IDENTIFICATION OF THE GnRH-(1-5) RECEPTOR AND SIGNALING PATHWAY

By

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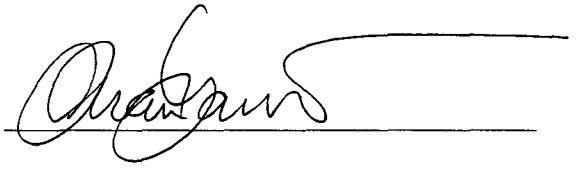
[Signature] 

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DEDICATION

This dissertation is dedicated to my parents, Darwin and Carmen Larco, whose love and support gave me the confidence to pursue my dreams.

ABSTRACT

Title of Dissertation: Identification of the GnRH-(1-5) receptor and signaling pathway

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The decapeptide gonadotropin-releasing hormone (GnRH) is a central regulator of pubertal onset and reproductive function. In mammals, GnRH is released into the median eminence of the hypothalamus to stimulate the pituitary release of the gonadotropins. In turn, these hormones regulate the gonadal production of steroid hormones, which act as feedback modulators of the GnRH neuroendocrine system. Developmental defects in this regulation can lead to hypogonadotropic hypogonadism where the onset of puberty and fertility are impaired. Such is the case in patients suffering from Kallmann Syndrome (KS) where GnRH neurons emanating from the olfactory placode during development do not reach their destination within the central nervous system. Despite ongoing research in this field, a complete molecular understanding in the development of KS remains elusive.

The first five amino acids of the full-length GnRH are highly conserved across nonmammalian and mammalian species. In the extracellular space GnRH is metabolized by the endopeptidase EC3.4.24.15 (EP24.15) to generate the pentapeptide GnRH-(1-5). Our previous studies have shown that GnRH-(1-5) has functional roles including the ability to regulate GnRH mRNA expression in immortalized GnRH neurons and to facilitate lordosis behavior in female rats. Interestingly, EP24.15 colocalizes with

migrating GnRH neurons during development. Thus the availability of GnRH-(1-5) mediated by EP24.15 suggests that GnRH-(1-5) may play a role in the development of the GnRH neuroendocrine system.

This dissertation focuses on elucidating the receptor and signaling mechanism mediating the effects of GnRH-(1-5) on GnRH neuronal migration using the immortalized GnRH-secreting cell line, the GN11 cell. We demonstrate that GnRH-(1-5) inhibits GN11 cellular migration in both the wound healing assay and Boyden chamber assay. Interestingly, we show this inhibition is mediated by the activation of the orphan G protein-coupled receptor 173 (GPR173), which subsequently leads to an inhibition of the signal transducer and activator of transcription 3 (STAT3) pathway. Additionally, we sought to identify the G protein-mediated mechanism regulating the action of GnRH-(1-5) on cell migration. Our results show that GnRH-(1-5) does not activate the cyclic adenosine monophosphate or inositol triphosphate second messenger systems, but rapidly recruits β-arrestin 2 upon GnRH-(1-5) binding to GPR173. Collectively, these findings demonstrate that the downstream signaling of GnRH-(1-5) binding to GPR173 deviates from the canonical GPCR signaling cascade. This is the first time to our knowledge that an endogenous ligand is implicated in the activation of the orphan receptor GPR173. An overall better understanding of the GnRH-(1-5) and GPR173 interaction may have clinical implications in determining the molecular pathogenesis of KS, which is attributed to a defect in GnRH neuronal migration.

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LIST OF ABBREVIATIONS

AC	Adenylyl Cyclase
AT1A	Angiotensin 1 Receptor
ACTH	Adrenocorticotropic hormone
αMSH	α-Melanocyte-Stimulating Hormone
βAR	β Adrenergic Receptor
βARK	β Adrenergic Receptor Kinase
BCA	Bicinchoninic Acid Assay
cAMP	Cyclic Adenosine Monophosphate
cDNA	Complementary Deoxyribonucleic Acid
CNS	Central Nervous System
co-IP	Coimmunoprecipitation
DAG	Diacylglycerol
DNA	Deoxyribonucleic Acid
DR	Aspartic Acid/ Aspargine Motif
ED	Embryonic Day
ELISA	Enzyme-Linked Immunosorbent Assay
EP24.15	Zinc Metalloendopeptidase EC 3.4.24.15
FGF8	Fibroblast Growth Factor 8
FGFR	Fibroblast Growth Factor Receptor
FSH	Follicle-Stimulating Hormone
GAP	GnRH-Associated Peptide
GDP	Guanosine Diphosphate

GnRH	Gonadotropin-Releasing Hormone
GnRH-(1-5)	Gonadotropin-Releasing Hormone-(1-5)
GnRHR	GnRH Receptor
GPAnt2	G Protein Anagonist 2
GPR27	G Protein Coupled Receptor 27
GPR85	G Protein Coupled Receptor 85
GPR101	G Protein Coupled Receptor 101
GPR119	G Protein Coupled Receptor 119
GPR148	G Protein Coupled Receptor 148
GPR173	G Protein Coupled Receptor 173
GPCR	G Protein Coupled Receptor
GTP	Guanosine Triphosphate
HH	Hypogonadotropic Hypogonadism
HPG	Hypothalamic-Pituitary-Gonadal
IB	Immunoblot
IF	Immunofluorescence
IP_1	Inositol phosphate
IP_3	Inositol 1,4,5-Trisphosphate
KAL1	Anosmin-1 Gene
KS	Kallmann Syndrome
LIF	Leukemia Inhibitory Factor
LIFR	Leukemia Inhibitory Factor Receptor
LH	Luteinizing Hormone

LHR	Luteinizing Hormone Receptor
LTV	Left Telencephalic Vesicle
ME	Median Eminence
mRNA	messenger Ribonucleic Acid
MV	Mesencephalic Vesicle
nHH	Normosmic Hypogonadotropic Hypogonadism
OB	Olfactory Bulb
OE	Olfactory Epithelium
pERK1/2	Phosphorylated Extracellular Signal-Regulated Kinases 1/2
PLC	Phospholipase C
POMC	Proopiomelanocortin
PKA	Protein Kinase A
PKC	Protein Kinase C
PROKR2	Prokineticin Receptor 2
PTEN	Phosphatase and Tensin Homolog
PTM	Post-Translation Modification
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
siRNA	Small Interfering Ribonucleic acid
STAT3	Signal Transducer and Activator of Transcription 3
SREB	Super Conserved Receptor Expressed in Brain
T	Tectum
TM	Transmembrane
VNO	Vomeronasal Organ

CHAPTER 1: Introduction

The gonadotropin-releasing hormone (GnRH) is a decapeptide critical for reproductive behavior and function. In mammals, GnRH is released into the median eminence (ME) of the hypothalamus to stimulate the pituitary release of gonadotropins. In turn, these hormones regulate the gonadal production of steroid hormones, which act centrally to modulate reproductive behavior. Defects in the development of the GnRH neuroendocrine system can lead to hypogonadism, delayed pubertal onset, and infertility. This is evident in patients suffering from Kallmann Syndrome (KS) where GnRH neurons born in olfactory placode do not properly migrate to the central nervous system (CNS). Our understanding in the pathogenesis of KS remains in its infancy. In approximately 75% of patients with KS a molecular explanation for the development of this defect is unknown (3).

Even though GnRH is highly conserved across nonmammalian and mammalian species, the first five amino acids of the decapeptide GnRH are even more conserved. This high degree of conservation suggests that this peptide sequence is functionally critical across species. In the extracellular space GnRH is metabolized by the endopeptidase EC3.4.24.15 (EP24.15) to generate the pentapeptide GnRH-(1-5). Our previous studies have shown GnRH-(1-5) regulates GnRH mRNA expression in immortalized GnRH neurons (73) and facilitates lordosis behavior in female rats (72). Interestingly, EP24.15 is expressed along the migratory path of GnRH-secreting neurons during development (65). Thus the availability of GnRH-(1-5) mediated by EP24.15 suggests that GnRH-(1-5) may play a role in the migration of GnRH neurons. Additionally, GnRH-(1-5) likely signals via a G protein coupled receptor (GPCR) since

many neuropeptides including the parent peptide GnRH bind and activate a GPCR. *Therefore, the over-arching hypothesis of this dissertation is that GnRH-(1-5) regulates the migration of GnRH neurons by activating a GPCR*. Two aims are proposed to identify the GnRH-(1-5) receptor and the signaling mechanism to regulate GnRH neuronal migration.

AIM 1: To determine whether GnRH-(1-5) activates a GPCR to regulate GnRH neuronal migration. The goal of this aim was to identify the GPCR that mediates the GnRH-(1-5) regulation of migration in GnRH-secreting neurons. We narrowed our search for candidate GnRH-(1-5) receptors by using a high-throughput enzyme fragment complementation assay (DiscoveRx, Fremont, CA). The results from the assay returned the following candidate orphan GPCRs: GPR101, GPR119, and GPR173. Subsequent experiments involved measuring the expression of the candidate GPCRs in the *in vitro* model used in these studies, the GN11 cell line. GN11 cells are immortalized GnRH-secreting cells extensively used to study cellular migration. Additionally, the effect of GnRH-(1-5) on migration was tested using established migration assays such as the wound closure assay and Boyden chamber. These experiments were followed by siRNA-mediated down-regulation of the candidate receptors to determine whether the effect of GnRH-(1-5) was reversed.

AIM 2: To determine the GnRH-(1-5) signaling mechanism on GnRH neuronal migration. The goal of this aim is to identify the regulators of migration induced by GnRH-(1-5). The second messengers cyclic adenosine monophosphate and inositol triphosphate were measured to identify the G protein complex recruited due to GnRH-(1-

5) treatment. Additionally, co-immunoprecipitation assays and western blots were used to verify the GnRH-(1-5) effectors coupling to the receptor.

In summary, completion of both aims will identify the receptor and signaling pathway mediating the action of GnRH-(1-5) to regulate neuronal migration in an *in vitro* model. These studies will set the foundation for future experiments investigating the role played by GnRH-(1-5) during the development of the GnRH neuroendocrine system. Furthermore, the implication of an endogenous ligand for an orphan GPCR is of paramount significance since there are greater than 100 orphan GPCRs considered potential targets for the development of therapeutic agents to combat disease.

CHAPTER 2: Literature Review

The Hypothalamic-pituitary-gonadal (HPG) Axis

The regulation of reproductive function is dependent on the proper coordination between the 3 axes of reproduction. These comprise the hypothalamus, anterior pituitary and the gonads. GnRH is a key regulator of reproductive function and behavior. Neurons that synthesize GnRH are widely distributed in the basal forebrain and project to the ME where GnRH is released in a pulsatile manner into the hypophyseal portal vessels. Secreted GnRH stimulates the synthesis and secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), in the anterior pituitary. These gonadotropins, subsequently exert their effects at the gonadal level to regulate the secretion of steroid hormones. In the female, LH and FSH act on the ovary to facilitate follicular maturation and to regulate the secretion of steroid, estrogen and progesterone, which act as feedback modulators of the hypothalamic-pituitary axis. Estrogen has been shown to both stimulate and inhibit GnRH release depending on the stage of the estrus cycle (13; 42; 46). Furthermore, GnRH can regulate its own synthesis and secretion via an autocrine mechanism, which can be influenced by other neuropeptides (28; 29). One such peptide that has emerged as a major regulator of GnRH secretion is kisspeptin, which has been shown to be critical in the onset of puberty and gonadal function (26; 27). Figure 1 illustrates how all these factors may contribute to GnRH secretion in the hypothalamus. However, the mechanism mediating the integration of all signals required for normal function of the GnRH neuroendrocrine axis remains to be completely addressed.

Development of the GnRH Neuroendocrine System

Initial studies by Schwanzel-Fukuda demonstrated that GnRH neurons are born with olfactory neurons outside the CNS in the nasal region (54). Subsequent studies validated these observations showing GnRH neurons migrate from the nasal placode traversing the cribiform plate to target the basal forebrain (69; 70). In the developing mouse, GnRH immunoreactivity is detected as early as embryonic day (ED) 11 in the vomeronasal region (54). By ED 16, most GnRH neurons have already migrating along vomernasal tract to the prepoptic area (54; 69). Interestingly, a subpopulation of GnRH neurons migrate to the tectum near the mesenchepalic vesicle peaking at ED 15; however, they cease to exist in the adult and their function has not been elucidated (71). Once adulthood is reached the primary GnRH population is estimated to be near 800 cells distributed throughout the basal forebrain (61; 69; 70). Figure 2A shows the GnRH neuronal migratory route during different developmental stages in the mouse. At approximately ED 15.50 most GnRH neurons have crossed the cribiform plate into the basal forebrain. Interestingly, the remaining GnRH positive cells near the nasal region migrate radially, which is in contrast to their tangential migratory pattern once these neurons are in the CNS (Figure 2B). The proper migration of GnRH neurons during development requires the integration of multiple guidance cues in part mediated through the activation of GPCRs, including the prokineticin receptor-2 (PROKR2) and fibroblast growth factor receptor 1 (FGFR1) (49).

Hypogonadotropic Hypogonadism

Defects in the HPG axis lead to the development of various types of hypogonadotropic hypogonadism (HH) where the onset of puberty and fertility are impaired. There are a number of genes implicated in the pathogenesis of HH, including mutations in the genes encoding the GnRH peptide or its receptor. Mutations associated with these genes are generally classified as norsosmic HH (nHH) where GnRH signaling is impaired yet olfactory senses remain intact (60). Other forms of HH exist where patients suffer from reduced olfaction in addition to deficiencies in pubertal development and fertility. This form of HH also called Kallmann Syndrome (KS) is attributed to a defect in GnRH neuronal migration. Specifically, precursor olfaction and GnRH neurons emanating from the olfactory placode remain arrested in the cribiform plate during development, never entering the CNS to reach their target sites (4). Patients diagnosed with nHH or KS have reduced levels of circulating gonadotrophins, LH and FSH, which would normally stimulate the secretion of steroid hormones from the testes or ovaries. In turn, these patients suffer from delayed pubertal onset and reduced gonadal function among other pathologies (35; 44; 51; 53; 60).

Recent studies focusing on the molecular basis for KS implicate genes believed to aid in neuronal migration during development such as KAL1, FGFR1 and FGF8 (56). *In vitro* studies indicate the product of KAL1 acts as a chemoattractant for immortalized premigratory GnRH neurons (9) by associating with cell surface heparin sulphate proteoglycans (62). Interestingly, many signaling cues required for normal GnRH neuronal migration activate GPCRs such as PROKR2, which has been linked to the development of KS (21). On the other hand, patients with nHH harboring mutations in genes encoding GnRH or the GnRHR do not have obvious anatomical aberrations of the

hypothalamus that would suggest a defect in neuronal migration; yet continue to suffer from delayed pubertal development. Recently, a frameshift mutation in the GnRH gene was identified in teenage siblings who had intact olfaction but suffered from underdeveloped gonadal function. Interestingly, GnRH treatment in one of the siblings increased circulating LH levels (6; 7), indicating that the GnRHR was responsive to exogenous GnRH administration. However, a significant number of other patients with nHH have been shown to contain various types of mutations in the gene encoding the GnRHR leading to complete or partial loss of receptor activity (2; 15; 60). Furthermore, depending on the severity of hypogonadism, these patients may or may not be responsive to GnRH treatment (40; 55).

GnRH Metabolism

A number of neuropeptides are processed prior to being biologically active. For example, the precursor polypeptide proopiomelanocortin (POMC) can undergo several post-translation modifications that give rise to different mature peptides such as ACTH, α-MSH, and β-endorphin (48). Although these peptides are synthesized from the same parent peptide they do not necessarily mediate the same functions. Furthermore, their processing is dependent on the tissue where they are expressed (5). Similarly, the prepro-GnRH undergoes several modifications before giving rise to the mature form of the GnRH peptide. The prepro-GnRH is 92 amino acids with an N-terminal signal sequence that is removed to generate the pro-GnRH peptide. The decapeptide GnRH is located closer to the N-terminus while the C-terminal region contains the GnRH-associated peptide (GAP). Both fragments are separated by a cleavage site that allows for further

processing to generate the mature forms of the GnRH and GAP peptides (68)(Figure 3). The function of GAP is not clear but it has been shown to regulate prolactin, LH, and FSH secretion (47). Furthermore, it is possible GAP is metabolized into smaller peptide fragments that have biological activity; however this remains to be determined.

The previous notion that neuropeptidases degrade peptides to cease further biological activity has been modified due to the growing evidence that suggests otherwise (14). Metabolized peptide products have been shown to regulate a variety of other functions independent of the parent peptide. For example, the C-terminal fragments degraded from the full-length arginine vasopressin have no effect on water retention but modulate learning behavior and memory formation (19; 20). Furthermore, there is evidence suggesting that metabolized fragments can regulate a more specific function from that of the parent peptide. This is the case for bradykinin-(1-5), which only inhibits thrombin-induced platelet aggregation, but does not induce vasodilation like its parent peptide bradykinin (43). Collectively, these findings highlight another level of complexity where peptidases can be viewed as activating enzymes that participate in the regulation of a wide variety of processes in the CNS.

In the extracellular matrix, GnRH is cleaved by the endopeptidase EP24.15 to generate the pentapeptide GnRH-(1-5) (Figure 3). We have previously demonstrated that GnRH-(1-5) is biologically active by facilitating lordosis behavior in ovariectomized estrogen-primed rats (72); and can increase the mRNA expression of GnRH in immortalized GnRH neurons (73). The full-length GnRH peptide is processed in a two-step mechanism. First, the glycine residue at the 10th position is removed by a proplyl endopeptidase to generate GnRH-(1-9). Subsequently, EP24.15 cleaves GnRH-(1-9) at

the 5th and 6th position to form the metabolite GnRH-(1-5) (14). Apart from GnRH processing, EP24.15 is also involved in the metabolism of other peptides including bradykinin, neurotensin, and angiotensin I (10; 14). Interestingly, EP24.15 cleaves angiotensin I to angiotensin-(1-7), which has the opposite effect on blood pressure relative to angiontensin-(1-8) (10). This finding suggests that EP24.15 can process various peptides that can oppose the actions of the parent peptide. Similarly, we have shown that GnRH-(1-5) stimulates GnRH mRNA expression in GnRH neurons while treatment with the full-length GnRH inhibits its expression (73). Therefore in mammals EP24.15 may have a role in regulating reproductive behavior by processing GnRH to GnRH-(1-5). In a previous study, we have shown immunoneutralization of EP24.15 in animals treated centrally with GnRH reduced lordosis behavior compared to those treated with GnRH alone. Likewise, female rats treated with GnRH-(1-5) facilitated the lordosis response (72), suggesting that this behavior can indirectly be regulated by EP24.15 via the availability of GnRH-(1-5). This is further reinforced by evidence indicating that exposure to the steroid hormone 17β-estradiol regulates EP24.15 levels in brain regions implicated in reproductive function such as the ventromedial nucleus of the hypothalamus (17). However, a clear link between ovarian steroids and EP24.15-mediated generation of GnRH-(1-5) remains elusive in addition to the mechanism of GnRH-(1-5) to facilitate lordosis behavior.

GnRHR Signaling

GPCRs make up a large protein family characterized by receptors with seven transmembrane domains. Each receptor responds to an extracellular event by triggering a

signal transduction pathway within the cell. These receptors are grouped into 5 sub families based on sequence similarity and functionality consisting of the Rhodopsin, Secretin, Adhesion, Glutamate, and the Frizzled families. The Rhodopsin family is the largest of the five groups with 672 members mostly being odorant receptors. Currently, there are 63 orphan receptors in the Rhodopsin family since their corresponding ligands have yet to be discovered (41). All members of the Rhodopsin family share certain amino acid residues such as the well-conserved aspartic acid/arginine motif present between the transmembrane (TM) region 3 and the intracellular loop 3, which is implicated in receptor activation (1). Interestingly, the GnRHR can undergo alternative splicing to generate different transcripts; however, only one sequence codes for a functional receptor (75). Furthermore, although the GnRHR has many characteristics shared by all Rhodopsin members including the DR motif mentioned, the GnRHR is missing an intracellular C-terminal tail (12). The lack of a C-terminal tail allows the GnRHR to be resistant to internalization and desensitization, which would normally be mediated by recruiting β-arrestin to the C-terminal tail upon sustained activation of the receptor (45). Hanyaloglu and colleagues demonstrated that the GnRHR is capable of interacting with β-arrestin as long as the GnRHR was modified to express the C-terminal tail of the thyrotropin-releasing hormone receptor (25). These observations suggest that the GnRHR is likely regulated by another mechanism independent of the β-arrestin pathway underscoring its unique properties as a receptor relative to the other members of the Rhodopsin family.

GPCRs mediate their actions through G proteins, which are composed of the following three subunits: Gα, β, and γ. Of the 20 known Gα isoforms, the GnRHR

activates the $G\alpha_q$ subunit, which activates the inositol 1,4,5-trisphosphate (IP_3)/ diacylglycerol (DAG) second messenger system to increase intracellular Ca^{2+} levels. In the pituitary, gonadotropic cells expressing the GnRHR bind GnRH released from GnRH-synthesizing neurons in the hypothalamus. Once activated, the GnRHR changes conformation allowing the $G\alpha_q$ subunit to exchange normally bound GDP for GTP. The $G\alpha_q$-GTP complex, subsequently, dissociates from the receptor and the $G\beta\gamma$ subunits to activate the enzyme phospholipase C (PLC). Active PLC cleaves its substrate, phosphatidylinositol 4,5-bisphosphate, to generate the effector molecules, IP_3 and DAG. IP_3 diffuses to the endoplasmic reticulum where it binds an IP_3 receptor, which leads to an increase in intracellular Ca^{2+}. This increase in cytosolic Ca^{2+} levels facilitates the secretion of vesicles containing LH and FSH (45; 67). Additionally, DAG and Ca^{2+} activate protein kinase C (PKC), which also contributes to the regulation of gonadotropin secretion (45). However, it is important to note that the gene expression and the amount of each gonadotropin released are differentially dependent on the GnRH pulse frequency; for example, an increase in GnRH secretion will release more LH than FSH (22; 50). The intrinsic GTPase activity of the $G\alpha_q$ subunit allows the hydrolysis of GTP to GDP, thus reconstituting the inactive receptor/$G\alpha_q\beta\gamma$ complex.

Apart from the GnRH-mediated activation of the $G\alpha_q$ cascade in the pituitary, there is evidence suggesting that the GnRHR can interact with other $G\alpha$ isoforms, including $G\alpha_i$ and $G\alpha_s$ (63). These G proteins modulate the activity of adenylate cyclase (AC), which is the enzyme responsible for the synthesis of the second messenger, cyclic adenosine monophosphate (cAMP). Changing the availability of intracellular cAMP can alter the activity of certain kinases such as protein kinase A (PKA) and in turn regulate

other signaling pathways. Interestingly, GT1-7 cells treated with low levels of a GnRHR agonist stimulated cAMP production while more concentrated levels of this agonist inhibited this effect. This inhibition was blocked by the addition of pertussis toxin, which prevents activation of the $G\alpha_i$, suggesting that the GnRHR is associating with this G protein complex to inhibit cAMP production in cases of high agonist concentration (31). Additionally, the initial increase in cAMP can be attributed to the activation of the $G\alpha_s$ subunit, suggesting that GnRH levels regulate the selectivity of the G protein signaling cascade (28; 30; 31). Modulation of the cAMP second messenger system is likely more relevant to the autocrine mechanism by which GnRH regulates its own production and secretion in the hypothalamus (30). These studies underscore the complexity of GnRHR signaling. The mechanism of how the GnRHR couples to different G protein to regulate process independent of reproduction remains to be examined, which can further be extended to include other tissues that express the GnRHR and how the signaling is coupled to different G proteins, which may regulate processes independent of reproduction.

GnRH-(1-5) Binds GPR173 to Regulate GnRH Neuronal Migration

Our previous studies demonstrating the biological action of GnRH-(1-5) indicate that its actions involve the activation of a GPCR independent of the GnRHR. Recently, we implicated GnRH-(1-5) to have a role in the regulation of GnRH neuronal migration (34) [Chapter 3: Manuscript 1]. Using the immortalized GnRH-secreting cell line, the GN11 cell, we showed GnRH-(1-5) inhibited migration in a wound closure assay and in the Boyden chamber (34). Next, we sought to identify the signal transducing element

required for the action of GnRH-(1-5) on GN11 cellular migration. Since many neuropeptides including the parent peptide GnRH bind and activate a GPCR, we hypothesized that GnRH-(1-5) likely binds a previously uncharacterized GPCR. We narrowed our search for candidate GnRH-(1-5) receptors by using a high-throughput enzyme fragment complementation assay that measures receptor/β-arrestin proximity by generating a luminescent signal in the presence of an activating ligand (DiscoveRx, Fremont, CA). The results from this assay returned the following putative orphan GPCRs: GPR101, GPR119, and GPR173. Interestingly, we found that GPR173 was the only candidate receptor expressed in GN11 cells. Subsequently, by using an siRNA approach we blocked the effect of GnRH-(1-5) on migration, demonstrating that GPR173 was required for this action. All the experiments regarding these results are explained in further detail in chapter 3 of this dissertation.

GPR173 belongs to a small subfamily of GPCRs known as the Super Conserved Receptor Expressed in Brain (SREB) family. Only two other SREB proteins have been identified: GPR27 and GPR85 (39). The SREB proteins are expressed primarily in the brain and genital organs (39). Functional studies reveal that GPR27 regulates the production of insulin (32; 33) while GPR85 is thought to play a role in neurogenesis (11). Although no known ligands have been found to activate the SREB receptors with GPR173 as the exception, they are thought to be aminergic receptors due to their sequence similarity to other such GPCRs (39). GnRH-(1-5) is a pentapeptide with an imidazole functional group provided by the second N-terminal amino acid histidine, which may serve as the function group required for the activation of GPR173.

One of the most intriguing aspects of GPR173 is its conservation among vertebrate species. Orthologues of GPR173 have been identified in rats, mice, bovine and the zebrafish (Figure 4). At the mRNA level, the protein-coding regions of many species share a significant degree of homology relative to the human GPR173 (Table 1). Similarly, peptide sequence alignments of the human, mouse, rat, and zebrafish orthologues of GPR173 are highly homologues revealing multiple putative sites of post-translation modifications (PTM) (Figure 5). Like other highly conserved peptides, such as the GnRH, this degree of retention throughout evolutionary history suggests an imperative physiological purpose. GnRH is a neuropeptide known to be essential to reproduction in vertebrates – a function, without which, there would be no propagation. Due to its critical function, GnRH arose 540 million years ago and has been identified in every vertebrate and even some invertebrates (66). By the same logic, the conservation of GPR173 throughout the evolution of vertebrates suggests its function to be important, if not indispensible.

Using a bioinformatic approach, we examined the peptide sequence of GPR173 that are likely sites for PTM thereby regulating receptor activity and function. There are seven putative phosphorylation sites (Figure 5), four of which are in the large third cytoplasmic loop (Figure 6). This is of significance since phosphorylation typically negatively regulates GPCRs by desensitization and endocytosis, best exemplified by the β-adrenergic receptor (βAR). Upon epinephrine binding to the βAR, a cascade of events occur that lead to the activation of PKA, β-adrenergic-receptor kinase (βARK), and others. These kinases phosphorylate βAR leading to its internalization and desensitization

(57-59). Whether this is the case for GPR173 remains to be investigated since there is a growing body of evidence suggesting that GPCRs are regulated by multiple mechanisms.

Another potential site for PTM within the accessible areas of GPR173 is an aspargine residue in the extracellular N-terminal tail (Figure 6). This site is a candidate for N-glycosylation, which may facilitate receptor interaction with the extracellular milieu or assist in ligand recruitment. Previous studies have identified two glycosylation sites (Asn4 and Asn18) within the GnRHR to be critical for receptor expression and stability in the plasma membrane (18). Additionally, N-glycosylation sites on the Luteinizing Hormone Receptor (LHR) at Asn173 and Asn152 were demonstrated to be essential in the binding of LH to its receptor (74). Lastly, there is a lysine in the third cytoplasmic loop that could potentially be a target of ubiquitination (Figure 6). Ubiquitination has long been associated with the down regulation of GPCR activity via receptor endocytosis and degradation, but recent studies suggest that ubiquitination plays a role in the trafficking and positive regulation of certain GPCRs (38). In some instances, ubiquitination-mediated internalization of GPCRs facilitates the formation of complexes important in propagating a signal rather than ceasing activity (38). This is best exemplified by the co-internalization of the angiotensin (AT1A) receptor and activated pERK-1/2 into an endosome to allow for sustained activity of pERK-1/2 (38). Whether this type of regulation or any of the PTMs mentioned are related to GPR173 function and activity remains to be determined.

Table 1. Percent Homology of the GPR173 mRNA Sequence across Species.

Species	Mouse NM_027543.3	Rat NM_022255.1	Bovine NM_001015604.1	Zebrafish NM_131498.1
Human NM_018969.5	95%	95%	95%	77%

The percent homology was calculated using the coding region of the putative GPR173 mRNA sequence of each species relative to the human sequence. Sequence information and the calculation of homology were obtained from the NCBI website (http://www.ncbi.nlm.nih.gov/pubmed) and the NCBI Blast website (http://blast.ncbi.nlm.nih.gov/Blast.cgi), respectively. The NCBI accession ID is indicated below each species.

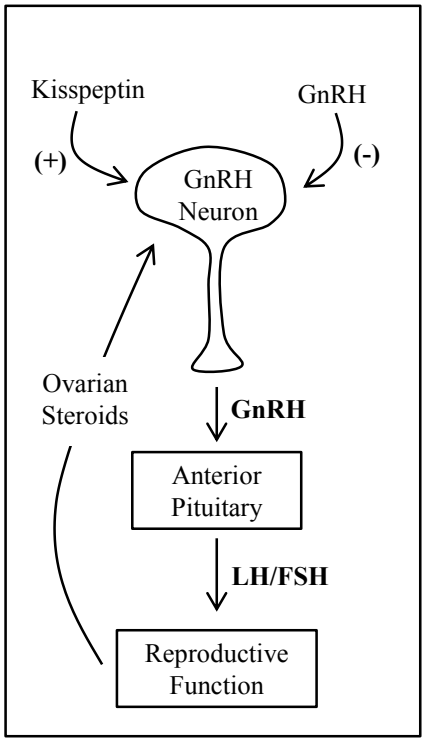

Figure 1. The Hypothalamic-Pituitary-Gonadal Axis.
Different factors regulate GnRH neuronal function. The decapeptide GnRH is released from the hypothalamus to stimulate LH and FSH release from the pituitary to regulate reproductive function.

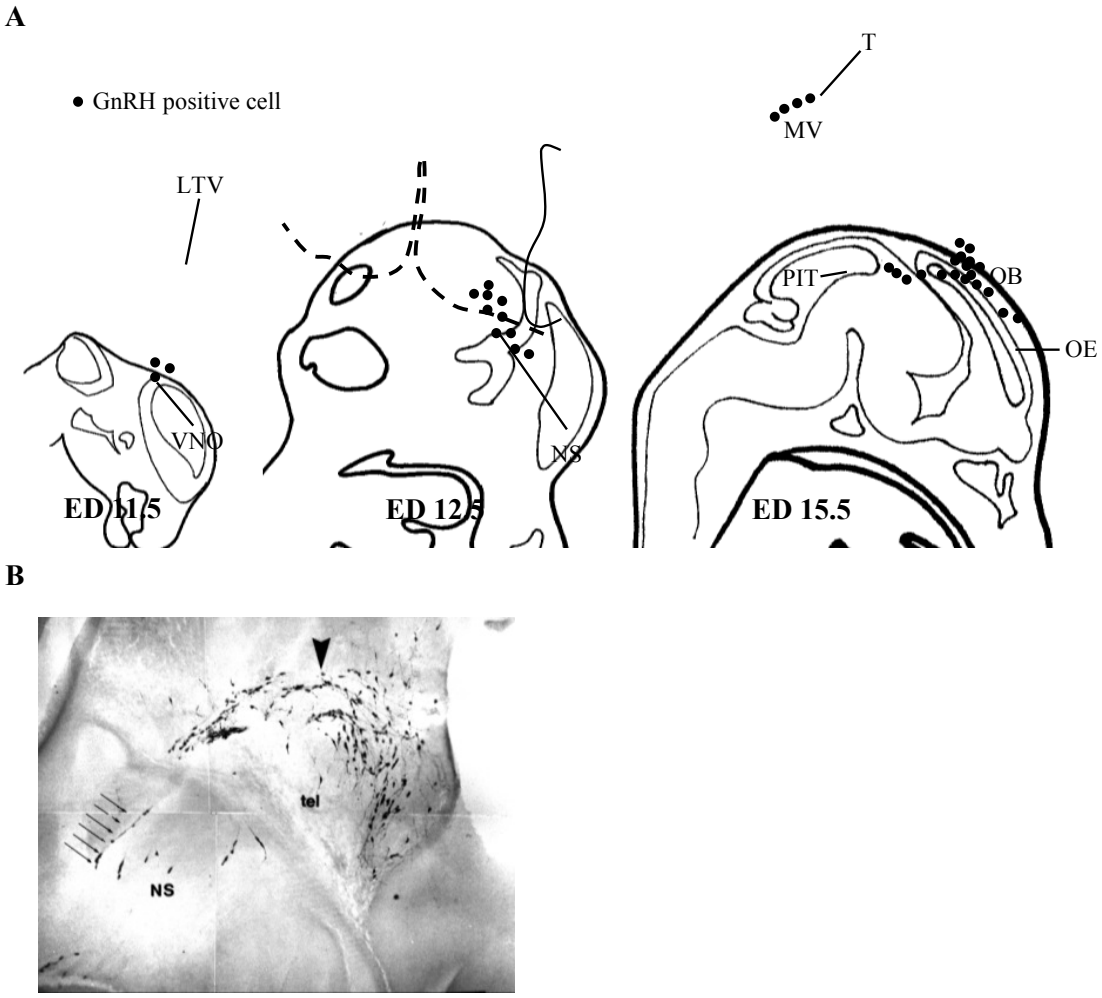

Figure 2. GnRH neuronal migratory route during development.
A) GnRH neurons are born in the nasal placode near the vomernasal organ (VNO) at approximately embryonic day (ED) 11.5 in the mouse. As development progresses GnRH neurons begin to enter the CNS (dashed lines) at ED12.5 and begin to descend within the basal forebrain region at ED15.5. B) Most GnRH neurons are within the CNS at ED15.5 however the remaining GnRH neurons in the nasal septum (NS) have a morphologically distinct migratory pattern relative to neurons found near the telencephalon (tel). Arrow indicates descending GnRH neurons of a mouse ED15.5 brain (unpublished data; Wu, TJ). LTV: left telencephalic vesicle; OB: olfactory bulb; OE: olfactory epithelium; PIT: pituitary; MV: mesencephalic vesicle; T: tectum

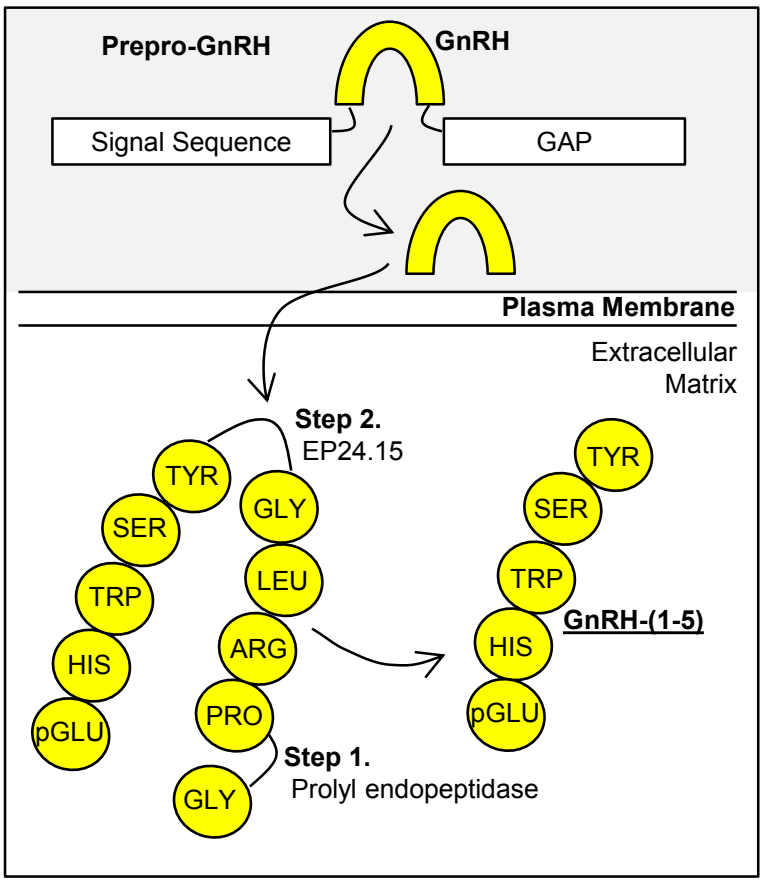

Figure 3. GnRH Peptide Processing.
The prepro-GnRH is processed intracellularly to generate the mature GnRH peptide. In the extracellular matrix is metabolized in a 2 step mechanism generating the metabolite GnRH-(1-5). Modified from Wetsel, 2002 (68) and Roberts and Wu, 2007 (52).

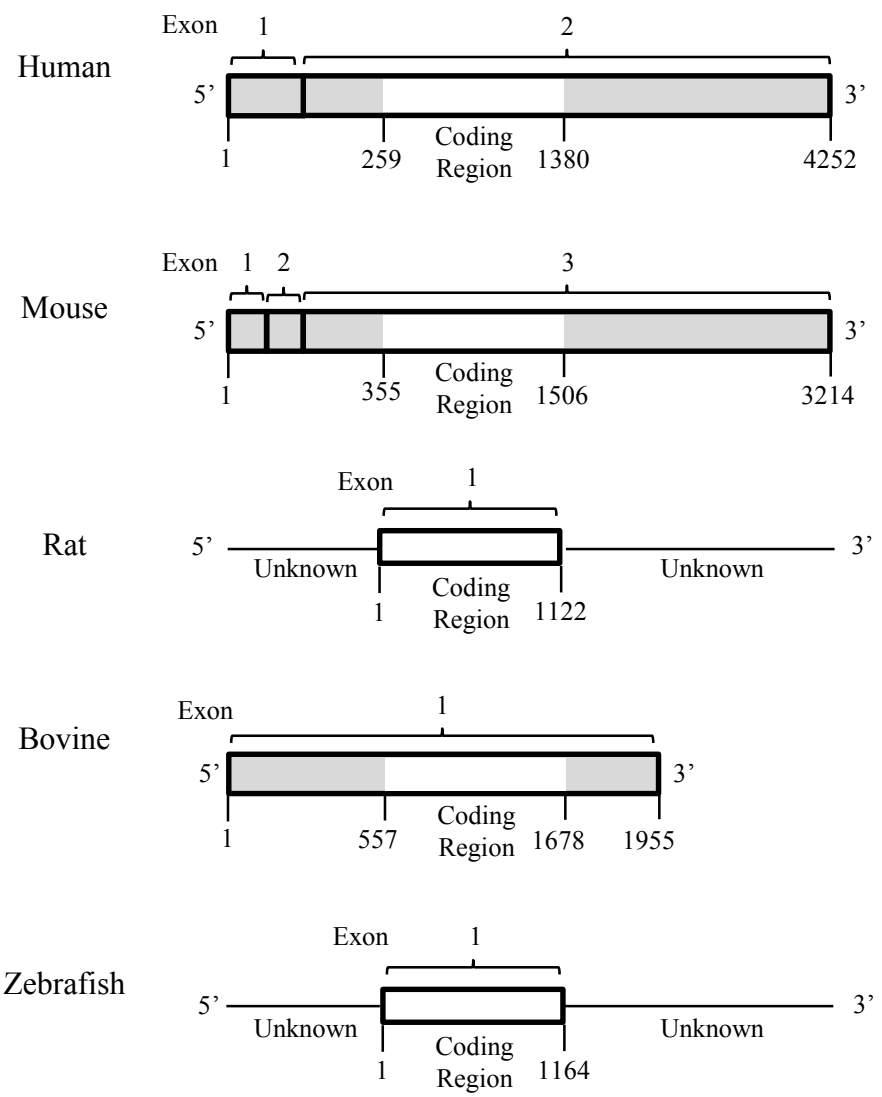

Figure 4. Putative mRNA Structure of GPR173 across Species.
Analysis of the putative GPR173 mRNA structure across species reveals a significant degree of conservation within the coding region. These structures were generated from NCBI website (Bethesda, MD). The mRNA accession number can be found in table 1.

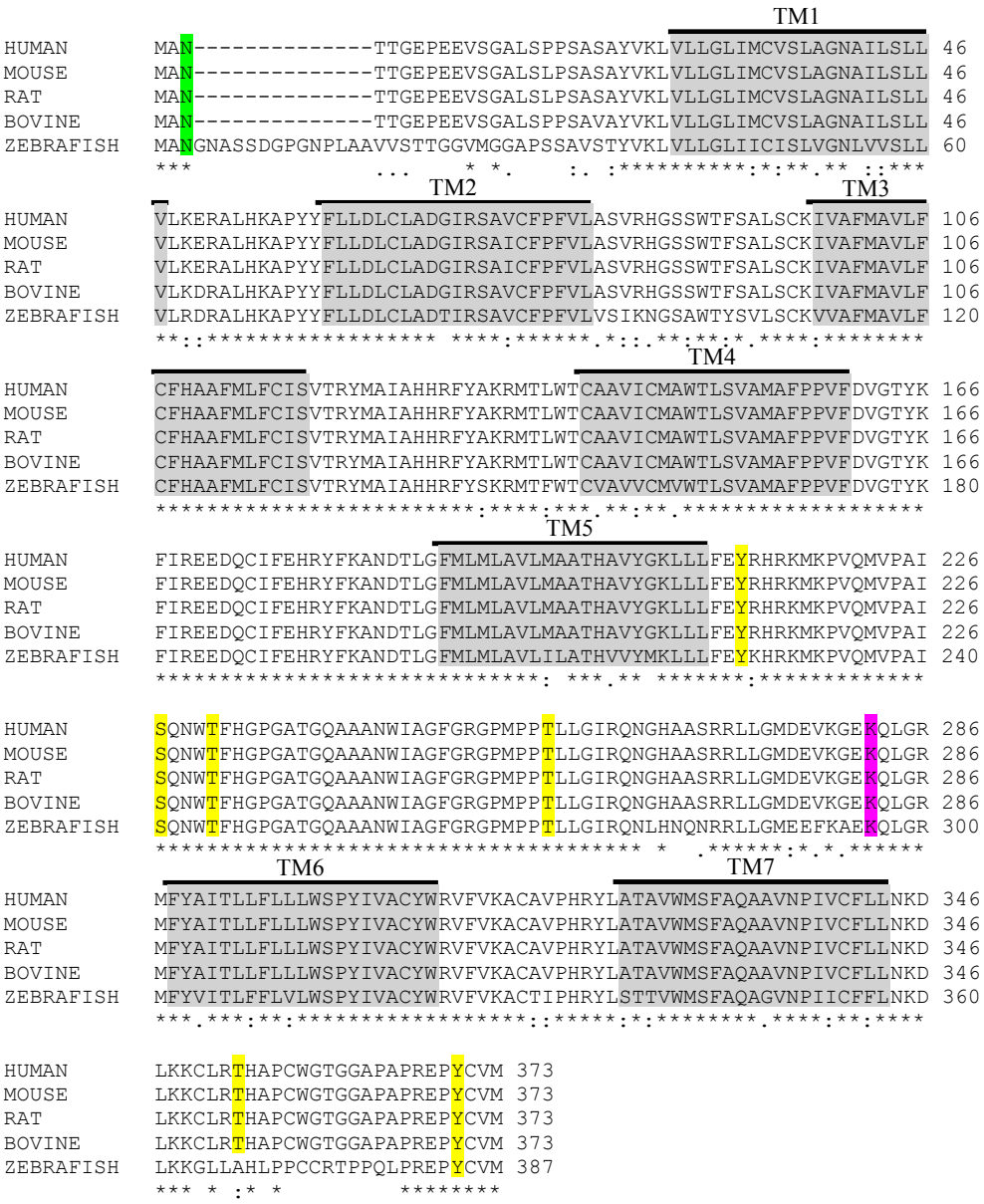

Figure 5. GPR173 Peptide Sequence Alignment.
A peptide sequence analysis of GPR173 reveals a high degree of conservation across species. Additionally, many sites within the intracellular loop 3 are putative phosphorylation sites (yellow) with a conserved lysine residue (purple), which may undergo ubiquitination. A highly conserved arginine residue (green) in the N-terminal tail indicates a putative site for N-glycosylation. '*' indicate conserved residues; '.' or ':' indicate similarity between residues. Shaded regions indicate putative transmembrane spanning domains (TM). Sequence alignment analysis was conducted by using the uniprot consortium (www.uniprot.org).

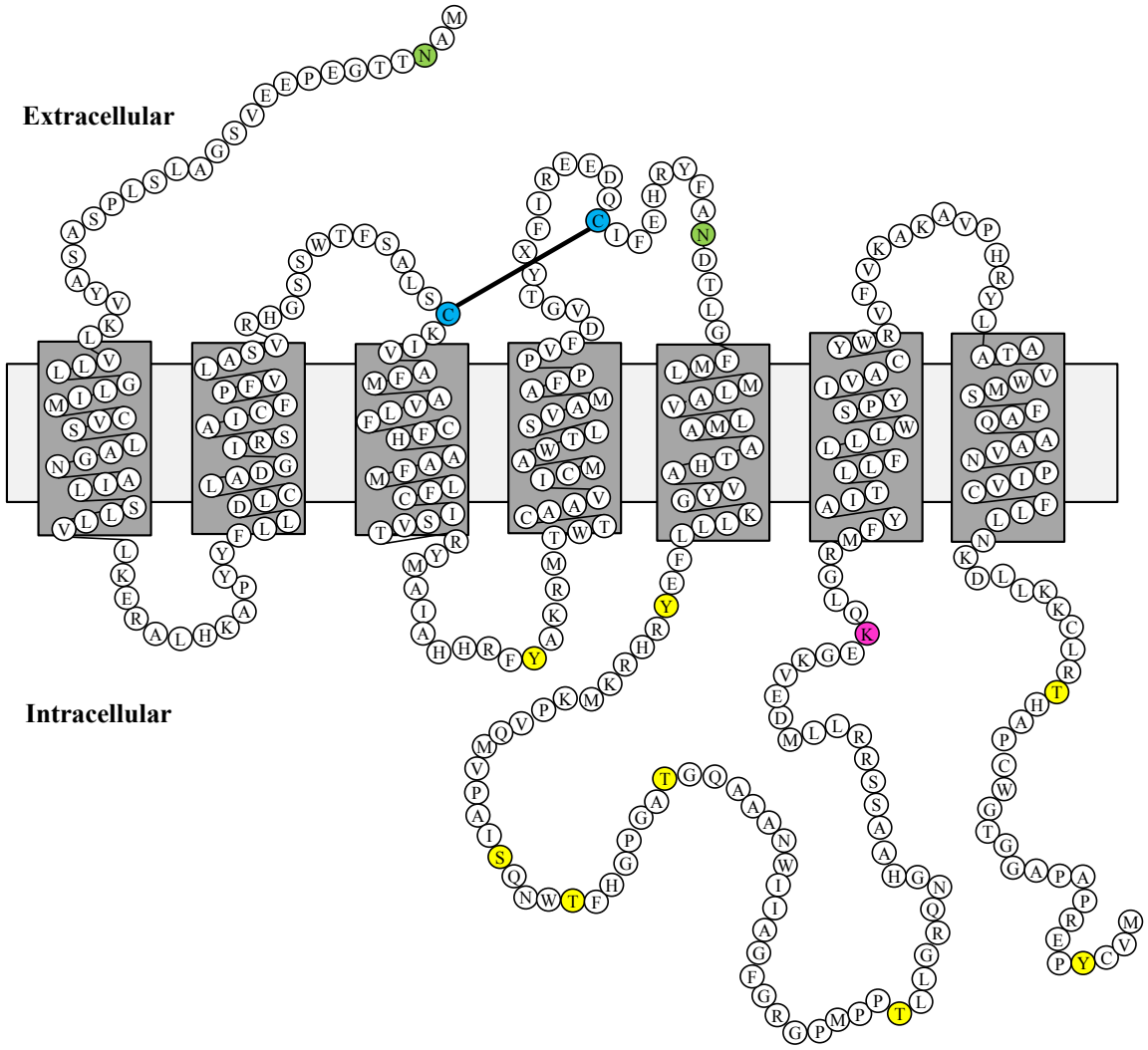

Figure 6. Putative Secondary Structure of the Mouse GPR173.
The secondary structure of the mouse GPR173 suggests there is a large intracellular loop with multiple sites that may undergo post-translational modification. Blue denotes putative cysteine residues, which may serve as a disulfide bridge. Yellow denotes putative phosphorylation site; purple denotes putative ubiquitination site; and green denotes putative N-glycosylation site. Secondary structure was generated with the aid of the uniprot consortium (www.uniprot.org).

CHAPTER 3: Manuscript 1

Title:	The metabolite GnRH-(1-5) inhibits the migration of immortalized GnRH neurons.
Abbreviated Title:	GnRH-(1-5) regulates the migration of GN11 neurons
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Disclosure Statement:	The authors have nothing to disclose.
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Key Words: GnRH, GPCR, migration, GN11, GPR173

Abstract

The decapeptide gonadotropin-releasing hormone (GnRH) is an important regulator of reproductive behavior and function. In the extracellular matrix, GnRH is metabolized by the endopeptidase EC3.4.24.15 (EP24.15) to generate the pentapeptide GnRH-(1-5). In addition to its expression in the adult hypothalamus, EP24.15 is expressed along the migratory path of GnRH-expressing neurons during development. While we have previously demonstrated a role for EP24.15 in the generation of the biologically active pentapeptide GnRH (1-5) in regulating GnRH expression and mediating sexual behavior during adulthood in rodents, the modulatory role of GnRH-(1-5) in the migration of GnRH neurons during development remains unknown. To address this information gap, we examined the effect of GnRH-(1-5) on the cellular migration of a pre-migratory GnRH-secreting neuronal cell line, the GN11 cell, using a wound-healing assay. Dose and time response studies demonstrated that GnRH-(1-5) significantly delayed wound closure. We then sought to identify the mechanism by which GnRH-(1-5) inhibits migration. Since the cognate GnRH receptor (GnRHR) is a G protein-coupled receptor (GPCR), we examined whether GnRH-(1-5) regulates migration by also activating a GPCR. Using a high-throughput β-arrestin recruitment assay we identified an orphan G protein-coupled receptor 173 (GPR173) that was specifically activated by GnRH-(1-5). Interestingly, siRNA to GPR173 reversed the GnRH-(1-5)-mediated inhibition on migration of GN11neurons. Furthermore, we also demonstrate that the GnRH-(1-5)-activated GPR173-dependent signal transduction pathway involves the activation of the signal transducer and activator of transcription 3 (STAT3) in GnRH

migration. These findings indicate a potential regulatory role for GnRH-(1-5) in GnRH neuronal migration during development.

Introduction

The neuropeptide GnRH is an important regulator of reproductive function and behavior. GnRH-secreting neurons populate the basal forebrain and target the median eminence of the hypothalamus where GnRH is released in a pulsatile manner. Subsequently, GnRH acts in the anterior pituitary to control the release of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). These hormones in turn act on the gonads to regulate the production of steroid hormones, estrogen and progesterone, which act centrally to modulate reproduction.

The factors that enable the proper migration of GnRH neurons from the nasal placode to the central nervous system (CNS) are still largely unknown despite ongoing research in this field (34; 41). Defects in this process can impair the establishment of the hypothalamic-pituitary-gonadal axis (HPG) and result in delayed pubertal onset, hypogonadism, and infertility (33; 35). This is the case in Kallmann Syndrome (KS) in which precursor olfaction and GnRH neurons emanating from the olfactory placode remain arrested in the cribriform plate, never entering the CNS to reach their target sites (5; 33). A complete molecular understanding of the pathogenesis leading to KS is still in its infancy. It is estimated that 60-75% of cases associated with KS are not linked to any currently known genetic defect (4).

In addition to the actions of the full-length GnRH, there are also biologically active effects of its cleavage products. We have previously shown that GnRH-(1-5), produced by the zinc metalloendopeptidase, EP24.15, facilitates lordosis behavior in ovariectomized estrogen-primed rats (42). Also, GnRH-(1-5) stimulates GnRH mRNA expression in GnRH neurons while treatment with the full-length GnRH inhibits its own

expression (43). EP24.15, the enzyme that is responsible for generating GnRH-(1-5), is expressed in the CNS during development along the migratory path of GnRH neurons (37). It is possible that the availability of GnRH-(1-5) during development may contribute to the migration of GnRH neurons prior to their entering the CNS through the cribriform plate.

The pathway mediated by the Signal Transducer and Activator of Transcription 3 (STAT3) plays a critical role in cell survival and differentiation. STAT3 activation is induced by phosphorylation at the residue Y705 leading to subsequent dimerization with another phosphorylated-STAT3 (pSTAT3). This event leads to nuclear translocation and initiation of transcription (1). In the CNS, STAT3 signaling has been implicated in neurite outgrowth (44; 45) and in neuronal pathfinding (8). Furthermore, the developmental presence of the cytokine leukemia inhibitory factor (LIF), which induces the phosphorylation of STAT3, has been shown to promote GnRH neuronal migration *in vitro* (19). Therefore modulation of STAT3 activity may serve as a mechanism by which the extracellular environment can in part regulate the migration rate of GnRH neurons.

In this study, we investigated whether GnRH-(1-5) regulates GnRH neuronal migration using the GnRH-secreting cell line, the GN11 cell, as a model (18; 30; 32). GN11 cells were isolated from a mouse olfactory tumor induced by the SV40 T-antigen driven by the human GnRH promoter (32). These cells are considered an immature GnRH-secreting cell line that has extensively been used to study GnRH neuronal migration *in vitro* (17-19). Our results demonstrate that GnRH-(1-5) inhibits GN11 cellular migration by inhibiting STAT3 signaling. Furthermore, we identify the orphan G

protein-coupled receptor GPR173 as the signal transducing element mediating the actions of GnRH-(1-5) on migration in GN11 cells.

Materials and Methods

Cell Culture

GN11 cells (generously donated by Dr. S. Radovick; Johns Hopkins University School of Medicine, Baltimore, MD) were cultured as previously described (27; 48) with some modification (personal communication with Dr. H. Novaira). Briefly, cells were grown in Dulbecco's modified Eagle's medium (Mediatech Inc., Herndon, VA) without antibiotics supplemented with 7% fetal bovine serum (FBS; Hyclone, Logan, UT), 3% newborn calf serum (Hyclone, Logan, UT), 25 mM glucose, and 5 mM L-glutamine. Cells were maintained at 37 C in an atmosphere with 5% CO_2.

Wound Closure Assay

A wound closure assay was used to determine the effect of GnRH-(1-5) on GN11 migration. GN11 cells were plated on plastic uncoated 6-well plates (Costar; Corning, NY) and allowed to reach 80% confluency. The cells were starved for 24 h in 10% charcoal-stripped dextran-treated FBS (Atlanta Biologicals; Lawrenceville, GA) and tested for migration using *in vitro* wound closure assay. The scratch was made through a cell monolayer with a sterile 1000 uL pipette tip. The cells were washed 3 times with PBS to remove detached cells and incubated with 10% stripped FBS containing the indicated treatment condition. Images were taken using a phase contrast microscope (Olympus Ix71; Olympus America Inc, Center Valley, PA) of each wound and its location was noted. Subsequent pictures were taken at 24 or 48 h after the addition of treatments depending on the experiment. The wound area was measured using the Multi Gauge software (Fujifilm; Valhalla, NY). The percent wound closure was calculated with the following formula: % wound closure = $((area_{t=0h} - area_{t=24h})/area_{t=0h})$ X 100%. Similar

protocols have been previously described to measure cell migration in cancer cells (10; 38) and in GN11 cells (17; 36; 46).

Boyden Chamber Assay

The QCM Chemotaxis 96-well Migration Assay (Millipore; Temecula, CA) was employed as an alternative method to determine the effect of GnRH-(1-5) on GN11 cell migration. This assay based on the Boyden chamber assay, has previously been used to characterize GN11 cellular migration (6; 19). GN11 cells were grown in complete media to 80% confluency and then starved in serum-free media for 24 h. Subsequently, cells were harvested with 2mM EDTA/PBS, centrifuged for 5 min at 1500 RPM, resuspended in serum-free media. Cells were loaded in the upper compartment of the well separated by an 8 μm porous membrane precoated with 0.2 mg/mL gelatin. The lower compartment contained the specific treatment condition [VEH, 1 nM GnRH-(1-5), 100 nM GnRH-(1-5)] in 10% charcoal-stripped dextran-treated FBS. GN11 cells were allowed to migrate through the pores for 3 h at 37 C in a cell culture incubator. This time point has been previously established to measure GN11 migration (18; 19). Following the incubation period, migrating cells attached to the bottom of the membrane were lysed and incubated with the supplied nucleic acid-binding fluorescent dye (CyQuant GR; Molecular Probes, Eugene, OR) according to the manufacturer's instructions (Millipore; Temecula, CA). Fluorescence was read using a Victor X5 2030 Multilabel plate-reader (Perkin Elmer; Shelton, CT) with a 480/535 filter set. VEH-treated cells were set at 100 % for comparison with GnRH-(1-5)-treated cells.

Proliferation Assay

Experiments were conducted to determine whether the effect of GnRH-(1-5) on migration was dependent on cell proliferation. The proliferation assay used a colorimetric assay based on measuring the reduction of 3-(4,5-dimethythiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) (Promega CellTiter 96 AQueous One Solution Cell Proliferation Assay; Madison, WI). Briefly, GN11 cells were plated at 20% confluency on 96-well plates and the following day the media was replaced with 10% charcoal-stripped dextran-treated FBS. After 24 h, cells were treated with VEH, 1 nM, or 100 nM GnRH-(1-5) and allowed to grow for either 24 h or 48 h. At the end of each experiment, 20 uL of the CellTiter MTS solution was added to each well and incubated for 30 min at 37 C. The absorbance of each plate was read at 490 nm with a Victor X5 2030 Multilabel plate-reader (Perkin Elmer; Shelton, CT). The experiments for each assay were performed with at least 8 replicates for each treatment condition and repeated at least 4 times using different cell passages.

Screening for Putative GnRH-(1-5) Receptors

We narrowed our search for putative GnRH-(1-5) receptors by using the PathHunter Orphan GPCR Biosensor Panel (DiscoveRx, Fremont, CA). In this primary screen, 30 μM GnRH-(1-5) (n=2/cell line) was used to assess ligand activity in an array of identified human orphan G-protein coupled receptors using standard DiscoveRx protocols (24; 39) (Table 1). All activity was compared to the respective VEH (1% DMSO) after treatment. This technology uses enzyme fragment complementation of the β-galactosidase (β-gal) enzyme, which upon formation of the complete enzyme generates a luminescent signal that can be measured by spectrometry. Specifically, orphan GPCRs where modified as fusion proteins to a portion of the β-gal enzyme. The complementing

fragment of β-gal is fused to β-arrestin, which is recruited upon ligand binding to the modified GPCR. In our primary screen, a total of 74 GPCRs were assayed in the presence of GnRH-(1-5) (24; 39). Once we determined that GPR173 was the only receptor expressed in GN11 cells, a secondary screen was implemented using the GPR173-expressing cell line (PathHunter™ HEK 293 GPR173 Orphan GPCR β-arrestin High Expression Cell Line; Cat #93-0380C1A, DiscoveRx). These cells were grown and maintained in DMEM (Cat# 12430-054, Invitrogen) containing 10% heat inactivated FBS (Cat # 10082-147, Invitrogen), Pen/Strep/Glu (Cat# 10378-016, Invitrogen), 200 μg/ml Hygromycin (Cat # 10678-010, Invitrogen) and 800 μg/ml Geneticin (Cat #10131-035, Invitrogen). A time course study was initially conducted to determine the optimal time course for this cell line ranging from 15 min to 90 min. We found that 15 min yielded the optimal results (data not shown) and subsequently conducted a dose response curve ranging from 0.01 nM to 1000 nM GnRH-(1-5). β-gal activity was determined using the PathHunter™ detection kit (Cat #93-0001, DiscoveRx) and measured on a Victor X5 2030 Multilabel plate-reader (Perkin Elmer; Shelton, CT). The EC_{50} was calculated by applying a 4-parametric logistic curve using Sigma Plot (San Jose, CA).

Reagents

GnRH-(1-5) was synthesized on-site (Biomedical Instrumentation Center, Uniformed Services University, Bethesda, MD) as previously described (3). Briefly, the peptide was synthesized by Fmoc chemistry using an Applied Biosystems 433A Peptide Synthesizer (Foster City, CA). Synthesized GnRH-(1-5) was aliquoted into 10-20 mg aliquots and stored at – 80 C with a desiccant. Prior to use, GnRH-(1-5) was dissolved in water to 1 mM and subsequently serially diluted in culture media to its final

concentration. D-ser GnRH was purchased from Bachem (catalog no. H-6395; Torrance, CA). The STAT3 inhibitor WP1066 was purchased from EMD Millipore (catalog no. 573097; Darmstadt, Germany) and dissolved in 100% ethanol to 100 mM. Control experiments included the appropriate solvent (water or ethanol) not exceeding more than 0.01% indicated as vehicle (VEH).

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

To determine the expression of target GPCRs in the GN11 cell line, total RNA was extracted with trizol reagent (Invitrogen, Carlsbad, CA) and 2 μg of RNA was reverse transcribed (Maxima First Strand cDNA synthesis kit; Fermentas; Glen Burnie, MD) for PCR analysis. In parallel, RNA samples in the absence of reverse transcriptase (-RT) were used as a negative control (-RT) to exclude genomic contamination during cDNA synthesis. PCR reactions used cDNA templates equivalent to 50 ng of RNA using the Promega PCR Master Mix (Promega; Madison, WI) and 200 nM of each primer. The PCR conditions were: initial denaturation at 95 °C for 2 min, followed by 35 cycles of denaturation (95 C, 30 s), annealing (55 C, 30 s), and extension (72 C, 30 s). There was a final extension period of 5 min at 72 C. Amplicons were visualized by agarose gel electrophoresis (1 %) using the GelStar Nucleic Acid Gel Stain (Lonza; Rockland, ME) and verified by sequencing. Primers specific to each target gene were generated with the NCBI Primer Blast website (http://www.ncbi.nlm.nih.gov/tools/primer-blast; NCBI, Bethesda, MD) with the exception of GPR119 (29) and GnRH (20), which were previously described. All primers were made to span at least one intron on the corresponding gene (See Table 2 for more details). Mouse hypothalamic tissue was used

as a positive control for each primer pair. The putative mRNA structure for GPR173 was obtained from the NCBI website (http://www.ncbi.nlm.nih.gov; Bethesda, MD).

GPCR silencing by siRNA

GN11 cells were plated at 20% confluency and transfected with a cocktail of siRNA (25 nM) specific to GPR173 (SMARTpool; Cat. No. L-059639-00-0005, Dharmacon, Lafayette, CO). Control cells were transfected with a non-targeting siRNA mixture (25 nM) (SMARTpool; Cat. No. D-001810-10-05, Dharmacon). Dharmafect4 (Dharmacon) was used as the transfection reagent. All treatments were made in 10% charcoal/dextran stripped FBS (Atlanta Biologicals; Lawrenceville, GA). Cells were incubated with the siRNA treatments for 48 h and then a scratch was made as described above in our wound closure assay protocol. Cells were washed 3 times and the media replaced either with VEH or 100 nM GnRH-(1-5). Images were taken immediately after the addition of GnRH-(1-5) treatment and 24 h later with a phase contrast microscope (Olympus 1x71).

Quantitative RT-PCR (qPCR)

The efficiency of GPR173 silencing by siRNA was measured in GN11 cells by qPCR. Total RNA was harvested 48 h post transfection with siRNA as described above. 2 μg of RNA was reverse transcribed using the Maxima First Strand cDNA synthesis kit (Fermentas; Glen Burnie, MD) and the cDNA was analyzed by qPCR using the FAST SYBR Green Mastermix (Cat. No. 330609, Qiagen, Valencia, CA) using 200 nM of the appropriate primer pair. All samples were assayed in duplicate using the CFX Connect Real-time System (Bio-Rad; Hercules, CA). The qPCR conditions were performed as follows: initial denaturation and enzyme activation at 95 °C for 10 min followed by 40

cycles of denaturation (95 C, 15 s), annealing and reading (60 C, 30 s). Melt curve analyses was conducted after each qPCR reaction to demonstrate the presence of a single amplicon. Additionally, qPCR products were visualized by agarose gel electrophoresis (1 %) using the GelStar Nucleic Acid Gel Stain (Lonza; Rockland, ME) under UV light and verified by sequencing. Primers specific to GPR173 and the reference gene Cyclophilin A are shown in table 2. qPCR conditions were optimized to produce a greater than 95% efficiency for both primer pairs as determine by a 10-fold serial dilution of cDNA (40). The delta delta ct method was used to measure fold change in GPR173 expression relative to levels obtained from VEH-treated cells. This experiment was repeated over 3 consecutive passages.

Mouse Brain and Nasal Tissue

Experiments using RNA from animal tissues were samples from previously completed studies. For adult mouse brain, RT-PCR (with subsequent sequencing) was conducted on hypothalamic RNA extracted from an 8 wk old C57bl/6 male mouse (Jackson Laboratories, Inc., Bar Harbor, ME). Briefly, the mouse was killed by carbon dioxide overdose with rapid decapitation. The brain was quickly removed and the whole hypothalamus was dissected on ice. The RNA was immediately extracted by trizol reagent (Invitrogen) and processed as described in our RT-PCR protocol.

RNA samples from mouse noses were obtained at embryonic days 11.5 to 12.5 (E11.5 or E12.5, respectively). These samples were generously donated from Dr. Susan Wray (National Institute of Health, Bethesda, MD) and previously described by Wray and colleagues (11; 12). All experimental procedures were conducted in accordance to the NIH guidelines (NIH guide for the Care and Use of Laboratory Animals, NIH

Publication) and were approved by the USUHS Institutional Animal Care and Use Committee.

Western Blot Analysis

Total protein lysates were harvested from cells with lysis buffer (O'Dell's Lysis Buffer containing 10mM EGTA; 10mM EDTA; 80uM Na_2MoO_4; 5mM $NaPO_4$; 1mM Na_3VO_4; 1mM PMSF; 4mM pNPP; 1% Triton; Sigma Inhibitor Cocktail I and II and Roche Complete Protease Inhibitor, Indianapolis, IN). Cell lysates were briefly sonicated and supernatant (20 ug) were subjected to SDS-PAGE (4-12%; Lonza; Rockland, ME) after centrifugation (20,000g for 20 min). After electrophoresis, the proteins were transferred onto a polyvinyldifluoride membrane (Bio-Rad). Western blot analysis was conducted using previously established methods (32). Briefly, the membranes were sequentially incubated with specified primary antibodies (pSTAT3 1:2000; STAT3 1:2000; caspase-7 1:2000; Cell Signaling Technology, Inc., Danvers, MA; or β-actin 1:2000; Sigma, St. Louis, MO) overnight at 4 C followed by incubation at room temperature with the appropriate secondary antibody conjugated to horseradish peroxidase (Bio-Rad; Hercules, CA). The blots were visualized with a chemiluminescent signal (Immobilon Western HRP Substrate Peroxide Solution; Millipore Corp, Billerica, MA) subsequently digitized (Fujifilm LAS-3000 imager; Stamford, CT) and analyzed (Fujifilm Image Gaug; Valhalla, NY).

Immunofluorescent Studies

After treatments, GN11 cells were washed with ice-cold 0.1 M phosphate-buffered solution containing 0.9% saline (PBS) and fixed with buffered 4% paraformaldehyde (pH7.4; Fisher) for 10 min. Subsequently, cells were washed 3 times

for 5 min with PBS and incubated with methanol for 10 min. After the formalin/methanol fixation, the cells were sequentially washed with PBS (3 times, 5 min each), followed by blocking with 5% normal goat serum (1 h; Sigma) at room temperature followed by an overnight incubation at 4 C with pSTAT3 primary antibody (1:1000; Cell Signaling), additional washes and incubated with the Alexa Fluor-488 conjugated secondary antibody (1:1000; Jackson ImmunoResearch Labs, West Grove, PA) for 2 h. After washing with PBS, the cells were coverslipped. Nuclear staining was performed with a DAPI counterstain (Invitrogen, Eugene, Oregon). Images were taken with the Leica AF6000 microscope (Allendale, New Jersey).

Time-lapse Imaging System

To better visualize the effect of GnRH-(1-5) on GN11 cellular migration, a wound closure assay was examined by time-lapse video microscopy using a Leica AF600 Time-lapse microscope (Allendale, New Jersey) equipped with CO_2 and temperature controls. GN11 cells were cultured on a Lab-Tek II Chamber Slide System (Nunc; Rochester, NY) following the same protocol for the wound closure assay as mentioned above. However the scratch was made with a 200 uL sterile pipette tip to see the width of the wound at the magnification used. Cells treated with VEH or 100 nM GnRH-(1-5) were run in parallel and pictures of the same wound area were taken every 10 min for 24 h using the Leica AF600 Imaging software (Allendale, New Jersey). All pictures were converted to a Quicktime AVI file and processed with Adobe Premier Pro (San Jose, CA). The resultant video is labeled as Supplemental Video 1.

Statistical Analysis

All statistical analyses were conducted using SPSS (Version 16.0, IBM Corp., Armonk, NY). Wound closure assay data were analyzed by a one-way or two-way ANOVA depending on the experiment followed by Fisher's least significant difference (LSD) post-hoc test. Proliferation assay data were analyzed by a two-way ANOVA followed by an LSD post hoc test. Western blot and qPCR data were analyzed by a one-way ANOVA followed by an LSD post hoc test. A value of $P<0.05$ was considered significant.

Results

Effect of GnRH-(1-5) on Migration

The ability of GnRH-(1-5) to regulate GN11 neuronal migration was examined using a wound closure assay. GN11 cells treated with 1 nM and 100 nM GnRH-(1-5) significantly ($p < 0.05$) delayed wound closure at 24 h and 48 h treatments (Figure 1A). Additionally, we wanted to better visualize cell morphology and movement so we generated a time-lapse video of the wound closure assay in cells treated with VEH and 100 nM GnRH-(1-5). Again, there is a delay in wound closure with GnRH-(1-5) treatment showing GN11 cells seem to have a lower propensity to extend their filopodia and migrate into the wound (Supplemental Video 1). We also confirmed the effect of GnRH-(1-5) on migration using the secondary method, the Boyden chamber assay. A significant dose dependent decrease ($p < 0.05$) in migration across the porous membrane was observed in GN11 cells treated with 100 nM GnRH-(1-5) compared to VEH treatment; Figure 1B). Although there was a reduction in migration with 1 nM GnRH-(1-5) treatment, this difference did not reach statistical significance (Figure 1B). Additionally, the inhibitory effect of GnRH-(1-5) on migration was independent of cellular proliferation as determined by the MTS-based colorimetric assay, which showed no statistical differences in growth between VEH and GnRH-(1-5)-treated cells at 24 h and 48 treatments (Figure 1C).

Screening for Putative GnRH-(1-5) Receptors

The primary screen of the panel of orphan human GPCRs showed that GnRH-(1-5) had increased binding activity for GPR101, GPR119, GPR148 and GPR173 while negligible activity was observed in cell lines expressing other GPCRs (Table 1). The cell

lines used initially for the primary screen displayed high constitutive activity (DiscoveRx, personal communication) that resulted in inverse agonism for the receptors. Of the 4 receptors identified in the primary screen, GPR148 was omitted as this receptor is not present in rodents (14). Only GPR173 mRNA was present in GN11 cells (see below). Thus, GPR173 was the focus for the secondary screen. GPR173 expressing cells were treated for 15 min with GnRH-(1-5) (0.01 nM – 1000 nM) to construct a binding curve. In this screen, GnRH-(1-5) caused a dose dependent increase in β-arrestin binding as measured by increased β-gal activity with an EC_{50} of 1.9 nM GnRH-(1-5) (Figure 2D).

To determine whether the GPCRs identified to bind GnRH-(1-5) in the primary screen were expressed in the GN11 cell line, we generated specific primers against each GPCR (Table 2) and measured their expression by RT-PCR and subsequently visualized by agarose gel electrophoresis (Figure 2A), along with RNA obtained from mouse hypothalamic tissue as positive controls (Figure 2A). Interestingly, we found that only GPR173 was expressed in GN11 cells while GPR101 and GPR119 were not detected (Figure 2A). As indicated above, GPR148 was not measured because it is not known to exist in rodents (14). No bands were observed in PCR samples in which the RNA was not treated with reverse transcriptase (-RT) (Figure 2A).

GPR173 Expression in Embryonic Nasal Tissue

To examine the physiological relevance of GPR173 in GnRH neuronal migration, RNA from E12.5 mouse nasal tissue and E11.5 nasal explant tissue cultured 3 days *in vitro* (3 div) were used to detect the expression of GPR173 in addition to GPR101 and GPR119 (Figure 2B). These time points have been previously characterized to contain migrating GnRH neurons (12). Furthermore, we confirmed GnRH expression in both

time points by RT-PCR (Figure 2B). Interestingly, only GPR173 mRNA was highly expressed in the nasal tissues examined but not GPR101 or GPR119 mRNAs.

Effect of GPR173 Silencing on GnRH-(1-5)-mediated Inhibition of GN11 Migration

Since it was determined that GPR173 is expressed in GN11 cells and in nasal tissue containing migrating GnRH neurons, we examined whether down-regulation of GPR173 blocks the effect of GnRH-(1-5) on GN11 cellular migration. We used an siRNA-based approach to silence GPR173 expression in GN11 cells. The efficacy of GPR173 knockdown was measured by qPCR using primers that span exons 2 and 3 of the full length GPR173 mRNA (Figure 2C) in GN11 cells treated with either the transfection reagent only (VEH), the GPR173 siRNA pool (25 nM), or a non-targeting siRNA pool (25 nM). GN11 cells treated with the GPR173 siRNA pool had an approximately 80% reduction in GPR173 mRNA levels relative to VEH and cells treated with the non-targeting siRNA (Figure 2E). We also conducted a wound closure assay to examine whether GPR173 silencing blocked GnRH-(1-5) inhibition of GN11 migration. Cells treated with 100 nM GnRH-(1-5) or 100 nM GnRH-(1-5) + non-targeting siRNA had significantly ($p < 0.05$) reduced wound closure relative to control cells (Figure 3). Conversely, wound closures in GN11 cells treated with 100 nM GnRH-(1-5) and GPR173 siRNA were statistically indistinguishable from control cells (Figure 3).

Effect of GnRH-(1-5) on STAT3 phosphorylation

Previous work has implicated STAT3 pathway in regulating GN11 migration (19); thus we examined whether GnRH-(1-5) alters the levels of phosphorylated STAT3 (pSTAT3). GN11 cells were treated for 5, 15, and 30 min with VEH or 100 nM GnRH-(1-5) and the relative levels of pSTAT3 (Y705) and STAT3 were measured by western

blot analysis. GnRH-(1-5) decreased the levels of pSTAT3 in time dependent manner reaching significance at 15 min (Figure 4A). This effect was specific to GnRH-(1-5) since treatment with the full-length GnRH did not alter the levels of pSTAT3 (Figure 4B).

To further reinforce the significance of STAT3 signaling in GN11 migration, we used a pharmacological inhibitor of STAT3, WP1066 (15). We found that WP1066 treatment decreased pSTAT3 levels (Figure 5A) without inducing apoptosis as measured by cleaved caspase 7 levels at 30 min, 18 h, or 24 h treatments (Figure 5B). Furthermore, WP1066 robustly delayed wound closure at 1 uM similar to GnRH-(1-5) treatment; however, we did see inhibition at lower concentrations (Figure 5C).

Finally, we investigated whether GPR173 mediates the GnRH-(1-5) decrease in pSTAT3 levels using siRNA to GPR173. We tested this effect using two methods: immunofluorescent analysis and with western blots. Our results indicate that the GnRH-(1-5)-mediated decrease in pSTAT3 levels was blocked by silencing GPR173 expression in GN11 cells (Figure 6A,B). This effect was specific to GPR173 since treatment with the non-targeting siRNA had no effect on the GnRH-(1-5) decrease in pSTAT3 levels (Figure 6A,B).

Discussion

The present study show that GnRH-(1-5), a metabolic product of GnRH, regulates the migration of GN11 neurons by attenuating FBS-stimulated GN11 neuronal migration. Furthermore, we have demonstrated that the orphan receptor GPR173 and the STAT3 signaling pathway mediate this inhibition.

Our previous studies have shown that GnRH-(1-5) can regulate GnRH mRNA expression (43) and facilitates lordosis behavior in estrogen-primed ovarectomized rats (42). Interestingly, EP24.15 is expressed along the migratory path of GnRH-secreting neurons between the olfactory placode and CNS during development (37). Furthermore, previous studies have shown that migrating GnRH neurons secrete GnRH; and that the levels of secretion increase as development progresses reaching the picogram range (21; 26). Thus the availability of GnRH-(1-5) mediated by EP24.15 suggests that GnRH-(1-5) may play a role in the development of the GnRH neuroendocrine system. In this study we tested whether GnRH-(1-5) regulates the migration of GN11 neurons, a GnRH-secreting cell line extensively used to study the mechanism of GnRH neuronal migration (6; 13; 19; 32). GnRH-(1-5) treatment inhibited the ability of GN11 neurons to migrate into a wound in an *in vitro* wound closure assay. This effect seems to be specific to GnRH-(1-5) since treatment with the full-length GnRH had no effect on GN11 cell migration compared to control cells (data not shown). We performed this type of migration assay since it is a simple experiment that has been previously used to test the motility/mobility of GN11 cells in response to a treatment (2; 25). GnRH neurons during development are exposed to a variety of signaling cues that regulate the rate of migration (41); and the proper coordination of these signals is critical for GnRH neurons to locate their

destination in a timely manner dependent on the stage of development (34). Although the local physical and chemical environment of the wound closure assay utilized in the present study is simpler than the environment encountered by migrating GnRH neurons during development, this assay and the GN11 cell line offer an initial starting point to test the effects of GnRH-(1-5) on migration. Our results suggest that GnRH-(1-5) decreases GnRH neuronal motility, which could potentially serve to balance the effects of other factors that enhance the migration of GnRH neurons during development. This is more evident from our time-lapse video demonstrating the effect of GnRH-(1-5) to inhibit radial migration of GN11 cells across the wound area. Furthermore, we used the Boyden chamber assay as an alternative assay to test the effect of GnRH-(1-5) to inhibit migration. These results mirrored our wound closure experiments where GnRH-(1-5) inhibited the migration of GN11 cells. In both these experiments, charcoal-stripped FBS was used as a stimulator of migration, which leads us to speculate that the effect of GnRH-(1-5) on migration is complex and involves the integration of multiple signaling factors. We also found that the effect of GnRH-(1-5) is directly on migration and not proliferation since our proliferation assay results showed no differences in growth in response to GnRH-(1-5) treatment.

It is well established that many neuropeptides including the decapeptide GnRH bind and activate a GPCR to elicit their function. The orphan receptor GPR173 is a member of the small GPCR family called Super Conserved Receptor Expressed in Brain (SREB), which includes the orphan receptors GPR27 and GPR85 (22). A recent report suggests that GPR27 may regulate insulin production (16) while GPR85 has been implicated in neurogenesis (7) and in the development of schizophrenia (23); however,

like GPR173 no known ligands have been implicated in their activation. In our study we sought to determine whether GnRH-(1-5) activated a GPCR to regulate GN11 migration. In our primary screen using a panel of 74 orphan GPCRs, GnRH-(1-5) showed specific activity with GPR101, GPR119, GPR148, and GPR173. In this screen we decided to use a high dose of GnRH-(1-5) (30 uM) due to the nature of the high-throughput assay having constitutive β-arrestin recruitment activity depending on the GPCR. Therefore, GnRH-(1-5) in this system may act as an inverse agonist. Nevertheless, changes in β-arrestin recruitment indicate specific binding to the GPCR. Once it was determined that GPR173 was the only receptor expressed in GN11 cells, a secondary screen was conducted using the GPR173 cell line to induce a concentration dependent increase in β-arrestin recruitment by GnRH-(1-5) indicating the binding specificity. We further examined whether GPR173 is required for the effect of GnRH-(1-5) on GN11 cellular migration using an siRNA-based approach to block GPR173 expression. Interestingly, we found that the GnRH-(1-5)-mediated inhibition of migration was reversed only when GPR173 expression was silenced. This suggests that GPR173 is the signal transducing element for the effect of GnRH-(1-5) on migration. It is still not clear however whether GnRH-(1-5) is the only endogenous ligand for GPR173 since many GPCRs have been known to bind multiple ligands leading to different biological functions. Furthermore, the other candidate receptors cannot be overlooked and GnRH-(1-5) may play a tissue-specific role depending on the expression of certain GPCRs. GPCR ligand promiscuity is not uncommon and such is the case with the osteocalcin-sensing receptor GPRC6A, which is a GPCR with multiple structurally dissimilar ligands (29). A more thorough

analysis of the GnRH-(1-5) and GPR173 interaction is underway in addition to the biological relevance of the other candidate receptors.

Previous studies have shown that the STAT3 pathway is important for axonal growth (8) and migration in GN11 neurons (19). Therefore to determine whether GnRH-(1-5) regulates the activation of STAT3, we measured the phosphorylation of STAT3 (pSTAT3) in GN11 neurons following treatment with GnRH-(1-5). Interestingly, GnRH-(1-5) potently reduced the levels of pSTAT3 in a time-dependent manner. This is in contrast to GN11 neurons treated with the decapeptide GnRH, which had no significant effect on pSTAT3 levels. These results suggest that the effect of migration and the inhibition of phosphorylation of STAT3 are specific to GnRH-(1-5). Furthermore, we expected GnRH-(1-5) to decrease pSTAT3 levels since a previous report by Magni and colleagues showed that GN11 cells treated with the cytokine leukemia inhibitory factor (LIF) had increased pSTAT3 levels (19). Additionally, they showed that LIF stimulated GN11 neuronal migration (19), which is in agreement with our results showing GnRH-(1-5) inhibits the phosphorylation of STAT3 and subsequently migration. To further reinforce the link between the STAT3 pathway and migration, we used a pharmacological inhibitor of STAT3, WP1066, which inhibits the phosphorylation of STAT3 (15). Interestingly, we found that WP1066 not only inhibited the phosphorylation of STAT3 but also delayed GN11 migration in a wound healing assay, thereby mimicking the effect of GnRH-(1-5). We determined whether GPR173 mediates the effect of GnRH-(1-5) to decrease pSTAT3 levels using our siRNA mediated approach. In accordance with the results from our migration study, silencing GPR173 expression also blocked the GnRH-(1-5)-mediated down-regulation of pSTAT3 levels.

The present study indicates that GnRH-(1-5) decreases pSTAT3 levels and inhibits GN11 neuronal migration via GPR173; yet, the intracellular mechanism mediated by the interaction of GnRH-(1-5) and GPR173 leading to the dephosphorylation of STAT3 remains to be determined. Previous work with the GPCR, angiotensin II type 1 receptor (A1R), showed that it can induce the phosphorylation of certain members of the STAT family including STAT3 when activated by angiotensin II (20; 28). Furthermore, it has been shown that agonist activation of A1R is mediated in part by the activation of certain Janus kinases (JAK) and depends on Rac recruitment in smooth muscle cells (28), suggesting some GPCRs can deviate from the canonical heterotrimeric G protein signaling pathway. However, our results indicate that GnRH-(1-5) decreases pSTAT3 levels suggesting that GPR173 is likely not interacting with members of the JAK family, which function to phosphorylate and therefore activate STAT proteins (9). In our *in vitro* model, treatments were administered after dilution in 10% charcoal-dextran stripped FBS to remove endogenous steroid influence; however, GN11 neurons still proliferate in this environment albeit at a slower rate. We chose this protocol since GnRH-expressing neurons during development are exposed to a variety of factors that aid in their migration prior to entering the CNS (41). Using a wound closure assay, GN11 cells invade the wound area rapidly in our culture conditions, yet following GnRH-(1-5) treatment there is a delay in wound closure suggesting that GnRH-(1-5) is likely attenuating the effect of certain cytokines/growth factors to stimulate migration. Interestingly, the cytokine LIF has been shown to enhance GN11 neuronal migration in part by up-regulating the levels of pSTAT3 (19). Furthermore, LIF expression is present early in development as GnRH neurons are migrating from the nasal placode to the CNS (19; 31), therefore LIF, among

other factors, may act to stimulate migration along the vomeronasal tract while GnRH-(1-5) via GPR173 plays a modulatory role in fine-tuning the migration of GnRH neurons prior to entering the CNS through the cribiform plate (37).

Our findings demonstrate that GPR173 may contribute to the migration of GnRH neurons. Previous work characterizing the GN11 cell line offer evidence for its neuronal phenotype (30; 48); yet it is still considered a heterogeneous population of cells in which the majority are GnRH positive (30; 47). Therefore in our model it is not clear whether GnRH-(1-5) is acting directly on GnRH neurons via GPR173. We began addressing this issue by detecting the expression of GPR173 in mouse embryonic nasal tissue from E12.5 and nasal explant tissue from E11.5 cultured for 3 days *in vitro*. These tissues have been previously verified to contain migrating GnRH neurons (12). Interestingly, we found that GPR173 was highly expressed at both time points measured while we could not detect GPR101 or GPR119 expression. These results indicate that GPR173 is present during critical periods where GnRH neurons are migrating along the vomeronasal tract; and that GnRH-(1-5) influence on their migration is plausible.

It is possible that GnRH-(1-5) could be acting in an autocrine or paracrine manner to regulate GnRH neuronal migration during development. Our results suggest an autocrine mechanism since we used GN11 cells, which have extensively been used as a model for GnRH neuronal migration. However, due to the immortalization of this cell line, the exact nature of GnRH neurons *in vivo* may not be reflected. Therefore, GnRH-(1-5) acting as a paracrine regulator via GPR173 to indirectly influence the migration of GnRH neurons cannot be ruled out. GnRH-(1-5) acting on neighboring cells expressing GPR173 may alter the microenvironment by regulating the availability of certain growth

factors/cytokines that directly act on GnRH neurons. The selective activation of GPR173 to regulate migration is an important finding as this type of regulation may contribute to the transitioning of migrating GnRH neurons from a radial migratory trajectory to a tangential one as they are leaving the nasal region to enter the CNS. Future work will focus on determining the localization of GPR173 relative to GnRH neurons; and how the microenvironment may be altered in the absence of GPR173 during development.

In summary, we demonstrate that the GnRH metabolite GnRH-(1-5) regulates GN11 cell migration by acting though intracellular STAT3 pathway. These processes are dependent on the activation of GPR173 since silencing GPR173 expression in GN11 cells reversed any GnRH-(1-5) effect. The mechanism of GnRH-(1-5) is likely complex involving the interaction of other growth factors that serve to stimulate migration. Further investigation is certainly warranted in elucidating the role of GnRH-(1-5) in the development of the GnRH neuroendocrine system and the role played by the orphan receptor GPR173 in the action of this metabolite.

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Figure Legends

Figure 1. Effect of GnRH-(1-5) treatment on GN11 cellular migration. A) GN11 cells treated with 1 nM or 100 nM GnRH-(1-5) delayed wound closure relative to VEH treatment at 24 h and 48 h treatments. Left panel indicates the representative photomicrographs of each treatment in the wound closure assay. Right panel indicates the quantification of the wound closure assay (n=4-8). Data were analyzed by a two-way ANOVA followed by LSD post hoc test. Scale bar indicates 250 μm. B) The Boyden chamber assay was used as a secondary method to measure migration. Treatment with 100 nM GnRH-(1-5) for 4 h decreased GN11 cellular migration (n=7). Data were analyzed by a one-way ANOVA followed by LSD post hoc test. C) GnRH-(1-5) treatment did not alter GN11 proliferation at 24h and 48h treatments (n=4). Data were analyzed by a two-way ANOVA followed by LSD post hoc test. Bars indicate SEM. * $p < 0.05$ vs VEH-treated cells.

Figure 2. GPR173 expression in GN11 cells. A) RT-PCR analysis of GPR101, GPR173, and GPR119 in GN11 cells showing GPR173 was the only GPCR detected. Adult mouse hypothalamic tissue was used as positive controls for each primer pair. As negative controls, RNA samples processed without reverse transcriptase (-RT) were used for PCR analysis to exclude genomic contamination. B) RT-PCR analysis on E12.5 nasal tissue and E11.5 nasal explant tissue cultured for 3 days in vitro (3 div) indicate GPR173 expression but not GPR101 or GPR119. C) The putative mRNA structure for GPR173 is shown with the location of PCR primers (indicated by arrows). Shaded gray indicate untranslated regions. White area indicates the coding region (CDS) between bases 355 –

1506. The whole mRNA sequence is estimated to be 3214 bases in length. D) The PathHunter™ GPR173-expressing cell line was used to show a dose dependent increase in GnRH-(1-5) binding to GPR173 as measured by β-arrestin recruitment activity. An EC_{50} value of 1.9 nM GnRH-(1-5) was obtained from this assay (n=3). E) The efficacy of GPR173 silencing using an siRNA mediated approach was measured by quantitative PCR. GN11 cells were treated with 25 nM of the GPR173 siRNA pool for 48 h and the total RNA was harvested. Control cells (VEH) were treated with the transfection reagent alone or a non-targeting siRNA pool. GN11 cells transfected with GPR173 siRNA had greater than 80% knockdown of GPR173 mRNA levels compared to VEH (n=3). Cyclophilin A was used as a reference gene. Data were analyzed by a one-way ANOVA followed by an LSD post hoc test. * $p<0.05$ relative to VEH.

Figure 3. Effect of GPR173 silencing on the GnRH-(1-5)-mediated inhibition of migration. GN11 cells were transfected with siRNA targeting GPR173 expression and the ability of GnRH-(1-5) to inhibit wound closure was measured. GN11 cells treated with 100 nM GnRH-(1-5) or 100 nM GnRH-(1-5) + non-targeting siRNA pool delayed wound closure. This effect was blocked in cells where GPR173 expression was down-regulated. Upper panel indicates representative photomicrographs of treatments. Scale bar indicates 250 μm. Lower panel indicates the quantification of the wound closure assay. Bars indicate SEM (n = 4). Data were analyzed by a one-way ANOVA followed by an LSD post hoc test. * $p < 0.05$.

Figure 4. Effect of GnRH-(1-5) treatment on pSTAT3 levels. A) GN11 cells were treated for 5, 15, and 30 min with 100 nM GnRH-(1-5) and the relative levels of pSTAT3(Y705) and STAT3 were measured by western blot analysis. GnRH-(1-5) treatment decreased pSTAT3 levels in a time-dependent manner reaching significance at 15 min. B) Conversely, treatment with the full-length decapeptide GnRH had no effect on the levels of pSTAT3. Bars indicate SEM (n=4). Data were analyzed by a one-way ANOVA followed by LSD pos hoc test. * $p < 0.05$ relative to VEH.

Figure 5. Effect of WP1066, an inhibitor of STAT3 phosphorylation, on GN11 cellular migration. A) GN11 cells treated for 30 min with increasing doses of WP1066 had reduced pSTAT3 levels. B) The doses of WP1066 did not induce apoptosis at 30 min, 18 h, or 24 h treatments as cleaved caspase-7 levels were not detected. C) A wound closure assay was used to determine the effect of WP1066 on migration. GN11 cells treated with increasing doses of WP1066 for 24 hrs delayed wound closure with a robust effect seen at 1uM. Scale bar indicates 250 μm.

Figure 6. Effect of GPR173 silencing on the GnRH-(1-5)-mediated decrease in pSTAT3 levels. A) GN11 cells were treated with the indicated siRNA treatment and treated with VEH or 100 nM GnRH-(1-5) for 30 min. Cells were fixed and immunostained for pSTAT3. Down-regulation of GPR173 reverses the GnRH-(1-5)-mediated decrease in pSTAT3 levels. Scale bar indicates 50 μm. B) Western blots showing GN11 cells treated for 30 min with 100 nM GnRH-(1-5) or 100 nM GnRH-(1-5) + non-targeting siRNA

pool had reduced pSTAT3 levels; however, silencing of GPR173 expression reversed this GnRH-(1-5) effect.

Figure 1

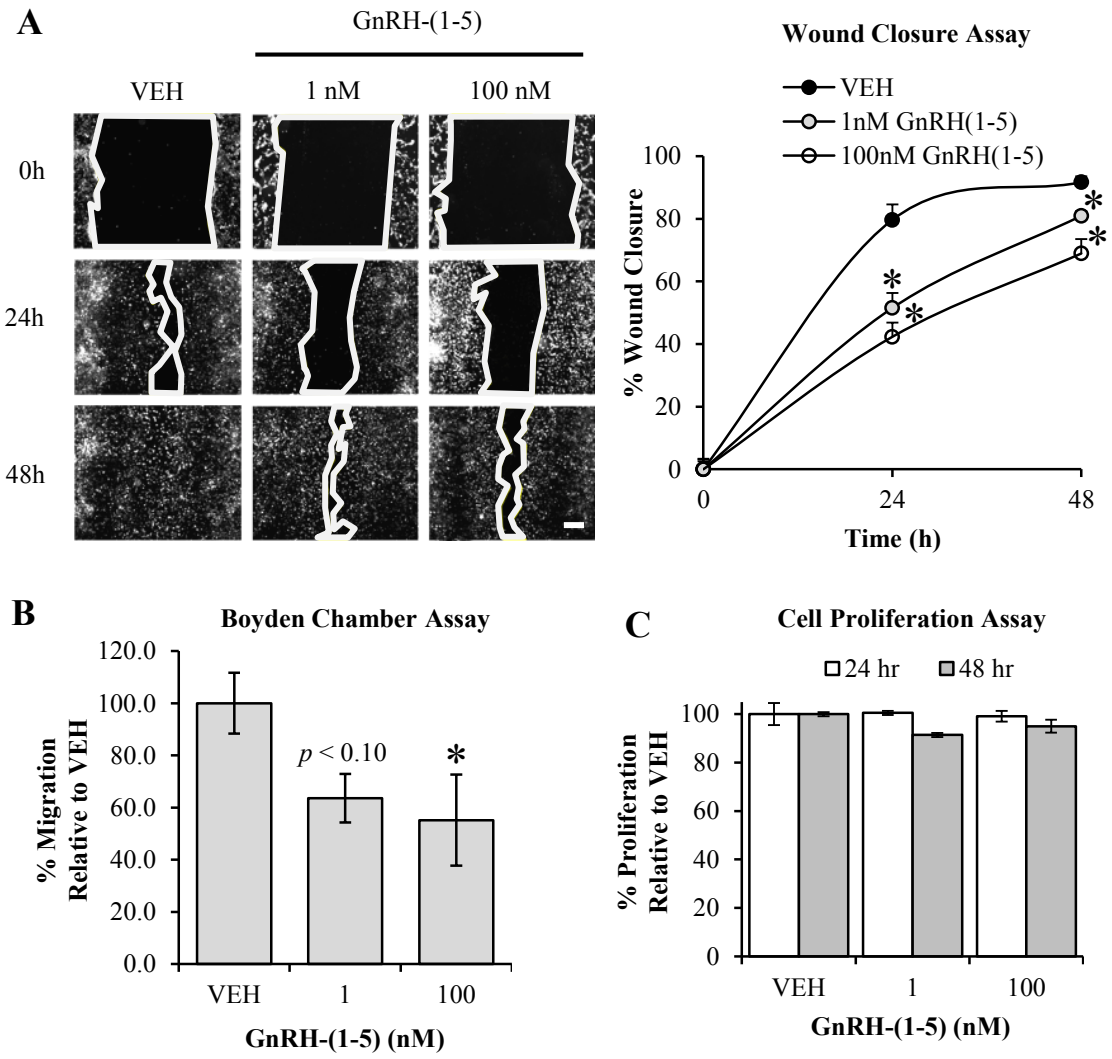

A
GnRH-(1-5)
VEH
1 nM
100 nM
0h
24h
48h
Wound Closure Assay
VEH
1nM GnRH(1-5)
100nM GnRH(1-5)
% Wound Closure
Time (h)
B
Boyden Chamber Assay
% Migration Relative to VEH
p < 0.10
GnRH-(1-5) (nM)
C
Cell Proliferation Assay
24 hr
48 hr
% Proliferation Relative to VEH
GnRH-(1-5) (nM)

Figure 2

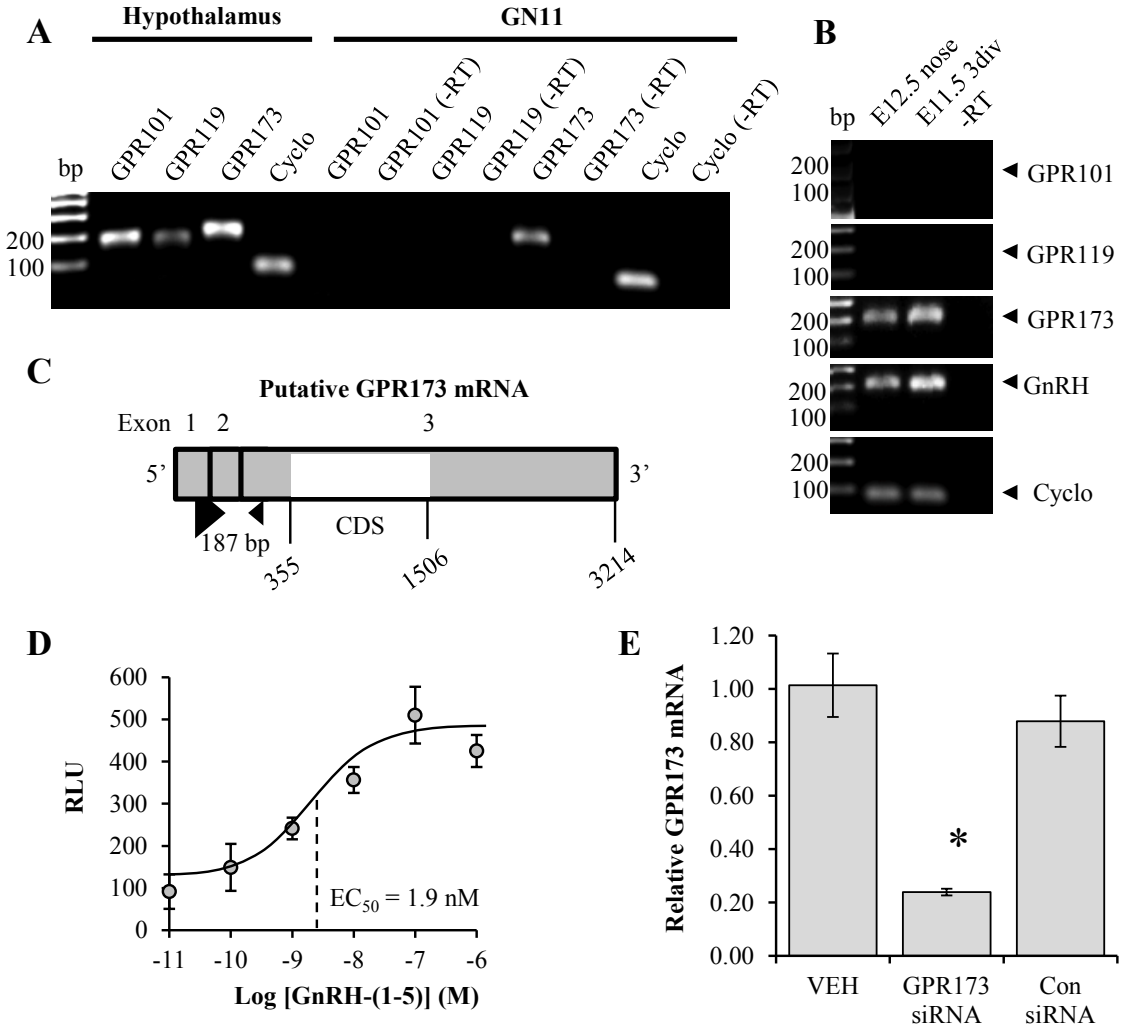

A
Hypothalamus
GN11
bp
GPR101
GPR119
GPR173
Cyclo
GPR101 (-RT)
GPR119 (-RT)
GPR173 (-RT)
Cyclo (-RT)
200
100
B
bp
E12.5 nose
E11.5 3div
-RT
GPR101
GPR119
GPR173
GnRH
Cyclo
C
Putative GPR173 mRNA
Exon 1 2 3
5'
3'
187 bp
CDS
355
1506
3214
D
RLU
600
500
400
300
200
100
0
-11 -10 -9 -8 -7 -6
Log [GnRH-(1-5)] (M)
EC50 = 1.9 nM
E
Relative GPR173 mRNA
1.20
1.00
0.80
0.60
0.40
0.20
0.00
*
VEH
GPR173 siRNA
Con siRNA

Figure 3

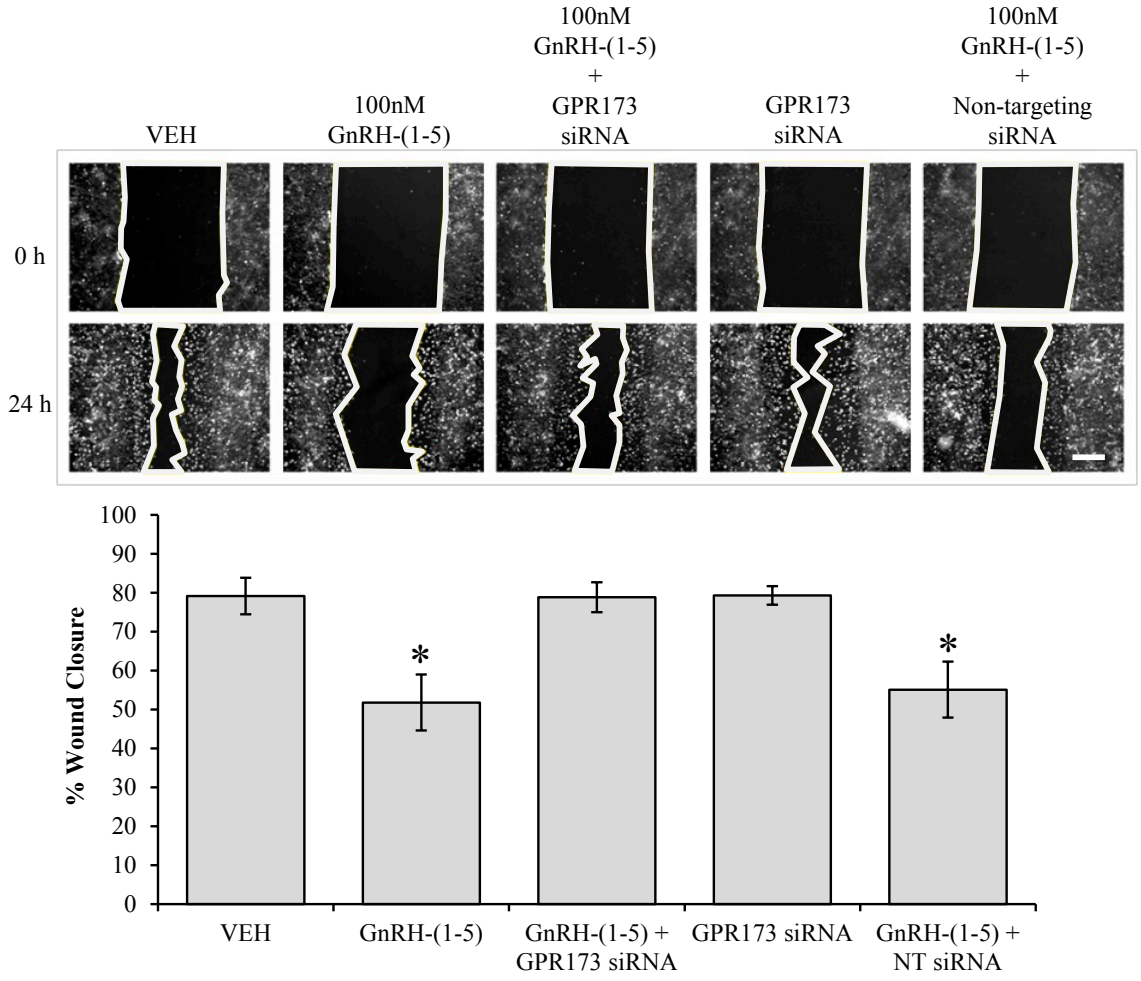

VEH
100nM GnRH-(1-5)
100nM GnRH-(1-5) + GPR173 siRNA
GPR173 siRNA
100nM GnRH-(1-5) + Non-targeting siRNA
0 h
24 h
% Wound Closure
100
90
80
70
60
50
40
30
20
10
0
*
*
VEH
GnRH-(1-5)
GnRH-(1-5) + GPR173 siRNA
GPR173 siRNA
GnRH-(1-5) + NT siRNA

Figure 4

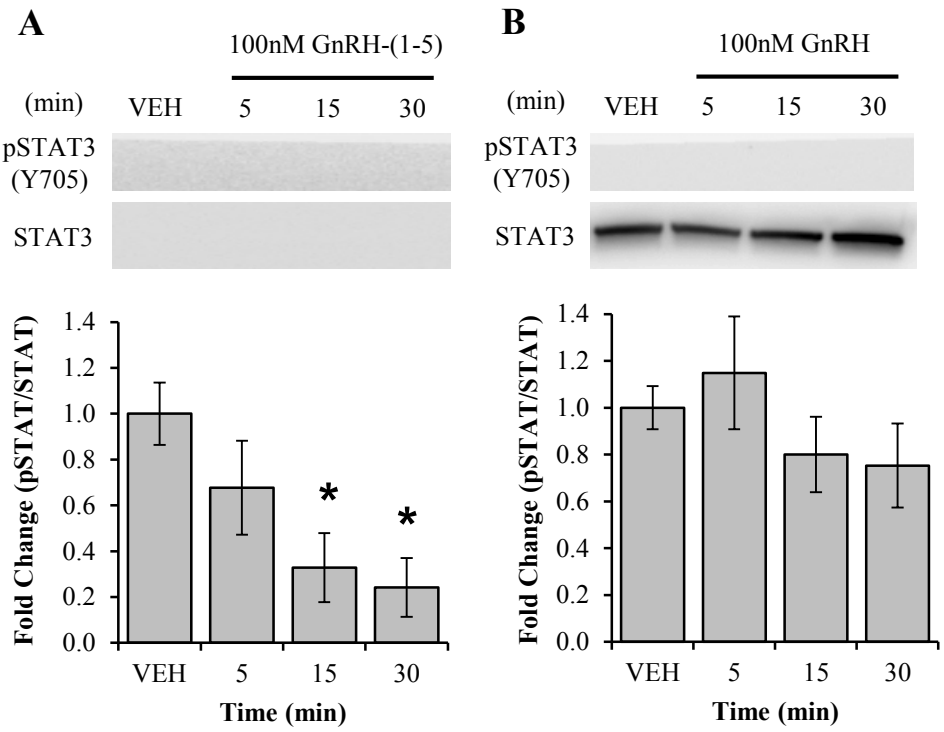

A
100nM GnRH-(1-5)
(min)
VEH
5
15
30
pSTAT3 (Y705)
STAT3
Fold Change (pSTAT/STAT)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
*
*
VEH
5
15
30
Time (min)
B
100nM GnRH
(min)
VEH
5
15
30
pSTAT3 (Y705)
STAT3
Fold Change (pSTAT/STAT)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
VEH
5
15
30
Time (min)

Figure 5

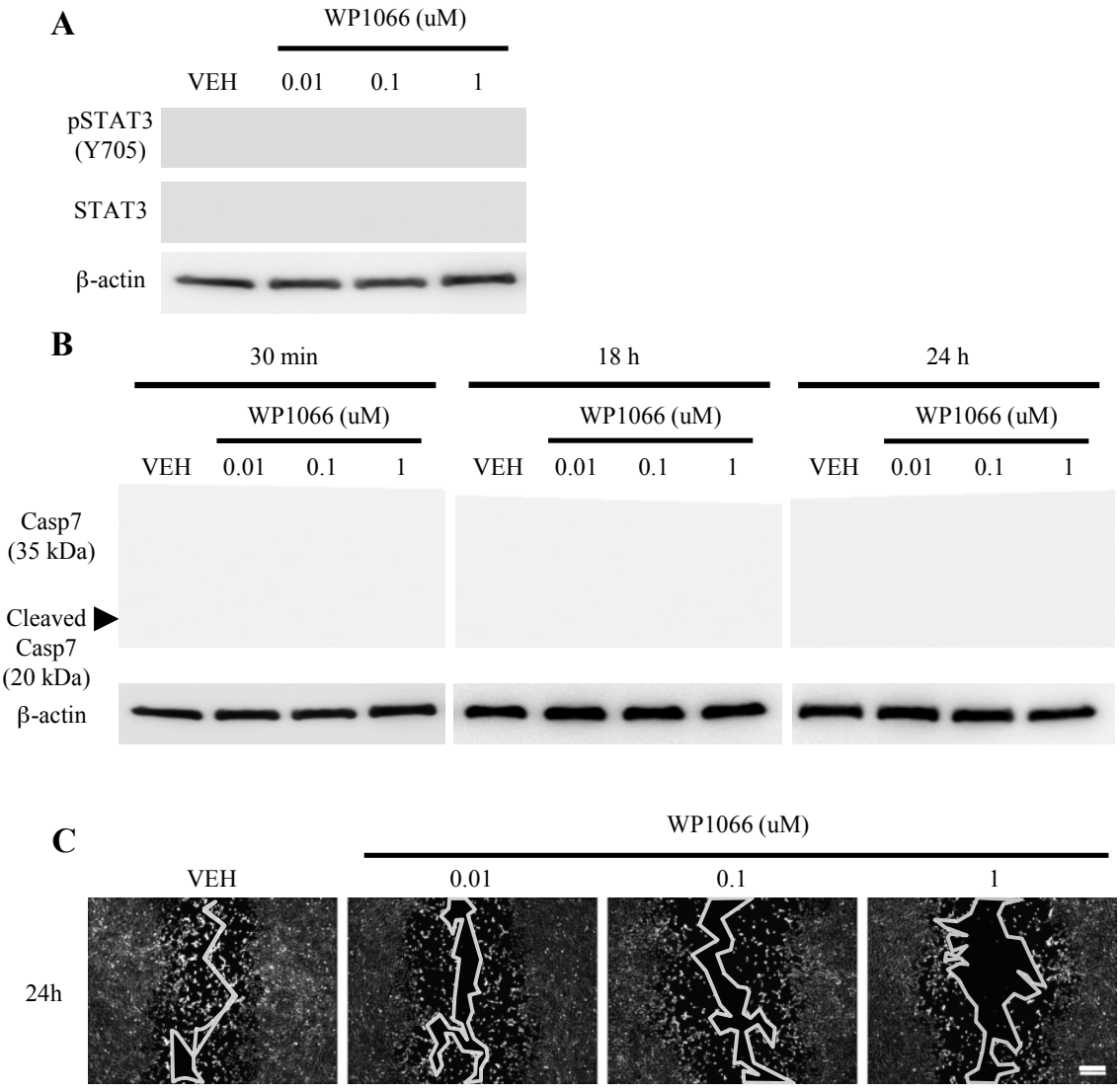
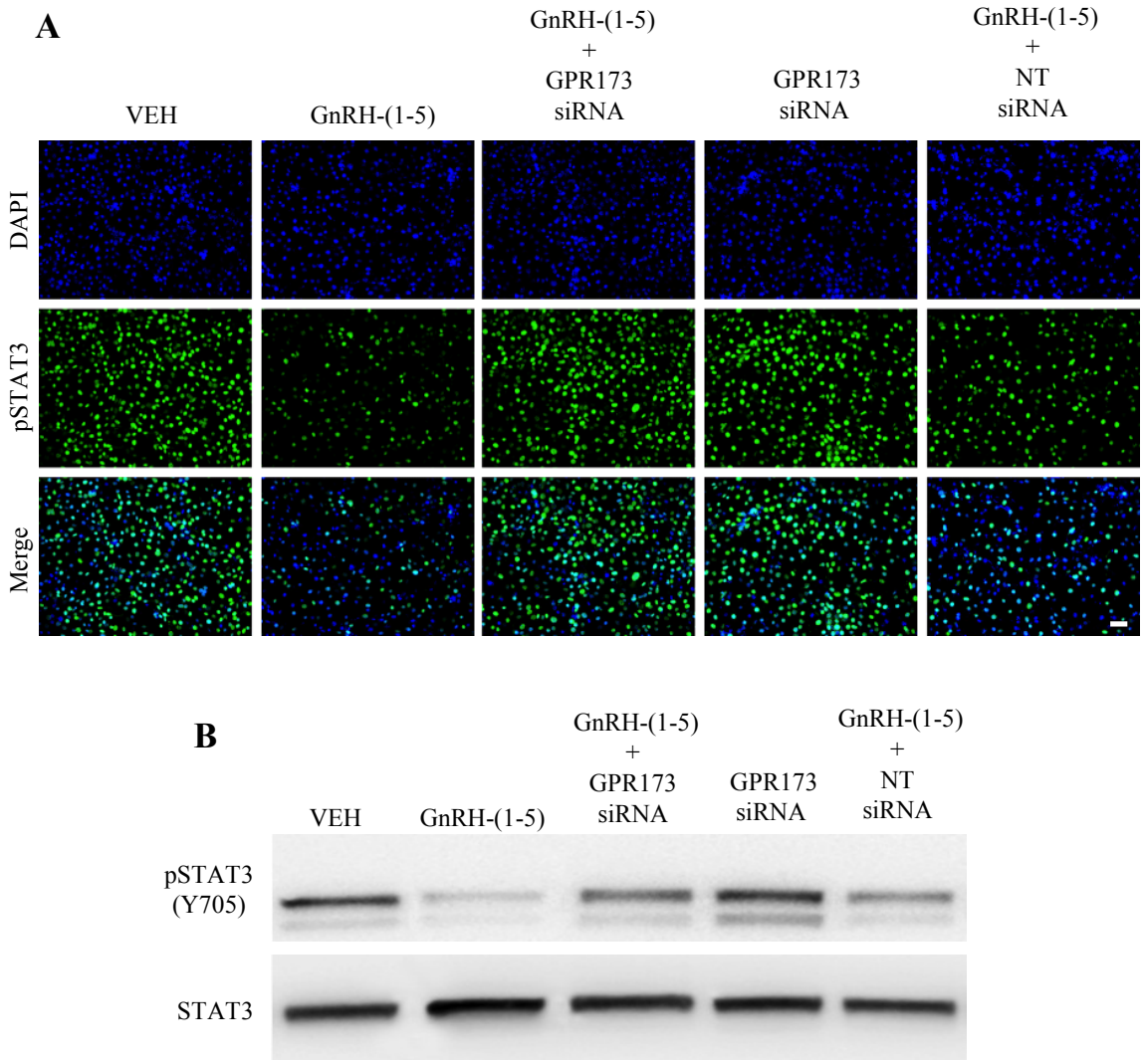

A
WP1066 (uM)
VEH
0.01
0.1
1
pSTAT3 (Y705)
STAT3
β-actin
B
30 min
18 h
24 h
WP1066 (uM)
VEH
0.01
0.1
1
Casp7 (35 kDa)
Cleaved Casp7 (20 kDa)
β-actin
C
WP1066 (uM)
VEH
0.01
0.1
1
24h

Figure 6


A
VEH
GnRH-(1-5)
GnRH-(1-5) + GPR173 siRNA
GPR173 siRNA
GnRH-(1-5) + NT siRNA
DAPI
pSTAT3
Merge
B
VEH
GnRH-(1-5)
GnRH-(1-5) + GPR173 siRNA
GPR173 siRNA
GnRH-(1-5) + NT siRNA
pSTAT3 (Y705)
STAT3

CHAPTER 4: Manuscript 2

Title:	Identification of the GnRH-(1-5) signaling pathway in immortalized GnRH neurons.
Abbreviated Title:	GnRH-(1-5) regulates the migration of GN11 neurons
Authors:	Darwin O. Larco, Nina N. Semsarzadeh, Madelaine Cho-Clark, Shaila K. Mani and T. John Wu
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Disclosure Statement:	The authors have nothing to disclose.

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Key Words: GnRH, GPCR, migration, GN11, GPR173

Abstract

We have previously demonstrated that the cleavage product of the full-length GnRH, GnRH-(1-5), is biologically active, binds G protein-coupled receptor 173 (GPR173), and inhibits the migration of cells in the immortalized GnRH-secreting GN11 cell. In this study, we attempted to characterize the GnRH-(1-5) intracellular signaling mechanism. To determine whether the signaling pathway mediating GnRH-(1-5) regulation of migration involves a G protein-dependent mechanism, cells were treated with a generic G protein antagonist (GPAnt2) in the presence and absence of GnRH-(1-5) and a wound healing assay was conducted to measure migration. GPAnt2 treatment abolished the GnRH-(1-5) inhibition of migration, indicating the mechanism of GnRH-(1-5) is G protein-coupled. To identify the potential Gα subunit recruited by GnRH-(1-5) binding GPR173, we measured the second messengers cyclic adenosine monophosphate (cAMP) and inositol triphosphate (IP3) levels. GnRH-(1-5) treatment did not alter cAMP levels relative to cells treated with vehicle or forskolin, suggesting GnRH-(1-5) does not couple to the Gαs or Gαi subunits. Similarly, IP3 levels remained unchanged with GnRH-(1-5) treatment, indicating a mechanism not mediated by the Gαq/11 subunit. Therefore, we also examined whether GnRH-(1-5) activating GPR173 deviated from the canonical GPCR signaling pathway by coupling to β-arrestin 1/2 to regulate migration. Our co-immunoprecipitation studies indicate that GnRH-(1-5) induces the rapid interaction between GPR173 and β-arrestin 2 in GN11 cells. Furthermore, we demonstrate that this association recruits PTEN to mediate the downstream action of GnRH-(1-5). These findings suggest that the GnRH-(1-5) mechanism deviates from the canonical GPCR pathway to regulate cell migration in immortalized GnRH neurons.

Introduction

G protein-coupled receptors (GPCRs) are key regulators of cell signaling and have been shown to respond to a variety of ligands. All GPCRs share a seven transmembrane-spanning topology linked together by intracellular and extracellular loops. The GPCR signaling pathway is commonly thought to involve the recruitment of the heterotrimeric G protein complex composed of the Gα, β, and γ subunits. Subsequently, depending on the Gα isoform, the levels of certain second messengers can be mobilized to regulate intracellular processes. In the pituitary, the gonadotropin-releasing hormone receptor (GnRHR), a GPCR belonging to the Rhodopsin family of receptors, recruits the $G\alpha_{q/11}$ complex, which initiates a cascade of events leading to an increase in intracellular Ca^{2+} levels facilitating gonadotropin secretion (18; 28).

The decapeptide gonadotropin-releasing hormone (GnRH) is a central regulator of reproduction mediated by the well characterized GnRHR (5; 18). Interestingly, we have previously demonstrated that metabolic product of GnRH, GnRH-(1-5), is biologically active by regulating GnRH-related functions. These include regulating GnRHmRNA expression (33) and reproductive behavior in female rats (32). Furthermore, our studies also show GnRH-(1-5) to be involved in a developmental function by regulating the migration of immortalized GnRH neurons, the GN11 cell, via the activation of a GPCR, GPR173 (10). During development, GnRH neurons are born outside the central nervous system (CNS) and migrate along the vomeronasal nerve, and subsequently entering the cribiform plate to reach their final destinations within the basal forebrain region (25). A number of GPCRs have been implicated in the proper migration of GnRH neurons, such as the fibroblast growth factor receptor (FGFR) and PROKR (19), since mutations in

these receptors lead to hypogonadotropic hypogonadism (HH) or Kallmann syndrome (KS). Our previous work with the GN11 cell line implicated GPR173 as a regulator of GnRH neuronal migration; however, the downstream mechanism has not been completely elucidated.

In this study, we investigated the intracellular mechanism by which GnRH-(1-5) activates GPR173 in immortalized GnRH neurons, the GN11 cell (21). We measured the levels of the second messengers in response to GnRH-(1-5) treatment to determine the specific G protein involved. Additionally, we assessed whether GPR173 recruits β-arrestin due to GnRH-(1-5) treatment and whether this interaction is required for GnRH-(1-5)-mediated regulation of migration. GPCR recruitment of β-arrestin has been shown to be important for receptor internalization and desensitization (1); however, there is evidence suggesting β-arrestin may play a role in other functions including cellular migration (2; 12; 15; 35). We demonstrate that GnRH-(1-5) activation of GPR173 deviates from the canonical signaling pathway; yet, rapidly recruits β-arrestin to inhibit GN11 cellular migration.

Methods

Cell Culture and Transfection

GN11 cells (generously donated by Dr. S. Radovick; Johns Hopkins University School of Medicine, Baltimore, MD) were grown in Dulbecco's modified Eagle's medium (Mediatech Inc., Herndon, VA) without antibiotics supplemented with 7% fetal bovine serum (FBS; Hyclone, Logan, UT), 3% newborn calf serum (Hyclone), 25 mM glucose, and 5 mM L-glutamine (10; 20). Cells were maintained at 37º C in an atmosphere with 5% CO_2.

Reagents

D-Trp GnRH (Cat. No. H4075) and GnRH-(1-5) were purchased (Bachem, Torrance, CA). Peptides were dissolved in water to 1 mM and subsequently serially diluted in culture media to its final concentration. Phosphatase and tensin homolog (PTEN) protein inhibitor (bpV(HOpic)) was purchased (EMD Millipore, Billerica, MA) and dissolved in water to 5 mg/mL. G protein antagonist-2 (GPAnt2) was purchased from Tocris (Cat. No. 1931; Bristol, United Kingdom) and dissolved in 100% DMSO to 1 mg/mL. Control experiments included the appropriate solvent (water or DMSO) not exceeding more than 0.01% indicated as vehicle (VEH). A complete table of antibodies used in these experiments are included in the supplemental section.

Wound Closure Assay

A wound closure assay was used to determine the effect of GnRH-(1-5) on GN11 migration as previously described (10). Briefly, GN11 cells were plated on plastic uncoated 6-well plates (Costar; Corning, NY) and allowed to reach 80% confluency. The cells were starved for 24 h in 10% charcoal-stripped dextran-treated FBS (Atlanta

Biologicals; Lawrenceville, GA) and tested for migration using *in vitro* wound closure assay. In each well 2 scratches were made through a cell monolayer with a sterile 1000 uL pipette tip. The cells were washed 3 times with phosphate buffered saline (PBS) to remove detached cells and incubated with 10% stripped FBS containing the indicated treatment condition. Multiple images were taken using a Leica AF6000 microscope (Leica Microsystems; Allendale, NJ) of each scratch area and its location was noted. Subsequent pictures were taken at 24 after the addition of treatments. The wound area was measured using the ImageJ software (National Institutes of Health; Bethesda, Maryland)(24). The percent wound closure was calculated with the following formula: % wound closure = $((area_{t=0h} - area_{t=24h})/area_{t=0h})$ X 100%. Similar protocols have been previously described using cancer cell models (7; 29) and with the GN11 cell line (11; 26; 34).

Subcellular Fractionation

Subcellular fractions were isolated as previously described with some modification (6; 23). GN11 cells were rinsed twice with ice cold PBS. The cells were then lysed in buffer containing 25 mM sucrose, 1.5 mM MgCl2, 50 mM NaCl, 25 mM HEPES (pH 7.2). The cells were subsequently homogenized by sonication and centrifuged at 800 g for 5 minutes. The supernatant was retained and the resulting pellet was reconstituted in buffer, then the homogenization and centrifugation steps were repeated. This process was repeated one more time to generate a third supernatant fraction and pellet containing the nuclear portion of the cell. All three supernatants were combined and centrifuged at 20,000 g for 15 minutes at 4º C. The supernatant, which contains the cytosolic fraction of the cells, was collected. The remaining pellet,

containing the membrane fraction, was homogenized in Immunoprecipitation (IP) lysis/Wash Buffer [0.025 M Tris, 0.15 M NaCl, 0.001 M EDTA, 1% NP-40, 5% glycerol; pH 7.4 (Thermo Scientific)]. All fractions were saved for western blot analysis or co-immunoprecipitation (co-IP) studies.

Co-Immunoprecipitation (co-IP) Assay

To determine which proteins interact directly with the GPR173 receptor, co-IP assays were performed using the Pierce Co-Immunopreciptiation Kit (Thermo Scientific, Rockford, IL) according to manufacturer's instructions. The appropriate antibody (anti-rabbit GPR173 1:5000; Sigma-Aldrich, St Louis, MO; anti-rabbit β-Arrestin 1/2; Cell Signaling Technology, Inc., Danvers, MA) was covalently coupled to agarose resin in a spin column using AminoLink Plus coupling resin (Thermo Scientific). 275 µg of protein, determined using the Pierce BCA Protein Assay Kit (Thermo Scientific), was incubated with the antibody-coupled resin overnight in 4º C with gentle rocking. The resin was washed seven times with PBS before the protein complexes were eluted with supplied amine-based elution buffer (pH 2.8). The elutant fractions were checked for protein by measuring the absorbance at 280 nm with a NanoDrop (Thermo Scientific).

Western Immunoblotting

Protein samples were loaded onto a 4-15% Tris-HCL gels (BioRad, Hercules, CA) and resolved by SDS-PAGE. The gel was transferred onto a polyvinyldifluoride membrane using a Trans-Blot Turbo Transfer System (BioRad) and incubated in 5% non-fat dry milk at room temperature for 1 hour to block non-specific antibody binding. The membrane was then rinsed with TBS-Tween (TBST) and incubated with the primary antibody overnight at 4º C. The membrane was subsequently rinsed 3 x 10 minutes with

TBST, incubated with the secondary antibody at an appropriate dilution conjugated to horseradish peroxidase (Bio-Rad) for 1 hr at room temperature, then rinsed 5 x 15 min with TBST. The blots were visualized by chemiluminescence (Millipore, Billerica, MA) using a Fujifilm LAS-3000 imager (Fujifilm; Stamford, CT). Densitometry was measured using Fujifilm Image Gauge Software (Valhalla, NY).

Measuring cAMP and IP_1 levels

A competitive enzyme-linked immunosorbent assay (ELISA) kit (CIS Bio International, France) was used to determine IP_1 levels in GN11 cells treated with GnRH-(1-5). GN11 cells were plated in a 24-well plate and incubated overnight at 37º C and 5% CO2. Cells were subsequently treated with vehicle, 1 nM, or 100 nM GnRH-(1-5) for 1 hour. Following treatment, the cells were lysed with 1% lysis reagent supplied by the assay manufacturer in 5% CO_2 at 37 °C for 30 min. A competitive ELISA (Caymen Chemical, Ann Arbor, MI) was performed to determine the concentrations of cAMP levels. Confluent GN11 cells were treated for 5 minutes with VEH, 1 nM, or 100 nM GnRH-(1-5) for 5 min. Samples were normalized to protein content using the Pierce BCA Protein Assay Kit (Thermo Scientific). Samples for each ELISA were run in duplicate and each ELISA was repeated at least four times.

Immunofluorescent (IF) Studies

GN11 cells were washed with ice-cold PBS and fixed by methanol fixation for 10 min at -20 C. Subsequently, the cells were sequentially washed with PBS (3 times, 5 minutes each), followed by blocking with 10 % normal goat serum (1 hour; Sigma-Aldrich) at room temperature. Cells were then incubated overnight in a PBS solution containing 0.1% triton in addition to anti-GPR173 (1:5000; Sigma) and anti-GnRH

(1:2000; SMI-41). The following day cells underwent additional washes and were incubated with the appropriate Alexa Fluor conjugated secondary antibody (1:2000; Jackson ImmunoResearch Laboratories, West Grove, PA) for 2 hours. After washing with PBS, the cells were coverslipped. Nuclear staining was performed with a 4,6-diamidino-2-phenylindole counterstain (Invitrogen, Eugene, Oregon). Images were taken with a Leica AF6000 microscope (Leica).

Statistical analysis

Statistics were conducted using the analysis software SPSS (version 16.0; IBM Corp, Armonk, NY). Wound closure data and ELISA results were analyzed by a 1-way ANOVA, followed by the Fisher least significant difference (LSD) post hoc test. A value of $p < 0.05$ was considered significant.

Results

Effect of G Protein Antagonist-2 (GPAnt2) on the GnRH-(1-5) Regulation of Migration

We have previously demonstrated that GnRH-(1-5) inhibits the migration of GN11 cells in a wound closure assay (10). To examine whether the parent peptide GnRH regulates migration, GN11 cells were treated with vehicle (VEH), 100nM GnRH-(1-5), and 100 nM GnRH (D-Trp) and conducted a wound closure assay. GnRH-(1-5) treatment significantly ($p < 0.05$) delayed wound closure; however, treatment with GnRH had no effect relative VEH (Figure 1A). Treatment of GN11 cells with a broad G protein antagonist, GPAnt2, in the presence or absence of GnRH-(1-5) followed by the wound closure assay demonstrated that GPAnt2 reversed the GnRH-(1-5)-mediated inhibition of migration while GPAn2 alone had no significant effect on migration relative to cells treated with VEH (Figure 1B).

Effect of GnRH-(1-5) on cAMP and IP_3 Levels

To examine whether GnRH-(1-5) activates a second messenger system to regulate migration, we measured the levels of cAMP and IP_3 in GN11 cells treated with VEH, 1 nM, or 100 nM GnRH-(1-5). Treatment for 5 min did not significantly alter cAMP levels. Additionally, we treated cells with 10 uM forskolin to induce an increase in cAMP levels (Figure 2A). Acute treatment with forskolin significantly ($p < 0.05$) increased cAMP levels; however, GnRH-(1-5) treatment did not change the effect of forskolin (Figure 2A). Next, we determined whether IP_3 levels were regulated by GnRH-(1-5) treatment. Since IP_3 has a very short half-life (30), we decided to measure its degradation product IP_1, which is stable in the presence of LiCl. Treatment with GnRH-(1-5) treatment did not

significantly change the levels of IP_1, indicating that IP_3 levels were also not altered (Figure 2B). As a control experiment, GN11 cells were also treated for different time points up to 1 hr and the levels of IP1 were measured; however, no effect was observed (data not shown).

Identification of the GnRH-(1-5) Downstream Signaling Pathway

Since a G protein mechanism could not be implicated in GnRH-(1-5) action, we used a specific GPR173 antibody to identify interacting proteins involved in cellular migration, which also deviate for the GPCR signaling pathway. First, we wanted to determine the specificity of the GPR173 antibody by western blot and IF analysis. A crude membrane fraction of GN11 cells was prepared and probed for GPR173. A single band corresponding to GPR173 was observed by western blot (Figure 3A). Subsequently, we conducted a peptide preabsorption experiment with the antigen used to make the GPR173 antibody. In this experiment, 2 concentrations of peptide were used and in both cases the band corresponding to GPR173 was no longer detected, indicating the specificity of the antibody (Figure 3A). Next, we determined the cellular distribution of GPR173 in GN11 cells by IF relative to GnRH staining. GnRH localization was more diffuse and characteristic of cytoplasmic distribution; however, GPR173 levels were more punctate and broadly distributed throughout the GN11 cell (Figure 3B).

To investigate the cellular localization of GPR173 and whether its levels are regulated by GnRH-(1-5), GN11s were treated with VEH or 100 nM GnRH-(1-5) for 5, 15, and 30 min. GnRH-(1-5) treatment did not alter the levels of GPR173 in the membrane or soluble fraction while GPR173 expression was not detected in the nuclear fraction (Figure 4). Flotillin 1 was used as a membrane marker to demonstrate the

efficiency of the subcellular fractionation. We detected flotillin 1 only in the plasma membrane fraction while no detection was observed in the soluble and nuclear fractions (Figure 4). Additionally, we saw no changes in flotillin 1 due to GnRH-(1-5) treatment (Figure 4). Next, since we have previously determined that GnRH-(1-5) decreases the basal level of phosphorylated STAT3 (pSTAT3) (10), we measured the levels of pSTAT3 in the fractions isolated. The levels of pSTAT3 were decreased beginning at 15 min with GnRH-(1-5) treatment in both the membrane and nuclear fraction (Figure 4).

With the availability of a specific GPR173 antibody, we wanted to determine whether GnRH-(1-5) binding to GPR173 recruits a G protein. Since we saw no changes in the second messengers associated with the canonical GPCR signaling pathway, we began our search for potential interacting G proteins by looking at the $G\alpha_{12}$ and $G\alpha_{13}$ subunits using a co-immunoprecipitation (co-IP) approach. GN11 cells were treated with VEH or 100 GnRH-(1-5) for 5 min and the plasma membrane fraction was analyzed by co-IP. GnRH-(1-5) treatment did not alter the levels of membrane localized GPR173. Furthermore, the G protein subunits $G\alpha_{12}$ or $G\alpha_{13}$ did not co-IP with GPR173 due to GnRH-(1-5); however protein levels were detected in the input (membrane fraction) as demonstrated by flotillin 1 protein expression (Figure 5). In the absence of detectable G protein recruitment, we decided to examine whether β-arrestin 1 or 2 were recruited upon GnRH-(1-5) treatment since we have previously demonstrated this association in a GPR173 over-expressing cell line (10). Interestingly, we found GN11 cells treated with GnRH-(1-5) robustly increased β-arrestin relative to VEH treatment (Figure 5). Additionally, this was specific to β-arrestin since pSTAT3 or its negative regulator SOCS3 did not co-IP with GPR173 (Figure 5). Nor was there an interaction with

receptors established as activators of STAT3 signaling such as the type 1 cytokine receptors, leukemia inhibitory factor receptor (LIFR) or the leptin receptor (Ob-R) (Figure 5). Subsequently, we wanted to confirm the interaction between GPR173 and β-arrestin is dependent on GnRH-(1-5) by conducting a co-IP with the β-arrestin antibody. As expected, GnRH-(1-5) robustly enhanced the association between GPR173 and β-arrestin confirming the interaction (Figure 6A). Since we used an antibody that detects both β-arrestin 1 and β-arrestin 2, we wanted to identify which β-arrestin member was specific to GnRH-(1-5) signaling. Interestingly, we found that only β-arrestin 2 co-IPs with GPR173 while β-arrestin 1 was not detected in the GN11 cell (Figure 6B).

Since the membrane levels of GPR173 did not change due to GnRH-(1-5) treatment, it is likely the recruitment of β-arrestin 2 plays a role independent of receptor internalization and desensitization. Recent work by Lima-Fernandes and colleagues give evidence that β-arrestin 1 and 2 may act as a scaffold for the phosphatase and tensin homolog (PTEN) to regulate cellular migration (12). To test this effect in our model, GN11 cells were treated with VEH or 100 nM GnRH-(1-5). We found that PTEN co-IPs with β-arrestin 2 only with GnRH-(1-5) treatment (Figure 6A). Subsequently, we examined whether PTEN activity is required for the action of GnRH-(1-5) on migration. GN11 cells were treated with a potent PTEN inhibitor (bpV(HOpic)) at 12 nM and 120 nM doses in the absence or presence of GnRH-(1-5) and a wound closure assay was conducted. Incubation with the PTEN inhibitor at the 12nM or 120 nM bpV(HOpic) blocked ($p < 0.05$) the GnRH-(1-5)-mediated inhibition of GN11 cellular migration (Figure 7).

Discussion

We have previously demonstrated that GPR173 is required for the GnRH-(1-5)-mediated inhibition of GN11 cellular migration (10). In the present study we identify key effectors induced by the binding of GnRH-(1-5) to GPR173. Our findings demonstrate that the effect of GnRH-(1-5) is G protein-dependent since co-treatment with GPAnt2, a G protein antagonist with broad specificity (17), reversed the inhibition on migration; however, identification of the Gα subunit remains to be addressed. Interestingly, we found that GnRH-(1-5) binding to GPR173 rapidly recruits β-arrestin 2 and likely serves as a scaffold for proteins important for cytoskeletal rearrangement. We also show that β-arrestin 2 interacts with PTEN, an observation previously described by Lima-Fernandes and colleagues (12), in a GnRH-(1-5)-dependent mechanism. Our results demonstrate that GnRH-(1-5)-mediated activation of GPR173 deviates from the canonical GPCR signaling cascade by recruiting β-arrestin 2 to regulate GnRH neuronal migration.

The metabolite GnRH-(1-5) may likely play different roles during development and in adulthood. Our previous work demonstrates that GnRH-(1-5) can regulate GnRH mRNA levels (33) and facilitates lordosis behavior in the female rat independent of GnRHR signaling (32). In addition we recently gave evidence to suggest that GnRH-(1-5) is involved in the development of the GnRH neuroendocrine system by modulating the migration of GnRH neurons; and that this effect required the ability of GnRH-(1-5) to bind the orphan receptor, GPR173 (10). In this study, we extend our findings by characterizing the intracellular signaling pathway mediated by the interaction of GnRH-(1-5) with GPR173.

As previously described, GnRH-(1-5) significantly delayed the migration of GN11 cells in a wound closure assay (10); an effect that was specific to GnRH-(1-5) since treatment with a GnRH analogue resistant to cleavage between the 5^{th} and 6^{th} amino acid did not alter GN11 cellular migration. Subsequently, we examined whether the effect of GnRH-(1-5) was dependent on G protein signaling using the antagonist GPAnt2, which broadly blocks G protein recruitment by GPCRs (17). Interestingly, we found that GPAnt2 blocked the ability of GnRH-(1-5) to inhibit migration, indicating that the mechanism involves G protein activation. The canonical GPCR signaling pathway involves the mobilization of a heterotrimeric G protein complex, which are composed of the following three subunits: Gα, β, and γ. Depending on the specific Gα subunit, the levels of the second messengers cAMP or IP_3/diacylglycerol (DAG) can change leading to a cascade of intracellular events. Thus to identify the G protein involved we measured the levels of cAMP; however GN11 cells treated with GnRH-(1-5) had unaltered levels of cAMP. In addition GnRH-(1-5) did regulate the levels of cAMP in response to forskolin treatment. Collectively, these results suggest a mechanism independent of the $G\alpha_s$ and $G\alpha_i$ subunits as these are implicated in the regulation of adenylyl cyclase, which is the enzyme responsible for the synthesis of cAMP (22). Similarly, we found the levels of IP_1, an indirect method of assessing IP_3 levels, remained unchanged with GnRH-(1-5) treatment ruling out the $G\alpha_{q/11}$ subunit. In the absence of signaling through the GPCR canonical pathway, we also looked at the $G\alpha_{12/13}$ subunits since they have been implicated in the regulation of neuronal migration (16). Unfortunately, we could not co-IP these subunits in response to GnRH-(1-5) treatment. However, we cannot completely exclude recruitment of these subunits since it is possible that our current methods of

measuring G protein activation are not sensitive enough or within the optimal time frame to detect an interaction. Current advances in fluorescence and bioluminescence resonance energy transfer technologies have allowed investigators to overcome these obstacles (3; 14). Our future work will begin to implement these techniques to identify the G protein mediating the actions of GnRH-(1-5).

There is a growing body of evidence demonstrating that GPCRs can signal through myriad mechanisms. For example gonadotropic cells in the pituitary expressing the GnRHR upon GnRH binding can increase intracellular Ca^{2+} levels to facilitate the vesicular release of LH and FSH (18; 28). However, in the hypothalamus the mechanism of GnRHR can be quite different. Using the hypothalamic GnRH-secreting cell line, the GT1-7 cell, GnRHR activation can act as a positive and negative regulator of cAMP levels (9). Furthermore, other effectors, independent of the heterotrimeric G protein complex, can mediate the actions of certain GPCRs. This has been the case for β-arrestin, which was initially discovered to be a negative regulator of the β-adrenergic receptor (13) and other GPCRs. However, more recently, β-arrestin has been implicated as a scaffold to localize molecules essential for cytoskeletal reorganization required for cell migration (8). Interestingly, our results demonstrate that GnRH-(1-5) binding to GPR173 rapidly recruits β-arrestin. This was a surprising interaction since the level of GPR173 in the membrane was unaltered, indicating that the function of β-arrestin is independent of GPR173 desensitization but may serve as a scaffold for signal transducers. Additionally, our co-IP experiments suggest that the GPR173 interaction is specific to β-arrestin 2 since β-arrestin 1 protein levels were not detected in the GN11 cell. This is an exciting finding demonstrating that GnRH-(1-5) binding to GPR173 deviates from the canonical

GPCR signaling pathway by recruiting β-arrestin 2. Furthermore, these results reinforce the role played by β-arrestin 2 in other intracellular events independent of GPCR desensitization.

The mechanism of GnRH-(1-5) inhibiting GN11 cellular migration is in part mediated by decreasing the levels of pSTAT3 (10). We have previously shown that this effect requires GPR173 (10); however the mechanism of GnRH-(1-5) decreasing pSTAT3 levels has not been identified. In an effort to address this gap, we examined whether GPR173 recruitment of β-arrestin 2 acts as a scaffold for PTEN. Previous work by Lima-Fernandes and colleagues elucidated the novel function of β-arrestin 2 to interact with PTEN to regulate cellular migration (12). Furthermore, this interaction may relate to STAT3 signaling since PTEN has been shown to negatively regulate the phosphorylation of STAT3 (27). Interestingly, we found that PTEN associates with β-arrestin 2 in a GnRH-(1-5)-dependent manner, reinforcing the role of β-arrestin 2 to act as a scaffolding protein. Furthermore, we show that a PTEN specific inhibitor can block the GnRH-(1-5)-mediated inhibition on GN11 cellular migration, demonstrating that PTEN activity is necessary for this action. However, the direct mechanism of how PTEN decreases STAT3 signaling is unclear. The interaction of β-arrestin and PTEN in other cell models used suggest that PTEN is sequestered by β-arrestin, which blocks the ability of PTEN to inhibit pathways aimed at promoting migration (12). This is in contrast to what we propose since GnRH-(1-5) decreases pSTAT3 levels and cellular migration while facilitating the interaction of β-arrestin 2 and PTEN. It is possible that PTEN retains its ability to negatively regulate the STAT3 pathway even if the interaction between PTEN and β-arrestin 2 persists. Additionally, these discrepancies may in part be

explained by the different *in vitro* models utilized. GN11 cells endogenously express β-arrestin 2 and are considered neuronal in phenotype while other models used to study the interaction between PTEN and β-arrestin do not endogenously express β-arrestin 1or 2. (12). In GN11 cells, GnRH-(1-5) binding to GPR173 enhances the association of β-arrestin 2 with PTEN, leading to an inhibition of cellular migration. Thus the role played by β-arrestin 2 interacting with PTEN is likely cell type dependent.

The guidance cues enabling the proper migration of GnRH neurons from the nasal region to the CNS remain largely unknown (25; 31). Interestingly, many receptors implicated in the development of KS and hypogonadism are GPCRs (4; 19), underscoring the significance of these receptors in the proper function of the reproductive system. Our current studies implicate GnRH-(1-5) acting through GPR173 regulates the migration of GnRH neurons by enhancing the recruitment of β-arrestin 2, which subsequently interacts with PTEN to inhibit STAT3 signaling and migration. Formation of this complex is G protein-dependent; however identification of the specific Gα subunit remains to be elucidated. Physiologically GnRH-(1-5) may mediate a transient change in the migration rate of GnRH neurons during development. This change in rate is likely important when GnRH neurons are transitioning from a radial migratory trajectory along the vomeronasal tract to a tangential one as they begin to enter the CNS through cribiform plate. Future work will continue to elucidate the significance of the GnRH-(1-5) and GPR173 interaction in regulating GnRH neuronal migration using animal models.

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Figure Legends

Figure 1. Effect of GPAnt2 on the GnRH-(1-5)-mediated inhibition of GN11 cellular migration. A) GN11 cells treated with 100 nM GnRH-(1-5) for 24 h delayed wound closure relative to VEH and GnRH (D-trp) treatment. Left panel indicates the representative photomicrographs of each treatment in the wound closure assay. Right panel indicates the quantification of the wound closure assay (n=6). B) GN11 cells with the GPAnt2 reversed GnRH-(1-5)-mediated inhibition of migration. Left panel indicates the representative photomicrographs of each treatment in the wound closure assay. Right panel indicates the quantification of the wound closure assay (n=4). Data were analyzed by a one-way ANOVA followed by an LSD post hoc test. Scale bar indicates 100 μm. * $p < 0.05$ relative to VEH.

Figure 2. Effect of GnRH-(1-5) on second messenger systems. A) GN11 cells were treated with the indicated treatments for 5 minutes. GnRH-(1-5) treatment did not alter the levels of cAMP while forskolin treatment robustly increased cAMP levels regardless of GnRH-(1-5) treatment. Bars represent cAMP levels normalized to VEH-treated cells (n=4). B) The levels of IP_3 were measured indirectly by assaying for its metabolite, IP_1. GN11 cells were treated for 1 h with the indicated treatments. GnRH-(1-5) treatment had no effect on IP_1 levels. Data were analyzed by a one-way ANOVA followed by an LSD post hoc test. * $p < 0.05$ relative to VEH.

Figure 3. GPR173 distribution in the GN11 cell. A) The plasma membrane of GN11 cells was extracted and probed for GPR173. A single band corresponding to GPR173 was

present. Preabsorption experiments with the peptide used to make the GPR173 antibody removed the correct band corresponding to GPR173 demonstrating its specificity. B) GN11 cells were methanol-fixed and stained for GPR173 (green) and GnRH (red). GPR173 staining was more punctate while GnRH was more diffuse, which indicative of a cytoplasmic protein. Scale bar indicates 10 μm.

Figure 4. Effect of GnRH-(1-5) on GPR173 and pSTAT3 levels. A) GN11 cells were treated for 5, 15, and 30 min with 100 nM GnRH-(1-5) followed by subcellular fraction. The indicated proteins were analyzed by western blot in within the nucleus, cytoplasm, and membrane fractions. Interestingly, GPR173 and β-arrestin levels remained unchanged due to GnRH-(1-5) treatment in all fractions. As expected, GnRH-(1-5) treatment decreased pSTAT3 levels in a time-dependent manner. These blots are representative of three separate experiments.

Figure 5. Effect of GnRH-(1-5)-mediated recruitment of β-arrestin by GPR173. GN11 cells were treated with VEH or 100 nM GnRH-(1-5) for 5 min and lysates were subjected to immunoprecipitation (IP) assays with the anti-GPR173 antibody. Blots were subsequently immunoblotted (IB) with the indicated antibody. GnRH-(1-5) robustly enhanced the interaction between GPR173 and β-arrestin while no other association was detected.

Figure 6. Identification of the β-arrestin GnRH-(1-5)-dependent pathway. A) To confirm the interaction between GPR173 and β-arrestin, GN11 cells were treated with VEH or

100 nM GnRH-(1-5) for 5 min and lysates were subjected to immunoprecipitation (IP) assays with the anti-β-arrestin monoclonal antibody. Blots were subsequently immunoblotted (IB) with the anti-GPR173 antibody. Interestingly, β-arrestin association with GPR173 is enriched with GnRH-(1-5) treatment. Additionally, β-arrestin recruited PTEN only with GnRH-(1-5) treatment, offering a possible mechanism by which GnRH-(1-5) inhibits the STAT3 pathway. B) Next, we wanted to identify the specific β-arrestin since the antibody we used in our experiments recognized both β-arrestin 1 and 2. GN11 cells were treated with VEH or 100 nM GnRH-(1-5) for 5 min and lysates were subjected to immunoprecipitation (IP) assays with the anti-GPR173 antibody. Blots were subsequently immunoblotted (IB) with an anti-β-arrestin 1 or anti-β-arrestin 2 specific antibodies. GnRH-(1-5) treatment enhanced GPR173 recruitment of β-arrestin 2 while β-arrestin 1 was not detected.

Figure 7. Effect of bpV(HOpic) on the GnRH-(1-5)-mediated inhibition of GN11 cellular migration. GN11 cells were treated with the PTEN inhibitor, bpV(HOpic), at 12 nM or 120 nM doses in presence or absence of 100 nM GnRH-(1-5) and a wound closure assay was conducted. PTEN inhibition blocked the GnRH-mediated inhibition of GN11 cellular migration. Upper panel indicates the representative photomicrographs of each treatment in the wound closure assay. Lower panel indicates the quantification of the wound closure assay (n=4). Data were analyzed by a one-way ANOVA followed by an LSD post hoc test. Scale bar indicates 100 μm. * $p < 0.05$ relative to VEH.

Figure 1

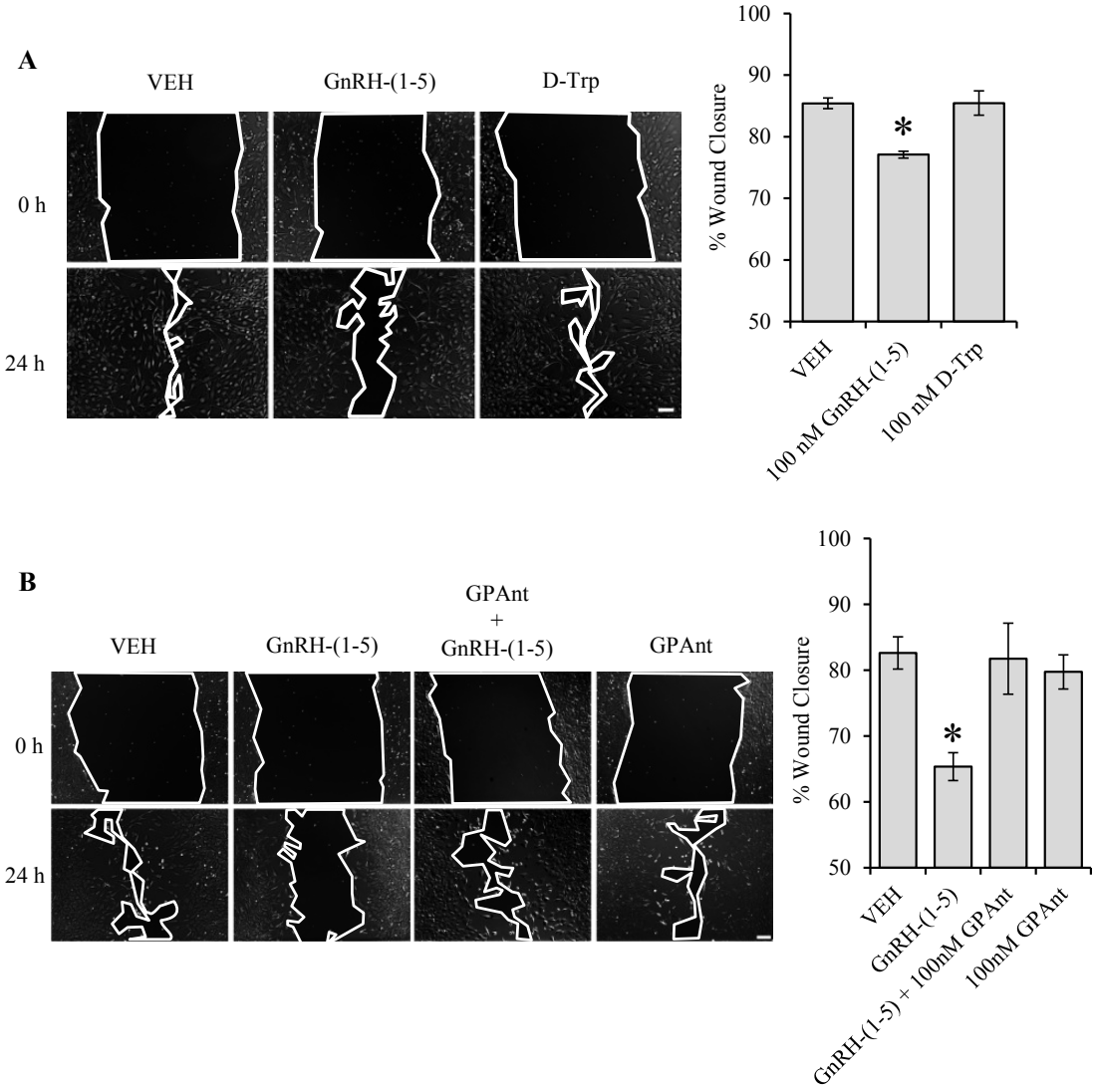

A
VEH
GnRH-(1-5)
D-Trp
0 h
24 h
% Wound Closure
100
90
80
70
60
50
*
VEH
100 nM GnRH-(1-5)
100 nM D-Trp
B
VEH
GnRH-(1-5)
GPAnt
+
GnRH-(1-5)
GPAnt
0 h
24 h
% Wound Closure
100
90
80
70
60
50
*
VEH
GnRH-(1-5)
GnRH-(1-5) + 100nM GPAnt
100nM GPAnt

Figure 2

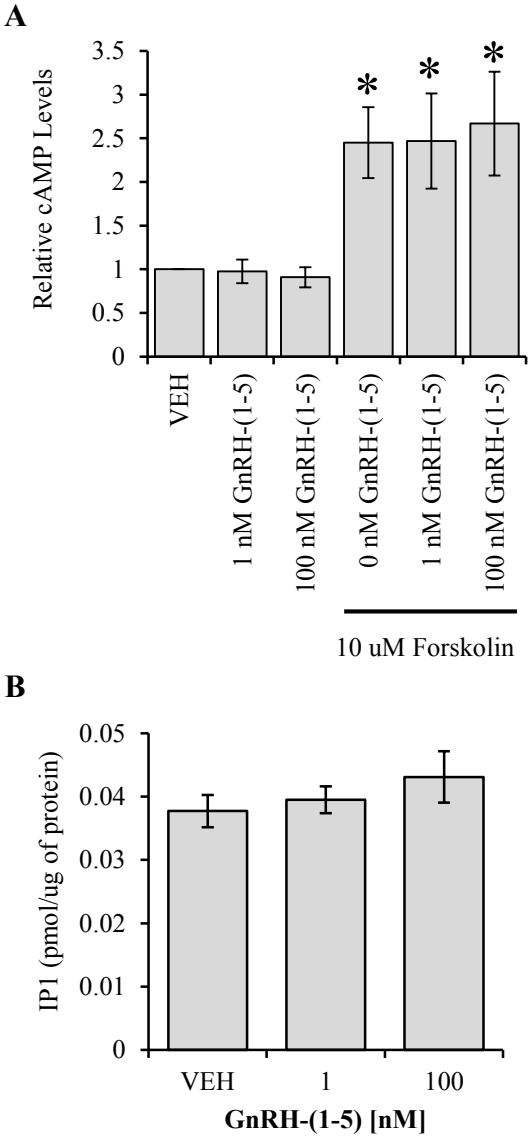

A
Relative cAMP Levels
3.5
3
2.5
2
1.5
1
0.5
0
*
*
*
VEH
1 nM GnRH-(1-5)
100 nM GnRH-(1-5)
0 nM GnRH-(1-5)
1 nM GnRH-(1-5)
100 nM GnRH-(1-5)
10 uM Forskolin
B
IP1 (pmol/ug of protein)
0.05
0.04
0.03
0.02
0.01
0
VEH
1
100
GnRH-(1-5) [nM]

Figure 3

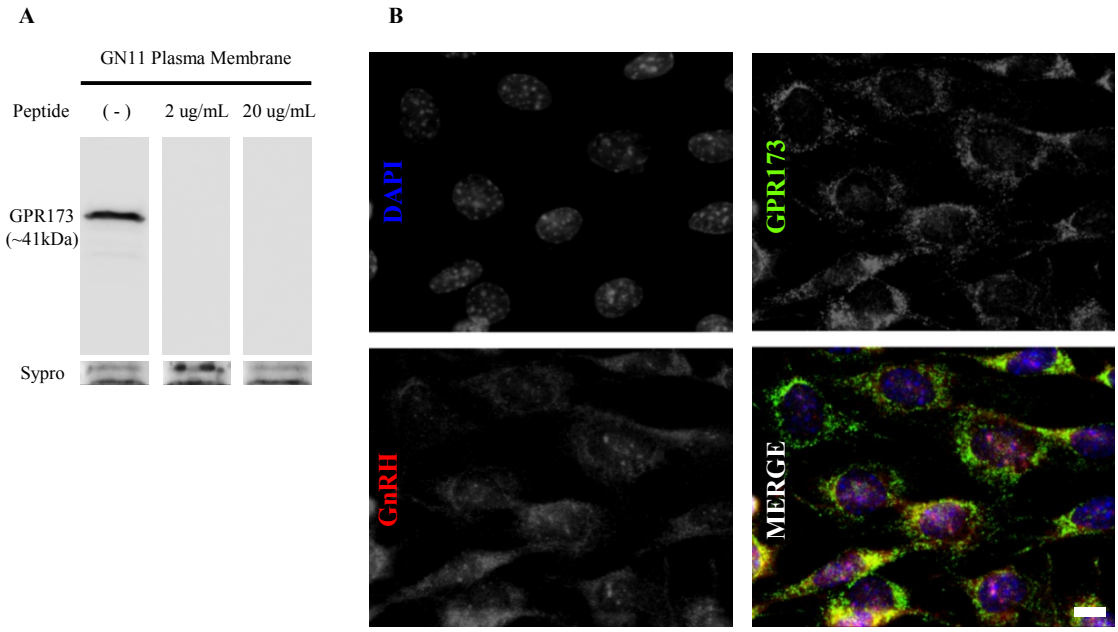

A
GN11 Plasma Membrane
Peptide
(-)
2 ug/mL
20 ug/mL
GPR173
(~41kDa)
Sypro
B
DAPI
GPR173
GnRH
MERGE

Figure 4

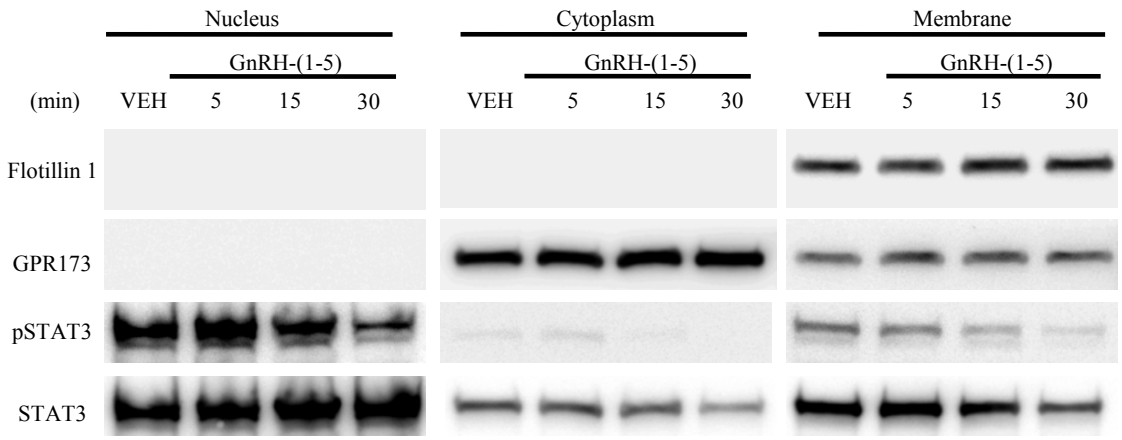

Nucleus
Cytoplasm
Membrane
GnRH-(1-5)
GnRH-(1-5)
GnRH-(1-5)
(min)
VEH
5
15
30
VEH
5
15
30
VEH
5
15
30
Flotillin 1
GPR173
pSTAT3
STAT3

Figure 5

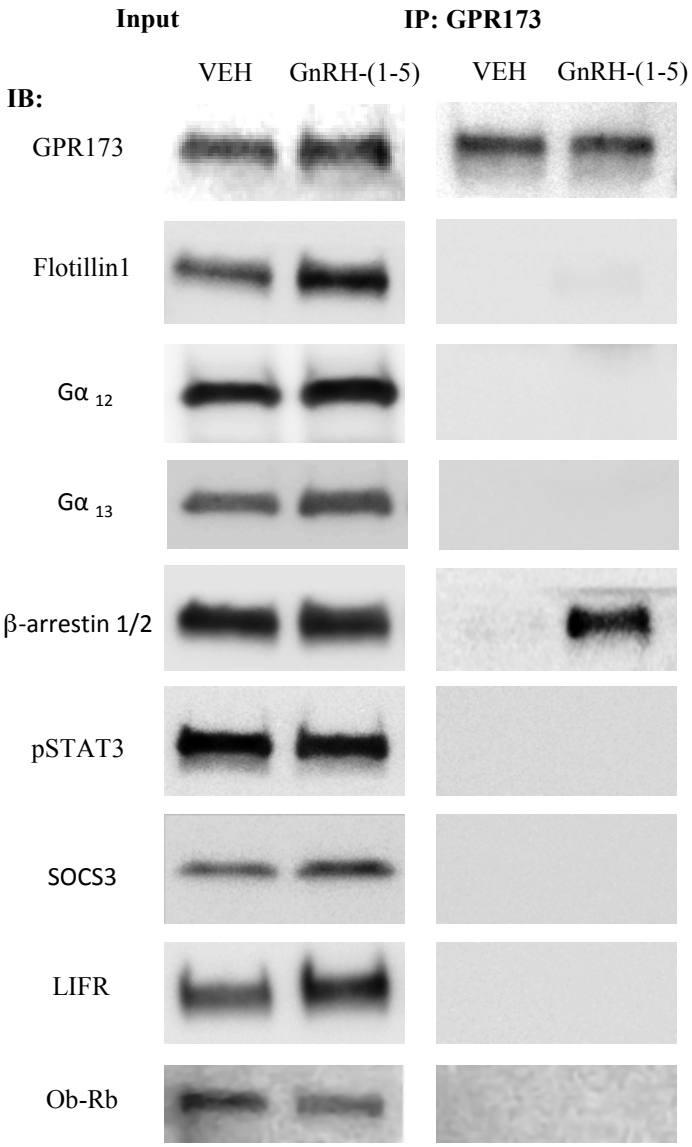

Input
IP: GPR173
VEH
GnRH-(1-5)
VEH
GnRH-(1-5)
IB:
GPR173
Flotillin1
Gα 12
Gα 13
β-arrestin 1/2
pSTAT3
SOCS3
LIFR
Ob-Rb

Figure 6

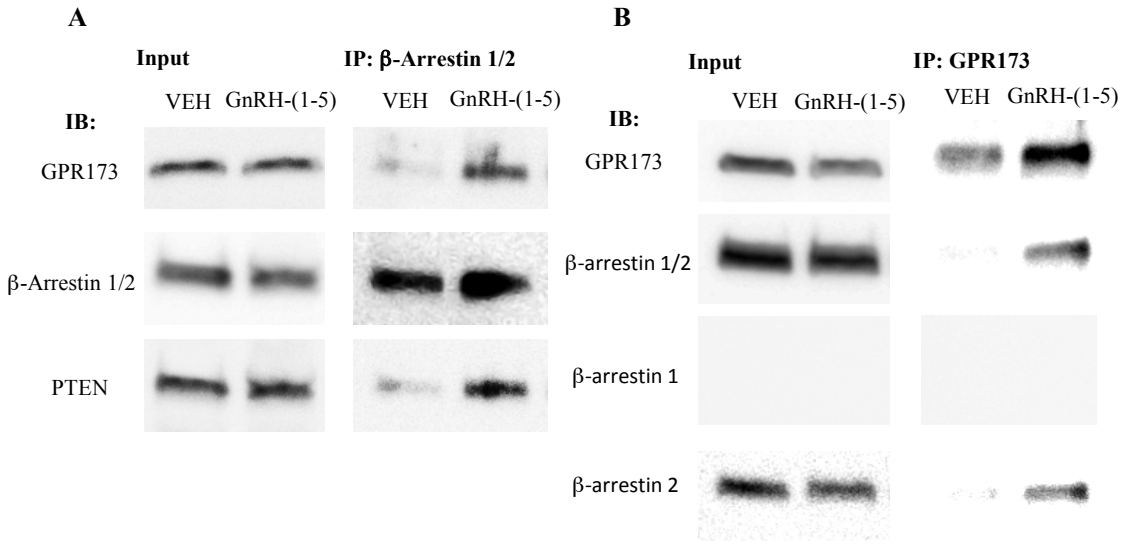
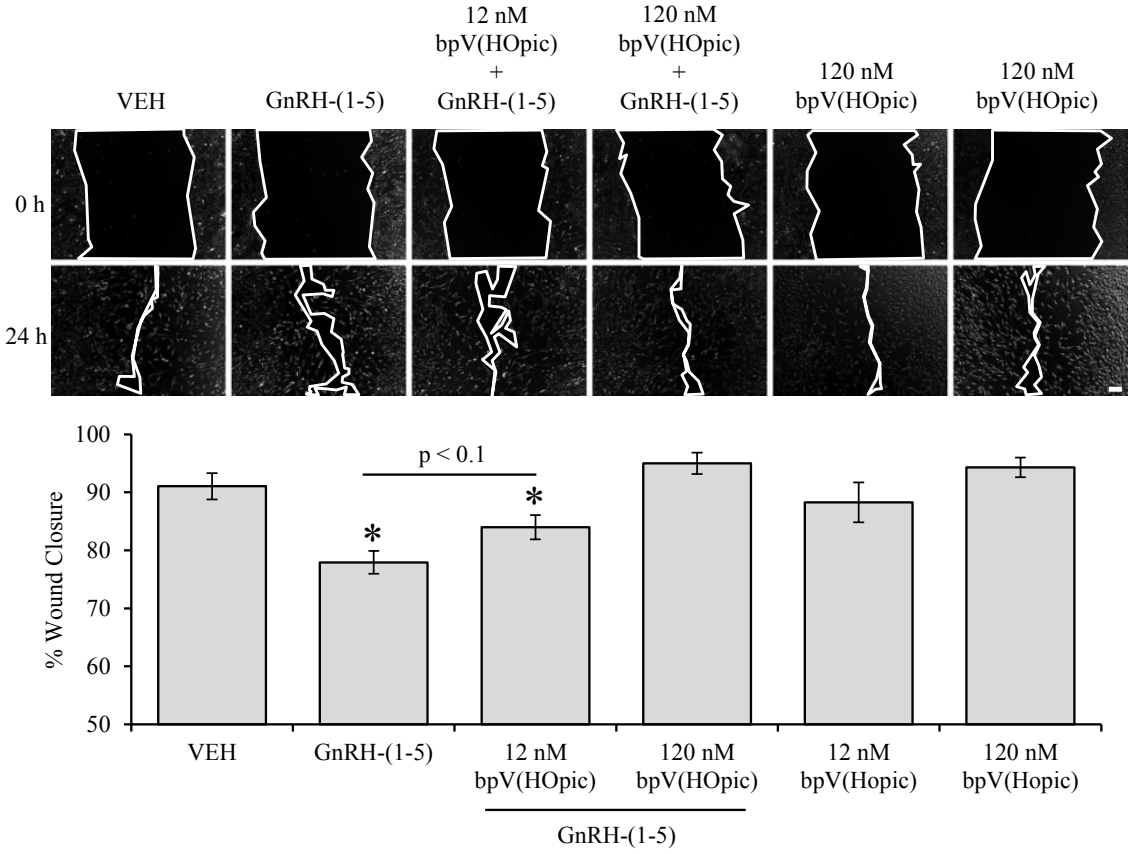

A
Input
IP: β-Arrestin 1/2
VEH
GnRH-(1-5)
VEH
GnRH-(1-5)
IB:
GPR173
β-Arrestin 1/2
PTEN
B
Input
IP: GPR173
VEH
GnRH-(1-5)
VEH
GnRH-(1-5)
IB:
GPR173
β-arrestin 1/2
β-arrestin 1
β-arrestin 2

Figure 7


VEH
GnRH-(1-5)
12 nM bpV(HOpic) + GnRH-(1-5)
120 nM bpV(HOpic) + GnRH-(1-5)
120 nM bpV(HOpic)
120 nM bpV(HOpic)
0 h
24 h
% Wound Closure
100
90
80
70
60
50
p < 0.1
*
*
VEH
GnRH-(1-5)
12 nM bpV(HOpic)
120 nM bpV(HOpic)
12 nM bpV(Hopic)
120 nM bpV(Hopic)
GnRH-(1-5)

CHAPTER 5: Summary

Our studies elucidate a novel function for the metabolite GnRH-(1-5) to regulate GnRH neuronal migration. Moreover, we demonstrate that this regulation requires the activation of a GPCR, GPR173. This receptor belongs to a family of receptors including GPR27 and GPR85, which are all highly expressed in the brain (39). The anatomical distribution of these receptors suggests that their function is critical for the development and/or maintenance of the CNS. Discovery of the endogenous ligands activating GPR27 and GPR85 remains to be addressed. Nevertheless, our work implicates GnRH-(1-5) as an activator of GPR173 [Chapter 3; manuscript 1] (34). Physiologically this interaction may contribute to the development of the GnRH neuroendocrine axis.

Using the GN11 cell line as a model to examine the effects of GnRH-(1-5), we found that cells exposed to GnRH-(1-5) treatment had reduced migration in separate assays of migration. Additionally, when we conducted a high-throughput assay to identify candidate GnRH-(1-5) receptors, of the 3 positive results indicative of GnRH-(1-5) binding, only GPR173 was expressed in our cell model allowing us to test whether this receptor is required for the action of GnRH-(1-5). Using an siRNA approach, silencing GPR173 reversed the GnRH-(1-5)-mediated inhibition on GN11 cellular migration. This is an exciting finding in that GN11 cells have been extensively used to elucidate the mechanism of factors associated with the regulation of GnRH neuronal migration (8; 23; 24; 37). Yet, an analysis of the genetic profiles of patients suffering from KS reveals that a significant percentage of these cases do not implicate a previously characterized gene in the pathogenesis of this disease, indicating that there a many factors that remain to be identified (3). Our studies on the GnRH-(1-5)-mediated activation of GPR173 and

subsequent signaling pathway may offer insight not only into the development of the GnRH neuroendocrine system but also the molecular pathogenesis of certain cases of KS.

GPCRs can trigger any number of complex signaling cascades to regulate cellular function. Thus we sought to identify the intracellular mechanism mediated by GnRH-(1-5) binding to GPR173. Interestingly, previous studies have shown that the STAT3 signaling pathway regulates neuronal migration (16) and promotes GN11 migratory activity (37). In congruence with previous findings, our results demonstrated that the GnRH-(1-5)-mediated activation of GPR173 leads to the inhibition of the STAT3 pathway to inhibit cellular migration. In our model, STAT3 phosphorylation and subsequent activation is mediated by the presence of growth factors found within the charcoal-stripped fetal bovine serum. In this environment, GN11 cells have relatively high basal levels of phospho-STAT3 (pSTAT3) and are highly migratory while GnRH-(1-5) treatment served to diminish pSTAT3 levels and migration. We decided to use this paradigm in all migration assays tested since GnRH neurons are exposed to a variety of factors during development. Although our model is likely simpler than that of the GnRH neuronal microenvironment early in development, it allowed us to examine the role played by GnRH-(1-5) relative to other growth factors. Thus we propose that early in development, GnRH neurons are exposed to a variety of factors that serve to stimulate radial migration from the nasal septum to the CNS. However, as GnRH neurons begin to mature and secrete more GnRH, the interaction of GnRH-(1-5) with GPR173 may play a modulatory role to aid in the transition of GnRH neurons from the nasal region to the CNS.

Identification of the intracellular pathway bridging the interaction of GnRH-(1-5) with GPR173 to STAT3 signaling remained to be addressed. Interestingly, we found that GnRH-(1-5) binding to GPR173 deviates from the canonical GPCR signaling pathway [Chapter 4; manuscript 2]. Our co-IP studies revealed that GPR173 interacts with β-arrestin 2 in a GnRH-(1-5)-dependent mechanism. This association was rapid and allowed β-arrestin 2 to associate with PTEN, a phenomenon previously characterized (36). This complex induced by GnRH-(1-5) likely leads to the decrease in pSTAT3 levels since increased PTEN activity has been shown to decrease STAT3 signaling (64). Furthermore, blocking PTEN activity reversed the GnRH-(1-5)-mediated inhibition of migration, reinforcing its role in the GnRH-(1-5) mechanism. Figure 1 shows our proposed model integrating GnRH-(1-5) binding to GPR173; subsequently leading to β-arrestin 2 recruitment and its association with PTEN to inhibit growth fractor-stimulated STAT3 signaling and cellular migration.

Future work will need to address the functional significance of GnRH-(1-5) binding to GPR173 using animal models to verify its role in the development of the reproductive axis. An immunohistological approach will be required to prove that the expression of GPR173 colocalizes with migrating GnRH neurons at different developmental stages. Additionally, generation of a GPR173-null mouse model will allow us to investigate the anatomical distribution of GnRH neurons during development; and whether these neurons follow the correct migratory route to reach their targets within the CNS. These experiments will not be limited to the developmental stage since we have previously shown that GnRH-(1-5) facilitates lordosis behavior in adult ovariectomized female rats (72); and whether GPR173 mediates this behavior remains to be tested.

In addition to the *in vivo* experiments, a comprehensive molecular analysis of the GPR173 gene, mRNA, and protein will need to be conducted. A preliminary mRNA sequence alignment demonstrates that the coding region of GPR173 is highly conserved across species (Chapter 2; Table 1 and Figure 4). This degree of conservation is more pronounced when analyzing the GPR173 peptide sequences between species, indicating that the functional significance of this receptor is likewise highly conserved (Chapter 2; Figure 5). Apart from the evolutionary implications of GPR173, elucidating the mechanism by which GPR173 activity is regulated needs to be investigated. Using a bioinformatic approach, there are several motifs that are conserved across species and are candidates to undergo post-translation modifications (Chapter 2; Figure 5 and 6). These modifications are critical for receptor internalization and/or facilitating protein interactions, which lead to changes in receptor activity or the iniation of an intracellular event. Mutagenesis studies will be instrumental in examining the role played by each of these motifs and how they regulate GPR173 activity in response to GnRH-(1-5).

Complete identification of the factors that serve to facilitate the migration of GnRH neurons during development remains elusive. In a subset of patients suffering from KS, certain GPCRs have been linked to the proper development of the GnRH neuroendocrine system. Our current studies implicate another GPCR, GPR173, which may contribute to the development of this system mediated by its interaction with the metabolite GnRH-(1-5). Using an immortalized GnRH-secreting cell line we demonstrate that GnRH-(1-5) activates GPR173 and recruits β-arrestin 2, which serves as a scaffolding protein to regulate STAT3 and cellular migration. Identification of the

GnRH-(1-5) receptor has set the foundation for our future work regarding the physiological significance of this metabolite throughout different stages of development.

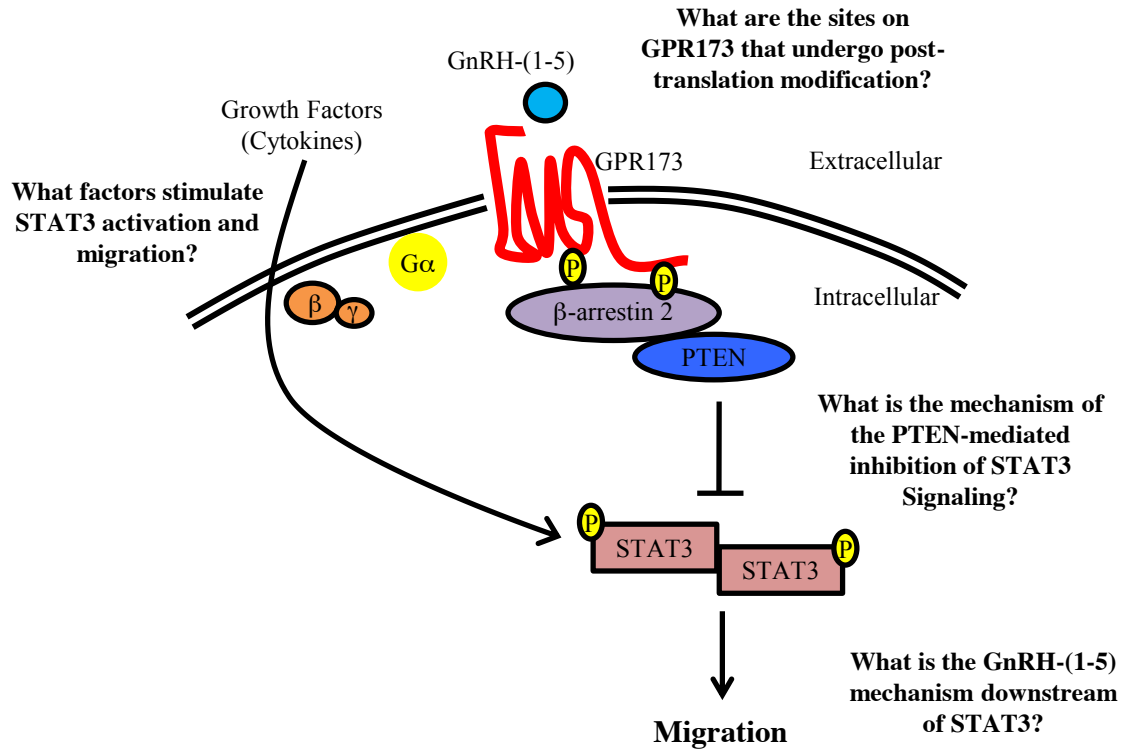


Figure 1. The Mechanism of GnRH-(1-5) Action.
The proposed model of the GnRH-(1-5) mechanism to regulate GN11 cellular migration. GnRH-(1-5) binds its receptor GPR173, which induces the rapid G protein-mediated recruitment of β-arrestin 2. Subsequently, formation of this complex allows the association of β-arrestin 2 with PTEN. This interaction may lead to the inhibition of growth factor-stimulated activation of the STAT3 pathway to inhibit migration. Additionally, there are some key questions (indicated in the figure) that need to be addressed to completely elucidate the mechanism of GnRH-(1-5).

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