

DCC/NTN1 complex mutations in patients with congenital hypogonadotropic hypogonadism impair GnRH neuron development

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Abstract

Congenital hypogonadotropic hypogonadism (CHH) is a rare genetic disease characterized by absent puberty and infertility due to GnRH deficiency, and is often associated with anosmia (Kallmann syndrome, KS). The genetic etiology of CHH is heterogeneous, and more than 30 genes have been implicated in approximately 50% of patients with CHH. We hypothesized that genes encoding axon-guidance proteins containing fibronectin type-III (FN3) domains (similar to *ANOS1*, the first gene associated with KS), are mutated in CHH. We performed whole-exome sequencing in a cohort of 133 CHH probands to test this hypothesis, and identified rare sequence variants (RSVs) in genes encoding for the FN3-domain encoding protein DCC and its ligand Netrin-1 (*NTN1*). *In vitro* studies of these RSVs revealed altered intracellular signaling associated with defects in cell morphology, and confirmed five heterozygous *DCC* mutations in 6 probands—5 of which presented as KS. Two KS probands carry heterozygous mutations in both *DCC* and *NTN1* consistent with oligogenic inheritance. Further, we show that Netrin-1 promotes migration in immortalized GnRH neurons (GN11 cells). This study implicates *DCC* and *NTN1* mutations in the pathophysiology of CHH consistent with the role of these two genes in the ontogeny of GnRH neurons in mice.

Introduction

In vertebrates, puberty onset and maintenance of reproductive competence depends on a small population of hypothalamic neurons which secrete gonadotropin releasing hormone (GnRH) (1). This neuropeptide controls, in the anterior pituitary, the production and release of the gonadotropins LH (luteinizing hormone) and FSH (follicle stimulating hormone) (2) which stimulate gametogenesis and sex steroids production in the gonads.

During embryonic development, GnRH neurons originate in the olfactory placode and migrate through the nasal mesenchyme using the olfactory/vomer nasal axons as a scaffold to reach their final destination in the basal forebrain (3, 4). This migratory process has been well-described in rodents, and is tightly controlled by a complex network of genes encoding molecular cues that drive GnRH neuron motility, cell adhesion and directionality (4). Defects disrupting the genesis, migration of GnRH neurons and/or synthesis and secretion of GnRH in humans can lead to congenital hypogonadotropic hypogonadism (CHH, MIM #146110, (5, 6)).

CHH is a rare endocrine disorder (1 in 4,000) characterized by absent or incomplete puberty resulting in infertility (5). CHH associated with anosmia (the lack of sense of smell) occurs in 50% of CHH patients and is termed Kallmann Syndrome (KS) (7). CHH is genetically heterogeneous, and both sporadic and familial cases are observed. To date, more than 30 genes have been implicated in the pathophysiology of the disease. The underlying biological mechanisms have been classified into two predominant categories: (I) alteration of the prenatal development and/or migration of GnRH neurons, generally associated with KS, and (II) disruption of the synthesis, release or signaling of GnRH, commonly associated with normosmic CHH (nCHH) (5). The low penetrance of most CHH genes combined with variable expressivity among affected individuals carrying the same gene defect suggests that CHH is not a strictly monogenic disorder. Indeed, patients harboring mutations in two or more CHH loci (oligogenicity) have been described (8, 9).

Additionally, the genetic cause remains unknown in half of cases, indicating that additional CHH loci are yet to be discovered.

The first gene found to be mutated in this disease, *ANOS1* (previously known as *KAL1*), encodes for an extracellular cell adhesion protein containing fibronectin type-III (FN3) domains—an adhesive motif typical of extracellular matrix molecules (6, 10). This protein, Anosmin-1, plays a key role in axonal guidance and is involved in the migration of GnRH neurons from the olfactory placode to the brain during development (10, 11). The biological relevance of FN3-domain containing proteins in GnRH neuron development is well-illustrated by the fact that the majority of *ANOS1* mutations identified in KS are located in regions encoding for FN3 domains. Defects in additional genes encoding these critical domains have also been identified in CHH (i.e. *AXL* (12), *FLRT3* (13)), as well as in other diseases associated with altered neuronal development such as *L1CAM* in MASA syndrome (14), *DCC* in congenital mirror movement (15) and agenesis of the corpus callosum (16), and *PTPRQ* in autosomal recessive non-syndromic sensorineural deafness (17).

Therefore, we hypothesized that genes encoding axon-guidance proteins containing FN3 domains may be mutated in CHH patients. We used whole exome sequencing (WES) to screen a large cohort of CHH probands and identify novel genetic causes of CHH among genes encoding axon-guidance proteins containing FN3 domains. Our results demonstrate for the first time several rare and likely pathogenic variants in *Deleted in Colorectal Cancer (DCC)*, a gene encoding FN3-domain protein, and in *Netrin-1 (NTN1)*, its ligand in patients with congenital hypogonadotropic hypogonadism. *In vivo* expression of these proteins along the GnRH migratory route in human fetuses and the cellular and molecular functional assays, together with a relevant prevalence of these variants, strongly support the pathogenicity of these mutations in CHH patients.

RESULTS

***DCC* is a novel candidate gene implicated in CHH**

Whole-exome sequencing of 133 CHH patients (73 KS and 60 normosmic CHH [nCHH]) revealed 74,326 high-quality transcript variants. The first step, we evaluated all probands for the presence of mutations in the known CHH genes. According to the hypothesis that genes encoding proteins containing FN3 domains are potentially mutated in CHH, we filtered our results to include (Supplemental Figure 1): (I) genes encoding proteins containing FN3-domains (Supplemental Table 1), (II) involved in axon guidance (GO: 0007411, Supplemental Table 2), (III) genes implicated in human genetic diseases characterized by alterations in central nervous system neurobiological processes according to OMIM (Table 1), (IV) genes with rare variants (minor allele frequency (MAF) less than 0.1% in ExAC controls), and conserved nonsense, splice-site, or missense variants predicted to be damaging in at least one of three protein prediction programs (see Methods section). This strategy identified five candidate genes, including *ANOS1* (Table 1). Notably, CHH is often associated with specific non-reproductive phenotypes such as congenital mirror movement (CMM), hearing loss and split-hand/foot malformations, providing a useful tool to improve the identification of mutations in causal genes (5). In our CHH cohort, all the rare variants identified in potential candidate genes are found in a heterozygous state. Interestingly, *DCC* was the only candidate gene for which mutations were inherited autosomal dominantly related to a CHH-associated phenotype (CMM, (18)). According to the literature related to the associated phenotype of orthologous mouse genes, we found that *Dcc* heterozygous knockout mice exhibit impaired GnRH neuron migration (19-21). Overall, these evidences prioritized *DCC* as a novel candidate gene implicated in CHH.

CHH probands harbor rare sequence variants in *DCC* and its ligand *NTN1*

After prioritizing *DCC*, we next selected variants from within our study population to further evaluate *in vitro*. This included not only those variants identified and filtered as noted above, but also those with MAF up to 0.5% in ExAC reference data, given that mutations in other CHH genes with higher MAF up to 0.5% have been functionally validated for contributing to oligogenic inheritance. Five heterozygous rare sequence variants (RSVs) were identified in *DCC* (Table 2). Three of these RSVs are found in probands that also have mutations in known CHH genes. (Table 3) Two of them, p.Asn176Ser (N176S) and p.Gly470Asp (G470D), have been associated with CMM in public variant databases (ClinVar; rs138724679 and rs141813053, respectively) and recent publications (22).

p.Gly649Glu (G649E) and p.Ser876Tyr (S876Y) were not found in any of the control cohorts, while the N176S, G470D and p.Pro645Ser (P645S) were present in controls at a low frequency (Table 2). Notably, the N176S *DCC* variant is significantly enriched in our CHH cohort when compared to ExAC controls ($p=0.016$). This variant maps to the second Immunoglobulin-like domain that affects protein stability, while the remaining (G470D, P645S, G649E and S876Y) are located in FN3 domains (Fig. 1A). Interestingly, the S876Y variant maps to the fifth FN3 domain, which is implicated in the interaction of *DCC* with its ligand Netrin-1, encoded by *NTN1* (Fig. 1A). Therefore, we expanded our analysis to include *NTN1* and identified two heterozygous variants — p.Arg362Cys (R362C) and p.Thr525Arg (T525R). Both *NTN1* variants were absent in the control cohorts, and located in the EGF-like and NTR-like domains, respectively (Table 2, Fig. 1B). All of these missense variants are predicted by at least one protein prediction algorithm as damaging to protein function, and occur in conserved residues suggesting these variants likely impact protein function (Table 2 and Fig. 1).

DCC and NTN1 RSVs cause signaling defects

In vitro analysis revealed that all identified *NTN1* and *DCC* variants do not impair transcript or protein expression (Fig. 2A, B and Supplemental Fig. 2A, B, Supplemental Fig. 3). To assess the biological activity of the variants, we first tested the binding properties of DCC and its ligand NTN1, as previously described (15). Two *DCC* variants, N176S and S876Y, displayed impaired binding to wild-type (WT) Netrin-1 (Fig. 2C), while both NTN1 mutants exhibited a significantly reduced binding to DCC WT (Fig. 2D). The binding of Netrin-1 to the DCC receptor activates several signaling pathways important for cell migration and proliferation, including ERK signaling (23). We used an *Egr1*-promoter luciferase reporter assay to measure DCC-induced ERK signaling pathway activation (Supplemental Fig. 2C). All *NTN1* and *DCC* variants displayed a significantly decreased *Egr1*-promoter activation, reflecting a blunted DCC signaling (Fig. 2E and F). These results confirm that our RSVs are indeed loss-of-function mutations, and suggest a pathogenic role in our patients.

DCC mutations lead to cell morphology defects in GnRH neuron cells

Cell migration relies on the protrusion of actin-rich organelles such as lamellipodia and filopodia as the first and essential step of the cell motility cycle. To further explore the impact of the observed signaling defects on cellular behavior, we examined the effect of DCC mutations in cell morphology of immortalized GnRH neurons (GN11 cells) using immunofluorescence (Fig. 3A and Supplemental Fig. 4). As previously shown in other neuronal cell lines, overexpression of DCC-WT in GN11 cells produced severe morphological changes due to the activation of cytoskeletal remodeling including lamellipodia and filopodia formation (24) (Fig. 3A). Quantitatively, this was reflected by a significant increase in cell spreading (i.e. cell surface area and cell perimeter) and cell shape complexity (i.e. complexity index, Ci) in DCC-WT cells compared to control GFP-transfected cells (Fig. 3B, C) without modification of cell polarity

(i.e. aspect ratio) (Fig. 3D). Globally, all mutants were unable to reproduce the morphological changes seen with DCC-WT. Indeed, when compared to DCC-WT, three mutants (N176S, G470D and G649E) displayed a significantly lower cell area (Fig. 3B), while all mutants showed a reduction in cell perimeter (Fig. 3C). Finally, all DCC mutants also exhibited significantly reduced induction of cell shape complexity compared to the DCC-WT, revealing a strong defect in the induction of cell protrusions (Fig. 3A, E and Supplemental Fig. 4). Together, these results confirm that the identified *DCC* mutations significantly impair the cytoskeletal and membrane rearrangement involved in cell protrusions formation, consistent with an impairment of GnRH neuron migration.

NTN1 mutations affect GnRH neuron migration

To test the impact of NTN1 mutants on immortalized GnRH neurons cell motility, we performed wound healing migration assays using conditioned media (CM) containing WT or mutant recombinant Netrin-1 proteins. As expected, NTN1 WT-CM treatment significantly increased GN11 cell migration by 55% compared to mock-CM. This effect was significantly impaired for both R362C and T525R NTN1 mutants with a migration rate reduced to the level of mock-CM treated cells (Fig. 3F, J). These results confirm NTN1 RSVs as loss-of-function mutations, and point towards a cell-autonomous role for Netrin-1 in GnRH neuronal migration.

Mutations in *DCC* and *NTN1* are associated with severe GnRH deficiency

In total, 4 male and 3 female unrelated CHH probands (7/133) harbor heterozygous *DCC* and/or *NTN1* mutations (Fig. 4, Table 3, Supplementary data). One mutation was *de novo* (Family 7), and 3 out of 7 cases the mutation was inherited from an unaffected parent (Families 1, 2, and 3). In the remaining 3 families, no parental DNA was available for testing (Families 4, 5, and 6). Notably, all probands had severe

GnRH deficiency with absent puberty, and all males had a history of micropenis with/without cryptorchidism. The majority of probands harboring mutations in *DCC* had been diagnosed with KS based on abnormal olfactory function (5/7). This includes a neonatal KS proband (Family 2, Subject II-1) who was unable to complete formal smell testing, however cranial MRI revealed unilateral right olfactory bulb agenesis consistent with KS.

Additional CHH-associated phenotypes were highly variable (Table 3 and Supplementary Data) including CMM (probands in families 1 and 2); bilateral sensorineural hearing loss confirmed by audiogram (proband in family 4); mild facial asymmetry with unilateral auricular hyperplasia (proband family 1); and developmental delay and a mid-brain malformation (cavum septum pellucidum, proband family 2). Three of the 7 probands with *DCC/NTN1* mutations are obese, one has confirmed glucose intolerance, and two presented with reduced bone density (osteopenia/osteoporosis).

Family members were available for genetic analysis in five cases and revealed asymptomatic carriers consistent with reduced penetrance in families 1, 2 and 3 (Fig. 4). Mutations in other CHH loci in addition to the *DCC/NTN1* mutations (oligogenicity) were identified in 3/7 families (Fig. 4, Table 3). In Pedigree 1, the proband and the unaffected brother exhibit digenicity with mutations in *DCC* (N176S), as previously associated with CMM (22), and a known founder mutation in *PROKR2* (p.Leu173Arg) (25). The proband in Pedigree 3 harbors mutations in *CHD7* (p.Tyr1616Cys) and *SEMA3A* (p.Arg66Trp) in addition to *DCC* (G649E). All of the mutations in known CHH genes are predicted to be deleterious *in silico*, and two of them (*PROKR2* p.Leu173Arg and *SEMA3A* p.Arg66Trp) have been previously demonstrated to be loss-of-function by *in vitro* experiments (Table 3) (26-28). In family 2, paternal DNA was not available, however the presence of an asymptomatic carrier together with the CMM observed in the proband are consistent with the previously described incomplete penetrance of the observed *DCC* mutation in CMM (29). In family 4 and 6, parental DNAs were unavailable, however several evidences suggest an oligogenic

inheritance including the presence of milder phenotypes in both paternal and maternal families (Family 4) or the presence of an additional *NTN1* mutation (Family 6) (Figure 4). The proband in Pedigree 7 harbors a *de novo NTN1* mutation and a previously-reported homozygous frameshift mutation in *GNRH1* (30).

DCC and Netrin-1 are expressed in human GnRH neurons and fetal olfactory system

Several lines of evidence implicate *DCC* and *NTN1* as two novel genes involved in CHH pathogenesis: (i) CHH probands harbor rare mutations in *DCC* and/or its ligand *NTN1*, (ii) mutations in *DCC* and *NTN1* are associated with defects in cell protrusion formation and cell migration, and (iii) *Dcc* and *Ntn1* deficient mice exhibit impaired GnRH neuron migration (19, 20).

To further support the biological function of DCC and NTN1 in GnRH neuron migration in humans, we evaluated their expression patterns in GnRH neurons and along their migratory route during human fetal development at 10th gestational week (10 GW) (see schematic in Fig. 5A). Triple-immunofluorescence staining of coronal sections of 10 GW fetuses (n=2) revealed that Netrin-1 and DCC are expressed in the developing vomeronasal organ (VNO) (Fig. 5B-C), the surrounding tissue extending from the VNO toward the forebrain (Fig. 5B, C), and in GnRH neurons (Fig. 5D-I). Moreover, Netrin-1 was also expressed in other cell types belonging to the migratory mass—a heterogeneous population of placode—derived and neural crest-derived migratory cells (31-35), (Fig. 5F, H). We further evaluated whether Netrin-1 and DCC expression was retained by GnRH neurons that entered the brain, however at this developmental stage Netrin-1 was too heavily expressed in the forebrain to properly assess its expression and distribution specifically in GnRH neurons (data not shown).

DISCUSSION

We performed whole-exome sequencing in a large cohort of CHH probands (n=133) and their family members (when available) focusing on genes encoding FN3-domain proteins involved in axonal guidance to identify *DCC* and *NTN1* as candidate genes for CHH. In addition to demonstrating Netrin-1 and DCC expression along the GnRH migratory pathway in human fetal development, we report the first clinical cases of CHH patients (n=6) harboring loss-of-function mutations in *DCC*, a gene encoding FN3-domain protein, along with two mutations in its ligand, *NTN1*.

Several lines of evidence support the role of *NTN1* and *DCC* as promising candidate CHH genes: (I) Netrin-1 and its receptor DCC play a critical role in axonal guidance during brain development (23, 36, 37); (II) mouse genetic studies showed that both *NTN1* and *DCC* are essential for GnRH neuron migration (19-21); (III) *DCC* mutations are also implicated in human CMM (15, 22, 38), a CHH-associated phenotype (5). Our data exemplify these observations, where DCC and NTN1 distribution along the GnRH neurons migratory route suggests an analogous mechanism in human GnRH neuron migration and vomeronasal axon guidance. The majority of probands (5/6) harboring mutations in these genes presented with KS (6.8% of KS cohort), a condition more frequently associated with developmental defects than normosmic CHH (5). Two out of five *DCC* mutations (N176S, S876Y) and one *NTN1* mutation (R362C) are located in regions already known to be critical for structural integrity and/or for binding (39-42), and our experiments confirmed that these mutations abolish the Netrin-1/DCC interaction. Interestingly, although located in a region with unknown function, the T525R *NTN1* mutation also displays a clear loss of binding to DCC, suggesting a potentially novel biological function of this NTR-like domain. Consistent with the binding assay results, these mutations showed loss of ERK signaling *in vitro*. Interestingly, the three remaining DCC mutants (G649E, P645S and G470D) also demonstrated a loss of ERK signaling, however their binding to Netrin-1 was intact which is consistent with a recent report in which artificial DCC mutants with normal binding to Netrin-1 displayed defective signaling (39).

Lamellipodia and filopodia are membrane and actin-based cell protrusions essential for cell migration and neurite outgrowth (43, 44). DCC signaling controls both of these cellular processes by promoting lamellipodia and filopodia formation in neuronal cells (24, 45). In this respect, our morphological analyses showing that all DCC mutants result in a significant defect in cytoskeleton remodeling provide functional evidence linking *DCC* mutants with the alteration of cellular structures essential for cell migration and axonal guidance in GnRH neurons.

Netrin-1 and DCC do not exhibit an exclusive ligand/receptor relationship. Draxin and Cerebelin-4 have been identified as DCC ligands playing a key role in axon guidance (46, 47). Similarly, additional receptors such as neogenin, $\alpha 3\beta 1$ -integrin, UNC5b or DSCAM, were described as critical mediators of Netrin-1 effect on cell migration and guidance (41, 48, 49). Our identified *NTN1* mutations also significantly decreased cell migration in immortalized GnRH neurons. Since GN11 cells also express neogenin and $\alpha 3\beta 1$ -integrin ((45) + unpublished observations), it suggests that our *NTN1* mutants may also affect these alternative netrin receptors.

DCC and *NTN1* are genes known to be involved in axonal guidance during embryonic development as demonstrated by *Dcc* and *Netrin-1* null mice exhibiting severe defects of commissural development in the brain and spinal cord with particular defects in axonal projections (50, 51). Subsequent studies have shown that DCC/Netrin-1 signaling is essential for the proper development of olfactory/vomer nasal fibers, a scaffold for GnRH neuron migration. Indeed, GnRH neuron migration is profoundly impaired due to the mistargeting of the migratory scaffold in both *Dcc* and *Netrin-1* knockout mice. A similar phenotype has been observed in *Dcc +/-* and *Netrin-1 +/-* mice suggesting a gene dosage effect (19-21). This data strongly supports the concept that the heterozygote variants found in our patients may be implicated in the physiopathology of CHH. Furthermore, heterozygous mutations in *DCC* have been previously implicated in CMM (15, 22, 38), a CHH-associated phenotype (5). Interestingly, a recent report also

demonstrated that heterozygote mutations in *DCC* (protein truncating mutations) are associated with isolated ACC (agenesis of corpus callosum) with an incomplete penetrance (29). Similarly, biallelic mutations in *DCC* have recently been found in association with a new genetic syndrome of HGPPS (horizontal gaze palsy, scoliosis), ACC, and midline brainstem cleft (52). The severity (missense vs. protein-truncating) and inheritance (heterozygous vs. biallelic) of *DCC* mutations only partially explains the phenotypic overlap and variability between CMM, ACC, HGPPS and CHH suggesting a significant contribution of additional genetic defects or environmental factors.

The genetic architecture of CHH is complex, as mutations in most known CHH genes exhibit incomplete penetrance and variable expressivity both within and between families carrying the same mutations (5). Consistent with this, incomplete penetrance has also been observed for CMM families segregating the N176S and G470D mutations in *DCC* (22). The CHH families 1 and 2 in the current study harbor these same *DCC* mutations and also exhibit incomplete penetrance. This is similarly demonstrated by Marsh et al. (29) in regards to *DCC* mutations in ACC.

Oligogenic inheritance of mutations is observed in the known CHH genes, and this can at least partially explain the low penetrance and variable expressivity observed both within and across CHH families (8). In particular, we observed mutations in *DCC* in patients who also harbor pathogenic variants in known CHH genes. Further, digenic mutations in *NTN1* (T525R) and *DCC* (G470D) were found in monozygotic twins (Family 6) with severe KS associated with cryptorchidism, suggesting a synergistic effect. Finally, based on the incomplete penetrance and the clinical family histories, an oligogenic inheritance implicating the presence of mutations in unknown CHH loci cannot be ruled out. A particular case of digenic inheritance was observed in family 7. The normosmic proband in this family harbors a *de novo NTN1* (R362C) mutation in addition to previously described homozygous *GNRH1* mutations (p.Leu30fs) (30). One copy of the *GNRH1* mutation is inherited from his asymptomatic mother, and the other is inherited from the father

who has a history of delayed puberty. The nCHH phenotype could be *a priori* explained by the deleterious genetic defect in *GNRH1* gene, however following the The American College of Medical Genetics and Genomics (ACMG) guidelines for the interpretation of sequence variants (53) (absence in control cohort, confirmed *in vitro* deleterious effect, and *de novo* inheritance), the *NTN1* (R362C) mutation is classified as pathogenic. Combined with the previous studies, our results illustrate the complexity of *DCC* and *Netrin-1* mutations in the genetic architecture of human disorders of axon guidance, and stress the relevance of considering the potential physiopathological consequences of these mutations when providing genetic counseling.

In summary, we report loss-of-function mutations in *DCC*, a gene encoding FN3-domain protein, and its ligand, *Netrin-1*, establishing a mutation prevalence of 5.2% for these two genes in our CHH cohort. The prevalence of *DCC/NTN1* mutations is similar to another FN3-containing gene, *ANOS1* (5), suggesting that prevalence of this family of genes is about 10% in CHH, equivalent to *FGF8/FGFR1*. The latter were reported as the first genes to be associated with both KS and nCHH with a prevalence of 10-12% (54, 55). Our findings of (I) high prevalence of *DCC/NTN1* mutations in CHH, (II) impaired cytoskeletal reorganization and cell migration induced by signaling defect, and (III) the expression in the developing human GnRH system are consistent with the altered migration of GnRH neurons previously observed in *DCC* and *Netrin-1* deficient mice models (19, 20) and provide a functional link with the phenotype of our CHH patients. These results further implicate genes encoding FN3-domain protein in the pathophysiology of CHH, and KS in particular. Finally, this study highlights the clinical relevance of the Netrin-1/DCC pathway in CHH, confirms their essential role in signaling in human GnRH biology, and supports the inclusion of *DCC* and *NTN1* in the list of genes implicated in human CHH.

MATERIALS AND METHODS

Subjects

The CHH cohort included 133 European probands (73 KS and 60 nCHH). The diagnosis of CHH was made on the basis of: I) absent or incomplete puberty by 17 years of age; II) low/normal gonadotropin levels in the setting of low serum testosterone/estradiol levels; and III) otherwise normal anterior pituitary function and normal imaging of the hypothalamic-pituitary area (56). Olfaction was assessed by self-report and/or formal testing (57). Neonatal CHH is diagnosed in male infants with micropenis and/or cryptorchidism in the setting of low sex steroid and gonadotropin concentrations during “minipuberty” (58). When available, family members were included for genetic studies. The control population cohort consisted of 405 participants from the Cohorte Lausannoise (CoLaus) study, recruited and phenotyped as previously described (59-62). This study was approved by the Institutional Review Board of Partners Healthcare and the ethics committee of the University of Lausanne. All participants provided written informed consent prior to study participation.

Genetic studies

Genomic DNA was extracted from peripheral-blood samples using the Puregene Blood Kit (Qiagen), following the manufacturer protocol. The coding exons and intronic splice regions (≤ 6 bp from the exons) of genes encoding proteins containing FN3-domains, as well as all CHH known genes (5) were analyzed by exome sequencing. Exome capture was performed using the SureSelect All Exon capture v2 and v5 (Agilent Technologies, Santa Clara, CA USA) and sequenced on the HiSeq2500 (Illumina, San Diego CA USA) at BGI (BGI, Shenzhen, PRC). Raw sequences (fastq files) were analyzed using an in-house pipeline that utilizes the Burrows-Wheeler Alignment algorithm (BWA) (63) for mapping the reads to the human reference sequence (GRCh37), and the Genome Analysis Toolkit (GATK) (64) for the detection of single nucleotide

variants (SNVs) and insertion/deletions (Indels). The resulting variants for both exome and Sanger sequences were annotated using SnpEff version 4.0 (65) and dbNSFP version 2.9 (66) to calculate minor allele frequency (MAF) and protein functional predictions. Variants were confirmed by Sanger sequencing on both strands with duplicate PCR reactions and are described according to HGVS nomenclature (67). Exome sequences from all probands were evaluated for mutations in the known CHH genes as previously described (68). The known genes evaluated are: *ANOS1* (NM_000216.2), *FGFR1* (NM_023110.2), *FGF8* (NM_033163.3), *PROKR2* (NM_144773.2), *PROK2* (NM_001126128.13), *GNRHR* (NM_000406.2), *GNRH1* (NM_000825.3), *KISS1R* (NM_032551.4), *KISS1* (NM_002256.3), *TACR3* (NM_001059.2), *TAC3* (NM_013251.3), *NSMF* (NM_001130969.1), *HS6ST1* (NM_004807.2), *DUSP6* (NM_001946.2), *FGF17* (NM_003867.2), *FLRT3* (NM_198391.2), *IL17RD* (NM_017563.3), *WDR11* (NM_018117.11), *SOX10* (NM_006941.3), *FEZF1* (NM_001024613.3), *CHD7* (NM_017780.3) and *SPRY4* (NM_030964.3)]. In addition, we screened the *PLXNA1* gene (NM_032242.3), recently shown to be implicated in CHH (69).

Bioinformatic filtering of rare non-synonymous variants

A comprehensive list of genes (n=203) encoding proteins containing FN3-domains was extracted from the Prosite database (<http://prosite.expasy.org/PS50853>, accessed on February 9, 2017) and served as a reference for the subsequent filtering processes (Supplemental Table 1). Genes known to be involved in axon guidance (GO:0007411, Supplemental Table 2) were selected and prioritized using OMIM database according to their implication in human genetic diseases characterized by clinical features related to alterations in central nervous system neurobiological processes and CHH-associated phenotypes. Data for CNS phenotype and/or CHH-associated phenotype was obtained from OMIM (<http://omim.org/>). Nonsense, missense, and splice-site rare variants (MAF<0.1% in ExAC controls) identified from exome sequencing were cross-referenced with this list.

The OMIM database (<http://omim.org/>) was used to investigate the presence of CNS phenotypes and/or CHH-associated phenotypes in humans harboring mutations in the individual candidate genes. A central nervous system (CNS) phenotype was considered positive (Table 1) if the “Gene Function” section of the OMIM entry described functional studies that link defects in the gene to alterations in CNS development processes. CHH-associated phenotypes have been previously described in detail (Boehm, Endocrine Reviews), and a positive entry in Table 1 indicates the presence of this feature in humans with mutations in the respective gene. After identifying a candidate gene for further *in vitro* studies, variants with MAF up to 0.5% were identified in the CHH cohort to account for similar MAF’s observed for mutations in other CHH genes that have been functionally validated for contributing to oligogenic inheritance

***In silico* prediction**

To assess the possible functional effect of amino acid variants, PolyPhen-2 v.2.2.5 (<http://genetics.bwh.harvard.edu/pph2/>), SIFT (<http://sift.jcvi.org/>) and CADD (<http://cadd.gs.washington.edu/info>) algorithms were used. Missense RSVs were also evaluated for conservation across species (GERP++ (70)).

Collection and processing of human tissues

Tissues were made available in accordance with French bylaws (Good practice concerning the conservation, transformation and transportation of human tissue to be used therapeutically, published on December 29, 1998). The studies on human fetal tissue were approved by the French agency for biomedical research (Agence de la Biomédecine, Saint-Denis la Plaine, France, protocol n°: PFS16-002). Non-pathological human fetuses (10 weeks post-aménorrhée, n=2) were obtained from voluntarily terminated pregnancies after obtaining written informed consent from the parents (Gynecology

Department, Jeanne de Flandre Hospital, Lille, France). Fetuses were fixed by immersion in 4% paraformaldehyde (PFA) at 4°C for 3 days. The tissues were then cryoprotected in 30% sucrose/PBS at 4°C overnight, embedded in Tissue-Tek OCT compound (Sakura Finetek, USA), frozen in dry ice and stored at -80°C until sectioning. Frozen samples were cut serially at 20 µm using a Leica CM 3050S cryostat (Leica Biosystems Nussloch GmbH, Germany).

Immunohistochemistry

Immunohistochemistry for GnRH was performed as previously reported (35) using a guinea-pig anti-GnRH (EH#1018; 1:10000), produced by Dr. Erik Hrabovszky (Laboratory of Endocrine Neurobiology, Institute of Experimental Medicine of the Hungarian Academy of Sciences, Budapest, Hungary) and previously characterized in post-mortem human hypothalami (71). Antibodies against DCC (Goat IgG, sc-6535; Santa Cruz, (72)) and Netrin-1 (Monoclonal Rat IgG2A; MAB1109; R&D System, (73)) were used at a dilution of 1:500. Samples were rinsed in TBS (TRIS buffer saline, pH 7.6) and subsequently blocked for 2-hrs at room temperature in blocking solution (TBS+0.3% Triton X-100; Sigma), 0.25% BSA (Bovine Serum Albumin; Sigma) and 10% normal donkey serum (D9663; Sigma). Sections were incubated in a cocktail of primary antibodies (guinea pig anti-GnRH, rat anti-Netrin-1, goat anti-DCC) diluted in blocking solution for 48-hrs at 4 °C. Sections were then rinsed in TBS and incubated in a cocktail of fluorochrome-conjugated secondary antibodies (all raised in donkey; Alexa-Fluor 488-, 568-, 647-conjugated secondary antibodies; Molecular Probes, Invitrogen) diluted at 1:400 in TBS for 2 hrs at room temperature. Sections were then rinsed in TBS, coverslipped with Mowiol, and imaged using an inverted laser scanning Axio observer confocal microscope (LSM 710, Zeiss; Imaging Core Facility of IFR114 of the University of Lille 2, France).

Molecular characterization of *DCC* and *NTN1* variants

Plasmids. pCMV-hDCC-HA and pCMV-hNTN1-V5 was obtained from Addgene. All variants were generated using QuickChange XLII Kit (Stratagene) and confirmed by Sanger sequencing. Primers flanking the mutations (available upon request) were used for subsequent PCR amplifications (5' → 3').

Cell culture and transfection. CHO and COS7 cells were grown in DMEM-F12 and DMEM-high glucose medium, respectively, and supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and antibiotics (Gibco, Carlsbad, CA, USA) in humidified air containing 5% CO₂ at 37°C. Transient transfections of cells were carried out using FuGENE 6 reagent (Promega), according to the Manufacturer's protocol.

Protein Expression. After transient transfection in CHO cells, protein expression of tagged wild-type and mutated proteins was detected by Western blotting. Briefly, cells were lysed in RIPA buffer and separated with 8% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by electro-transfer onto a nitrocellulose membrane. For immunoblotting, the membranes were probed overnight at 4°C with anti-HA (Roche) and anti-V5 (Invitrogen) antibodies and then incubated with appropriate infrared fluorescent secondary antibodies (Licor).

RNA Expression. Total RNAs were extracted from CHO cells after transient transfection using Trizol reagent (Ambion) according to the manufacturer's recommendations and processed for RT-PCR as previously described (74).

Transcriptional assays. Cells were transfected with plasmids encoding the different wild-type and mutant DCCs, Netrin-1, appropriate firefly luciferase reporters, and β-Galactosidase (internal control) in 96-well plates, using the FuGENE 6 transfection reagent (Promega). Luciferase activity was measured using NOVOstar (BMG LABTECH), normalized to β-galactosidase activity and analyzed using Synergy™ Mx (BioTek). All experiments were performed three times in sextuplicates and are expressed as relative light units (RLU).

Binding Assays. Briefly, COS7 cells were plated in 24-well dishes and cultured in DMEM-high glucose supplemented with 10% FBS and penicillin/streptomycin. After 24h, cells were transfected with 1µg of control vector, pCMV-hDCC-HA WT or mutants using Lipofectamine 2000 reagent (Invitrogen). Culture media was removed 24h after transfection and replaced by Netrin-1-V5 protein in DMEM; 10% FBS for 2h at 37°C. Cells were then washed, fixed 5 min with 4% PFA, blocked using PBS-10% goat serum, and permeabilized with 0.1% triton X-100. Bound Netrin-1-V5 protein was detected using a monoclonal primary antibody (Invitrogen) in 10% goat serum-PBS and a horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody in 1% goat serum-PBS (Thermo Fisher). HRP activity was measured using SigmaFast OPD peroxidase substrate (Sigma) and read at 492 nm using a microplate spectrophotometer. The signal is calculated relative to protein concentration.

Functional characterization of DCC and NTN1 mutants

Immunocytofluorescence. GN11 cells were grown in DMEM-high glucose supplemented with 10% FBS and penicillin/streptomycin. Briefly, after 48h of transfection with the expression vectors coding for DCC-HA WT and mutants, cells were fixed with 4% paraformaldehyde, permeabilized for 15 min with 0.1% Triton X100 in PBS and blocked with Superblock 100% (Invitrogen) for 20 min. Cells were then incubated for 2h at room temperature with an anti-HA antibody (Sigma), followed by 1h incubation with an anti-rat Alexa 488-coupled secondary antibody (Life Technologies). After primary and secondary antibody labeling, cells were incubated with anti-phalloidin-594 (Thermo Scientific) and coverslips were mounted on slides with Fluoromont GTM with DAPI (Interchim)

Image acquisition and morphological analysis. Confocal images were acquired using a Zeiss LSM 780 Quasar inverted microscope (Plan Apochromat 63x /1.40 NA / oil immersion). For morphological analysis, images were taken using a Nikon Eclipse 90i microscope (CFI Plan Fluor 20x: N.A. 0.50; W.D.

2.1mm). ImageJ was used to analyze the cell spreading (cell surface area and cell perimeter) and aspect ratio (cell polarity) of WT and mutants DCC. To quantify the effect of DCC-WT or mutants on cytoskeleton/membrane plasticity, as lamellipodia and filopodia formation, we used imageJ to measure the Complexity Index (CI) with a morphological parameter measuring cell surface and perimeter roughness as defined in figure 3.

Migration assays. GN11 cells were plated in 6-wells with silicone reservoirs separated by a 500 μ m thick wall (Ibidi) and incubated 24h in serum-containing medium. After 2 hours of serum starvation, Ibidi chambers were removed to create a wound field and cells incubated with FBS-free medium, Mock-CM; Netrin-1-WT-CM or Netrin-1 T525R-CM for 6h. Images were acquired at different time points using a Nikon TS100 inverted microscope (CFI Plan Fluor DL 10x: N.A. 0.30, W.D. 15.2mm), and cell migration quantified as previously reported (75).

Statistics. PRISM 7 software (GraphPad) was used for statistical analysis (one way ANOVA followed by Tukey's post hoc test). Data are shown as mean \pm SEM and P values <0.05 were considered to be statistically significant.

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CONFLICT OF INTEREST STATEMENT.

None declared.

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LEGENDS TO FIGURES

Figure 1. Schematic representing of *DCC* and *Netrin-1*. (A, B) Schematic representation of *DCC* (A) and *NTN1* (B) genes and sequence alignment of amino acid variants from *D. melanogaster* to *H. sapiens*. SP: Signal peptide; Ig: Immunoglobulin domain; Fn: Fibronectin domain; TM: Transmembrane domain; EGF-L: EGF-like domain; NTR-like: Netrin-like domain.

Figure 2. Signaling potency of *DCC* and *Netrin-1* variants. (A) Western blot showing DCC-HA protein expression in transiently transfected CHO cells. (B) Western blot showing V5 tag Netrin-1 Protein (NTN-V5) expression in cell lysates and in conditioned-media (CM) in transiently transfected CHO cells. (C) Analysis of Netrin-1 binding with the WT or variant DCC receptors. CHO cells are transfected with plasmid encoding DCC-WT or variants. 48h post-transfection, transfected cells are treated with Netrin-1 WT or Mock-CM. ELISA assays showing the binding of DCC/Netrin-1 is visualized by absorbance, and expressed in relative units. (D) Analysis of WT and mutants Netrin-1 binding. Cells are transfected with plasmid encoding NTN1-WT, mutants or empty vector. 24h post-transfection, we collected the supernatant (conditioned-medium) which was used to stimulate CHO transiently transfected cells with DCC-WT. ELISA assays showing the binding of DCC/Netrin-1 is visualized by absorbance, and expressed in relative units. (E, F) Transcription reporter assay of WT and mutated DCC or Netrin-1. CHO cells were transiently cotransfected with or without DCC, Netrin-1, Egr1-luciferase reporter and B-galactosidase plasmid. ERK signaling is shown as luciferase activity above baseline, defined as the activity observed with the empty vector alone (black box). Results are presented as mean \pm SEM of 3 independent experiments each performed in sextuplicate. Statistical significance was evaluated with one way ANOVA followed by Tukey's post hoc test. Different letters indicate significant differences between groups ($p < 0.05$).

Figure 3. Effect of *DCC* and *Netrin-1* on morphometric aspects and migration in GN11 cells. (A) Immunocytofluorescence of DCC in GN11 cells. GN11 cells are transfected with GFP or DCC plasmid.

Twenty-four hours post-transfection, cells are plated onto poly-lysine coated coverslips for 24h. Cells are stained for DCC (green), phalloidin (red), nucleus (blue). **(B, C, D, E)** Quantification of cell area, cell perimeter, aspect ratio and complexity index in GN11-cells transfected with indicated plasmids. $n \approx 100$ cells per mutants. A: area, P: perimeter, x: major axis, y: minor axis, AR: aspect ratio and Ci: Complexity index. Results reflect three independent experiments. Error bars represent SEM, $P < 0.01$. Different letters indicate significant difference between groups. **(F)** Migration assays were performed on GN11 cells stimulated with FBS-free medium, Mock-CM or Netrin-1-CM. Images were taken 0h and 6h after treatment, image in black and white corresponding to binarized image use to determine the percentage of migration. **(J)** Percentage of GN11 migration after treatment with or without Netrin-1. Results are presented as mean \pm SEM of 3 independent experiments each performed in sextuplicate. Statistical significance was evaluated with one way ANOVA followed by Tukeys posthoc test. Different letters indicate significant differences between groups ($p < 0.05$).

Figure. 4. *DCC* and *NTN1* heterozygous variants identified in CHH probands. Pedigrees of CHH probands harboring variations in *DCC* and *Netrin-1*. Genotypes are indicated below each individual. Squares depict males, circles depict females and the proband is indicated by an arrow. Solid symbols indicate affected individuals (CHH and KS), while hatched symbols indicate partial phenotypes, as shown in figure legend. The horizontal line connecting siblings in Family 6 indicates monozygotic twins.

Figure.5. GnRH, Netrin-1 and DCC expression in coronal sections of a GW10 human fetus.

(A) Schematic representation of a GW10 human fetus head (sagittal view) illustrating the plane of section (red dotted line) used for immunofluorescence. **(B)** Netrin-1 (green) is expressed in the VNO. **(B-C)** Netrin-1 and DCC (red) are both expressed in the VNO and in cells of migratory mass (arrowheads). **(D-F)** Single-optical confocal planes showing migrating GnRH neurons (blue,D) co-expressing DCC (E) and Netrin-1 (F).

(G-I) High-power photomicrograph of the white box in D, confirming the co-localization of the three antigens. OE: olfactory epithelium. VNO: Vomeronasal organ. Scale bars: 20 μ m.

TABLES

Table 1. Potential candidate genes encoding FN3-containing protein in CHH

Gene	# rare variants	# probands (KS/nCHH)	Phenotype	Phenotype MIM number	Inheritance	CNS development phenotype	CHH associated phenotype
<i>ANOS1</i>	3	3 (3/0)	Hypogonadotropic hypogonadism 1 with or without anosmia (Kallmann syndrome 1)	308700	X-linked	yes	yes
<i>CNTN2</i>	7	12 (6/6)	Epilepsy, myoclonic, familial adult, 5	615400	Autosomal recessive	yes	no
<i>DCC</i>	5	7 (6/1)	Mirror movements 1	157600	Autosomal dominant	yes	yes
<i>ROBO2</i>	2	5 (5/3)	Vesicoureteral reflux 2	610878	-	no	no
<i>ROBO3</i>	2	4 (1/3)	Gaze palsy, horizontal, with progressive scoliosis	607313	Autosomal recessive	no	yes

*please see Methods section for a detailed description of these features.

Table 2. Genotypes and functional *in silico* prediction of *DCC* and *NTN1* variants found in CHH patients.

	Nucleotide change	Amino acid change	CHH patients (%)	MAF (%)		Prediction			Conservation	Variant-level association (p-value)
				ExAC controls	CoLaus controls	PPhen-2	SIFT	CADD	GERP	
<i>DCC</i>	c.527A>G	p.Asn176Ser	0.75	<0.005	0.120	P	D	D	5.71	0.016
	c.1409G>A	p.Gly470Asp	1.5	0.320	0.370	P	D	D	5.4	0.170
	c.1933C>T	p.Pro645Ser	0.75	0.019	-	D	D	D	5.45	0.334
	c.1946G>A	p.Gly649Glu	0.75	-	-	B	B	D	4.54	-
	c.2627C>A	p.Ser876Tyr	0.75	-	-	P	D	D	5.42	-
<i>NTN1</i>	c.1084C>T	p.Arg362Cys	0.75	<0.001	-	D	D	D	4.36	0.002
	c.1574C>G	p.Thr525Arg	0.75	-	-	B	B	D	4.62	-

cDNA and protein changes are based on reference cDNA sequence NM_005215.3 (*DCC*) and NM_004822.2 (*NTN1*). CHH cohort included 133 patients. MAF, minor allele frequency; CoLaus, Cohorte Lausannoise study (405 participants)—an ethnically-matched cohort of European controls with detailed phenotypic information (59-62) ; SIFT, scale-invariant feature transform; PPhen-2, polymorphism phenotyping v2; CADD, Combined Annotation Dependent Depletion; Pr, Private; D, Deleterious or Damaging; P, Possibly damaging; B, Benign. The Genomic Evolutionary Rate Profiling (GERP) score was obtained from the GERP website in September of 2011. It ranges from -12.3 to 6.17, with 6.17 being the most conserved. Variant-level association in CHH cases vs. ExAC controls were performed with a two-sided Fisher's exact test. *although not meeting the MAF criteria, this variant was included as it was inherited in a digenic fashion with the *NTN1* T525R variant.

Table 3. Clinical phenotypes of congenital hypogonadotropic hypogonadism patients with heterozygous *DCC* and/or *NTN1* mutations.

Family	Subject	Diagnosis	<i>DCC</i> Mutations	<i>NTN1</i> Mutations	Sex	Inheritance	Puberty	Additional reproductive phenotypes	Associated phenotypes	Mutations in other CHH genes	MRI Results
1	II-1	KS	p.Asn176Ser		M	S	A	Cryptorchidism Micropenis Hypospadias	Mild CMM Mild facial asymmetry Right ear hypoplasia	<i>PROKR2</i> p.Leu173Arg ¹	MRI not performed*
2	II-1	KS (neonatal)	p.Gly470Asp		M	S	A	Micropenis	Mental retardation CMM Obesity Glucose intolerance	None	Cavum septum pellucidum, reduced pituitary volume, decreased Rathke's cyst OB asymmetry
3	III-1	KS	p.Pro645Ser		F	F	A	None	Obesity	None	MRI not performed*
4	II-2	KS	p.Gly649Glu		F	F	A	None	Bilateral sensorineural hearing loss Osteopenia (hip)	<i>CHD7</i> p.Tyr1616Cys ¹ <i>SEMA3A</i> p.Arg66Trp ¹	Absence of OB
5	II-1	nCHH	p.Ser876Tyr		F	F	A	None	Obesity Osteoporosis	None	Reduced size of pituitary, thinning of distal pituitary stalk
6	II-1	KS	p.Gly470Asp	p.Thr525Arg	M	F	A	Cryptorchidism	None	None	Normal pituitary, shallow olfactory sulci, normal OB
	II-2	KS	p.Gly470Asp	p.Thr525Arg	M		A	Cryptorchidism	Pulmonary stenosis		Data not available
7	II-1	nCHH		p.Arg362Cys	M	F	A	Cryptorchidism Micropenis	None	<i>GnRH1</i> p.G29GfsX12 ²	Data not available

¹heterozygous or ²homozygous mutation

CHH: congenital hypogonadotropic hypogonadism; DCC: deleted in colorectal cancer; NTN1: netrin 1; Sex: F: female; M: male; Inheritance: F: familial, S: sporadic; Puberty: A: absent puberty. Olfactory phenotype: OB: olfactory bulb, MRI: magnetic-resonance imaging. *see Supplementary Data for detailed explanation

