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

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Abstract

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

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

Materials and Methods

Chick embryos

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

RNA extraction from chick olfactory placodes and microarray analysis

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

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

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

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

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

Whole mount *in situ* hybridisation

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

Injection of chick embryos and immunohistochemistry

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

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

Chick HTR1A miR RNAi was designed according to the software at Invitrogen and cloned into the BLOCK –iT^{TM} Pol II miR RNAi Expression Vector (Invitrogen). Double stranded oligonucleotides were generated using the forward primer 5' -

CCTGTTCGCTTGTTGATAGTCGAGTCAGTCAGTGGCCAAAACTCGACTATGTCAACAAGCGA AC - 3' and reverse primer 5' - TGCTGTTCGCTTGTTGACATAGTCGAGTTTTGGCCACTGACTGACTCGACTATCAACAAGCGA A - 3', cloned and sequenced. The pcDNA^{TM}6.2-GW/± EmGFP-miR-negative control plasmid used contained an insert that can form a mature miRNA but is predicted not to target any known vertebrate gene. The sequence of the insert used was 5'-GAAATGTACTGCGCGTGGAGACGTTTTGGCCACTGACTGACGTCTCCACGCAGTACATTT-3'.

Electroporation (10) was used to introduce chick HTR1A miR RNAi along with chick ß-actin RFP or chick ß-actin eGFP or pcDNA^{TM}6.2-GW/± EmGFP-miR-negative control plasmid together with chick ß-actin RFP or chick ß-actin eGFP *in ovo* into olfactory placodes at stages 16 (HH16) using 5×20 millisecond pulses of 10 volts. DNA samples were used at a concentration of 1.5 – 2ug mixed with trace amounts of Fast Green. Again, at least three dozen embryos were analysed for each construct.The embryos were then incubated for a further 48 hours prior to fixation in either 4% (w/v) PFA for *in situ* hybridisation or in 4% (w/v) EDC for GnRH immunohistochemistry. Embryos that had undergone *in situ* hybridisation were stained with anti-GFP antibodies (Abcam) (1:200), detected with goat anti-rabbit 488 (Molecular Probes) (1:1000). Embryos were then embedded in gelatin, and sectioned.

Results

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

In embryos electroporated with pcDNA^{TM}6.2-GW/± EmGFP-miR-negative control plasmid, the GnRH positive population were also analysed at HH24 (Figure 4E and E'). In these embryos, RFP+ve and GnRH+ve cells were found at the four distinct locations, olfactory placode, migratory stream, base of the forebrain and inside the forebrain. Electroporated GnRH neurons were analysed based on the dual expression of RFP and GnRH in all cases.

Olfactory sensory neuronal development is not affected by HTR1A knockdown

Embryos electroporated with both HTR1A miR RNAi and pcDNA^{TM}6.2-GW/± EmGFP-miR-negative control plasmid were subsequently analysed using antibodies to neurofilament to assess if there were any consequence for the formation of the olfactory nerve following electroporation. This was performed to make sure that the failure of GnRH positive neurons to enter into the forebrain was intrinsic to the GnRH neurons and not because of a secondary failure as a result of olfactory sensory axonal pertubations. Embryos were electroporated at HH16 and harvested at stage 28 (HH28) and immunostained with antibodies to neurofilament. Olfactory sensory neurons stain with antibodies to neurofilament and their axonal bundles can be seen projecting out from the olfactory placode and towards the forebrain in untreated embryos (Figure 5A). When the electroporated embryos were stained with antibodies to neurofilament and analysed, with both the constructs, HTR1A miR RNAi (Figure 5B and 5B') and pcDNA^{TM}6.2-GW/± EmGFP-miR-negative control plasmid (Figure 5C and 5C'), the normal pattern of

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

Discussion

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

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

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

The identification of HTR1A as a player in GnRH migration is also significant as it suggest that molecules acting downstream of this receptor and mediating its function may also be important for this process. With regards to this point, it is known that phospholipase A_2 (PLA_2) is activated downstream of HTR1A (23), and significantly we found that this gene was also upregulated in the transcriptome analysis (by approximately 30 fold between HH16 and HH20). Furthermore, binding of GnRH to the GnRH receptor, stimulates the release of phosphoinositide (IP3) which along with diacylglycerol (DAG) is required for intracellular calcium mobilisation and subsequent activation of PKC, phospholipase D (PLD) and the activation of PLA_2 (24; 25). Thus, it is possible that both serotonin and GnRH signalling converge on PLA_2.

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

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

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

Figure legends

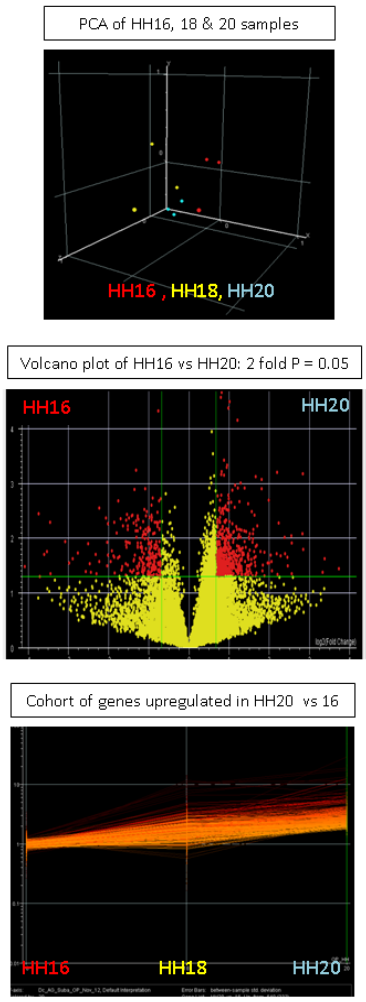


Figure 1

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.

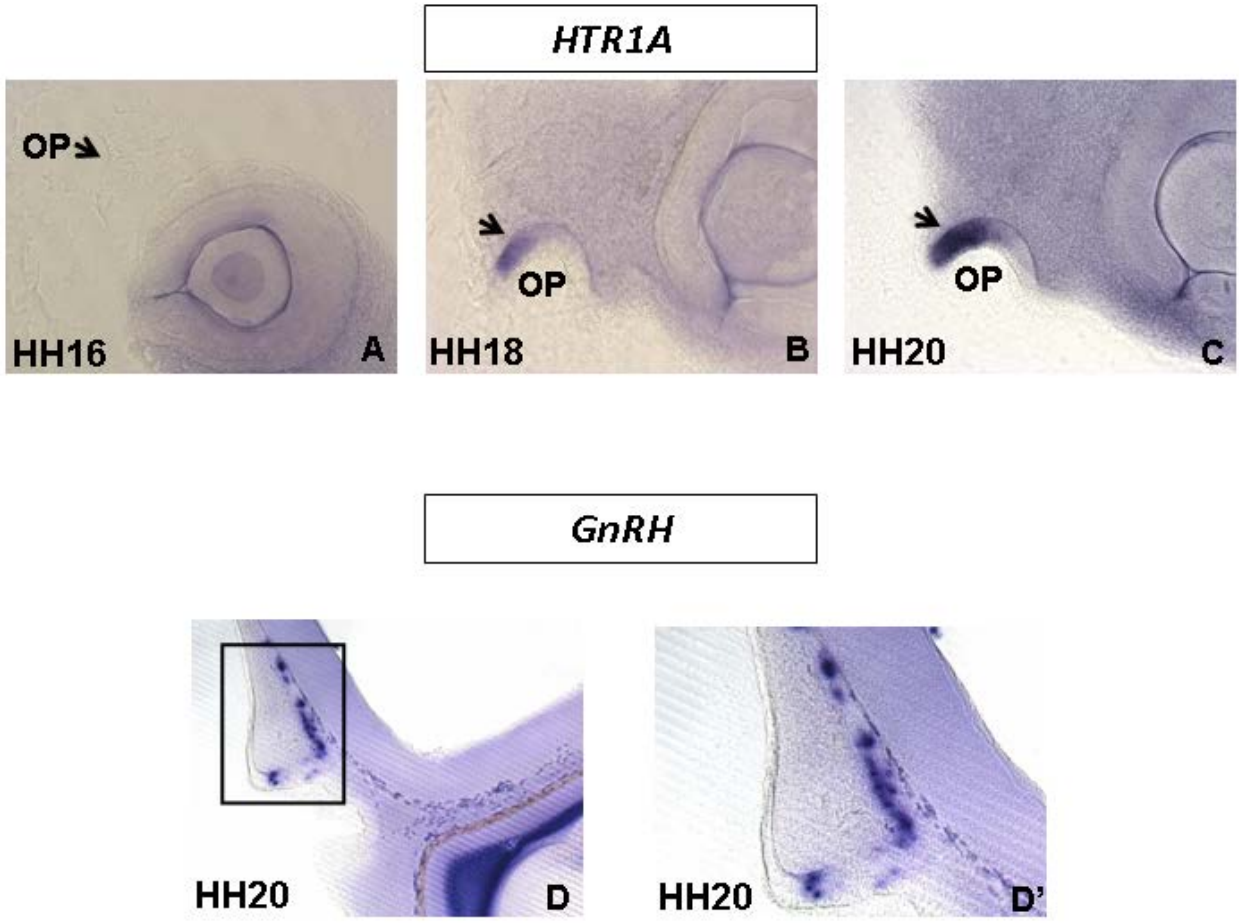


Figure 2

***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.

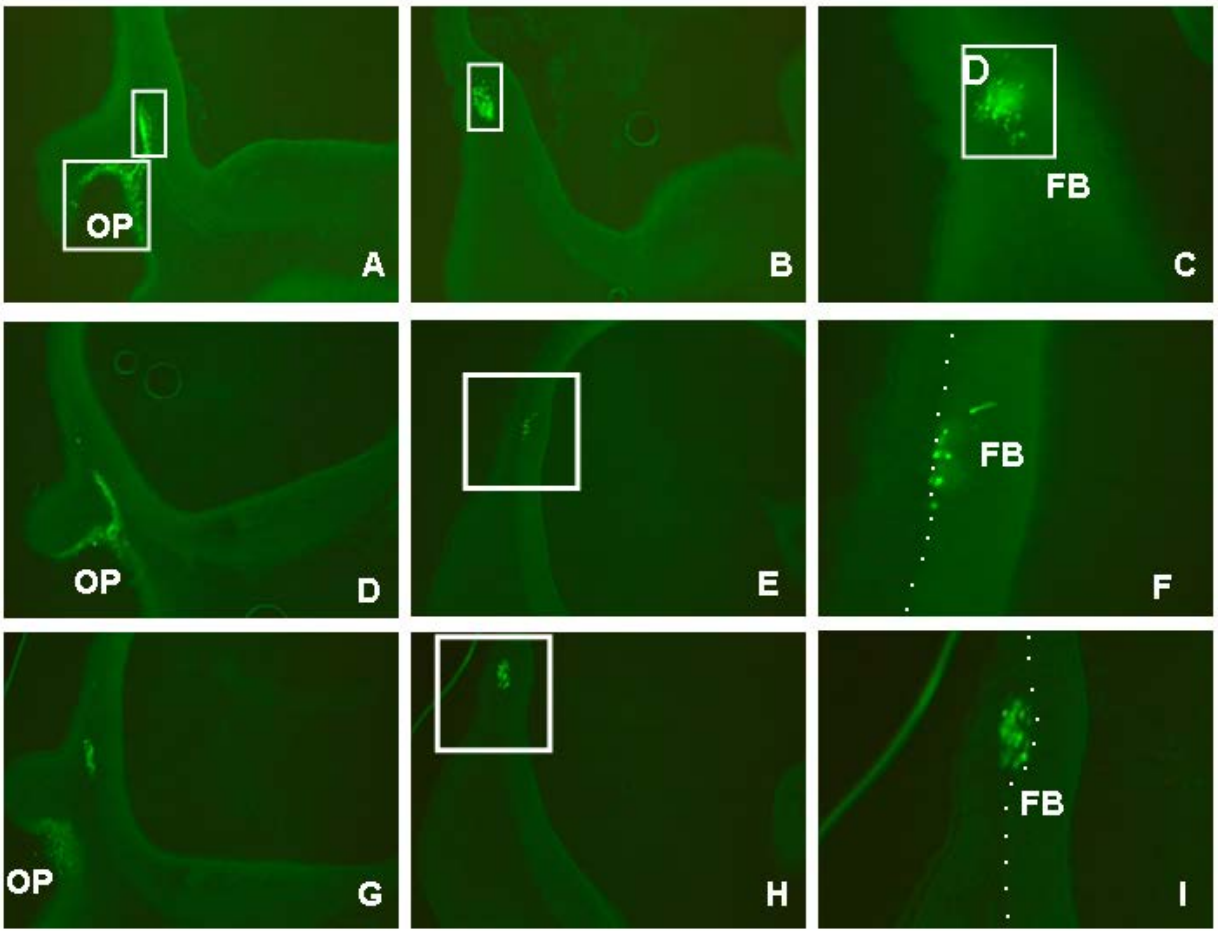
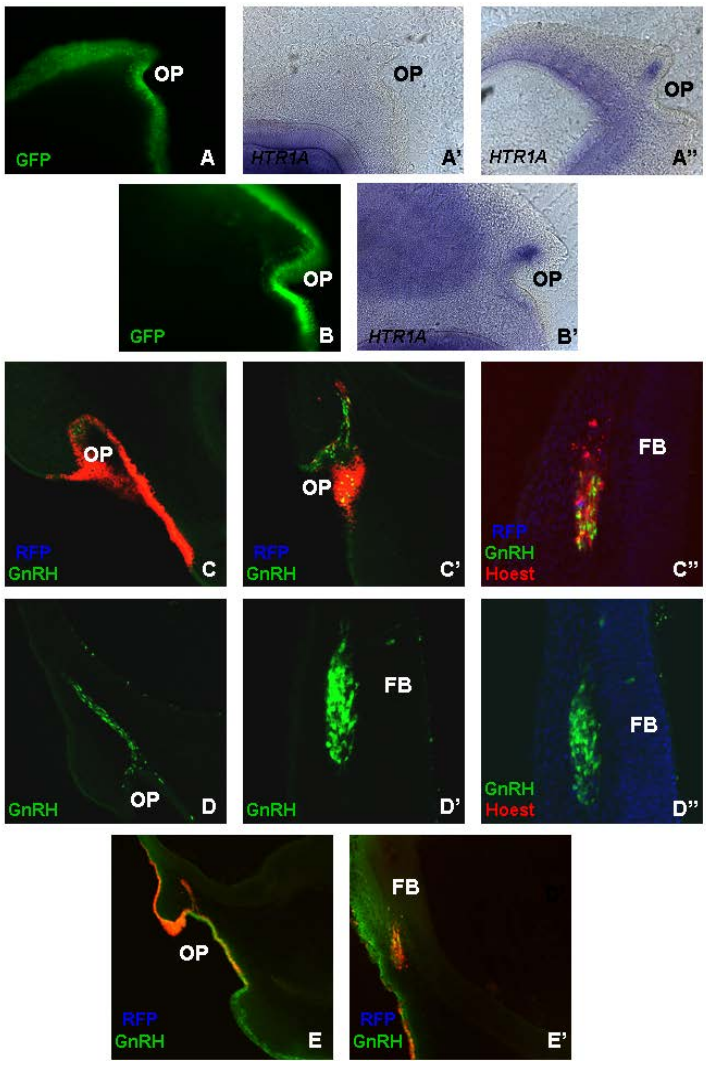


Figure 3

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.



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 unelectroported 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

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

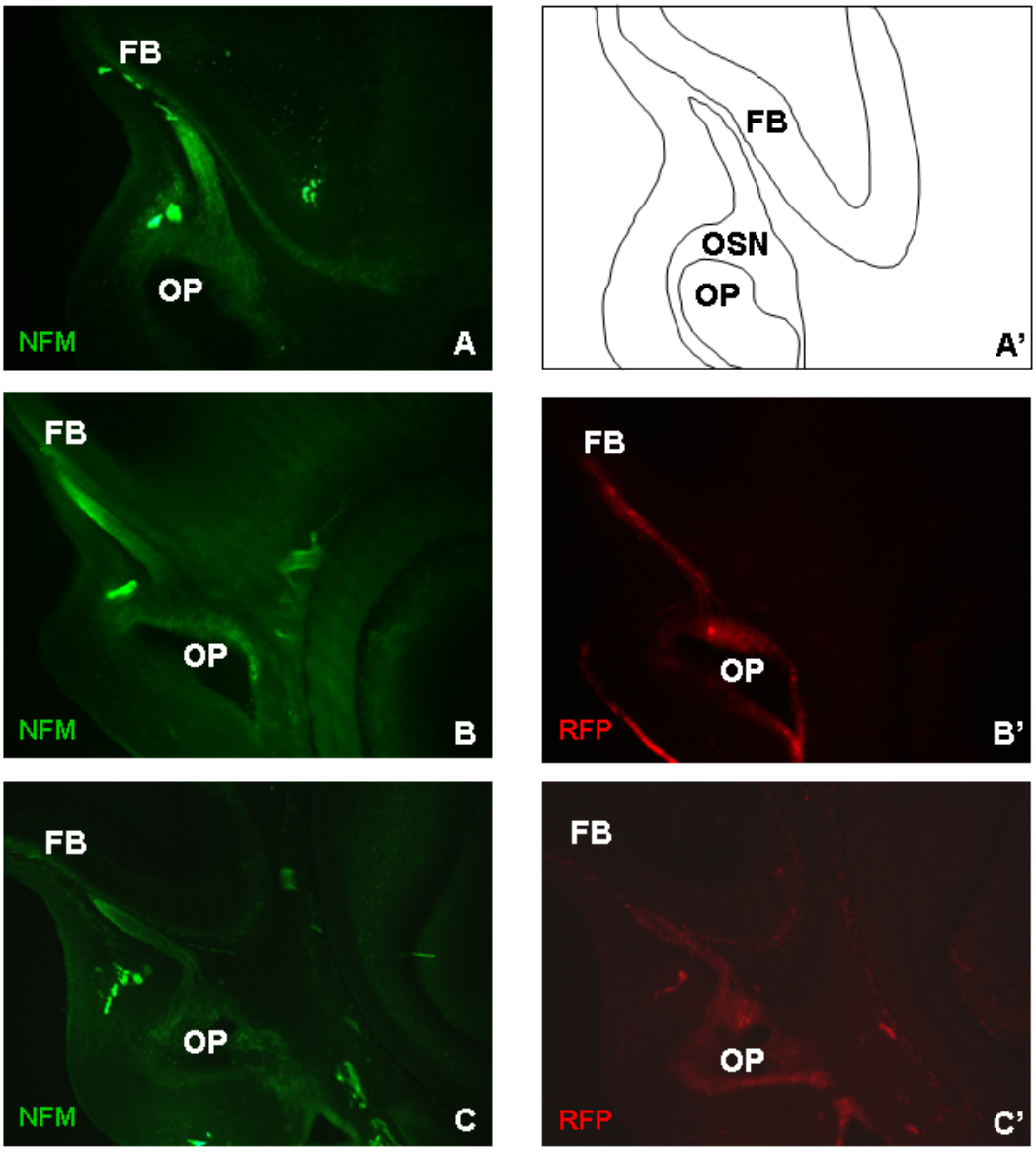


Figure 5

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).

Abbreviations

CNS	central nervous system
EDC	N-Ethyl-N′-(3-dimethylaminopropyl)carbodiimide
FGF	fibroblast growth factor
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GnRH	gonadotrophin releasing hormone
HH	hypogonadotrophic hypogonadism
5-HT	5-hydroxytrytamine
HTR1A	serotonin receptor 1A

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Disclosure Summary

The authors have nothing to disclose.

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