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

2

3 Subathra Poopalasundaram[1*], David Chambers[2], Anthony Graham[2*] and Pierre-Marc Bouloux[1]

4

5 [1]Centre for Neuroendocrinology, University College London Medical School, Royal Free Campus,

6 London NW3 2PF, United Kingdom and [2]Centre for Developmental Neurobiology, Kings College

7 London, London SE1 1UL, United Kingdom

8

9 *Address correspondence to: Subathra Poopalasundaram, Centre for Neuroendocrinology, University

10 College London Medical School, Royal Free Campus, London NW3 2PF or Anthony Graham, Centre for

11 Developmental Neurobiology, Kings College London, London SE1 1UL, United Kingdom

12

13 Tel. +44-20-7794 0500 ext: 38158; Fax: +44-20-7317 7625

14 E-mail: subathra.poopalasundaram@ucl.ac.uk or anthony.graham@kcl.ac.uk

15

16 Running title: Serotonin receptor 1A in GnRH neuronal migration

17 Total number of words: 6,198

18 Key words: GnRH neurons, serotonin receptor 1A, hypogonadotropic hypogonadism

19

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.

40

41

42

43

44

Introduction

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

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

To address this problem, we conducted a transcriptomic analysis of the olfactory placode during the period covering the initial specification of the GnRH neurons, their delamination and their migration from the olfactory placode in the chick embryo. This resulted in the identification of 332 transcripts that were 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 it 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 38ºC 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

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 70ºC 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 70ºC, 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

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 piperazinyl] 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 4ºC.

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

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 –iT™ Pol II miR RNAi Expression Vector (Invitrogen). Double stranded oligonucleotides were

153 generated using the forward primer 5' -

154   CCTGTTCGCTTGTTGATAGTCGAGTCAGTCAGTGGCCAAAACTCGACTATGTCAACAAGCGA

155   AC - 3' and reverse primer 5' -

156   TGCTGTTCGCTTGTTGACATAGTCGAGTTTTGGCCACTGACTGACTCGACTATCAACAAGCGA

157   A - 3', cloned and sequenced. The pcDNA^{TM}6.2-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 ß-actin RFP or

162   chick ß-actin eGFP or pcDNA^{TM}6.2-GW/± EmGFP-miR-negative control plasmid together with chick ß-

163   actin RFP or chick ß-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 its 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 unelectroprated 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 pcDNA^{TM}6.2-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 pcDNA^{TM}6.2-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 pertubations.

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   pcDNA^{TM}6.2-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 A_2 (PLA_2) 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 PLA_2 (24; 25). Thus, it is possible that both serotonin and GnRH signalling converge on

349 PLA_2.

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 adenyl 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.
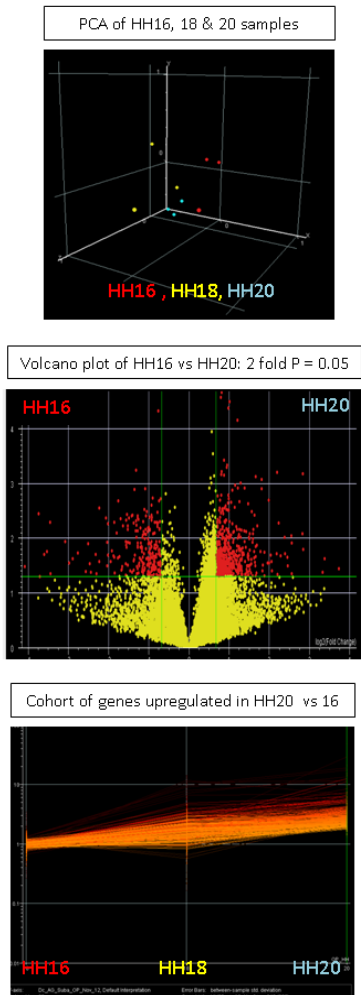
357 It is perhaps also worthwhile noting that the effects of serotonin signaling on mature GnRH neuronal

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

Figure legends

PCA of HH16, 18 & 20 samples

HH16 , HH18, HH20

Volcano plot of HH16 vs HH20: 2 fold P = 0.05

HH16   HH20

Cohort of genes upregulated in HH20 vs 16

HH16   HH18   HH20

373

Figure 1

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

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

14

381
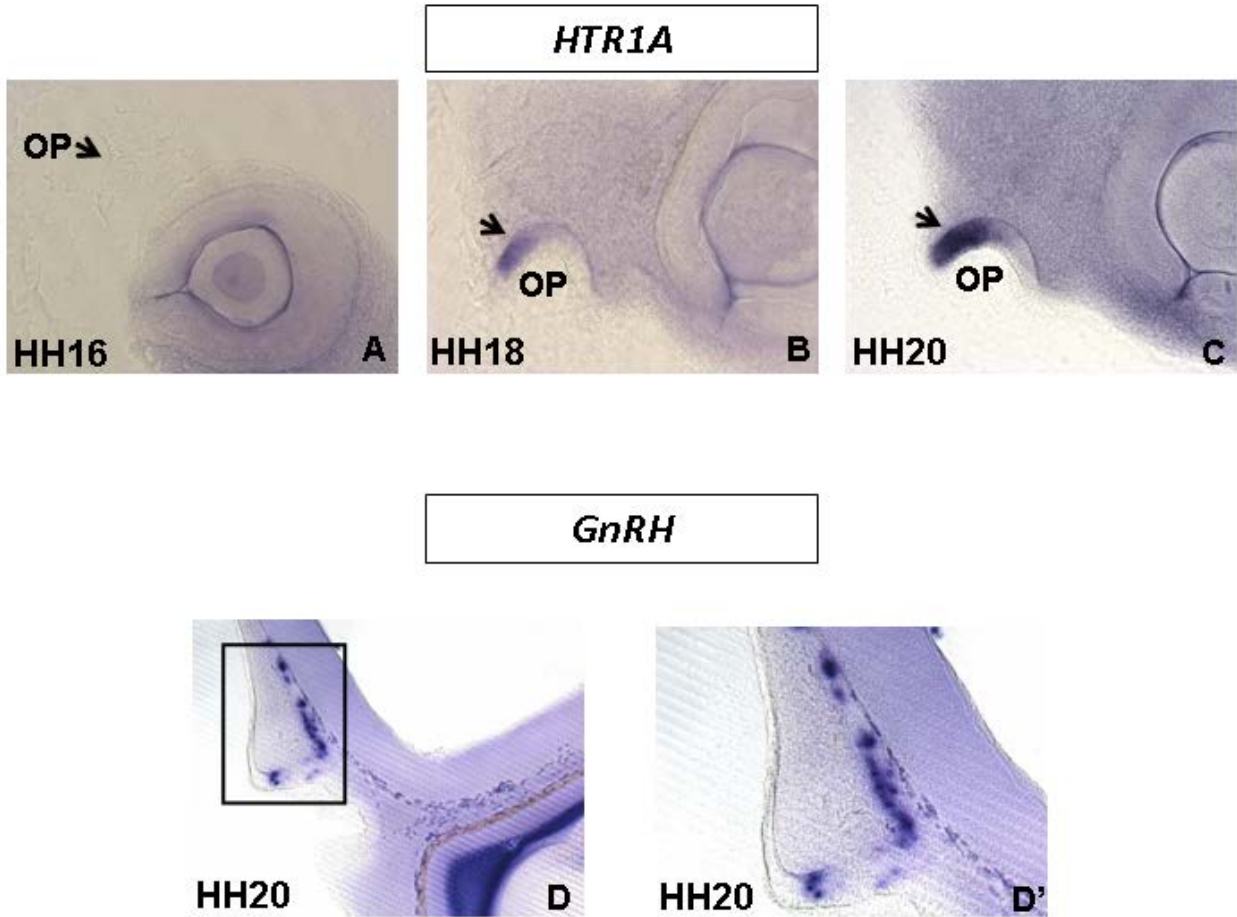
Figure 2

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

(A) At stage 16 (HH16) there was no expression of *HTR1A* in the olfactory placode. However, by stage 18
(HH18) (B) *HTR1A* expression could be seen to be localised to the anterior portion of the placode
(arrowhead). This same domain of expression was still evident at stage 20 (HH20) (C; arrowhead).
Importantly, this is the same region of the placode in which the GnRH neurons are generated, as shown by
GnRH mRNA expression at stage 20 (D). D' indicates the magnified region boxed in D.

389
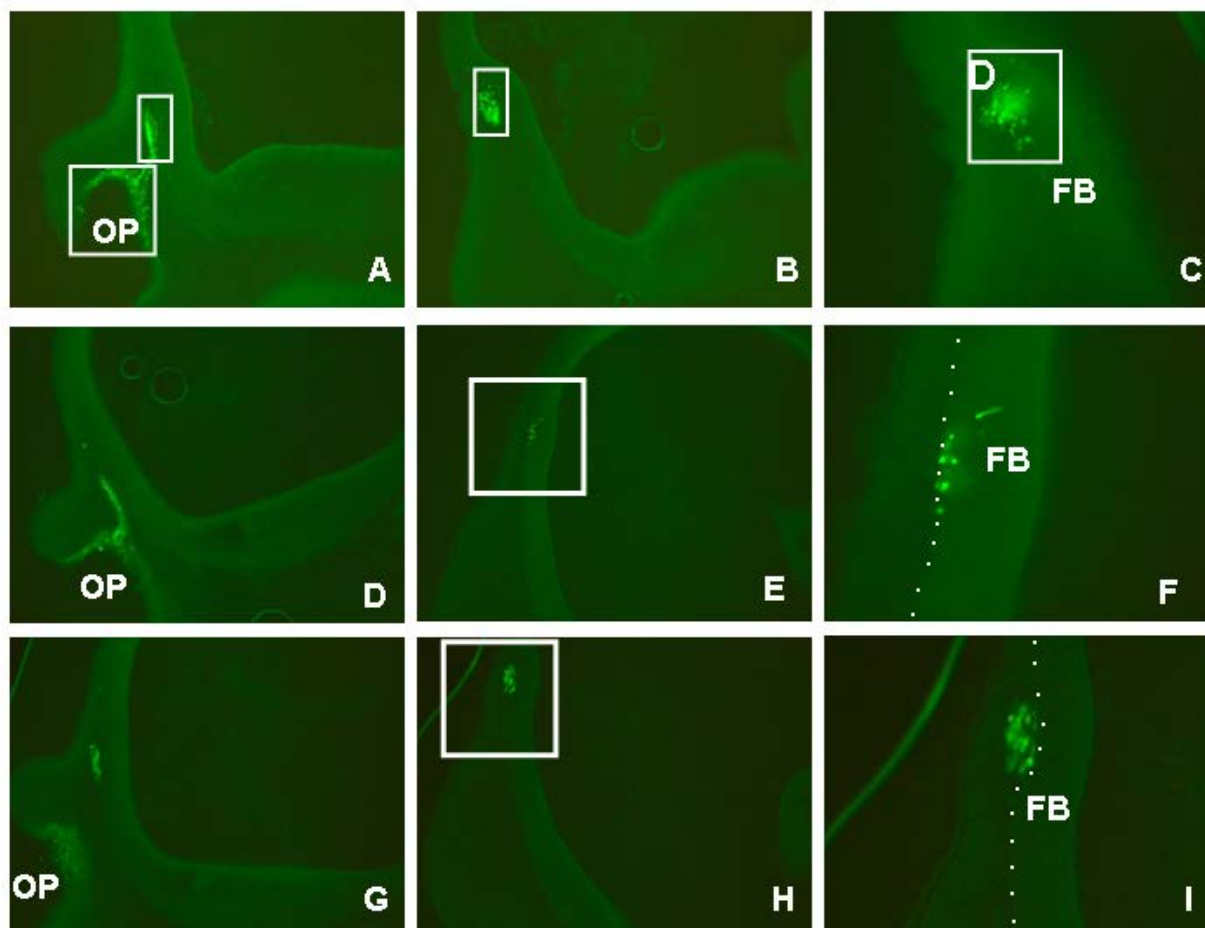
Figure 3

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

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

400

401
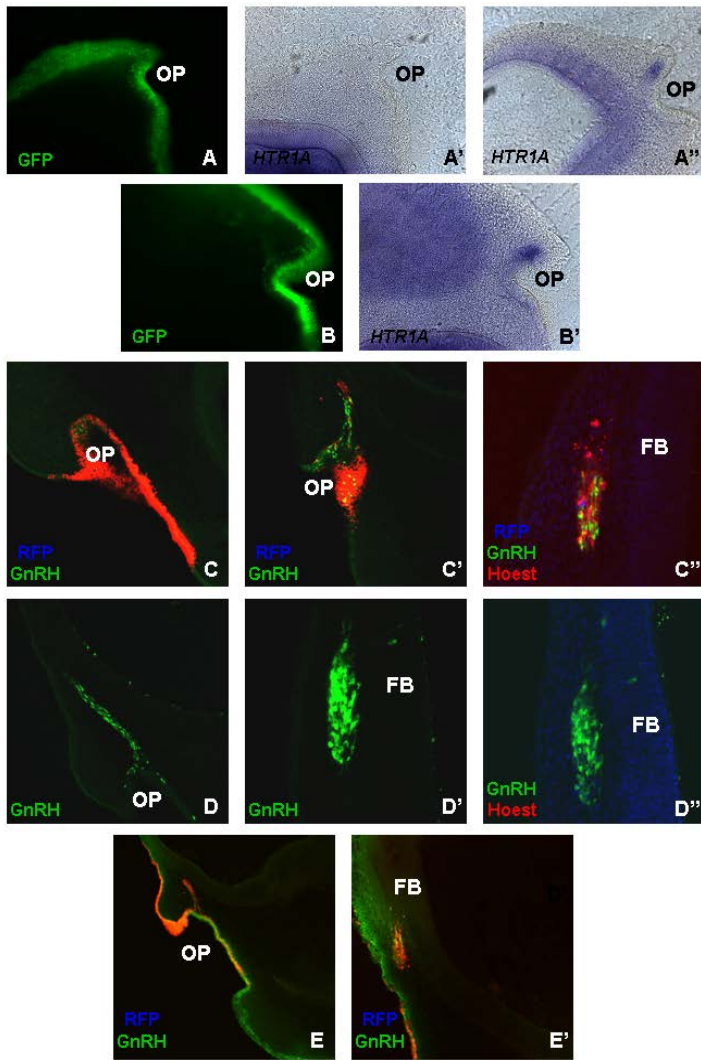
Figure 4

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

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

17

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

419

420

421

422
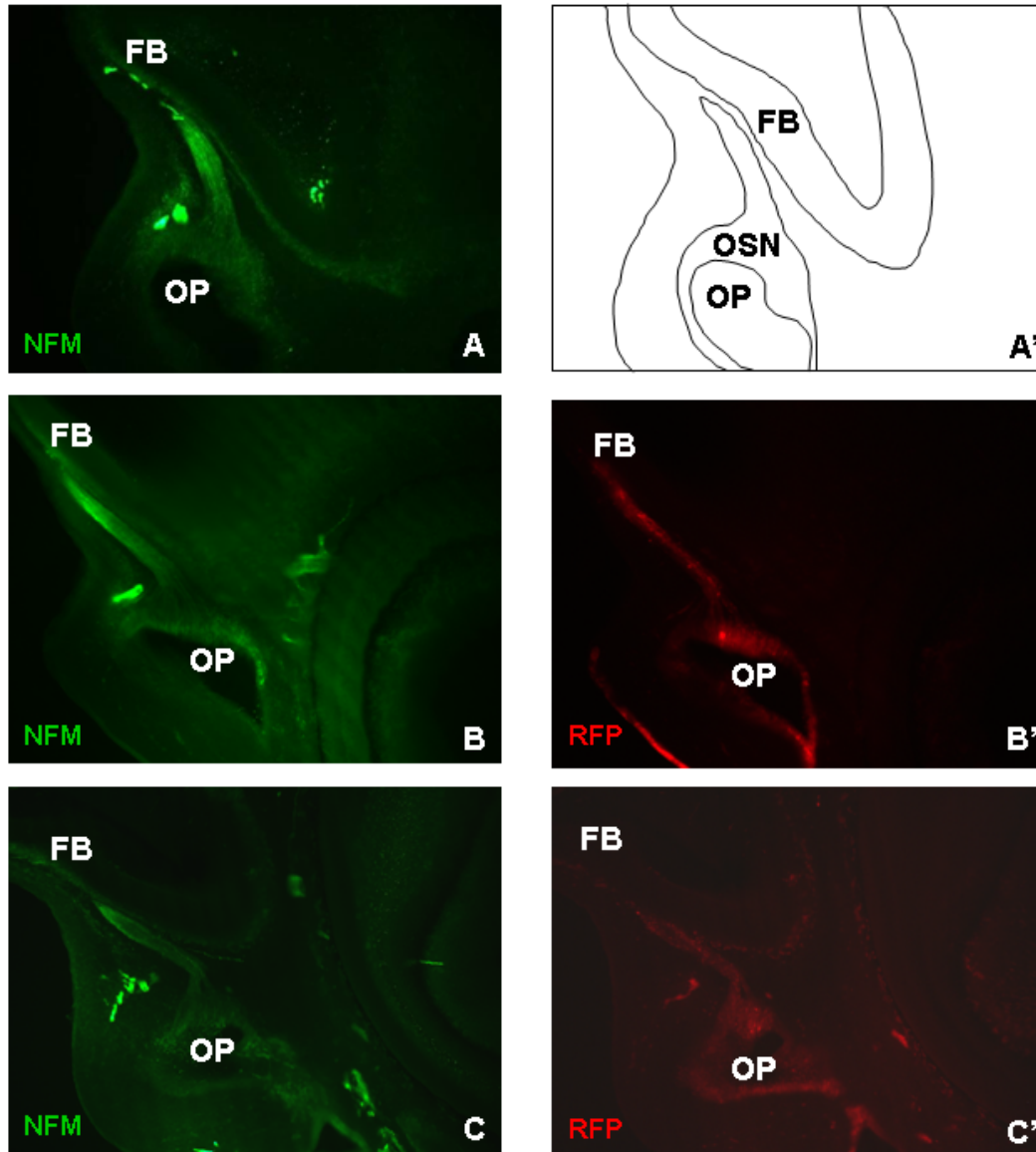
Figure 5

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

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

19

431

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	hypogonadotrophic hypogonadism
439	5-HT	5-hydroxytrytamine
440	HTR1A	serotonin receptor 1A

441

Acknowledgments

443   We thank the Royal Free Charity for funding this research.

444

Disclosure Summary

446   The authors have nothing to disclose.

447

448

449

450

451

452

References

453

1. Sower SA, Freamat M, Kavanaugh SI. The origins of the vertebrate hypothalamic-pituitary-gonadal
454
(HPG) and hypothalamic-pituitary-thyroid (HPT) endocrine systems: new insights from lampreys. Gen
455
Comp Endocrinol. 2009; 163(3): 342-3.
456

2. Sabado V, Barraud P, Baker CV, Streit A. Specification of GnRH-1 neurons by antagonistic FGF and
457
retinoic acid signaling. Dev Biol. 2012; 362(2): 254-62.
458

3. Graham A, Shimeld SM. The origin and evolution of the ectodermal placodes. J Anat. 2013; 222(1):
459
32-40.
460

4. Forni PE, Wray S. GnRH, anosmia and hypogonadotropic hypogonadism - where are we? Front
461
Neuroendocrinol. 2015; 36: 165-77.
462

5. Hamburger V, Hamilton HL. A series of normal stages in the development of the chick embryo. Dev
463
Dyn. 1992; 195(4): 231-72.
464

6. Thompson H, Blentic A, Watson S, Begbie J, Graham A. The formation of the superior and jugular
465
ganglia: insights into the generation of sensory neurons by the neural crest. Dev Dyn. 2010; 239(2): 439-
466
45.
467

7. Middlemiss DN, Fozard JR. 8-Hydroxy-2-(di-n-propylamino)-tetralin discriminates between subtypes
468
of the 5-HT1 recognition site.Eur J Pharmacol. 1983; 90(1): 151-3.
469

8. Forster EA, Cliffe IA, Bill DJ, Dover GM, Jones D, Reilly Y, Fletcher A. A pharmacological profile of the
470
selective silent 5-HT1A receptor antagonist, WAY-100635. Eur J Pharmacol. 1995; 281(1): 81-8.
471

9. Park MK, Wakabayashi K. Preparation of a monoclonal antibody to common amino acid sequence of
472
LHRH and its application. Endocrinol Jpn. 1986; 33(2): 257-72.
473

10. Itasaki N, Bel-Vialar S, Krumlauf R. 'Shocking' developments in chick embryology: electroporation
474
and in ovo gene expression. Nat Cell Biol. 1999; 1(8): E203-7.
475

11. Mulrenin EM, Witkin JW, Silverman AJ. Embryonic development of the gonadotropin-releasing
476
hormone (GnRH) system in the chick: a spatio-temporal analysis of GnRH neuronal generation, site of
477
origin, and migration. Endocrinology. 1999; 140(1): 422-33.
478

479   12. Fornaro, M., Geuna, S., Fasolo, A., and Giacobini-Robecchi, M.G. (2003). HuC/D confocal imaging

480   points to olfactory migratory cells as the first cell population that expresses a post-mitotic neuronal

481   phenotype in the chick embryo. Neuroscience 122, 123-128.

482   13. Graham, A., Blentic, A., Duque, S. and Begbie, J. (2007). Delamination of cells from neurogenic

483   placodes does not involve an epithelial-to-mesenchymal transition. Development **134**, 4141-4145.

484   14. Gaspar P, Cases O, Maroteaux L. The developmental role of serotonin: news from mouse molecular

485   genetics. Nat Rev Neurosci. 2003; 4(12): 1002-12.

486   15. Fukumoto T, Kema IP, Levin M. Serotonin signaling is a very early step in patterning of the left-right

487   axis in chick and frog embryos. Curr Biol. 2005; 15(9): 794-803.

488   16. Palen K, Thornby L, and Emanuelsson H. Effects of serotonin and serotonin antagonists on chick

489   embryogenesis. Wilhelm Roux' Arch. 1979; 187: 89-103.

490   17. Yavarone MS, Shuey DL, Sadler TW, Lauder JM. Serotonin uptake in the ectoplacental cone and

491   placenta of the mouse. Placenta. 1993; 14(2): 149-61.

492   18. Emanuelsson, H. Serotonin in chick embryo cells during early morphogenesis. J. Cell Biol. 1976; **70**,

493   131a.

494   19. Strudel G, Recasens M, Mandel P. Identification of catecholamines and serotonin in the notochords of

495   chick embryos. C R Acad Sci Hebd Seances Acad Sci D. 1977; 284(11): 967-9.

496   20. Wallace JA. Monoamines in the early chick embryo: demonstration of serotonin synthesis and the

497   regional distribution of serotonin-concentrating cells during morphogenesis. Am J Anat. 1982; 165(3):

498   261-76.

499   21. Walther DJ, Bader M. A unique central tryptophan hydroxylase isoform. Biochem Pharmacol. 2003

500   Nov 1; 66(9): 1673-80.

501   22. Patel PD, Pontrello C, Burke S. Robust and tissue-specific expression of TPH2 versus TPH1 in rat

502   raphe and pineal gland. Biol Psychiatry. 2004; 55(4): 428-33.

503 23. Claustre Y, Benavides J, Scatton B. Potential mechanisms involved in the negative coupling between

504 serotonin 5-HT1A receptors and carbachol-stimulated phosphoinositide turnover in the rat hippocampus. J

505 Neurochem. 1991; 56(4): 1276-85.

506 24. Ishige T, Okamura HO, Kitamura K. Immunohistochemical localization of phospholipase A2 in the

507 guinea pig nasal mucosa. J Med Dent Sci. 2008; 55(1): 29-32.

508 25. Negro-Vilar A, Conte D, Valenca M. Transmembrane signals mediating neural peptide secretion: role

509 of protein kinase C activators and arachidonic acid metabolites in luteinizing hormone-releasing hormone

510 secretion. Endocrinology. 1986; 119(6): 2796-802.

511 26. Haase A, Bicker G. Nitric oxide and cyclic nucleotides are regulators of neuronal migration in an

512 insect embryo. Development. 2003; 130(17): 3977-87.

513 27. Kumada T, Lakshmana MK, Komuro H. Reversal of neuronal migration in a mouse model of fetal

514 alcohol syndrome by controlling second-messenger signalings. J Neurosci. 2006; 26(3): 742-56.

515 28. Li S, Pelletier G. Involvement of serotonin in the regulation of GnRH gene expression in the male rat

516 brain. Neuropeptides. 1995; 29(1): 21-5.

517 29. Héry M, François-Bellan AM, Héry F, Deprez P, Becquet D. Serotonin directly stimulates luteinizing

518 hormone-releasing hormone release from GT1 cells via 5-HT7 receptors. Endocrine. 1997; 7(2): 261-5.

519 30. Jennes L, Beckman WC, Stumpf WE, Grzanna R. Anatomical relationships of serotoninergic and

520 noradrenalinergic projections with the GnRH system in septum and hypothalamus. Exp Brain Res. 1982;

521 46(3): 331-8.

522 31. Kiss J, Halász B. Demonstration of serotoninergic axons terminating on luteinizing hormone-releasing

523 hormone neurons in the preoptic area of the rat using a combination of immunocytochemistry and high

524 resolution autoradiography. Neuroscience. 1985; 14(1): 69-78.

525

526

527