Ca\(^{2+}\) free Tyrode’s). Fig. 3.3A shows representative data of the LA-induced [Ca\(^{2+}\)], increase in PrV-labeled TGNs in normal saline followed by Na\(^+\)-free and Ca\(^{2+}\)-free Tyrode’s solutions. To eliminate the cell-to-cell variability and the underlying differences between TGNs from adult and neonatal rats (Fig. 3.2D), I normalized each cell’s LA-induced [Ca\(^{2+}\)], increase in Na\(^+\)/Ca\(^{2+}\) free solution with the LA-induced [Ca\(^{2+}\)], increase in regular Tyrode’s solution in the same cell. The resulting percentages were used as the functional output to further compare across groups. Fig. 3.3B showed the comparison of the normalized percentage in Na\(^+\) free Tyrode’s between adult TGNs and PrV-labeled TGNs. In PrV-labeled TGNs, the [Ca\(^{2+}\)], increase in Na\(^+\) free Tyrode’s was 78.0 ± 3.0% (n=101) of the response magnitude in regular Tyrode’s, which was not significantly different from our previous result obtained in adult rats (P>0.05). Interestingly, the effects of Ca\(^{2+}\) substitution yielded results different from that in adult TGNs. In adult TGNs, there was significant variation in the extracellular Ca\(^{2+}\) dependence of the LA responses, which was taken as evidence of multiple cell types (Fig. 2.2 D-F). However, in PrV-labeled TGNs, the LA responses were much more consistent, with cells showing little dependence upon extracellular Ca\(^{2+}\). As shown in Fig. 3.3C, LA-induced [Ca\(^{2+}\)], increase in Ca\(^{2+}\) free Tyrode’s composed of 89.2 ± 5.6% (n=100) of the magnitude of responses in normal Tyrode’s in PrV-labeled TGNs, compared with averaged 43.1 ± 2.6% (n=177; P<0.001) in the adult TGNs. Thus, in the PrV-labeled population of lingual-innervated TGNs, the
LA-induced \([\text{Ca}^{2+}]_i\) increase was mostly independent of extracellular Na\(^{+}\) and Ca\(^{2+}\), and largely dependent on the release of Ca\(^{2+}\) from intracellular stores.

**Thapsigargin treatment blocked LA-induced \([\text{Ca}^{2+}]_i\) increases in PrV-labeled TGNs.** To further confirm the LA-induced \([\text{Ca}^{2+}]_i\) increase is mainly coming from intracellular Ca\(^{2+}\) stores, I used the SERCA inhibitor thapsigargin to deplete the Ca\(^{2+}\) store, and tested if LA could still activate the neurons. TGNs were pretreated with thapsigargin (1 \(\mu\)M) for 1 hour before plating onto the imaging chamber. Again, similar to the observations in adult TGNs, most thapsigargin pretreated TGNs including PrV-labeled and PrV-unlabeled ones showed basal Ca\(^{2+}\) level increases, which returned to near control levels by LA stimulation (Fig. 3.4A and B). The activation of store operated calcium entry (SOCE) during the intracellular Ca\(^{2+}\) store depletion might still be involved in the LA-induced signaling (10-12). In this present study, the PrV-unlabeled TGNs (Fig. 3.4A) were regarded as the control group, because of the obvious underlying differences between the TGNs from adult and neonatal rats (Fig. 3.2D). In PrV-unlabeled TGNs (Fig. 3.4A), application of LA still elicited a decrease in basal Ca\(^{2+}\) concentration as previously because of its ability to inhibit SOCE (11), followed by the responses induced by LA displayed their characteristic slow \([\text{Ca}^{2+}]_i\) increases. In PrV-labeled TGNs (Fig. 3.4B), thapsigargin treatment inhibited the LA-induced \([\text{Ca}^{2+}]_i\) increase in the regular Tyrode’s saline. In the Ca\(^{2+}\) removal experiments, however, in both PrV-labeled and PrV-unlabeled TGNs, the LA-induced \([\text{Ca}^{2+}]_i\) increase was abolished (Fig. 3.4 A and B). Fig. 3.4C shows the mean responses in Tyrode’s and Ca\(^{2+}\) free Tyrode’s in both groups of PrV-
labeled and PrV-unlabeled TGNs. Thus, I concluded that the \([\text{Ca}^{2+}]_i\) increase initiated by LA stimulation was mainly coming from the intracellular \(\text{Ca}^{2+}\) stores.

Discussion

Different strains of PrV inserted with marker protein genes have been developed as molecular pathfinders in nervous system circuits. The host range of PrV has made it a useful tool in a wide range of animal models but remains safe for laboratory workers. Studies have been performed to determine the connections of the central nervous system (CNS) to many peripheral organs and tissues, such as tongue (7), nose (4), adipose tissues (13-16), muscle (17), heart (18), stomach (19), etc. In 2006, Damann and colleagues utilized PrV as a ‘live-cell’ tracing tool to functionally separate a subpopulation of specific organ-innervated TGNs (4). Besides the significant efforts they made to study the conditions of using PrV as a reliable tracer for trigeminal nerve, they also made the further attempts to investigate the physiological properties of TGNs after PrV infection. According to their pioneering results, within carefully manipulated post-inoculation-time-window, PrV was tracing from the peripheral injection sites to the first primary ganglia faithfully. Specifically, although wild type Kaplan strain elicits a powerful inflammatory response that minimizes the post-infectious duration of animal survival, this strain has a much more rapid labeling time course and a much more effective labeling profile. Therefore, as verified in the recent study, PrV-Kaplan based tracers appeared as a good choice for functionally separating a population of specific tissue-innervated TGNs.

In my studies, I observed about 10% of TGNs labeled by PrV-KaΔgGfp. It is difficult to state how many TGNs have nerve fibers innervating oral cavity theoretically,
since the adult trigeminal system still retains considerable plasticity (20). Specific marker expression and physiological studies provided some degrees of classification information, e.g. 25% of sensory neurons that are TRPA1 and TRPV1 positive nociceptors, while TRPM8 appeared in a separate 20% unmyelinated C fiber and myelinated Aδ fibers neurons that does not express either TRPA1 or TRPV1 (1, 21). However, a particular receptor expressed neuron does not necessarily respond only to the specific chemical stimuli that activate the receptor (22). PrV tracing, on the other hand, separates a subpopulation of TGNs in the functional level that provides us different insights of the problem. Interestingly enough, from my qRT-PCR results, TRPM8 was commonly expressed in PrV-labeled TGNs, but TRPA1 was rarely seen, which was consistent with the known expression pattern. These results further indicate that the connection between the functional nerve innervations and the receptor expression pattern might exist in the sensory system.

My goal with the PrV labeling was to validate my findings of FA responsiveness in lingual-innervated TGNs. Thus, I closely examined the FA-induced $[\text{Ca}^{2+}]_i$ in PrV-labeled TGNs. Compared to my previous studies on pooled TGNs from adult rats, the general response profile did not change significantly, although a small increase on responding percentage was observed for each of the FAs tested. Moreover, LA seemed to activate the PrV-labeled and PrV-unlabeled TGNs similarly both in terms of the percentage of responding cells and the magnitude of the LA-induced responses. There are two obvious conclusions from this finding. First, PrV labeled a subpopulation of TGNs that innervate the oral cavity. Second, TGNs that innervate other areas of the head, such
as the face or nasal cavity that are not labeled by this approach, respond to FAs in a similar fashion as those that innervate the tongue.

Indeed, the ion-substitution experiments in PrV-labeled TGNs suggested similar conclusions. The variation of the LA-induced \([\text{Ca}^{2+}]_i\) increase in Ca\(^{2+}\)-free Tyrode’s disappeared. Extracellular Ca\(^{2+}\) did not play a big role in PrV-labeled TGNs, whereas the previous data showed about 70% of TGNs were highly dependent on extracellular Ca\(^{2+}\). On the other hand, the role of extracellular Na\(^+\) was consistent between PrV-labeled and pooled adult TGNs. Removal of extracellular Na\(^+\) consistently lead to only about a 10% decrease in the change in \([\text{Ca}^{2+}]_i\) initiated by LA. These experiments, along with the previous data from pooled adult rat TGNs suggested that LA activation of TGNs is largely dependent upon Ca\(^{2+}\) release from the intracellular Ca\(^{2+}\) stores.

Pharmacological studies with thapsigargin further confirmed the importance of Ca\(^{2+}\) release from stores in the LA-induced responses in TGNs. In PrV-labeled TGNs treated with thapsigargin, LA was not able to induce the robust \([\text{Ca}^{2+}]_i\); increase in Tyrode’s solution, and subsequent substitution for Ca\(^{2+}\) completely abolished the response. However, in PrV-unlabeled TGNs, the LA-induced \([\text{Ca}^{2+}]_i\) increases were dependent on Ca\(^{2+}\) from extracellular environment. Interestingly, in pooled TGNs from adult rats, a subpopulation of neurons that was apparently thapsigargin insensitive as thapsigargin treatment has little effect on LA-induced \([\text{Ca}^{2+}]_i\); in those TGNs (see Chapter 2 for details). In my studies using PrV as tracing tool, the number of thapsigargin-insensitive TGNs was negligible indicating that this population of TGNs found in the adult was either not of lingual origin or not found in neonates.
Taken together, I have been able to use PrV-KaΔgGfp as a ‘live-cell’ tracer to label a portion of the lingual-innervated TGNs. This has allowed the verification of my previous conclusions regarding the ability of FAs to activate the somatosensory system and illustrates that these pathways are active in those TGNs that functionally innervate the tongue. Thus, it allows the conclusion to be drawn that FAs may be effective stimuli in the oral cavity to convey some of the somatosensory information of fats. Clearly, however, lingual-innervated TGNs do not compose the entire population of FA-sensitive neurons as similar responses were seen in those cells not labeled by PrV. Thus, TGNs that innervate other areas may also respond to FAs and/or the efficiency of PrV labeling of the lingual TGNs may be low. Future studies will be required to discern about these possible conclusions. Nonetheless, having access to a population of lingual TGNs has allowed the elucidation of some aspects of the FAs signaling pathway, one goal of this dissertation research. The data shown in this study demonstrate conclusively that responses to LA are largely dependent upon release of intracellular Ca\(^{2+}\) from stores and are consistent with our current model of FAs transduction (cf. Fig, 1.3 in Chapter 1).

References


Table 3.1: Individual TGNs expressed TRPM8 and TRPA1 channels. TRP channels expression was determined using quantitative real time PCR in PrV-labeled (i.e. lingual-innervated) large and small TGNs. +, positive; -, negative; n=positive or negative number out of total number of assays.

<table>
<thead>
<tr>
<th>Single cell RT-PCR</th>
<th>Cell Size</th>
<th>TRPM8</th>
<th>TRPA1</th>
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<tbody>
<tr>
<td></td>
<td>Large</td>
<td>&gt;30 µm</td>
<td>+ (n=4/6)</td>
</tr>
<tr>
<td></td>
<td>Small</td>
<td>&lt;30 µm</td>
<td>+ (n=5/7)</td>
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Figure 3.1: Pseudorabies virus injected to the tongue successfully labeled a subpopulation of neurons in the isolated TGNs. Fluorescence image showing PrV-KaΔgGfp labeled TGNs ~24 h after injection. Arrows point to TGNs that were obviously infected by PrV-KaΔagGfp.
Figure 3.2: Fatty acids activated a subpopulation of PrV-labeled, lingual-innervated TGNs. (A) and (B), bath application of linoleic, oleic and myristic acids (each at 30 µM) activate PrV-labeled TGNs. (A), this PrV-labeled TGN respond to linoleic and myristic acid, while (B) shows another PrV-labeled TGN that respond to linoleic and oleic acids. (C) The proportion of fatty acids responsiveness seen in PrV-labeled neurons. In PrV labeled TGNs, 67% (n=186) of TGNs respond to linoleic acid, while 41% (n=128) and 31% (n=107) of linoleic acid responsive neurons respond to oleic acid and myristic acid, respectively. (D), Mean responses (rise in $[Ca^{2+}]_i$) ± SEM induced by linoleic acid (30 µM) in adult TGNs, total number of neonatal TGNs, PrV-unlabeled TGNs and labeled TGNs. *, P<0.005.
Figure 3.3: LA-induced \([\text{Ca}^{2+}]\) increase in PrV-labeled TGNs was not largely dependent on extracellular Na\(^+\), but showed dependence on intracellular Ca\(^{2+}\) release. Two sample graphs of LA-induced \([\text{Ca}^{2+}]\), increase in regular Tyrode’s saline, Na\(^+\) free Tyrode’s saline and Ca\(^{2+}\) free Tyrode’s saline in PrV-unlabeled TGNs (A) and PrV-labeled TGNs (B). (C) Mean normalized percentages (LA-induced [Ca\(^{2+}\)] increase in Na\(^+\) or Ca\(^{2+}\) free Tyrode’s / in LA-induced [Ca\(^{2+}\)], increase in Tyrode’s ± SEM, %) in PrV-unlabeled TGNs and PrV-labeled TGNs.
Figure 3.4: Thapsigargin treatment blocked LA-induced [Ca^{2+}]i increase in PrV-labeled TGNs. (A)(B) Representative data showing LA-induce [Ca^{2+}]i increases in Tyrode’s and calcium free Tyrode’s in PrV-unlabeled TGNs (A) and PrV-labeled TGNs (B) after 1h thapsigargin (1 µM) treatment. (C) Mean responses (rise in [Ca^{2+}]i) ± SEM induced by LA in Tyrode’s and Ca^{2+} free Tyrode’s in both PrV-labeled and PrV-unlabeled TGNs.
CHAPTER 4

LINOLEIC ACID INDUCES INWARD SODIUM CALCIUM PERMEABLE CATION CURRENT IN TRIGEMINAL GANGLIONIC NEURONS

Abstract

Transient receptor potential (TRP) channels are involved in the transduction cascade of many sensory perception processes. Recent studies in our lab have shown that the transient receptor potential melastatin 5 channel (TRPM5) is involved in the fatty acids (FAs)-induced signaling pathway in taste receptor cells and in an enteroendocrine cell line, STC-1. In this present study, I have begun to characterize the downstream conductance that is activated by FAs (e.g. linoleic acid, LA) in rat trigeminal ganglionic neurons (TGNs). Using perforated patch clamp recording, I have recorded LA-induced current that exhibited many of the properties of a TRP-like current. The inward current activated by LA is both calcium (Ca\(^{2+}\)) and sodium (Na\(^{+}\)) permeable, which could be inhibited by the general TRP channel blocker ruthenium red (10 µM). TRPM5 channel is not found in a detectable level in trigeminal ganglia, nonetheless, the specific TRPM5 inhibitor, triphenylphosphine oxide (TPPO), did inhibit a significant proportion of this current. Although the lack of a specific target and the specific pharmacological tools are preventing me from drawing any definite conclusions concerning the nature of the TRP channel activated by LA, the features of this current are consistent with their roles in the transduction pathway for FAs in the somatosensory system.
Introduction

The TRP channels are known to constitute important components of sensory systems. They work as some of the primary cellular sensors in virtually all organisms. In human, activation of a specific TRP channel might represent the detection of tastants, temperature or pungent reagents (1). TRP channels contain a large and functionally diverse family of ion channels that have distinct activation mechanisms and biophysical properties. In general, the mammalian TRP superfamily is divided into six subfamilies based on the sequence homology, which includes TRPC (canonical, TRPC1-7), TRPM (melastatin, TRPM1-8), TRPV (vanilloid, TRPV1-6), TRPA (ankyrin, TRPA1), TRPP (polycystin) and TRPML (mucolipin) families (2).

These subfamilies of TRP channels have their own permeability and selectivity properties while sharing some common features. For example, all TRP channels are known to be cation-selective; most functionally characterized TRP channels are permeable to Ca\(^{2+}\) ions except for TRPM4 and TRPM5, which are permeable to monovalent cations (3). Other TRP channels are permeable to both Ca\(^{2+}\) and Na\(^{+}\) ions with the different permeability and activation mechanisms. For example, TRPV1 is a Ca\(^{2+}\)-permeable channel with a rather low discrimination between Ca\(^{2+}\) and Na\(^{+}\) (P_{Ca}/P_{Na}~10), which is activated directly by heating or agonists like capsaicin and H\(^{+}\) (2). The TRPC family, however, is activated by stimulation of G protein-coupled receptors (GPCRs), and mainly functions as store- and/or PLC-dependent Ca\(^{2+}\)-permeable cation channels (2). Although the TRPC family contains the first recognized mammalian TRP channel TRPC1, these channels are still enigmatic. For example, homomeric TRPC1 has
been proposed to function as a store-operated, diacylglycerol (DAG)-activated, inositol 1,4,5-trisphosphate (IP3R)-gated cation channel, downstream of Gq/11 activation (2). There is another report stated that TRPC1 could form a stretch-activated channel in frog oocyte (4). TRPC1 was initially regarded as a mediator in the process of store-operated calcium entry (SOCE) (5), recent discoveries brought ER membrane protein STIM1 and plasma membrane protein Orai1 into view. Whether TRPC1 is working with STIM1 and Orai1 or independently is still under intense discussion in the field (6, 7). To make the situation even more complicated, several subtypes of TRPCs could work together to form a complex in the physiological state, which would have quite different electrophysiological features (8, 9).

TRPM4/5, on the other hand, is not permeable to Ca2+, and is activated downstream of other membrane proteins by some second messengers as TRPCs do. More specifically, TRPM5 is selectively expressed in taste-related tissues, and works downstream of taste receptors for sweet, bitter and umami (a group of GPCRs, called T1Rs and T2Rs). TRPM4, which is the most closely related channel to TRPM5 is expressed ubiquitously and appears likely to mediate so-called Ca2+-activated monovalent cation channels (10).

There is evidence showing a member of TRP channels might be involved in the FA-induced signaling transduction pathway in somatosensory system. In Chapter 2, I showed LA’s ability to depolarize TGNs, which involved the G protein/PLC pathway. In Chapter 2 and Chapter 3, I concluded that LA-induced [Ca2+]i increase was dependent upon the release of the Ca2+ from intracellular stores in a lingual-innervated population of
TGNs. However, the link between the rise in intracellular Ca\textsuperscript{2+} and depolarization was not elucidated. In taste receptor cells, TRPM5 has been shown to be the downstream of the membrane taste receptors T1Rs and T2Rs where it provides the taste-induced receptor potential (11, 12) and appears to play a similar role in FAs taste transduction (P. Liu and T. A. Gilbertson, unpublished data). In sensory neurons like TGNs and DRGs, TRP channels are directly involved in the transduction pathway for temperature and pungent reagents (13-17). Whether a TRP channel is involved in the FA-induced activation of TGNs is presently unclear. In this dissertation study, I have attempted to record the putative FA-activated TRP-like current in TGNs, and begin to characterize the current’s properties.

**Materials and Methods**

**Acute isolation of trigeminal ganglionic neurons.** All surgical and experimental procedures were reviewed and approved by the Institutional Animal Care and Use Committee, Utah State University. TGNs were isolated according to methods modified from Simon and colleagues (18, 19). Four- to 8-week-old male Sprague-Dawley rats were deeply anaesthetized by 50 mg/kg i.p. injection of sodium pentobarbital. The paired trigeminal ganglia were then harvested and diced into small pieces into Hank’s balanced salt solution (HBSS), followed by a 45-min incubation with 2.5 mg/ml collagenase (Type XI-S) at 37°C in HBSS also and a 10-min incubation at 37°C in 100 μg/ml DNase I in culture medium (DMEM/F-12 1:1 medium supplemented with 10% FBS). The neurons were centrifuged and resuspended in culture medium by gentle trituration with a flame-polished Pasteur pipette. Subsequently, neurons were plated onto poly-D-lysine (0.1
mg/ml)-coated glass coverslips (12-15 mm in diameter) and cultured at 37°C in a water-saturated atmosphere with 5% CO₂. For electrophysiology, neurons with soma diameters ranging between 18 to 25 µm were used within 8 hours after plating on coverslips. For calcium imaging, neurons were cultured 36-48 hours in culture medium before use. These cell based assays were handled in the room temperature unless stated.

**Calcium imaging.** TGNs were loaded with fura-2/AM (5 µM; Molecular Probes, Eugene, OR) for 1 hour in Tyrode’s saline solution with 10% pluronic acid at 37°C in the dark. Cells were then rinsed and placed in culture medium for 30 minutes to allow de-esterification of the acetoxymethyl ester group from fura-2. The coverslips were mounted into an imaging chamber (RC-25F and RC-26Z, Warner Instruments, Hamden, CT), placed on an inverted microscope (Nikon, Eclipse TS100, Japan) and perfused continuously with Tyrode’s solution. Neurons were illuminated with a 100-watt xenon lamp and excitation wavelengths (340/380 nm) were delivered by a monochromator (Bentham FSM150, Intracellular Imaging Inc., Cincinnati, OH) at a rate of 20 ratios per minute. Fluorescence was measured by a CCD camera (pixelFly, Cooke, MI) coupled to a microscope and controlled by imaging software (Incyt Im2™, Intracellular Imaging). The ratio of fluorescence (340/380 nm) was directly converted to Ca²⁺ concentrations using a standard curve generated for the imaging system using a fura-2 calcium imaging calibration kit (Molecular Probes, Eugene, OR). FAs were applied extracellularly with a bath perfusion system at a flow rate of 4 ml/min permitting complete exchange of the extracellular solution in less than 20 seconds.
**Electrophysiological recording and analysis.** We performed perforated whole-cell patch clamp recording to record FFA-induced currents in TGNs with an Axopatch-200B amplifier (Molecular Devices, Sunnyvale, CA). Patch pipettes were fabricated from borosilicate glass on a Flaming-Brown micropipette puller (model P-97; Sutter Instrument, Novato, CA) and subsequently fire-polished on a microforge (model MF-9, Narishige, Japan) to a resistance of 3-4 MΩ. Commands were delivered and data were recorded using pCLAMP software (v. 10, Molecular Devices, Sunnyvale, CA) interfaced to an Axopatch-200B amplifier with a DigiData 1322A analog-to-digital board. Data were filtered online at 1 kHz and sampled at 2-4 kHz. Nystatin was made into a 50 mg/ml stock with DMSO. Fresh intracellular solution with nystatin was made every 2 hours at a concentration of 200 µg/ml, supplemented with 0.2% pluronic acid. The time for backfill the tip is around 1 minute for each pipette. The voltage clamp mode is used to record the inward current, with a holding potential of -60 mV. LA was applied by a bath application system.

**Solutions.** Standard extracellular saline solution (Tyrode’s) contained (in mM): 140 NaCl, 5 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, 10 glucose, and 10 Na pyruvate; pH 7.40 adjusted with NaOH; 310 mOsm. Sodium-free saline (Na⁺ free Tyrode’s) contained (in mM): 280 mannitol, 5 KCl, 1 CaCl₂, 1 MgCl₂, 10 HEPES, 10 glucose; pH 7.40 adjusted with TrisOH; 310 mOsm (adjusted with mannitol). Calcium free saline (Ca²⁺ free Tyrode’s) contained (in mM): 140 NaCl, 5 KCl, 1 EGTA, 1 MgCl₂, 10 HEPES, 10 glucose, and 10 Na pyruvate; pH 7.40 adjusted with NaOH; 310 mOsm. The calcium-sodium free saline (Ca²⁺-Na⁺ free Tyrode’s) contained: 280 mannitol, 5 KCl, 1 EGTA, 1
MgCl₂, 10 HEPES and 10 glucose; pH 7.40 adjusted with TrisOH; 310 mOsm (adjusted with mannitol). The cesium based intracellular solution was used for recording the LA-induced inward current (in mM): 130 Cs acetate, 8 MgCl₂, 10 HEPES; pH 7.2 adjusted with TrisOH; 310 mOsM. Ruthenium red, the general TRP channel blocker, was obtained from MP Biomedicals (Solon, OH). The TRPM5 channel blocker triphenylphosphine oxide (TPPO) and linoleic acid were obtained from Sigma (St. Louis, MO). TPPO and linoleic acid stocks were made with 100% ethanol. Ruthenium red was directly dissolved into Tyrode’s. All the working solutions were made from stock solutions immediately prior to use.

**Quantitative real-time PCR.** For tissue level RT-PCR, the paired trigeminal ganglion was either stored at 20°C in RNAlater (Ambion, Austin, TX) or placed in TRI Reagent (MRC, Cincinnati, OH) for immediate extraction of RNA. Extraction was done according to manufacturer’s protocol. First strand cDNA was synthesized using the iScript cDNA synthesis kit from Bio-Rad (Hercules, CA). To get a final volume of 20 µl, 50 ng trigeminal RNA was used for the reaction. DNA contamination was evaluated by setting up a reaction where the reverse transcriptase was omitted. After first-strand synthesis, 2 µl cDNA was added to a PCR reaction mix. The HotMaster Taq DNA polymerase kit (5 Prime, Gaithesburg, MD) was used for the PCR reaction (final concentration: 1X reaction buffer, 5.0 mM Mg²⁺, 200 µM dNTPs, 300-900 nM forward and reverse primers, and 1.25 U/µl HotMaster Taq). Primer sequences are shown in Table 4.1.
In Chapter 3, I adopted and refined the technique of using pseudorabies virus Kaplan strain with the GFP gene inserted (PrV-KaΔgGgfp) as a ‘live-cell tracing tool’ to identify a subpopulation of TGNs that innervate the oral-cavity (see Chapter 3 for details). Here, I collected a limited number of identified TGNs for quantitative RT-PCR (qRT-PCR) analysis. 6-10 TGNs were collected under an inverted microscope using borosilicate hematocrit capillary pipettes. The capillary pipettes were pulled and fire-polished to a bore slightly smaller that the size of the cell to guard against collection of cell debris. Target cells were sucked into unfilled collection pipettes, which were controlled by a micromanipulator. After capture, the tip of the pipette was broken into a 0.5 ml microfuge tube containing 50 µl RLT buffer (supplemented with B-mercaptoethanol) from the RNeasy Micro Kit (Qiagen) and kept on ice. Total RNA isolated using the RNeasy Micro kit and MessageBOOSTER kit was used for amplification. In this study, I collected PrV-labeled medium to large cells (soma diameter >20 µm), PrV-labeled small cells (soma diameter < 20 µm) or non-fluorescent mixed cells to assay for TRPM8 or TRPA1 expression. The HotMaster Taq DNA polymerase kit was used for the PCR reaction, 1 µl cDNA per reaction was amplified using a 2-step PCR (6 s denaturation, 60 s annealing and extension). A TaqMan (ABI, Foster City, CA) detection system was used to detect the specific targets and a housekeeping gene GAPDH, for comparison of expression levels. The primers were from either TaqMan Gene Expression Assays or designed by ourselves.
Statistical analysis. The significant effects of all the treatments were determined by a two-tailed Student’s t-test (α=0.05) compared with their controls as described in the text. Data are presented as mean ± SEM, unless otherwise indicated.

Results

TGNs express several TRP channels. Table 4.1 shows the expression of several common TRP channels in trigeminal ganglia. Using conventional RT-PCR on RNA extracted from the trigeminal ganglia, TRPV1, TRPA1 and TRPM8 are expressed in the trigeminal ganglia as expected. These TRP channels are responsible for the detection of capsaicin, mustard oil and menthol, respectively (13, 20, 21), which are well established to activate TGNs. One of our initial targets of interest, TRPM5, the TRP channel involved in taste perception of sweet, bitter, umami and FA tastes, is apparently not expressed at detectable levels in these assays. However, TRPM4, which is closely related to TRPM5 channel by sequence homology, is expressed in trigeminal ganglia. Because of their suspected role in store operated calcium entry (SOCE) (22-26) and my data supporting SOCE in TGNs (Chapter 2), I also tested for expression of several members of the TRPC family. Interestingly, several members in TRPC family that have been implicated in SOCE also showed up in this assay, including TRPC1, TRPC3, TRPC4 and TRPC7. These, like TRPM4, remain potential candidates for underlying FA-induced receptor potential in TGNs.

In Chapter 3, I utilized a neurotropic virus (i.e. pseudorabies virus; PrV), to trace the lingual-innervated population of TGNs. To validate that the PrV-labeled TGNs were a chemosensitive subpopulation of sensory neurons, I checked the expression of TRPM8
and TRPA1 in individual PrV-labeled TGNs using qRT-PCR. As I expected, TRPM8 and TRPA1 were expressed. However, I was unable to find a consistent relationship between neuron soma size and TRP channel expression in these PrV-labeled TGNs. TRPM8 were consistently found in both PrV-labeled medium to large (soma diameter $> 20 \, \mu m$) and small (soma diameter $< 20 \, \mu m$) neurons. In 2 out of 5 assays with medium to large neurons, TRPA1 was also detected. In small neurons, however, TRPA1 was not found in any assays ($n=6$), but 5 out of 7 assays still showed TRPM8 expression. The non-labeled neurons showed TRPA1 and TRPM8 expression (data not shown). Because of this inconsistent pattern of expression with known targets at the single cell level, it was not possible to probe at this level for the TRP channels of interest (TRPM4, TRPC1, TRPC3, TRPC4 and TRPC7) in the FAs signaling pathway. Additional refinements of these single cell PCR approaches in PrV-labeled TGNs will be required before I am able to draw meaningful conclusions regarding which TRP channel types are expressed in lingual-innervated TGNs.

**LA activates inward, cation-selective current in TGNs.** Because of their involvement in chemosensory pathways and their expression patterns in TGNs, I hypothesized that a TRP channel might be involved in the FA-induced signaling pathway in TGNs. In order to minimize wash-out of second messengers and to preserve the integrity of the intracellular contents of the cells, nystatin-mediated perforated patch clamp recording (27, 28) was used to record the TRP-like inward currents activated by LA, the prototypical FA stimulus.
This technique was chosen to record the LA-activated TRP-like inward current since the intracellular Ca\(^{2+}\) mobilization and some other second messengers seem to play an important role in FAs signaling (see Chapter 2 and Chapter 3 for details). In the whole-cell voltage clamp experiments (\(V_h = -60\) mV), LA elicited a slowly activating, inward current averaging 1107.5 ± 116.7 pA (\(n=20\); Fig. 4.1A) that had a time course similar to that of the LA-induced intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) increase and depolarization.

To define the permeability properties of this LA-activated inward current, I performed a series of perforated whole-cell patch clamp recording in which the extracellular Ca\(^{2+}\) or Na\(^+\) was replaced with EGTA or mannitol, respectively. Ca\(^{2+}\) removal from extracellular saline solution decreased the average peak inward current ~60\% to a value of 447.4 ± 97.9 pA (Fig. 4.1B and E; \(n=13\); \(P<0.001\)). Substitution for extracellular Na\(^+\) had a similar effect on the LA-induced inward current. The peak inward current decreased to 261.5 ± 55.5 pA (\(n=11\)) in Na\(^+\) free Tyrode’s, which is about 24\% of the control current (\(P<0.001\)). Further, I replaced both cations from the extracellular solution. The current further decreased to 145.2 ± 45.3 pA (\(n=6\)), but was not completely abolished. Therefore, I concluded that the LA-induced inward current is a non-selective cation current carried by both Ca\(^{2+}\) and Na\(^+\) consistent with many TRP-like channels. The generally slow time course of the current and the nature of these experiments precluded me from conducting a more systematic analysis of the permeability properties of the LA-induced inward current.
The non-specific TRP channel blocker ruthenium red inhibited the LA-activated inward current and [Ca\(^{2+}\)]\(_i\) increase. Ruthenium red is known as a general TRP channel blocker as well as an inhibitor of ryanodine receptors (13, 29, 30). It blocks every known TRPV channel at concentrations as low as 100 nM to 1 µM (31, 32). It was also reported that ruthenium red was able to inhibit TRPA1 and TRPM6 currents (13, 15, 33, 34).

The non-selective and non-voltage activated natures displayed in Fig. 4.1 suggest that this current could be through a TRP channel. As the initial attempt, I used the general TRP channel blocker ruthenium red (10 µM) to see if this LA-induced inward current could be inhibited. Indeed, the mean inward current was decreased to 304.9 ± 81.3 nM (n=12; P<0.001).

In addition, I carried out calcium imaging experiments with ruthenium red (10 µM) also. Similarly to the results using patch clamp recording, the [Ca\(^{2+}\)]\(_i\) increase was diminished when ruthenium red was present (Fig. 4.2C). Average [Ca\(^{2+}\)]\(_i\) rise decreased from 193.2 ± 15.0 nM (n=45) to 24.8 ± 8.2 nM (n=45; P<0.001) (Fig. 4.2D).

The TRPM5 inhibitor TPPO partially blocks the LA-activated inward current. Triphenylphosphine oxide (TPPO) is the recently reported blocker with apparent specificity for TRPM5 channels (35). Current studies in our laboratory have shown that TRPM5 is involved in FA-induced signaling pathways in both taste receptor cells and the enteroendocrine cell line, STC-1. However, the specificity of TPPO to TRPM5 has not been externally validated to our knowledge. Since TRPM5 was not found to be detectable in mRNA from the trigeminal ganglia in my assays, I sought to validate
this finding using TPPO to try and inhibit the LA-activated inward currents. My expectations were that TPPO should be ineffective in inhibiting the LA-induced currents in TGNs. In whole-cell voltage clamp experiments, I compared LA-induced inward currents in control TGNs and in TPPO treated (100 µM, 2 min pre-treatment) TGNs. Fig. 4.3A is a typical LA-induced inward current in the presence of TPPO. Compared to the control mean peak current in regular Tyrode’s solution (1107.5 ± 116.7 pA; n=20), the LA-induced inward current in the presence of TPPO was significantly reduced (570.8 ± 91.9 pA; n= 9; Fig. 4.3B). Although the inward current is significantly decreased (P<0.005), the remaining current still composed of 52% of the control current. TPPO treatment partially blocked the LA-induced inward current in TGNs.

Discussion

In this present study, I have shown for the first time that FAs activate TGNs with an inward current that has several characteristics of a channel in TRP family. First, the inward current is evoked at a negative voltages and would be expected to contribute to the LA-induced depolarization reported previously (Chapter 2), by extracellular agonists. Second, the inward current is carried by Na⁺ and Ca²⁺. This nonselectivity among cations is a hallmark of many members in TRP channel family. Third, a relatively nonspecific TRP channel blocker ruthenium red inhibits both the [Ca²⁺]i increase and the inward current induced by LA. Fourth, the specific blocker for TRPM5 partially inhibited but did not completely block the LA-induced inward current. Taken together, these features lead to the conclusion that this FA-induced inward current is likely carried through one or more types of TRP channels.
To date, however, I have been unable to identify unequivocally the subtype(s) of TRP channels that may be activated following FAs stimulation in TGNs. Based upon my previous data (Chapters 2 and Chapter 3), I have shown that intracellular Ca\(^{2+}\) stores play an important role in the FA-induced signaling pathway. It led me to the initial idea that FA-activated TRP channels were functionally related to SOCE activity, a domain of the TRPC subfamily. TRPC is a unique family of TRP channels in that its members are not only responsible for agonist-activated nonselective cation currents, but they also participate in the so-called slow sustained mode of Ca\(^{2+}\) signaling, which requires sustained elevations of intracellular Ca\(^{2+}\) (36, 37). Signaling process mediated by Ca\(^{2+}\) could either be fast or slow. Fast responses usually involve the voltage-dependent Ca\(^{2+}\) channels, while slow response is generally seen in the case of receptor triggered activation, with phospholipase C cleaving phosphatidylinositol 4,5-bisphosphate (PIP\(_2\)) into inositol 1,4,5-trisphosphate (IP\(_3\)) and DAG. This process is sometimes called receptor-operated calcium entry (ROCE) (38).

My recordings of the \([\text{Ca}^{2+}]\text{i}\) rise initiated by polyunsaturated FAs was always in a slow sustained manner; and my data supports a model where the G protein/PLC pathway is involved (see Chapter 2 for details). Thapsigargin treatment increases the resting \([\text{Ca}^{2+}]\text{i}\) level 2-3-fold which may be subsequently inhibited by LA application (see Chapter 2 for details). More interestingly, LA-induced \([\text{Ca}^{2+}]\text{i}\) rise is not dependent on extracellular Na\(^{+}\), but the LA-induced inward current is dependent on both extracellular Ca\(^{2+}\) and Na\(^{+}\), which implicates that this current is likely downstream of the intracellular Ca\(^{2+}\) stores release (39-41).
The inhibition of LA-induced currents by TPPO, the TRPM5 specific inhibitor, was surprising. The fact that the LA-induced current was carried, at least partially, by Ca\(^{2+}\) is inconsistent with the properties of TRPM5 (or TRPM4) channels (3). Further, my RT-PCR assays failed to find detectable levels of expression of TRPM5 in mRNA isolated from trigeminal ganglia. The reason for this discrepancy is unclear but it is possible that TPPO is not entirely specific for TRPM5 channels as it has not apparently been screened for its ability to inhibit TRPC channels. Alternatively, there may be multiple channel types activated downstream of G protein/PLC activation that may include TRPM5 expressed at low levels in TGNs. Obviously, additional experiments aimed at testing among these possibilities are needed.

Given the fact that multiple TRP channels are expressed in TGNs and that there is not strong evidence implicating any single channel type in the LA-induced pathway in TGNs, it is not possible to draw any meaningful conclusions regarding the identity of the channel that mediates this inward current in TGNs. Due to the lack of specific pharmacological tools and the nature of TRPCs, which can exist in a variety of heteromeric forms in native cells, identifying the source of these currents in TGNs has proven to be overly complicated. The conventional approaches like using RNA interference are practically impossible. Further, the mechanisms of SOCE/ROCE are still obscure. Recent discoveries of STIM1 and Orai proteins as mediators of SOCE processes and the possible interaction of these proteins with TRPC channels directly further complicates the issue (6, 7, 42, 43). How TRPCs fit into the whole signaling pathway is
still under debate. Future studies will depend on the new information of the TRPC channels, SOCE and specific targets that might involve in the FAs signaling pathway.

References


Table 4.1: TGNs expressed several TRP channels. TRP channels expression was determined using conventional RT-PCR and in individual neurons using quantitative real time RT-PCR in PrV-labeled (i.e. lingual-innervated) TGNs. +, positive; -, negative; NT, not tested.

<table>
<thead>
<tr>
<th>TRP</th>
<th>Regular RT-PCR</th>
<th>Individual PrV-labeled neurons RT-PCR</th>
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<tr>
<td>TRPV1</td>
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<td>TRPA1</td>
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<td>TRPM4</td>
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<td>TRPC4</td>
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<td>TRPC7</td>
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Figure 4.1: LA-induced inward currents are dependent upon extracellular Ca$^{2+}$ and extracellular Na$^{+}$. LA-induced inward currents ($V_h$ = -60 mV) in a single TGNs in Tyrode’s (A), Ca$^{2+}$ free Tyrode’s (B), Na$^{+}$ free Tyrode’s (C) and Ca$^{2+}$-Na$^{+}$ free Tyrode’s (D). (E) Mean responses (inward currents) ± SEM to 30 µM LA in TGNs in Tyrode’s, Ca$^{2+}$ free Tyrode’s, Na$^{+}$ free Tyrode’s and Ca$^{2+}$-Na$^{+}$ free Tyrode’s. *, P<0.001.
Figure 4.2: The nonspecific TRP channel blocker ruthenium red inhibits the LA-activated inward current and \([\text{Ca}^{2+}]_i\) increases. 30 µM LA-induced inward current is inhibited by 10 µM ruthenium red (A). (B) Mean peak responses (inward currents) ± SEM to 30 µM LA in TGNs with and without ruthenium red’s presence. 30 µM LA-induced \([\text{Ca}^{2+}]_i\), increase is blocked by 10 µM ruthenium red (C). (D) Mean \([\text{Ca}^{2+}]_i\), increase ± SEM to 30 µM LA in TGNs with and without ruthenium red’s presence. *, P<0.001.
Figure 4.3: TRPM5 blocker TPPO partially blocked the LA-activated inward current. 30 µM LA-induced inward current is inhibited by 100 µM TPPO (A). (B) Mean responses (inward currents) ± SEM to 30 µM LA in TGNs with and without TPPO. *, P=0.0013
CHAPTER 5
SUMMARY AND FUTURE DIRECTIONS

In this dissertation research, I basically asked two questions. First, could fatty acids (FAs) work as effective tactile stimuli to activate the somatosensory system; and second, what are the underlying mechanisms of FAs’ activating somatosensory system? To answer the first question, I used acutely isolated trigeminal ganglionic neurons (TGNs) as the cellular model and performed a series of cell-based assays with different FAs (i.e. different carbon length, double bond number and position) as the stimuli for fats. Importantly, I did see initial evidence of TGN activation by FAs, including increasing intracellular calcium concentration ([Ca^{2+}]_i) and inducing membrane depolarization and the ability of FAs to elicit action potentials in TGNs (J. T. Klein and T.A. Gilbertson, unpublished data). These data led me to answer the second question: what are the mechanisms underlying the ability of FAs to activate TGNs. More specifically, what are the important players in this FA-induced signaling pathway in TGNs? While initially exploring the details of FAs signaling in this system, I encountered a big obstacle: the polymodal feature of TGNs. Although some conclusions could be drawn (Chapter 2), the variable responses from cell-to-cell prevented me from generating a comprehensive and definite answer to this question. Because of this, the dissertation research took a new direction in the hope of answering the following question: how to functionally isolate a subpopulation of TGNs that innervate the tongue that might be responsible for somatosensation specifically in the oral cavity?
I solved this problem by adopting and refining a new technique into our lab, using pseudorabies virus (PrV) as a ‘live-cell’ tracer to trace the lingual-innervated population of TGNs. Successful implementation of this approach lead me to be able to use PrV to label ~10% of TGNs isolated from the trigeminal ganglia, the majority of which appeared to represent TRPM8 positive nociceptors (Fig. 3.1, Table 3.1). Clearly, this approach does not label the entire populations of lingual-innervated TGNs but does allow us to identify those neurons that do innervate the tongue (Fig. 3.2 C and D). To this end, I have used cell-based approaches (functional calcium imaging and patch clamp recording) to study FA-induced signaling transduction pathways in both pooled TGNs from adult and PrV-injected neonate Sprague-Dawley rats.

My data are the first showing that FAs are capable of activating TGNs directly by eliciting a rise in [Ca^{2+}]_{i} (Fig. 2.1A). Further imaging studies with removal of extracellular sodium (Na\(^+\)) and calcium (Ca^{2+}) on pooled TGNs implicated the important role of intracellular Ca^{2+} stores in this process (Fig. 2.3). The parallel ion removal experiments and the other experiments using pharmacological tools in PrV-labeled TGNs have further demonstrated that this [Ca^{2+}]_{i} rise was specifically dependent on the release of intracellular Ca^{2+} stores (Fig. 3.3 and Fig. 3.4).

Using quantitative real time PCR, I have shown that TGNs express a variety of FAs activated receptors including GPR40, GPR43, GPR84, GPR120 and CD36 (Fig. 2.5F). Our current clamp recording data with GDP-\(\beta\)-S and U73122 inhibiting the linoleic acid (LA)-induced depolarization suggested that the G protein/PLC pathway was
involved in the FA-induced signaling pathways (Fig. 2.5A-E). This was the first clue showing the involvement of FA-sensitive GPCRs in fat somatosensory perception.

Another important molecular sensor in many sensory systems is the group of TRP channels. Many sensory transduction cascades utilize a member of TRP channel families on the cell membrane to either reinforce the signaling (taste) or act directly as the receptor (e.g. TRPV1 for capsaicin, TRPM8 for menthol and TRPA1 for mustard oil (1-4)). In our laboratory, TRPM5 has been shown to play a role in the FA-induced signaling pathway in both taste receptor cells and an enteroendocrine cell line, STC-1. During the course of this research, significant effort was put into finding evidence of the involvement of a specific TRP channel in the FA-induced signaling pathways in TGNs. However, as I have learned, the identification of the specific channel is very difficult without a specific target of interest. It was simply not practical in terms of time, effort and expense to sort through the numerous channels expressed by TGNs that had the properties we had identified for the LA-induced inward current. Attempts to identify a target through the use of single cell PCR in PrV-labeled (i.e. lingual-innervated) TGNs proved too technically challenging. The carefully designed voltage-clamp recording studies revealed a TRP-like inward current was activated by LA. This inward current is both Na$^+$ and Ca$^{2+}$ permeable, sensitive to a non-specific TRP channel blocker ruthenium red, and partially blocked by a TRPM5 inhibitor TPPO. This was also the first gathered data showing that TRP-like channels might be involved in the FA-induced signaling pathway, where they are downstream of the release of intracellular Ca$^{2+}$ from stores.
Although my studies at this point are complete, there are several questions rising immediately from my research. First, although I studied the abilities of several types of FAs to activate TGNs initially, the transduction pathway described in this research was almost entirely studied with LA as the stimulus. The different time course of FA-induced $[\text{Ca}^{2+}]$, increase indicated that different mechanisms or receptors might be involved with different FAs. Future research focused upon the applicability of these pathways to other FAs is warranted. Second, I have provided some evidence of the involvement of FA-sensitive GPCRs. However, further studies will be needed to prove 1) the direct binding/interaction of FAs and GPCRs; 2) whether GPCRs are the sole membrane proteins that interact with FAs. The answers will bring up the third question, as lipids themselves, how FAs interact with cell membrane and what are the details of this interaction process. Are lipid rafts required for these receptors to work? Forth, exactly which TRP channel is involved in FA signaling pathway, or there are several TRP channel working together? Are these TRP channels solely activated as downstream player of GPCRs, or could they be directly activated by extracellular or intracellular FAs? I will expand these problems in detail below.

**What Causes the Variable Time Courses for Different Fatty Acids?**

The FAs in the oral cavity comprise different classes of FAs which vary in their carbon length, number and position of double bonds. I checked some representative FAs in my dissertation research, including LA and docosahexaenoic acid (DHA; long-chain polyunsaturated FA), oleic acid (OA; long-chain monounsaturated FA) and myristic acid (MA; medium-chain saturated FA). In calcium imaging studies, these FAs displayed very
different response profiles. Specifically, polyunsaturated FAs almost always have a
delayed and gradual slow response, while OA and MA have faster responses. These
phenotypic differences lead to ask two basic questions: What are the mechanistic
differences underlying these responses?; and, what is the physiological underpinning of
the delayed responses seen for the polyunsaturated FAs?

In recent years, a group of previously orphan G protein-coupled receptors
(GPCRs) have emerged and been proposed to function as FAs receptors in different
systems. GPR40 and GPR120 are receptors for long chain FAs (5-8); GPR43 is a
receptor for short chain FAs (9); GPR84 is the receptor for medium chain FAs (10). Are
these receptor differences the reason for various types (i.e. fast or slow, adapted or not
adapted) of responses? While this is certainly feasible, the molecular evidence to support
this is still lacking. Heterologous expression systems revealed specificity of these
GPCRs, but lacking the physiological downstream players. In my observation, a single
neuron could respond to several FAs stimuli with different responding time and
amplitudes, which indicate the “cross-fiber” coding theory fitting into the somatosensory
system. The interactions of downstream players, along with the membrane FAs receptors,
contribute to the finally different responding profile.

My studies consistently showed a delayed response for LA in all physiological
assays. Interestingly, this delay does not exist in taste receptor cells and STC-1 cells,
although they express similar FA-sensitive GPCRs. What is the physiological reason
behind this delay and does it play a role in normal somatosensory processing?
Unfortunately, the molecular/cellular knowledge of somatosensation is still in a gathering
stage, no clear evidence showed the physiological role of a delayed response. To answer this question, I will need the additional understanding of fat somatosensory perception as well as the fat taste perception at the *in vitro* and *in vivo* levels. The information on the specific molecular underpinnings that lead to the slow response in TGNs is needed.

**What Is the Direct Evidence that FA-activated GPCRs Are Involved?**

Using pharmacological tools (GDP-β-S, the G protein blocker and U73122, the phospholipase C (PLC) blocker), I have provided the first evidence that the G protein/PLC pathway is involved in the FA-induced signaling pathway. This conclusion further suggested the involvement of a membrane GPCR. As stated above, this group of GPCRs is reported to be activated by different types of FAs in a variety of tissues. Obviously different from the GPCRs for sweet, bitter and umami in taste, this functional class of GPCRs is not specific for taste/sensory detection. The convincing evidence supporting the claim that these GPCRs are acting as FAs receptors in the somatosensory system or taste system is still lacking.

To obtain direct evidence, animals with targeted gene deletion of these GPCRs would help validate their roles in the chemosensation of fat. Since these GPCRs have different FA ligands, the thorough studies would include both cell-based assays and behavioral assays for each GPCR knockout model. To date, the GPR40 knockout animals have already been generated. However, reports showed that GPR40 is partially required in FA-stimulated insulin secretion (11). Although I haven’t tried the knockout animals in my research, I would doubt this single GPCR knockout would give us clean deletion data on either cell-based assays or behavior studies. Of course, other GPCR knockout animals
should be tested. For example, GPR40 and GPR120 are both long chain FAs receptors with significant overlap in their ligand profiles as shown by heterologous expression studies. GPR120 might be compensated when GPR40 is functional. In this case, double knockout animals might be needed.

Secondly, whether this group of FA-activated GPCRs works independently is still very controversial in the field. CD36 is a glycoprotein which is considered a transmembrane protein that binds lipids including long chain FAs (12, 13). One of the roles proposed for CD36 is transporting FAs across plasma membranes from the extracellular to the intracellular side (14). Then, another important question that I would ask is, whether CD36 is working by itself or is cooperating with other fatty acid activated membrane proteins like GPCRs. To answer this question, a series of biophysical studies would be needed. Do CD36 and GPCRs colocalize or become a complex when FAs are present? Since FAs themselves are lipids, do FAs have interactions with the plasma membrane when they are presented to the extracellular environment? Furthermore, are there any interactions between these membrane proteins and the lipid membrane itself? These are some important questions to answer in order to accept these GPCRs as FAs receptors in somatosensory or taste perception.

Which TRP Channel Is Involved in FA-induced Signaling Pathways in TGNs?

In Chapter 4, I first checked the mRNA expression of several TRP channels in trigeminal ganglia. A series of TRP channels showed up, including four TRPC channels. I further recorded a TRP-like inward current which was activated by FA. This current is carried by both Ca\(^{2+}\) and Na\(^{+}\), inhibited by non-specific TRP channel blocker ruthenium
red. Although there are hints pointing this current to be a TRPC current, no clear evidence supported this conclusion in my dissertation work. There are several technical obstacles that prevented me from identifying the TRP channel(s) in FAs transduction in the somatosensory system. First, I was unable to narrow down the list of possible TRP targets. 2-APB was thought to block TRPC channels specifically at concentrations from 50 µM to 80 µM (15-17). Using 2-APB in both calcium imaging and inward current patch clamp recording might give us a better idea whether the current is mostly through a TRPC channel. Second, I had attempted to use single cell real-time PCR to narrow the list of potential TRP channels in this pathway. However, technical difficulties prevented me from accumulating significant amount of data, especially for PrV-labeled TGNs. This might actually be an indication of the low quality of mRNA when TGNs were infected by PrV. Alternatively, the virus infection itself might be interfering with the real time-PCR assay. The exact reasons are still under investigation.

Therefore, if the technique of real time-PCR on individual PrV-labeled TGNs is well-established in the future, the additional markers could be verified. Some of the initial markers would be TRPC1, STIM1 and Orai genes, which were candidates of calcium released activated calcium channel (CRAC). The immunocytochemistry assay on TGNs with these markers would further help me visualize the protein expression patterns. I would be very curious to see if these potential CRAC players were physically located close to other FA-sensitive membrane proteins, including FA-sensitive GPCRs and FA-sensitive delayed rectifying potassium channels (DRKs). Since I don’t have direct evidence showing the TRP current is activating after the activation of another membrane
protein (i.e. GPCRs), a very interesting question would be whether these CRAC players were working independently or closely cooperating with other membrane proteins. To answer this question, more interdisciplinary approaches would be needed (i.e. detailed structure information of these proteins and proof of protein-protein interaction in physiological state).

Additional Electrophysiological Assays Would Help Clarify Remaining Details of the FA-induced Signaling Pathways in the Somatosensory System.

The previous studies on primary neurons provide me some good candidate players in the FA-induced signaling pathway in the TGNs. These primary polymodal neurons are preventing me from looking into the signaling mechanisms in a detailed way free of confounding variables. In the future studies, I would like to utilize several cell lines which express many of the putative proteins involved in this pathway. For example, hypothalamic GT1-7 and hippocampal HT22 cell lines would be viable options. We have some preliminary data of the expression of FA-sensitive GPCRs in GT1-7 and HT22 cells (data not shown). Further, preliminary calcium imaging studies revealed that these cells were capable of being activated by FAs (data not shown). If I was to tackle this approach, I would like to further check the expression of some other important players indicated in my previous research, including the TRPCs, STIM1 and Orai genes. The cell line model will help me to unravel the signaling pathway in a more efficient way. First, knockdown experiments can be carried out without any hesitation. For example, FA-sensitive GPCRs knockdown will give us a clear idea whether these GPCRs are involved in the FA-induced signaling transduction pathway. The second interesting line of
experiments will be focused on the involvement of SOCE activity in the FA-induced signaling pathway. Since these cell lines are endogenous cell lines that represent a much realistic physiological state, the studies of TRPCs, STIM1 and Orai proteins interaction downstream of FAs activation would help us understand the FAs signaling in TGNs. Moreover, if hints show the involvement of TRPC channels, the patch clamp recording studies on these cells would be wonderful follow up experiments.

The ultimate goal in this series of studies will be to elucidate the FA-induced signaling pathways in somatosensory system, including confirming the FAs membrane receptors, the involvement of SOCE activity, and exactly which channel generates the FA-induced receptor potential. Answering these questions will provide us a brand new view of molecular/cellular understanding of fat somatosensory perception, and what lead us to a new stage of somatosensory research.

References


CURRICULUM VITAE

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ACADEMIC PROJECTS


2. Utilize Fluorescent Pseudorabies Virus to identify a sub-population of trigeminal ganglionic neurons.

3. Utilize combined atomic force microscopy and Raman microspectroscopy to characterize the toxic effect of diesel exhaust particles on the cell-surface biochemical and biomechanical properties of single cancer cell. (Collaborated with Dr. Anhong Zhou from Department of Biological Engineering, Utah State University)

TECHNICAL EXPERTISE

1. Patch clamp recording
2. Calcium imaging
3. Cell & tissue culture
4. Heterologous gene expression (transfection)
5. Animal care
TEACHING EXPERIENCE

Teaching assistant, Department of Biology, Utah State University
2006-2007: General Biology BIOL1610,
2008: Human Physiology BIOL2420
2009: Animal Cell Culture Methods BIOL/ADVS5160

AWARDS

2005: Vice-President for Research Fellowship, School of Graduate Studies, Utah State University
2007: Keystone Symposium Scholarship, National Institute of Deafness and other Communication Disorders (NIDCD) of NIH
2007: Greaves Fellowship, Department of Biology, Utah State University
2009: The Dissertation Fellowship, School of Graduate Studies, Utah State University

INVITED PRESENTATIONS

The obesity epidemic: Are our taste buds to blame? (2006)
Gilbertson TA, Yu T, Shah BP. Old Main Weekend, Utah State University, Logan, UT

Fatty acid transduction in chemosensory cells (2009)
Making Sense of Fat Taste Symposium, Association of Chemoreception Sciences 31st Annual Meeting, Sarasota, FL

Taste or Texture? – Fatty acids activate the somatosensory system (2009)
Biology Department, Johns Hopkins University, Baltimore, MD

Taste or Texture? – Fatty acids activate the somatosensory system (2010)
Department of Pharmacology, University of Texas Southwestern Medical Center, Dallas, TX

PUBLICATIONS


Shah BP, Liu P, Yu T, Hansen DR, and Gilbertson TA. (2009) TRPM5 is critical for linoleic acid-induced CCK secretion from the enteroendocrine cell line, STC-1. (Submitted)


**Yu T**, Shah BP and Gilbertson TA. (2010) Fatty acids activate a TRP-like current in trigeminal neurons that innervate the oral cavity. (manuscript in preparation)


**ABSTRACTS**


