Physiological Characterization and Transcriptomic Properties of GnRH Neurons
Derived from Human Stem Cells

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Disclosure Statement: The authors declare no conflicts of interest.

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Note that Gonadotropin Releasing Hormone (GnRH) in this article is synonymous of Gonadotropin Releasing Hormone-1 (GnRH-1).
ABSTRACT:

Gonadotropin releasing hormone (GnRH) neurons in the hypothalamus play a key role in the regulation of reproductive function. In this study, we sought an efficient method for generating GnRH neurons from human embryonic and induced pluripotent stem cells (hESC and hiPSC, respectively). First, we found that exposure of primitive neuroepithelial cells, rather than neuroprogenitor cells, to FGF8, was more effective in generating GnRH neurons. Second, addition of kisspeptin to FGF8 further increased the efficiency rates of GnRH neurogeneration. Third, we generated a fluorescent marker m-Cherry labeled human embryonic GnRH cell line (mCh-hESC) using CRISPR-Cas9 targeting approach. Fourth, we examined physiological characteristics of GnRH (mCh-hESC) neurons: Similar to GnRH neurons in vivo, they released the GnRH peptide in a pulsatile manner at ~60 min intervals, GnRH release increased in response to high potassium, kisspeptin, estradiol and neurokinin B challenges, and injection of depolarizing current induced action potentials. Finally, we characterized developmental changes in transcriptomes of GnRH neurons using hESC, hiPSC, and mCh-hESC. The developmental pattern of transcriptomes was remarkably similar among the three cell lines. Collectively, human stem cell-derived GnRH neurons will be an important tool for establishing disease models to understand diseases, such as Idiopathic Hypothalamic Hypogonadism, and testing contraceptive drugs.

Keywords: GnRH, human stem cell, induced pluripotent stem cell, kisspeptin
INTRODUCTION:

The gonadotropin releasing hormone (GnRH) neuron plays a central role in reproductive function. GnRH neurons in the hypothalamus release the decapeptide hormone from the median eminence (ME) into the portal circulation in a pulsatile manner and stimulate pituitary LH and FSH release, which controls gamete production and gonadal steroid secretion in the gonads. Consequently, patients with Idiopathic Hypogonadotropic Hypogonadism (IHH) due to carrying defective genes for the generation or migration of GnRH neurons, or for upstream regulatory neurons of normal GnRH function, suffer from abnormal GnRH release and therefore an absence of reproductive function. For example, patients with Kallmann syndrome (no GnRH neurons in the hypothalamus due to failure of GnRH neurons to migrate from the olfactory placode) do not undergo puberty and exhibit IHH, and therefore they are infertile [1-4]. Presently, symptomatic improvement in Kallmann syndrome patients can be achieved by treatments with a GnRH agonist and gonadal steroids, but life-long treatments are required, as a tool for permanent cure has not been found [5,6]. If GnRH neurons were generated from IHH patient’s somatic cells, this may not only provide a new tool for understanding the underlying pathophysiology of this disease but also provide a treatment tool for individual IHH patients, such as a cell replacement therapy. Overall, there is an urgent need to create a model to study GnRH neuronal biology, particularly as it relates to both rare and common diseases of human reproduction that occur across our reproductive life.

For many years we and others have been studying primary GnRH neurons derived from the monkey and mouse embryonic nasal placode [7,8]. Similar to neurons in in vivo [9,10], they release GnRH peptide in a pulsatile manner [11,12], exhibit periodical intracellular calcium $[Ca^{2+}]$, oscillations [13,14], and respond to external signals such as ATP, estradiol, and kisspeptin [14-18]. However, there are some species differences in physiological characteristics of GnRH neurons from rodents to monkeys and even monkeys to humans, and examination of primary GnRH neurons derived from human fetuses is ethically not possible in the US. In fact, to date molecular and cellular studies of...
human GnRH neurons have not been conducted and therefore many human disease states have not been easily studied. Accordingly, several years ago we started to generate GnRH neurons from human embryonic stem cells (hESC) and induced pluripotent stem cells (hiPSC). Unlike most neurons in the CNS which arise from the neural tube, GnRH neurons originate from the olfactory placode [19,20]. Genetic analysis of Kallmann syndrome patients [21] and transgenic mice [22] indicate that the presence of FGF8 and its receptor FGFR1 are critical for GnRH neurogenesis. Moreover, during embryonic development, neurogenesis of the GnRH niche is regulated by BMP4, Nog, and FGF8 [23], and neural crest cell signaling to the olfactory placode is a key for GnRH neurogeneration [24]. Based on these developmental characteristics, we have developed a reliable method to generate GnRH neurons from human hESC and hiPSC, generated an ESC line carrying the mCherry-labeled hGnRH gene from which we can identify GnRH neurons in live culture, and compared physiological characteristics between stem cell derived human GnRH neurons and olfactory placode derived monkey primary GnRH neurons. Additionally, we characterized developmental changes in GnRH neurons transcriptomes using hESC, hiPSC, and mCh-hESC.

This work provides a tool to study human GnRH neurons in vitro, by which we can understand the normal state of human GnRH neurons including the differentiation process, migration patterns, and neurocircuitry formation, and then compare these processes to disease states, such as IHH, for comparison.
METHODS:

Cell cultures:

In the present study pluripotent H9 hESC (WA09, WiCell Research Institute, Madison, WI) and hiPSC (IMR90 clone #4, WiCell Research Institute, Madison, WI) were used. All work with H9 was reviewed and approved by the Stem Cell Oversight Committee, University of Wisconsin.

Maintenance of hESC and hiPSC: H9 and IMR90 cells were maintained in the presence of 5% CO\textsubscript{2} at 37 °C on 6-well tissue culture plates (Greiner Bio-One Cellstar, Monroe, NC) coated with matrigel (WiCell) in mTeSR1 media (Stem Cell Technologies). The culture medium was changed daily and cells were passaged with versene (Fisher Scientific, Hampton, NH) every 4–6 days.

Generation of GnRH neurons: Exposure to FGF8 at two different developmental stages:

Protocol 1: In general, treating neuroepithelial (NE) cells with small molecules results in a specific type of neurons [25]. Three to 4 days after passaging ESC, adherent cells were exposed to dual SMAD inhibitors (10µM SB431542 and 100nM LDN-193189) in cell differentiation medium (CDM, see Supplementary Table 1 [26]) for 10 days. This procedure led to stem cells transforming predominantly to NE cells. Subsequently, cells were lifted from the culture plate with 2 mg/ml dispase (17105-041, Fisher) and left to become predominantly neural progenitor (NP) cells i.e., neurospheres (NS), in the presence of neural induction medium (NIM, Supplementary Table 1 [26]) in suspension for 7-10 days. Next, cells were dispersed with accutase (SCR005, Sigma), plated on matrigel and exposed to FGF8 (10 ng/ml) in neural differentiation medium (NDM, Supplementary Table 1 [26]) for 3-6 weeks to induce GnRH neurons. The first day of exposure to FGF8 was designated as Day 0 (Fig. 1A, top). Protocol 2: As stated earlier, GnRH neurons originate from nasal placode, yet need a signal from neural crest cells [24]. As such, exposure of primitive NE cells in the presence of various types of undifferentiated cells to FGF8 would be helpful for GnRH neurogeneration. After stem cells were exposed to dual SMAD inhibitors in CDM for 10 days
resulting in primitive NE cells, they were dispersed with accutase, replated on matrigel and exposed to FGF8 (10 ng/ml) in NDM for a similar period to Protocol 1. Again, the first day of exposure to FGF8 was designated as Day 0 (Fig. 1A, bottom).

**Differentiation of hESC and human hiPSC and molecules that facilitate GnRH neurogenesis:** We first examined FGF8 at different doses (2, 10, 20, and 100 ng/ml) and time periods, and FGF21 (10ng/ml, AF10042; PeproTech). Next, the additive effect of various small molecules including kisspeptin (0.1 µM; Phoenix Pharm., Burlingame, CA), Sonic Hedgehog (SHH, 20 ng/ml; Fisher), and retinoic acid (RA, 3 ng/ml; Fisher), with or without FGF8 was examined. Additionally, the effects of two notch inhibitors were examined: Compound E (0.5 ng/ml; Fisher), DAPT (20 µM; Sigma); and the antimitotic drug fluorodeoxyuridine (FdUR, 30 µM; Sigma). The doses of DAPT and FdUR were the same or 50% lower as those described for stem cell derived GnRH cultures [27,28] and for primary cultures of GnRH neurons [8,17], respectively.

**RT-PCR:** hESC at Day 0, 5, 10 and 24 of FGF8 treatment (n=4 for all) from Protocol 2 were frozen and isolated using RNA STAT-60 (Tel-Test, Friendswood, TX) according to the manufacturers instructions. Individual RNA samples were then analyzed for the presence of KISS1 using RT-PCR with primer sequences shown in **Supplementary Table 2** [26]. RT-PCR was conducted as described previously [15]. For RT 1 µg of total RNA and random hexamer primers were used with the GeneAmp RNA PCR Core Kit (Applied Biosystems, Branchburg, NJ) according to manufacturer’s specifications. For PCR, primers were synthesized by the University of Wisconsin Biotechnology Center. Aliquots (3 µl) of each RT reaction were used for PCR and combined with 2.5 mm deoxynucleotide triphosphates (Amersham Biosciences, Princeton, NJ), 1× PCR Buffer II with MgCl, 1.25 U AmpliTaq (Applied Biosystems, Branchburg, NJ), and 12.5 pmol of primer in a final volume of 50 µl. Water mixed with each PCR/primer cocktail served as a negative control. PCR amplification conditions consisted of one cycle at 95 °C for 2 min; 45 cycles at 95 °C for 45 sec and 58 °C for 45 sec; and an incubation at 72 °C
for 7 min. After PCR amplification, 20-μl aliquots of each reaction were loaded on a 1 or 2% agarose TBE gel, and products were visualized using ethidium bromide staining.

**Immunohistochemistry:** Neurons grown on glass coverslips were fixed with 4% paraformaldehyde for 20 min. Coverslips were washed with PBS for 5 min three times and blocked for 1 h in 0.5% normal goat serum before incubation with the primary antibody in 0.1% Triton X-100 at 4 °C overnight. Cells were subsequently washed with PBS and stained with Alexa Fluor-conjugated secondary antibodies. Coverslips were washed and mounted onto glass slides using Vectashield mounting media with DAPI (Vector Labs., Burlingame, CA). To ensure the consistency of the GnRH staining, we used 3 primary antibodies (the polyclonal antibodies, GF6 and SW1 and the monoclonal antibody LRH13). A complete list of antibodies is seen in Supplementary Table 3 [26]. Images were taken using a Nikon TiE2 fluorescence microscope or a Nikon Confocal TiE microscope (Nikon Instruments, Tokyo, Japan).

**Cell counts:** The efficiency rate of GnRH neurogeneration was calculated by manual GnRH cell count over the number of the total cells (DAPI stained cells). For example, the comparison between Protocols 1 and 2 was made by counting cell bodies of GnRH positive neurons on the entire coverslip and the total cell number was assessed by DAPI staining and NIS elements software (Nikon), obtained from Day 24-27 of the FGF8 treatment. For the GnRH cell count we were not able to use computer-based programs including a high-content imaging platform, as GnRH neurons are not uniform in shape and size (Figs. 1C, D, F, G, H, I and K) and they aggregated and formed thick clusters (see Figs. 1D, I and K). As total numbers increased, however, the approach to count the entire slide was inefficient. Accordingly, we decided to count a portion of the coverslip to extrapolate the total number. Using previously assessed GnRH positive cells in the entire slide, we placed a 20 x 20 grid (i.e. a 6x6 mm section with 300 μm²) and counted the number of GnRH positive cells in randomly selected 5, 10, 20, and 30 grid sections. We found that counting 30 grid sections (~1% of the total area of the coverslip) accurately reflected the entire coverslip. This method was applied to all
statistical analyses except for comparison between the two Protocols in which we counted the entire slide, and developmental changes in mCherry. Numbers of mCherry GnRH (mCh-GnRH) neurons, were determined by counting all red cells in an 8 mm² area per slide. For statistical analysis, we always counted at least 3 independent coverslips from at least 2 different non-consecutive passages per treatment and derived the average ± SEM. For all stem cell cultures, we used passage 39-49 in H9 cells or mCherry labeled H9 cells, and passage 56-66 in IMR90. Note that cell count of GnRH neurons in older cultures, i.e., over FGF8 Day 30, was not easy to perform, as they aggregate forming thick clusters and fair cell count was not possible, therefore we did not count above this age (see Results).

**Generation of a stem cell line carrying the fluorescent marker mCherry labeled GnRH gene.** The transgenic mCh-GnRH cell line was generated by inserting a P2A-mCherry transgene downstream of the GnRH coding sequence, as schematically illustrated in **Supplementary Fig. 1** [26] at the Human Stem Cell Core at the University of Wisconsin-Madison using a CRISPR/Cas9 targeting approach [29-31].

**CRISPR/Cas9 targeting design and plasmid generation:** sgRNA design targeting the C-terminal locus of GnRH was performed using the crispr.mit.edu design tool. To utilize a Cas9D10A nickase, two sgRNAs targeting opposite DNA strands (sgRNA-1A: TACTTAAGTCATGTTAGTAA.TGG and sgRNA-1B: ACCCATTTAATACCTGGATAAA.TGG) were cloned into an sgRNA expression plasmid from the laboratory of Su-Chun Zhang (Addgene #68463) [32].

**Electroporation, selection, and growth:** hESC (H9) were generally cultured and electroporated as described in Chen et al. [32], although this protocol was modified for standard feeder-free maintenance conditions. Human ESCs were cultured in TeSR-E8 media (StemCell Technologies) on matrigel substrate (Corning). Twenty-four hours prior to electroporation, when cells were approximately 80% confluent, media was supplemented with Rho Kinase (ROCK)-inhibitor (0.5 µM, Calbiochem, H-1152P). Cells were lifted and singularized with 0.5 mM EDTA in PBS (GIBCO) for 3-4
minutes, washed two times with DMEM/F12, and harvested in PBS. Cells were dispersed into single cells, and $1 \times 10^7$ cells were electroporated with appropriate combination of plasmids using the Gene Pulser Xcell System (Bio-Rad) at 250 V, 500 μF in a 0.4 cm cuvettes (Phenix Research Products). The plasmid cocktail contained 15 μg of CAG-Cas9$^{D10A}$ plasmid (Kiran Musunuru, Addgene #44720), 6 μg each sgRNA plasmid (sgRNA-1A and sgRNA-1B), and 30 μg of a donor plasmid designed to insert a P2A-mCherry construct downstream of the GnRH coding sequence. This donor plasmid has approximately 1 kb homology arms consisting of the sequence upstream and downstream of the GnRH stop codon. Between the homology arms, the plasmid is designed to remove the stop codon and insert a P2A linker sequence and the mCherry gene (Clontech) along with a LoxP-flanked PGK-puromycin cassette to facilitate selection of cells with proper homologous repair [33]. Following electroporation, cells were re-plated on matrigel in 0.5 μM ROCK inhibitor at a low density (original $1 \times 10^7$ cells re-plated to 3 6-well plates). After approximately 5 days, cells were treated with puromycin (0.5 μg/mL, Invivogen, ant-pr-1) to select for cells incorporating the plasmid. Puromycin was re-supplemented daily until hESC colonies grew to sufficient size (approximately 10 days). At this point, puromycin was removed and 0.5 μM ROCK inhibitor was added 24 hours prior to clone picking.

**Genotyping:** Single-cell colonies were manually selected and mechanically disaggregated. Genomic DNA was isolated from a portion of these colonies using QuickExtract DNA Extraction Solution 1.0 (Epicentre). All genotyping and subsequent sequencing was performed via Q5 polymerase-based PCR (NEB). For genotyping, three pairs of primers were used to assess genomic integration of the transgenic construct. The transgene integration PCR (Geno 2F with 3’ close 2R) identified clones with correct targeting of the transgenic construct, the homozygous/heterozygous PCR (Geno 2F with 3’ close 2R) identified whether these clones exhibited monoallelic or biallelic transgenic integration, and the plasmid retention PCR (T3 with PGK up 2R) identified whether there was retention or non-specific integration of the plasmid that may erroneously express the mCherry protein (Supplementary Fig. 1 [26]). Clone 1 was identified as a heterozygous transgenic line, and clone 6
was identified as a homozygous transgenic line without non-specific plasmid retention, so the transgenic integration was sequence validated for these clones. Purified PCR fragments, identified via agarose gel and purified using a Zymoclean Gel DNA Recovery Kit (Zymo Research), identified via agarose gel and purified using a Zymoclean Gel DNA Recovery Kit (Zymo Research), were submitted to Quintara Biosciences for Sanger sequencing to identify clones with the proper genetic modification and to ensure sequence fidelity of the reporter construct.

**Off-target analysis:** To identify whether the CRISPR-Cas9 system produced any non-specific genome editing, we analyzed suspected off-target sites for genome modification in Clone 6.

Using the 5 highest-likelihood off-target sites predicted by the crispr.mit.edu algorithms for both of the sgRNAs that were used (sgRNA 1A and sgRNA 1B), we designed genotyping primers (see Supplementary Table 2 [26]) to amplify these regions via Q5-polymerase PCR. PCR products were identified via agarose gel, purified using a Zymoclean Gel DNA Recovery Kit, and submitted to Quintara Biosciences for Sanger sequencing. No off-target CRISPR activity was observed.

**CRE/LoxP recombination:** To ensure proper GnRH-P2A-mCherry expression, we wanted to remove the floxed PGK-puromycin cassette. For this experiment, cells from Clone 1 and Clone 6 were lifted and singularized with 0.5 mM EDTA for 3-4 minutes, washed two times with DMEM/F12, and harvested in TeSR-E8 media with 0.5 µM ROCK-inhibitor. Cells were pelleted by centrifugation (1000 rpm for 1.5 minutes). Remaining media was aspirated, leaving approximately 50 µL of media in the tube. 10 µL of a recombinant TAT-CRE recombinase (Excellgen) was added to the cells and the pellet was re-suspended. The cells were allowed to sit in the media-CRE solution for 20 minutes at 37°C. After this time, cells were triturated to ensure single-cell dispersion and plated in 6-well plates for clonal selection in TeSR-E8 media with 0.5 µM ROCK-inhibitor. Following growth of clones, individual colonies were selected as previously described and PCR verified to exhibit loss of the PGK-puromycin cassette. Final clones were sequenced to again reconfirm sequence fidelity of the GNRH-P2A-
mCherry expression construct, and karyotyping was performed by WiCell (Madison, WI). Clone 6, Cre subclone 1 was expanded and used for all subsequent fluorescent reporter experiments.

**Physiological characterization of generated GnRH neurons**

**Electrophysiological recording:** Whole cell patch recordings were performed on 10 mCh-GnRH neurons between 20-34 days after the start of FGF8 exposure. Coverslips were immersed in HEPES based aCSF recording solution consisting of (mM): 128 NaCl, 5 KCl, 2 CaCl$_2$, 2 MgSO$_4$, 30 Glucose, 25 HEPES. Solutions were adjusted to 7.3 pH and 300-310mOsm. Patch pipettes made by borosilicate glass were fire polished to resistances of 5-10MΩ. Recording pipettes were filled with a K-gluconate based intracellular solution consisting of (mM): 140 K-gluconate, 10 NaCl, 10 HEPES, 5 EGTA, 0.5 CaCl$_2$, 10 Na$_2$ phosphocreatine, and 4 MgATP. Intracellular recording solutions were corrected to 7.2 pH and 290 mOsm. mCh-GnRH cells were identified using an Olympus BX50WI microscope under 40x immersed-lens objective and visualized through a U-MW1Y cube (EX 545-580; EM 600). Recordings were amplified and telegraphed in current clamp mode via MultiClamp 700B (Axon Instruments, San Jose, CA) and digitized by Digidata1440A (Axon Instruments). Data were analyzed online and post-hoc using pClamp 10 (Axon Instruments) software, Clampex and Clampfit 10.

**Calcium imaging:** [Ca$^{2+}$]$_i$ levels were assessed by the method described previously [13, 15, 17] with some modifications. Culture medium (2 ml) was mixed with 18 µM fur-a-2 AM (Life Technologies) and 6 µl of a mixture of pluronic F-127 (BASF Corp., Parsippany, NY) and dimethylsulfoxide (1:2) by vortexing for 15 sec. Cells on a coverslip were incubated in fur-a-2 for 20–40 min at 37 °C under 5% CO$_2$. The coverslip was then placed in a Dvorak-Stotler recording chamber. Fluorescence imaging of the dye-loaded cells was achieved with an inverted microscope. A culture was viewed through a x20 objective lens, and a 750x750 µm recording field with mCh-GnRH cells were selected for data capture. Cultures were continuously perfused at a speed of 50 µl/min with Brain Phys medium without Phenol Red (05791, Stemcell Tech., Cambridge, MA), under 95 % O$_2$ and 5 % CO$_2$ at room temperature (22–25 °C).
[Ca^{2+}]_i was monitored as a function of the ratio of the 510-nm fura-2 emission excited by illuminations at 340 and 380 nm with a Lambda DG-4 light source and filter exchanger (Sutter Instruments, Novato, CA). Fura-2 fluorescence was recorded at 10s intervals with a CCD camera (Hamamatsu, Hamamatsu City, Japan) and Nikon NIS imaging software (Nikon). The ratio of the fluorescence intensities (ΔF/F0) from the 340- and 380-nm excitation wavelength-evoked images were used to calculate the free [Ca^{2+}]_i levels as described previously [13,15,17]. All [Ca^{2+}]_i oscillation experiments were conducted between Day 20 and 50 of FGF8 treatment.

**Perifusion experiments and GnRH measurements:** To examine the spontaneous GnRH release pattern and responses of GnRH neurons to various secretagogues, perifusion experiments were conducted using Sykes-Moore chambers as described previously [11,17]. Briefly, GnRH neurons on two thermanox coverslips were placed face-to-face, separated by a rubber O-ring, forming a chamber with a volume of 200 µl. Cultured cells were perifused with a modified Krebs-Ringer phosphate buffer [11,17] with 0.1% glucose (pH=7.4) under 95 % O₂ and 5% CO₂ at 37 °C. Perifusates were collected at 30 µl/min in 10 min fractions for 6 h using the ACUSYST (Endotronics, Minneapolis, MN) perifusion system. Various known exocytotic stimuli, such as human kisspeptin-10 (hKP10, 1-100 nM), senktide (1-100 nM, neurokinin B agonist), and estradiol (10 nM) for GnRH neurons, or vehicle for control, were applied for 20 min periods at approximately 2 h intervals. To test the viability of cells, cultures were challenged with 56 mM KCl at the end of experiments. All samples were stored at -80 °C for later GnRH RIA. GnRH concentrations in media samples were measured in 200 µl aliquots (single) with RIA using antiserum R42 (see Supplementary Table 3 [26]) as described previously [34]. After the perifusion experiment, cells were fixed with 4 % paraformaldehyde (pH=7.6) and immunostained for GnRH and β-tubulin or the presence of mCh-GnRH neurons. This experiment was conducted between Day 22 and 63 of FGF8 treatment.
Developmental changes in the transcriptome of human GnRH neurons

Sample preparations: To uncover the genes up-regulated and down-regulated during the stem cell differentiation of GnRH neurons, we prepared human stem cells (H9, IMR90, mCh-H9) treated with FGF8, FGF8+KP or NDM alone and collected at day 0, 8, 15 and 25. This generated a total 12 groups with n=3 in each group (n consists of 3 different passages). Total RNA was extracted from all 108 samples, standard mRNA libraries were prepared, and subjected to RNAseq analysis.

RNA sequencing was conducted by the University of Wisconsin, Biotechnology Center using an Illumina NovaSeq 6000 platform.

RNASeq preprocessing: The average size of RNAseq libraries (n=108) was 20,329,051 reads with standard deviation 2,941,894 and the read length was 151 base pairs. FASTQC was used for quality control of the sequencing data [35]. The read pairs were aligned to the human reference genome (GRCh37 Ensembl release 75) using STAR 2.5.3 [36] with the following parameters “--outFilterMultimapNmax 1 --outFilterMismatchNoverLmax 0.1 --alignEndsType local”. STAR was also used to quantify read counts per gene based on GTF file (GRCh37 Ensembl release 75). Picard’s CollectInsertSizeMetrics, CollectRnaSeqMetrics, and EstimateLibraryComplexity were used for quality control of the aligned data. Based on GTF definition, non-protein-coding genes were filtered out. These filtered genes were used for differential expression analysis. To perform quality control on the samples, the correlation between each sample using hierarchical clustering and Principal Component Analysis (PCA) plots were tested for confounding effects of metadata for the samples.

Differential Gene Expression analysis: Differential expression (DE) analysis for RNAseq data from H9 samples between points of the time series experiment was performed using the R package DESeq2 version 1.26.0 [37]. To determine the strongest individual gene effects in GnRH differentiation, DE analysis was performed for FGF8-treated samples between Day 8 and Day 15, as well as between Day 15 and Day 25. Counts per million (CPM) were calculated based on the number of uniquely
mapped reads and 0.1 CPM cutoff threshold was used to filter the genes in less than 50% of the samples within the respective comparison groups, resulting in 14,260 genes for Day 8 and Day 15 that were included in the DESeq2 analysis and 14,785 genes for Day 15 and Day 25 included in the analysis. To correct for the hidden confounding variables, Surrogate Variable Analysis (SVAseq) version 3.34.0 [38] was used. The hidden variables estimated using SVAseq were regressed out in the differential expression analysis by incorporating surrogate variables into the DESeq2 model, and log2-fold changes and p values (corrected for multiple testing using Benjamini-Hochberg adjusted p-values; adj. p-value) were estimated. Significant differentially expressed genes (DEGs) with adj. p-value < 0.05 and absolute log2-fold change (lfc) > 1. To understand the uniqueness of transcriptomes associated with GnRH neurogenesis in vitro from those in other types of neurons derived form hiPSC, we compared the genes showing differential expression between Day 25 and Day 0 of FGF8-induced GnRH neurons to hiPSC derived Ngn2-induced neuron (iNs) from the Talkowski Lab. For Ngn2-induced neuron, RNAseq was performed on both the hiPSCs and iN samples (Day 21 post Ngn2-induction), and differential gene expression analysis was performed between the hiPSCs and iN samples using the same methods as described in this study.

Time-series expression analysis for FGF8-treated H9 cell line samples from Day 0 to Day 25 was also performed using DESeq2. CPM-filtered and SVAses corrected 15798 genes were used in the time-course differential expression analysis. The significant time-series DEGs were selected through Likelihood ratio test (LRT). The DEGs with adj. p-value < 0.05 were used for GO Term and KEGG pathway enrichment analyses. Gene ontology (GO) term enrichment analysis was performed using R package topGO version 2.24 [39]. KEGG pathway enrichment analysis was performed using the R package clusterProfiler version 3.16.1 [40]. The significantly enriched GO classes and pathways were selected (Benjamini-Hochberg adj. p-value < 0.05).
Statistical analysis:

For all statistical analysis except for RNAseq analyses (described above) we used GraphPad-Prism (San Diego, CA) statistical software. The efficiency rate between protocols 1 and 2, kisspeptin and PCR data were examined using two-way ANOVA followed by Bonferroni’s post hoc analysis. For time-lapse analysis of mCh-GnRH neurons and perifusion experiments (percent values over baseline level, which was defined as the average of the 3 points before challenges) were subjected to two-way ANOVA repeated measures with Bonferroni’s post hoc analysis. Pulsatility of GnRH release was determined with Pulsar algorithm as described previously [11]. For analysis of calcium imaging data, identification of [Ca^{2+}]_i peaks and synchronization of [Ca^{2+}]_i peaks among individual GnRH neurons in a culture were conducted as described for primary GnRH neurons derived from the monkey placode, previously [13,41]. Subsequently, IPI in single GnRH neurons and synchronization interval among individual GnRH neurons were calculated as described previously [13,41]. For remaining data analysis, a Students t-test was applied. Significance was attained at p<0.05.

RESULTS:

1. Generation of GnRH neurons: Exposure to FGF8 at two different developmental stages:

Immunohistochemical staining data indicated FGF8 treatment of hESC with both Protocols 1 and 2 resulted in GnRH neurogeneration (Figs. 1C, D, H, I, and K). Similarly, the same FGF8 treatment of hiPSC with Protocols 1 and 2 resulted in GnRH neurogeneration (Figs. 1F and G). We confirmed GnRH staining with several antibodies to GnRH, including polyclonal antibodies GF6 (Figs. 1C-G) and SW1 (Figs. 1I and K), as well as monoclonal antibody LRH13 (Fig. 1H). A comparison of the efficiency rate between the two Protocols by cell count further indicated that Protocol 2 yielded 5-fold more GnRH neurons than Protocol 1 (Fig. 1B). Note that the cell count was conducted in cultures younger than FGF8 treatment Day 28, as GnRH neurons had a propensity to form thick multilayer clusters when they reached 4-6 weeks of FGF8 treatment and an accurate cell cell count became considerably...
difficult. Importantly, with either hESC or hiPSC Protocol 2 (Figs. 1D and G) was far more efficient than Protocol 1 (Figs. 1C and F). Regardless of the origin (hESC or hiPSC), in both protocols there were small numbers of GnRH neurons without any FGF8 treatment, as a result of self-patterning (Figs. 1B and E). Collectively, we concluded that the FGF8 exposure of less differentiated NE cells is more efficient in generating GnRH neurons.

2. Small molecules that facilitate GnRH neurogenesis:

   From this point we decided to use Protocol 2 exclusively. Comparing the effectiveness of FGF8 doses ranging from 2 to 100 ng/ml we found that 10 ng/ml was optimal for GnRH neurogenesis (Fig. 2). Increasing the dose to 20 ng/ml did not increase the number of GnRH neurons and 100 ng/ml was ineffective over control (Fig. 2). Moreover, we examined the effects of FGF21, as it has been reported that FGF21 is also involved in IHH disease [42]. The results suggested that FGF21 did not have any trophic effect resulting in GnRH neurogeneration (Fig. 3B).

   Next we examined an additive effect of various molecules with FGF8 that potentially enhance GnRH neurogenesis. First we examine the effects of kisspeptin, one of the most important upstream regulators for GnRH release [43], which was reported to increase the number of hypothalamic GnRH neurons in embryonic zebra fish [44]. We also observed that KP mRNA levels in cultures were generally elevated during the differentiation of stem cells, and specifically on Day 5 in the presence of FGF8, kisspeptin expression was significantly higher than other time periods measured (Fig. 3A). Results indicated that hKP10 treatment between Days 10 and 15 in the presence of FGF8 increased the efficiency rate almost double over FGF8 alone, whereas hKP10+FGF8 treatments between Days 0 and 5, or 15 and 20 were effective, but not as effective as hKP10+FGF8 treatment between Days 10 and 15 (Fig. 3B). Addition of hKP10 for the entire FGF8 treatment period did not show further improvement over FGF8 alone (data not shown). hKP10 treatment alone between Days 15 and 20 had a trend to increase the efficiency rate over NDM control, although it
was not statistically significant (Fig. 3B). Second, we examined the effects of RA and SHH, as they have been implicated in olfactory epithelial differentiation [45]. Neither RA nor SHH were effective in increasing GnRH neurogeneration (Supplementary Fig. 2 [26]).

**Notch inhibitors and antimitotic substances**

It has been reported that after a fair amount of a specific type of neurons are generated, weeding mitotic cells or using notch inhibitors to synchronize neurogeneration helped the efficiency rate [21,22]. As such, we tested the effects of two notch inhibitors, CE and DAPT, and the antimitotic compound, FdUR, on Days 14-18 and Days 18-22 of FGF8 treatment. The results indicated that DAPT at 2 and 10 µM doses for Days 14-18 had a tendency to increase efficiency rates over those without the notch inhibitors, although the effect was statistically insignificant (Supplementary Fig. 3 [26]). CE at 0.05 and 0.1 µM did not help the efficiency rate (Supplementary Fig. 3 [26]). Applications of both DAPT and CE at both doses examined sporadically reduced the total number of cells on coverslips, resulting in irregular results. FdUR at 10 and 20 µM doses on either Days 14-18 or Days 18-22 completely failed to improve the efficiency rate (Data not shown). The effectiveness of the notch inhibitors needs further examination by changing different concentrations and/or the day of application.

3. Time course of GnRH neurogeneration:

Upon GnRH expression in this cell line, the P2A peptide self-cleaves, resulting in separate GnRH and mCherry expression in the cell. Genotyping efforts revealed clones 1 (heterozygous) and 6 (homozygous) as transgenic line without confounding plasmid retention, so we performed CRE recombination to remove the PGK-puromycin resistance cassette (Supplementary Fig. 1A and B [26]). Subsequent PCR confirmed successful Cre recombinase activity and excision of the resistance cassette (Supplementary Fig. 1C [26]). Clone 1, Cre subclone 1 and Clone 6, Cre subclone 1 were tested for transgenic sequence validation (and WT allele sequence validation for Clone 1 subclone 1),
karyotyped, and screened for off-target Cas9 activity to verify this clone met our quality standards (Supplementary Fig. 1D [26]). Clone 6 Cre subclone 1 was expanded and used for all subsequent fluorescent reporter experiments. We refer to this cell line as “mCh-GnRH”. Differentiation of mCh-GnRH ESCs using protocol 2 confirmed mCherry expression in GnRH peptide-containing neurons (Fig. 4B). Quantitative analysis indicated that 591 out of 600 mCherry positive cells on 6 coverslips (100 cells/coverslip) also expressed the GnRH peptide (98.5±0.5%). Subsequent real time live cell image analysis (Fig. 4A) indicated that mCh-GnRH neurons started to appear on Days 11-15 after FGF8 exposure and the number of GnRH neurons continued to increase up to Day 24 and reached a plateau on Day 26 under Protocol 2 (Fig. 4C and Supplementary Table 4 [26]). The results from two-way ANOVA indicated that there were significant interactions (p<0.05), treatment effects (p<0.0001), and time effects (p<0.05). As expected from the results seen in Fig. 1E, without any FGF8, a small number of GnRH neurons appeared between Days 15 and 26, although they were not significant (Fig. 4C and Supplementary Table 4 [26]).

4. Physiological characterization of generated GnRH neurons

The generation of mCh-GnRH neurons allowed us to characterize electrophysiological properties, intracellular calcium [Ca\(^{2+}\)] signaling, and GnRH release pattern in human GnRH neurons.

a. Electrophysiological characteristics of GnRH neurons derived from human ES cells.

We recorded electrical activity from 10 mCh-GnRH neurons between Day 20 and Day 34. Recordings from cells younger than Day 20 were unsuccessful, due to weaker mCherry fluorescence under a conventional microscope used for electrophysiological recording and to the fragility of the cells. The summary of electrophysiological characteristics of the 10 GnRH neurons and examples from which we successfully obtained stable recordings are shown in Table 1 and Fig. 5, respectively.
Several findings are noteworthy. First, the differentiated human GnRH neurons had much more hyperpolarized resting membrane potentials and smaller action potential amplitudes compared with GnRH neurons reported for other mammalian species (Table 1) [46-49]. Second, among the human differentiated GnRH cells between Day 20 and Day 34, there were no significant age-related differences in resting membrane potential, input resistance, membrane capacitance, or in action potential threshold, amplitude, or half-width (Fig. 5D). Third, depolarizing current pulses induced trains of action potentials in all cells except one, and in that cell, which had a relatively depolarized resting membrane potential, hyperpolarizing current pulses induced ‘rebound’ action potentials (Figs. 5A and B). Thus, action potentials could be induced by current injection in all cells. Fourth, all cells displayed spontaneous action potentials, whether occurring individually or in short bursts (Fig. 5C). Finally, there were negative correlations between action potential half width and input resistance (p=0.012, Fig. 5Di) and between membrane potential and input resistance (p=0.007, Fig. 5Dii).

One additional striking property that was observed in 6 of the 10 cells was a propensity to switch between two semi-stable “resting” membrane potentials (i.e. i=0 under current clamp). These cells were silent, or nearly so, at their hyperpolarized states, but they jumped spontaneously to a depolarized level marked by abundant bursts of spontaneous activity (Fig. 5Ci and ii), and then returned suddenly back to their hyperpolarized state. The depolarized states lasted for several seconds up to tens of seconds, and they could sometimes be induced by hyperpolarizing current pulses (Fig. 5Ci and iv), but never by depolarizing pulses.

Taken together, these data indicate that by the time or shortly after the GnRH gene turns on, as judged by mCherry expression, GnRH neurons appear to have mature cell membrane characteristics and firing patterns.
b. Intracellular calcium signaling

Previously, we have shown that primary GnRH neurons derived from monkey embryos exhibit periodical [Ca$^{2+}$]$_{i}$ oscillations and they synchronize at an interval of approximately 53 min [13,41]. As such, we examined whether similar [Ca$^{2+}$]$_{i}$ oscillations are seen in stem cell derived GnRH neurons. Systematic analysis with 33 cultures indicated that individual GnRH neurons exhibit periodical [Ca$^{2+}$]$_{i}$ oscillations with an inter-pulse-interval (IPI) of 18.3±1.3 min and they also synchronized with an IPI of 46.5 ±3.0 min, although there were considerable variations among GnRH neurons (Fig. 6A). Neither the IPI of the periodical [Ca$^{2+}$]$_{i}$ oscillations nor their synchronization changed over culture days after Day 20 up to Day 50 (Supplementary Fig. 4 [26]). Similarly, modifications with culture media, such as addition of Neural Supplement B or Brain Phys medium, did not change the IPIs of [Ca$^{2+}$]$_{i}$ oscillations or their synchronization intervals. Moreover, stem cell derived GnRH neurons readily responded to hKP10 and K$^{+}$ challenges (Figs. 6B and C).

c. GnRH release

Release of the decapeptide hormone from hESC- (Figs. 7A and B) and hiPSC- (Fig. 7C) derived GnRH neurons are both pulsatile with IPI of 59.5±3.3 (n=17) and 62.5±6.7 (n=3) min, respectively. They also respond to known GnRH secretagogues, such as the kisspeptin agonist, hKP10 (Fig. 7D), the NKB agonist senktide (Fig. 7E), estradiol (E$_2$, Fig. 7F), and high K$^{+}$ (Figs. 7B-F). Group data indicate that the hKP10 and senktide stimulated GnRH release was significantly elevated for the first 20 min, while the effects of E$_2$ and K$^{+}$ lasted for 30 min (Fig. 7G). Vehicle challenges did not induce any changes (Fig. 7G).
5. Developmental changes in the transcriptome of human GnRH neurons

To interrogate developmental transcriptional changes in human stem cell-derived GnRH neurons, we performed gene expression analysis of the FGF8-treated human stem cell (H9)-derived GnRH neurons on Day 0, 8, 15, and 25. To compare the Day 8 (n=3) to Day 15 (n=3) samples, gene expression CPM filtered counts were normalized using DESeq2, which resulted in 3,883 DEGs; 2,850 up-regulated DEGs and 1,033 down-regulated DEGs (Fig. 8A, Supplementary Table 5A [26]). These up-regulated DEGs from Day 8 to Day 15 were enriched for GO terms associated with regulation of transcription by RNA polymerase II (Fig. 8B, Supplementary Table 5B [26]), and KEGG Pathways associated with herpes simplex virus 1 infection, circadian entertainment, glutamatergic synapse, dopaminergic synapse and amphetamine addiction (Fig. 8C, Supplementary Table 5C [26]).

Comparison of Day 15 (n=3) and Day 25 (n=3) samples found 14,785 genes expressed at measurable abundance (as stated in methods section), with 2,590 DEGs (1,489 up-regulated genes and 1,101 down-regulated DEGs, Fig. 8D; Supplementary Table 5D [26]). The GO terms associated with up-regulated genes from Day 15 to Day 25 were glutamate secretion, chemical synaptic transmission, gamma-aminobutyric acid signaling pathway and neurotransmitter secretion (Fig. 8E, Supplementary Table 5E [26]), whereas KEGG Pathway terms included glutamatergic synapse, retrograde endocannabinoid signaling, dopaminergic synapse, GABAergic synapse and oxytocin signaling pathway (Fig. 8F, Supplementary Table 5F [26]). Interestingly, the GnRH signaling pathway was also an associated with up-regulated DEGs from Day 15 to Day 25 and included 15 GnRH-relevant DEGs (adj. p-value 0.027; Supplementary Table 5F [26]). These unbiased analyses strongly supported the experimental validation of GnRH neuron generation.

We next evaluated the changes in DEGs from Day 8 to Day 25. Among the 2,850 DEGs that were up-regulated between Days 8 and 15 comparison, 28% (n = 804) were also up-regulated from Day 15 to Day 25 (Fig. 9A, Supplementary Table 6A [26]), while 35% (n = 364) of the 1,033 down-regulated DEGs from Day 8 and Day 15 were consistently reduced from Day 15 to Day 25 (Fig. 9D,
Supplementary Table 6A [26]). Results were even more consistent when comparing later stages of development, as over half (53%) of 1,489 up-regulated DEGs from Day 15 and Day 25 were also up-regulated from Day 8 to Day 15 (Supplementary Table 6A [26]), and 33% of 1,101 down-regulated DEGs were commonly down-regulated across these comparisons (Supplementary Table 6A [26]). This overlap of up-regulated and down-regulated DEGs is highly significant (hypergeometric test p-value = 1.6x10^{-213} and 1.6x10^{-174} respectively). For the 804 overlapping up-regulated DEGs, the top associated GO terms were regulation of dopamine secretion, glutamate secretion and learning (Fig. 9B, Supplementary Table 6B [26]), and associated KEGG Pathways included glutamatergic synapse, dopaminergic synapse, circadian entrainment, retrograde endocannabinoid signaling, GABAergic synapse, synaptic vesicle cycle, and GnRH secretion (Fig. 9E, Supplementary Table 6C [26]). When considering time-specific characteristics of gene regulation, we observed enrichment of pathways associated with regulation of transcription by RNA polymerase II, regulation of transcription, DNA-templated and regulation of DNA replication among DEGs uniquely up-regulated from Day 8 to Day 15 but not from Day 15 to Day 25 (Fig. 9C, Supplementary Table 6D-F [26]), while neuroactive ligand-receptor interaction, GABAergic synapse, nicotine addiction, glutamatergic synapse and retrograde endocannabinoid signaling KEGG pathways were enriched for DEGs specifically up-regulated from Day 15 to Day 25 but not Day 8 to Day 15 (Fig. 9F, Supplementary Table 6G [26]).

The time-course analysis of FGF8 treatment samples from day 0 to day 25 identified 1,355 genes that displayed increased expression levels with adj. p-value < 0.05. From these 1,355 genes, we further subdivided the genes using hierarchical clustering with three branches and focused on the cluster of 439 genes that peak in expression levels on Day 25 (Fig. 10A, Supplementary Table 7A [26]). From GO enrichment analysis, these up-regulated genes were associated with memory, synaptic vesicle cycle, glutamatergic synaptic transmission and axon guidance (Supplementary Table 7B [26]), while the 1,403 down-regulated genes (Fig. 10B) were associated with protein sumoylation, tRNA export from the nucleus and mRNA export from the nucleus (Fig. 10C, Supplementary Table 7C [26]). The top KEGG Pathway terms associated with these 439 up-regulated genes were morphine
addiction, circadian entrainment, nicotine addiction, GABAergic synapse, retrograde endocannabinoid signaling and glutamatergic synapse (Supplementary Table 7D [26]), while the 1,403 down-regulated genes were not significantly associated with any statistically significant pathway terms (Fig. 10D). Furthermore, we confirmed the expression pattern of known genes involved in GnRH neurogeneration from stem cells with FGF8 treatment [50] (Fig. 11A). Importantly, despite the methodological differences, including the difference in the stem cell differentiation date/age and RNAseq with mix cell population vs. single cell RNAseq between the present study and Lund et al. [50], almost all (44 of the top 50, hypergeometric test p-value = 5.3x10^{-63}) of the up-regulated genes from Lund et al. [50] displayed similar expression pattern changes this study, while the down-regulated genes did not significantly overlap. Furthermore, we reached a similar conclusion from an additional analysis by testing if the transcriptome signals originate from “non-GnRH cells” in the culture. We compared the expression profiles for different neurotransmitter receptors, namely GABA, glutamate, and acetylcholine receptors, along with neuropeptides such as kisspeptin, neurokinin B, and dynorphin. We observed that 57 of such genes were found in the RNAseq data at the detection limits, 19 of which were differentially expressed between Day 25 and Day 0. These differentially expressed genes showed both up (n=15) and down-regulation (n=4, Supplementary Fig. 5 [26]). Finally, we compiled the developmental expression pattern of all genes based on publications from IHH patients and development of GnRH neurons for future studies and provide these data in Fig. 11B [2,21,51-58].

We evaluated DEGs (|lfc| > 1 and FDR < 0.05) from iPSC to iNs in comparison with those from GnRH maturation, revealing 935 (overlap p-value = 5.4x10^{-73}) and 755 (overlap p-value=2.9x10^{-13}) genes up- and down-regulated respectively common to both neuron maturation procedures. These overlaps are highly significant and suggest that both procedures result in neuronal cell types, as shown by the functional and pathway enrichments (Fig. 12A and B). Interestingly, the GnRH signaling pathway that was significantly enriched in DEGs between Day 15 and Day 25 in FGF8-induced GnRH neurons (adj. p-value 0.027, 15 genes corresponding to this
pathway), was not significant in genes overlapping between the GnRH and iN maturation protocol. This indicates that GnRH maturation protocol leads to a different type of neurons as compared to iNs.

**DISCUSSION:**

In the present study we were successful in establishing a reliable method for generation of GnRH neurons from both hESC and hiPSC. We further observed that SC derived GnRH neurons exhibit physiological characteristics similar to those seen in olfactory placode derived primary GnRH neurons. Importantly, although the treatment with FGF8 alone was effective in generating GnRH neurons, FGF8 plus kisspeptin yielded a higher efficiency rate, on average 10-16% of GnRH decapeptide expressing neurons over the total cells (Figs. 1B and 3B). These numbers were far from a “pure population”. There might be, however, a potential limitation to reach close to 100%. As seen in the results of notch inhibitors and an antimitic drug, elimination of non-GnRH cells dramatically reduced the total number of cells, resulting in a significant loss of GnRH neurons in the culture. We have also noticed that stem cell derived GnRH neurons prefer to grow on the top of non-neuronal cells and form thick multilayered clusters (Fig. 1D and 4A), but not form ganglia. This characteristic is very similar to that in primary GnRH neurons. They always grow on the top of non-neuronal cells (e.g., fibroblasts or epithelial cells) [41], which differ from other types of neurons, such as spinal motor neurons [59]. Whether the efficiency rate improves by modifications of plating substrates other than matrigel, such as neural cell adhesion molecule (NCAM) [60], or by three-dimensional cultures with proper scaffolds remains to be investigated.

When we started this project, there was no publication on this topic. Since then, two groups have reported the generation of human GnRH neurons from stem cells using FGF8 [27,28]. The basic method of Lund et al. [27] is similar to our Protocol 2, while our Protocol 1 has some similarities with Poliandri et al. [28]. In Protocol 2, we could count nearly 40% efficiency rate in parts of cultures, but in other parts of the same cultures the efficiency rate was less than 1-2%, yielding an overall average
of 10%. Nonetheless, this study is unique, as we focused on the developmental window of GnRH neurogeneration using m-Cherry labeled GnRH neurons and RNAseq, and physiological characterization of stem cell derived GnRH neurons using electrophysiological analysis, calcium imaging, and pulsatile GnRH release.

Mutations in FGFR1 and FGF21 result in IHH [52]. FGF8 action is mediated largely through FGFR1 and some through FGFR3 in rodents [61,62]. FGF21 action is also mediated through FGFR1, but it requires the co-receptor β-Klotho [63]. In the present study we found that while the exposure of hESCs to FGF8 generated GnRH neurons, FGF21 was ineffective (Fig. 3). The results from this study, then, indicate that β-Klotho is unlikely required for GnRH neurogeneration. Whether additional members of FGF family, such as FGF17, are involved in the GnRH neurogeneration remain to be examined.

Among small molecules tested, we found that addition of hKP10 supplementation during Day 10-15 FGF8 exposure had a synergistic action with FGF8. Apparently, kisspeptin possesses a neuro-proliferative action. Exposure of embryonic, not adult, zebrafish to kisspeptin increases the number of hypothalamic GnRH neurons [44]. In our study kisspeptin alone was not as effective as FGF8. Neuro-proliferative action of kisspeptin during the GnRH neurogeneration appears to be an evolutionally well-conserved feature. Nevertheless, a question arises: Why is kisspeptin supplement on Day 10-15 FGF8 most effective? Regardless of FGF8 treatment KISS1 expressions assessed by RT-PCR were significantly elevated on Day 5 (Fig. 3A) and RNAseq analysis indicates that both KISS1 and KISS1R expressions appeared to be elevated between Day 8 and 15 (Fig. 11B). Perhaps, hKP10 supplementation during Day 0-5 is less effective, because kisspeptin in stem cell cultures is already high, whereas KISS1R between Day 0 and 8 is low (Fig. 11B). Interestingly, transgenic male mice studies indicate that highly specific transsynaptic neural pathways from kisspeptin neurons to GnRH neurons are established by the mid-point of GnRH neurogeneration in vivo [64,65], suggesting kisspeptin can influence the ontogeny of GnRH neurons in vivo. Among other small molecules,
failure of RA and SHH to increase GnRH neurogeneration needs further examination, as the doses and timing of drug applications require a delicate balance.

Generation of mCh-GnRH carrying hESC allowed us to find the exact time course necessary for stem cell transformation into GnRH neurons, to count GnRH producing cells more accurately, and to conduct experiments for physiological characterization of GnRH neurons. Live cell imaging suggests that expression of the GnRH gene was visible as early as Day 11 FGF8 exposure and the number progressively increased for an additional 2 weeks until it reached a plateau. Importantly, RNAseq analysis also indicates that GnRH neurogenesis start between Day 8 and Day 15 FGF8 exposure, then it accelerated between Day 15 and Day 25 (Fig. 11B). Available reports in vivo show that GnRH neurons in human fetuses are not visible on E28-E32, but they are seen in the olfactory placode between E42 and E56 [27,60]. Thus, the in vitro maturation of GnRH neurons from hESCs seems to recapitulate in vivo maturation of human GnRH neurons.

Because of the fluorescent mCherry marker labeling, we were able to conduct extensive physiological characterization of hESC derived GnRH neurons. In fact, to our knowledge, these are the first successful single cell patch clamp recordings from human GnRH neurons reported. We found that human GnRH neurons had more hyperpolarized resting membrane potentials (approximately -90 mV) and smaller action potential amplitudes (approximately 50 mV) compared with GnRH neurons of other mammalian species (approximately -55 mV and 80 mV, respectively, see Table 1) [46-49]. The majority of these cells did, however, exhibit spontaneous depolarizing plateaus that triggered trains of action potentials. All GnRH neurons older than Day 20 FGF8 exposure, that we were successful in recording, exhibited similar electrophysiological characteristics and included spontaneous action potentials occurring individually and in bursts, indicating that Day 20 FGF8 exposure may be sufficient to achieve GnRH neural maturation. Because in this study we did not examine GnRH neurons older than Day 34, questions of whether highly hyperpolarized resting membrane potentials and whether the propensity of membrane potentials switching between two
semi-stable “resting” potentials, change in older GnRH neurons (over Day 35) remain to be investigated. The exceptionally hyperpolarized resting membrane potentials in human GnRH neurons need further examination. We can, however, add our preliminary observations that monkey primary GnRH neurons also display highly hyperpolarized resting membrane potentials, such as -100 mV (Abe and Terasawa, unpublished observation).

For $[\text{Ca}^{2+}]_i$ imaging analysis, we applied the same method and criteria used for the primary GnRH cell cultures in non-human primates, as described previously [13,41]. We found that similar to primary GnRH neurons, individual GnRH neurons exhibited periodical $[\text{Ca}^{2+}]_i$ oscillations with the IPI of $18.3 \pm 1.3$ min and individual $[\text{Ca}^{2+}]_i$ oscillations synchronized periodically at the IPI of $46.5 \pm 3.0$ min. However, in contrast to primary GnRH neurons that exhibit relatively uniform oscillatory $[\text{Ca}^{2+}]_i$ patterns [13], the individual pattern of stem cell derived GnRH neurons was highly variable (Fig. 6), synchronization peaks were not as conspicuous as seen in primary GnRH neurons, and the IPI was ~2 fold longer than that in primary GnRH neurons ($8.2 \pm 0.7$ min). Importantly, however, the synchronization interval of stem cell derived GnRH neurons was very similar to that in primary GnRH neurons ($52.8 \pm 3.0$ min) [13], as well as the IPI of GnRH release in vitro (46.6±4.0 min) [11] and in vivo (~60 min) [9,66,67]. We hypothesize that GnRH neurons release the decapetide hormone at the time of their synchronization.

While primary GnRH neurons derived from embryonic olfactory placode exhibit relatively uniform $[\text{Ca}^{2+}]_i$ patterns [13,41], they are quite variable among individual hESC-derived GnRH neurons. We speculate that the highly variable $[\text{Ca}^{2+}]_i$ patterns in hESC-derived GnRH neurons as compared to those in placode derived GnRH neurons are attributable to the difference in the cell composition in the two culture systems. Primary GnRH neurons derived from embryonic monkey olfactory placode contain a lot of non-neural cells, but they do not contain other type of neurons or glia [7,41], whereas cultures of stem cell-derived GnRH neurons contain a substantial number of glia, and other types of neurons including $\gamma$-amino butyric acid (GABA), neuropeptide Y (NPY), and...
tyrosine hydroxylase positive neurons (Fig. 13). As such, the presence of other types of neurons and glia adjacent to GnRH neurons is the more likely explanation, as they can influence GnRH neuronal activity synaptically or non-synaptically. Species differences (i.e., human GnRH neurons in this study vs. non-human primate GnRH neurons in the primary culture) cannot be excluded. Nevertheless, despite highly variable \([\text{Ca}^{2+}]_i\) patterns in individual GnRH neurons in this study, they exhibited a synchronization interval similar to that seen in primary GnRH neurons, suggesting that GnRH neurons themselves are the pacemaker for the pulsatile peptide release (see Terasawa, 2019 [68] for further discussion).

In this study we have further shown that stem cell-derived GnRH neurons release the 10 amino acid GnRH peptide spontaneously or in response to known secretagogues, such as kisspeptin and NKB. Moreover, direct stimulatory action of estradiol on GnRH neurons is consisted with previous observations in the median eminence \textit{in vivo} [69]. Importantly, the IPIs of hESC and hiPSC are both \(~60\) min \((59.5\pm3.3\) for hESC and \(62.5\pm6.7\) min for hiPSC), which are very similar to the reported IPI in pulsatile LH release in humans [70,71], release of LH and GnRH and multiple unit activity associated with LH pulses in monkeys \textit{in vivo} [9,66,67,72,73], and cultured placode-derived GnRH neurons [11]. Additionally, we have previously shown that GnRH release occurs in response to kisspeptin, NKB, and \(\text{E}_2\) [17,74-78] \textit{in vivo}. Stimulatory actions of kisspeptin and NKB on GnRH/LH release have also been reported in many other mammalian species, including in rhesus monkeys [78-81]. Note that the secretagogue-induced GnRH release could be indirectly through other inter-neurons, as stem cell derived cultures contain other neurons (Fig. 13).

In the present study, we designated the first day of FGF8 exposure as Day 0, not counting the first 7-10 days of the CDM exposure (and 7-10 days of NIM in Protocol 1), whereas works by Lund et al. [27,50] designated the first day of the CDM exposure as Day 0 and 10 days later epithelial cells are exposed FGF8 (Day 10). As such, there is a 10-day difference in FGF8 exposure between our study and Lund et al. [27,50]. Additionally, while for the RNAsesq analysis we used mixed cell groups...
throughout the stem cell differentiation by FGF8 exposure, i.e., Day 0, 8, 15, and 28, Lund et al. [50] used mixed cell groups for their Day 20 (Day 10 of FGF8 exposure) and Td-Tomato-labeled GnRH neurons only for Day 27 (Day 17 of FGF8 exposure) in the RNAsesq analysis. Despite these differences between the present study and Lund et al. [50], the profile of the top 44 up-regulated genes is essentially identical.

Prior to the GnRH neurogeneration the presence of genes Sox2, Ascl1 and Sox2 in the developing lateral olfactory epithelial and Fgf8 in the medical olfactory epithelial has been described [82,83]. Indeed, the results from RNAseq (Fig. 11B) indicate that GSX2, ASCL1, and SOX2 were elevated on Day 15 and Day 25. In contrast, while GnRH was low on Day 8, slightly elevated on Day 15, and reached the highest level on Day 25. These observations are consistent with developmental changes in the generation of olfactory placode followed by the birth of GnRH neurons [23,45,84]. Importantly, the intensity of elevated GnRH-1 gene expression is parallel to the increased number of mCh-GnRH neurons.

Genetic analyses of IHH patients suggest that the mutations in ANOS1 (KAL1), PROKR2, PRK2, FGF17, FGF19, TAC3R, TAC3, SPRY4, and SEMA3A, 3C, 3E are all involved in infertility or abnormal timing of puberty [2,51,52,54,56,85-88]. The developmental profiles of the present study suggest that expressions of these genes were elevated on Day 15 and Day 25 along with the GnRH gene. In contrast, both FGF8 and FGFR1 were elevated on Day8, but on Day 15 they started to decline reaching their lowest levels on Day 25. Although this might indicate the timing of the importance of FGF8 and FGFR1 presence olfactory placode/GnRH neurogeneration, it is also possible that the presence of FGF8 in media might have changed the expression pattern. Addtionally, a highly similar differential gene expression pattern between FGF8-derived GnRH neurons and iNs (Fig. 12 B and C) suggest that during the differntiaion and maturation the FGF8-derived GnRH neuron acquires pan-neuronal characteristics. On the other hand, the absence of GnRH signalling genes in the DEGS shared between two maturation protooolcs indicate that the timing and dose of FGF8 treatment
protocol applied to hiPSC are unique to GnRH neurogeneration when compare to neurogeneration by Ngn2 treatment.

In the present study we observed that the rate limiting enzyme for GABA synthesis from glutamate, \textit{GAD1} and \textit{GAD2} prominently increased along with GnRH gene and genes involved in IHH, and the importance of GABA and glutamate \textit{GAD1} and \textit{GAD2} in regulation of GnRH release and puberty has been well documented (see Terasawa and Fernandez [89]). In fact, the prominent increase in \textit{GAD1} and \textit{GAD2} might be related to GnRH neuronal function, such as cell migration, as GABA is involved in the cell migration of GnRH neurons [90-92]. Similarly, genes RELN and LRRTM that significantly activated on Day 15 and Day 25, are likely involved in synaptic formation [93,94] as well as neuronal cell migration [95] after GnRH neuron are generated.

The developmental profiles of the genes that involved in GnRH transcription, such as \textit{OTX1}, \textit{OTX2}, \textit{TP53}, \textit{SIX3}, and \textit{SIX6} [57,96-100] were weakly associated with changes in GnRH neurogeneration, although they are not as prominent as the genes associated with IHH. Interestingly, on Day 25 the expression of \textit{SIX3} was slightly elevated, whereas \textit{SIX6} was suppressed (Fig. 11B). It has been reported that \textit{Six6} is activational, while \textit{Six3} appears to be a repressional, transcription factor for the \textit{GnRH} gene [57].
In summary, here we report a reliable method for generation of human GnRH neurons from hiPSC as well as ESC. Importantly, with our method we can harvest approximately 10,000-20,000 GnRH neurons per single slide. This is equivalent to having 5 to 10 primate brains, as human and macaque brains contain a total of ~2,000 GnRH neurons [101]. We also generated a hESC line, in which mCherry fluorescent marker is expressed when the GnRH gene turns on. Furthermore, we conducted extensive physiological characterization experiments in stem cell derived GnRH neurons and found that they exhibit characteristics similar to GnRH neurons in vivo and primary GnRH neurons in vitro. Finally, we characterized developmental profiles of the GnRH transcriptome. Collectively, benefits of human GnRH neurons generated by the method described in this article are multi-fold: 1) they will help with research on human GnRH neurobiology, 2) they will be useful for establishing a disease model for IHH, 3) they will provide a potential tool for cell transplantation therapies in IHH patients, and 4) they will be useful for contraceptive drug screening.
Acknowledgments:

The authors thank Drs. Su-chun Zhang, Department of Neuroscience and Waisman Center, University of Wisconsin-Madison for his comments on this project, Dr. Stephanie Seminara, Harvard Reproductive Sciences Center for her comments on the manuscript, and Dr. Nelly Pitteloud, Lausanne University Hospital, Switzerland for her comments regarding FGF21.

Funding:

This work was supported by Grant R21HD092009 (to E.T.) and R01HD096326 (to M.E.T.) from the Eunice Kennedy Shriver Institute of Child Health and Human Development and by UW-ICTR grant (UL1TR002373) from the NIH National Center for Advancing Translational Sciences. The work was made possible by support from the NIH Office of the Director for the Wisconsin National Primate Research Center (P51OD011106). Generation of the fluorescent reporter cell line was supported in part by a core grant to the Waisman Center from the NICHD (U54HD090256) and by a UW2020 Grant from the University of Wisconsin and the Wisconsin Alumni Research Foundation (to A.B. and Su-Chun Zhang).
Author Contributions:

ET, RAP, AB and KLK designed experiments, KLK, AJP, AGF, and BIF conducted experiments, JS, RY and SE conducted differential gene expression analysis, and ET, KLK and AJP, and MET wrote the manuscript.

The Data Availability Statement:

All data presented in this manuscript are available upon request.
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**Figure Legends**

**Figure 1.** Comparison of two protocols for FGF8-induced GnRH neurogeneration from hESC or hiPSC. Schematic illustrations of two protocols that we examined for the efficiency rates of GnRH neurogeneration (A); Comparison of the efficiency rate between immunohistochemically stained GnRH neurons generated by the two protocols (B); and Examples of immunopositive GnRH neurons from the two protocols (C-K) are shown. **A.** The first day of FGF8 treatment is designated Day 0. In Protocol 1, after dual SMAD inhibition in cell differentiation media (CDM), cells were exposed to neural induction media (NIM) for at least 7 days resulting in neurospheres [neuroprogenitor (NP) cells]. Then NP cells were exposed to FGF8 in neural differentiation media (NDM). In Protocol 2, after dual SMAD inhibition neuroepithelial (NE) cells were exposed to FGF8 in NDM. These cultures contain multiple types of cells at multiple developmental stages. **B.** Whisker plot shows Protocol 2 yielded a significantly higher efficiency rate of GnRH neurogeneration than Protocol 1 (Left). *** p<0.001 vs. control (NDM alone); ††† p<0.001 vs. Protocol 1. n=5-20. Exposure of hESC (C and D) or hiPSC (F and G) to FGF8 resulted in GnRH neurogeneration. Comparison between Protocol 1 (C and F) and Protocol 2 (D and G) by immunohistochemical staining with the polyclonal antibody GF6 (Red, C-G) indicates that Protocol 2 yielded a larger number of GnRH neurons in both hESC and hiPSC. **E.** Without FGF8 (control) only a few GnRH neurons were occasionally seen in control hESC culture. For C to G Green = β-tubulin. **H.** GnRH neurogeneration by FGF8 is also shown with immunostaining using the monoclonal antibody LRH13 (Green) and the neural marker MAP2 (Red). Insets in C and H are a high power (2.5x) magnification of GnRH neurons with arrows. **I-K.** GnRH neurons (Red) derived from hESC by FGF8 treatment, immunostained by polyclonal antibody SW1 (I), double stained with β-tubulin (Green, J) and overlay (K). For all: Blue = DAPI nuclear staining.

**Figure 2.** Determination of FGF8 doses for GnRH generation from hESC using Protocol 2. FGF8 at 10 μg/ml is sufficient. Total cell number of a single coverslip was ~200,000. **p <0.01 vs. control (NDM alone). n=3 to 6.

**Figure 3.** **A.** Changes in kisspeptin mRNA expressions during GnRH neurogeneration by FGF8 treatment. mRNA levels on Day 0 (green), 5 (pale pink), 10 (dark pink), and 24 (Red) in the presence of FGF8 and Day 5 (pale blue),10 (blue), and 24 (dark blue) in the absence of FGF8 (NDM control) were assessed by RT-PCR (all, n=4). aaa = p<0.001; aa= p <0.01; and a=p<0.05 vs. FGF8 Day 0 (NE stage). Note that regardless of the presence or absence of FGF8, kisspeptin mRNA expressions on Day 5 were significantly higher than Day 0. **B.** Effects of various molecules on GnRH neurogeneration from hESC. The Mean ± SEM efficiency rate (n=6, green). Exposure of NE cells to FGF8, but not to FGF 21, was effective in GnRH neurogeneration and addition of KP10 to FGF8 in NDM for a 5-day period, especially, Day 10-15, significantly increased the efficiency rate of GnRH neurogeneration as well as the maximum efficiency rate. **p<0.01, *** p<0.001 vs. control (NDM alone); † = p< 0.05 vs. FGF8 alone.

**Figure 4.** Fluorescent mCherry labeled GnRH neurons derived from hESC allowed us to clarify the time course of GnRH neurogeneration. Using the CRISPR/Cas9 gene editing system, hESCs were modified such that m-Cherry is expressed when the GnRH gene turns on. **A.** Live cell imaging (top row) and phase contrast imaging (second raw)
were taken on Days 7, 11, 13 and 20 of the FGF8 exposure at the same slide position, as indicated by a black marker with a white shadow circle. Note that because of the migratory nature of GnRH neurons, GnRH neurons seen one day are not visible in the same location in the later days. B. An overlay microphotograph (bottom panel) indicates that m-Cherry expressing cells (red, middle panel) are also GnRH peptide positive (green, top panel). Note that while the peptide staining is seen in the cell body as well as in neurites, mRNA expression is limited to the cell body. C. GnRH neurons counted in an 8 mm² area showed that GnRH neurons are seen as early as Day 11-12 of FGF8 exposure, and the number of GnRH expressing cells progressively increased between Days 12 and 24, reaching the plateau. Red bars indicate cultures exposed to FGF8, whereas gray bars indicate cultures exposed to NDM alone. n=4/group. Statistical analyses are seen in Supplementary Table 4 [26].

**Figure 5.** Electrophysiological characteristics of GnRH-expressing neurons derived from hESC. A and B. Examples of voltage responses to 50 pA (A) or 10 pA (B) current steps in two different neurons. (right) I-V plots corresponding to traces to the left. In one of the cells (A, 28 days, \(V_m = -48 \text{ mV}\)), no action potentials were elicited by depolarizing current steps, but rebound action potentials followed termination of hyperpolarization. In the other cell (B, 34 days, \(V_m = -72 \text{ mV}\)), depolarizing steps elicited action potential trains, but not rebound potentials. C. Examples of bimodal resting membrane potentials (i.e. \(i = 0 \text{ pA}\)) in four cells. Traces i (28 days) and ii (22 days) show spontaneous transitions between hyperpolarized and depolarized levels, with activity consisting of single action potentials or brief bursts of action potentials. Traces iii (30 days) and iv (21 days) show transitions to sustained depolarized levels induced by brief 100 pA hyperpolarizing current pulses (arrows). Note that broken lines in C indicate the resting membrane potentials. D. Relationship between electrophysiological characteristics of generated GnRH neurons. The age of the cell when recorded is labeled on each point. Although there were negative correlations between action potential half width and input resistance (i, \(r^2 = 0.62, F_{1,7} = 11.45, p = 0.012\)) and between membrane potential and input resistance (ii, \(r^2 = 0.67, F_{1,7} = 14.63, p = 0.007\)), both were developmental age independent.

**Figure 6.** An example of the pattern of \([\text{Ca}^{2+}]_i\) oscillations in a GnRH neuron (top row) and the raster plot of significant \([\text{Ca}^{2+}]_i\) peaks in all 15 neurons (third row) in the field on a single coverslip (A) are shown. Similar to primary GnRH neurons, GnRH neurons derived from hESC exhibited periodical synchronizations of \([\text{Ca}^{2+}]_i\) oscillations, shown by the colored heat map above the raster plot (second row). The shaded area (bottom row) shows a close up profile of synchronized pulses from five cells during the second synchronization. The \([\text{Ca}^{2+}]_i\) response to 56 mM K\(^+\) (B) and kisspeptin (C) are also shown. The \([\text{Ca}^{2+}]_i\) responses to high K\(^+\) and KP10 were uniform among the cells.

**Figure 7.** GnRH neurons derived from hESC (A and B) and hiPSC (C) released the GnRH decapetide in a pulsatile manner. They also responded to 10 nM KP10 (D), 10 \(\mu\)M senktide (E), 100 nM estradiol (E2, F) and 56 mM K\(^+\) (B, C, D, E and F). Arrows indicate GnRH peaks identified by Pulsar algorithm. Results of statistical analyses for all secretagogues are shown in G. n= 9-20. * = \(p<0.05\), ** = \(p<0.01\), *** = \(p<0.001\) vs. baseline values; † = \(p<0.005\), †† = \(p<0.01\), ††† = \(p<0.001\) vs. vehicle at the corresponding time. Note that while examples of GnRH release
were shown as actual concentrations (pg/ml), the data of statistical analysis were shown as percent change from
the baseline (an average of the 30 min prior to the secretagogue challenge).

Figure 8. Differential Expression Analysis of Day 8, Day 15, and Day 25. (A) Volcano plot for differentially
expressed genes from Day 8 to Day 15. The top 20 genes with adj. p-value < 0.05 and highest log2 (fold change)
are labeled. The number of up-regulated genes is 2,960, while the number of down-regulated genes is 1,075. (B)
GO enrichment terms for differentially expressed genes from Day 8 and Day 15 (up and down-regulated genes
are defined the same way as above). The numbers at the end of the bars indicate the number of genes
corresponding to the respective GO enrichment terms. (C) KEGG pathways for differentially expressed genes
from Day 8 to Day 15. The numbers at the end of the bars indicate the number of genes corresponding to the
respective KEGG pathway terms. (D) Volcano plot for differentially expressed genes between Day 15 and Day 25.
Top 20 genes with adj. p-value < 0.05 and highest log2 (fold change) are labeled. The number of up-regulated
genes is 1,475, while the number of down-regulated genes is 1,143. (E) GO enrichment terms for differentially
expressed genes from Day 15 and Day 25. (F) KEGG pathways for differentially expressed genes from Day 15 to
Day 25.

Figure 9. Differences in the differentially expressed genes between Day 8 and Day 15 and between Day 15 and
Day 25. (A) Overlapping set of up-regulated genes between Day 8 and Day 15 and the set of up-regulated genes
between Day 15 and Day 25. (B) GO enrichment terms for overlapping up-regulated genes (n=840). (C) GO
enrichment terms for genes that are up-regulated between Day 8 and Day 15, but not between Day 15 and Day
25 (n=2,120). (D) Venn diagram for the set of down-regulated genes between Day 8 and Day 15 and the set of
up-regulated genes between Day 15 and Day 25. (E) KEGG pathways for overlapping up-regulated genes
(n=840). (F) KEGG pathways for genes that are up-regulated between Day 15 and Day 25, but not between Day 8
and Day 15 (n=635).

Figure 10. Genes with expression pattern during time-course treatment with FGF8 from day 0 to
day 25. (A) Gene expression patterns for 439 up-regulated genes from Day 0 to Day 25. (B) Gene
expression patterns for 1,403 down-regulated genes from Day 0 to Day 25. (C) GO enrichment terms for
genes with correlated expression pattern during time-course FGF8 treatment. (D) KEGG pathway terms
for genes with correlated expression pattern during time-course FGF8 treatment

Figure 11. Gene expression changing during the GnRH development. (A) Gene expression heatmap
for top 49 up and 42 down-regulated genes, aligned with 50 up and down genes (each) differentially
expressed between day 20 and day 27 in FGF8-treated mCh-expressing GnRH neurons reported by
Lund et al (2020). (B) Gene expression heatmap for the genes of our interests during the GnRH neuronal
differentiation from stem cells. The color scale of heatmaps represents row-scaled z-scored DESeq2
normalized counts.

Figure 12. Comparison of FGF8 treated GnRH maturation protocol with iPSC-derived Ngn2-induced
neurons (iN). (A) Number of genes overlapping between up and down-regulated differentially expressed
genes from the GnRH protocol from Day 0 to Day 25 and iPSC to iN development. (B) GO Enrichment Terms for overlapping up and down-regulated genes between the GnRH protocol and iPSC-iN development. (C) KEGG Pathway Terms for overlapping up-regulated genes between the GnRH protocol and iPSC-iN development.

**Figure 13. Left column:** GFAP positive cells (A) and neurons immunopositive to antibodies against tyrosine hydroxylase (TH, D), γ-aminobutyric acid (GABA, G), and neuropeptide Y (NPY, J) are shown in Green. **Middle column:** Red =mCherry positive GnRH neurons (B, E, H, and K). **Right column:** overlay. Note that a subset of GABA neurons co-localize with GnRH neurons (I). Also, while glia, GABA and NPY neurons are closely associate with GnRH neurons, TH positive neurons are located in a completely different location within the slide.
### Table 1: Electrical properties of human GnRH neurons derived from embryonic stem cells.

|                         | Human     | Mouse     | Guinea pig | Mouse     |
|-------------------------|-----------|-----------|------------|-----------|
|                         | ES derived (n=10) | GFP-labeled (n=26)<sup>a</sup> | Hypothalamus (n=8)<sup>b</sup> | Olfactory placode (n=13)<sup>c</sup> |
| Resting potential (mV) | -88.1±8.5 | -55.7±3.7 | -55.0±8.5  | -50.8±3.6 |
| Input resistance (GΩ)  | 3.3±0.4   | 1.2±0.5   | 0.5±0.2    | 2.4±2.0   |
| Membrane capacitance (pF)| 21.8±3.8  | 25.9±11.3 | 46.0       | 10        |
| AP half width (msec)   | 2.9±0.3   | 1.2±0.3   | 2.5±0.7    | 2         |
| AP Amplitude (mV)      | 54.3±2.7  | 79.5±9.8  | NR         | 80        |
| Threshold (mV)         | -45.4±1.5 | -32.2±3.9 | NR         | -37.5     |

<sup>a</sup> Spergel et al., 1999<sup>38</sup>  
<sup>b</sup> Lagrange et al., 1995<sup>39</sup>  
<sup>c</sup> Kusano et al., 1995<sup>40</sup>

Note that the information from b and c were extracted from Spergel et al., 1999<sup>38</sup>. 
