1 Generation of kisspeptin-responsive GnRH neurons from human pluripotent

2 stem cells

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7 KEY WORDS

8 GnRH neurons, pluripotent stem cells, embryonic stem cells, kisspeptin.

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10 SUMMARY STATEMENT (15-30 words)

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12 ABSTRACT

13

14 GnRH neurons are fundamental for reproduction in all vertebrates ultimately

15 integrating all reproductive inputs. The inaccessibility of human GnRH-

16 neurons has been a major impediment to studying the central control of

17 reproduction and its disorders. Here, we report the efficient generation of

18 kisspeptin responsive GnRH-secreting neurons by directed differentiation of

19 human Pluripotent Stem Cells. The protocol involves the generation of

20 intermediate Neural Progenitor Cells (NPCs) through long-term Bone

21 morphogenetic protein 4 inhibition followed by terminal specification of these

22 NPCs in media containing FGF8 and a NOTCH inhibitor. The resulting GnRH

23 expressing and secreting neurons display a neuroendocrine gene expression

24 pattern and present spontaneous calcium transients that can be stimulated by

25 kisspeptin. These *in vitro* generated GnRH expressing cells provide a new

26 resource for studying the molecular mechanisms underlying the development

27 and function of GnRH neurons.

28

29   Introduction

30

31   GnRH neurons are fundamental for reproduction in all vertebrates. They not

32   only determine the timing of puberty but ultimately integrate all reproductive

33   inputs (Romanelli et al., 2004; Stevenson et al., 2013). In humans, the GnRH-

34   neuronal network is composed of a few hundred neurons distributed as

35   clusters located from the preoptic area to the anterior hypothalamic regions

36   making *in vivo* or *ex vivo* experimental studies of GnRH neurons extremely

37   difficult (Maggi et al., 2000; Wang et al., 2015). The molecular ontogeny of

38   GnRH neurons remains elusive. It is generally accepted that most

39   hypothalamic GnRH neurons originate in the olfactory placode (OP) but

40   compelling evidence suggests that a significant proportion of GnRH neurons

41   may descend from neural crest progenitors (either directly or via their early

42   migration to the OP) (Forni et al., 2011; Forni and Wray, 2012). Bone

43   morphogenetic protein-4 (BMP4) plays an essential role in the formation of

44   the olfactory placode (Leung et al., 2013) and GnRH neurons arise from the

45   olfactory placode in a niche defined by gradients of BMP4/Noggin and FGF8

46   (Forni et al., 2013; Sabado et al., 2012). After arising in the olfactory placode,

47   GnRH neurons migrate to their final location in the anterior hypothalamus

48   during embryonal development. Failure of these neurons to reach their

49   appropriate place in the hypothalamus or to form functional networks has

50   been associated with many reproductive phenotypes including delayed or

51   absent puberty (Boehm et al., 2015; Howard et al., 2016). Some pubertal

52   disorders such as Kallmann syndrome (where puberty is never or only

53   partially completed) are often associated with anosmia (the inability to

54   perceive odours) pointing to the common origin of olfactory and GnRH

55   neurons (Forni and Wray, 2015; Herbison, 2007; Tucker et al., 2010).

56   GnRH neurons secrete GnRH peptide in a pulsatile fashion. The frequency

57   and amplitude of these secretory pulses changes during reproductive

58   development and reproductive cycles, for example during puberty or the

59   menstrual cycle (Apter, 1997; Barbieri, 2014; Ojeda et al., 2010). The pulsatile

60   release of GnRH is controlled by the periodicity and amplitude of action

61   potentials and calcium transients. These are regulated by several

62   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.

88   Results

89

90   Generation of Neural Progenitor Cells (NPCs)

91   To generate human GnRH-expressing cells we started by producing NPCs

92   adapting a protocol previously published by Gerrard *et al.* (Gerrard et al.,

93   2005). This protocol involves culturing ESCs under neurogenic conditions for

94   several passages using Basal Neuro-Epithelial (BNE) medium supplemented

95   with the BMP inhibitor noggin (Fig 1A). As expected, during the development

96   of NPCs, pluripotency markers were downregulated whereas neuronal

97   markers were upregulated (Fig 1B). NPCs readily differentiated into neurons

98   after withdrawal of growth factors (Suppl Fig 1). The differentiation process

99   produced NPCs with similar efficiencies from human Embryonic Stem Cells

100   (ESCs) and induced Pluripotent Stem Cells (iPSCs) derived from individuals

101   with Kallmann Syndrome and their healthy family members (Fig 1 C and D).

102   After passage 4 (P4) noggin was withdrawn and the cells were expanded in

103   BNE medium supplemented with bFGF and EGF for up to 4 passages. It has

104   been reported that the development of GnRH neurons in the olfactory

105   epithelium depends on a neurogenic environment provided by an interplay

106   between BMP4 and FGF8 signalling (Forni et al., 2013; Leung et al., 2013;

107   Sabado et al., 2012). FGF8 is essential for GnRH neurons development *in*

108   *vivo* and it has been used previously to differentiate chicken olfactory placode

109   progenitors into GnRH neurons (Sabado et al., 2012).

110

111   GnRH neuronal specification

112   For the second stage of our differentiation protocol we removed bFGF and

113   EGF from our medium and incubated the NPCs in medium supplemented with

114   10 ng/ml FGF8 for 21 days. This led to significantly elevated levels of GnRH

115   mRNA and protein (Fig 2 B-D). GnRH was also detected by ELISA in the cell

116   media (Fig 2 E). In addition, NPCs in FGF8 supplemented medium adopted a

117   mature neuronal morphology forming neurospheres and generating

118   projections (Fig 2A). After plating the NPCs in FGF8 for differentiation, the

119   cells still went through several rounds of division. We found that a correct

120   initial cell density was essential for achieving an efficient

121   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).

Gene expression profiling of PSCs-derived GnRH expressing cells.

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

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

159 To further investigate the phenotype of our GnRH-ECs we performed a global
160 mRNA expression analysis comparing ESCs, NPCs and GnRH-ECs. Because
161 cells subjected to 21 days differentiation continued to express genes present
162 in very immature GnRH neurons a 35 day differentiation point was included in
163 the   analysis   (Suppl   Fig   2   and
164 http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE86990). All neuronal
165 groups clearly diverged from ESCs (Suppl Fig 1) therefore we concentrated
166 on just NPCs, 21 days GnRH-ECs, and 35 days GnRH-ECs to look for a
167 specific gene expression profile for the differentiated GnRH neuronal
168 phenotype. We performed a hierarchical clustering of differentially expressed
169 genes using Cluster 3 software. Selecting for probes with variability greater
170 than 3 SDs between groups we ascribed 1081 differentially expressed entries
171 (Fig 5 A). As expected, GnRH-ECs clustered together (Fig 5 A). Two groups
172 of genes were highly expressed in GnRH-ECs as compared with NPCs (Fig 5
173 A and B, groups a and b). Group a (which contains group b, Suppl Table 1)
174 was highly expressed mainly in 21 days GnRH-ECs whereas group b was
175 highly expressed in 35 days GnRH-ECs. The differentially expressed clusters
176 were   analysed   using   DAVID   functional   annotation   tool   (DAVID,
177 https://david.ncifcrf.gov/). The analysis revealed that 35 days GnRH-ECs
178 display a slightly more neuroendocrine phenotype than 21 days GnRH-ECs,
179 with top upregulated pathways related to response to hormonal stimulus,
180 steroid biosynthesis, and multicellular organism reproduction (Fig 5 D). In
181 addition, the NF1 family of transcription factors has been implicated in
182 controlling specificity of GnRH1 expression in GnRH neurons (Givens et al.,
183 2004). Investigation of the tissue expression pattern for these genes carried
184 out using DAVID associates them with the olfactory bulb (Fig 5 C) from which
185 GnRH neurons derive and migrate and shares many of their developmental
186 signals. The olfactory bulb could be one of the closest matches to GnRH
187 neurons provided by DAVID's GNF_U133A_QUARTILE database as it does
188 not contain specific information about GnRH neurons or the embryonic

189 olfactory epithelium. Pathway analysis of the same differentially expressed
190 genes using DAVID functional annotation clustering showed a clear induction
191 of neuronal differentiation pathways. Induction of genes associated with
192 response to hormonal signalling was among the 10 highest scoring pathway
193 clusters affected by terminal differentiation (Fig 5 D, Suppl table 2).
194
195 <u>GnRH-EC display spontaneous and kisspeptin-stimulated calcium transients</u>
196 To investigate whether ESCs-derived GnRH-ECs produced calcium transients
197 and expressed functional kisspeptin receptors we loaded 35 days GnRH-ECs
198 with calcium dye Fluoro4 and measured fluorescence using confocal
199 microscopy. Approximately 20% of the differentiated cells displayed calcium
200 transients which were increased in frequency and amplitude by Kisspeptin-10
201 (Fig 6). This behaviour resembles that of functional GnRH neurons which
202 produce spontaneous calcium transients that can be stimulated by kisspeptin
203 both in frequency and amplitude (Han et al., 2005; (Constantin et al., 2009).
204 Taken together these results show that our protocol efficiently and
205 consistently generates ESCs and iPSCs-derived GnRH-secreting neurons
206 with characteristics of true -albeit somehow immature- GnRH neurons.
207
208 Discussion
209
210 The inaccessibility of human GnRH-neurons has been a major impediment to
211 studying the hypothalamic control of reproduction and its disorders. Here, we
212 report the efficient generation of GnRH-secreting neurons (GnRH-ECs) from
213 human ESCs by directed differentiation. Notably, this protocol also works
214 efficiently and consistently with human iPSCs lines derived from both subjects
215 with Kallmann syndrome and healthy controls.
216 Establishing a directed differentiation protocol relies upon understanding the
217 details of cellular ontogenesis. Our differentiation strategy was based on the
218 current knowledge about the *in vivo* development of GnRH neurons. GnRH
219 neurons arise in the anterior area of the nasal placode (Wierman et al., 2011)
220 in a niche defined by a gradient of BMP4 and its own target and antagonist
221 noggin (Forni et al., 2013; Leung et al., 2013). We modified a protocol that
222 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

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

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

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

281
282

283   Materials and Methods

284

285

286   Work with human embryonic stem cells was reviewed and approved by the

287   UK's Steering Committee For The Stem Cell Bank And For The Use Of Stem

288   Cell Lines. Use of patient samples was approved by the UK's National

289   Research Ethics Service (13/LO/0224).

290

291   Pluripotent stem cells (PSCs) culture

292   Hues7 Human embryonic stem cells (hESCs) and human induced pluripotent

293   stem cells (hiPSCs) were maintained on plates coated with Growth Factor

294   Reduced Matrigel (BD Biosciences) in chemically defined PluriSTEM medium

295   (Merck Millipore) or mTeSR-1 (Stemcell Technologies) and were passaged by

296   mechanical dissociation (scrapping) after 5 min incubation in 0.5 mM EDTA

297   PBS.

298

299   iPSC Reprogramming of human dermal fibroblasts

300   Human dermal fibroblasts (hDFs) were dissociated using TrypLE (Gibco),

301   plated on gelatin-coated 6 well plates at a density of 5×10^4 cells/well in

302   10% FBS-DMEM. Twenty four hours after plating hDFs were transduced with

303   a STEMCCA lentiviral vector (Sommer et al., 2012) using a MOI of 10. Three

304   days after transduction, hDFs were passaged 1:10 on matrigel coated plates

305   and incubated in PluriSTEM. Colonies with stem cell-like morphology usually

306   started to appear at day 14 post-transdution and were picked for expansion

307   and characterisation of pluripotency from day 21.

308

309   Neuronal differentiation

310   Neural Progenitor Cells (NPCs) were generated by an adaptation of a

311   previously published protocol (Gerrard et al., 2005), briefly: PSCs were split

312   mechanically with 0.5 mM EDTA PBS in 1:4 ratios into culture dishes coated

313   with matrigel and incubated in Basal Neuro Epithelial (BNE) medium (1:1 mix

314   of D-MEM/F12 and Neurobasal medium supplemented with N2 and serum-

315   free B27, all from Gibco) supplemented with 100 ng/ml mouse recombinant

316   noggin (Peprotech). At this stage cells were considered to be at passage 1

317   (P1). Cells were allowed to grow until confluence and split at 1:3 ratios using
318   EDTA PBS and cultured using the same conditions until passage 4 (P4).
319   Some neuronal rosettes appeared at P3. At P4 plenty of neuronal rosettes
320   usually took over the culture. If this didn't happen, cultures were discarded.
321   From P4 onwards cells were completely dissociated using TrypLE and plated
322   1:10 on matrigel-coated plates in BNE medium supplemented with 20 ng/ml
323   human FGFb and 20 ng/ml human EGF (both Peprotech) without noggin.
324   NPCs were kept in these conditions until P10-11 at which point cells were
325   discarded. For terminal differentiation, NPCs between P6-P11 were plated at
326   a density of 5000 cells/cm^2 and incubated either in BNE supplemented with
327   10 ng/ml FGF8 (Peprotech) or 10 ng/ml FGF8 plus 5 □M N-[N-(3,5-
328   Difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT, Sigma-
329   Aldrich) for 21 days.

330

331   RT-QPCR
332   Total RNA was extracted using TriReagent (Sigma-Aldrich). For reverse
333   transcription (RT), RNA (0.5 μg) was reverse transcribed to cDNA using M-
334   MLV Reverse Transcriptase (Promega) and random hexamers (Invitrogen).
335   Quantitative PCR (QPCR) was performed on diluted cDNA samples with
336   SYBR Green JumpStart Taq ReadyMix (Sigma-Aldrich) in an ABI Step One
337   Plus QPCR system (Applied Biosystems, Carlsbad, CA) using the following
338   program: 95 °C for 10 minutes, 40 cycles at 95 °C for 15 seconds, 60 °C for
339   30 seconds, and 72 °C for 30 seconds. Primers used are listed in
340   Supplementary Table SX and were designed using Primer3 (http://frodo.
341   wi.mit.edu/primer3). RT-QPCR results were analyzed using the 2–ΔΔCt
342   method as described by Livak and Schmittgen (2001). The geometric mean of
343   3 housekeeping genes -Glyceraldehyde-3-phosphate dehydrogenase
344   (GAPDH), Ribosomal Protein L19 (RPL19), and beta-Actin (bACTIN)- was
345   used as a calibrator after confirming that the genes were not affected by the
346   treatment (Livak and Schmittgen, 2001).

347

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

351   0.1% Triton X100, 2% w/v bovine serum albumin (BSA), and 5% normal goat

352   serum (NGS) in PBS and 1 h at room temperature with secondary antibodies

353   in 2% w/v BSA PBS. Stained cells were mounted in Vectashield Antifade

354   Mounting Medium with DAPI (vectorlabs). Antibodies used were: mouse anti-

355   Tra1-60 (Cat. num. MAB4770, R&D Systems), mouse anti-beta III tubulin

356   (TUJ1) (Cat. num. MAB1195, R&D Systems), rabbit anti-GnRH (Cat. num.

357   20075, Immunostar), mouse anti-GFAP (Cat. num. G3893, Sigma-Aldrich),

358   Alexa 488 conjugated goat anti-mouse antibody (Cat. num. A10684,

359   Invitrogen) and Alexa 568 goat anti-rabbit antibody (Cat. num. A21069,

360   Invitrogen).

361

362   Flow cytometry

363   Cells were differentiated as described above. Cells where dissociated by

364   incubation in 0.1 mg/mL DNase I (D5025 - Sigma-Aldrich) 1X TrypLE

365   (12605028 - GIBCO) for 30 min at 37°C and gently pipetting up and down

366   every 5 min. After dissociation cells were washed twice in PBS. Visible

367   clumps were removed by pipetting them out. Cells were fixed in chilled 4%

368   formaldehyde PBS for 10 min and washed twice in PBS. Cells were

369   permeabilized on ice in 100% methanol which had been previously chilled to -

370   20°C. After permeabilization, cells were washed twice in 1% BSA PBS. Cells

371   were counted using a hemocytometer and 0.5-1 x 10^6 cells were incubated in

372   a 1:100 dilution of rabbit anti-GnRH (Cat. num. 20075, Immunostar) in 1%

373   BSA PBS for 1 h at RT. Cells were washed twice in PBS and incubated in a

374   1:50 dilution of Alexa 568 goat anti-rabbit antibody (Cat. num. A21069,

375   Invitrogen) for 1 h. After incubation with secondary antibody cells were

376   washed three times, resuspended in 1% BSA PBS and analysed in a BD

377   LSR-Fortessa Cell Analyzer (BD Biosciences) using a blue laser and a YG

378   610/20A filter. Figures were generated with Flowing Software v2.5.1

379   (http://www.uskonaskel.fi/flowingsoftware/).

380

381   Immunoblotting

382   Cell lysates were harvested by the addition of SDS lysis buffer (2% SDS, 30

383   mM NaCl, 10 mM HEPES, pH 7.4, 20 mM NaF, 1 mM NaPPi, 1 mM PMSF,

384   and 1X Complete Protease Inhibitor Cocktail [Roche]). Equal amounts of

385 protein from lysates were resolved by SDS-PAGE, immunoblotted, and
386 detected in an Odyssey Imaging System (Li-Cor Biotechnology). Rabbit anti-
387 GnRH antibody was purchased from Immunostar (Cat. Num. 20075).
388 Secondary IRDye 680RD Goat anti-Rabbit was purchased from Li-Cor
389 Biotechnology.
390
391 GnRH release
392 GnRH release to the media was detected using an Enzyme-linked
393 Immunosorbent Assay (ELISA) Kit (Biomatik) according to manufacturer's
394 instructions. Briefly: media was collected, centrifuged 10 min at 1000 xg and
395 stored at -20oC until measurement. For measurements, standard GnRH curve
396 was prepared in fresh cell media (BNE medium) and 50 □l of undiluted
397 samples were used in duplicates.
398
399 Microarray
400 Total RNA was extracted using TriReagent (Sigma-Aldrich). RNA QC was
401 performed in a 2100 Bioanalyzer (Agilent Technologies). RNA was labelled
402 using Ambion Total Prep kit and hybridized on a human Illumina genome wide
403 gene expression array (HT12v4). Raw data was quartile normalised and
404 analysed using Illumina's Genome Studio software. P-values were calculated
405 using the Illumina Custom method followed by a Bonferroni correction to
406 account for multiple testing. Hierarchical clustering of differentially expressed
407 genes was performed with Cluster 3 software
408 (http://bonsai.hgc.jp/~mdehoon/software/cluster/software.htm) and displayed
409 using Java TreeView software (https://sourceforge.net/projects/jtreeview/).
410 Log-transformed row-centred data was used after selecting rows with
411 standard deviations at least 3 times higher than the array's SD. The data
412 discussed in this publication have been deposited in NCBI's Gene Expression
413 Omnibus (Poliandri et al., 2016) and are accessible through GEO Series
414 accession number GSE86990
415 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE86990)
416
417 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°C in 5% CO_2 before measurements. Measurements were performed at 37°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.

440   Figure 1. Generation of Neural Progenitor Cells (NPCs)

441

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

461

462

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

465

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

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

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480   Figure 3. GnRH expression in different cell lines

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482   NPCs incubated for 21 days in BNE supplemented with 10 ng/ml FGF8
483   differentiate into TUJ1-positive neurons and express GnRH. (A)
484   Immunofluorescence images of GnRH-Expressing Cells (GnRH-ECs) stained
485   for TUJ1 (green) and GnRH (red). Nuclei were stained with DAPI (blue).
486   iPSCs-derived NPCs incubated for 21 days in BNE supplemented with 10
487   ng/ml FGF8 also differentiate into TUJ1-positive neurons and express GnRH.
488   (B) Immunofluorescence images of ESCs-derived neurons (GnRH-ECs, top
489   row), healthy iPSCs-derived neurons (H-GnRH-ECs, middle row), and
490   Kallmann syndrome iPSCs-derived neurons (P-GnRH-ECs, bottom row)
491   stained for TUJ1 (green) and GnRH (red). Nuclei were stained with DAPI
492   (blue). (C) Isotype controls: GnRH-ECs were stained without primary
493   antibodies (top row). P-iPSCs were stained for the pluripotency marker TRA1-
494   60 (green) and GnRH (red) (middle row). GnRH expressing GN11 cells were
495   stained for TUJ1 (green) and GnRH (red). Nuclei were stained with DAPI
496   (blue). (D) Flow-cytometry quantification of the number of cells expressing
497   GnRH. Cells were stained with anti-GnRH antibody and analysed in a BD
498   LSR Fortressa cell analyser. HEK293(Da) and GN11(Db) cells were used as
499   negative and positive controls respectively. Up to 90% of ESCs-derived
500   GnRH-ECs were positive for GnRH (Dc and Dd). Up to 50% of iPSCs-derived
501   GnRH-ECs were positive for GnRH (De and Df). Few undifferentiated NPCs
502   were positive for GnRH (Dg).

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

507   Differentiation in BNE supplemented with 10 ng/ml FGF8 of ESCs-derived

508   NPCs for 21 days induced the expression of markers present in GnRH neuron

509   KISS1R, TAC3R, GAD65, and ESR2. There was also an induction of nasal

510   epithelium markers GATA2 and EYA1 together with a strong induction of the

511   neuronal marker TUJ1. There was also an induction of the glial marker GFAP.

512   The pluripotency marker NANOG was strongly downregulated. Top right

513   panel: image of 21 day neurons. UND = undetected. *p<0.05 vs control,

514   ***p<0.001 vs control, student's t-test, N=3.

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517   Figure 5. Global expression analysis of GnRH-ECs shows tissue expression

518   patterns consistent with GnRH neurons' origin in the nasal epithelium.

519   Global mRNA expression was measured in NPCs and GnRH-ECs at days 21

520   and 35 of differentiation using Ilumina HT12v4 microarray. (A) Row-centred

521   heat map of hierarchical clustering carried out on the differentially expressed

522   gene probes is shown. Probe sets are coloured according to the average

523   expression level across all samples, with green denoting a lower expression

524   level and red denoting a higher expression level. (B) Two groups of genes (a

525   and b) highly expressed in GnRH-ECs were investigated for tissue expression

526   pattern and physiological pathways using DAVID Functional Annotation tool.

527   (C) Top tissue expression patterns enriched in each of the different clusters.

528   The bars represent significance of enrichment. (D) Top pathways that are

529   enriched in each of the different clusters. The bars represent significance of

530   enrichment, % shows the percentage of genes involved in the pathway out of

531   the total list of genes.

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533

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

535   transients.

536   Representative experiments of cells responding to Kisspeptine: Cells were

537   recorded for 3 min before adding 200 µl of buffer (blue dot) to control for

538   possible effect on calcium, after 1 min 200 µl of Kisspeptine (final

539   concentration 100 nM) were added (red dot), after 3 min glutamate (final

540   concentration 1 mM) was added (green dot). Finally KCL (75 mM) was added.

541   Inset: image of cells loaded with Fluo4.

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545 citations in text are as follows:

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

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