Reproductive dysfunction and decreased GnRH neurogenesis in a mouse model of CHARGE syndrome

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Received January 30, 2011; Revised and Accepted May 11, 2011

CHARGE is a multiple congenital anomaly disorder and a common cause of pubertal defects, olfactory dysfunction, growth delays, deaf-blindness, balance disorders and congenital heart malformations. Mutations in CHD7, the gene encoding chromodomain helicase DNA binding protein 7, are present in 60–80% of individuals with the CHARGE syndrome. Mutations in CHD7 have also been reported in the Kallmann syndrome (olfactory dysfunction, delayed puberty and hypogonadotropic hypogonadism). CHD7 is a positive regulator of neural stem cell proliferation and olfactory sensory neuron formation in the olfactory epithelium, suggesting that the loss of CHD7 might also disrupt development of other neural populations. Here we report that female Chd7Gt/+ mice have delays in vaginal opening and estrus onset, and erratic estrus cycles. Chd7Gt/+ mice also have decreased circulating levels of luteinizing hormone and follicle-stimulating hormone but apparently normal responsiveness to gonadotropin-releasing hormone (GnRH) agonist and antagonist treatment. GnRH neurons in the adult Chd7Gt/+ hypothalamus and embryonic nasal region are diminished, and there is decreased cellular proliferation in the embryonic olfactory placode. Expression levels of GnRH1 and Otx2 in the hypothalamus and Gnrhr in the pituitary are significantly reduced in adult Chd7Gt/+ mice. Additionally, Chd7 mutant embryos have CHD7 dosage-dependent reductions in expression levels of Fgfr1, Bmp4 and Otx2 in the olfactory placode. Together, these data suggest that CHD7 has critical roles in the development and maintenance of GnRH neurons for regulating puberty and reproduction.

INTRODUCTION

In humans, heterozygous mutations in chromodomain helicase DNA binding protein 7 (CHD7) cause CHARGE syndrome, a clinically variable, multiple congenital anomaly condition with an estimated incidence of 1:8500–1:12 000 in newborns (1–3). CHARGE is characterized by ocular coloboma, heart defects, atresia of the choanae, retarded growth and development, genital hypoplasia and ear abnormalities including deafness and vestibular disorders (4). Genital hypoplasia occurs in ~62% of CHARGE individuals with confirmed CHD7 mutations, and gonadotropins [luteinizing hormone (LH) and follicle-stimulating hormone (FSH)] are deficient in 81% of males and 93% of females (5–12).

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The gonadotropic hormones LH and FSH are generated and secreted from the pituitary in response to gonadotropin-releasing hormone (GnRH) from the median eminence (13–16). GnRH production is dependent upon multiple signaling mechanisms, including sex-steroid feed-back regulation, kisspeptin-GPR54 signaling and leptin signaling (14,15,17). Transcription of GnRH1 requires the paired-like homeodomain transcription factor OTX2 (18–20). OTX2 is also required for neurogenesis in multiple tissues (21,22). GnRH neurogenesis is partially regulated by fibroblast growth factor (FGF) signaling, and mutations in FGF8 and FGFR1 cause hypogonadotropic hypogonadism and olfactory dysfunction in humans and mice (23–28).

Mice with the heterozygous loss of Chd7 (Chd7Gt/+ ) have olfactory defects (29) similar to those reported in CHARGE
RESULTS

Analysis of puberty and estrus cycles

Endocrine defects including delayed puberty, hypogonadotropic hypogonadism and genital hypoplasia have been reported in CHARGE individuals (5–11). Prior studies of the reproductive system in Chd7Whi/+ mice, a model of the CHARGE syndrome, reported an increased time to first litter and hypoplasia of the testes, clitoris and uterus compared with wild-type mice (30). Our data cannot completely rule out a more subtle defect in metabolism or nutrition that may influence reproduction in Chd7Gt/+ mice.

Chd7Gt/+ mice have decreased levels of circulating gonadotropic hormones

CHARGE individuals are reported to have hypogonadotropic hypogonadism and variable response to GnRH stimulation (5–11). To test the integrity of the hypothalamic-pituitary axis, we assayed circulating levels of LH and FSH in 3–4-month-old wild-type (males n = 4, females n = 5) and Chd7Gt/+ (males n = 5, females n = 6) sex-matched littermate mice (Fig. 2A and B). Circulating levels of female gonadotropic hormones fluctuate dependent upon stage of estrus (57); therefore, we collected serum from all females at late estrus. We found that Chd7Gt/+ female mice have reduced levels of circulating LH (74% reduction) and FSH (83% reduction) in comparison to wild-type littermates (Fig. 2A and B). Chd7Gt/+ male mice also have reduced levels of circulating LH (86% reduction), whereas levels of FSH are normal (Fig. 2A and B). Interestingly, studies of Fshβ−/− mice reported that FSH is not required for male fertility but is required for female fertility (58,59). In contrast, Fshβ mutations in human males cause azoospermia and infertility (60,61). Fshβ is also expressed independent of GnRH signaling in an activin-dependent pathway (62,63), and LH and FSH are differentially regulated by GnRH during development (64).

Chd7Gt/+ mice respond normally to GnRH agonist stimulation

Hypogonadotropic hypogonadism can be caused by defects in the hypothalamus, the pituitary or both. Mutations in the
GnRH receptor gene, GnRHR, cause pituitary gonadotrope insensitivity to GnRH stimulation and subsequent hypogonadotropic hypogonadism (65,66), whereas mutations in genes such as GPR54 or GnRH1 affect GnRH neuronal function at the level of the hypothalamus (67,68). Mutations in genes affecting GnRH neuronal function could therefore mask a normal ability of pituitary gonadotropes to respond to GnRH. To test this, we administered the GnRH agonist, leuprolide, to 3–4-month-old wild-type (males \( n = 4 \), females \( n = 3 \)) and Chd7Gt/+/ females (males \( n = 4 \), females \( n = 4 \)) sex-matched, late estrus-matched (females) littermate mice. In mice, leuprolide causes a rapid (between 70 and 180 min) increase in production and circulation of LH and FSH (69,70). Wild-type and Chd7Gt/+/ mice had similar circulating levels of LH and FSH measured 2 h following leuprolide administration (Fig. 2C and D). These observations are consistent with normal pituitary gonadotrope responsiveness to GnRH agonist in Chd7Gt/+/ mice.

Figure 1. Chd7Gt/+ females have delayed puberty and erratic estrus cycles. (A) Wild-type and Chd7Gt/+/ female littermates were examined for vaginal opening and first estrus. (B) In wild-type females, estrus cyclicity is obtained at postnatal day 39, 9 days after first estrus. (C and D) Chd7Gt/+/ female littermates never achieve cyclicity and have erratic estrus cycles. (E) Pubertal markers [vaginal opening (VO), first estrus and cyclicity] in relation to body size are depicted for wild-type and Chd7Gt/+/ females. (F) Circulating levels of insulin and leptin are similar in wild-type and Chd7Gt/+/ female littermate mice. (G and H) Circulating levels of GH or IGF1 are similar in wild-type and Chd7Gt/+/ female littermate mice. ∗\( P < 0.05 \), ∗∗\( P < 0.01 \) by unpaired Student’s \( t \)-test. NS, not significant.
Male Chd7GT/+ mice have normal baseline circulating levels of FSH (Fig. 2B). To determine whether pituitary gonadotropes in Chd7GT/+ males constitutively produce FSH independent of GnRH signal, we administered the GnRH antagonist antide to 3-month-old male wild-type (n = 4) and Chd7GT/+ (n = 4) littermate mice. In mice, antide causes a rapid (2 h) decrease in the production and circulation of LH and FSH (71, 72). Wild-type and Chd7GT/+ mice had no difference in circulating levels of FSH or LH 2 h following antide administration (Fig. 2E and F). Additionally, no abnormalities in pituitary histology were found by hematoxylin and eosin staining of sections from E10.5, E14.5, E18.5 and adult Chd7GT/+ mice (data not shown; 30).

**GnRH neurons are decreased in Chd7-deficient mice**

Reduced levels of circulating LH and FSH could reflect defects in GnRH neurons in the hypothalamus, as a result of reductions in GnRH neuronal number, defective migration to the hypothalamus and/or aberrant hormone production and/or release (14). To measure GnRH neurons in Chd7GT/+ mice, immunofluorescence was performed with anti-GnRH antibody on hypothalamic sections from 3–4-month-old wild-type (males n = 4, females n = 4) and Chd7GT/+ (males n = 4, females n = 4) sex-matched, late estrus-matched (females) littermates. A visually apparent decrease in GnRH immunofluorescence was found in the median eminence in both male and female Chd7GT/+ mice compared with wild-type littermates (Fig. 3A and B). In contrast, there was no difference between wild-type and Chd7GT/+ mice in immunofluorescence of arginine vasopressin (AVP)-positive fibers in the median eminence (Fig. 3C and D), providing evidence against a general defect in neural development or maintenance. Quantitation of anti-GnRH immunofluorescence in the median eminence using ImageJ software showed a 54% reduction in GnRH neurons in Chd7GT/+ females and a 51% reduction in Chd7GT/+ males compared with wild-type littermates (Fig. 3K).

Reduced GnRH in the median eminence could be a result of fewer GnRH neurons in the hypothalamus or defects in GnRH

Figure 2. Circulating LH and FSH are decreased in Chd7GT/+ mice. (A) Chd7GT/+ mice analyzed for circulating LH have decreased serum levels compared with wild-type littermates. The LH reportable range was 0.04–37.4 ng/ml. (B) Chd7GT/+ females have decreased levels of circulating FSH but Chd7GT/+ males have normal serum levels. The FSH reportable range was 2.3–20.0 ng/ml. (C and D) GnRH agonist, leuprolide, administered to wild-type and Chd7GT/+ mice caused similar responses in the production and circulation of LH and FSH. The reportable range for LH was 0.04–37.4 ng/ml and for FSH was 6.7–75.0 ng/ml. (E and F) Wild-type and Chd7GT/+ males were administered GnRH antagonist, antide. Wild-type and Chd7GT/+ males have similar decreases in circulating levels of LH and FSH. The reportable range for LH was 0.04–37.4 ng/ml and for FSH was 6.7–75.0 ng/ml. ∗∗P < 0.01, ∗∗∗P < 0.001 by unpaired Student’s t-test. NS, not significant.
neuronal function. Adult GnRH neurons have a rostral–caudal distribution that spans from the nasal region to the hypothalamus, but the vast majority of GnRH neurons reside in specific regions of the hypothalamus (73). To quantify GnRH neurons in the hypothalamus, immunofluorescence was performed with anti-GnRH antibody on hypothalamic sections from 3–4-month-old wild-type (males $n = 4$, females $n = 4$) and Chd7Gt/+ sex-matched (males $n = 4$, females $n = 4$), late estrus-matched (for females) littermates. GnRH neuronal cell counts taken from the hypothalamus including the medial septal nucleus (MSN) (Fig. 3E and F) and preoptic area (POA) (Fig. 3G and H) showed a significant (35%) reduction in the number of GnRH-positive cell bodies in the hypothalamus of Chd7Gt/+ females and Chd7Gt/+ males compared with wild-type littermates (Fig. 3L). The average total number of GnRH-positive neurons counted in the hypothalamus is: (for females) wild-type $= 487$ (standard error $= 26$), Chd7Gt/+ $= 348$ (standard error $= 21$; $P < 0.001$); (for males) wild-type $= 473$ (standard error $= 24$), Chd7Gt/+ $= 361$ (standard error $= 18$; $P < 0.001$). To determine whether GnRH neurons exhibit migration abnormalities in Chd7Gt/+ mice, immunofluorescence was performed with anti-GnRH antibody on both coronal and sagittal sections from 3–4-month-old wild-type (males $n = 4$, females $n = 4$) and Chd7Gt/+ (males $n = 4$, females $n = 4$) sex-matched, late estrus-matched (females) littermates. GnRH neurons were distributed similarly throughout the rostral–caudal axis from the nasal region to the cerebellum in wild-type and Chd7Gt/+ mice. Thus, we did not identify a major defect in GnRH neuronal migration in Chd7Gt/+ mice.

Chd7 is expressed in immortalized GnRH neuronal cell lines (8), but Chd7 expression in GnRH neurons in vivo had not been previously examined. We analyzed adult Chd7Gt/+ hypothalamic sections using anti-β-galactosidase (β-gal) and anti-GnRH showed that most GnRH-positive neurons in the hypothalamus are also positive for the β-galactosidase reporter for Chd7 expression. White box in (I) indicates the co-labeled neuron magnified in (J). (K) Quantitation of anti-GnRH immunofluorescence in the median eminence using ImageJ software showed significantly decreased GnRH in Chd7Gt/+ mice compared with the wild-type. (L) Quantitation of GnRH neurons per hypothalamus showed decreased numbers of GnRH neurons in both male and female Chd7Gt/+ mice compared with sex-matched wild-type littermates. Sections are in the coronal plane. **$P < 0.01$, ***$P < 0.001$ by unpaired Student’s t-test. 3v, third ventricle.
studied (74). In mice, GnRH neurons arise from the E9.5–11.5 olfactory placode and then migrate along olfactory tracts leading to the forebrain (32). GnRH neurons reside in the nasal region until E13.5, at which time most GnRH neurons have migrated into the forebrain; GnRH neurons continue to migrate until E16.5 when they reach the hypothalamus and have assumed their adult-like distribution (32).

Hypogonadotropic hypogonadism is often associated with abnormalities in GnRH neurons, and can be caused by defects in neurogenesis, migration or survival (75). Mouse models with mutations affecting genes such as Pkr2, EphA5 and Nhlh2 are reported to have defects in GnRH neuronal migration and/or survival (76–78), while mutations in Fgf8 and Fgfr1 cause reduced neurogenesis (26–28). To determine whether GnRH neuronal numbers are altered in Chd7Gt/+ embryos, GnRH-positive cells were counted in wild-type (n = 4 each time point) and Chd7Gt/+ (n = 4 each time point) littermate embryos at E11.5 and E12.5. We found a 30% reduction in the number of GnRH neurons in the nasal region of Chd7Gt/+ embryos at both E11.5 and E12.5 (Fig. 4). The average total number of GnRH-positive neurons counted in the embryonic nasal region is: (for E11.5) wild-type = 793 (standard error = 42), Chd7Gt/+ = 565 (standard error = 19; P < 0.001); (for E12.5) wild-type = 882 (standard error = 40), Chd7Gt/+ = 622 (standard error = 32; P < 0.001). Decreased GnRH neurons in Chd7Gt/+ embryos are consistent with fewer GnRH neurons in the hypothalamus of adult Chd7Gt/+ mice.

Chd7Gt/+ embryos have defects in cellular proliferation in the olfactory placode

Chd7 is highly expressed in the olfactory placode by E10.5 (30), and continues to be expressed in olfactory tissues throughout development and into adulthood (29). To test cellular proliferation in the olfactory placode, immunofluorescence was performed with anti-CHD7 and the proliferation marker anti-phospho-histone H3 in wild-type (n = 4, Chd7Gv/+ (n = 4) and Chd7Gt/+ (n = 3) littermate embryos at E10.5 (Fig. 5A–I). A 49% reduction in phospho-histone H3-positive cells was observed in E10.5 Chd7Gt/+ embryos compared with the wild-type (Fig. 5A–F and J). The vast majority (97%) of phospho-histone H3-positive cells in the olfactory placode were CHD7 positive, similar to previously published results showing CHD7 in 98% of proliferating cells in the adult olfactory epithelium (29). In Chd7Gv/Gv embryos, we found a 90% reduction in H3-positive cells compared with the wild-type, and an 80% reduction in H3-positive cells compared with Chd7Gv/+ embryos (Fig. 5A–J). The olfactory placode was also reduced in size in Chd7Gv/Gv embryos but appeared unchanged in Chd7Gv/+ compared with the wild-type. Together, these data suggest that Chd7 deficiency negatively impacts cellular proliferation in the developing olfactory placode, consistent with fewer GnRH neurons available for migration into the hypothalamus and with our earlier observation that there are fewer olfactory sensory neurons in the adult Chd7Gv/+ olfactory epithelium (29).

Decreased numbers of adult GnRH neurons could also result from defects in cell survival (78). To test this, wild-type (n = 4 each time point) and Chd7Gv/+ (n = 4 each time point) littermate embryos at E10.5, E11.5 and E12.5 were analyzed for apoptosis by the TUNEL assay. Chd7Gv/+ embryos have significantly fewer TUNEL-positive cells in the nasal region compared with wild-type embryos at all time points analyzed [E10.5 (21% reduction), E11.5 (11% reduction) and E12.5 (13% reduction)] (Fig. 5K). The reduction in TUNEL-positive cells at all three time points may reflect fewer cells in the nasal region of Chd7Gv/+ embryos, or decreased susceptibility to apoptosis. In E10.5 Chd7Gv/+ embryos, the 49% reduction in H3-positive cells in the olfactory placode is greater than the 21% reduction in E10.5 TUNEL-positive cells, consistent with a net loss of GnRH precursors. Wild-type and Chd7Gv/+ embryos at E10.5 appear to have normal invagination of the olfactory placode to form the olfactory pit (Fig. 5A–F). However, Chd7Gv/Gv embryos appear to lack olfactory pits, suggesting that invagination of the olfactory placode is disrupted (Fig. 5G–I). Altogether, our data suggest that defects in GnRH neurogenesis (rather than increased cell death) lead
to reduced GnRH neurons in embryonic and adult Chd7Gt/Gt mice.

Reduced CHD7 dosage alters gene expression in olfactory placode, pituitary and hypothalamus

CHD7 may regulate neural progenitors by directly influencing the expression of morphogens such as the bone morphogenetic proteins (BMPs) and FGFs. CHD7 may also regulate the expression and/or function of transcription factors involved in neurogenesis. Chd7 is highly expressed throughout the entire olfactory placode, whereas the expression of morphogens is often regionally restricted within the olfactory placode (23,79). To determine whether CHD7 regulates the expression of genes required for normal adult hypothalamic-pituitary signaling, we analyzed the expression of GnRH1, Otx2, Kiss1 and Gnrhr using TaqMan gene expression assays. We analyzed RNA isolated from the hypothalamus and pituitary of 3–4-month-old wild-type (n = 4) and Chd7Gt/Gt (n = 4) littermate mice. We found decreased expression of Gnrhr (fold change: −2.34 ± 0.22) in Chd7Gt/Gt pituitary, consistent with decreased GnRH signal (80–82) rather than a direct effect of reduced Chd7 dosage. We also found decreased expression of Gnrh1 (fold change: −3.61 ± 0.63) and Otx2 (fold change: −4.09 ± 0.43) in Chd7Gt/Gt hypothalamus compared with wild-type littermates (Fig. 6B). There was no significant difference in expression of Kiss1 in the Chd7Gt/Gt hypothalamus compared with wild-type littermates (Fig. 6B).

DISCUSSION

We report here that Chd7Gt/Gt mice have delayed puberty, erratic estrus cycles, decreased levels of circulating gonadotropins and reduced GnRH neurons in the hypothalamus. Chd7Gt/Gt embryos also have fewer GnRH neurons and reduced cellular proliferation in the olfactory placode. Additionally, reduced CHD7 dosage impacts expression of genes involved in proliferation and/or neurogenesis (Fgf8, Bmp4 and Otx2) in the olfactory placode and GnRH signaling (Otx2 and Gnrh1) in the adult hypothalamus. Together, our data suggest that defects in neural progenitor proliferation in the developing olfactory epithelium may underlie the Kallmann-like features found in Chd7Gt/Gt mice and in

Figure 5. Chd7Gt/+ embryos have reduced cellular proliferation in the olfactory placode. (A–F) Immunofluorescence of the wild-type and Chd7Gt/+ at E10.5 using antibodies against CHD7 (red) and phospho-histone H3 (H3) (green). (G–I) Immunofluorescence of Chd7Gt/Gt embryos at E10.5 using antibodies against CHD7 (red) and phospho-histone H3 (H3) (green) counterstained with DAPI (blue). (J) Cell counts showed decreased cellular proliferation in the olfactory placode in Chd7Gt/+ and Chd7Gt/Gt embryos compared with wild-type littermates. (K) Cell counts showed a decrease in TUNEL-positive cells at E10.5, E11.5 and E12.5 in Chd7Gt/+ embryos compared with the wild-type. Sections are in the transverse plane. **P < 0.01, ***P < 0.001 by ANOVA (proliferation) and unpaired Student’s t-test (TUNEL).
CHD7 mutation-positive humans with the CHARGE syndrome. These observations also provide evidence that CHD7 positively regulates cellular proliferation potentially by either directly or indirectly regulating transcription of Fgfr1, Bmp4 and Otx2. Combined with previous published results in the olfactory system (29) and the developing inner ear (83), our data imply a broad role for CHD7 in promoting neurogenesis in multiple tissues.

Although no binding site consensus sequences have been identified for CHD7, we compared our gene expression results to CHD7 ChIP-seq data (derived from embryonic stem cells) deposited in GEO by Schnetz et al. (84). We found CHD7-binding sites at the medium threshold for Otx2 (one site inside the gene and one upstream close to the transcriptional start site), Bmp4 (three sites downstream of the gene) and Fgfr1 (five sites in the second intron and several sites both upstream and downstream of the gene). We also found a potential CHD7-binding site for Fgf8 in the adjacent gene Mgea5, which could contain an enhancer region for Fgf8. There were no CHD7-binding sites at the low, medium or high thresholds for GnRH1, GnRHHR or Kiss1. It is important to note that comparison of our data to that of prior ChIP-seq experiments is limited due to differences in cell types analyzed. Moreover, our results do not provide information on whether changes in gene expression result from direct or indirect effects of CHD7.

Roles for CHD7 in regulation of gene expression during tissue development and maintenance are emerging for multiple organs. In a study of neural crest formation in Xenopus, CHD7 was implicated to regulate multipotent neural crest-like cells by binding to PBAF components, including BRG1, BAF170, BAF155, BAF57, PB1, ARID2 and BRD7 (85). In mesenchymal stem cells, CHD7 regulates cell fate specification during osteoblast and adipocyte differentiation (86). CHD7 forms a complex with NLK, SETDB1 and PPAR-γ, and binds to methylated lysine 4 and lysine 9 residues on histone H3 at PPAR-γ target promoters, which suppresses ligand-induced transactivation of PPAR-γ target genes (86). Additionally, the CHD7 Drosophila orthologue, Kismet, is involved in transcriptional elongation by RNA polymerase II through recruitment of ASH1 and TRX, and may help maintain stem cell pluripotency by regulating methylation of histone H3 lysine 27 (87). These data provide evidence that the role of CHD7 in tissue development and maintenance is dependent upon coordination of multiple components. Our results showing that CHD7 affects GnRH neurogenesis and signaling, potentially by influencing transcriptional regulation of key target genes, add to this emerging evidence.

Previous reports of reproductive phenotypes in Chd7 haploinsufficient mice using the Chd7Whi allele (which contains a nonsense mutation) (39) showed that Chd7Whi/+ mice have genital hypoplasia, hypogonadism and increased time to first litter (38). Similar to Chd7Whi/+ mice, Chd7Whi/+ mice were also reported to have reduced GnRH neurons in the adult hypothalamus (38). However, the mechanism underlying decreased GnRH neurons in Chd7Whi/+ mice was not evaluated. Our data provide a potential cellular mechanism for the reduced gonadal size and decreased fertility associated with CHD7 haploinsufficiency. Compared with wild-type littersmates, Chd7Whi/+ mice have reduced capacity for GnRH signal to pituitary gonadotropes, leading to less LH and FSH available to act on the gonads for sex-steroiod production. Reductions in circulating LH and FSH negatively affect both gonad size and fertility (88). Although our data do not exclude the possibility of a subtle defect in GnRH migration, reduced GnRH neurons in Chd7Whi/+ mice are explained at least in part by defects in GnRH neurogenesis during development. Together, these data support our model for an underlying intrinsic cellular mechanism for hypogonadotropic hypogonadism in Chd7 haploinsufficient mice.

Neural progenitors must be tightly regulated during development by factors that are likely to be temporally and spatially restricted (89). The neurogenic potential of progenitors is influenced by both BMP and FGF signaling in the central and peripheral nervous systems (90–94). The opposing effects of FGF and BMP signaling must be tightly regulated during olfactory development to obtain the proper proportions of olfactory sensory and olfactory respiratory cell types; notably, reductions in either FGF8 or BMP4 can cause decreased cellular proliferation in the olfactory placode (95). In addition, BMP4 and BMP7 have dosage- and threshold-dependent effects on neurogenesis in the olfactory epithelium (90–92,96,97). Although BMP activity is generally thought to inhibit neurogenesis, low doses of BMP4 are required for promoting neurogenesis in multiple tissues.
including the olfactory placode, hippocampus and subventricular zone (95–100). Neurogenesis in the olfactory epithelium is also regulated by FGFR8 and FGFR1, and mutations in Fgf8 or Fgfr1 in humans and mice cause reduced numbers of olfactory sensory neurons and GnRH neurons (23–28). FGF2 induces neurogenesis and increases cellular proliferation of progenitors in olfactory tissues (96), and FGF2 signals via FGFR1 to induce proliferation of olfactory bulb progenitors in the subventricular zone (101). Our data provide the first evidence that reduced CHD7 dosage results in decreased expression of Fgfr1 and Bmp4, and that CHD7 is required for proper cellular proliferation and genesis of GnRH neurons during development.

Proper CHD7 dosage is critical for development in humans and mice (30,31,39,102). However, CHD7 function in adult tissues and post-mitotic cells has not been fully explored. Although adult Chd7 expression in the brain is mostly restricted to proliferative regions, cells expressing Chd7 are scattered throughout the adult mouse hypothalamus [data available on GENSAT website (http://www.gensat.org/index.html)]. CHD7 is also expressed in GnRH neurons in vivo (Fig. 3I and J) and in GnRH neuronal cell lines (8), but it is unknown whether CHD7 has a functional role in post-mitotic GnRH neurons. Our data provide the first evidence that CHD7 haploinsufficiency in mice results in decreased GnRH1 and Otx2 expression in the adult hypothalamus. These data are consistent with a recent report showing that the loss of CHD7 in the developing mouse inner ear causes decreased expression of Otx2 (83). Interestingly, humans with OTX2 mutations have some clinical features similar to those seen in CHARGE, including pituitary hormone deficiency, short stature and ocular colobomata (103,104). Thus, fewer GnRH neurons in Chd7+/- mice and defects in GnRH signaling may be exacerbated by decreased expression of GnRH1. Together, these results provide evidence that CHD7 controls multiple facets of normal hypothalamic-pituitary signaling, by regulating GnRH neuronal cell number as well as expression of transcription factors and morphogens required for proper GnRH signaling.

MATERIALS AND METHODS

Mice

Chd7+/- mice were generated and characterized as previously described (30). Mice were maintained by backcrossing with 129S1/SvImJ (Jackson Laboratory) mice to generation N6–N8. Mice are housed with a 10/14 h dark/light cycle and fed ad libitum. Serum was collected in the afternoon following paraffin or cryosectioning, sections were processed for immunofluorescence with antibodies against CHD7 (1:1000; Abcam, Cambridge, MA, USA), GnRH (1:150; Abcam (ab5617)), β-galactosidase (1:200; Vector Laboratories, Burlington, CA, USA), AVP (1:500; Abcam) or phospho-histone H3 (1:200; Millipore, Temecula, CA, USA). Secondary antibodies were used at 1:200 and were conjugated with Alexa 488, Alexa 555 or biotin with streptavidin-HRP (Vector Laboratories) and or biotinylated secondary antibodies conjugated with streptavidin-Alexa488 or streptavidin-Alexa555 (Molecular Probes, Eugene, OR, USA and Invitrogen, Carlsbad, CA, USA). Images were captured by single channel fluorescence microscopy on a Leica upright DMRB microscope and processed in Photoshop v.9.0 (San Jose, CA, USA). NIH ImageJ software (Bethesda, MD, USA) was used to analyze anti-GnRH fluorescence intensity over at least 30 images of the median eminence per animal. Fluorescence intensity was measured in restricted areas of the median eminence and background was subtracted to obtain the final measurements. GnRH neuronal cell counts were performed by counting cell bodies only on at least four adults (4 pairs male and 4 pairs female) and four embryos of each genotype at each time point. A minimum of 30 sections per animal were used for all cell counts. Cell counts were analyzed by Student’s t-test using two-tailed unequal variance.

Mouse insulin and leptin ELISA assay

Circulating serum levels of insulin and leptin were assayed by ELISA from 3–4-month-old Chd7+/- and Chd7+/- stage of estrus cycle-matched littermate mice. Insulin was measured using the Ultra Sensitive Mouse Insulin ELISA Kit (Crystal Chem, Downers Grove, IL, USA). Leptin was measured using the Mouse Leptin ELISA Kit (Crystal Chem).
Absorbance (A450) for insulin and leptin was measured and analyzed using a standard curve. Differences in absorbance were analyzed for significance by Student’s t-test using two-tailed unequal variance.

Mouse GH and IGF1 ELISA assay

Circulating levels of GH and IGF1 were assayed by ELISA. GH was measured in serum collected from 3–4-month-old Chd7+/+ and Chd7Gt/+ stage of estrus cycle-matched littermate mice using the ELISA Kit Cat# EZRMRG-45K (LINCO Research, St Charles, MO, USA). IGF1 was measured in serum using the ELISA Kit Cat# EZRMGH-45K (LINCO Research, St Charles, MO, USA). Absorbance (A450) for GH and IGF1 was measured and analyzed using a standard curve. Differences in absorbance were analyzed for significance by Student’s t-test using two-tailed unequal variance.

Mouse LH sandwich assay (MLHS)

Circulating levels of LH were assayed at the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core using the following methods: LH was measured in serum by a sensitive two-site sandwich immunoassay (105,106) using monoclonal antibodies against bovine LH (no. 581B7) and against the human LH-beta subunit (no. 5303: Medix Kauniainen, Finland) as previously described (106). The tracer antibody (no. 518B7) was kindly provided by Dr Janet Roser (107) (Department of Animal Science, University of California, Davis) and iodinated by the chloramine T method and purified on Sephadex G-50 columns. The capture antibody (no. 5303) was biotinylated and immobilized on avidin-coated polystyrene beads (7 mm; Epitope Diagnostics, Inc., San Diego, CA, USA). Mouse LH reference prep (AFP5306A; provided by Dr A.F. Parlow and the National Hormone and Peptide Program) was used as standard. The mouse LH sandwich assay (MLHS) has a sensitivity of 0.07 ng/ml.

Mouse FSH radioimmunoassay (RIA)

Circulating levels of FSH were assayed at the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core using the following methods: FSH was assayed by radioimmunoassay (RIA) using reagents provided by Dr A.F. Parlow and the National Hormone and Peptide Program, as previously described (108). Mouse FSH reference prep AFP5308D was used for assay standards and mouse FSH antisera (guinea pig; AFP-1760191) diluted to a final concentration of 1:400 000 was used as primary antibody. The secondary antibody was purchased from Equitech-Bio, Inc. and diluted to a final concentration of 1:25. The assay has a sensitivity of 2.0 ng/ml and <0.5% cross-reactivity with other pituitary hormones.

GnRH agonist and antagonist assays

Three- to 4-month-old Chd7+/+ and Chd7Gt/+ sex-matched littermate mice were given a subcutaneous injection of either the GnRH agonist leuprolide (Sigma, St Louis, MO, USA) or the GnRH antagonist antide (Sigma). Leuprolide was dissolved in 0.9% saline and administered at a dose of 1 mg/kg body weight 2 h prior to cardiac exsanguination (69,70). Antide was dissolved in 20% propylene glycol and 0.9% saline and administered at a dose of 3.0 mg/kg body weight 2 h prior to cardiac exsanguination (71,72). Serum was collected and analyzed by MLHS and mouse FSH RIA.

Cellular proliferation assays

Wild-type, Chd7Gt/+ and Chd7Gt/Gt E10.5 embryos were processed as above for immunofluorescence. Serial adjacent sections were labeled with rabbit anti-H3 (1:200, Millipore) followed by incubation with anti-rabbit AlexaFluor488 conjugated secondary antibody (Invitrogen). A minimum of 30 sections per embryo were photographed by single channel fluorescence microscopy on a Leica upright DMRB microscope and processed for dual channel imaging with Adobe Photoshop software v9.0 (San Jose, CA, USA). Cell counts were performed on at least four embryos of each genotype. Statistical significance was determined using one-way analysis of variance (ANOVA) analysis with GraphPad Prism 5 software.

TUNEL assays

Wild-type and Chd7Gt/+ E10.5, E11.5 and E12.5 embryos were processed as above for immunofluorescence. Serial adjacent sections were assayed for apoptosis using Fluorescein-FragEL DNA Fragmentation Detection Kit (Calbiochem, Darmstadt, Germany). A minimum of 30 sections per embryo were photographed by single channel fluorescence microscopy on a Leica upright DMRB microscope and processed for dual channel imaging with Adobe Photoshop software v9.0. Cell counts were performed on at least four embryos of each genotype at each time point. Cell counts were analyzed for significance by Student’s t-test using two-tailed unequal variance.

RNA isolation and real-time PCR

Three- to 4-month-old Chd7+/+ and Chd7Gt/+ sex-matched littermate mice were euthanized by cervical dislocation and decapitated. The brain and pituitary were removed from the head. The hypothalamus was microdissected from whole brain and placed in ice-cold TRIzol (Invitrogen) and mechanically homogenized prior to RNA isolation. Pituitary RNA was isolated using the RNAqueous-Micro RNA Isolation Kit (Ambion, Austin, TX, USA). Wild-type, Chd7Gt/+ and Chd7Gt/Gt E10.5 embryos were harvested as described above. The olfactory placode was microdissected and RNA isolated using the RNAqueous-Micro RNA Isolation Kit (Ambion). Isolated RNA from adult and embryonic tissues was treated with DNase I prior to cDNA synthesis. cDNA was generated using Superscript First-Strand cDNA Synthesis system for reverse transcriptase-polymerase chain reaction (RT-PCR) (Invitrogen) with random primers.

Relative expression levels were assayed utilizing TaqMan Gene Expression Master Mix and TaqMan probes (Applied Biosystems, Foster City, CA, USA) for GnRH1, GnRHR, Kiss1, Otx2, Bmp4, Fgf4r1, Fgf8 and Gapdh. Reactions were...
run in triplicate in an Applied Biosystems StepOne-Plus Real-Time PCR System. The level of Gapdh was used as an internal control. The difference in $C_T$ between the assayed gene and Gapdh for any given sample was defined as $\Delta C(T)$. The difference in $\Delta C(T)_x$ between two samples was defined as $\Delta\Delta C(T)_x$, which represents a relative difference in expression of the assayed gene. The fold change of the assayed gene relative to Gapdh was defined as $2^{-\Delta\Delta C(T)}$ (109). DataAssist software (Applied Biosystems) was used for statistical analysis and to confirm $\Delta\Delta C(T)_x$ calculation.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Sally Camper for insightful discussions and critical reading of the manuscript. We also thank Gina Leinninger for help with ELISA assays and Joseph Micucci for help analyzing ChIP-seq data sets.

Conflict of Interest statement. None declared.

FUNDING

This work was supported by the National Institutes of Health (F31DC010955-01 to W.S.L., R01DC009410 and an ARRA supplement to D.M.M.).

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