Generation of kisspeptin-responsive GnRH neurons from human pluripotent stem cells

Ariel Poliandri, Duncan Miller, Sasha Howard, Muriel Nobles, Steven Harmer, Andy Tinker, Tristan McKay, Leonardo Guasti, and Leo Dunkel.

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SUMMARY STATEMENT (15-30 words)

ABSTRACT

GnRH neurons are fundamental for reproduction in all vertebrates ultimately integrating all reproductive inputs. The inaccessibility of human GnRH-neurons has been a major impediment to studying the central control of reproduction and its disorders. Here, we report the efficient generation of kisspeptin responsive GnRH-secreting neurons by directed differentiation of human Pluripotent Stem Cells. The protocol involves the generation of intermediate Neural Progenitor Cells (NPCs) through long-term Bone morphogenetic protein 4 inhibition followed by terminal specification of these NPCs in media containing FGF8 and a NOTCH inhibitor. The resulting GnRH expressing and secreting neurons display a neuroendocrine gene expression pattern and present spontaneous calcium transients that can be stimulated by kisspeptin. These *in vitro* generated GnRH expressing cells provide a new resource for studying the molecular mechanisms underlying the development and function of GnRH neurons.

Introduction

GnRH neurons are fundamental for reproduction in all vertebrates. They not only determine the timing of puberty but ultimately integrate all reproductive inputs (Romanelli et al., 2004; Stevenson et al., 2013). In humans, the GnRH-neuronal network is composed of a few hundred neurons distributed as clusters located from the preoptic area to the anterior hypothalamic regions making *in vivo* or *ex vivo* experimental studies of GnRH neurons extremely difficult (Maggi et al., 2000; Wang et al., 2015). The molecular ontogeny of GnRH neurons remains elusive. It is generally accepted that most hypothalamic GnRH neurons originate in the olfactory placode (OP) but compelling evidence suggests that a significant proportion of GnRH neurons may descend from neural crest progenitors (either directly or via their early migration to the OP) (Forni et al., 2011; Forni and Wray, 2012). Bone morphogenetic protein-4 (BMP4) plays an essential role in the formation of the olfactory placode (Leung et al., 2013) and GnRH neurons arise from the olfactory placode in a niche defined by gradients of BMP4/Noggin and FGF8 (Forni et al., 2013; Sabado et al., 2012). After arising in the olfactory placode, GnRH neurons migrate to their final location in the anterior hypothalamus during embryonal development. Failure of these neurons to reach their appropriate place in the hypothalamus or to form functional networks has been associated with many reproductive phenotypes including delayed or absent puberty (Boehm et al., 2015; Howard et al., 2016). Some pubertal disorders such as Kallmann syndrome (where puberty is never or only partially completed) are often associated with anosmia (the inability to perceive odours) pointing to the common origin of olfactory and GnRH neurons (Forni and Wray, 2015; Herbison, 2007; Tucker et al., 2010).

GnRH neurons secrete GnRH peptide in a pulsatile fashion. The frequency and amplitude of these secretory pulses changes during reproductive development and reproductive cycles, for example during puberty or the menstrual cycle (Apter, 1997; Barbieri, 2014; Ojeda et al., 2010). The pulsatile release of GnRH is controlled by the periodicity and amplitude of action potentials and calcium transients. These are regulated by several neuropeptides including kisspeptin, neurokinin B, and GABA (Constantin et

al., 2009; Ronnekleiv and Kelly, 2013; Verma et al., 2014). Of particular interest, kisspeptin and its receptor -the GPCR Kiss1R- have been implicated in several reproductive disorders (Clarke et al., 2015). Activation of Kiss1R causes an increase in the frequency and amplitude of calcium transients in GnRH neurons partly by inhibiting A-type and inwardly rectifying K^+ currents and activating non-selective cation (TRPC) currents (Lee et al., 2010; Ronnekleiv and Kelly, 2013).

Cell line models of GnRH neurons exist and they have proven useful for unpicking mechanisms of GnRH regulation. These cell lines however have been generated from tumours and represent static models of neurons at a fixed developmental stage (Constantin et al., 2009; Radovick et al., 1991; Romanelli et al., 2004).

Pluripotent Stem Cells (PSCs)-derived neurons have been used successfully to model several human disease conditions in the lab (Hibaoui et al., 2014; Liu and Deng, 2016; Mattis and Svendsen, 2015). An *in vitro* model of PSCs-derived GnRH neurons could help not only in understanding the normal development of this unique subset of neurons but it could be used to investigate aberrant development of conditions such as Kallmann syndrome and as a platform for drug discovery and functional genomics.

Here we describe a protocol that consistently and efficiently generates GnRH-expressing and secreting cells (GnRH-ECs) from human PSCs. Analysis of their gene expression profile and response to kisspeptin suggest that these *in vitro* generated neurons functionally resemble GnRH neurons.

Results

Generation of Neural Progenitor Cells (NPCs)

To generate human GnRH-expressing cells we started by producing NPCs adapting a protocol previously published by Gerrard *et al.* (Gerrard et al., 2005). This protocol involves culturing ESCs under neurogenic conditions for several passages using Basal Neuro-Epithelial (BNE) medium supplemented with the BMP inhibitor noggin (Fig 1A). As expected, during the development of NPCs, pluripotency markers were downregulated whereas neuronal markers were upregulated (Fig 1B). NPCs readily differentiated into neurons after withdrawal of growth factors (Suppl Fig 1). The differentiation process produced NPCs with similar efficiencies from human Embryonic Stem Cells (ESCs) and induced Pluripotent Stem Cells (iPSCs) derived from individuals with Kallmann Syndrome and their healthy family members (Fig 1 C and D). After passage 4 (P4) noggin was withdrawn and the cells were expanded in BNE medium supplemented with bFGF and EGF for up to 4 passages. It has been reported that the development of GnRH neurons in the olfactory epithelium depends on a neurogenic environment provided by an interplay between BMP4 and FGF8 signalling (Forni et al., 2013; Leung et al., 2013; Sabado et al., 2012). FGF8 is essential for GnRH neurons development *in vivo* and it has been used previously to differentiate chicken olfactory placode progenitors into GnRH neurons (Sabado et al., 2012).

GnRH neuronal specification

For the second stage of our differentiation protocol we removed bFGF and EGF from our medium and incubated the NPCs in medium supplemented with 10 ng/ml FGF8 for 21 days. This led to significantly elevated levels of GnRH mRNA and protein (Fig 2 B-D). GnRH was also detected by ELISA in the cell media (Fig 2 E). In addition, NPCs in FGF8 supplemented medium adopted a mature neuronal morphology forming neurospheres and generating projections (Fig 2A). After plating the NPCs in FGF8 for differentiation, the cells still went through several rounds of division. We found that a correct initial cell density was essential for achieving an efficient differentiation/specification. We determined an optimal plating density of

between 5000 to 7500 cells/cm^2. The notch inhibitor DAPT has been used previously to induce mitotic arrest and to promote neuronal differentiation from ESCs (Borghese et al., 2010). We therefore decided to treat the cells during the first week of differentiation with 5 μM DAPT to induce mitotic arrest and aid differentiation.

To investigate the proportions of GnRH-Expressing Cells (GnRH-ECs) after 21 days of differentiation we stained the cells with an anti-GnRH antibody which has been consistently used to visualise GnRH neurons in both human and mouse hypothalami (Howard et al., 2016). The majority of TUJ1 positive cells expressed GnRH (Fig 3 A). Furthermore, GnRH staining was presented in a punctate pattern typical of neuropeptides which are packaged into vesicles. Our differentiation protocol also produced GnRH-expressing neurons from iPSCs derived from both Kallmann syndrome patients and their healthy family members (Fig 3 B). To quantify the proportion of GnRH positive neurons we analysed 21 days differentiated cells using flow cytometry (Fig 3C). The proportion of GnRH positive cells derived from ESCs varied between experiments but it was always over 60% (Fig 3 Dc and Dd). Cultures of mouse GN11 cells -a model for immature, migratory GnRH neurons- contained similar proportions of positive cells (Fig 3 Db). The proportion of GnRH-positive cells varied between 40 and 50% in GnRH-ECs derived from iPSCs (Fig 3 De and Df). Human Embryonic Kidney-derived cells (HEK293) did not stain positively for GnRH (Fig 3 Da).

<u>Gene expression profiling of PSCs-derived GnRH expressing cells.</u>

After confirming that our protocol reliably produced neuronal cultures with high numbers of GnRH-positive neurons we looked for other genes known to be expressed in GnRH neurons (Fig 4). In addition to a complete downregulation of pluripotency marker NANOG and inducing a 600-fold upregulation of the neuronal marker TUJ1, 21 days differentiation induced the expression of KISS1R and upregulation of TAC3R, ESR2, and GAD65, factors playing essential roles in regulating GnRH neurons. In addition, differentiation also induced the expression of markers associated with nasal placode GABA2 and EYA1 (Sabado et al., 2012) possibly indicating immaturity (or incomplete

differentiation) of some of the cells. The increase in GFAP could indicate the presence of Olfactory Ensheathing Cells, glial cells which have been shown to arise in the nasal placode and to be important in GnRH neurons development and migration (Geller et al., 2013).

To further investigate the phenotype of our GnRH-ECs we performed a global mRNA expression analysis comparing ESCs, NPCs and GnRH-ECs. Because cells subjected to 21 days differentiation continued to express genes present in very immature GnRH neurons a 35 day differentiation point was included in the analysis (Suppl Fig 2 and http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE86990). All neuronal groups clearly diverged from ESCs (Suppl Fig 1) therefore we concentrated on just NPCs, 21 days GnRH-ECs, and 35 days GnRH-ECs to look for a specific gene expression profile for the differentiated GnRH neuronal phenotype. We performed a hierarchical clustering of differentially expressed genes using Cluster 3 software. Selecting for probes with variability greater than 3 SDs between groups we ascribed 1081 differentially expressed entries (Fig 5 A). As expected, GnRH-ECs clustered together (Fig 5 A). Two groups of genes were highly expressed in GnRH-ECs as compared with NPCs (Fig 5 A and B, groups a and b). Group a (which contains group b, Suppl Table 1) was highly expressed mainly in 21 days GnRH-ECs whereas group b was highly expressed in 35 days GnRH-ECs. The differentially expressed clusters were analysed using DAVID functional annotation tool (DAVID, https://david.ncifcrf.gov/). The analysis revealed that 35 days GnRH-ECs display a slightly more neuroendocrine phenotype than 21 days GnRH-ECs, with top upregulated pathways related to response to hormonal stimulus, steroid biosynthesis, and multicellular organism reproduction (Fig 5 D). In addition, the NF1 family of transcription factors has been implicated in controlling specificity of GnRH1 expression in GnRH neurons (Givens et al., 2004). Investigation of the tissue expression pattern for these genes carried out using DAVID associates them with the olfactory bulb (Fig 5 C) from which GnRH neurons derive and migrate and shares many of their developmental signals. The olfactory bulb could be one of the closest matches to GnRH neurons provided by DAVID's GNF_U133A_QUARTILE database as it does not contain specific information about GnRH neurons or the embryonic

olfactory epithelium. Pathway analysis of the same differentially expressed genes using DAVID functional annotation clustering showed a clear induction of neuronal differentiation pathways. Induction of genes associated with response to hormonal signalling was among the 10 highest scoring pathway clusters affected by terminal differentiation (Fig 5 D, Suppl table 2).

GnRH-EC display spontaneous and kisspeptin-stimulated calcium transients

To investigate whether ESCs-derived GnRH-ECs produced calcium transients and expressed functional kisspeptin receptors we loaded 35 days GnRH-ECs with calcium dye Fluoro4 and measured fluorescence using confocal microscopy. Approximately 20% of the differentiated cells displayed calcium transients which were increased in frequency and amplitude by Kisspeptin-10 (Fig 6). This behaviour resembles that of functional GnRH neurons which produce spontaneous calcium transients that can be stimulated by kisspeptin both in frequency and amplitude (Han et al., 2005; (Constantin et al., 2009).
Taken together these results show that our protocol efficiently and consistently generates ESCs and iPSCs-derived GnRH-secreting neurons with characteristics of true -albeit somehow immature- GnRH neurons.

Discussion

The inaccessibility of human GnRH-neurons has been a major impediment to studying the hypothalamic control of reproduction and its disorders. Here, we report the efficient generation of GnRH-secreting neurons (GnRH-ECs) from human ESCs by directed differentiation. Notably, this protocol also works efficiently and consistently with human iPSCs lines derived from both subjects with Kallmann syndrome and healthy controls.
Establishing a directed differentiation protocol relies upon understanding the details of cellular ontogenesis. Our differentiation strategy was based on the current knowledge about the *in vivo* development of GnRH neurons. GnRH neurons arise in the anterior area of the nasal placode (Wierman et al., 2011) in a niche defined by a gradient of BMP4 and its own target and antagonist noggin (Forni et al., 2013; Leung et al., 2013). We modified a protocol that used long term exposure of human ESCs to noggin in a neurogenic medium

for generating neural progenitor cells (Gerrard et al., 2005). These NPCs proved to be capable of differentiating into GnRH neurons. FGFs mediate a vast range of central nervous system developmental processes including neural induction, proliferation, migration, and cell survival (Mott et al., 2010). BMP4 and FGF8 are thought to have opposing roles in defining epithelial versus neurogenic fate in the developing olfactory/vomeronasal system (Forni et al., 2013). In particular, FGF8 has been implicated in specification of olfactory and GnRH neurons (Sabado et al., 2012). In addition, it is likely that some yet-to-be-determined factor(s) could block neural progenitor cells form developing into GnRH neurons *in vivo* (Markakis et al., 2004). After exposing PSCs-derived NPCs to FGF8 for 21 days in basic neuro-epithelial medium, the cells terminally differentiated into neurons which express and secrete GnRH. The proportion of terminally differentiated neurons expressing GnRH varied between experiments (40-90%). It was higher for ESCs-derived NPCs (60-90%) than for iPSCs-derived NPCs (40-50%), a modest difference which falls well within the bounds of normal hPSC line variability (Bock et al., 2011).

In vivo, GnRH neurons are phenotypically heterogeneous, complicating the use of defining markers other than GNRH1 (Han et al., 2005). GnRH-ECs expressed markers present both in nasal (immature) and hypothalamic GnRH neurons (Fig 4). In order to obtain a better insight into the phenotype of these secreting neurons we carried out a global expression analysis. In mammals, GnRH cells are distributed in a continuum from the olfactory bulbs to the hypothalamus (Wray, 2002). We used DAVID's GNF_U133A_QUARTILE tissue expression database. This database does not contain specific data on GnRH neurons. To the best of our knowledge there is no available data base containing specific information on GnRH neurons gene expression patterns. Our analysis linked GnRH-ECs' differentially expressed genes to the olfactory bulb, a rostral brain area proximal to the preoptic area where most GnRH neuronal bodies reside and intrinsically linked to GnRH neurons development and migration (Berghard et al., 2012; Hu et al., 2013; Teixeira et al., 2010; Wray, 2010). In addition to the expected enrichment of pathways associated to neuronal differentiation, GnRH-ECs presented an enrichment of pathways related to response to hormones, steroid biosynthesis, and multicellular organism reproduction. Differentiated cells also showed an increase of

Nuclear Factor 1 family members. These transcription factors have been implicated in controlling specificity of GnRH1 expression in GnRH neurons (Givens et al., 2004).

The production of spontaneous calcium transients whose frequency and amplitude can be modulated by neuropeptides such as kisspeptin is a hallmark of GnRH neurons (Constantin et al., 2009; Constantin et al., 2012; Han et al., 2005). In accordance with this, our differentiated neurons displayed both spontaneous and kisspeptin-stimulated calcium transients. This is the first report of *in vitro* generated, kisspeptin-responsive, GnRH neurons displaying spontaneous calcium transients.

Taken together, these findings suggest that we have generated cells that closely resemble GnRH neurons. Our claim that GnRH neurons can be generated from PSCs is supported by a very recent report by Lund et al. These researchers produced GnRH-secreting neurons from hPSCs by using a protocol similar to ours (including BMP4 inhibition followed by FGF8 (Lund et al., 2016). In our view, our method presents a number of advantages: not only can NPCs be expanded before differentiation, but our protocol seems to achieve higher differentiation efficiencies. Crucially cells obtained here are endowed with a calcium oscillator responsive to Kisspeptin and Glutamate. These *in vitro* generated GnRH expressing cells provide a new resource for studying the neuromolecular mechanisms underlying the development of GnRH neurons. In addition, these GnRH neurons will enable several new lines of research including disease modelling, cell transplantation and drug screening.

Materials and Methods

Work with human embryonic stem cells was reviewed and approved by the UK's Steering Committee For The Stem Cell Bank And For The Use Of Stem Cell Lines. Use of patient samples was approved by the UK's National Research Ethics Service (13/LO/0224).

Pluripotent stem cells (PSCs) culture

Hues7 Human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) were maintained on plates coated with Growth Factor Reduced Matrigel (BD Biosciences) in chemically defined PluriSTEM medium (Merck Millipore) or mTeSR-1 (Stemcell Technologies) and were passaged by mechanical dissociation (scrapping) after 5 min incubation in 0.5 mM EDTA PBS.

iPSC Reprogramming of human dermal fibroblasts

Human dermal fibroblasts (hDFs) were dissociated using TrypLE (Gibco), plated on gelatin-coated 6 well plates at a density of 5 × 10^4 cells/well in 10% FBS-DMEM. Twenty four hours after plating hDFs were transduced with a STEMCCA lentiviral vector (Sommer et al., 2012) using a MOI of 10. Three days after transduction, hDFs were passaged 1:10 on matrigel coated plates and incubated in PluriSTEM. Colonies with stem cell-like morphology usually started to appear at day 14 post-transdution and were picked for expansion and characterisation of pluripotency from day 21.

Neuronal differentiation

Neural Progenitor Cells (NPCs) were generated by an adaptation of a previously published protocol (Gerrard et al., 2005), briefly: PSCs were split mechanically with 0.5 mM EDTA PBS in 1:4 ratios into culture dishes coated with matrigel and incubated in Basal Neuro Epithelial (BNE) medium (1:1 mix of D-MEM/F12 and Neurobasal medium supplemented with N2 and serum-free B27, all from Gibco) supplemented with 100 ng/ml mouse recombinant noggin (Peprotech). At this stage cells were considered to be at passage 1

(P1). Cells were allowed to grow until confluence and split at 1:3 ratios using EDTA PBS and cultured using the same conditions until passage 4 (P4). Some neuronal rosettes appeared at P3. At P4 plenty of neuronal rosettes usually took over the culture. If this didn't happen, cultures were discarded. From P4 onwards cells were completely dissociated using TrypLE and plated 1:10 on matrigel-coated plates in BNE medium supplemented with 20 ng/ml human FGFb and 20 ng/ml human EGF (both Peprotech) without noggin. NPCs were kept in these conditions until P10-11 at which point cells were discarded. For terminal differentiation, NPCs between P6-P11 were plated at a density of 5000 cells/cm^2 and incubated either in BNE supplemented with 10 ng/ml FGF8 (Peprotech) or 10 ng/ml FGF8 plus 5 □M N-[N-(3,5-Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT, Sigma-Aldrich) for 21 days.

RT-QPCR

Total RNA was extracted using TriReagent (Sigma-Aldrich). For reverse transcription (RT), RNA (0.5 µg) was reverse transcribed to cDNA using M-MLV Reverse Transcriptase (Promega) and random hexamers (Invitrogen). Quantitative PCR (QPCR) was performed on diluted cDNA samples with SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) in an ABI Step One Plus QPCR system (Applied Biosystems, Carlsbad, CA) using the following program: 95 °C for 10 minutes, 40 cycles at 95 °C for 15 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds. Primers used are listed in Supplementary Table SX and were designed using Primer3 (http://frodo.wi.mit.edu/primer3). RT-QPCR results were analyzed using the 2–ΔΔCt method as described by Livak and Schmittgen (2001). The geometric mean of 3 housekeeping genes -Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), Ribosomal Protein L19 (RPL19), and beta-Actin (bACTIN)- was used as a calibrator after confirming that the genes were not affected by the treatment (Livak and Schmittgen, 2001).

Immunostaining

Cells were fixed for 10 min in 4% formaldehyde (Fisher Scientific) at room temperature. Fixed cells were incubated over night with primary antibodies in

0.1% Triton X100, 2% w/v bovine serum albumin (BSA), and 5% normal goat serum (NGS) in PBS and 1 h at room temperature with secondary antibodies in 2% w/v BSA PBS. Stained cells were mounted in Vectashield Antifade Mounting Medium with DAPI (vectorlabs). Antibodies used were: mouse anti-Tra1-60 (Cat. num. MAB4770, R&D Systems), mouse anti-beta III tubulin (TUJ1) (Cat. num. MAB1195, R&D Systems), rabbit anti-GnRH (Cat. num. 20075, Immunostar), mouse anti-GFAP (Cat. num. G3893, Sigma-Aldrich), Alexa 488 conjugated goat anti-mouse antibody (Cat. num. A10684, Invitrogen) and Alexa 568 goat anti-rabbit antibody (Cat. num. A21069, Invitrogen).

Flow cytometry

Cells were differentiated as described above. Cells where dissociated by incubation in 0.1 mg/mL DNase I (D5025 - Sigma-Aldrich) 1X TrypLE (12605028 - GIBCO) for 30 min at 37^{o}C and gently pipetting up and down every 5 min. After dissociation cells were washed twice in PBS. Visible clumps were removed by pipetting them out. Cells were fixed in chilled 4% formaldehyde PBS for 10 min and washed twice in PBS. Cells were permeabilized on ice in 100% methanol which had been previously chilled to -20^{o}C. After permeabilization, cells were washed twice in 1% BSA PBS. Cells were counted using a hemocytometer and 0.5-1 x 10^{6} cells were incubated in a 1:100 dilution of rabbit anti-GnRH (Cat. num. 20075, Immunostar) in 1% BSA PBS for 1 h at RT. Cells were washed twice in PBS and incubated in a 1:50 dilution of Alexa 568 goat anti-rabbit antibody (Cat. num. A21069, Invitrogen) for 1 h. After incubation with secondary antibody cells were washed three times, resuspended in 1% BSA PBS and analysed in a BD LSR-Fortessa Cell Analyzer (BD Biosciences) using a blue laser and a YG 610/20A filter. Figures were generated with Flowing Software v2.5.1 (http://www.uskonaskel.fi/flowingsoftware/).

Immunoblotting

Cell lysates were harvested by the addition of SDS lysis buffer (2% SDS, 30 mM NaCl, 10 mM HEPES, pH 7.4, 20 mM NaF, 1 mM NaPPi, 1 mM PMSF, and 1X Complete Protease Inhibitor Cocktail [Roche]). Equal amounts of

protein from lysates were resolved by SDS-PAGE, immunoblotted, and detected in an Odyssey Imaging System (Li-Cor Biotechnology). Rabbit anti-GnRH antibody was purchased from Immunostar (Cat. Num. 20075). Secondary IRDye 680RD Goat anti-Rabbit was purchased from Li-Cor Biotechnology.

GnRH release

GnRH release to the media was detected using an Enzyme-linked Immunosorbent Assay (ELISA) Kit (Biomatik) according to manufacturer's instructions. Briefly: media was collected, centrifuged 10 min at 1000 xg and stored at -20oC until measurement. For measurements, standard GnRH curve was prepared in fresh cell media (BNE medium) and 50 □l of undiluted samples were used in duplicates.

Microarray

Total RNA was extracted using TriReagent (Sigma-Aldrich). RNA QC was performed in a 2100 Bioanalyzer (Agilent Technologies). RNA was labelled using Ambion Total Prep kit and hybridized on a human Illumina genome wide gene expression array (HT12v4). Raw data was quartile normalised and analysed using Illumina's Genome Studio software. P-values were calculated using the Illumina Custom method followed by a Bonferroni correction to account for multiple testing. Hierarchical clustering of differentially expressed genes was performed with Cluster 3 software (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) and displayed using Java TreeView software (https://sourceforge.net/projects/jtreeview/). Log-transformed row-centred data was used after selecting rows with standard deviations at least 3 times higher than the array's SD. The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (Poliandri et al., 2016) and are accessible through GEO Series accession number GSE86990
(http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE86990)

Calcium Imaging

NPCs were differentiated as described above. Cells were grown on glass-bottom petri dishes (Met Tek Corporation, Ashland, MA 01721, USA) for 35 days.

A stock solution of fluo-4 acetoxymethyl ester (Fluo4-AM) (Molecular Probes) was prepared by adding 50 µl of an 80% DMSO 20% pluronic acid mix to 50 µg of Fluo4-AM. The stock solution was diluted 1:100 in calcium buffer (135 mm NaCl, 2.7 mm KCl, 2 mm CaCl2, 1 mm MgCl2, 0.33 mm NaH2PO4, 5 mm HEPES, 10 mm glucose, pH 7.4) and this dilution was used to load the cells for 40 min at 37oC in 5% CO2. After loading cells were rinsed in calcium buffer and incubated for another 40 min at 37^{o}C in 5% CO_2 before measurements. Measurements were performed at 37^{o}C in calcium buffer in a Zen LSM 510 confocal microscope in line scan mode. A series with a scan time of 600 msec, 1000 cycles, and an interval of 1 sec between scans was used.

Images were analysed using ImageJ software with Multi Selection plugin. The mean background of 3 different regions was subtracted before analysis. At least 20 cells per field were randomly selected for analysis from the pool of cells which displayed a calcium increase after a final KCl (100 mM) challenge.

Figure 1. Generation of Neural Progenitor Cells (NPCs)

(A) Schematic diagram of how NPCs are generated. Briefly: ESCs are resuspended in clumps using 5 mM EDTA and a cell scraper. Resuspended ESCs are then plated at a 1:4 ratio onto matrigel-coated wells with BNE medium supplemented with 100 ng/ml noggin. At this stage cells are considered NPCs passage 1 (P1). NPCs are passaged 3 more times using 5 mM EDTA and mechanical dispersion and cultured in BNE medium supplemented with 100 ng/ml noggin. After P4 cells are completely dissociated using 1X TripLE and plated onto matirgel-coated wells in BNE medium supplemented with 20 ng/ml bFGF and 20 ng/ml EGF (P5). During the differentiation process pluripotency markers are downregulated and neuronal markers upregulated. (B) QPCR results showing downregulation of pluripotency markers NANOG and OCT4 (top row) and upregulation of neuronal markers PAX6 and TUJ1 (bottom row). NPCs can be generated with similar efficiency using iPSCs derived from healthy subjects (H-iPSCs > H-NPCs) or patients with Kallmann syndrome (P-iPSCs > P-NPCs). (C) Representative image of P4 H-NPCs and QPCR data showing downregulation of NANOG and upregulation of PAX6 and TUJ1. (D) Representative image of P4 P-NPCs and QPCR data showing downregulation of NANOG and upregulation of PAX6 and TUJ1. Bars represent mean ± SEM.

Figure 2. Terminal differentiation of NPCs induces expression and secretion of GnRH

Plating NPCs at a low density in BNE medium supplemented with 10 ng/ml FGF8 for 21 days causes neuronal differentiation and induces the expression and secretion of GnRH. (A) Terminally differentiated (TD) NPCs. RT-QPCR and end-point RT-PCR showing an increase of GNRH1 mRNA in Terminally Differenciated NPCs (TD-NPCs) (A) and (B). ***$p<0.001$ vs NPCs. (C) Western blot showing the presence of GnRH peptide in whole-cell lysates of TD-NPCs. Whole-cell lysates of HEK293 cells and GT1-7 cells were used as negative and positive controls respectively. NPCs and TD-NPCs secrete

GnRH. Cells were incubated for 48 h in BNE alone and GnRH accumulation in the media was measured using an ELISA kit (D). GT1-7 cells were used as a positive control and all samples were blanked against media alone. *p<0.05 vs NPCs, Student's t-test, N=3.

Figure 3. GnRH expression in different cell lines

NPCs incubated for 21 days in BNE supplemented with 10 ng/ml FGF8 differentiate into TUJ1-positive neurons and express GnRH. (A) Immunofluorescence images of GnRH-Expressing Cells (GnRH-ECs) stained for TUJ1 (green) and GnRH (red). Nuclei were stained with DAPI (blue). iPSCs-derived NPCs incubated for 21 days in BNE supplemented with 10 ng/ml FGF8 also differentiate into TUJ1-positive neurons and express GnRH. (B) Immunofluorescence images of ESCs-derived neurons (GnRH-ECs, top row), healthy iPSCs-derived neurons (H-GnRH-ECs, middle row), and Kallmann syndrome iPSCs-derived neurons (P-GnRH-ECs, bottom row) stained for TUJ1 (green) and GnRH (red). Nuclei were stained with DAPI (blue). (C) Isotype controls: GnRH-ECs were stained without primary antibodies (top row). P-iPSCs were stained for the pluripotency marker TRA1-60 (green) and GnRH (red) (middle row). GnRH expressing GN11 cells were stained for TUJ1 (green) and GnRH (red). Nuclei were stained with DAPI (blue). (D) Flow-cytometry quantification of the number of cells expressing GnRH. Cells were stained with anti-GnRH antibody and analysed in a BD LSR Fortressa cell analyser. HEK293(Da) and GN11(Db) cells were used as negative and positive controls respectively. Up to 90% of ESCs-derived GnRH-ECs were positive for GnRH (Dc and Dd). Up to 50% of iPSCs-derived GnRH-ECs were positive for GnRH (De and Df). Few undifferentiated NPCs were positive for GnRH (Dg).

Figure 4. Differentiated neurons express markers present in hypothalamic and nasal GnRH neurons.

Differentiation in BNE supplemented with 10 ng/ml FGF8 of ESCs-derived NPCs for 21 days induced the expression of markers present in GnRH neuron KISS1R, TAC3R, GAD65, and ESR2. There was also an induction of nasal epithelium markers GATA2 and EYA1 together with a strong induction of the neuronal marker TUJ1. There was also an induction of the glial marker GFAP. The pluripotency marker NANOG was strongly downregulated. Top right panel: image of 21 day neurons. UND = undetected. *p<0.05 vs control, ***p<0.001 vs control, student's t-test, N=3.

Figure 5. Global expression analysis of GnRH-ECs shows tissue expression patterns consistent with GnRH neurons' origin in the nasal epithelium.

Global mRNA expression was measured in NPCs and GnRH-ECs at days 21 and 35 of differentiation using Ilumina HT12v4 microarray. (A) Row-centred heat map of hierarchical clustering carried out on the differentially expressed gene probes is shown. Probe sets are coloured according to the average expression level across all samples, with green denoting a lower expression level and red denoting a higher expression level. (B) Two groups of genes (a and b) highly expressed in GnRH-ECs were investigated for tissue expression pattern and physiological pathways using DAVID Functional Annotation tool. (C) Top tissue expression patterns enriched in each of the different clusters. The bars represent significance of enrichment. (D) Top pathways that are enriched in each of the different clusters. The bars represent significance of enrichment, % shows the percentage of genes involved in the pathway out of the total list of genes.

Figure 6. GnRH-ECs display spontaneous and kisspeptin-stimulated calcium transients.

Representative experiments of cells responding to Kisspeptine: Cells were recorded for 3 min before adding 200 µl of buffer (blue dot) to control for possible effect on calcium, after 1 min 200 µl of Kisspeptine (final concentration 100 nM) were added (red dot), after 3 min glutamate (final

concentration 1 mM) was added (green dot). Finally KCL (75 mM) was added.
Inset: image of cells loaded with Fluo4.

citations in text are as follows:

(Jones et al., 1995) or (Jones et al., 1995a,b; Smith et al., 1994, 1995).

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