Honors Project of Excellence
Decoding GnRH neurohormone pulse frequency and amplitude with mathematical model on GnRHR activation and expression revealed convergent signaling modules of ERK and NTAF

Thanh Pham

June 15th, 2014
Honor Program - Cohort 9

Primary Investigator: Craig Mc.Ardle, PhD – Professor of Molecular Pharmacology Laboratories for Integrative Neuroscience and Endocrinology, School of Clinical Science, University of Bristol, United Kingdom.

Honor Faculty/Home institution mentor: Gregory Buck, Ph.D - Associate Professor of Biology and Biomedical Sciences, Texas A&M University-Corpus Christi

Institutions that would be involved in the project:

1. Texas A&M University – Corpus Christi
2. Laboratories for Integrative Neuroscience and Endocrinology, School of Clinical Science, University of Bristol, Whitson Street, Bristol BS1 3NY, United Kingdom
3. Bristol Centre for Applied Nonlinear Mathematics
4. Bristol Centre for Complexity Sciences

Abstract

GnRH is a hypothalamic neuropeptide that stimulates the synthesis and secretion of LH and FSH and thereby mediates central control of reproduction. It is secreted in brief pulses at intervals that vary (30 min-16 hr) under different physiological conditions, such as puberty and during the menstrual cycle . GnRH effects on its target cells (pituitary gonadotropes) are also dependent on pulse frequency, with effects on gene expression and hormone secretion often being maximal at sub-maximal pulse frequency. This phenomenon is exploited therapeutically but the molecular mechanisms are poorly understood. At the cellular level GnRH acts via G-protein coupled receptors that mediate activation of phospholipase C, causing a largely PKC-mediated activation of the MAPK ERK, as well as Ca^{2+} mobilization and activation of the Ca^{2+}-dependent transcription factor NFAT. The McArdle lab recently developed a mathematical model of GnRH signaling that was trained against wet-laboratory data for GnRH-stimulated nuclear translocation of ERK2-GFP and NFAT-EFP, used as readouts for ERK and NFAT activation. This model predicts that the characteristic feature of maximal transcription activation at sub-maximal GnRH pulse frequency could reflect the co-operative convergence of these two pathways at the transcriptome (Tsaneva-Atanasova et al., 2012). The purpose of the research is to use the model simulations to determine if increased GnRH transcription results from the level of signaling through merging of ERK and NFAT pathways, not from each individual pathway.

Introduction

Normal reproductive function requires the precise temporal and quantitative regulation of hormone secretion at all levels of the hypothalamic–pituitary–gonadal axis. The hypothalamus contains gonadotropin-releasing hormone (GnRH) neurons which secrete pulsatile GnRH into the hypophyseal portal blood system through which it is transported to the anterior pituitary gland. GnRH binds to its G- protein receptor on gonadotrope cells, stimulating the biosynthesis and secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH travel through the peripheral circulation, acting at the gonads to stimulate gametogenesis (i.e., the development of mature eggs and sperm) and steroidogenesis (i.e., synthesis of the gonadal hormones – estrogen, progesterone, and androgens). In the majority of physiological conditions, the gonadal steroids send negative feedback to the hypothalamus and pituitary to decrease GnRH and gonadotropin secretion. An exception is at the time of the periovulatory LH surge in females, believed to be due to positive feedback by rapidly rising estradiol levels.

GnRH consists of 10 amino acids which have been highly conserved over millions of years of evolution (Figure 1). In common with other neuropeptides, GnRH is synthesized as part of a large pro-hormone that is cleaved enzymatically and further modified within the secretory granules. This 92 amino acid precursor protein is proteolytically cleaved to generate four isoforms: the GnRH decapeptide, a 23 amino acid signaling sequence which directs intracellular packaging and secretion, a three amino acid (Gly-Lys-Arg) proteolytic processing site, and a 56 amino acid GnRH-associated protein (GAP) that is secreted with GnRH. The function of GAP is unknown but it has been proposed to inhibit prolactin secretion in some species (Ehlers et al, 2013).

GnRH has a short half-life of approximately 2–4 min due to rapid cleavage by peptidases. As a result of this rapid degradation as well as massive dilution, the peripheral circulation does not contain biologically active concentrations of GnRH. Serum LH and FSH levels are used clinically as surrogate markers for the presence of pulsatile GnRH secretion. LH is a more accurate indicator of GnRH pulse characteristics (i.e., frequency and amplitude) than is FSH, which has a longer half-life.

With brief pulses secreted from the hypothalamus, GnRH acts via seven transmembrane receptors on pituitary gonadotrophs to stimulate phospholipase C, mobilize calcium ions and activate protein kinase C isozymes, resulting in activation of mitogen-activated protein kinase (MAPK) pathways and Ca^{2+} effectors, such as calmodulin, which in turn mediate the effects of GnRH on exocytotic gonadotropin secretion as well as its effects on expression of many genes including those for gonadotropin subunits (Millar et al, 2004; Burger et al, 2008; Naor et al, 2000). GnRH pulse frequency changes under different physiological conditions as well as species. GnRH pulse frequency can vary with intervals from 1-6 hours in women and as low as eight minutes in mice (Ferris et al, 2006). Also, pubertal increases in

gonadotropin secretion and the gonadotropin surge pre-ovulation are both driven by increases in GnRH pulse frequency (Ferris et al, 2006). Moreover, according to Belchetz et al (1978), this characteristic of GnRH can be exploit by using agonists to increase circulating gonadotropin and gonadal steroids, which would then increase fertility, and vice versa. The sustained stimulation of GnRH can cause chemical contraception and can be used therapeutically in treatment of hormone dependent cancers (Conn et al, 2010).

With these crucial characteristics and application, frequency decoding of GnRH is fundamental to the physiology and pharmacology of this system. However, the mechanism is poorly understood. Some recent publications have focused on transcription and posttranscriptional control of GnRH on gonadotropin gene expression (in both gonadotrophs and LbT2 cells). Predictably, these genes were found to be sensitive to GnRH pulse frequency. In many systems, increasing pulse frequency simply increases output until maximal response is maintained with continuous stimulation. On the other hand, the pulsatile stimuli might stimulate maximal response at sub-maximal frequency, and generate bell-shaped frequency-response relationships. This type of behavior is called genuine frequency decoding.

Just as any signaling cascade in the biology, GnRH signaling requires negative feedback loops. However, the mechanism of GnRH feedback loops is unclear. According to current literature, evidence exists for at least three possibilities: feedback to upstream components of GnRH, feedback to the decoding process of transcription factor in pituitary cells, or transcription-dependent negative feedback on upstream pathways

First of all, the feedback on upstream components of GnRH would help GnRH receptor (GnRHR) reduce the information transfer from cytoplasm to nucleus at high pulse frequency. GnRH causes downregulation of cell surface GnRHR, which is caused by pulse-frequency-dependent desensitization of upstream signals (McArdle et al, 1999). The second possibility is the down-regulation of transcription factor to decode GnRH signaling and pulse frequency. The third possibility included GnRHR-mediated induction of the regulator of G-protein signaling (RGS-2), which has the potential to inhibit all responses distal to induction of mitogen-activated protein kinase (MAPK) phosphatases (MKPs). These enzymes would then potentially regulate GnRHR-mediated extracellular signal regulated kinase (ERK) signaling (Lim et al, 2009). Recently, there is a focus on nuclear factors of activated T-cells (NFAT) and ERK because both would decode pulse frequency in other cellular models as well as gene regulation (, Hanson et al, 1994; Macian et al, 2000; Sanna et al, 2005; Tomida et al, 2003).

Most of the data on GnRH pulsatile frequency decoding in the literature were obtained in HeLa cell lines engineered to express GnRHR. Interestingly, frequency-response relationships for GnRH effects of LHβ and FSHβ transcription were bell-shaped according to luciferase reporters containing LHβ and FSHβ promoters. These data demonstrate that genuine GnRH frequency decoding is not restricted to gonadotrope cells and such behavior can occur in the absence of negative feedback loops implicated in GnRH frequency decoding.

This project would use a mathematical equation model of GnRH signaling based on the ordinary differential equations describing GnRHR occupancy,

activation and downstream signaling. This differs from earlier models (, Washington et al, 2004; Lim et al, 2009) that it extends signaling to ERK and NFAT, includes cellular compartmentalization (nucleus or cytoplasm) and importantly lacks upstream negative feedback. This model would be used as readouts for ERK and NFAT activation, accurately predicting wet-laboratory data. Therefore, this model would be used as inputs to the transcriptome. In many other physiological systems, ERK and NFAT might converge to regulate transcription and this is predicted to be relevant to GnRH signaling as well, because LHβ and FSHβ genes contained sites for regulation by both NFATs and ERK-dependent transcription factors. Therefore, the model can be used to predict the characteristic feature of maximal transcription activation at sub-maximal GnRH pulse frequency, whether it would reflect the cooperative stimulation between these two pathways at transcriptome. The model would also be used to predict plasticity, which the frequency response relationships will be altered by the changes of signaling via ERK and NFAT. The experiment work plan would also try to confirm this.

Hypothesis:
NFAT and ERK pathways will not work alone in decoding GnRH pulsatile frequency but mediate a genuine frequency decoding when converging in a cooperative manner at transcription.

Methods of Approach
The project will provide practical experience in cell culture, transfection, transduction, luciferase activity assays, high imaging content and associative data analysis using the mathematical modeling of ERK and Ca^{2+}/NFAT signaling pathways. The mathematical model can be used as framework for wet-laboratory experiment plan. Effects of these manipulations on upstream pathways will also be determined by fluorescence microscopy with a high content imaging platform. Effects of pulsatile GnRH will be determined using luciferase reporters (Egr1-luc, NFAT-RE-luc, FSHβ-luc, LHβ-luc, αGSU-luc) in cells where ERK signaling and NFAT signaling are prevented, increased or reduced (i.e. by partial or complete inhibition, by over-expression etc.) so that GnRH pulse frequency-response relationships can be related to transcriptional effects.

1. Cell Culture, transfection and transduction
HeLa cells and MCF7 cells (from European Collection of Cell Cultures) will be cultured in 10% FCS-supplemented Dulbecco's modified Eagle's medium (DMEM). For experiments they will be harvested by trypsonization and seeded at 3–5 × 10^3 cells/well in 96-well plates. For some experiments ERK will be knocked down by reverse transfection using RNAiMAX (Invitrogen) and two siRNA duplexes (Qiagen, Crawley, UK) each for ERK1 and ERK2 A mixture of all four ERK duplexes or control siRNA against GFP (Ambion, Warrington, UK) will be used (at 2.5 nm total). Where ERK will be knocked down, recombinant adenovirus (Ad) will be used to add back

previously characterized imaging reporters consisting of wild-type (WT) ERK2 in tandem with green fluorescent protein (ERK2-GFP) or a catalytically inactive mutant (K52R ERK2-GFP). Cells will be transduced with Ad ERK2-GFP in DMEM with 2% FCS 16 h after siRNA transfection. The Ad-containing medium will be removed after 4–6 h and replaced with fresh DMEM with 0.1% FCS. For some experiments, an alternative approach will transduce cells with Ad for an Egr-1 promoter driving expression of dsRED or zsGREEN. Ad5 zsGREEN and DsRedExpress vectors will be made by digesting pzsGREEN1-DR and pDsRed-Express-DR (Clontech) with BamHI/NotI and subcloning fluorescent protein cDNAs into a corresponding digest of promoterless pAd5K-NpA vector (a gift from Prof. Beverly Davison, Gene Transfer Vector Core, University of Iowa). Egr-1 promoter will be amplified using 5′-tat gta ctc gag acg gag gga ata gcc ttt cg-3′ forward primer and 5′-tat gta gaa ttc gag aac tga tgt tgg gtg gtg-3′ reverse primer using Egr-1-promoter-Luc vector as template. The product will be digested with XhoI and EcoRI and subcloned into the corresponding digests of pAd5 zsGREEN1-DR and pAd5 DsRed-Express-DR. All Ad will be generated from shuttle vectors and added to cells at the same time using 0.5–10 plaque-forming units (pfu)/nl. In the HeLa cell system used here this yields multiplicity of infection values of 10–100 and transduction efficiency approaching 100%. The cells will be cultured for 16–24 h after transduction and stimulated with EGF or PDBu (Sigma).
For some experiments the cells may also be treated with a selective ERK inhibitor, FR180204 (Tocris Bioscience, Bristol, UK), or the structurally related negative control compound, 328008 (Calbiochem) .

2. Engineering of Plasmids and Viruses - Ad-expressing wild-type (WT) and D319N ERK2-GFP, mGnRHR, and Egr-1 promoter luciferase reporter will prepared, grown to high titer, and purified as described (Macian et al, 2001). Ad K52R ERK2-GFP will be prepared using 5′-CAA AGT TCG AGT TGC TAT CAG GAA AAT CAG TCC TTT TGA GC-3′ forward and complementary reverse primers to mutate WT ERK2-GFP template with a Stratagene QuikChange mutagenesis kit before Ad manufacture. Briefly, 4.5 μg of viral shuttle vectors will be digested alongside 1.5 μg of pacAd5 9.2–100 sub360 backbone vector (donated by Prof. Beverly Davidson, University of Iowa, IA) with PacI or NheI. Cut shuttle and backbone vectors will be then mixed and transfected into low passage HEK293 cells using Superfect (Qiagen, Crawley, UK). Cells will be left for 7–10 days to allow recombination between shuttle and backbone vectors, and after cytopathic effects, lysates were collected for further bulking. Ad vectors were grown to high titer and purified according to standard protocols (Macian et al, 2001).

3. Luciferase reporter assays:
Cells will be plated and transfected or transduced with luciferase reporter as above. Cells will be washed in ice-cold phosphate buffered saline and lysed, and luciferase activity determined. Data will be reported as relative to light units normalized as a fold change over control except where indicated

5. High content Image acquisition and analysis

Cells will be cultured, plated, transfected, and transduced on Costar black-wall 96-well plates (Corning, Arlington, UK). After stimulation they will be fixed in 4% paraformaldehyde (in PBS) and permeabilized in −20 °C methanol. In most experiments cells will be stained for ppERK by blocking in 5% normal goat serum, PBS and probing with mouse anti-ppERK monoclonal antibody (MAPK-YT, 1:200, Sigma) in PBS and visualization with Alexa 488- or 546-conjugated goat anti-mouse antibodies (1:200 Invitrogen). Total ERK will be stained with rabbit anti-ERK monoclonal (137F5, 1:100, Cell Signaling Technology, Hutchin, UK) and Alexa 546-conjugated goat anti-rabbit antibody (1:200, Invitrogen). Nuclei will also be stained (600 nm DAPI in PBS), and for most experiments the fluorescence of ERK2-GFP, dsRED and/or zsGREEN also visualized. Image acquisition will be automated using an IN Cell Analyzer 1000 (GE Healthcare) microscope with a ×10 objective and excitation and emission filters of 360 and 460 nm for DAPI, 475 and 535 nm for Alexa 488, GFP, and zsGREEN, or 535 and 620 nm for Alexa 546 and dsRED. Automated image analysis algorithms will be used to define perimeters and fluorescence intensity within regions of interest (nucleus and cytoplasm or nucleus and nuclear collar). The multi-target analysis algorithm in the IN Cell Analyzer Work station will be used to define nuclear perimeters from the DAPI stain. To control for cell masks, we will expand the nuclear perimeter (3 μm collar) to capture cytoplasmic fluorescence. Whole cell (nucleus plus cytoplasm) fluorescence measures will be used for ERK, ppERK, ERK2, ppERK2, dsRED, and zsGREEN and reported in arbitrary fluorescence units (AFU). In some experiments the proportion of ERK2-GFP in the nucleus will be calculated. For most experiments, replicate treatments will be applied in 2–4 wells and 4–9 fields of view collected per well, yielding data for >10,000 individual imaged cells (for each treatment in each experiment). These data will be used to produce population-averaged mean responses and for frequency distributions of individual cell measures or transformed and binned as described in the figure legends. Log concentration-response relationships will be fitted (GraphPad Prism, La Jolla, CA) to Sigmoid curves of variable slope to estimate EC50 values and Hill coefficients.

Outcome:
This project of excellence will be presented as a primary journal article and a Power Point presentation at the Honors symposium in May 2015. The final results will be incorporated into both the Power Point presentation, posters for several research symposia and possibly a primary literature journal article.

Bibliography

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Time line

Summer 2014

June 15,2014: Hand in Proposal

June 15- July 15, 2014

- settle funding (Honors scholarships, study abroad scholarship and Parents council funding)
- Visa application
- Prepare Accommodations/transportation/flights ticket for the UK trip
- Other relative paperwork

August 30st, 2014: Fly to University of Bristol, UK

Fall, 2014

September 1st: Start laboratory work at Dr. McArdle's lab.

September 30th: progress report to Dr. Gregory Buck

October 30th: second progress report to Dr. Gregory Buck

November 30th: third progress report to Dr. Gregory Buck

December 19th: last day of laboratory in the UK

Spring, 2015

January 19 – February 20th, 2015: Data analysis of results, continue to work on Mathematics model. Communicating with Dr. McArdle via email and video chat.

February 20th-February 31st, 2015: writing manuscript and final paper, first draft

March 1st, 2015: First draft due at Honors Senior seminar

March 10th, 2015: second draft due

April 15th, 2015 final draft due.

May 5th, 2015: Honor symposium and presentation

Fall, 2015:

May5th – November 1st, 2015 :

- Continue to working on mathemathical model/data analysis.
- 2nd draft manuscript writing for Neuroendocrinology peer- reviewed journal.

November 1st, 2015: Submit manuscript for publication

To whom This may concern:

My name is Thanh Pham, an Honors student in Cohort 9. I am deeply thankful for the generous resources that the Honors Program has been providing me for the past two years for my scientific and intellectual endeavors. The experience and knowledge I have gained from these trips are invaluable, which set a solid stepping-stone for me to strive further in science. In fact, despite my foreign citizenship, I was accepted to several elite summer programs at Baylor College of Medicine, UT Southwestern and Cold Spring Harbor Laboratory this summer. I am currently at Baylor College of Medicine, working under Dr. Theodore Wensel, a legendary scientist in Biochemistry/Molecular Biology.

My Project of Excellence proposal this time is indeed very ambitious, as it involves doing the laboratory work at the University of Bristol, United Kingdom under one of the best scientists in the field of Neuroendocrinology. This trip would be a unique experience for me, not only because I would be exposed to different laboratory work in another country, but also the project itself. Dr. McArdle has been a great scientist with such creative ideas and enthusiasm. The mathematic model he developed based on the pulsatile frequency of GnRH, which accurately predicts the data from wet-laboratory work, along with high imaging analysis and fascinating integration between engineering, non-linear mathematics, were actually a great breakthrough in the field. The massive amounts of data that these models generated were unbelievable. Dr. Mark Lawson from the University of California-San Diego and Dr. Robert Fowkes from Royal College of Veterinary, London have highly recommended me to him as Dr. McArdle is such "a great scientist in the field!"

As this exciting project would involve a hands-on laboratory work in Dr. McArdle's laboratory in University of Bristol, UK, I am sincerely hope to gather sufficient funding from many sources. I understand that the Honors Program provides approximately $4000 scholarship for POE project for two semesters. Due to the unique circumstances of my project, which involved a costly trip to the UK as well as my citizenship requirement for in-state tuition, I would like to propose an alternate breakdown of funding as below:

Fall, 2014: $3,000
Fall, 2015: $1,000

As for my trip, the expenses are below:

Round trip airplane tickets:	~$2,000
Accommodation:	~$3, 000 for 4 months
Lab bench fee (charged by University of Bristol for visiting research student) *__Note__: I'm communicating with the university to get a waiver for this fee	~$1,700
Food/other expenses:	~$1,300
TAMUCC tuition (12hours)	~$3,000
Total	~$11,000

I do understand your concerns with my taking such an expensive trip; however, as a student and a young scientist, I value the experience and knowledge above all other means. I have been working hard to have some sufficient but limited personal

savings to cover part of this cost. I also would like to use the majority of my Honors POE scholarship for this trip, utilize other available funding from Honors, the university and private scholarships as well.

I sincerely hope for your support to make this exciting opportunity happen. I am a young, enthusiastic and ambitious student who is eager to learn and explore all awaiting possibilities.

Sincerely,
Thanh Pham