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1 **Serotonin receptor 1A (HTR1A), a novel regulator of GnRH neuronal migration in chick embryo**

2

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21 analysed data and wrote the paper.

22 There is no conflict of interest.

23

24 **Abstract**

25 The hypothalamic gonadotrophin releasing hormone (GnRH) neurons are a small group of cells that
26 regulate the reproductive axis. These neurons are specified within the olfactory placode, delaminate from
27 this structure and then migrate to enter the forebrain before populating the hypothalamus. We have
28 employed microarray technology to analyse the transcriptome of the olfactory placode at a number of key
29 time points for GnRH ontogeny using the chick embryo. This resulted in the identification of a large
30 number of genes whose expression levels change significantly over this period. This repertoire include
31 those genes which are known to be important for GnRH neuronal development as well as many novel
32 genes, such as the serotonin (5-HT) receptor 1A, HTR1A. We find that HTR1A is expressed in the region
33 of the olfactory placode that generates GnRH neurons. We further show that when this receptor is
34 inactivated using a selective HTR1A antagonist as well as a gene knockdown approach using RNAi, this
35 resulted in delayed migration causing the GnRH neurons to stall just outside the forebrain. These findings
36 implicate HTR1A as being important for GnRH neuronal migration from the olfactory placode to the
37 forebrain. Our study thus extends the repertoire of genes involved in GnRH neuron biology and thus
38 identifies new candidate genes that can be screened for in patients who do not show mutations in any of
39 the previously identified HH/KS genes.

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44

45 **Introduction**

46 The hypothalamic gonadotropin releasing hormone (GnRH) neurons are a relatively small and dispersed
47 cell population essential for vertebrate reproduction (1). GnRH secretion from these neurons drives the
48 synthesis and secretion of the gonadotropins, follicle stimulating hormone (FSH) and luteinizing hormone
49 (LH) which in turn regulates sex steroid output and gamete formation in both sexes. GnRH neurons are
50 located in the hypothalamus in the adult but originate within and also outside the central nervous system
51 (CNS) in the olfactory placodes, from which they migrate to the anterior hypothalamus during
52 development (2). Olfactory placodes, paired thickenings of the embryonic ectoderm occupying a medial
53 frontal position in vertebrate embryos (3) generate the olfactory epithelium, olfactory sensory neurons and
54 the GnRH neurons. GnRH neuronal specification occurs within the placode, from which delamination and
55 axonophilic GnRH neuronal migration occur into the forebrain before dispersing and populating the
56 hypothalamus. Failure of normal GnRH neuronal ontogeny exerts profound effects on reproduction
57 resulting in delayed, reduced or absent puberty and fertility in a range of species (1). Mechanistically, this
58 may be the consequence of abnormal fate specification of precursor cells, defective proliferation or
59 premature apoptosis, faulty migration, or defective connectivity within the hypothalamus and
60 neurosecretory failure.

61 Molecular insights into GnRH neuronal ontogeny have come from ‘accidents of nature’ in humans, which
62 has driven much of the research into this area. Defects in the development of GnRH neurons result in
63 hypogonadotropic hypogonadism (HH) (4) characterised by low levels of circulating gonadotropins and
64 absent puberty, which when associated with anosmia (loss of smell), is termed Kallmann Syndrome (KS).
65 Over 26 genes associated with this developmental pathway have now been identified. These include *KAL-*
66 *1*, an X-linked gene encoding anosmin 1, *FGFR1* and other FGF signalling components as well as other
67 molecules implicated in axon guidance and neuronal migration, such as *Robo/Slit*, and *Semaphorins* (4).
68 However, only 60% of HH cases have a demonstrable genetic abnormality, and many other genes have yet
69 to be identified. In some cases, GnRH neuronal migratory arrest prior to entry into the CNS underpinned
70 the disorder. A significant number of individuals (40%) with HH present without anosmia; these cases are
71 presumably caused by defects specific to GnRH neuronal development and/or function. Considerably less
72 is known about the genes driving early GnRH neuronal ontogeny.

73 To address this problem, we conducted a transcriptomic analysis of the olfactory placode during the
74 period covering the initial specification of the GnRH neurons, their delamination and their migration from
75 the olfactory placode in the chick embryo. This resulted in the identification of 332 transcripts that were
76 differentially regulated during these stages and which displayed at least a twofold increase or decrease in

77 expression. These genes were subjected to further rounds of analysis using published data of gene
78 expression patterns and comparative genomics and this resulted in the identification of the serotonin (5-
79 HT) receptor, HTR1A, as a novel player in GnRH neuronal development and it is the analysis of this gene
80 and its role on GnRH neuronal migration that we present here. We find that HTR1A expression is
81 upregulated 12 fold during the period of GnRH neuronal specification, delamination and migration.
82 Furthermore, we show that HTR1A expression is restricted to the anterior region of the placode, the area
83 that generates GnRH neurons. We further show that inhibition of HTR1A activity, both by a
84 pharmacological antagonism and by siRNA knockdown, interferes with the migration of GnRH neurons
85 into the forebrain: when HTR1A function is perturbed the migration of GnRH neurons is also affected and
86 these cells stall outside of the forebrain. Thus, our studies highlight HTR1A, and more generally serotonin
87 signalling, as a novel player in the development of GnRH neurons.

88

89 **Materials and Methods**

90

91 **Chick embryos**

92 The stage of chick embryos used in this study is within half the gestation period [embryonic day (E) 11]
93 and is approved under the Animals (Scientific Procedures) Act 1986. Fertilised hen's eggs were incubated
94 at 38°C and staged according to Hamburger and Hamilton, 1992 (5), embryos will include both sexes.

95

96 **RNA extraction from chick olfactory placodes and microarray analysis**

97 Total RNA was extracted using the Stratagene MicroRNA kit and RNA resuspended in nuclease-free
98 water: triplicate extractions were performed. The integrity of the RNAs were assessed using a Bioanalyser
99 and 5 ng used for the preparation of biotin-labelled cell extract using the Nugen Ovation amplification
100 system (www.nugeninc.com). For each representative biological replicate group, 7 ug of labelled extract
101 was hybridised to Affymetrix Chicken GeneChips for 20 hours. The hybridised arrays were washed,
102 stained and scanned according to the protocols set out by Nugen and Affymetrix.

103

104 Data from individual GeneChip was MAS5 (www.affymetrix.com) pre-processed. Every signal from each
105 GeneChip was normalised to the median of the signal distribution on that array followed by individual
106 genes being normalised to the median of the distribution of their signal across the whole experiment. Gene
107 expression values were scaled and centred about 1, where N1 represents an enrichment of gene expression
108 in any given condition and b1 represents a rarefaction of expression relative to the other developmental
109 time points. Unreliable gene expression measurements were removed by the application of the Affymetrix
110 Flag filter prior to any statistical analysis. From these data, genes which did not vary beyond a 2 fold
111 range across the whole experiment were also removed to leave a set of genes defined as 'changing and
112 reliable'; these genes were subsequently used for all further analysis.

113

114 **Polymerase chain reaction (PCR) amplification of genes from microarray analysis**

115 Total RNA were extracted using Trizol reagent (Invitrogen) and reverse transcribed using Superscript II
116 RT (Invitrogen). Chick HTR1A (NM_001170528.1) sequence from NCBI was used to design primers for
117 the amplification of HTR1A from RNA extracted from chick olfactory placodes. The primers used were
118 for chick HTR1A forward 5'-TGTGGCCAACAACACTACCT-3', HTR1A reverse 5'-
119 GCCTCCGTGTTCTTCTCGTT-3'; Chick GAPDH primers were used as internal controls (chick GAPDH
120 forward 5'-GAGTCAACGGATTTGGCCGTATT-3' and GAPDH reverse 5'-

121 CACTCCTTGGATGCCATGTGGACCA-3'); the PCR products were cloned into pGEM®-T Easy vector
122 (Promega), sequenced and used to generate riboprobes for *in situ* hybridisation.

123

124 **Whole mount *in situ* hybridisation**

125 *In situ* hybridisation was performed as described (6) with slight modifications. Embryos were washed
126 twice for 20 minutes in detergent mix (1% (v/v) IGEPAL, 1% (w/v) SDS, 0.5% (w/v) deoxycholate, 50
127 mM Tris-HCl pH8, 1 mM EDTA, 150 mM NaCl) prior to post-fixation in 4% (w/v) PFA for 20 minutes.
128 Embryos were incubated at 70°C in hybridisation buffer containing digoxigenin-labelled riboprobes (1
129 ug/ml). Post hybridisation washes were performed twice in solution X (50% (v/v) formamide, 2X SSC,
130 pH 4.5 and 1% (w/v) SDS) for 30 minutes at 70°C, then three times for 30 minutes in MABT (100 mM
131 maleic acid, 150 mM NaCl, 1% (v/v) Tween-20; pH 7.5). Gene expression was either examined as whole
132 mounts or embryos were embedded in 20% (w/v) gelatin in PBS and sectioned using a vibratome.

133

134 **Injection of chick embryos and immunohistochemistry**

135 Hen's eggs were incubated to stage 16 (HH16), windowed and a small area of vitelline membrane torn.
136 The extra-embryonic membrane around the head was removed to expose the olfactory placode. Drugs
137 were injected at a concentration of 5mM, diluted in PBS containing 10% (v/v) glycerol and Fast Green
138 (Sigma) for visualisation directly into the olfactory placode. 8-OH-DPAT (8-hydroxy-2-(di-n-
139 propylamino) tetralin) , a specific HTR1A agonist (7), and WAY-100,635 (N-[2-[4-(2-Methoxyphenyl)-1-
140 piperaziny] ethyl]-N-(2-pyridyl) cyclohexanecarboxamide), a HTR1A specific antagonist (8) were used
141 in this study. At least three dozen embryos were used for each treatment. The window was resealed after
142 injection and returned to the incubator. Embryos were harvested at stage 24 (HH24), fixed in 4% (w/v)
143 EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride) overnight at 4°C.
144 Immunohistochemistry was carried out as previously described (6). Antibodies to GnRH (9) were used at
145 a dilution of 1:200, mouse anti-neurofilament at 1:500 (Sigma; RRID:AB_477261) and mouse anti-GFP at
146 1:200 (Molecular Probes; RRID:AB_221570) and visualised using Alexa conjugated secondary antibodies
147 (Molecular Probes; RRID:AB_141367 and RRID:AB_143165). Embryos were embedded in gelatin and
148 sectioned using a vibratome.

149

150 **Electroporation of chick embryos to knock down HTR1A expression in the olfactory placode**

151 Chick HTR1A miR RNAi was designed according to the software at Invitrogen and cloned into the
152 BLOCK -iTTM Pol II miR RNAi Expression Vector (Invitrogen). Double stranded oligonucleotides were
153 generated using the forward primer 5' -

154 CCTGTTCGCTTGTTGATAGTCGAGTCAGTCAGTGGCCAAAACCTCGACTATGTCAACAAGCGA
155 AC - 3' and reverse primer 5' -
156 TGCTGTTCGCTTGTTGACATAGTCGAGTTTTGGCCACTGACTGACTCGACTATCAACAAGCGA
157 A - 3', cloned and sequenced. The pcDNATM6.2-GW/± EmGFP-miR-negative control plasmid used
158 contained an insert that can form a mature miRNA but is predicted not to target any known vertebrate
159 gene. The sequence of the insert used was 5'-
160 GAAATGTACTGCGCGTGGAGACGTTTTGGCCACTGACTGACGTCTCCACGCAGTACATTT-3'.

161 Electroporation (10) was used to introduce chick HTR1A miR RNAi along with chick β-actin RFP or
162 chick β-actin eGFP or pcDNATM6.2-GW/± EmGFP-miR-negative control plasmid together with chick β-
163 actin RFP or chick β-actin eGFP *in ovo* into olfactory placodes at stages 16 (HH16) using 5×20
164 millisecond pulses of 10 volts. DNA samples were used at a concentration of 1.5 – 2ug mixed with trace
165 amounts of Fast Green. Again, at least three dozen embryos were analysed for each construct. The
166 embryos were then incubated for a further 48 hours prior to fixation in either 4% (w/v) PFA for *in situ*
167 hybridisation or in 4% (w/v) EDC for GnRH immunohistochemistry. Embryos that had undergone *in situ*
168 hybridisation were stained with anti-GFP antibodies (Abcam) (1:200), detected with goat anti-rabbit 488
169 (Molecular Probes) (1:1000). Embryos were then embedded in gelatin, and sectioned.

170

171 **Results**

172 **Transcriptomic analysis of the olfactory placode during the period of GnRH neuronal production**

173 To identify novel genes associated with the development of GnRH neurons we conducted a transcriptomic
174 analysis of the olfactory placode using the chick. This species is particularly useful as the embryos are
175 relatively large, and thus amenable to precise dissection. Moreover, the period covering the initial
176 specification of the GnRH neurons, their delamination and their migration from the olfactory placode has
177 been accurately mapped in the chick (11; 12) and we can, therefore, easily relate changes to gene
178 expression with the developmental sequence. Thus, olfactory placodes were dissected at stage 16 (HH16)
179 – prior to the specification of GnRH neurons, at stage 18 (HH18) – at the start of the specification of these
180 cells, and at stage 20 (HH20) – as these cells are still being produced and are delaminating and migrating
181 from the placode. This was done in triplicate and the samples subjected to microarray analysis. The data
182 was then analysed to identify genes that were differentially expressed between these key stages.

183
184 We first sought to determine the reproducibility of the biological replicates by Principal Component
185 Analysis (PCA) using the set of genes classified as ‘Changing and Reliable’ [i.e. all genes reliably
186 expressed within the biological system]. These data (see Figure 1A) revealed that the individual HH16, 18
187 and 20 samples were clearly demarcated from each other. In particular, the HH20 samples were tightly
188 grouped which is consistent with the tissue samples converging on a differentiated phenotype. Having
189 established the relationship of the replicates we next identified the cohort of differentially expressed genes
190 at each stage compared to the ‘founding’ HH16 samples. Our first analysis (T-Test; P value cut off 0.05; 2
191 fold change filter) revealed that 1373 genes significantly changed their expression (up and down) between
192 HH16 and 18. Similarly, using an identical approach we found that 640 transcripts significantly changed
193 their expression greater than twofold between HH16 and 20 (the list of all of these transcripts is included
194 in the supplementary material including the actual fold change values) (Figure 1B; Volcano plot of HH20
195 vs HH16). To expedite our studies, we further refined these set of genes into those transcripts (332) that
196 were exclusively upregulated at HH20 vs HH16 (Figure 1C). From this set we further investigated their
197 potential role in the system using multiple resources about their biological function and our current
198 understanding of the existing signalling pathways in the tissue under study.

199
200 This dataset has been particularly informative as it gives us an overview of the cellular processes that are
201 occurring in the placode during this key period. As anticipated, differentially expressed genes in this list
202 included those involved in neurogenesis, such as NEUROG1, axon guidance molecules, such as
203 SEMA3D, and components of the fibroblast growth factor (FGF) signalling system, FGFRs and their
204 ligands. Furthermore this data also confirmed that, as with the other neurogenic placodes, delamination of

205 cells from the olfactory placode does not involve an epithelial to mesenchymal transition (EMT) (13);
206 there is no upregulation of SNAIL genes or of the Rho small GTPases.

207
208 We then subjected this list of genes to further rounds of analysis drawing in on information from
209 published papers, gene expression patterns and comparative genomics. Table 1 lists the top 10 upregulated
210 and top 10 downregulated genes, obtained from comparing stage 16 (HH16) vs stage 20 (HH20) (refer to
211 Table 1). This then resulted in a list of genes which merited further investigation and one of the most
212 interesting of these proved to be the HTR1A gene, which was found to be expressed at 12.57 fold greater
213 at HH20 than it was at HH16 (Figure 1C).

214

215 **GnRH neurons arise from HTR1A mRNA positive region of the olfactory placode**

216 To gain spatial and temporal insights into the expression of HTR1A we conducted *in situ* hybridisation
217 analysis. This demonstrated that HTR1A expression was absent from the olfactory placode at HH16, but
218 increased dramatically at HH18 and remained high at HH20 (Figure 2A - C). Importantly, however,
219 HTR1A expression was spatially localised to the anterior olfactory placode at HH18 and HH20 (Figure 2B
220 and 2C; arrowheads); notably this region has also been documented to also give rise to GnRH neurons
221 (Figure 2D). At HH20, GnRH positive cells (Figure 2D, D') migrate out of the olfactory placodes and
222 navigate towards the brain.

223

224 **Inhibition of HTR1A activity delays migration of GnRH neurons from the olfactory placode** 225 **towards the forebrain**

226 We next examined whether the generation and migration of GnRH neurons from the olfactory placode
227 towards the forebrain was dependent on HTR1A activity. To resolve this issue we treated chick embryos
228 *in vivo* with pharmacological reagents that can modulate the HTR1A signalling pathway. Thus, chick
229 embryos were injected with 8-OH-DPAT and WAY-100,635. These injections were performed at HH16,
230 which is just prior to the generation GnRH neurons by the olfactory placode and their migration from this
231 structure. Control embryos were injected with PBS.

232

233 In all of the embryos injected, the overall anatomy of the entire embryo was analysed and no toxic side
234 effects were observed. Firstly, we noted that GnRH neurons were still generated when the HTR1A
235 signalling pathway was either downregulated or upregulated using these pharmacological reagents. In all
236 cases GnRH neurons could be seen to arise within the placode and to be delaminating from here. We
237 therefore analysed the migratory behaviour of these neurons to see if this was affected. Normal migration

238 of GnRH neurons is an ongoing process that involves their delamination from the olfactory placode
239 followed by their migration along the olfactory nerve. These cells then reach the outside of the forebrain
240 where they stall before entering and then migrating within the developing CNS to the hypothalamus. Thus,
241 to determine if interfering with HTR1A can affect their migration we assessed the extent to which GnRH
242 expressing cells could be found across their migratory path. To do this we assayed for the presence of
243 GnRH neurons at four different spatial positions in HH24 embryos: those present in the olfactory pit,
244 those found between the placode and the developing brain, those at the entry point into the forebrain and
245 those which had entered the forebrain. In each case, injected embryos were analysed and scored according
246 to the position of GnRH positive cells.

247
248 When embryos were treated with 8-OH-DPAT, an HTR1A agonist, or with PBS, GnRH positive neurons
249 were found at all four locations, olfactory pit, migratory stream, base of the forebrain and inside the
250 forebrain (Figure 3A, B, and C). This was seen in all 7 embryos treated with the agonist and in all 11 PBS
251 injected embryos and there was no noticeable difference between the embryos treated with the agonist and
252 those treated with PBS. For each injection, the uninjected side of the embryo served as stage matched
253 control. On the control side of the WAY-100,635 injected embryo, GnRH positive cells were seen in the
254 olfactory pit, migratory stream, base of the forebrain and inside the forebrain as described for the 8-OH-
255 DPAT and PBS injected control embryos (Figure 3D, E and F). However, when embryos were injected
256 with WAY-100,635, GnRH positive cells were only found in 3 locations on the injected side of the
257 embryo. Thus, GnRH positive cells were found in the olfactory pit, migratory stream and base of the
258 forebrain, but none were detected in the forebrain (in 5 out of 7 embryos analysed). Furthermore, the
259 GnRH positive cells that had migrated furthest were seen clumped together in a distinct structure at the
260 forebrain entry point, but not seen to penetrate the basal lamina and enter into the CNS (Figure 3G, H and
261 I). Thus, HTR1A activity is important for GnRH neuronal migration into the forebrain in chick embryos.

262

263 **Knockdown of HTR1A in chick embryos delays the migration of GnRH neurons into the forebrain**

264 To complement the pharmacological approach, miR RNAi was also used to knockdown HTR1A
265 expression in chick olfactory placodes. The embryos were electroporated at HH16, which is prior to the
266 initiation of HTR1A expression and thus ensures that the expression of this gene is knockdown from its
267 initial onset. Embryos were co-electroporated with a reporter construct expressing either GFP or RFP to
268 facilitate the visualisation of electroporated cells. Following electroporation, embryos were harvested at
269 HH24, stained for GnRH immunoreactivity and analysed for RFP expression to determine the extent of
270 electroporation. Gene knockdown was confirmed by *in situ* hybridisation for HTR1A (Figure 4A' and
271 4A''). As can be seen with the HTR1A miR RNAi electroporations (detected with GFP expression, Figure

272 4A), HTR1A mRNA levels were significantly reduced (Figure 4A') compared to the unelectroporated
273 control side (Figure 4A''). This demonstrates that the HTR1A miR RNAi construct can efficiently knock
274 down HTR1A, as the negative RNAi control electroporations showed no effect on HTR1A gene
275 expression (Figure 4B and 4B'). We then analysed the spatial distribution of GnRH positive cells in the
276 embryos after electroporation. When HTR1A miR RNAi was electroporated, we noticed that the number
277 of GnRH positive cells had decreased overall in the embryo. Furthermore, although GnRH positive cells
278 were localised in the olfactory placode, migratory stream and the base of the forebrain (Figure 4C – C'')
279 all of those cells that were RFP+ve, GnRH+ve, i.e. had been electroporated with the knockdown construct,
280 were only detected outside the forebrain and these cells failed to enter into the forebrain (Figure 4C'').
281 Tissue morphology via differential interference contrast (DIC) microscopy was used to confirm the
282 forebrain boundary. The path of GnRH neurons on the uninjected side of the embryo can be clearly
283 visualised at the olfactory placode, the migratory stream, and at the base of the forebrain as well as
284 penetrating into the forebrain (Figure 4D, D' and D''). RFP-ve GnRH+ve cells were seen in the forebrain,
285 showing that unelectroporated GnRH cells have migrated into the forebrain in these embryos suggesting
286 that the defective migration of cells electroporated with the miRNA construct is intrinsic to these cells.

287

288 In embryos electroporated with pcDNATM6.2-GW/± EmGFP-miR-negative control plasmid, the GnRH
289 positive population were also analysed at HH24 (Figure 4E and E'). In these embryos, RFP+ve and
290 GnRH+ve cells were found at the four distinct locations, olfactory placode, migratory stream, base of the
291 forebrain and inside the forebrain. Electroporated GnRH neurons were analysed based on the dual
292 expression of RFP and GnRH in all cases.

293

294 **Olfactory sensory neuronal development is not affected by HTR1A knockdown**

295 Embryos electroporated with both HTR1A miR RNAi and pcDNATM6.2-GW/± EmGFP-miR-negative
296 control plasmid were subsequently analysed using antibodies to neurofilament to assess if there were any
297 consequence for the formation of the olfactory nerve following electroporation. This was performed to
298 make sure that the failure of GnRH positive neurons to enter into the forebrain was intrinsic to the GnRH
299 neurons and not because of a secondary failure as a result of olfactory sensory axonal perturbations.

300 Embryos were electroporated at HH16 and harvested at stage 28 (HH28) and immunostained with
301 antibodies to neurofilament. Olfactory sensory neurons stain with antibodies to neurofilament and their
302 axonal bundles can be seen projecting out from the olfactory placode and towards the forebrain in
303 untreated embryos (Figure 5A). When the electroporated embryos were stained with antibodies to
304 neurofilament and analysed, with both the constructs, HTR1A miR RNAi (Figure 5B and 5B') and
305 pcDNATM6.2-GW/± EmGFP-miR-negative control plasmid (Figure 5C and 5C'), the normal pattern of

306 immunostaining was observed. RFP expression indicates the site of electroporation (Figure 5B' and 5C').
307 Utilising the RNAi plasmid to knock down HTR1A in chick olfactory placodes does not affect the
308 olfactory sensory neurons, either their formation or projection to the forebrain.

309

310 **Discussion**

311 In this study we present HTR1A as a novel gene implicated in the early development of GnRH neurons.
312 We arrived at this point through a transcriptomic analysis that was performed in order to identify
313 candidate molecules acting intrinsic to GnRH neurons, and which may be responsible for their
314 specification, delamination and migration from the olfactory placode. This resulted in the identification of
315 332 genes which were differentially expressed during the key early stages of GnRH neuronal
316 development. We focussed on the 10 genes whose expression was most upregulated and downregulated
317 (Table 1). This data set was further analysed and this highlighted HTR1A as a gene that both shows
318 significant up regulation during these early stages and which, importantly, is spatially localised to the
319 region of the olfactory placode that generates the GnRH neurons. We further show that inhibition of
320 HTR1A activity impairs GnRH neuronal migration. In embryos injected with the antagonist, WAY-
321 100,635, GnRH neurons were delayed in entering the forebrain. However, their specification and
322 delamination were not affected, but rather they clumped together at the entry point at the base of the
323 forebrain. Similarly, RNAi knockdown of HTR1A also resulted in a delay in GnRH neurons migrating
324 into the forebrain, an effect which was not observed with the negative control RNAi electroporations. In
325 addition, the effect of delayed migration into the forebrain was intrinsic to GnRH neurons themselves as
326 the olfactory neurons and their axonal projections were not affected in the knockdown experiments. Thus,
327 HTR1A function is required for the normal migration of GnRH neurons.

328 A number of studies have shown that the serotonergic machinery exist in early embryos from stages prior
329 to neurogenesis and onwards (14; 15). Serotonin has a maternal origin in the chick and is produced by the
330 placenta in mouse and rat (16; 17). Thus, serotonin is localised to the chick blastoderm and formed in the
331 yolk from tryptophan even at the early stages of embryogenesis (18) and endogenous stores of serotonin
332 have also been shown in the chick notochord (19). Moreover, chick embryos treated with serotonin
333 precursors have shown their ability to synthesise serotonin (20). We have also analysed the expression of
334 the serotonin synthetic enzyme tryptophan hydroxylase. Tryptophan hydroxylase (TPH) 1 and 2 are
335 enzymes involved in serotonin synthesis in peripheral and central nervous system respectively (21; 22).
336 Examination of our transcriptomic data sets revealed the expression of both enzymes in the olfactory
337 placode at all stages with the expression of TPH1 being at much lower levels than that of TPH2. The
338 expression of both synthetic enzymes was subsequently confirmed using RT-PCR on RNA extracted from

339 olfactory placodes (data not shown). Thus, it would seem that serotonin is generally available in the chick
340 embryo during the period of GnRH neuronal migration.

341 The identification of HTR1A as a player in GnRH migration is also significant as it suggest that molecules
342 acting downstream of this receptor and mediating its function may also be important for this process. With
343 regards to this point, it is known that phospholipase A₂ (PLA₂) is activated downstream of HTR1A (23),
344 and significantly we found that this gene was also upregulated in the transcriptome analysis (by
345 approximately 30 fold between HH16 and HH20). Furthermore, binding of GnRH to the GnRH receptor,
346 stimulates the release of phosphoinositide (IP3) which along with diacylglycerol (DAG) is required for
347 intracellular calcium mobilisation and subsequent activation of PKC, phospholipase D (PLD) and the
348 activation of PLA₂ (24; 25). Thus, it is possible that both serotonin and GnRH signalling converge on
349 PLA₂.

350 Increased levels of cAMP have been demonstrated to reduce neuronal migration in invertebrates and in the
351 cerebellum of mammals, and is associated with a reduction in intracellular calcium ion transients (26; 27).
352 HTR1A is coupled to adenylyl cyclase (AC) and inhibits the production of cAMP. Early in development,
353 when GnRH neurons are migrating, inhibition of cAMP production would be advantageous to these cells.
354 The upregulation of HTR1A in the transcriptome assay and the downregulation of HTR4 (which also
355 couples to AC, but stimulates the production of cAMP) at HH20 when GnRH neurons are migrating from
356 the olfactory placode to the forebrain is essential to GnRH neurons during these stages of development.

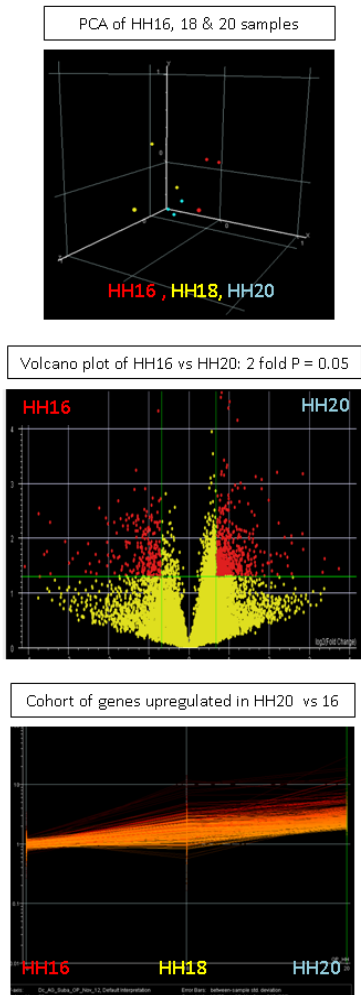
357 It is perhaps also worthwhile noting that the effects of serotonin signaling on mature GnRH neuronal
358 physiology in the adult has been well documented. Serotonin has been proposed as a regulator of GnRH
359 neurons due to the expression of serotonin receptors by these cells (28; 29). Serotonin containing axons
360 are found in close proximity to GnRH neurons in the hypothalamus and to GnRH containing axons in the
361 median eminence (30; 31). Synaptic contacts have been observed in serotonin-labeled boutons and GnRH
362 positive dendrites, serotonin containing neurons can act directly on GnRH release (31). Thus it would
363 seem that serotonin signalling has ongoing roles in GnRH neurons, from the earliest periods to adulthood.

364 We propose that activation of HTR1A and the signalling pathways that lie downstream of this are
365 important for the normal migratory behaviour of GnRH neurons from the olfactory placode towards and
366 into the forebrain. Our study thus identifies HTR1A as a novel gene involved in the regulation of the early
367 development of GnRH neurons and suggests that other novel insights into this system may also come from
368 the further analysis of molecules that are further involved in mediating the effects of this receptor. This in
369 turn may lead to the identification of novel candidate genes that may underlie HH/KS in humans.

370

371

372 **Figure legends**

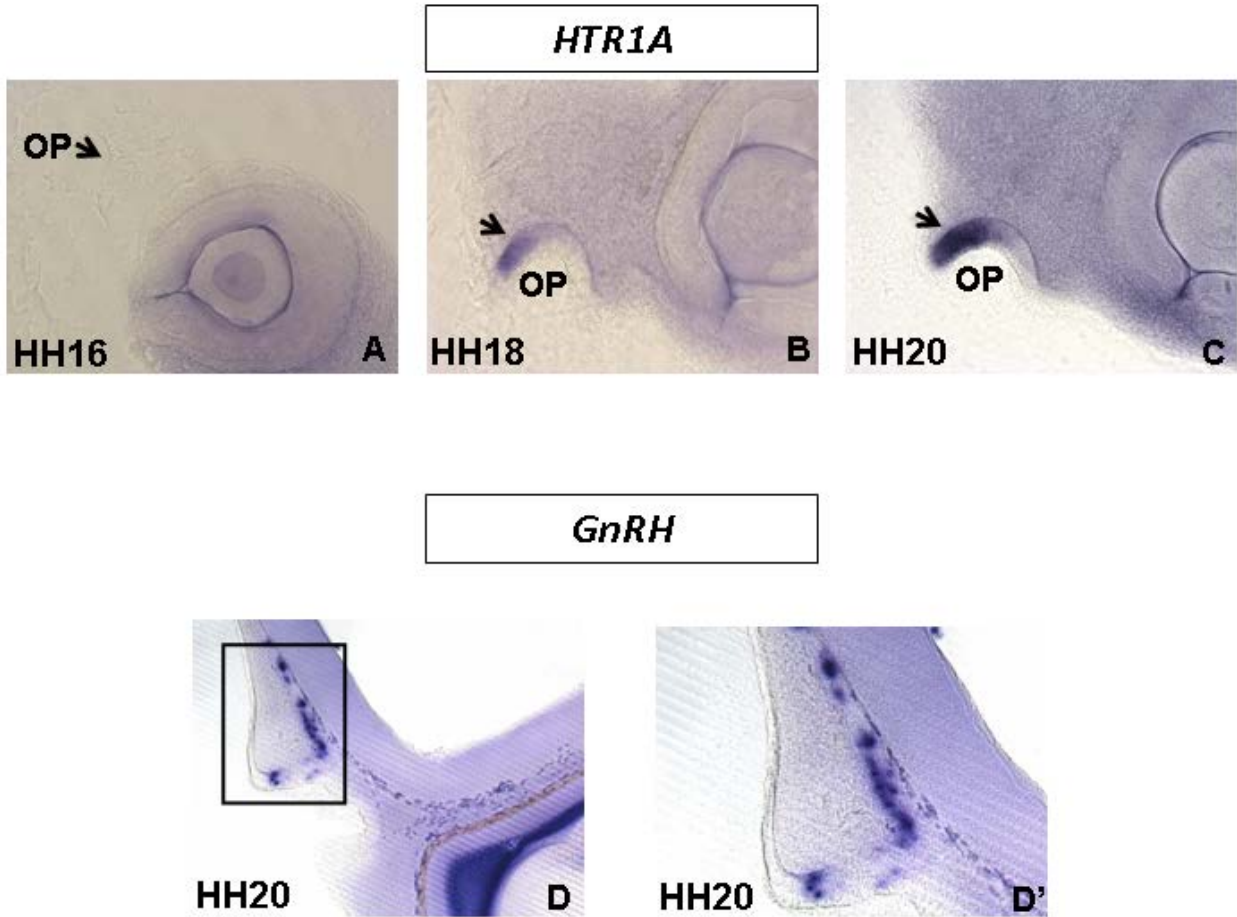


373

374 **Figure 1**

375 **A large number of genes were differentially expressed between key stages of olfactory placodal**
376 **development**

377 Principal Component Analysis (PCA) of the genetic profile of olfactory placodes at HH16 (red), HH18
378 (yellow) and HH20 (blue). (B) Volcano plot indicating genes which had significantly changed in
379 expression greater than twofold between HH20 and HH16. (C) Genes which were upregulated at least
380 twofold between HH20 and HH16 are indicated.



381

382 **Figure 2**

383 ***HTR1A* is differentially expressed in the olfactory placode during olfactory system development**

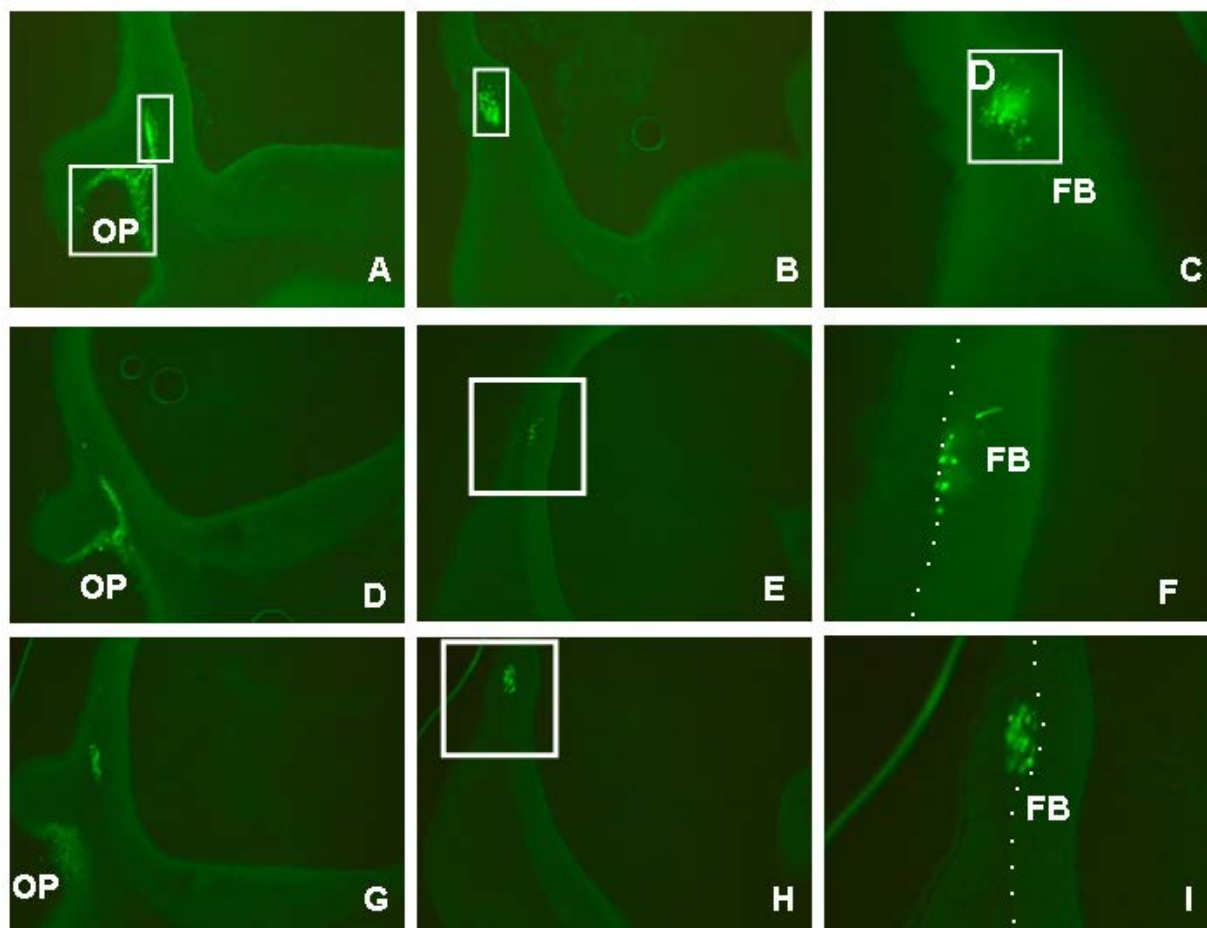
384 (A) At stage 16 (HH16) there was no expression of *HTR1A* in the olfactory placode. However, by stage 18

385 (HH18) (B) *HTR1A* expression could be seen to be localised to the anterior portion of the placode

386 (arrowhead). This same domain of expression was still evident at stage 20 (HH20) (C; arrowhead).

387 Importantly, this is the same region of the placode in which the GnRH neurons are generated, as shown by

388 GnRH mRNA expression at stage 20 (D). D' indicates the magnified region boxed in D.



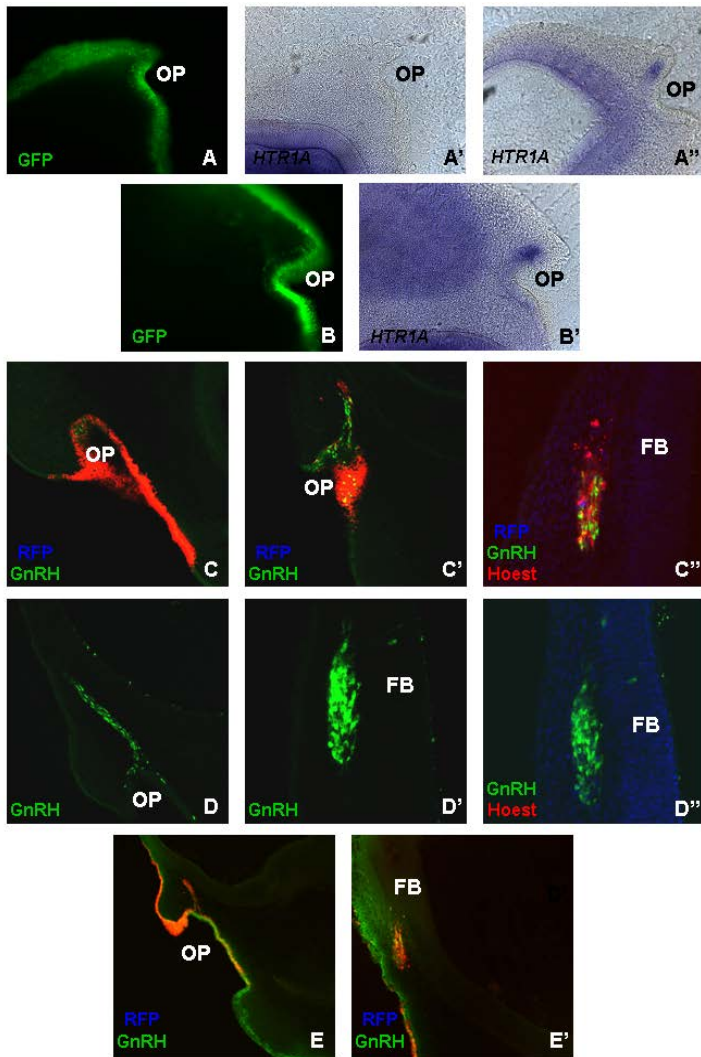
389

390 **Figure 3**

391 **HTR1A activity is required for GnRH neuronal migration into the forebrain in chick embryos**

392 The normal migratory path of GnRH neurons is indicated (A-C). During development, GnRH neurons are
 393 generated in the olfactory placode (A), from wherein they delaminate, migrate and enter the forebrain at
 394 stage 24 (HH24) (C). The contra-lateral side of the WAY-100,635 treatment is shown indicating the
 395 profile of GnRH neuronal populations (D, E and F). In embryos treated with the HTR1A antagonist
 396 (WAY-100,635) GnRH neurons are still generated in the olfactory placode and migrate from this region
 397 (G) but they stall outside of the forebrain (H and I). GnRH positive cells are indicated at the forebrain
 398 entry point. (F) and (I) are higher magnifications of the boxed regions in (E) and (H) respectively. Dotted
 399 lines indicate the forebrain boundary.

400



401

402 **Figure 4**

403 **Downregulating HTR1A expression in the chick olfactory placode affects the migration of GnRH**
 404 **neurons into the forebrain**

405 Chick olfactory placodes were electroporated at HH16 with RNAi specific to chick HTR1A or a negative
 406 control RNAi and a reporter construct (expressing either RFP or GFP) and left to develop until HH20 or
 407 HH24. Electroporated side of the embryo is shown by the expression of GFP in the olfactory placode (A)
 408 and subsequent knockdown of HTR1A mRNA expression (A') at HH20. The unelectroporated side of the
 409 same embryo showing expression of HTR1A mRNA is indicated (A''). Negative control RNAi and GFP
 410 co-electroporation (B) and *in situ* hybridisation for HTR1A expression (B') are shown. HTR1A and RFP
 411 co-electroporations are shown (C, C' and C''). After electroporation, the embryos were stained with
 412 antibodies to GnRH and Hoest and imaged using a confocal microscope. Electroporated cells are indicated

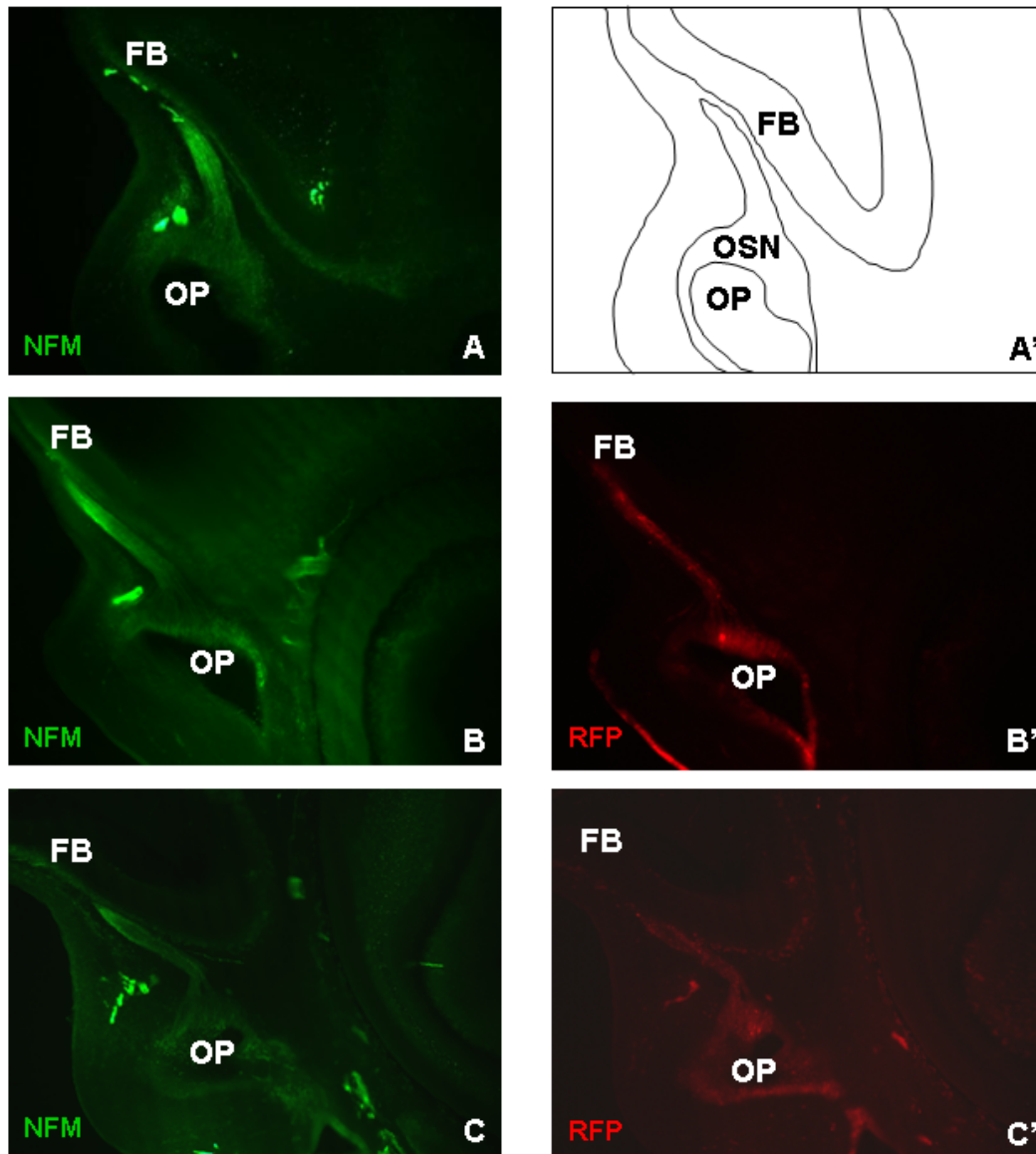
413 by the expression of RFP. The contra-lateral side of the electroporated embryo is shown (D, D' and D''),
414 indicating the presence, delamination and migration of GnRH neurons from the olfactory placode and into
415 the forebrain. Hoest staining shows the laminar organisation of the forebrain. Electroporated GnRH
416 neurons are seen delaminating from the olfactory placode and migrating towards the forebrain (E) and
417 subsequent migration of GnRH and RFP positive cells into the forebrain (E').

418

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422

423 **Figure 5**

424 **Olfactory sensory axonal projection to the forebrain is not affected by the knockdown strategy**

425 RNAi and RFP co-electroporated chick embryos were harvested, fixed and immunostained with
 426 antibodies to neurofilament to label the olfactory sensory axons. The contra-lateral side of the HTR1A
 427 RNAi electroporated embryo is shown (A), with neurofilament staining of the olfactory sensory axons. A
 428 schematic cross section through the head of a chick embryo at HH28 is shown in A'. The HTR1A RNAi
 429 and negative control RNAi electroporated side of the embryo is indicated by the expression of RFP (B'
 430 and C' respectively), and olfactory axons are seen projecting from the olfactory placode (B and C).

431

432 **Abbreviations**

433	CNS	central nervous system
434	EDC	N-Ethyl-N'-(3-dimethylaminopropyl)carbodiimide
435	FGF	fibroblast growth factor
436	GAPDH	glyceraldehyde 3-phosphate dehydrogenase
437	GnRH	gonadotrophin releasing hormone
438	HH	hypogonadotropic hypogonadism
439	5-HT	5-hydroxytryptamine
440	HTR1A	serotonin receptor 1A

441

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444

445 **Disclosure Summary**

446 The authors have nothing to disclose.

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