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Role of free fatty acid receptors in the regulation of brain-derived neurotrophic factor levels in the enteric glial and endocrine cells

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Role of long chain free fatty acid receptors in the regulation of brain-derived neurotrophic factor levels in the enteric glial and endocrine cells

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

By

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LIST OF ABBREVIATIONS

5-HT ………………………………………………………………Serotonin

Ach…………………………………………………………………..Acetylcholine

ATP……………………………………………………………Adenosine triphosphate

bp……………………………………………………………………….base pair

BSA………………………………………………………………...Bovine serum albumin

cAMP……………………………………………………….Cyclic Adenosine monophosphate

CCK……………………………………………………………………Cholecystokinin

cDNA………………………………………………….Complementary deoxyribonucleic acid

CGRP………………………………………………….....Calcitonin gene related peptide

cpm…………………………………………………………………Count per minute

CT……………………………………………………………………….Cycle threshold

DMEM………………………………………..……………….Dulbecco's Modified Eagle Medium

dNTP……………………………………………………..………Deoxynucleotide Triphosphate

DNA………………………………………………………………Deoxyribonucleic acid

ELISA………………………………………………………Enzyme linked immunosorbent assay

EEC……………………………………………………………...Enteroendocrine cell

PCR…………………………………………………………………Polymerase chain reaction

PI3K…………………………………………………………………...Phosphoinositide 3-kinase

PKC…………………………………………………………………………Protein kinase C

PLC……………………………………………………………………...…Phospholipase

PYY………………………………………………………………………..……Peptide YY

RT-PCR…………………………………………………………………Real time-PCR

SCFA…………………………………………………………………...Short chain fatty acid

SEM……………………………………………………...…………………standard error of the mean

SDS-PAGE…………………….Sodium dodecyl sulfate polyacrylamide gel electrophoresis

TBS-T…………………………………………………………Tris buffered saline tween 20

VIP………………………………………………………………..Vasoactive intestinal peptide

WT………………………………………………………………………………...Wild type

ABSTRACT

Role of long chain free fatty acid receptors in the regulation of brain-derived neurotrophic factor levels in the enteric glial and endocrine cells

Reem M Alkahtani

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University

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Recent deorphanization of several G-protein-coupled receptors (GPRs) as endogenous receptors for free fatty acids (FFAs) has increased our understanding of these nutrients as signaling molecules and the integral role of gut microbiota in the regulation of host defense mechanisms, energy metabolism, gastrointestinal motility and secretion. There are five distinct FFA receptors (FFRAs: FFAR1, FFAR2, FFAR3, FFAR4 and GPR84) that differ in molecular structure, ligand specificity, expression pattern, and functional properties. FFARs are grouped according to the chain length of FFAs that activate each FFAR.

Enteric glial cells and enteroendocrine cells play a pivotal role in the secretion of gliotransmitters and peptide hormones, respectively, in response to specific stimuli.

Neurotrophins such as brain-derived neurotrophic factor (BDNF) are essential for the development and integrity of enteric nervous system in the gut and play an important role in the regulation of gastrointestinal functions including motility and secretion.

The aim of this study was to identify the expression of FFARs in enteric glial cells and endocrine cells and determine the effects of fatty acids in the regulation of BDNF levels. Expression studies of FFARs by quantitative PCR, western blot and Immunohistochemistry from glial cells and enteroendocrine cells (STC-1 cells) demonstrated predominant expression of FFAR4 in STC-1 cells and predominant expression of FFAR1 in glial cells. Both FFAR1 and FFAR4 are activated by long-chain fatty acid.

Activation of specific G protein(s) coupled to FFAR1 and FFAR4 and signaling pathways downstream of G proteins in response to the endogenous long chain fatty acid, linolenic acid (100 µM), demonstrated both FFAR1 and FFAR4 are coupled to $G\alpha_q$ and stimulation of PLC-β/IP_3/ Ca^{2+} pathway. In enteroendocrine cells (STC-1) long chain fatty acids preferentially activate FFAR4, whereas in glial cells long chain fatty acids preferentially activate FFAR1. Furthermore, measurement of changes in BDNF levels in response to linolenic acid by immunocytochemistry and enzyme-linked immunosorbent assay (ELISA) demonstrated that in STC-1 cells, long chain fatty acids increased BDNF content via FFAR4, whereas in glial cells they increased BDNF content via FFAR1. The present study shed light on the expression and physiological role of long chain fatty acids and FFARs in the regulation of BDNF levels, and thus, gastrointestinal functions.

INTRODUCTION AND BACKGROUND

1. Introduction

The gastrointestinal tract is a long muscular tube; approximately 6.5 meters (20 feet) long, and is responsible for the digestion, absorption of nutrients, and expulsion of waste. The ingested food breaks down into small molecules and the movement of nutrients, water, and electrolytes from the lumen of the intestine into the blood require the major activities of gastrointestinal tract to be served. First of these is the gut motility, which is determined by the patterns of contraction and relaxation of the circular and longitudinal muscle layers to mix chyme with digestive enzymes and propel ingested food from the mouth toward the rectum. Second, the secretions of additional fluids, enzymes and mucus into the lumen from salivary glands, the cells of gastric mucosa, pancreas and liver are regulated. Motility and secretion further aid in digestion and absorption, which are the ultimate functions of the gastrointestinal tract.

I.1 The Structure of The Gastrointestinal Tract Wall.

The digestive system is characterized by a wall contains four layers, from inside lining of the tract to the outside lining: the mucosa is the innermost layer, underneath is submucosa which is a loose connective tissue layer with blood vessels, nerves, lymphatic and secreting gland. The submucosa is then followed by the muscular propria which contains two layers of the smooth muscle; circular smooth muscle cells is the inner layer,

and the outer layer is longitudinal smooth muscle cells. The main function of smooth muscle of the gut is to mix and propel intraluminal contents allowing effective digestion of food, and absorption of nutrients. Finally the outermost layer – the serosa, is a serous membrane that covers the muscularis externa and comprises a loose connective tissue that is covered by the visceral peritoneum that contains blood vessels, lymphatic, and nerves.

1.2 Enteric Nervous System

The human enteric nervous system (ENS) contains about 150 million nerve cells, comprising the largest division of the autonomic nervous system (Langley 1921). ENS provides confined neural control of the gastrointestinal tract and is essential in coordinating the digestive and defensive functions in the gut. A characteristic of ENS is that the nerve cell bodies located within the wall of the gut are organized into two ganglionated plexuses and nerve fibers throughout the external muscle layers, submucosa, and mucosa. The Myenteric plexus is located between the outer longitudinal and the inner circular smooth muscle layers of the muscularis externa and its neurons play a role in the gut motility. The submucosal plexus is located between the circular muscle layer and the muscularis mucosa and its neurons are involved in mucosal function, secretion, blood flow, and barrier function. Neurons of the ENS are supported by unique peripheral glial cells called enteric glia (Grundy and Brooks 1996). There are different classes of neurons in the ENS; motor neurons, classified as excitatory motor neurons that release acetylcholine or substance P as neurotransmitter or inhibitory motor neurons that release nitric oxide (NO) or vasoactive intestinal peptide (VIP) as transmitter. Both motor neurons innervate the muscle layer in the gut (Harmer, Fahrenkrug et al.

2012). In addition to motor neurons, there are sensory neurons, which have been classified as Dogiel type II neurons, which are characterized by multiple processes that via a complex of neuronal network that includs synaptic connections with interneurons, motor neurons and other sensory neurons (Grundy and Brooks 1996).

1.3 Cells in the Gastrointestinal Tract.

There are multiple type of cells that line the gastrointestinal tract wall from the stomach to the anal sphincter. In the mucosal layer there are; enteroendocrine cells, brush cells, goblet cells, and paneth cells. Inflammatory cells are in the lamina propria, and in the muscularis externa there are smooth muscle cells, which also includes interstitial cells of Cajal, enteric neurons and glial cells. Each of them has different function.

For example, the goblet cells are specialized cells scattered in the epithelium of the small intestine and colon. They are interposed between enterocytes and secret mucins which play role in mucosa curing and protection. Whereas, Paneth cells, are known for their location at the ends of the intestinal crypt. They play a role in the innate immunity system regulation of lamina microflora in the small intestine by secreting anti-microbial peptides into the crypt lumen, thereby providing protection for the stem cells that line the crypt walls. Other cells in mucosa such as brush cells are similar to other cells and they are specialized epithelial cells that are scattered within the epithelial lining of the gastrointestinal tract (Moore, B.A et al 2000; Kieta, A.V et al 2010). Moreover, smooth muscle cells in the gut are spindle shape cells, that contain actin and myosin, and are electrically coupled by gap junction for generating contractions. Also, interstitial cells of

Cajal (ICCs) are the gut pacemaker cells that creates electrical slow waves leading to smooth muscle contraction (Takaki M 2003).

However, two of the main cell types that have gained more attention recently are enteric glial cells and enteroendocrine cells.

2. Enteric Glial Cells

2.1 Historical Perspective on Enteric Glia Cells. More than 115 years ago, the first known description of the enteric glia was demonstrated by Dogiel in 1899. Yet, enteric glia were not clearly illustrated until just over 35 years ago when Giorgio Gabella for the first time addressed the structural relationship between enteric neurons and glia in enteric nervous system. He identified them as a unique class of cells and were name enteric glia cell (Gabella, 1981). The word "glia" derives from the Greek word for "glue". They were seen as a mere packing substance holding the enteric nervous system together (A Ruhl 2005).

The enteric glial cells are small cells with irregular stellate shape cell bodies and highly branched processes that extend from the cell body interposed throughout the neuropil. In addition, the composition of gliofilaments in enteric glia is predominantly composed of glial fibrillary acidic protein (GFAP) and vimentin (Jessen and Mirsky, 1983).

There are four major types of enteric glia: 1) type I "protoplasmic" enteric gliocytes, the star-shaped enteric glia within enteric ganglia, 2) type II "fibrous" enteric gliocytes, the elongated glia within interganglionic fiber tracts, 3) type III "mucosal" enteric gliocytes associated with nerve fibers and epithelial cells at the level of the mucosa, and 4) type IV

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"intramuscular" enteric gliocytes, the elongated glia associated with nerve fibers scattered between smooth muscle cells. (Liu et al, 2013).

2.2 Function Roles of Enteric Glia. For a long time, enteric glial cells were believed to play role mainly as support cells for neurons. Over the past five years the importance of glial cells in the gut has been studied and this dogma has been gradually abandoned. Enteric glia cells are actively involved in modulating gut processes. They are a significant regulator of gut barrier maintenance which is required to protect the gut lumen from the invasion by bacteria, viruses or foreign substances. Dr. Brian Gulbransen in his enteric glia chapter remark that Type III enteric glial cells in the intestinal mucosa have an important role in maintaining the integrity of the mucosal barrier of the gut (A Ruhl 2005). Type I and Type II enteric gliocytes are important in the support of enteric neurons, they are involved in homeostatic control that maintain enteric neurotransmission. (Gulbransen and Sharkey, 2012). Additionally, enteric glia actively is involved in the regulation of neurotransmission and gliotransmission by participating in neurotransmitter synthesis or inactivation, which influence the gut function. Enteric glial cells may also contribute in the regulation of intestinal epithelial function; cell bodies and processes of enteric glial cells extend to the mucosal plexus and come in close contact with the epithelial cell layer.

There are some neurotrophins as well as neurotransmitters that are produced from enteric glial cells, such as, nerve growth factor (NGF), and glial cell-derived neurotrophic factor (GDNF) (Boyen et al, 2006; Korsak et al. 2012). The most well characterized neurotransmitter released from enteric glial cells is ATP, which has a potential role to trigger intracellular calcium waves and influence adjacent neurons, thus modulating

enteric neurotransmission (Zhang et al. 2007). Enteric glial cells also release nitric oxide (NO), the inhibitory neurotransmitter that has a physiological role in modulating epithelial ion transport (MacEachern et al. 2011 and Esposito et al, 2013).

The role of enteric neurons in the regulation of gastrointestinal function is well known, however the role of enteric glial cells in the regulation of gastrointestinal function has only recently started to be elucidated. Moreover, the current knowledge about enteric glial in the human gut health and disease is rather poor.

3. Enteroendocrine Cells

3.1 Characteristics of the Enteroendocrine cells. The gastrointestinal tract is profoundly involved in the control of metabolism and motility via peptide hormones secreted from enteroendocrine cells scattered throughout the gut mucosa (Engelstoft MS, et al 2013). The endocrine system produces more than 20 different hormones from at least 10 distinct enteroendocrine cells that mediate effects through neuronal, auto, and paracrine mechanism.

Enteroendocrine cells constitute the largest endocrine organ of the human body, they are a group of specialized epithelial cells distributed among mucosal cells of the gastrointestinal tract, that represent less than 1% of the mucosal cell population (Tongzhi Wu et al. 2013). Enteroendocrine cells might be the first level of integration of information from the gut lumen, which act as specialized transducer of luminal factors, producing and secreting a variety of hormones or/and signaling molecules including: motilin, ghrelin, gastrin, somatostatin, cholecystokinin (CCK), glucose-dependent insulinotropic peptide

(GIP), glucagon-like peptide-1 (GLP-1), and peptide YY (PYY). Each one of these hormones are released from different type of enteroendocrine cells which are differentially distributed in the gut. Such as I-cells and K-cells, that release cholecystokinin and glucose-dependent insulinotropic peptide, respectively, predominate in the proximal small intestine. Whereas, L- cells are present in highest concentration in the distal small intestine and colon which are responsible in glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) release (Tongzhi Wu et al 2013).

3.2 Classification of The Enteroendocrine Cells. There are two types of enteroendocrine cells that are observed in the gut. The first is an open cell type. In most of enteroendocrine cells exhibit the characteristic of open type structure, as they have microvilli extending to the gut luminal surface. Microvilli are directly in contact with gut lumen, providing the ability to sense luminal contents or increased the level of absorbed substances across the epithelial layer (Duca FA and Lam TK 2014). In addition to open cell type, there are closed cells that do not reach the lumen. Such as, ghrelin- secreting cells that do not come into contact with the gastric lumen (Parker HE et al. 2008). Moreover, enteroendocrine cells are known as chemoreceptors that sense ingested substances (nutrient and non-nutrients). Upon activation they induce changes in gastrointestinal function and food intake through the release of signaling substances acting on a variety of targets locally or at a distance.

Hormones or peptides released from enteroendocrine cells possibly will communicate through paracrine fashion by interacting locally on specific receptors on vagal nerves or enteric neurons to activate afferent terminals or other cells. They can also

act in an endocrine fashion through the intestinal capillaries and the lymphatic system and on specific receptors located on more distant targets.

3.3 Function Roles of Enteroendocrine Cells Secretion. The enteroendocrine cells are the first level of integration of information from the gut and are responsible for secreting an array of gut hormones that induce changes in gastrointestinal physiological function through the release of signaling molecules. The gut peptides released from enteroendocrine cells play a pivotal role in the regulation of multiple physiological responses including gastrointestinal motility and secretion, glucose level control, food intake, and gastric emptying. K cells secret glucose-dependent insulinotropic peptide, an incretin hormone, which stimulates glucose induced insulin release. I cells secrets cholecystokinin, which "was the first GI peptide for which a satiating effect was described" (Mansouri A 2014) by acting as a hunger suppressant. CCK mediates digestion in the small intestine by slowing gastric emptying. L cells secrets glucagon-like peptide-1, an incretin hormone. It plays an important role in promoting glucose homeostasis by augmenting insulin secretion, suppressing glucagon release and slowing gastric emptying, as well as by reducing food intake. In addition to glucagon like peptide-1, L cells secret peptide YY hormone that has physiological function in inhibiting the gastric motility (Parker HE et al. 2014).

4.1 Neurotrophins

Originating from the greek words (trophe) meaning nourishment, neurotrophins are target-derived growth factors that regulate the survival, phenotypic differentiation, and

promote growth of axonal neurons in the central, peripheral, and enteric nerves system. There are four members of neurotrophic family; Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 NT3 and neurotrophin-4 NT4 (Prakash et al 2010).

4.2 Brain-derived Neurotrophic Factor. Brain derived neurotrophic factor was the second discovered member of neurotrophins family of growth factor, which regulates the neuronal differentiation, migration, synaptic function. It also plays an important role in memory processes. Physiological functions of BDNF are initiated by binding to two classes of cell surface receptors; the first class is tropomyosin-related kinase (TrkB) receptor that binds to the BDNF with high affinity, the second class is the pan-neurotrophin receptor (p75NTR) which binds to the BDNF with low affinity (Binder and Scharfman 2004) .

4.3 Synthesis of BDNF. BDNF is synthesized as pre-pro-BDNF. On the rough endoplasmic reticulum (ER), the pre-BDNF single peptide is cleaved into pro-BDNF that is packaged in the Golgi, transported to the nerve terminal, stored in presynaptic vesicles and secreted from terminals in a stimulus-dependent manner. Neurotrophin-induced BDNF effects involve activation of TrkB that mediate PLCγ1 activation and IP3-induced mobilization of Ca^{+2} from the endoplasmic reticulum. Increase in intracellular Calcium causes secretion of BDNF (Hasbi A et al 2009).

Changes in BDNF components are associated with pathological conditions. The high BDNF level is related to lower stroke rate, Alzheimer's, Parkinson's disease, and depression (Hashimoto et al 2005).

4.4 BDNF in the Gut. In the mature gastrointestinal tract of several species, such as human, rat, and mouse, the presence of BDNF and its receptors has been demonstrated. Expression of BDNF has been identified in enteroendocrine cells and enteric glial cells in the gut. Recent studies from Grider, et al 2006 have shown that stimulation of mucosa induces the release of BDNF which augments the release of 5-HT and calcitonin gene related peptide (CGRP), thus, enhancing the peristaltic reflex and gastrointestinal motility (Grider et al 2006).

Moreover, BDNF has an excitatory effect on colon, increase in the rate of colonic pellet propulsion has been observed. In human, BDNF increases gut motility, diarrhea, and bowel urgency. Changes in BDNF components in the gut are associated with inflammatory bowel disease, and it has been reported that increased BDNF levels were associated with ulcerative colitis (Massa SM et al 2010).

5.1 Free Fatty Acids and Their Receptors

Free fatty acids are essential nutrients that have very important function as a source of energy, structural contribution to cellular components, and maintaining homeostasis under physiological and pathophysiological states. This is due to the contribution to coordinating expression of proteins involved in lipid uptake, synthesis, transport storage and degradation (Nilsson NE et al 2003). There are two types of fatty acids based on their formation: short chain free fatty acids that are primarily produced through the fermentation of indigestible fiber such as carbohydrates, oligosaccharides and resistant starch by anaerobic gut flora (Paul LE et al. 2003). The second type of fatty

acids have longer-chain fatty acids. They are the products of dietary intake, adipose recycling, and hepatic turnover of neutral fats and phospholipids (Wong JM et al. 2006). Also FFAs are categorized by the length of their carbon chain; short-chain fatty acids with carbon length less than 6 carbons, medium-chain fatty acid with carbon length between 6-12 carbons, and long-chain fatty acids have more than 14 carbons.

The major component of short chain fatty acids in the human colon contains acetate (C2), propionate (C3), and butyrate (C4) with ratio of almost 3: 1: 1 respectively (Savage DC 1988). The total concentration of the short chain fatty acid is about 100 mM in the lumen of mammalian colon, yet the precise ratio is dependent on species and diet.

In past decade, it has been reported that the sensor of free fatty acids are nuclear receptors, including peroxisome proliferator-activated receptor (PPARs) (Chawla A et al. 2001). However, the free fatty acid effects on some biological processes have been suggested to involve cell surface receptors. Recent data have recognized free fatty acids as endogenous agonists for a small family of G-protein coupled receptors.

5.2 Free Fatty Acids Receptors (FFARs) Five different membrane-bound free fatty acid receptors have been recently identified upon successful deorphanization strategy. The free fatty acids receptor are G protein-coupled receptor (GPCRs) activated by free fatty acids. In the gastrointestinal tract these receptors for free fatty acids serve as nutrient sensor and differ in their ligand specificity, expression pattern, and functional properties (Kotarsky K et al. 2003). Short chain fatty acids (six or less carbon chain length) activate FFAR2, and FFAR3. GPR84 is activated by medium-chain free fatty acids, whereas both saturated and unsaturated medium- and long chain fatty acids activate FFAR1 and FFAR4 (Briscoe CP et al, 2003; Brown AJ et al. 2003; and Itoh Y et al. 2003).

5.3 FFAR1

FFAR1 is a G protein-coupled receptor recognized to be activated by medium to long chain saturated and unsaturated free fatty acids. The Briscoe group has reported that several free fatty acids work as agonists to FFAR1 in the micromolar concentration range. The potency of saturated free fatty acids to activate FFAR1 depends on their carbon chain length. However, the length of the carbon chain or the saturation degree doesn't correlate with the potencies of the unsaturated fatty acids (Briscoe CP et al, 2003; Brown AJ et al. 2003; Itoh Y et al. 2003).

5.3.1 Ligands. As mention previously, three separate groups reported that medium to long chain fatty acids activated FFAR1. The rank order of the agonists display most potency were as follows: docosahexaenoic acids (DHA, C22:6) > alpha-linolenic acid (C18:3) = oleic acid (C18:1) > palmitic acid (C16) and pentadeconic acid (C15). In addition to the natural ligands, several pharmacological studies reported novel series of FFAR1 agonists. GW9508 is a FFAR1 selective ligand used in many studies with 100-fold more potent than at FFAR4 (Hara T et al 2013). The agonist activity of free fatty acids ligands has been evaluated by intracellular calcium response. Besides GW9508, Medica 16, NCG75, PRARγ (thiazolidinedione) and TAK-857 have been reported as pharmacological ligands that activate FFAR1 (Hara T et al. 2011; Fukunaga S et al. 2006).

5.3.2 Expression. FFAR1 expression was analysed by reverse-transcription polymerase chain reaction (RT-PCR), immunohistochemistry, and in situ hybridization techniques and revealed high level of FFAR1 expression in various tissues. Ubiquitous distribution of FFAR1 but with the highest expression was observed in the brain, then in the pancreas (Briscoe 2003). Moreover, FFAR1 mRNA has been detected in mammary

gland such as bovine mammary epithelial gland. In particular FFAR1 shows high expression in the β cells (insulin producing pancreatic islet cells). Also, expression of the FFAR1 protein has been reported in the endocrine cells of gastrointestinal tract (Edfalk et al. 2008).

5.3.3 Signal Transduction and Physiological Function. FFAR1 plays an important physiological role in the intestine by contributing to insulin secretion. It has been reported that activation of FFAR1 by medium to long chain fatty acids promotes glucose-stimulated insulin secretion (GSIS) from pancreatic β cells. Various reports have confirmed that FFAR1 mediates the increase in intracellular calcium and the insulin secretion coupled to $G\alpha_q$ protein leading to activation of phospholipase C (PLC) and subsequent increase in the intracellular calcium concentration in β cells, which results in mobilization of calcium from endoplasmic reticulum (ER) leading to calcium influx through voltage-dependent L-type Ca^{2+} channel (VLTCC).

Furthermore, activation of FFAR1 on endocrine cells of the gastrointestinal tract enhances insulin secretion indirectly through the release of incretin hormones, since receptors for glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are expressed in pancreatic β cells (Itoh et al. 2003; Edfalk 2008). Moreover, Efdalk et al 2008, have shown that oral fat diet administration to FFAR1-null mice results in an impaired secretion of both peptides; GLP-1 and GIP with a decrease in insulin secretion (Steneberg P et al. 2005; Shapiro H et al. 2005; Fujiwara K et al. 2005).

Similarly, loss of the FFAR1 function via free fatty acids receptor 1 small interfering RNA (siRNA) treatment suppresses the insulin secretion in response to free fatty acid stimulation. Also, it has been reported that fatty acid stimulated-induced insulin secretion

was significantly decreased in FFAR1 knock out mice (Hara T et al. 2013). These reports suggest that FFAR1 plays a crucial role in the mechanism involved in the development of obesity and type 2 diabetes (Steneberg P et al. 2005; Shapiro H et al. 2005).

5.4.1 FFAR2

FFAR2 is a deorphanized G protein coupled receptor that has been identified to be activated with short chain free fatty acids for the first time in 2003. Two separate groups (Brown et al. 2003 and Nilsson et al. 2003) have demonstrated during ligand fishing that FFAR2 was activated by the short chain fatty acid acetate.

5.4.2 Ligands. Short chain fatty acid (SCFAs) with carbon length less than six carbons such as formate, acetate, propionate, butyrate, and pentanoate have been shown to be the endogenous agonists of FFAR2. However, this receptor shows differences in ligand specificity and tissue localization from FFAR3. The rank order of ligand potencies for FFAR2 activation was as follow: acetate = propionate > butyrate (Brown et al. 2003, Poul et al. 2003, and Nilsson et al. 2003). Recently it has been reported that different species show differences in the rank potency of short chain fatty acid ligands (Hansen et al 2012).

5.4.3 Expression. FFAR2 has been shown to be expressed in various tissues, in particular fat stores, inflammatory cells and the gastrointestinal tract (Hong 2005). Since short chain fatty acids are highly concentrated in the distal gastrointestinal tract it has been reported that FFAR2 is found in mucosal cells of the ileum and large intestine. Their pattern of expression is variable along the intestine and perhaps among various species (Tazo H et al. 2009 and Ulven T 2012).

5.4.4 Signal Transduction and Physiological Function. Several reports have confirmed that stimulation of FFAR2 by short chain fatty acids lead to inhibition of cAMP production. Even though FFAR2 and FFAR3 have similar endogenous ligands, their G-protein signaling mechanisms are different. Both the G_q pathway and the pertussis toxin sensitive $G_{i/o}$ pathway are activated by FFAR2 (le Poul et al 2003).

As mention above, FFAR2 is highly expressed in adipose tissue. A series of studies has been made to understand the role of FFAR2 in adipocytes by Hang et al. There results suggested that activation of FFAR2 by short chain fatty acids inhibits lipolysis, and increases adipogenesis.

Moreover, the Karaki group in 2008, by using reverse transcription polymerase chain reaction (RT-PCR) and immunohistochemistry detected colocalization of the FFAR2 with PYY (peptide YY) in mucosal epithelium and mast cells. These discoveries elucidate the crucial role of FFAR2 in the gastrointestinal tract. Prior studies have reported that short chain fatty acids stimulate the release of 5-HT and PYY from the ileum and colon (Karaki et al 2006). This led to the hypothesis that FFAR2 might contribute to these pathways as transduces between nutrient stimuli and satiety mediator release in distal gastrointestinal tract. Eventually FFAR2 might be a putative transducer of free fatty acid stimuli and have a therapeutic use in the treatment of numerous metabolic disorders, including diabetes and obesity (Samuel BS et al. 2008).

5.5.1 FFAR3

FFAR3 is a deorphanized G protein coupled receptor reported for the first time in 2003 by Brown et al. Similar to FFAR2, short chain fatty acids with carbon length less than six carbons have been demonstrated to be the endogenous agonist of FFAR3 (Tazoe H et al. 2009).

5.5.2 Ligands. FFAR3 was activated by short chain fatty acids. As mention in the previous section, the rank order of potencies with respect to activation of FFAR2 was as follow: acetate = propionate > butyrate. Whereas, that of FFAR3 was as follow: propionate > butyrate > acetate. Due to the low potency level of acetate in FFAR3 it has been used to distinguish between FFAR3 and FFAR2. Moreover, there are several pharmacological compound reported as FFAR3 agonist, such as, compound 5 (Leonard TW et al 2006).

5.5.3 Expression. The initial expression studies using reverse transcriptase polymerase chain reaction detected highest levels of FFAR3 mRNA expression in the adipose tissue across a panel of human tissue (Xiong et al. 2004). In addition to its extra-gastrointestinal localization, FFAR3 has been detected in the small and the large intestinal mucosa (Cohen P et al. 2001; Latour MG et al 2007). The FFAR3 expression also was found in the enteroendocrine cells of the distal small intestine (ileum) and the (proximal) colon (Karaki et al. 2006).

5.54 Signal Transduction and Physiological Function. Various reports have confirmed that FFAR3 mediated the release of intracellular calcium and the phosphorylation of ERK1/2 (Le Poul et al 2003). Pertussis toxin treatment completely

abolished the intracellular calcium and ERK1 phosphorylation. Therefore, it has been suggested that the signaling mechanism activated by FFAR3 was coupled to $G_{i/o}$.

The analysis of FFAR3-deficient mice demonstrated that FFAR3 deficiency is associated with reduction of PYY (peptide YY) expression, increased intestinal transit rate, and reduction of extraction of energy obtained from short chain free fatty acids that are produced by the microbial fermentation of indigestible dietary polysaccharide (Samuel BS et al. 2008). These results suggest that FFAR3 regulates host energy balance through mechanisms that are dependent upon the microbiota (Xiong Y et al. 2004; Samuel BS et al. 2008; Karaki S et al. 2006).

Moreover, it has been reported that short chain fatty acids enhance leptin production by adipocytes. Leptin is a 16 kDa potent anorexigenic hormone produced mainly by fat cells, It acts via its receptors in the central nervous system to reduce food intake. Also, by using small interfering RNA treatment to knockdown the expression of FFAR3 mRNA, it has been found that FFAR3 knockdown almost totally abolished the ability of short chain fatty acids to induce leptin receptor expression and thus production of leptin. These results suggested that FFAR3 has a potential role in the regulation of feeding and obesity (Cherbut C et al. 1998; Cohen P et al. 2001).

5.7.1 FFAR4

FFAR4 is a deorphanized G protein coupled receptor. By using a receptor internalization assay, medium to long chain free fatty acids were found to serve as endogenous ligands (Fukunaga et al 2006). The saturated free fatty acids with a carbon

length of 14 to 18 and unsaturated free fatty acids with a carbon length of 16 to 22 were detected to activate FFAR4 (Hirasawa et al 2005).

5.7.2 Ligands. As mention above, FFAR4 is activated by medium to long chain free fatty acids ligands. Although most fatty acids that were shown to activate FFAR4 were also able to activate FFAR1, the amino acids homology between both receptors was only 10% (Hara T et al. 2013). Several pharmacological research reported a novel series of FFAR4 agonists. NCG21 was developed as the selective agonist for FFAR4. Other synthetic FFAR4 ligands have been reported recently, such as, TUG891, Compound 8 and Compound 9 (Hara T et al. 2011; Fujiwara K et al. 2005).

5.7.3 Expression. Expression of FFAR4 has been abundantly found in the intestinal tract in both mice and human (Hirasawa et al 2005). FFAR4 is also expressed in the intestinal endocrine cell line STC-1 (Hirasawa et al. 2005; Tanaka et al. 2008; Miyauchi et al. 2009). Moreover, FFAR4 have been identified in adipocytes and macrophages (Oh DY et al. 2010). Activation of the receptor increases glucose uptake and adipogenesis in adipocytes cells whereas it exerted anti-inflammatory actions in macrophages (Gotoh et al. 2007). Moreover, FFAR4 expression has been reported in ghrelin cells and it may play a role in the lipid sensing cascade (Lu et al. 2012).

5.7.4 Signal Transduction and Physiological Function. Studies in HEK293 cells have shown that both natural polyunsaturated fatty acids and synthetic ligands mediated the release of intracellular calcium. However, they did not change the cAMP production. FFAR4 is similar to FFAR1 is coupled to the G_q protein family. Moreover, it has been reported that FFAR4 activation induces PI3 kinase.

Hirasawa et al. used the STC-1 cell line to study the secretion of glucagon like peptide (GLP-1). They found that fatty acids such as alpha linolenic acid, docosahexaoienic acid, or palmitoleic acid activated FFAR4 to promote the release of GLP-1 from the STC-1 (Dockray GJ 2003). Since FFAR1 is also expressed in STC-1, they used small interfering RNA (siRNA) treatment to knockdown both receptors in order to identify which receptor was being activated by fatty acids. The results showed that the cells transfected with FFAR1 siRNA had no change in ability to release GLP-1 (Dockray GJ 2003). However, there was a significant decrease in GLP-1 release detected in cells transfected with FFAR4 siRNA. The ability of free fatty acids to cause release of GLP-1 by the activation of FFAR4 introduced a new insight for alternative strategy in the treatment of diabetes (Hara T et al. 2011; Briscoe CP et al. 2006).

5.6.1 GPR84

GPR84 is a recently discovered G protein coupled receptor that is specifically activated by medium chain free fatty acids, with carbon chain length of C9 to C14. (Wang J et al 2006). In contrast, neither short nor long chain free fatty acid activated GPR84.

5.6.2 Ligands. GPR84 is activated by medium chain free fatty acids ligands. The most potent ligand with that activate for GPR84 are: capric acid (C10:0), undecanoic acid (C11:0), and lauric acid (C12:0) (Hirasawa A et al. 2008).

5.6.3 Expression and Physiological Function. Only few publications reported on the function of GPR84. It is selectively expressed in peripheral blood leukocytes. In addition, it has been reported that GPR84 is activated by medium chain free fatty acids

and is coupled to $G_{i/o}$ pathway (Wang J et al 2006). Studies have shown that in monocytes/macrophages, medium chain fatty acids act via GPR84 to augment the stimulation of IL-12 production by lipopolysaccharide. Therefore, GPR84 might have important role in the free fatty acids metabolism to immune system regulation (Bouchard C et al 2007).

SIGNIFCANCE

The importance of chemosensing in the gut to regulate several physiological functions is emerging with the advent of deorphanization of several G protein coupled receptors (GPCRs) for free fatty acids. There are five distinct free fatty acid receptors (FFAR1, FFAR2, FFAR3, GPR84, and FFAR4) that differ in ligand specificity, expression pattern, and functional properties. Recent studies have shown that these receptors serve as nutrient sensors and play an important role in a variety of physiological processes. In the gastrointestinal tract they contribute to the secretion of regulatory peptides from enteroendocrine cells. Therefore, free fatty acids are not only essential nutrients but also extracellular signaling molecules.

Recent studies from our lab have shown that the release of brain-derived neurotrophic factor (BDNF) from enteroendocrine cells in response to luminal stimulation enhances the peristaltic reflex and gut motility. Release of BDNF from enteric glial cells is important for neuronal survival. However, the specific FFA receptors types and the signaling pathways involved in the release of paracrine agent is not known. In addition, the role of FFA receptors in the expression of BDNF content from enteroendocrine cells and enteric glial cells of the gastrointestinal tract is not known. In this context, we used biochemical, molecular, and functional approaches to investigating the expression of FFA receptors, their signaling pathway and their role in the regulation of BDNF content. The knowledge we gained from this study will generate a better understanding the mechanism by which FFA receptors works and the role of FFA receptors mediating BDNF. This can also aid in the development of agonist which can help in the therapy of Gut motility diseases.

HYPOTHESIS AND SPECIFIC AIMS

The following are the Specific Aims and underlying hypotheses.

Specific Aim 1:   To determine the expression of the free fatty acids receptors (FFARs) in enteroendocrine cells (STC-1), glial cells, and in mouse intestinal cells.

Hypothesis. Receptors for free fatty acids are differentially expressed in enteroendocrine cells and glial cells and in different regions of the gastrointestinal tract.

Specific Aim 2:   To determine the downstream signaling pathway involved in long chain FFAR1 and FFAR4 in enteroendocrine cells (STC-1) and enteric glial cells.

Hypothesis. Signaling pathway of activated FFAR1 and FFAR4 in both STC-1 and enteric glial cells is mediated via stimulation of PLC/IP3/Ca^{+2} pathway.

Specific Aim 3:   To determine the role of long chain FFA receptors in the regulation of brain derived neurotrophic factor content in STC-1 and enteric glial cells.

Hypothesis. Activation of FFAR1 and FFAR4 induces increase in BDNF content in STC-1 and glial cells.

CHAPTER 2
MATERIAL AND METHODS

2.1.1 Materials

Linolenic acid (LA) and GW9508 were obtained from Sigma-Aldrich (St Louis, Mo), TUG891 was obtained from TOCRIS Bioscience (Bristol, UK) and GW1100 was obtained from Cayman Chemical Company (Ann Arbor, MI). [^{35}S] GTPγS, myo-[^{3}H] inositol were obtained from Perkin Elmer (Boston, MA); antibodies to FFAR1, FFAR2, FFAR3, , FFAR4, GPR84, , BDNF, GAPDH, β actin, GFAP, S100, Gαq, and Gα$_{i1-3}$, were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA); the BDNF Emax® ImmunoAssay System was obtained from Promega Corporation (Promega Corporation, WI); U73122 (PLC inhibitor) was obtained from Calbiochem (La Jolla, CA); Fura 2-AM, was obtained from Invitrogen (Eugene, OR); western blotting, Dowex AG-1 X 8 resin (100-200 mesh in format form), chromatography material and protein assay kit were obtained from Bio-Rad Laboratories (Hercules, CA); RNAqueousTM kit was obtained from Ambion (Austin, TX); PCR reagents were obtained from Applied Biosystems (Foster city, CA); SuperScript MT II Reverse Transcriptase was obtained from Invitrogen (Carlsbad, CA); Dulbecco's 25 modified Eagle's medium (DMEM) was obtained from Fisher Scientific. Fluorescence-conjugated species-specific secondary antibody Alexa 488 and Alexa 594 were obtained from Li-Cor (Lincoln, NE). siRNA FFAR1, siRNA FFAR4 and siRNA control were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). STC-1 cell line were obtained from mouse musculus and enteric glial cells line were obtained from rat jejunum from ATCC. All *other chemical and reagents were obtained from Sigma (St Louis, MO).*

2.1.2 Tissue Collection

Wild type adult C57BL/6J mice were purchased from Jackson Laboratories (Bar Harbor, ME). Animals were killed by CO_2 inhalation/asphyxiation by placing them in sealed chamber with a CO_2 flow into the chamber at ~20% volume displacement per minutes for ~ 5 minutes, Tissues (small intestine and colon) were immediately dissected and used for immunohistochemistry. The animal was housed in the animal facility administrated by the Division of Animal Resources, Virginia Commonwealth University. All procedures were conducted in accordance with the Institutional Animal Care and Use Committee of the Virginia Commonwealth University.

2.1.3 Expression of free fatty acid receptors (FFAR) in STC-1 and glial cells by RT-PCR

Total RNA was isolated from both STC-1 and enteric glial cells with Ambion RNA isolation kit. RNA from each preparation was reversely transcribed using the SuperScript™ II system containing 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM dithiothreitol (DTT), 0.5 mM deoxynucleoside triphosphates (dNTP), 2.5 µM random hexamers and 200 units of reverse transcriptase in a 20 µl reaction volume. The reactions were carried out at room temperature for 10 min at 37°C for 120 min, and terminated by heating at 85°C for 5 min. Three µl of the reversely transcribed cDNA was amplified in a final volume of 50 µl by PCR in standard conditions (2 mM $MgCl_2$, 200 µM dNTP, 2.5 units Q5 High-Fidelity DNA Polymerase) with specific primers designed based on rat and mouse cDNAs. PCR was performed for 35 cycles. For each experiment, a

parallel control without reverse transcriptase was processed. The amplified PCR products were analyzed on 1.5% agarose gel containing 0.1 µg/ml ethidium bromide.

2.1.4 Expression of FFAR1 and FFAR4 in STC-1 and glial cell by quantitative PCR (qRT-PCR)

Quantitative PCR was performed using StepOne™ Real-Time PCR System (Applied Biosystem, Foster city, CA) and the intercalating dye, SYBRgreen. For each cDNA sample, real-time PCR was conducted in a 20 µl reaction volume containing Quantitect™ SYBRgreen PCR Mastermix (Qiagen, Mississauga, ON). The following time and temperature profiles were used for the real-time PCR reactions: 95 °C for 5 min; 50 cycles of a series consisting of 15 s at 94 °C, 30 s at 52 °C, 30 s at 72 °C; and a final extension of 5 min at 72 °C. The optimal annealing temperatures were determined empirically for each target and endogenous control (housekeeping gene) primer set. Real-time PCR reactions were performed in triplicate. The fluorescent threshold value was calculated using the StepOne™ Real-Time PCR System software. The absence of peaks in water controls suggested a lack of primer-dimer formation.

Two general types of quantification strategies were performed in quantitative RT-PCR (qRT-PCR). The levels of expressed genes were measured by an absolute quantification or by a relative qRT-PCR. The absolute quantification approach relates the PCR signal to input copy number using a calibration curve. Relative quantification relates the PCR signal of the target transcript in a treatment group to that of another sample such as an untreated control. Relative quantification of a target gene in relation to another gene (reference gene or housekeeping gene) was calculated on the basis of delta delta CT

values (CT, called as well CP), is the cycle number at which the fluorescence generated within a reaction crosses the threshold. CT reflects the point during the reaction at which a sufficient number of amplicons has accumulated). The target-gene expression is

Delta delta CT method $\Delta\Delta CT = (C_{T, Tag} - C_{T, HKG})_{Treatment} - (C_{T, Tag} - C_{T, HKG})_{Control}$

Relative gene expression (Fold Change) = $2^{-\Delta\Delta CT}$

Where, HKG is the housekeeping gene and Tag, the evaluated gene.

After normalization, the data for free fatty acid receptors were expressed as the fold-change in mRNA expression relative to that obtained from control samples.

2.1.5 Protein extraction

STC-1 and enteric glial cells were homogenized with solubilizing buffer of the following composition, T-PER (tissue protein extraction reagent) plus protease and phosphatase cocktail inhibitors. The supernatant was collected after sonication for 15 seconds and centrifugation at 10000g for 10 minutes at 4 °C. Then the protein concentration was determined by DC protein assay kit from Bio-Rad (a colorimetric assay to detect protein concentration after detergent solubilization based on Lowery assay).

2.1.6 Expression of free fatty acid receptors by Western blot

Equivalent amount of proteins were fractionated by SDS/PAGE electrophoresis using 10% (w/v) acrylamide resolving gel and the separated proteins were electrophoretically transferred onto a nitrocellulose membrane. The membrane was blocked by incubation in 5% non-fat dried milk + Tris buffered saline with 0.1% tween-20 (TBST) for one hour at the room temperature. The membrane was incubated overnight

at 4 °C with various primary antibodies diluted in TBS-T 5% (w/v) non-fat dried milk (Table 2). The blots were washed three times for five minutes with TBS-T and then the membrane was incubated with horseradish-peroxidase-conjugated to IRDye 800CW or 680 (LI-COR) corresponding secondary antibodies diluted to 1:20000 in 5% non-fat dry milk + TBS-T for two hours with gentle shaking at room temperature. The membrane was washed three times for five minutes with TBS-T, then the immunoreactive proteins were visualized by using Super Signal Femto maximum sensitivity substrate kit (Pierce, IL).

Quantification of protein bands obtained on western blot was done using the ODYSSEY infrared imaging system (LI-COR Biosciences). To maintain equal amount of loaded proteins, the average intensity obtained of each band was normalized to its respective band of β-actin. The average bands intensity were then presented as relative fold changes compared with the control of the same lane.

2.1.7 Colocalization of free fatty acid receptors with BDNF in STC-1 and glial cells by Immunocytochemistry

STC-1 or glial cells were cultured in 8-well chamber slide for two days and treated with free fatty acid receptor agonists for 15 minutes. Cells were fixed in 4% paraformaldehyde for 30 minutes at room temperature. After permeabilization with 0.3% Triton X-100 for 5 minutes and blocking with 5% normal donkey serum (Jackson ImmunoResearch, PA) in PBS for 30 minutes (pH 7.4), cells were incubated with FFAR1 or FFAR4 antibodies along with BDNF antibodies for two hours at room temperature. Cells were rinsed twice in PBS and then incubated with fluorochrome-conjugated secondary antibodies Alexa 594 or 488 for one hour at room temperature. Cells were

rinsed four times with PBS, then mounted with mounting buffer with DAPI on glass microscope slides and analyzed under a Zeiss fluorescent photomicroscope. Immunostaining in the absence of primary or secondary antibody was assessed for background evaluation.

2.1.8 Histology and tissue Immunofluorescence

About 4 cm long pieces of mouse intestinal and colon samples were cleaned with smooth muscle buffer of the following composition (NaCl 120 mM, KCl 4 mM, KH2PO4 2.6 mM, CaCl2 2.0 mM, MgCl2 0.6 mM, HEPES (N-2-hydroxyethylpiperazine-N' 2-ethanesulfonic acid) 25 mM, glucose 14 mM, and essential amino mixture 2.1% (pH 7.4). and placed in 4% paraformaldehyde fixative solution overnight at 4 ˚C. After fixation the tissue was placed on to pre-labeled tissue base mold, the entire tissue block was covered with cryo-embedding media (OCT), and then stored in -80 ˚C. Then, cross sections (10 µm) were cut and mounted on gelatin-coated microscope slide. The slides were warmed at 37 ˚C for one hour and then fixed with cold acetone for 10 minutes. The tissue were blocked with blocking solution containing 3% of normal donkey serum in PBS. The slides then were incubated with primary antibodies (1:200 dilution) diluted in PBS containing 5% normal donkey serum at 4 ˚C overnight. After rinsing three times with 0.1% IHC-PBS, the sections were incubated with fluorescence-conjugated species-specific secondary antibody Alexa 488 for two hours at room temperature. Tissue sections were rinsed three times in IHC-PBS with and without 0.1% tween-20. Then slides were mounted with Citiflour antifadent mounting medium. Control sections were incubated without the

primary antibody to evaluate nonspecific binding. Prepared slides were viewed with an AxioCam Carl Zeiss microscope.

2.1.9. Assay for G protein activation

G proteins selectively coupled to FFAR1 and FFAR4 were identified from the increase in $G\alpha$ binding to the $[^{35}S]$ GTPγS (5'-O-3-thiotriphosphate) using the method of Okamoto et al 2000. Ten ml of STC-1 or glial cells suspension (3×10^6 cells/ml) were homogenized in 20 mM HEPES medium (pH 7.4) containing 2 mM MgCl$_2$, 1 mM EDTA and 2 mM DTT. After centrifugation at 30,000 g for 15 min, the crude membranes were solubilized for 60 min at 4 °C in 20 mM HEPES medium (pH 7.4) containing 2 mM EDTA, 240 NaCl, 0.5% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-pro-panesulfonate), 2 mM PMSF, 20 µg/ml aprotinin, and 20 µM leupetin. The membranes were incubated for 20 min at 37 °C with 60 nM $[^{35}S]$ GTPγS with 100 µM linolenic acid in the presence or absence of FFAR1 (GW1100, 10 µM) or FFAR4 (AH7614, 100 µM) antagonists in a solution containing 10 mM HEPES (pH 7.4), 100 µM EDTA and 10 mM MgCl$_2$. The reaction was terminated with 10 volumes of 100 mM of Tris-HCl medium (pH 8.0) containing 10 mM MgCl$_2$, 10 mM NaCl and 10 µM GTP, and the mixture was placed in wells precoated with specific antibodies to $G\alpha_q$ or $G\alpha_{i1\text{-}3}$. Coating with G protein antibodies (1:1000) was done after the wells were first coated with anti-rabbit IgG (1:1000) for two hours on ice. After incubation for two hours on ice, the wells were washed three times with phosphate buffer saline solution (PBS) containing 0.05% Tween-20 followed by solubilization with 0.1N NaOH and the radioactivity from each well was counted by liquid

scintillation. The amount of [^{35}S] GTPγS bound to the activated Gα subunit was expressed as counts per minute (cpm).

2.1.10. Assay for phosphoinositide (PI) hydrolysis (PLC-β activity)

PI hydrolysis (PLC-β activity) was determined in STC-1 and glial cells by measuring the formation of inositol phosphates using ion-exchange chromatography using the method of Berridge et al as described previously (Murthy and Makhlouf 1991; Murthy, Zhou et al. 2003). STC-1 and glial cells were labeled with [^{3}H] myo-inositols (0.5 µCi/ml) for 24 hours in inositol-free DMEM medium. The cultures were washed three times with phosphate-buffer saline (PBS) and treated with long-chain free fatty acids receptor agonists or antagonists; linolenic acid, GW9508, TUG891, GW1100, AH7614 or U73122 (PI hydrolysis inhibitor) for one minute. The reaction was terminated by the addition of 940 µl chloroform-methanol-HCl (50:100:1 v/v/v). After chloroform (310 µl) and water (310 µl) were added, the samples were vortexed and the phases were separated by centrifugation at 3000 rpm for 20 minutes. The upper aqueous phase was applied to the column containing 1 ml of 1:1 slurry of Dowex AG-1 X8 resin (100-200 mesh in formate form) and distilled water.

The column was washed with 10 ml of water followed by 10 ml of 5 ml sodium tetraborate-60 mM ammonium formate to remove [^{3}H] glycerophosphoinositol. Total inositol phosphates were eluted with 6 ml of 0.8 M ammonium formate-0.1 M formic acid. The eluates were collected into scintillation vials and counted in gel phase after addition of 10 ml of scintillant. The results were expressed as counts per minute.

2.1.11 Measurement of $[Ca^{2+}]_i$

Free fatty acid receptor agonists-induced increase in $[Ca^{2+}]i$ was measured by fluorescence in single STC-1 or glial cells loaded with fluorescent Ca^{2+} dye fura 2-AM. STC-1 or glial cells were plated on coverslips for 12 h in DMEM. After being washed with PBS, the cells were loaded with 5 µM fura 2-AM in HEPES containing 10% pluronic and DMSO for 3 hours at room temperature. Clusters of cells were selected for imaging and visualized. The cells were visualized through a 40 objective (ZEISS; 0.9 NA) with a Zeiss Axioskop 2 plus upright fluorescence microscope and imaged with a setup consisting of a charge coupled device camera (Imago, TILL Photonics, Applied Scientific Instrumentation, Eugene, OR) attached to an image intensifier. The cells were alternatively excited at 340 nm and 380 nm and the emitted light was detected using a dichroic mirror (410 nm) and emission filter (530). The increase in Ca^{2+} is related to an increase in FIR (FIR= Fluorescence Intensity Ratio). The increase in intracellular calcium in response to LA and FFAR1 (GW9508) and FFAR4 (TUG891) agonists was measured by determining the ratio of the fluorescence of fura-2 AM at 340 and 380 nm excitation (F340/F380).

2.1.12. Transfection of FFAR1 siRNA or FFAR4 siRNA

STC-1 or glial cells were plated in six-well plates and cultured until confluent. Antibiotic free medium was used one day before the transfection. The cells were then transiently transfected with the FFAR1 siRNA or FFAR4 siRNA by using Lipofectamine 2000 for 24 hours according to the manufacturer's instructions (Invitrogen). Briefly, 100 pmol of the siRNA in

125 µl OptiMEM medium were mixed with 5 µl of Lipofectamine 2000 in 125 µl of Opti-MEM. The mixture was incubated at room temperature for 20 min and added to wells containing 1.5 ml DMEM with 10% FBS for one day. The medium was then replaced with DMEM with 10% FBS plus antibiotics for two days. Cells were maintained for a final 24 hours in DMEM without FBS before experiments were started. Transfection efficiency was monitored by the expression of the red fluorescence protein using FITC filters. Control cells were transfected with vector alone. Analysis by psiREN DS-RED with fluorescence microscopy showed that approximately 80-90%.of the cells were transfected.

2.1.13. Measurement of BDNF content by enzyme-linked immunosorbent assay (ELISA)

STC-1 or glial cells were plated in 6 wells plate with DMEM containing 5% FBS and antibiotics and cultured until 80-90% confluent. The cells were washed with DMEM 0 for 30 minutes. Cells were then treated for 15 minutes in DMEM 0 containing 1% albumin, amastatin, and phosphoramidon with or without 100 µM linolenic acid, 10 µM GW9508, 10 µM GW1100, 100 µM TUG891, or 100 µM AH7614. Cells were washed with PBS and solubilized in Triton X-100 based lysis buffer plus protease and phosphatase inhibitors. After centrifugation of the lysates at 14000 rpm for 10 minutes at 4 °C, the protein concentration from the pellet were determined with a Dc protein assay kit from Bio-Rad.

BDNF content in the STC-1 cells and glial cells was measured via a sandwich ELISA using the Promega (Promega Corporation, WI) BDNF Emax immunoassay according to the manufacturer's directions. Polystyrene ELISA plates were coated with

anti-BDNF mAb (1:1000) by adding 100 μl of the antibody to each well and overnight incubation at 4 °C without shaking. The plate was vigorously washed on the next day with TBST and blocked by adding 200 μl of block & sample 1X buffer to each well using a multichannel pipettor and incubated for one hour at room temperature without shaking. One hundred microliters of treated samples and BDNF standards were added in duplicate and incubated with shaking for two hours at room temperature. The plate was washed for five times and then incubated with 100 μl of anti-human BDNF pAb (1:500) at room temperature for two hours with shaking. After five times washing of the plate with TBST, 100 μl of diluted anti-IgY HRP Conjugate (1:200) were added to each well and the plate incubated for another two hours with shaking at room temperature. Then the plate was washed five times and 100 μl of room-temperature TMB One solution (3,3′,5,5′-tetramethylbenzidine), the substrate was added and incubated for 10 minutes with shaking. One hundred microliters of 1N hydrochloric acid were added to wells to stop the reaction. Then the plate was scanned using a plate reader and the absorbance was measured at 450 nm within 30 minutes of stopping the reaction. The BDNF levels were expressed as pg BDNF per mg total protein calculated from the standard curve.

Statistical Analysis

The results were expressed as means $\pm$ SE and the experiments were performed at least five times. Statistical significance was analyzed using Student's t-test for paired and unpaired values. The results were statistically analyzed using GraphPad Prism software, San Diego, CA. A probability of $P < 0.05$ was considered significance.

Table 1. Sequence of primers used for PCR, GAPDH; Glyceraldehyde 3-phosphate dehydrogenase, β-actin, FFAR: free fatty acid receptor, m:mouse, r:rat.

Primer set	Forward Primer 5' → 3'	Reverse Primer 3' → 5'	Size (bp)
m/FFAR1	AATGCCTCCAATGTGGATAG	AGTCCTCGTCACACATATTG	159
m/FFAR3	TCCTGGCATCGGCTCACT	TGTAGGTTGCATTTCCCCAGTA	62
m/FFAR2	ACCAAATCACCTGCTATGAGAACTT	CACGGGCAGCACCACAT	58
m/GPR48	CCCCCTCTGGAATGTTTTTGT	TCGCATGCGTCAAAGC	63
m/FFAR4	GCATAGGAGAAATCTCATGG	GAGTTGGCAAACGTGAAGGC	335
r/β-actin	CAGGGTGTGATGGTGGGTATG	AGTTGGTGACAATGCCGTGTT	
r/GAPDH	AAGGTGGTGAAGGAGGCGGC	GAGCAATGCCAGCCCCAGGA	
r/FFAR1	ACTGGTCACTGGCTACTTGG	TTGGAGTCCTGGTCACACAT	65
r/FFAR3	GCAGTCTTTCCGTCATGCCT	TCCCTGAAGGTCTCTCCCAG	140
r/GPR84	AAGTGCATCGCAAGACTGGA	CTGAGGACGAAGCAAAGGAAC	156
r/FFAR4	AATCGCACCCACTTCCCTTT	GATGAGTCCCAGAACGGTGG	90
m/GAPDH	AGAAACCTGCCAAGTATGATG	GGAGTTGCTGTTGAAGTCG	122

Table 2. Source and concentration of primary antibodies used and the product size of the proteins identified

Antibody	Catalog #	Product Size (kDa)	Company name	Dilution
FFAR1	sc-32905	31	Santa Cruz	1:1000
FFAR3	sc-98332	39	Santa Cruz	1:1000
FFAR2	sc-32906	43	Santa Cruz	1:1000
GPR84	sc-99106	44	Santa Cruz	1:1000
FFAR4	sc-99105	52/42	Santa Cruz	1:1000
β-actin		42	Sigma	1:1000
BDNF			Promega	1:200

CHAPTER 3

RESULTS

3.1 Expression of Free Fatty Acid Receptors (FFARs)

Receptors for short-, medium- and long-chain free fatty acids (FFAs) belong to relatively newly deorphanized G protein coupled receptor family and recent evidence suggests that these receptors play a role in the regulation of gastrointestinal function such as motility and secretion, and energy metabolism. FFARS are grouped according to the chain length of FFAs that activate each FFARs. FFAR1 and FFAR4 are preferentially activated by medium- and long-chain FFAs, but preferentially activated by long-chain FFAs. FFA2 and FFA3 are activated by short-chain FFAs, whereas GPR84 is activated by medium-chain, but not long-chain FFAs.

In the present study, we analyzed the mRNA and protein expression of FFAR1, FFAR2, FFAR3, FFAR4 and GPR84 in mouse endocrine cells (STC-1 cells) and rat enteric glial cells by RT-PCR, real time RT-PCR, and Western blot, and by immunohistochemistry in mouse small intestine.

3.1A. Expression of FFAR1, FFAR2, FFAR3, FFAR4 and GPR84 mRNA in STC-1 and Enteric Glial Cells

(i) RT-PCR. Primers for RT-PCR analysis of FFARs in STC-1 cells and glial cells are based on rat FFARs sequences, The sequence of specific primers are listed in

chapter 2 table 1.Messenger RNA for FFAR1 (159 bp), FFAR2 (58 bp), FFAR3 (62bP), FFAR4 (335 bp) and GPR84 (63 bp) were detected by RT-PCR analysis of RNA isolated from STC-1 cells (Figure 3). Under similar condition, mRNA for FFAR1 (65 bp), FFAR3 (114 bp), FFAR4 (90 bp) and GPR84 (156 bp) were detected by RT-PCR analysis of RNA isolated from glial cells (Figure 4). The results suggest expression of all FFARs in both enteroendocrine cells and enteric glial cells.

(ii) Real-time RT-PCR. For comparative analysis of FFARs in STC-1 and glial cells, quantitative real time PCR was performed in triplicate in RNA isolated from both STC-1 and enteric glial cells. Expression of FFAR4 was 5.3 fold higher compared to other FFA receptors in STC-1 cells (Figure 5). In contrast, expression of FFAR1 was 2 fold higher compared to FFAR4 in glial cells. (Figure 5). These results suggest differential expression of FFAR1 and FFAR4 in in enteroendocrine and enteric glial cells.

3.1B. Expression of FFAR1 and FFAR4 protein in STC-1 and Glial Cells

Further confirmation for the differential expression of FFAR1 and FFAR4 in STC-1 and glial cells was obtained using western blot analysis and specific antibodies to FFAR1 and FFAR4. Expression of both FFAR1 (31 kDa) and FFAR4 (42 kDa) was detected in homogenates prepared from STC-1 cells (Figure 6). Similarly expression of both FFAR1 (31 kDa) and FFAR4 (42 kDa) was detected in homogenates prepared from glial cells (Figure 7). For comparative analysis of FFAR1 and FFAR4 expression in STC-1 and glial cells, densitometric analysis of FFARs normalized to loading control β-actin was performed. Expression of FFAR4 was 1.62 fold higher compared to FFAR1 in STC-1 cells. In contrast, expression of FFAR1 was 2.52 fold higher compared to FFAR4 in

glial cells. The pattern of FFAR1 and FFAR4 protein expression is consistent with the pattern of mRNA expression of these receptors in STC-1 and glial cells (Figure 8).

3.1C. Expression of FFAR1 and FFAR4 in Mouse Intestine

Immunohistochemical analysis of tissue cryosections obtained from mouse small intestine shows the presence of FFAR1 and FFAR4 in specific cells of the mucosal layer (Figure 7-11). FFAR1 and FFAR4 positive cells were located in the microvilli and crypts. The cells were triangular in appearance and extended from the basal lamina to luminal surface, typical of open type of enteroendocrine cells (EECs). Some FFAR1 and FFAR4 positive cells were located in close proximity to the intestinal epithelium, where enteric glial cells are located (Figures 9 and 10). FFAR1 and FFAR4 were also present in the core of the villi where subepithelial glial cells themselves are located. The immunohistochemistry data confirmed expression of FFAR1 and FFAR4 in native enteroendocrine cells and glial cells as well as the enteroendocrine cell line (STC-1) and the glial cell line.

In summary, FFARs are differentially expressed in STC-1 versus glial cells with predominant expression of FFAR4 in STC-1 cells and predominant expression of FFAR1 in glial cells.   Both FFAR1 and FFAR4 are also present in native enteroendocrine and glial cells

3.2 Signaling Pathways Coupled to FFAR1 and FFAR4

After demonstrating differential expression of FFAR1 and FFAR4 in STC-1 and glial cells, further studies were designed to examine the signaling pathways activated by

these receptors in both STC-1 and glial cells. Activation of specific G protein(s) and signaling pathways downstream of G proteins in response to the endogenous long chain fatty acid, linolenic acid (100 µM), was measured. Since linolenic acid activates both FFAR1 and FFAR4 receptors, agonist selective for FFAR1 (GW9508) and FFAR4 (TUG891), as well as antagonists selective for FFAR1 (GW1100) and FFAR4 (AH7614) were used to analyze receptor-selective signaling pathways in both STC-1 and glial cell.

3.2A. Signaling in STC-1 Cells

(i) Identification of G proteins coupled to FFAR4. Studies in various tissue and cell lines suggest that FFAR1 and FFAR4 are coupled to activation of G proteins (Salehi et al. 2005 and Hirasawa et al. 2005), but the specific G protein coupled to FFAR4 in enteroendocrine cells has not been identified. Membranes isolated from SCT-1 cells were incubated with [^{35}S]GTPγS (60 nM) in the presence or absence of linolenic acid (100 µM), and FFAR1 or FFAR4 specific inhibitors were added to wells precoated with different Gα antibodies. An increase in the binding of [^{35}S] GTPγS complexed to specific Gα antibody reflected the activation of the corresponding G protein. Incubation of membrane with linolenic acid in the presence of GTPγS caused a significant increase in the binding of [^{35}S] GTPγS to Gα$_q$ (3967±253 cpm, P< 0.0001, n=6) (Figure 11). There was no increase in the binding of [^{35}S]GTPγS to wells coated with antibody to Gα$_{i1-3}$ complex (Figure 12). These results suggest that linolenic acid activates Gq, but not Gi proteins. Linolenic acid-induced increase in the binding of [^{35}S]GTPγS to Gα$_q$ was blocked by a FFAR4 selective antagonist (AH7614, 100 µM), but not by a FFAR1 selective antagonist (GW1100, 10 µM) These results suggest that, in STC-1 cells, long chain fatty acid linolenic acid

preferentially activates FFAR4 receptors that are coupled to Gq. The results are consistent with the predominant expression of FFAR4 compared to FFAR1 in these cells.

(ii) Activation of PI hydrolysis. Previous studies (Kebeda et al. 2012 and Shah et al. 2012) in various tissue and cell lines have shown that activation of FFA receptors by polyunsaturated fatty acid resulted in stimulation of phosphoinositide (PI)-specific phospholipase C (PLC-β) activity, and generation of inositol 1,4,5-trisphosphate (IP$_3$). However, to deduce the signaling pathway involved in activation of long chain free fatty acid receptors in STC-1 cells, we measured the PLC activity in response to the long chain fatty acid natural ligand linolenic acid (100 µM), FFAR4 selective agonist TUG891 (100 µM), and FFAR4 selective antagonist AH7614 (100 µM). Addition of linolenic acid to STC-1 cells labeled with [^{3}H]myo-inositol, caused a significant increase in PI hydrolysis, measured as increase in water soluble [3H]inositol (410±40 cpm P<0.001, n=8). Similarly, selective agonist for FFAR4 caused an increased in PI hydrolysis (332±81 cpm, n=8). (Figure 13) Linolenic acid-induced increase in PI hydrolysis was significantly blocked by the selective FFAR4 antagonist (AH7614, 100 µM) (183±15 cpm, P<0.001, n=8) (Figure 13). These results suggest that, In STC-1 cells linolenic acid-induced increase in PI hydrolysis was mediated via FFAR4. The results are consistent with activation of Gq by linolenic acid via FFAR4 in these cells.

(iii) Increase in intracellular calcium Activation of PLC-β results in the generation of inositol 1,4,5-trisphosphate (IP$_3$) and IP$_3$-dependent Ca^{2+} release from intracellular sarcoplasmic reticulum stores. We next examined the effect of linolenic acid and a selective FFAR4 agonist (TUG891) on cytosolic Ca^{2+} ([Ca^{2+}]$_i$) by calcium imaging using Fura-2 AM. An increase in F340/F380 fluorescence intensity ratio reflected an increase

in intracellular Ca^{2+}. Consistent with the activation of PI hydrolysis, addition of linolenic acid (100 µM) or TUG891 to STC-1 cells resulted in a rapid increase in cytosolic Ca^{2+} , n=40 (Figures 14 and 15).

3.2B Signaling in Glial Cells

(i) Identification of G proteins coupled to FFAR1. Studies in various tissue and cells lines suggest that FFAR1 and FFAR4 are coupled to activation of G proteins, but the specific G proteins coupled to FFAR1 in enteric glial cells have not been identified. Membranes isolated from glial cells were incubated with [^{35}S]GTPγS (60 nM) in the presence or absence of linolenic acid, and FFAR1 or FFAR4 specific inhibitors and the aliquots were added to wells precoated with different Gα antibodies; an increase in the binding of [^{35}S]GTPγS complexes to specific Gα antibody reflected the activation of the corresponding G protein. Incubation of membrane with linolenic acid in the presence of GTPγS caused a significant increase in the binding of [^{35}S]GTPγS to $Gα_q$ (5097 cpm P< 0.001, n=6). (Figure 16). There was no increase in the binding of [^{35}S]GTPγS to wells coated with antibody to $Gα_{i1-3}$ complex (Figure 17). These results suggest that linolenic acid activates Gq, but not Gi proteins. Linolenic acid-induced increase in the binding of [^{35}S] GTPγS to $Gα_q$ was blocked by a FFAR1 selective antagonist (GW1100, 10 µM), but not by a FFAR4 selective antagonist (AH7614, 100 µM).   These results suggest that, in glial cells, long chain fatty acid linolenic acid preferentially activates FFAR1 receptors that are coupled to Gq. The results are consistent with the predominant expression of FFAR1 compared to FFAR4 in these cells.

(ii) Activation of PI hydrolysis. To deduce the signaling pathway involved in activation of FFAR1 in glial cells, we measured the PLC activity in response to long chain fatty acid natural ligand 100 µM linolenic acid, FFAR1 selective agonist GW9508 10 µM, and FFAR1 selective antagonist GW1100 10 µM. Addition of linolenic acid to glial cells labeled with [^{3}H]myo-inositol, caused a significant increase in PI hydrolysis, measured as increase in water soluble [^{3}H]inositol (3191 cpm, $P<0.0001$, n=8). (Figure 18) Similarly, selective agonist for FFAR1 (GW9508, 10 µM) caused an increased in PI hydrolysis (2700 cpm n=8). Linolenic acid-induced increase in PI hydrolysis was significantly blocked by the selective FFAR1 antagonist (GW1100, 10 µM) (1366 cpm, $P<0.001$, n=8), but not by FFAR4 antagonist (AH7614, 100 µM) (Figure 18). These results suggest that, in glial cells, linolenic acid-induced increase in PI hydrolysis was mediated via FFAR1. The results are consistent with activation of G_q by linolenic acid via FFAR1 in these cells. Control studies showed that linolenic acid induced increase in PI hydrolysis was blocked by the selective PLC inhibitor, U73122 (10 µM) (Figure 19).

(iii) Increase in intracellular calcium. Activation of PLC-β results in the generation of inositol 1,4,5-trisphosphate (IP$_3$) and IP$_3$-dependent Ca^{+2} release from intracellular sarcoplasmic reticulum stores. We next examined the effect of linolenic acid in the presence or absence of FFAR1 antagonist (GW1100, 100 µM) on cytosolic Ca^{+2} ([Ca^{+2}]$_i$) by calcium imaging using Fura-2 AM. An increase in the fluorescence intensity ratio (F340/F380) reflected an increase in intracellular Ca^{2+}. Consistent with the activation of PI hydrolysis, addition linolenic acid (50 µM and 100 µM) to glial cells resulted in a rapid increase in cytosolic Ca^{+2} (Figure 20). The effect of linolenic acid was inhibited in the presence GW1100 (Figure 21) suggesting that the effect of linolenic acid was mediated

via activation of FFAR1. In support of this notion, a selective FFAR1 agonist GW9508 (100 µM) caused an increase in Ca^{2+} (Figure 22) n=40.

In summary, both FFAR1 and FFAR4 are coupled to $G\alpha_q$ and stimulation of PLC-β/IP$_3$/ Ca^{+2} pathway. In enteroendocrine cells (STC-1) long chain fatty acids preferentially activate FFAR4, whereas in glial cells long chain fatty acids preferentially activate FFAR1. This is consistent with predominant expression of FFAR4 compared to FFAR1 in STC-1 cells, and predominant expression of FFAR1 compared to FFAR4 in glial cells.

3.3 Regulation of Brain-derived Neurotrophic Factor (BDNF) Content by Activation of Long Chain FFAR in STC-1 and Enteric Glial Cells.

There are many hormones, neurotransmitters and neurotrophins that are released in response to long chain free fatty acids in gastrointestinal tract. Activation of FFAR1 induces release of cholecystokinin (Elke Kaemmerer, 2010) and activation of FFAR4 activation induces release of incretin hormones (Frank Riemann, 2012). Of the several neurotrophins identified, brain-derived neurotrophic factor (BDNF) has been associated with regulation of gastrointestinal motility (Grider et al., 2006). However, the effect of long chain free fatty acids on the BDNF content is not known. Several studies have been addressed neurotrophins production using different approaches. In our study we used mainly two approaches; in the first approach BDNF tagged with green fluorescent protein was used to measure BDNF localization and expression in STC-1 and enteric glial cells, and in the second approach enzyme-linked immunosorbent assay (ELISA) was used to measure BDNF levels in the cell lysates.

3.3A. Regulation of BDNF Levels by FFAR4 in STC-1 Cells

First we examined the colocalization of BDNF with FFAR4 in cultured STC-1 cells. Some, but not all, cells show co-localization of BDNF with FFAR1 (Figure 23). Then, to evaluate whether BDNF levels are regulated by activation of FFAR4, STC-1 cells were treated for 15 minutes with linolenic acid (100 µM) and number of BDNF immunopositive cells were counted. As shown in Figure 24, treatment of cells with linolenic acid significantly increased the number of BDNF positive cell (four folds increase compared to control cells) ($P<0.0001$).

The immunocytochemistry study results were further confirmed by the measurement of BDNF content by ELISA. Linolenic acid (100 µM) caused a significant increase in BDNF ($P< 0.01$, n=7) (Figure 25). The increase was blocked by selective FFAR4 antagonist (AH7614, 100 µM), but not by a selective FFAR1 antagonist (GW1100, 10 µM), suggesting that, in STC-1 cells, linolenic acid selectively activates FFAR4 to increase BDNF levels. This conclusion was further supported by siRNA approach to suppress FFAR4 expression. As shown in Figure 26, linolenic acid-induced increase in BDNF levels was abolished in cells expressing FFAR4 siRNA. The selective involvement of FFAR4 receptors in the regulation of BDNF in STC-1 cells is consistent with the predominant expression of FFAR4 in these cells and selective activation of Gq/PLC/Ca2+ pathway coupled to FFAR4 by linolenic acid.

.3.3B. Regulation of BDNF Levels by FFAR1 in Glial Cells

First we examined the colocalization of BDNF with FFAR4 in glial cells. Some, but not all cells show co-localization of BDNF with FFAR1 (Figure 27). Then, to evaluate

whether BDNF levels are regulated by the activation of FFAR1, glial cells were treated for 15 minutes with linolenic acid (100 µM) and the number of BDNF immunopositive cells were counted. As shown in Figure 28, treatment of cells with linolenic acid significantly increased the number of BDNF positive cell (2.5 fold increase compared to control cells) (P<0.0001).

The immunocytochemistry study results were further confirmed by the measurement of BDNF content by ELISA. Linolenic acid (100 µM) caused a significant increase in BDNF (P< 0.01, n=7) (Figure 29). The increase was blocked by a selective FFAR1 antagonist (GW1100, 10 µM), but not by a selective FFAR4 antagonist (AH7614, 100 µM), suggesting that, in contrast to STC-1 cells, in glial cell linolenic acid selectively activates FFAR1 to increase BDNF levels. This conclusion was further supported by siRNA approach to suppress FFAR1 expression. As shown in Figure 30, linolenic acid-induced increase in BDNF levels was abolished in cells expressing FFAR1 siRNA. The selective involvement of FFAR1 receptors in the regulation of BDNF in glial cells is consistent with the predominant expression of FFAR1 in these cells and selective activation of Gq/PLC/Ca2+ pathway coupled to FFAR1 by linolenic acid.

In summary, in enteroendocrine cells (STC-1), long chain fatty acids increase BDNF content via FFAR4, whereas in glial cells they increase BDNF content via FFAR1. This is consistent with predominant expression FFAR4 and preferential activation of FFAR4 by long chain fatty acids in STC-1 cells and predominant expression of FFAR1 and preferential activation of FFAR1 in in glial cells.

Figure 1. Cell lines used in this study.

Photomicrographs of mouse STC-1 cells (enteroendocrine cell line) and rat glial cells (enteric glial line) in culture.
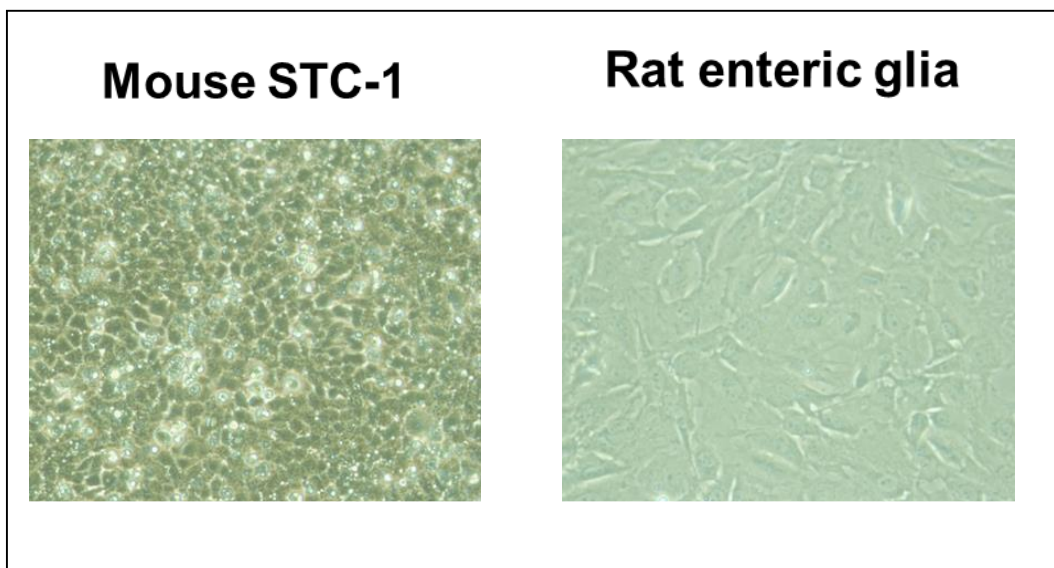
Figure 2. Immunohistochemical staining of glial cell markers.

Enteric glial cells were stained for glial cells markers, glial fibrillary acidic protein (GFAP, right panel) and S100 (left panel). Middle panel: Control staining without primary antibody Magnification 20X.
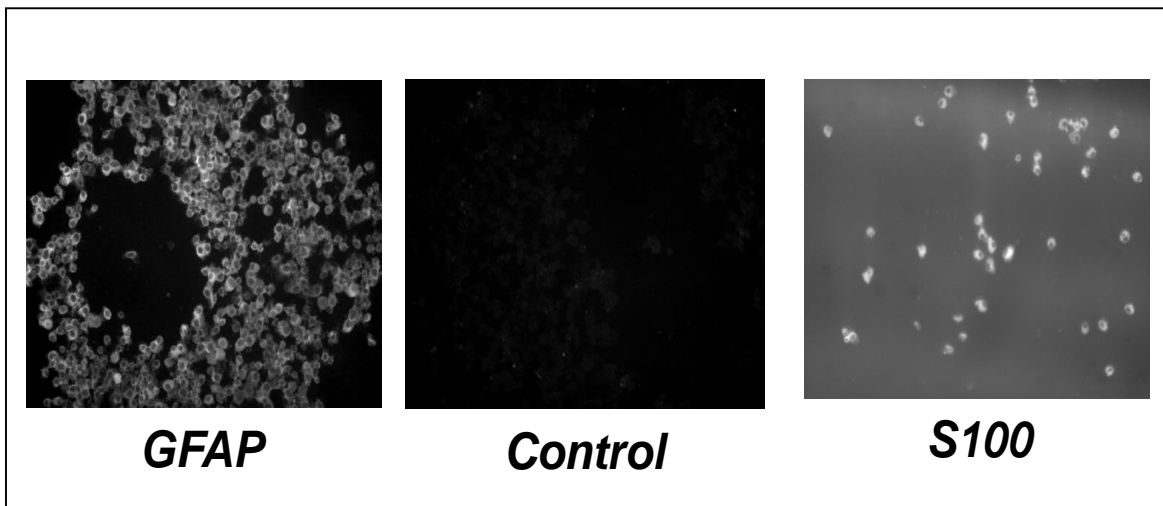

GFAP   Control   S100

Figure 3. Messenger RNA expression of free fatty acid receptors (FFARs) in STC-1 cells.

Total RNA isolated from mouse STC-1 cells was reverse transcribed using 2 µg of total RNA using qScript cDNA preparation kit. The cDNA was amplified with specific primers for FFAR1, FFAR2, FFAR3, FFAR4, and GPR84 and analyzed on 2% agarose gel containing ethidium bromide. The sequence of primers are listed in Table 1. PCR products of expected size were obtained with primers for FFAR1 (159 bp), FFAR2 (58bp), FFAR3 (62 bp), GPR84 (63 bp), and FFAR4 (335 bp).
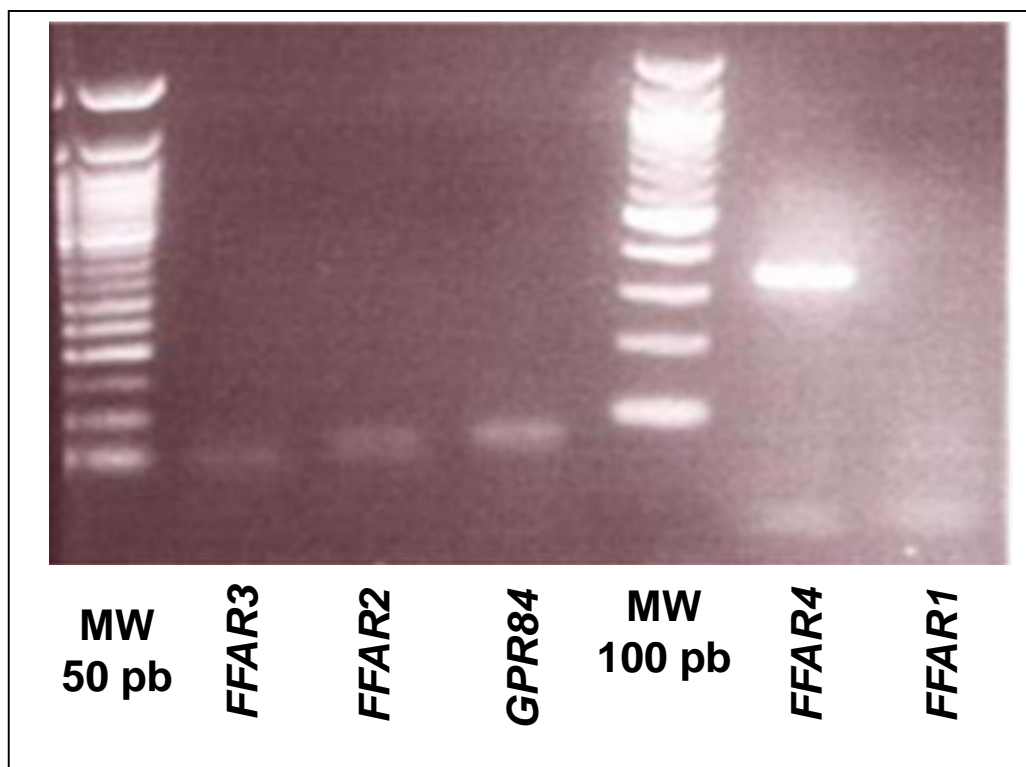
Figure 4. Messenger RNA expression of free fatty acid receptors (FFARs) in glial cells.

Total RNA isolated from rat enteric glial cells was reverse transcribed using 2 µg of total RNA using qScript cDNA preparation kit. The cDNA was amplified with specific primers for FFAR1, FFAR3, FFAR4, and GPR84 and analyzed on 2% agarose gel containing ethidium bromide. The sequence of primers are listed in Table 1. PCR products of expected size were obtained with primers for FFAR1 (65 bp), FFAR3 (114 bp), GPR84 (115 bp), and FFAR4 (90 bp).
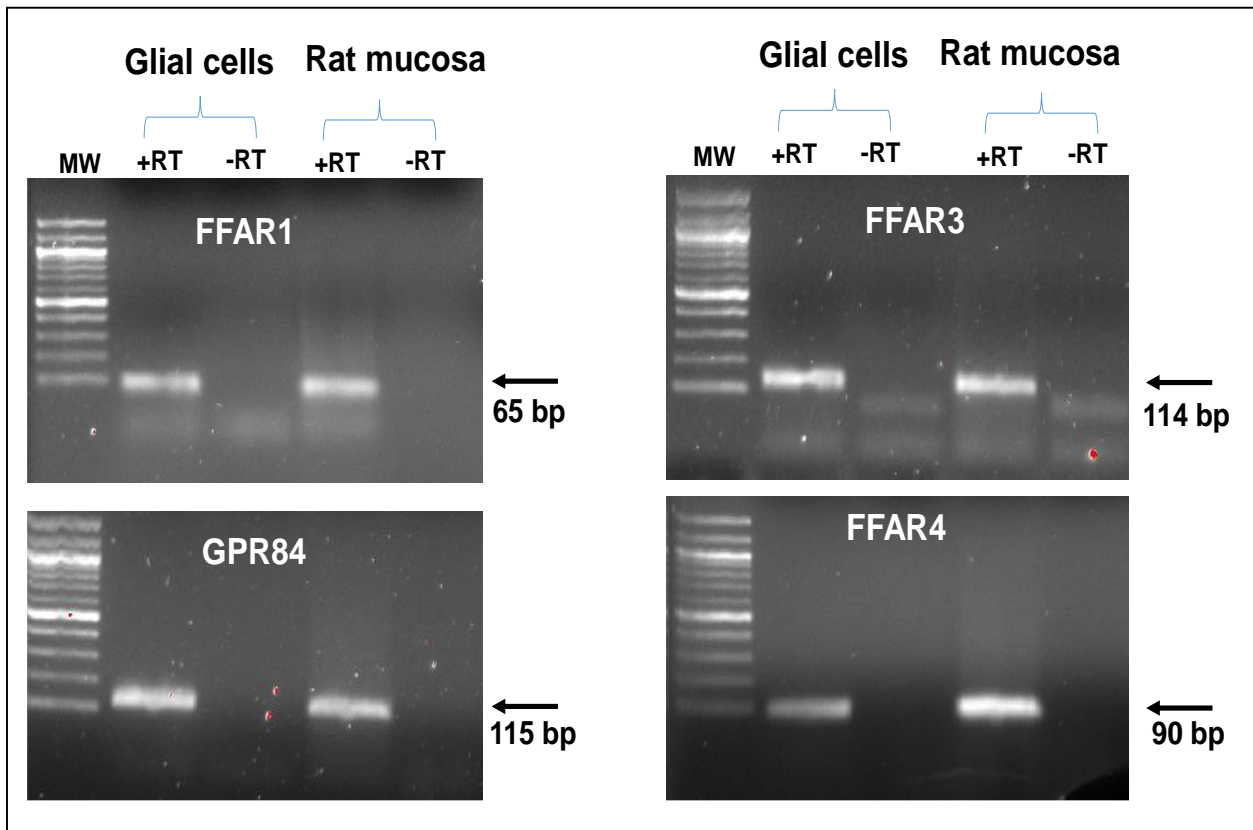
Figure 5. Quantitative PCR (qRT-PCR) of free fatty acid receptors in mouse STC-1 and rat glial cells.

Quantitative PCR was conducted in a 20 µl of reaction volume containing Quantitect ™SYBRgreen PCR Mastermix. Real-time PCR reactions were performed in triplicate. Results were expressed as fold change or fold differences in FFARs after normalizing with endogenous control, GAPDH or β-actin. Right panel: Expression in glial cell. GPR84 and FFAR4 expression levels with references to FFAR1 after normalizing with endogenous control, GAPDH or β-actin. Left panel: Expression in STC-1 cells. FFAR2, FFAR3, FFRA4 and GPR84 expression levels with references to FFAR1. Expression of FFAR1 is higher in enteric glial cells compared to other FFARs whereas expression of FFAR4 is higher in STC-1 cells compared to other FFARs. Values represent the means ±SEM of 4 separate experiments. In glial cells FFAR4 and GPR84 *$p < 0.05$ vs FFAR1 **$p < 0.001$ vs FFAR1.
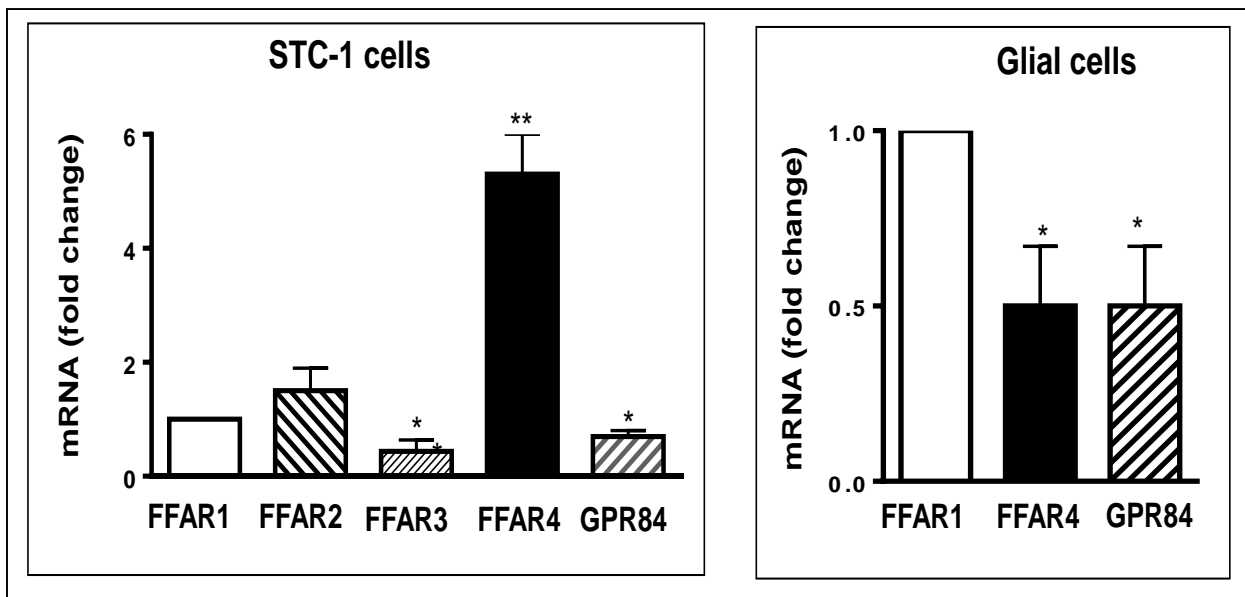
Figure 6. Protein expression of free fatty acid receptors (FFARs) in STC-1 cells. Cell lysates obtained from cultured mouse STC-1 cells. Equal amounts of total proteins were separated with SDS-PAGE and expression of FFAR1 and FFAR4 was analyzed by western blot using receptor-specific antibodies. β-actin was used as loading control. Protein band of expected size for both FFAR1 (31 kDa) and FFAR4 (42 kDa) was detected by western blot analysis.
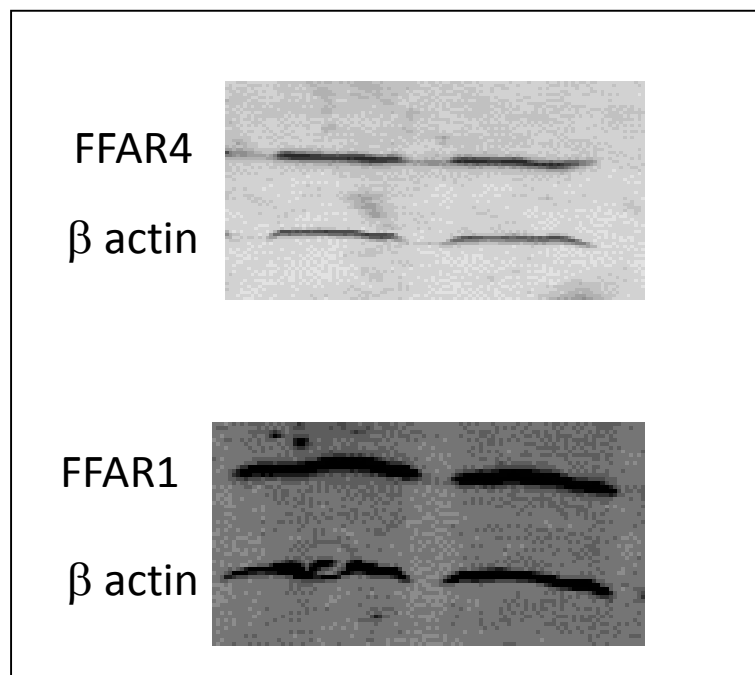
Figure 7. Protein expression of free fatty acid receptors (FFARs) in glial cells.

Cell lysates obtained from cultured rat glial cells. Equal amounts of total proteins were separated with SDS-PAGE and expression of FFAR1 and FFAR4 was analyzed by western blot using receptor-specific antibodies. β-actin was used as loading control. Protein band of expected size for both FFAR1 (31 kDa) and FFAR4 (42 kDa) was detected by western blot analysis.
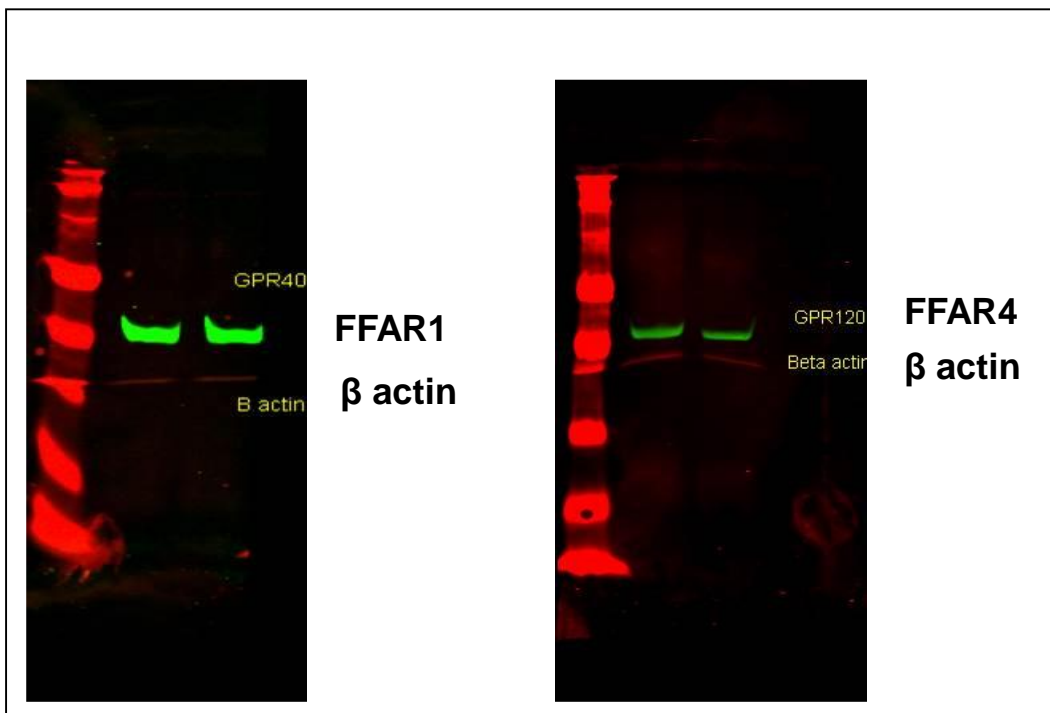
Figure 8. Comparitive quantitative analysis FFAR1 and FFAR4 expression in STC-1 and glail cells.

Densitometric analysis of the western blot of FFAR1 and FFRA4 expression Right panel: Relative abundance of FFAR4 compared with FFAR1 protein expression in STC-1. Left panel: Relative abundance of FFAR1 in glial cells compared with FFAR4 protein expression. **P< 0.001 vs FFAR1
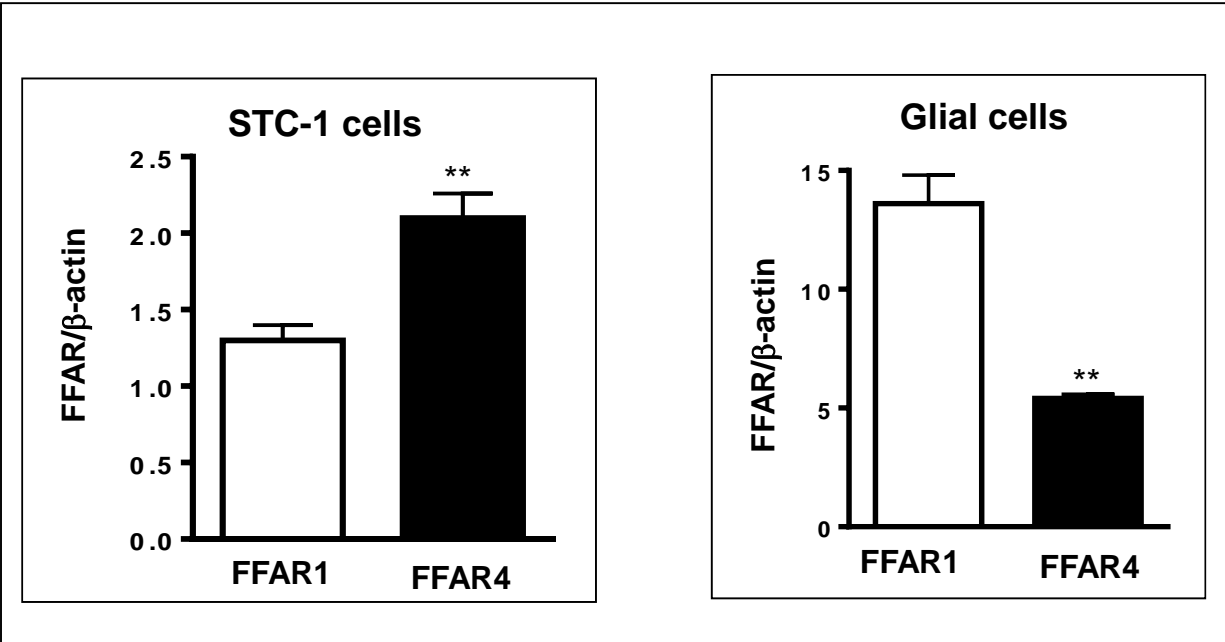
Figure 9. Expression of FFAR1 in mouse small intestine by Immunohistochemistry.
Cryosections of mouse small intestine was stained with FFAR1 antibody and imaged at 40x magnification. Some cells contained within submucosal epithelium area where enteric glial cells located are also positive for FFAR1. FFAR1-positive cells are located within mucosa of intestinal crypt. Arrows pointed to FFAR1 positively stained cells.
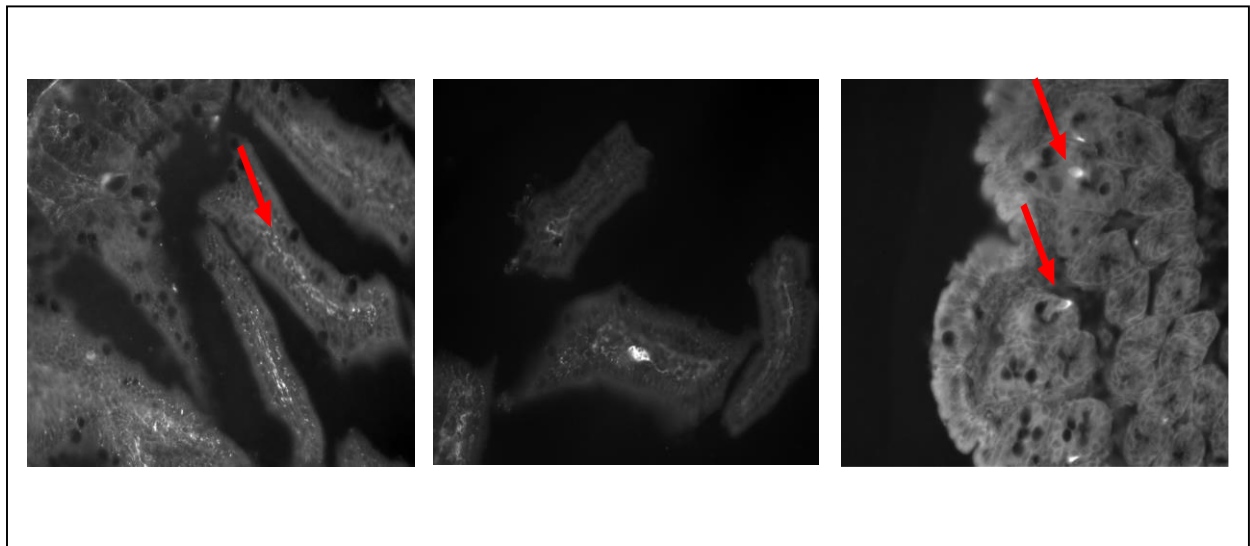
Figure 10. Expression of FFAR4 in mouse small intestine by Immunohistochemistry.

Cryosections of mouse small intestine was stained with FFAR4 antibody and imaged at 40x magnification. FFAR4-positive cells are located within mucosal of colonic crypt with enteroendocrine cell-like morphology. Some cells contained within submucosal epithelium area where enteric glial cells located are also positive for FFAR4. Arrows pointed to FFAR4 positively stained cells.
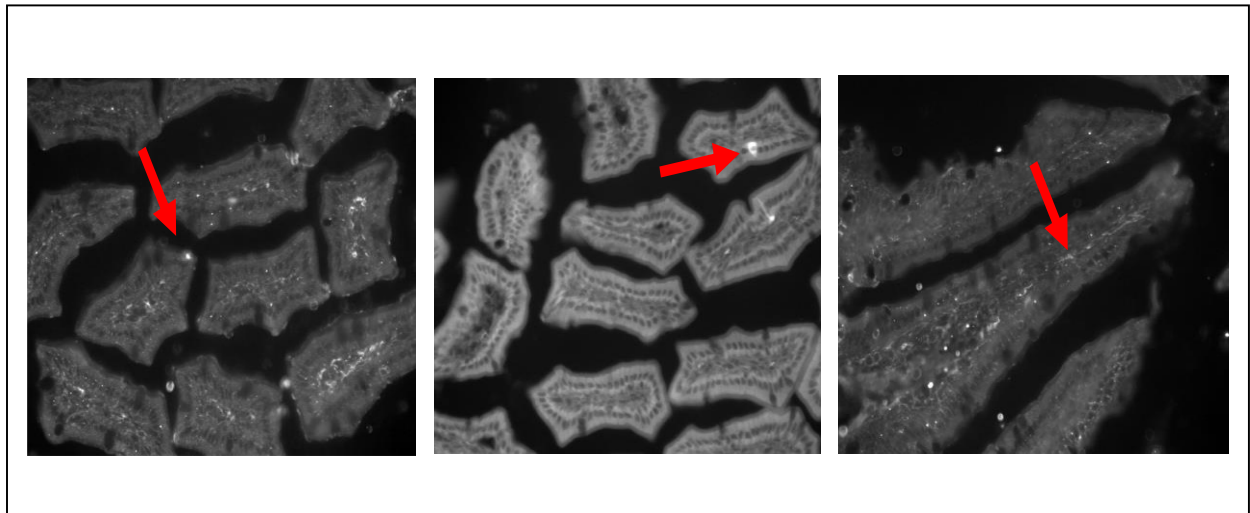
Figure 11. Activation of Gq protein by linolenic acid via FFAR4 in STC-1 cells.
Membranes isolated from STC-1 cells were incubated for 30 minutes at 37 °C with [^{35}S]
GTPγS and 100 μM linolenic acid (LA) in the presence or absence of antagonist for
FFAR1 (GW1100, 10 μM) or FFAR4 (AH7614, 100 μM). Aliquots quantity amount of
protein were added to the wells precoated with the specific antibody to Gα$_q$. The amount
of radioactivity bound to activate Gα$_q$ was measured by liquid scintillation and expressed
as counts per minutes (cpm). Linolenic acid (LA) caused a significant increase in the
binding of [^{35}S] GTPγS-Gα$_q$ that was blocked by the selective FFAR4, but not FFAR1,
antagonist. Values are expressed as mean ± SEM of 6 experiments. **P<0.001 vs basal.
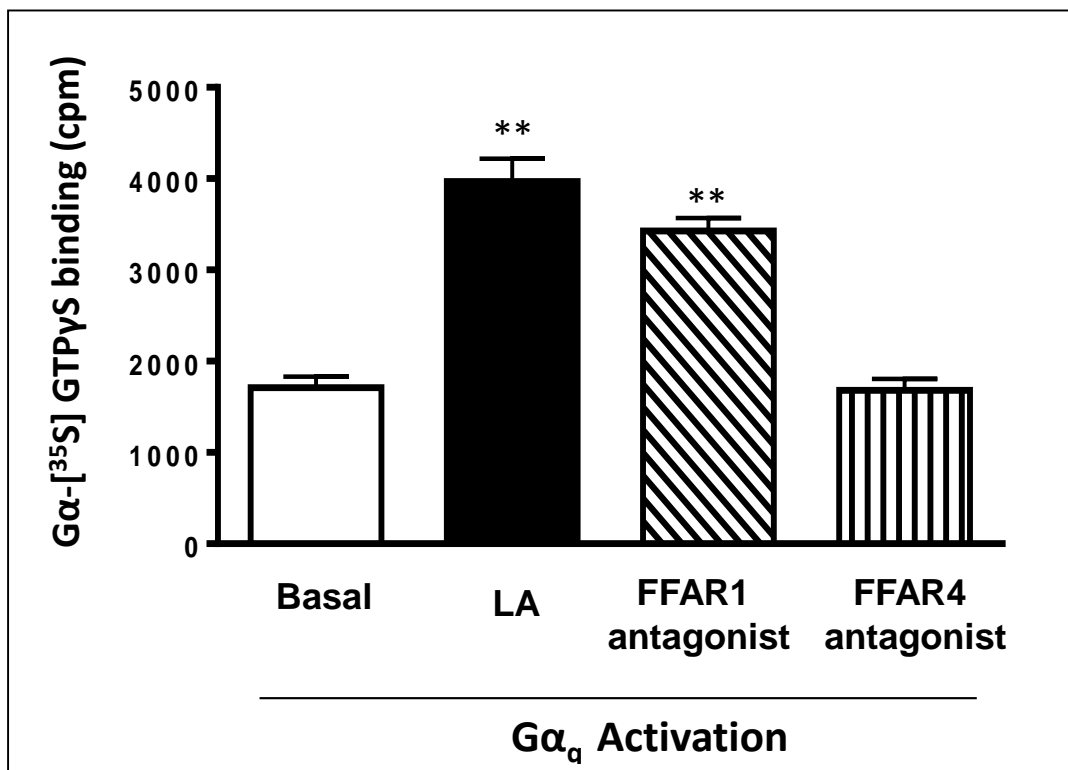
Figure 12. Effect of linolenic acid on the activation of Gi protein in STC-1 cells.

Membranes isolated from STC-1 cells were incubated for 30 minutes at 37 °C with [^{35}S] GTPγS and 100 μM linolenic acid (LA) in the presence or absence of antagonist for FFAR1 (GW1100, 10 μM) or FFAR4 (AH7614, 100 μM). Aliquots of equal amounts of protein were added to the wells pre-coated with the antibody to Gα$_{i1-3}$ and incubated for two hours. The amount of radioactivity bound to activate Gα$_{i1-3}$ was measured by liquid scintillation and expressed as counts per minute (cpm). Treatment with LA had no effect on the binding of [^{35}S] GTPγS-Gα$_{i1-3}$ Values are expressed as mean ± SEM of 6 experiments.
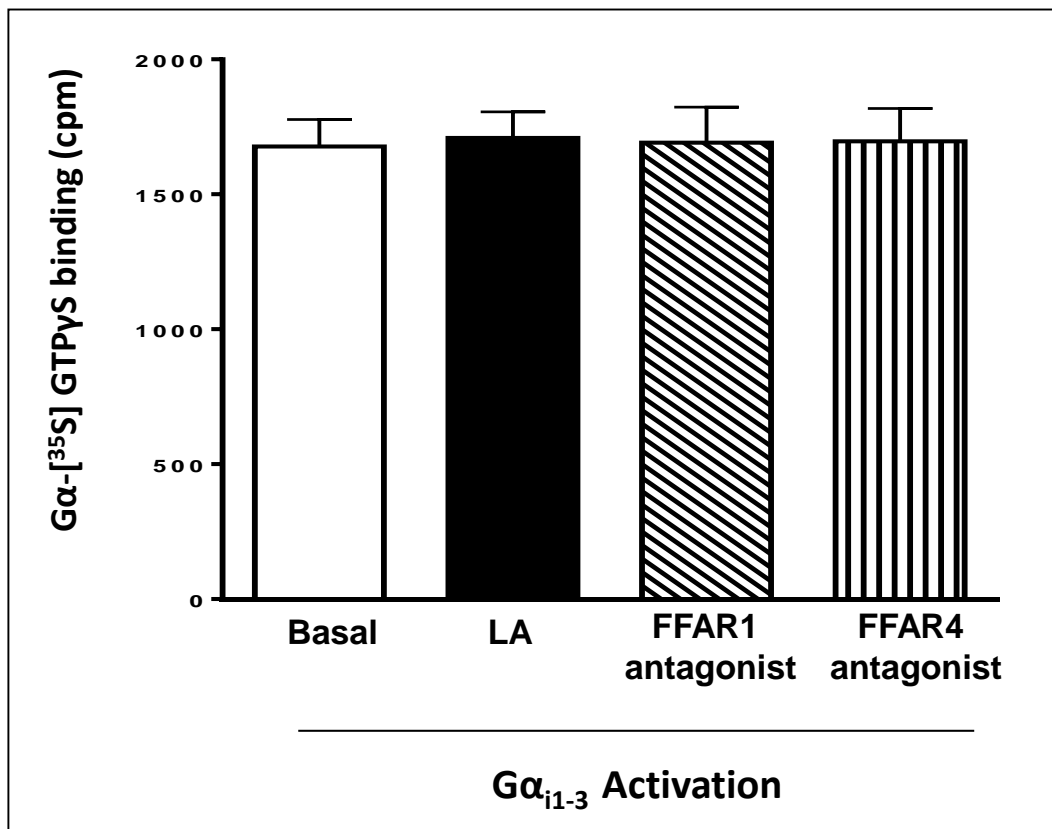
Figure 13. Activation of PI hydrolysis by linolenic acid (LA) via FFAR4 in STC-1 cells.

Cultured mice STC-1 cells were labeled with myo-[^{3}H] inositol for 24 hours and then treated with LA (100 μM), FFAR4 agonist TUG891 (100 μM), or FFAR4 antagonist AH7614 (100 μM). PI hydrolysis was measured as increase in water soluble [^{3}H] inositol by ion exchange chromatography. Result are expressed as count per minute. Treatment of cells with LA caused a significant increase in PI hydrolysis and the increase was blocked by the FFAR4 antagonist. FFAR4 agonist also caused an increase in PI hydrolysis. Values are expressed as mean ± SEM of 8 experiments. **P< 0.001; *p<0.01 significant increase in PI hydrolysis with LA vs. basal.
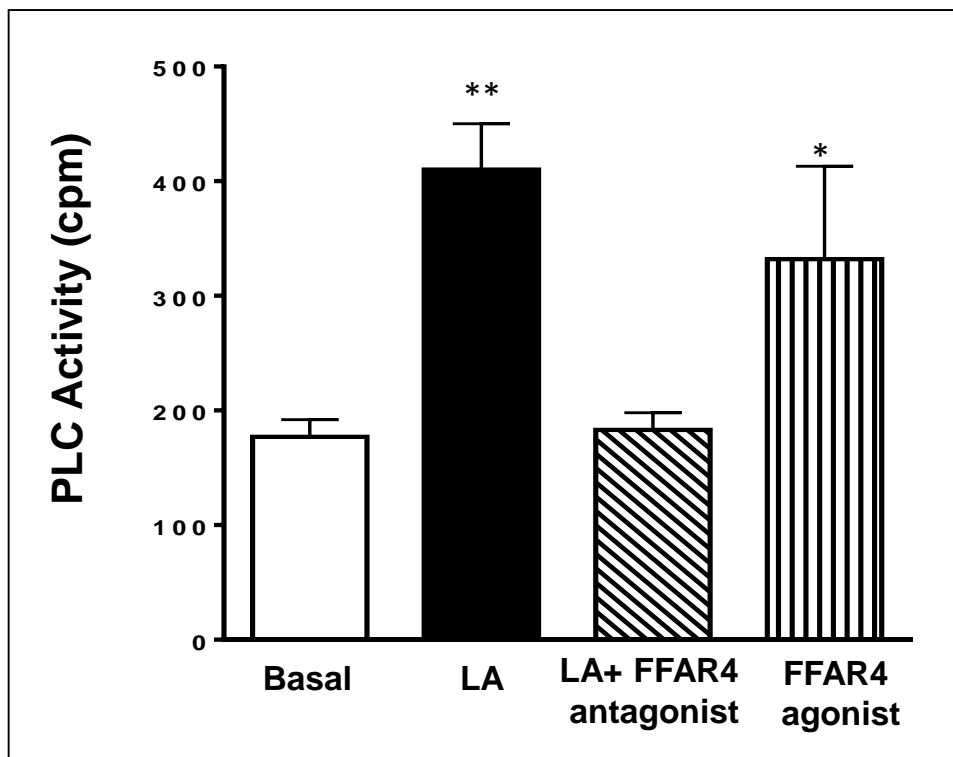
Figure 14. Increase in intracellular calcium by linolenic acid (LA) in STC-1 cells.
Cultured mouse STC-1 cells were loaded with fluorescent Ca^{2+} dye fura 2-AM (5 µM) in
in HEPES buffer containing 10% pluronic and DMSO for 3 hours at room temperature.
Clusters of cells were selected for imaging and visualization. The cells were alternatively
excited at 340 nm and 380 nm and the increase in cytosolic calcium in response to
linolenic acid was measured as the increase in the ratio of the fluorescence of fura-2 at
F340/F380 nm emission at 530 nm. Representative results demonstrated that addition of
LA to STC-1 cells resulted in a rapid increase in cytosolic Ca^{2+}.
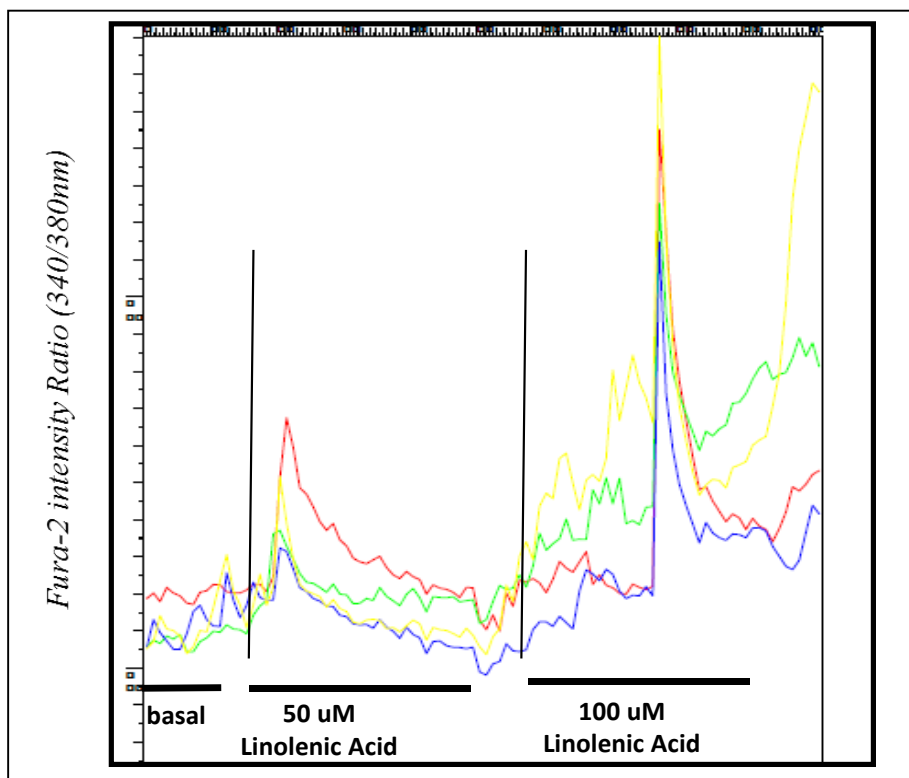
Figure 15. Increase in intracellular calcium by FFAR4 agonist TUG891 in STC-1 cells.

Cultured mouse STC-1 cells were loaded with fluorescent Ca^{2+} dye fura 2-AM (5 µM) in in HEPES buffer containing 10% pluronic and DMSO for 3 hours at room temperature. Clusters of cells were selected for imaging and visualization. The cells were alternatively excited at 340 nm and 380 nm and the increase in cytosolic calcium in response to FFAR4 agonist, TUG891 (100 µM) was measured as the increase in the ratio of the fluorescence of fura-2 at F340/F380 nm emission at 510 nm. Representative results demonstrated that addition of TUG891 resulted in a rapid increase in cytosolic Ca^{2+}.
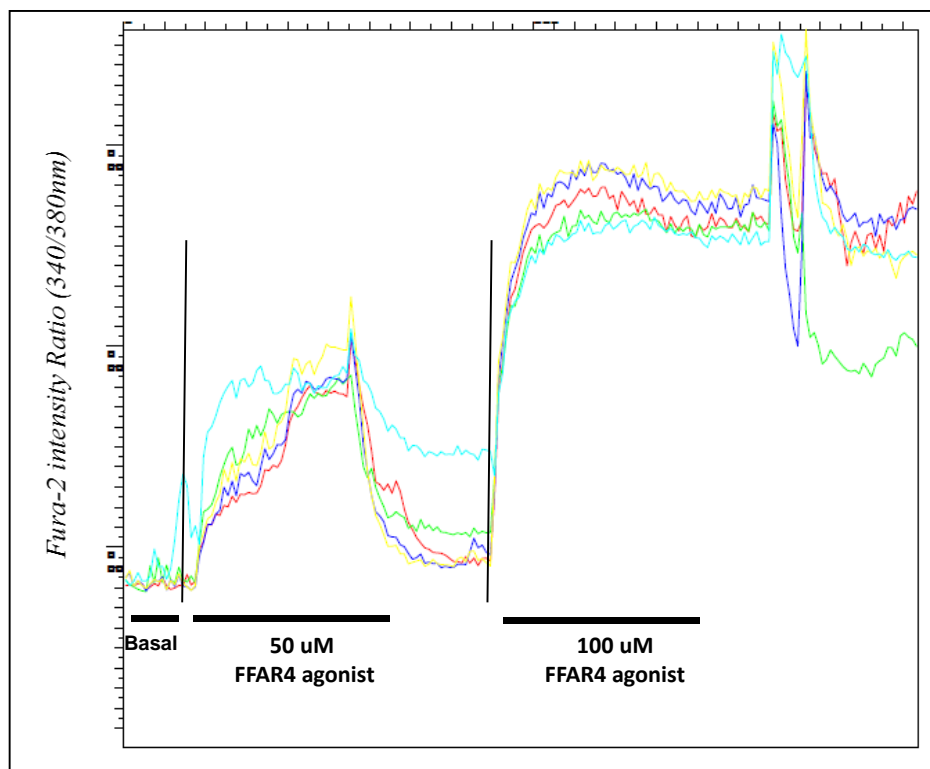
Figure 16. Activation of Gq protein by linolenic acid via FFAR1 in glial cells.
Membranes isolated from enteric glial cells were incubated for 30 minutes at 37 °C with
[^{35}S] GTPγS and 100 μM linolenic acid (LA) in the presence or absence of antagonist for
FFAR1 (GW1100, 10 μM) or FFAR4 (AH7614, 100 μM). Aliquots of equal amounts of
protein were added to the wells pre-coated with the specific antibody to $G\alpha_q$. The amount
of radioactivity bound to activated $G\alpha_q$ was measured by liquid scintillation and expressed
as counts per minute (cpm). Linolenic acid caused a significant increase in the binding
of [^{35}S] GTPγS-$G\alpha_q$ and the increase in the binding was blocked by the selective FFAR1,
but not FFAR4, antagonist. Values are expressed as mean ± SEM of 6 experiments.
**P<0.001 vs basal.
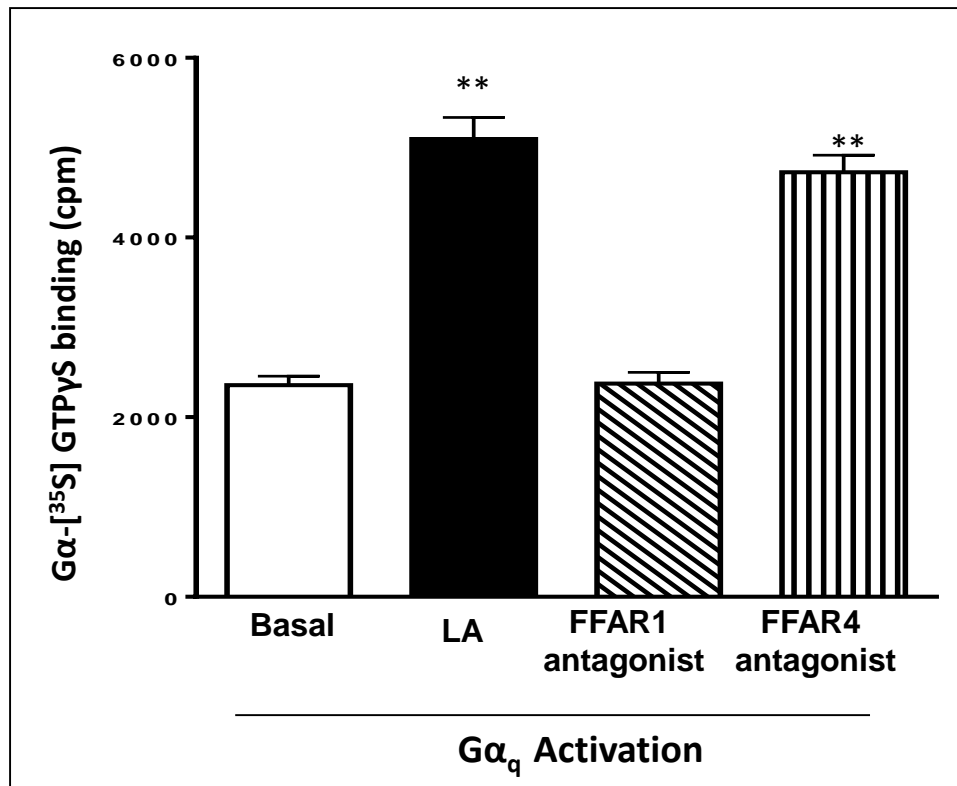
Figure 17. Effect of linolenic acid on the activation of Gi protein in glial cells.

Membranes isolated from glial cells were incubated for 30 minutes at 37 °C with [^{35}S] GTPγS and 100 µM linolenic acid (LA) in the presence or absence of antagonist for FFAR1 (GW1100, 10 µM) or FFAR4 (AH7614, 100 µM). Aliquots quantity equal amounts of protein were added to the wells precoated with the antibody to Gα$_{i1-3}$ and incubated for two hours. The amount of radioactivity bound to activated Gα$_{i1-3}$ measured by liquid scintillation and expressed as counts per minutes (cpm). Treatment with LA had not effect on the binding of [^{35}S] GTPγS-Gα$_{i1-3}$ Values are expressed as mean ± SEM of 6 experiments.
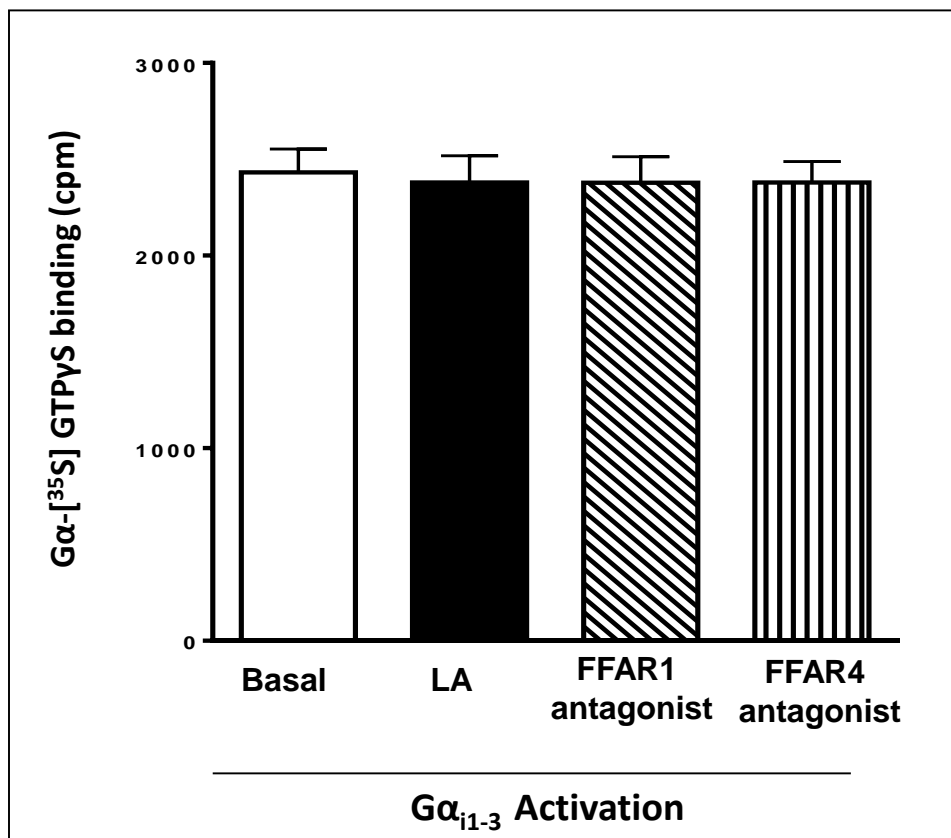
Figure 18. Activation of PI hydrolysis by linolenic acid (LA) via FFAR1 in glial cells.

Cultured glial cells were labeled with myo-[^{3}H] inositol for 24 hours and then treated with LA (100 µM) in the presence or absence of FFAR1 antagonist (GW100, 10 µM) or FFAR4 antagonist (AH7614, 100µM), or FFAR1 agonist GW9508 (10 µM).   PI hydrolysis was measured as increase in water soluble [3H]inositol by ion exchange chromatography. Result are expressed as count per minute (cpm). Linolenic acid caused a significant increase in PI hydrolysis and the increase was blocked by the FFAR1 antagonist, but not FFAR4 antagonist. FFAR1 agonist also caused an increase in PI hydrolysis. Values are expressed as mean ± SEM of 8 experiments. **P< 0.001; *P<0.01 significant increase in PI hydrolysis with LA vs basal.
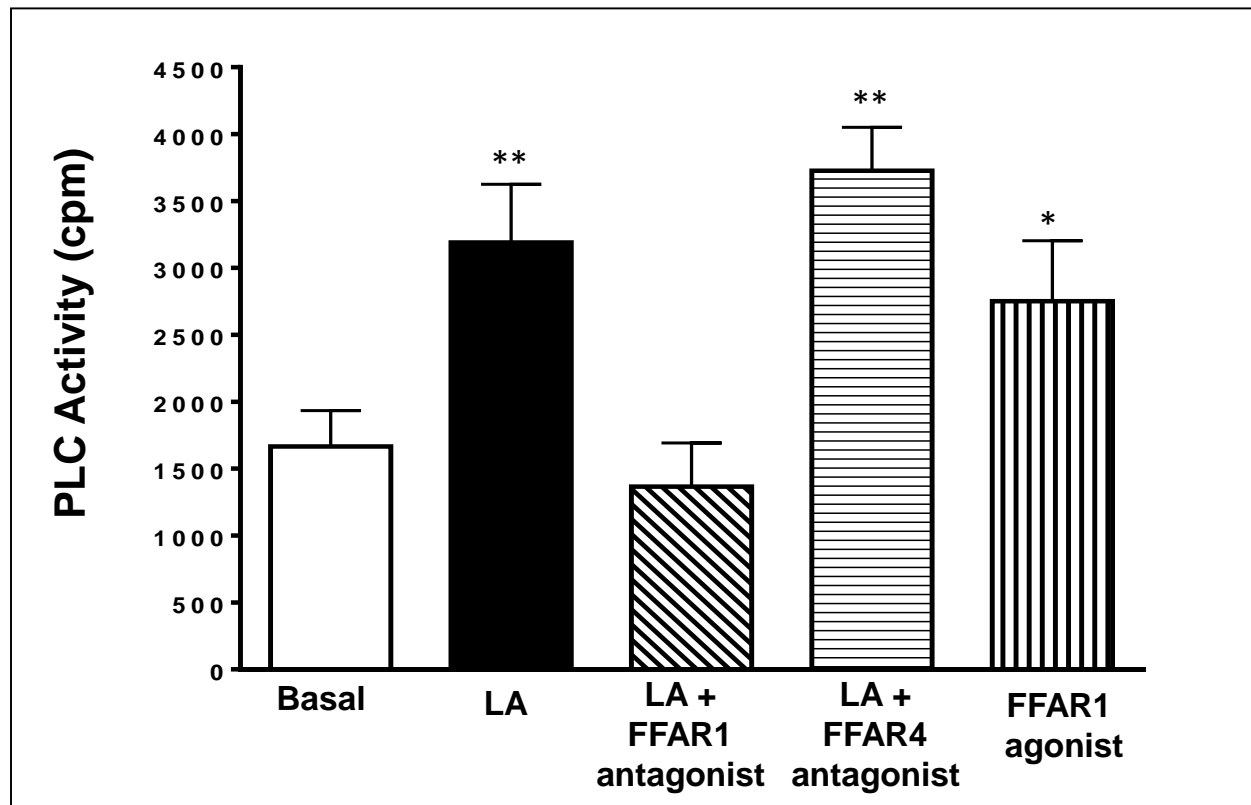
Figure 19. Inhibition of linolenic acid-induced PI hydrolysis by non-specifc PLC inhibitor U73122 enteric glial cells.

Cultured glial cells were labeled with myo-[^{3}H] inositol for 24 hours and then treated with linolenic acid (LA, 100 µM) in the presence or absence of PLC inhibitor U73122 (10 µM). PI hydrolysis was measured as increase in water soluble [^{3}H] inositol by ion exchange chromatography. Result are expressed as count per minute. Treatment of cells with LA caused a significant increase in PI hydrolysis and the increase was blocked by U73122. Values are expressed as mean ± SEM of 8 experiments. **P< 0.001 significant increase in PI hydrolysis with LA vs basal.

**Figure 20. Increase in intracellular calcium by linolenic acid (LA) in glial cells.**
Cultured glial cells were loaded with fluorescent Ca^{2+} dye fura 2-AM (5 µM) in in HEPES buffer containing 10% pluronic and DMSO for 3 hours at room temperature. Clusters of cells were selected for imaging and visualization. The cells were alternatively excited at 340 nm and 380 nm and the increase in cytosolic calcium in response to linolenic acid was measured as the increase in the fluorescence intensity ratio of fura-2 at F340/F380 nm emission. Representative results demonstrated that addition of LA to glia cells resulted in a rapid increase in cytosolic Ca^{2+}.

Figure 21. Inhibition of linolenic acid-induced Increase in calcium by FFAR1 antagonist in glial cells.

Cultured glial cells were loaded with fluorescent Ca^{2+} dye fura 2-AM (5 µM) in in HEPES buffer containing 10% pluronic and DMSO for 3 hours at room temperature. Clusters of cells were selected for imaging and visualization. The cells were alternatively excited at 340 nm and 380 nm and the increase in cytosolic calcium in response to linolenic acid in the presence or absence of FFAR1 antagonist GW1100 (10 µM) was measured as the increase in the fluorescence intensity ratio F340/F380 nm emission. Representative mean results of 40 cells demonstrated that addition of LA to glial cells resulted in a rapid increase in cytosolic Ca^{2+}.n=40.
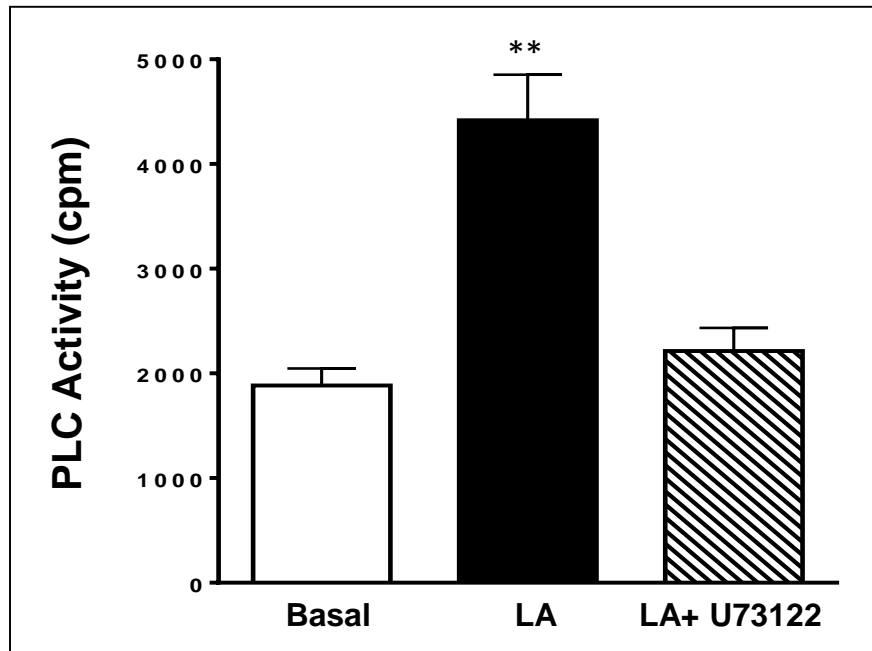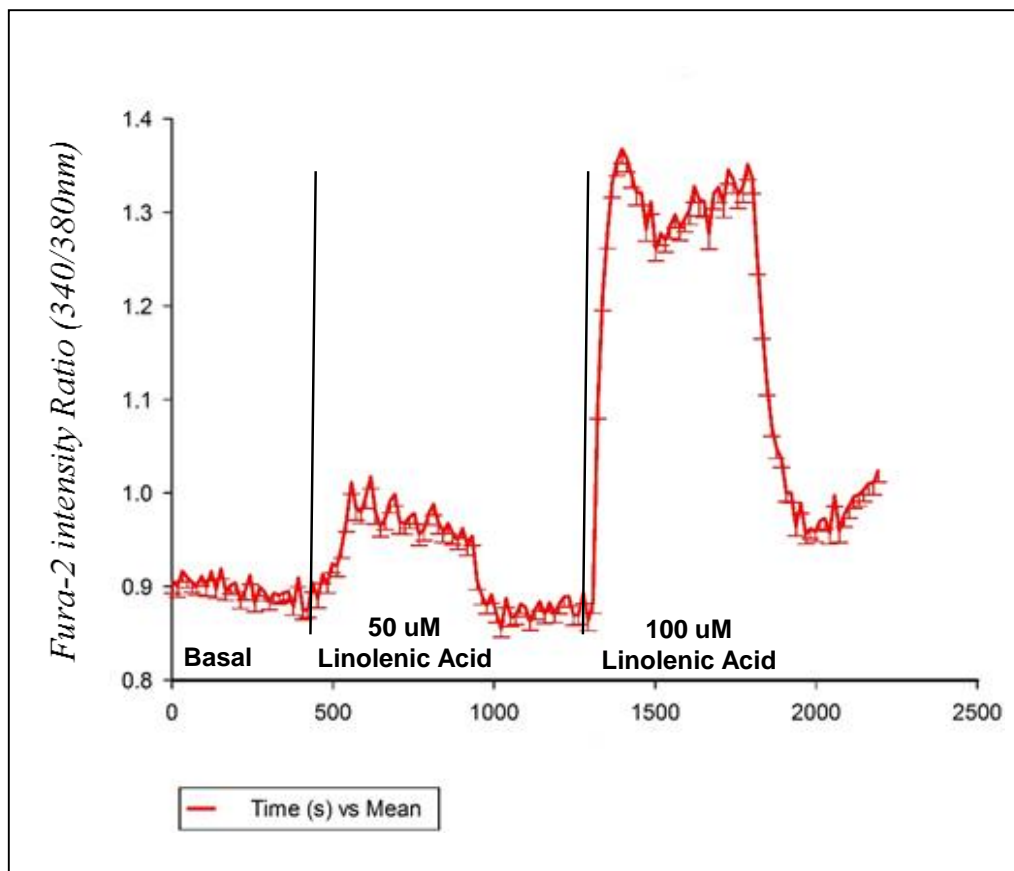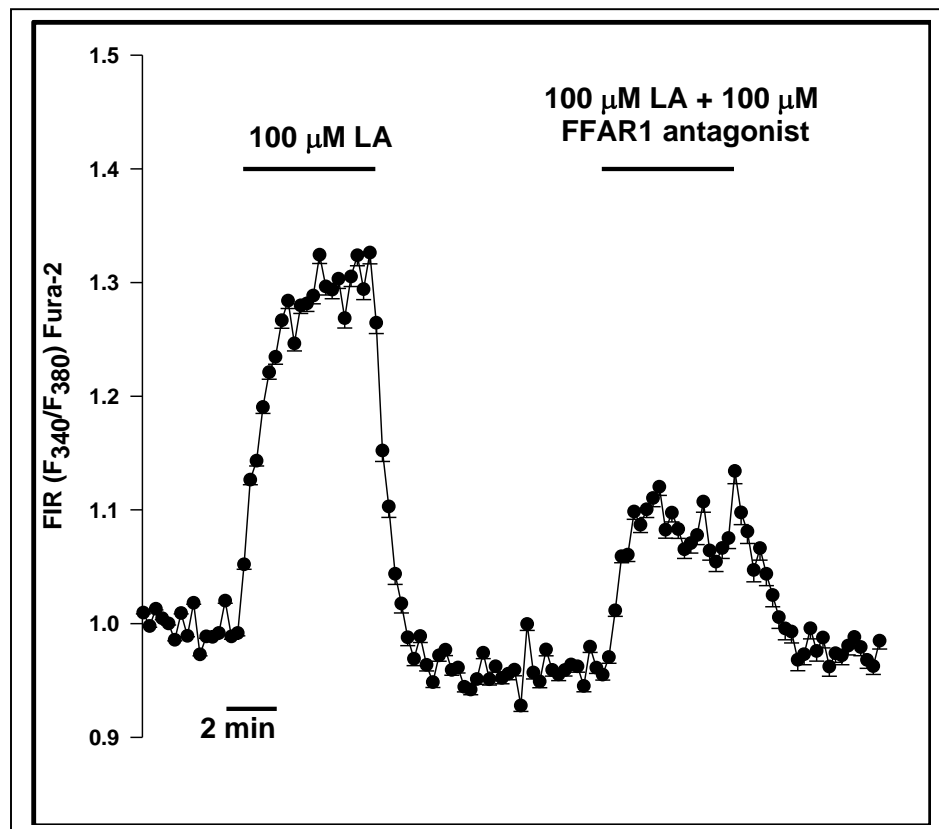
Figure 22. Increase in intracellular calcium by FFAR1 agonist, GW9508 in glial cells.

Cultured glial cells were loaded with fluorescent Ca^{2+} dye fura 2-AM (5 µM) in in HEPES

buffer containing 10% pluronic and DMSO for 3 hours at room temperature. Clusters of

cells were selected for imaging and visualization. The cells were alternatively excited at

340 nm and 380 nm and the increase in cytosolic calcium in response to FFAR1 agonist

GW9508 (100 µM) acid was measured as the increase in the fluorescence intensity ratio

at F340/F380 nm emission. Representative mean results of 40 cells demonstrated that

addition of FFAR1 agonist to glial cells resulted in a rapid increase in cytosolic Ca^{2+}.

Figure 23. Colocalization of FFAR4 and BDNF in cultured mouse STC-1 cells.
Cultured STC-1 cells were stained with primary antibody to BDNF or FFAR4 and secondary antibody Flexa 488 (green) for BDNF and Flexa 594 (red) for FFAR4. The cells were imaged at x400 magnification. Arrows point to the cells colocalization of BDNF and FFAR4. Left panel: Cells stained with nuclear stain dapi.

Figure 24. Increase in BDNF positive cells by linolenic acid in STC-1 cells.

Cultured STC-1 cells were stained with BDNF antibody before and after treatment with linolenic acid (100 µM) for 15 min. The cells were imaged at x400 magnification. Results demonstrated an increase in BDNF (green fluorescence) after linolenic acid (LA) treatment. Right panel: Quantitative analysis of BDNF positive cells before and after treatment with linolenic acid *P < 0.0001 versus control.

Figure 25. Increase in BDNF content by linolenic acid via FFAR4 in STC-1 cells.

Cultured STC-1 cells treated with 100 µM linolenic acid (LA) in the presence or absence of FFAR4 antagonist (AH7614, 100 µM) or FFAR4 agonist (TUG891, 100 µM) for 15 at 37°C. The amount of BDNF in the cell lysates was measured by ELISA. Linolenic acid-induced increase in BDNF content was inhibited by FFAR4-antagonist. Values are expressed as mean ± SEM of 3 experiments. *P< 0.05 significant increase of BDNF versus control.

Figure 26. Increase in BDNF content by linolenic acid via FFAR4 in STC-1 cells.

Cultured STC-1 cells were transfected with control siRNA for FFAR4 siRNA for 48 h and treated with 100 µM linolenic acid (LA) or FFAR4 agonist (TUG891, 100 µM) for 15 at 37°C. The amount of BDNF in the cell lysates was measured by ELISA. Linolenic acid or FFAR4 agonist caused an increase in BDNF content in cells transfected with control siRNA but not in cells transfected with FFAR4 siRNA. . Values are expressed as mean ± SEM of 3 experiments. *P< 0.05 significant increase of BDNF versus basal. #P< 0.05 significant inhibition of BDNF vs basal.   Inset showing transfection of pSIREN-DNR-DsRed vector verified by immunofluorescence
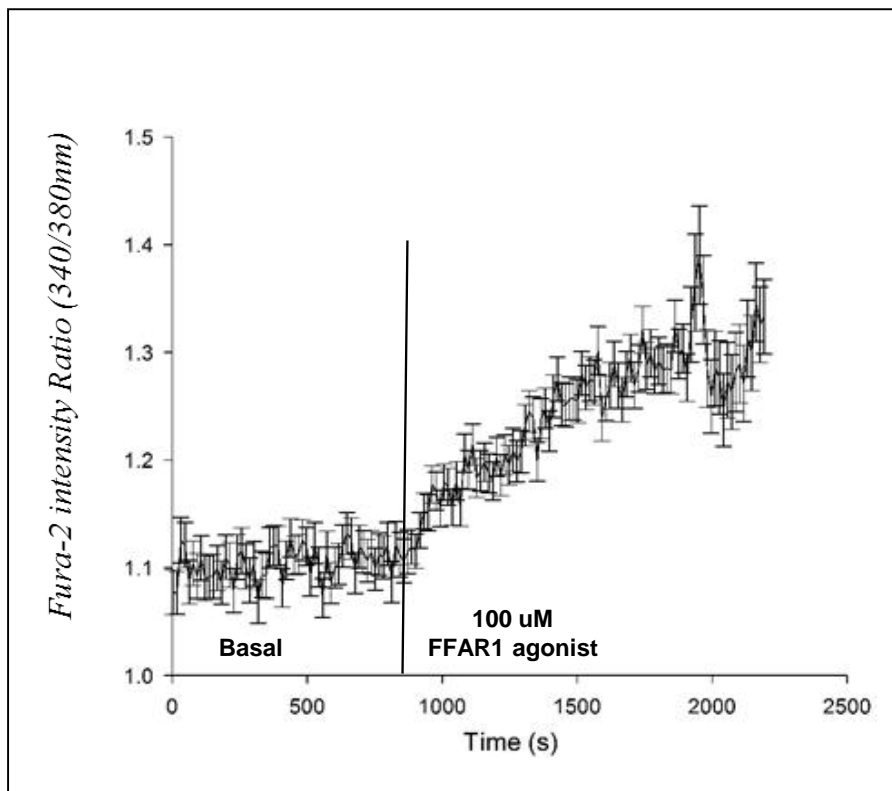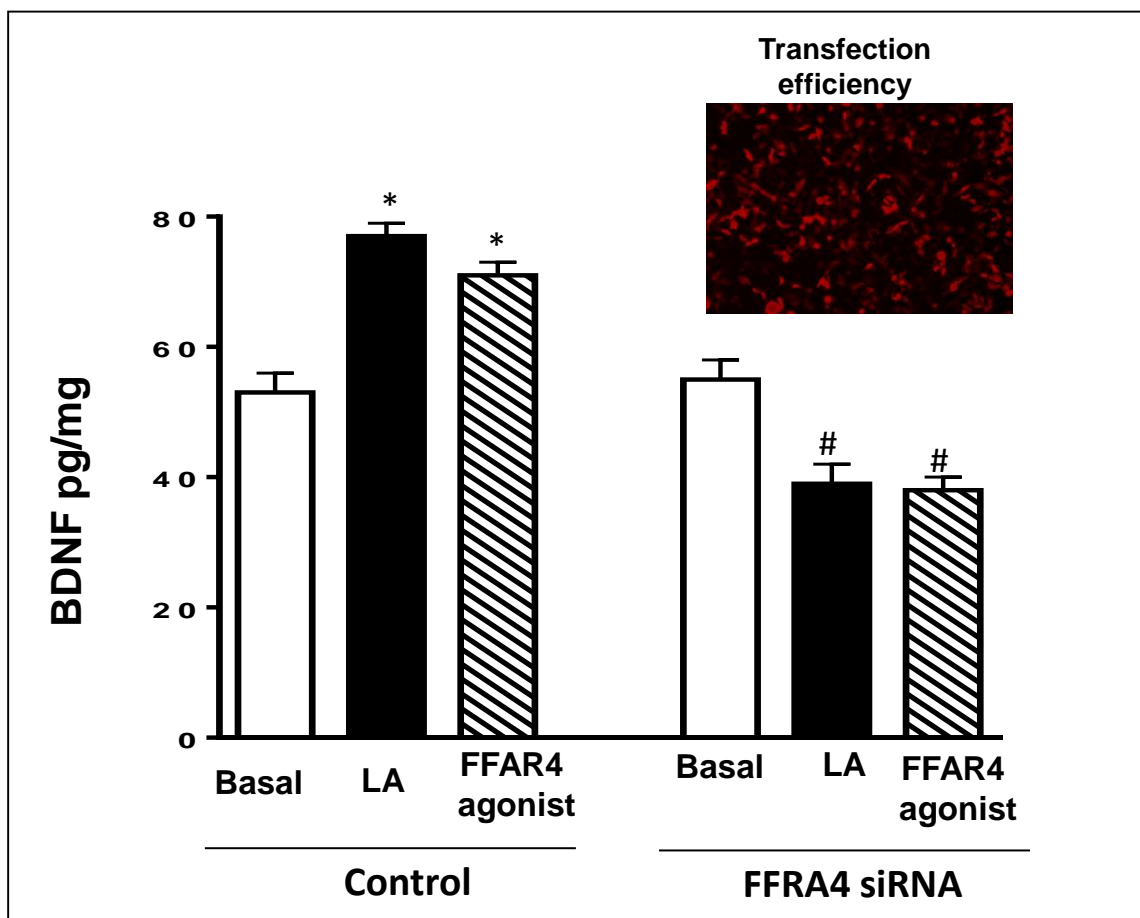
Figure 27. Colocalization of FFAR1 and BDNF in cultured enteric glial cells.

Cultured glial cells were stained with primary antibody to BDNF for FFAR1 and secondary antibody Flexa 488 (green) for BDNF and Flexa 594 (red) for FFAR1. The cells were imaged at x400 magnification. Arrows point to the cells colocalization BDNF and FFAR1. Right panel: cells stained with nuclear staining dapi.
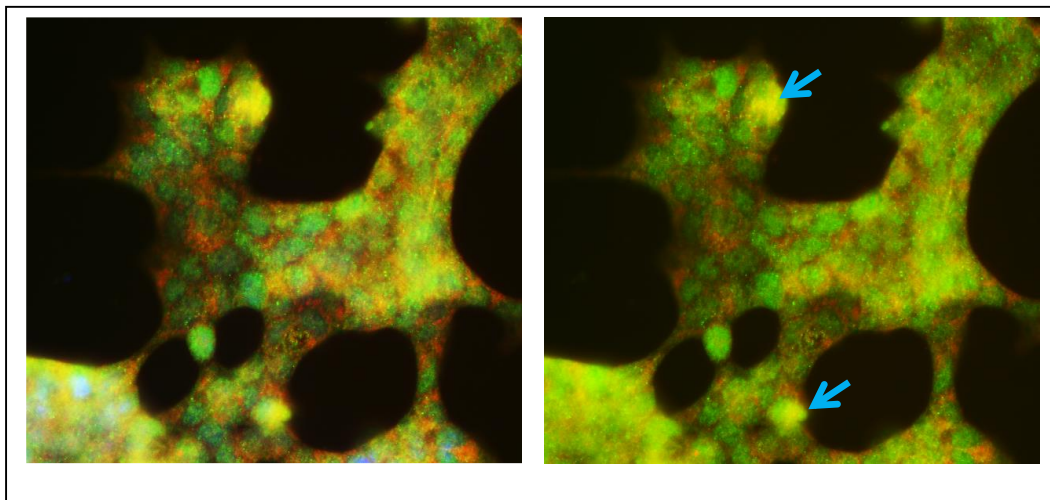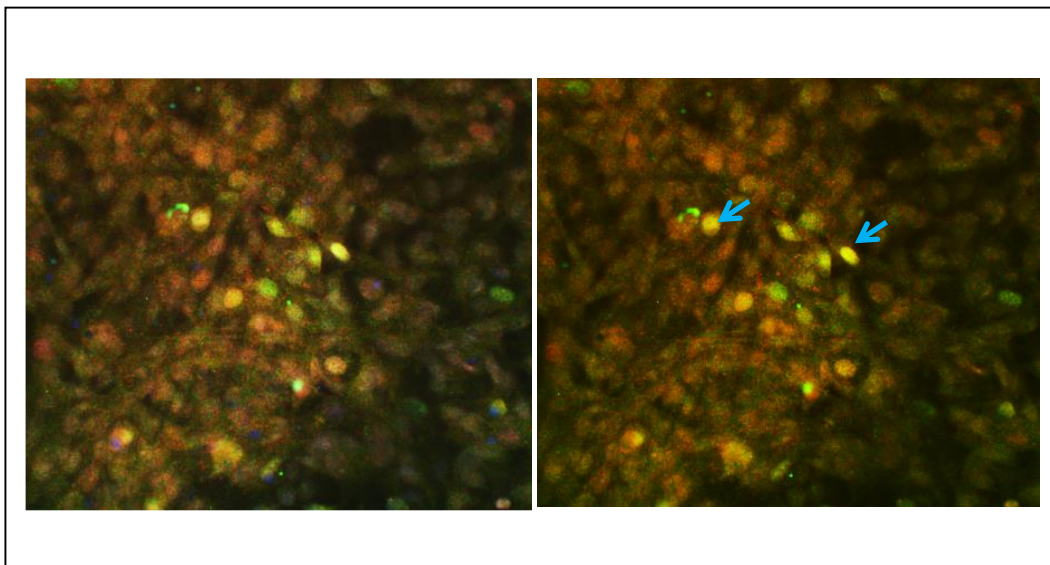
Figure 28. Increase in BDNF positive cells by linolenic acid in glial cells.

Cultured glial cells were stained with BDNF antibody before and after treatment with linolenic acid (100 µM) for 15 min. The cells were imaged at x400 magnification. Results demonstrated an increase in BDNF (green fluorescence) after linolenic acid (LA) treatment. Right panel: Quantitative analysis of BDNF positive cells before and after treatment with linolenic acid *P < 0.0001 versus control.
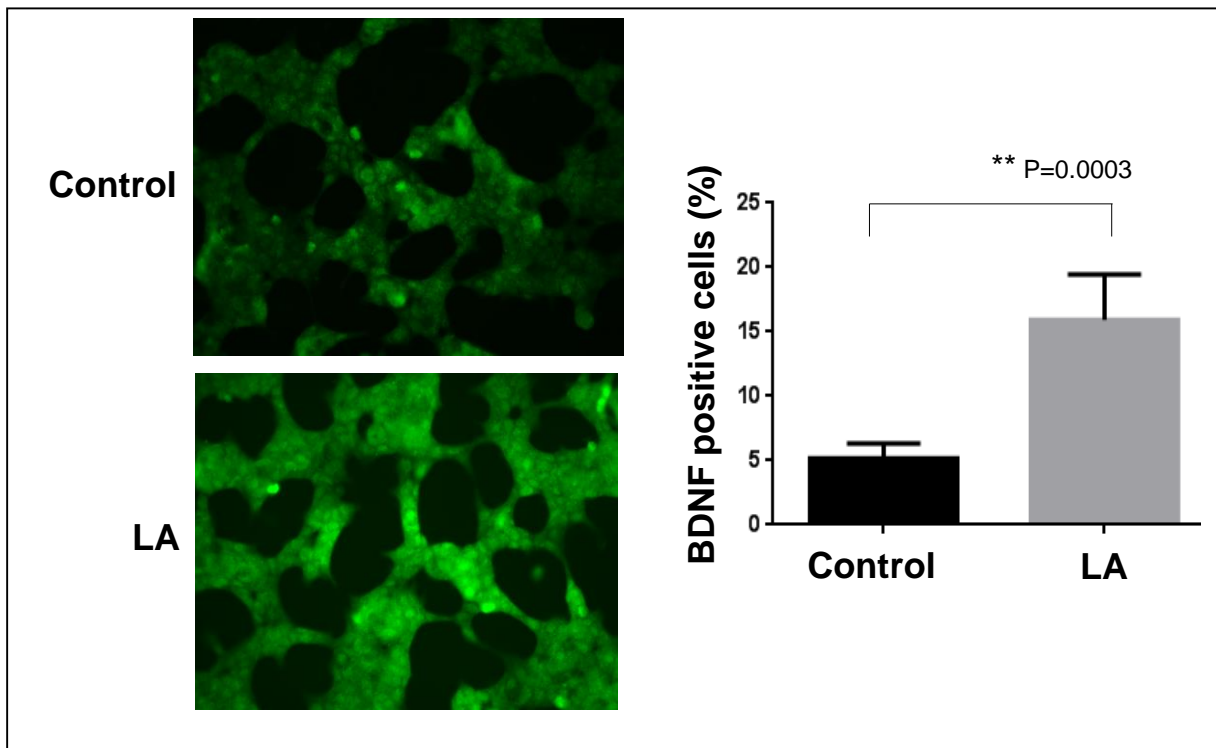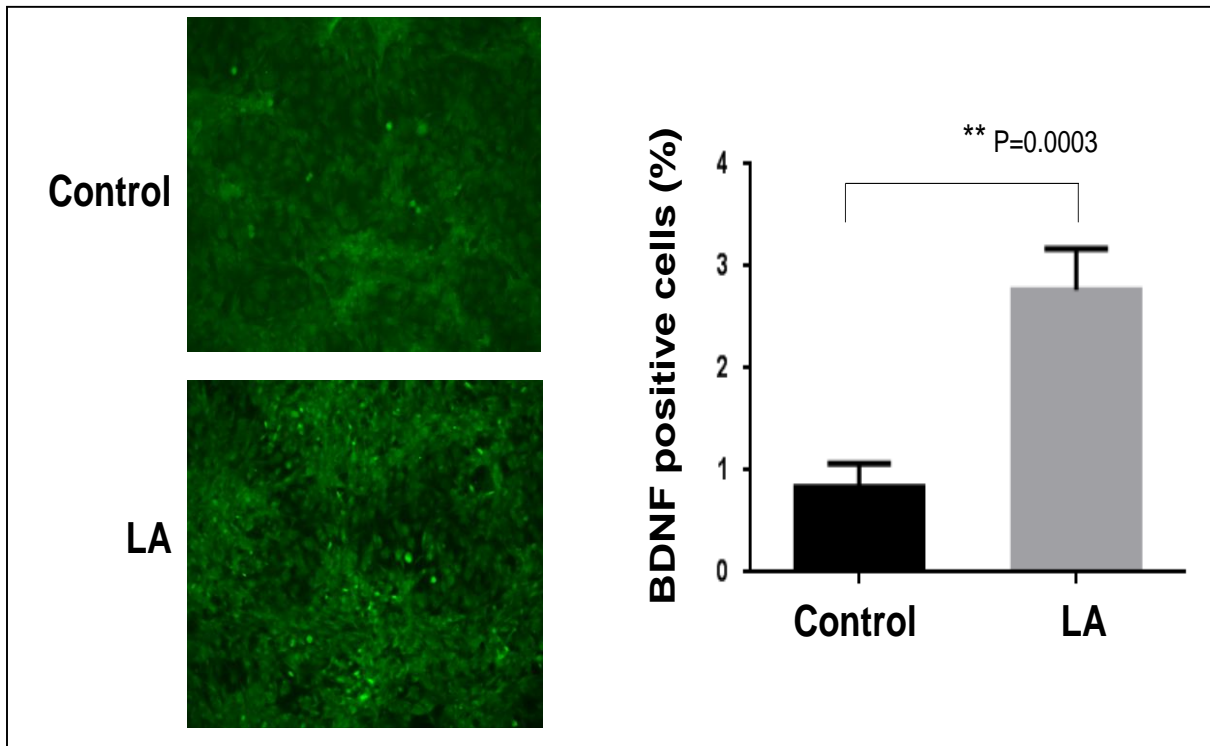
Figure 29. Increase in BDNF content by linolenic acid via FFAR1 in glial cells.

Cultured glial cells treated with 100 μM linolenic acid (LA) in the presence or absence of FFAR1 antagonist (GW1100, 10 μM) or FFAR1 agonist (GW9508, 10 μM) for 15 at 37°C. The amount of BDNF in the cell lysates was measured by ELISA. Linolenic acid-induced increase in BDNF content was inhibited by FFAR1. Values are expressed as mean ± SEM of 3 experiments. *P< 0.05 significant increase of BDNF versus basal.
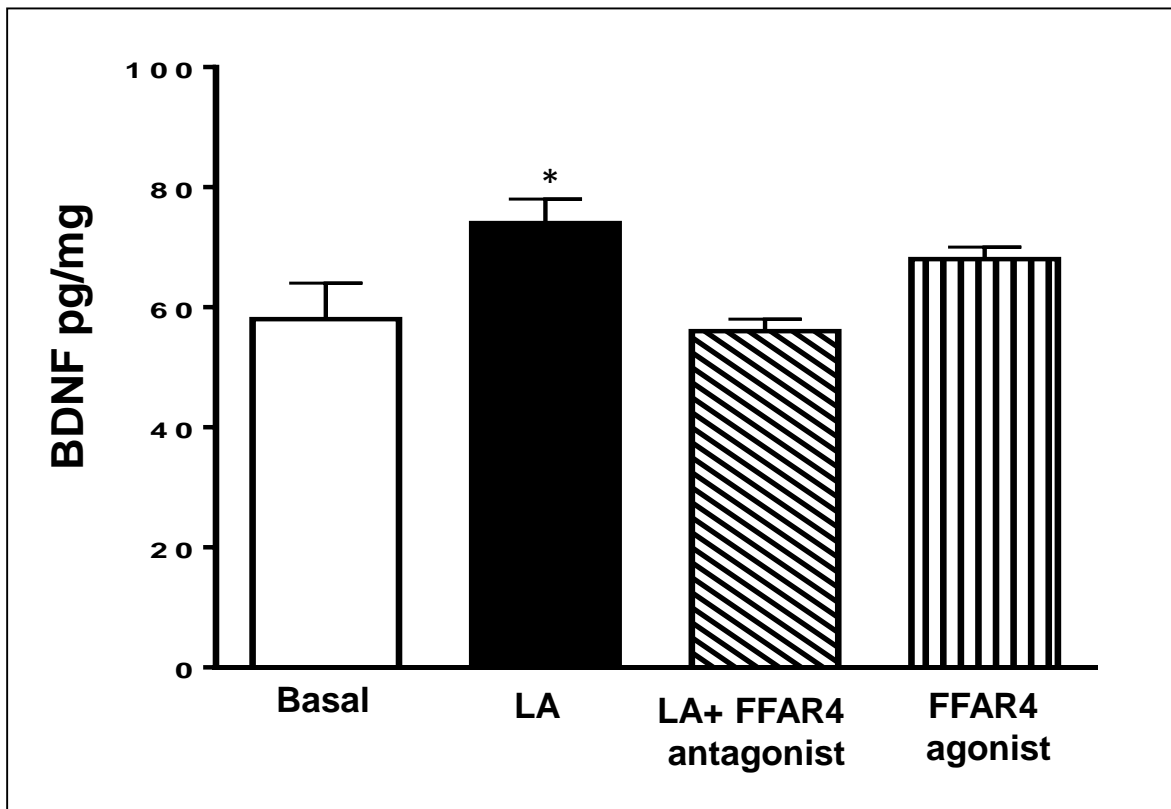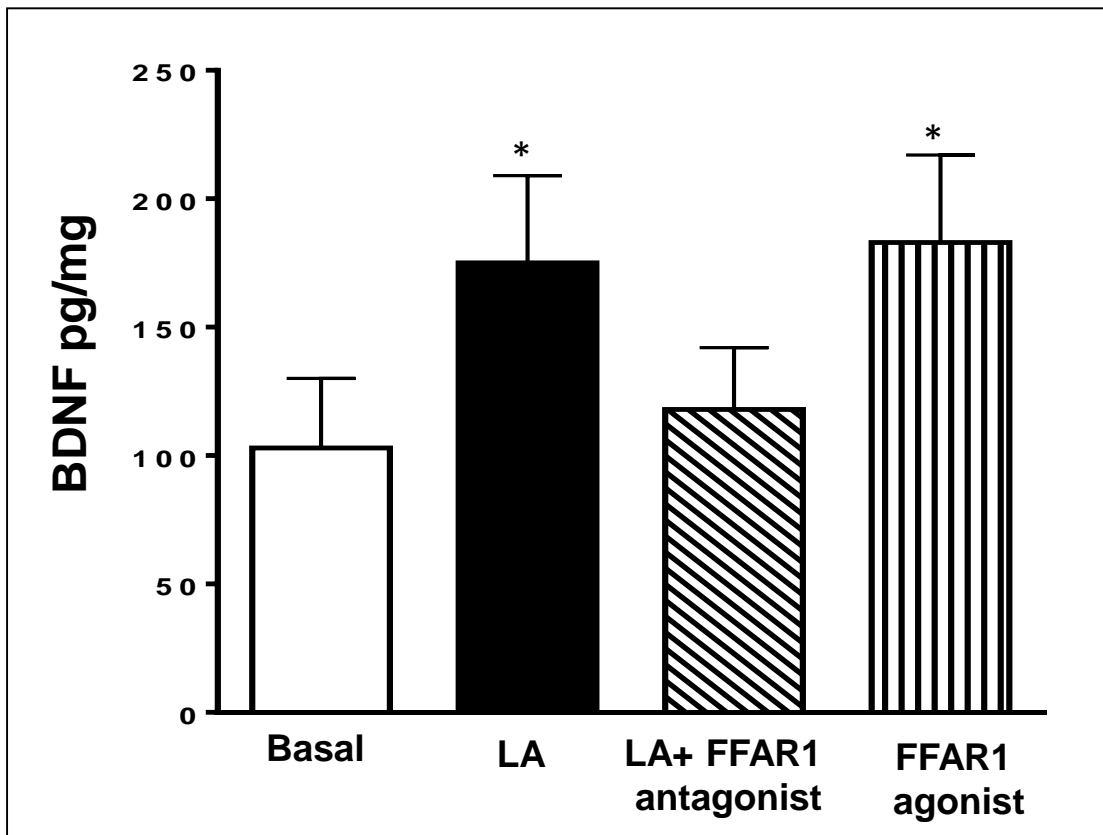
Figure 30. Increase in BDNF content by linolenic acid via FFAR1 in glial cells. Cultured glial cells were transfected with control siRNA or FFAR1 siRNA for 48 h and treated with 100 μM linolenic acid (LA) for 15 at 37°C. The amount of BDNF in the cell lysates was measured by ELISA. Linolenic acid caused an increase in BDNF content in cells transfected with control siRNA but not in cells transfected with FFAR1 siRNA. . Values are expressed as mean ± SEM of 3 experiments. *P< 0.05 significant increase of BDNF versus basal. #P< 0.05 significant inhibition of BDNF vs basal. Inset showing transfection of pSIREN-DNR-DsRed verified by immunofluorescence
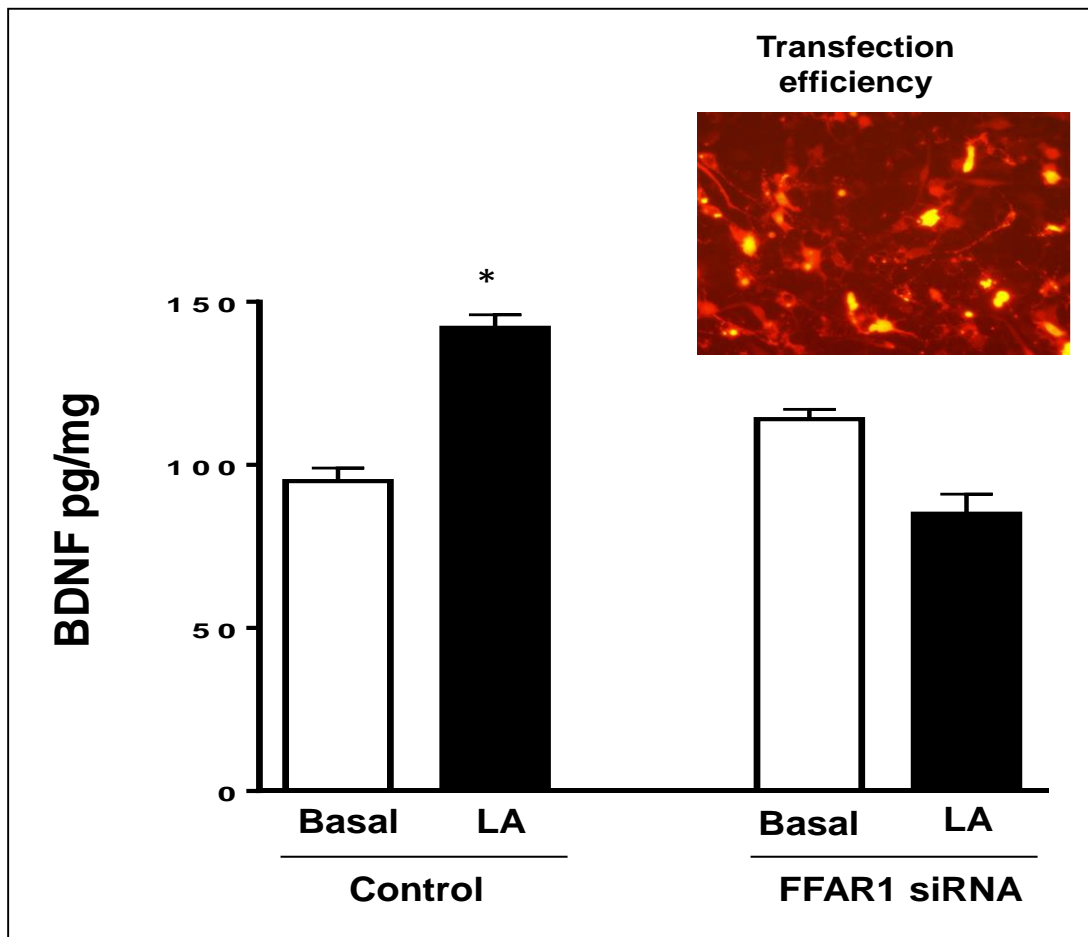

CHAPTER 4

DISCUSSION

Free fatty acids are essential dietary components that contribute to various cellular functions, provide energy to the cell, supply building material for cellular structure including membrane and organelles, and regulate cellular metabolism in various physiological and pathophysiological conditions. As mentioned in the first chapter, short chain fatty acids (SCFAs) are produced during fermentation of resistant starches and indigestible fiber by anaerobic gut flora, while long chain fatty acids are generated from dietary intake, lipolysis in adipose tissue, and hepatic forms of neutral fats, cholesteryl esters and phospholipids.

A series of experiments demonstrated that individual fatty acids have different effects in the nutrient sensing, gastrointestinal motility, hormone/paracrine release and function. For example, administration of the SCFA propionate (C3) caused decreased food intake in sheep (Masahito O and Michael A, 2003). Butyrate (C4), at physiological concentration of 10 to 30 mM, increased the proximal colon contraction, while acetate (C2) and propionate (C3) at the same concentration decreased proximal colon contraction, thus decreased the motility (Hurst et al 2014). Long chain fatty acids have been reported to play an important role in the regulation of insulin secretion in the pancreatic β-cells, in bone inflammation, and omega 3 restrain bone loss (Fabien CJ et al. 2013). However, chronic consumption of high fat diet has been attributed to the development of several diseases such as Type II Diabetes and obesity. Until the last decade, the roles of fatty acids were thought to be mediated exclusively through actions on cellular metabolism.

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In recent years, characterization and deorphanization of some seven transmembrane G-protein coupled receptors (GPCRs) by the identification of FFA ligands has provided a new development of the mechanism of action of free fatty acids. They serve as not only nutrients and metabolic substrates but also as extracellular signaling molecules.

Upon digestion of food, the gastrointestinal tract represents the first site of interaction between ingested nutrients and the host. The dietary fat and fatty acids are sensed via binding to specific free fatty acids receptors that are expressed on the surface of a variety of epithelial cells in the gut that mediate the release of gut peptides. It has been reported that ingested fat induces secretion of gut hormones; CCK from specialized enteroendocrine cells, I-cells, glucagon-like peptides and peptide YY from L cells, and glucose-dependent insulinotropic peptide from L cells (Liddle RA 1997, Strader AD et al. 2005 and Sternini C et al. 2008). Cholecystokinin (CCK) the upper small intestinal hormone is one of the primary endocrine regulators of small intestinal digestion. It was first described as a satiety hormone that plays a role in stimulation of pancreatic secretion, inhibition of gastric motility and food intake (Dockray GJ .2003).

In 2008 Toshiki Tanaka group has been using α-linolenic acid as a long chain free fatty acid to examine the role of free fatty acid receptors in secretion of CCK *in vitro*. They have demonstrated that long chain FFAs enhance the CCK secretion in STC-1 cells that mainly occurs through FFAR4.

Moreover, it has been reported that the secretion of GLP-1 from STC-1 is mediated via the activation of FFAR4 (Hirasawa et al. 2005). GLP-1 is a gut hormone is released from L cells and has a pivotal role in mediating food stimulated glucose

dependent secretion of insulin from pancreatic β cells. Also, GLP-1 has a role in stimulating β cells proliferation and differentiation. GLP-1 is considered the most potent insulinotropic incretin. Therefore, identification of FFAR4-mediating GLP-1 release induced by FFAs is very important in the treatment of diabetes. In addition, a number of studies have provided evidence for the importance of FFAR1 and FFAR4 in potentiating glucose stimulated insulin secretion via the secretion of GLP-1 and GIP hormones from enteroendocrine cells (Itoh et al 2003, Efdalk et al 2008).

Furthermore, FFAR2 and FFAR3 have been shown to stimulate the release of PYY (Kennedy RL et al, 2010). PYY is a neuroendocrine mediator is secreted from enteroendocrine cells (L cells) and plays a role in slow gastric emptying and stimulates digestion and nutrient absorption in the small intestine.

Most studies have focused on the role of free fatty acid receptors in energy intake, and regulating gastric and intestinal hormone/paracrine release and function. Little is known of the presence of free fatty acids receptors in the endocrine cell and enteric glial cells in the gut, and their role in regulation of brain derived neurotrophic factor. We hypothesize that free fatty acid receptors play a role in the regulation of brain-derived neurotrophic factor levels in the enteric glial and endocrine cells. Therefore, we examined *1)* FFAR1, FFAR2, FFAR3, FFAR4 and GPR84 expressions in enteroendocrine cells, glial cells and in mouse mucosal cells, *2)* the signaling pathway involved in long chain free fatty acid receptors FFAR1 and FFAR4 in enteroendocrine cells (STC-1) and enteric glial cells, *3)* the role of FFAR1 and FFAR4 in the regulation of brain derived neurotrophic factor contents in STC-1 and enteric glial cells.

Expression of FFARs in Enteroendocrine Cell and Enteric Glial Cells

In this study, we firstly reported that mRNA levels for FFAR1, FFAR2, FFAR3, FFAR4 and GPR84 are expressed differentially in enteroendocrine cell line STC-1 and enteric glial cells. Among several tissues and cell types in different species, various FFA receptor gene have been demonstrated. Our data showed an abundant expression of FFAR1 mRNA in enteric glial cells with two fold more expression than FFAR4 and GPR84. The study from Briscoe et al 2003 and Kotarsky et al 2003 revealed expression of FFAR1 in the skeletal muscle, liver and heart. TaqMan quantitative PCR showed that FFAR1 mRNA is predominantly expressed in insulin producing β cells (Itoh et al. 2003). Other studies have detected FFAR1 expression in the pancreatic β cell line MIN6, mouse βTC-3, and rat insulinoma INS-1E cells (Sharipo H et al. 2005). It was also detected in non β-cells, such as, human breast cancer cell line MCF-7 and enteroendocrine cells (Yonezawa T et al. 2004).

Furthermore, we have demonstrated the expression of FFAR2 mRNA in the enteroendocrine cell line STC-1. Other studies also have detected the level of FFAR2 mRNA in variety of tissues and cell types; such as neutrophils (Brown et al. 2003), skeletal muscle and heart (Nilsson et al, 2003), adipose tissue (Hong et al. 2005). FFAR2 has also been detected in the breast cancer cell line, MCF-7 (Yonezawa et al. 2006) and in rat distal ileum and colon (Karaki et al. 2006). We also reported the expression of other SCFA receptors such as FFAR3 mRNA in the enteroendocrine cells and enteric glial cells. Initial studies done on FFAR3 reported highest level of mRNA expression in adipose tissue (Brown et al. 2003). The expression was also seen in 3T3-L1 and 3T2-F442A adipose cell line (Xiong et al. 2004).

Our results and of others have shown FFAR4 mRNA expression was found in mouse STC-1 cells. Our studies demonstrated abundant expression of FFAR4 in STC-1 cells compared with other FFA receptors. This is in contrast to the abundant expression of FFAR1 compared to FFAR4 in enteric glial cells (Figure 5). Although the ligand profiles were similar for FFAR1 and FFAR4, both receptors were activated by long chain fatty acids, however, the amino acid homology between both receptors was only 10% (Hara T et al. 2009). Consistent with our data, FFAR4 was found to be expressed in the mouse intestinal tract with abundant expression in the murine enteroendocrine STC-1 cells (Hirasawa et al. 2005, Gotoh C et al. 2007, and Miyauchi et al. 2009).

Furthermore, in our studies the medium chain free fatty acid receptor, GPR84 mRNA was expressed in both enteroendocrine cells and enteric glial cells. Other studies have shown the expression of GPR84 in various tissues such as heart, lung, liver, and leucocytes. In particular, inflammatory cells, granulocytes, T and B cells also express GPR84 (Yousefi B et al. 2001; Venkataraman and Kuo 2005).

Consistent with differential expression of mRNA levels of FFAR1 and FFAR4 in our STC-1 and glial cells, results from protein expression showed the same distribution and differential expression. FFAR1 protein level was higher in enteric glial cells while FFAR4 protein level was more in STC-1 cells (Figure 8). Taken together, our expression results suggest that there is a different role and mechanism of each FFA receptors in different cells. Thus, we examined the signal transduction of long chain FFAR1 in enteric glial cells and FFAR4 in STC-1 cells.

Signaling Mechanisms Downstream of FFAR1 and FFAR4

This is the first study to demonstrate the effects of long chain fatty acids (linolenic acid) on enteric glial cells. Our studies demonstrate that FFAR1 are coupled to pertussis toxin (PTx)-insensitive Gq protein (Figure 16). FFAR1 activation caused an increase in PLC-β activity (PI hydrolysis) in enteric glial cells (Figure 18). Moreover, calcium release in response to linolenic acid and FFAR1 agonist was decreased with administration of FFAR1 antagonist (Figure 20-22). Several studies on the mechanism of activation FFAR1 via long chain fatty acid have been reported that FFAR1 is coupled to the G protein subunit Gαq, and leads to release of intracellular calcium (Fuijwara et al. 2005; Mancini AD and Poitout V 2013). Our studies provided a more detailed analysis of the signaling pathways and demonstrated for the first time that long chain fatty acids initiate signaling pathway via FFAR1 in enteric glial cells.

On the other hand, our studies demonstrate that activation of FFAR4 in enteroendocrine cells via long chain fatty acid linolenic acid leads to activation of the Gq/PLC-β/Ca^{2+} pathway. These conclusion were reached through several lines of evidence: (1) Abundance expression of FFAR4 in STC-1 cells (Figure 5 and 8), (2) selective activation of Gq and stimulation of PI hydrolysis (Figure 13). (3) Calcium release in response to linolenic acid and FFAR4 agonist (Figure 14 and 15). These data are consistent with previous studies that showed FFAR4 expression in HEK293 cells is coupled to Gq and promotes elevation of intracellular calcium. It is noteworthy, that in the preset study, we used linolenic acid, a natural agonist of long chain FFA receptors that can activate both FFAR1 and FFAR4.

Taken together, our studies imply that both FFAR1 and FFAR4 are coupled to $G\alpha_q$ that stimulates of PLC-β/IP$_3$/ Ca^{+2} pathway. In enteroendocrine cells (STC-1) long chain fatty acids preferentially activates FFAR4, whereas in glial cells long chain fatty acids preferentially activates FFAR1. This is consistent with the predominant expression of FFAR4 compared to FFAR1 in STC-1 cells, and predominant expression of FFAR1 relative to FFAR4 in glial cells.

Regulation of Brain-derived Neurotrophic Factor (BDNF) Content by FFAR1 and FFAR4 in STC-1 and Enteric Glial Cells

The discovery of FFAR1 and FFAR4 in the epithelial layer of the gastrointestinal tract has expanded the field of intraluminal nutrient sensing. Upon ingestion of food, chemosensory cells scattered throughout the gastrointestinal tract make direct contact with the gut lumen and their activation triggers the release of gastrointestinal regulatory peptides, neurotransmitters and neurotrophins. One of those is brain derived neurotrophic factor (BDNF).

As outlined in the introduction, BDNF is a neurotrophin present in the intestine where it participates in the survival and growth of enteric neurons, and stimulation of intestinal peristaltic reflexes and propulsion (Alqudah M et al. 2015). The physiological role of BDNF in the peristaltic reflex has been examined in genetically modified mouse. Mouse deficient in BDNF (BDNF$^{+/-}$) which have reduced peristalsis, release of 5-HT and slower velocity of propulsion of fecal pellets compared with wild type BDNF$^{+/+}$ mice (Goggi J et al. 2002 and Grider et al. 2006). Furthermore, BDNF release in the gut has been

demonstrated from mucosal enteroendocrine cells and from intrinsic primary afferent neurons innervating the mucosa of rat and mouse colon in response to mucosal mechanical stimulation (Grider et al. 2006). Within this context, the role of BDNF is to augment release of 5-HT and CGRP, thereby augmenting peristaltic reflex leading to enhanced propulsive motility (Grider et al. 2006).

Beside the motility effect of BDNF, the localization of BDNF in enteric neurons suggests that BDNF may acts as neurotransmitter or neuromodulator in the gut, similar to the notion of BDNF in the central nervous system. BDNF could be secreted from enteric neurons and can activate the release of CGRP in an autocrine manner, or it could be secreted from mucosal cells and act in a paracrine manner. Three different groups have identified BDNF in the glial cells and in endothelial cells (Lucini C et al, 2002, and Lommatzch M et al, 1999).

In the present study, we have demonstrated the colocalization of BDNF with the FFAR4 in the STC-1 cells (Figure 23) and colocalization of BDNF with the FFAR1 in the glial cells (Figure 27).

It is not known whether BDNF content level is regulated by the long chain free fatty acids via FFAR1 and FFAR4. The present study has demonstrated the functional role of activated FFAR1 and FFAR4 in BDNF regulation in both STC-1 and glial cells. The series of experiments demonstrated that the natural agonist, long chain fatty acid, linolenic acid, administration caused an increase in BDNF content level in STC-1 via FFAR4. The increase in BDNF in response to linolenic acid was demonstrated by both immunocytochemistry and measurements of BDNF by ELISA. The specific role of FFAR4 in the regulation of BDNF levels in STC-1 cells was demonstrated using both

pharmacological and molecular approaches. A selective FFAR4 antagonist, but not the FFAR1 antagonist, and silencing FFAR4 expression blocked the effect of LA on BDNF (Figures 25 and 26). Linolenic acid also caused an increase in BDNF content in glial cells. Increase in BDNF in response to linolenic acid was demonstrated by both immunocytochemistry and measurements of BDNF by ELISA. The specific role of FFAR1 in the regulation of BDNF levels in glial cells was demonstrated using both pharmacological and molecular approaches. A selective FFAR1 antagonist, but not the FFAR4 antagonist, and silencing FFAR1 expression blocked the effect of LA on BDNF (Figures 29 and 30). These results are consistent with the predominant expression of FFAR4 and selective activation of FFAR4 by linolenic acid in STC-1 cells, and with the predominant expression of FFAR1 and selective activation of FFAR1 by linolenic acid in glial cells.

In conclusion, the presented findings indicate the cell-specific expression of FFARs in STC-1 cells and glial cells, and their colocalization with BDNF, and the role of FFARS in the regulation of BDNF levels. Given the importance of enteroendocrine cells and glial cells as well as BDNF in the regulation of motility, secretion and energy metabolism, our studies underscore the physiological importance of free fatty acids and their receptors regulation of gastrointestinal functions. Our results potential suggest new therapeutics as FFARs to targets involved in gastrointestinal disorders.

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Vita

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