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The role of *chd7* in brain development in a Zebrafish model for CHARGE syndrome

By

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Here's hoping to work toward the Philosophy in Ph.D.

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Abstract

Pathogenic variants in the gene - chromodomain, helicase, DNA binding (CHD) 7, an ATP-dependent chromatin remodeller are a major cause of a developmental disorder called CHARGE syndrome. CHARGE is an acronym that describes the characteristic features presented by the patients: ***C***oloboma, ***H***eart defects, ***A***tresia choanae, ***R***etardation in growth and development, ***G***enital abnormalities, and ***E***ar defects. In severe cases of CHARGE, patients rarely survive beyond 5 years of age, and yet there is no known treatment to alleviate its defects beyond behavioural therapy. Although not prominently included in its diagnostic criteria, patients with CHARGE often present with brain developmental defects and behavioural anomalies that include microcephaly, intellectual disability, seizures and overlapping symptoms with autism spectrum disorders (ASD), hyperactivity, attention deficit hyperactivity disorder (ADHD) and obsessive-compulsive disorder (OCD). However, little is known about the molecular mechanisms that underlie these neurological symptoms upon pathogenic variations in *chd7*. Here, I have characterized the role of *chd7* in brain development using a CRISPR-cas9 *chd7* knockout zebrafish model. Our *chd7-/-* mutant zebrafish mimicked the characteristic features presented in CHARGE patients like heart developmental defects, cranio-facial defects, cranial nerve abnormalities, small head phenotype and were behaviourally hyperactive. We showed that this hyperactive phenotype in the *chd7-/- mutants* is underlined by a reduction in the number of GABAergic neurons. Employing an unbiased transcriptomic analysis in the *chd7-/- mutant* brains, we identified a gene -*paqr3b*-that is most downregulated. *paqr3b* (progestin and adipoQ receptor 3 protein) is an inhibitor of the MEK/ERK pathway. Interestingly, we report hyperphosphorylation of ERK (via increased p-ERK levels) in the mutant brains compared to the controls. An independent inhibition of the p-ERK in the mutant brains partially restores the number of GABAergic neurons and the hyperactivity. Further, overexpression of *paqr3b* also rescues the number of GABAergic neurons and restores the levels of p-ERK. Thus, *paqr3b* contributes to the GABA network defect and the hyperactivity via the MEK/ERK pathway. We could validate the reduced levels of *PAQR3* in *CHD7* mutation positive CHARGE syndrome patient derived lymphoblastoid cell lines (LCLs). And this reduction in the expression of *PAQR3* is in fact due to the reduced occupation of its proximal promoters by *CHD7.* Next, we report an increase in the number of Sox2 and GFAP positive neural stem/progenitor cells (NSPCs) in the *chd7-/-* mutant brains, with a reduction in the number of Sox2 positive progenitor cells that eventually differentiate into GABAergic neurons. An overexpression of *paqr3b* restores the number of both the Sox2 and GFAP positive NSPCs in the *chd7-/-* mutant brains along with the number of Sox2 positive progenitor cells that are determined into GABAergic neurons. This suggests a potential and novel role for the *chd7-paqr3b* regulatory axis in neurogenesis. Lastly, employing zebrafish

and C. *elegans chd7*-mut models in a high throughput drug screen we identified a small neuroprotective molecule called Ephedrine that partially rescues the hyperactivity and number of GABAergic neurons via the MEK/ERK pathway by partially restoring the p-ERK levels. In conclusion, this study has added to the understanding of the mechanistic link between *chd7* and the neurodevelopmental defects observed in CHARGE syndrome. This understanding can be potentially extrapolated to other neurodevelopmental disorders that have shared aetiology or symptoms with CHARGE syndrome.

Key words: chd7, brain, GABAergic neurons, zebrafish, neurogenesis

Resumé

Des mutations dans le gène - chromodomaine, hélicase, liaison à l'ADN (CHD) 7, un facteur de remodelage de la chromatine dépendant de l'ATP, sont une cause majeure de la maladie congénitale du syndrome de CHARGE (CS). CHARGE est un acronyme qui décrit les caractéristiques présentées par les patients : **C**olobome, malformations cardiaques (**H**eart), **A**trésie des choanes, **R**etard de croissance et de développement, anomalies **G**énitales et anomalies de l'oreille (**E**ar). Dans les cas graves de CS, les patients survivent rarement au-delà de 5 ans, pourtant, il n'existe aucun traitement connu pour atténuer ses effets mis à part la thérapie comportementale. Bien qu'ils ne représentent pas un critère majeur de diagnostic, les patients atteints de CHARGE présentent souvent des anomalies du développement cérébral ainsi que des anomalies comportementales. Ces anomalies cérébrales et comportementales montrent des symptômes tels que la microcéphalie, des retards intellectuels ainsi que des convulsions qui sont des symptômes communs avec le Trouble du Spectre Autistique (TSA), l'hyperactivité, le Trouble du Déficit de l'Attention avec/sans Hyperactivité (TDAH) ou les Troubles Obsessionnels Compulsifs (TOC). Cependant, le rôle du gène chd7 dans le développement du cerveau est encore peu connu et plus particulièrement son rôle dans la pathogénèse de CHARGE. Lors de mon doctorat, j'ai caractérisé le rôle de chd7 dans le développement cérébral à l'aide d'un modèle de poisson zèbre knock-out pour le chd7. Notre poisson zèbre mutant chd7 recapitule les caractéristiques présentées chez les patients CHARGE ainsi qu'un comportement hyperactif. Nous avons montré que ce phénotype hyperactif chez les mutants chd7 est dû à un défaut développemental et fonctionnel du réseau GABA. Une analyse transcriptomique non-biaisée des cerveaux mutants chd7 a permis d'identifier un gène -paqr3b comme étant parmi les gènes les plus fortement régulés négativement. Cette régulation négative de paqr3b contribue au défaut du réseau GABA et à l'hyperactivité via la voie de signalisation ERK/MEK. Paqr3b est une cible directe de remodelage de la chromatine par chd7 et la régulation négative de son expression a été validée dans une lignée cellulaire de lymphoblastoïde humaine mutante pour *CHD7*. Nous suggérons également que paqr3b joue un rôle dans le développement précoce du cerveau en affectant la différenciation des cellules souches neuronales puisqu'un nombre réduit de cellules positives pour Sox2 a été détecté dans le cerveau de nos mutants. Cela contribuerait donc au défaut du réseau GABA observé. Enfin, en utilisant des modèles de poissons zèbres et de nématode, c. elegans, mutants chd7 dans un criblage de molécules à haut débit, nous avons identifié une molécule neuroprotectrice, l'éphédrine, qui permet un sauvetage phénotypique partiel de l'hyperactivité et des défauts du réseau GABA via l'axe régulateur ERK/MEK. Cette étude a ajouté une connaissance cruciale des mécanismes impliqués dans les défauts du développement cérébral observés dans le syndrome CHARGE.

Ces mécanismes pourraient être extrapolés efficacement à d'autres troubles neurodéveloppementaux qui ont une étiologie ou des symptômes communs avec le syndrome CHARGE.

Mots clés : *chd7*, cerveau, neurones GABAergiques, poissons zèbres, neurogénèse.

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Abbreviations

ASD	Autism Spectrum Disorders
ADHD	Attention Deficit Hyperactivity Disorder
OCD	Obsessive Compulsive Disorder
ESCs	Embryonic Stem Cells
hESCs	human derived Embryonic Stem Cells
NPCs	Neural Progenitor Cells
NSPCs	Neural Stem/Progenitor Cells
NCCs	Neural Crest Cells
NSCs	Neural Stem Cells
OLs	Oligodendrocytes
OLPs	Oligodendrocyte Progenitors
OPC	Oligodendrocyte Precursor Cells
GNP	Granule Neuron Progenitors
DEE	Developmental Epileptic Encephalopathy
ENU	N-ethyl-N-nitrosourea
TALENS	Transcription activator-like effector nucleases
TILLING	Targeting Induced Local Lesions in Genomes
CRISPR	Clustered Regularly Interspaced Short Palindromic Sequences
ZFN	Zinc Finger Nucleases
GABA	Gamma aminobutyric acid
Dpf	days post fertilization
Hpf	hours post fertilization
RGC	Radial Glial Cells
rGC	retinal Ganglion Cells
BMP	Bone morphogenetic proteins
FgF	fibroblast growth factors
Wnt	wingless-related integration site
hNCLC	human Neural Crest-like Cells
LCLs	Lymphoblastoid cell lines

SECTION I: INTRODUCTION AND HYPOTHESIS

1 INTRODUCTION

1.1 CHARGE syndrome

CHARGE syndrome is a rare, autosomal dominant, congenital developmental disorder that is characterized as a conglomerate of multiple organ system defects. CHARGE itself is an acronym that stands for the most characteristic features presented by patients, that include but is not limited to **C**oloboma of the eye, **H**eart defects, **A**tresia choanae, **R**etarded growth and development, **G**enital abnormalities, and **E**ar defects. Individuals with CHARGE often visibly present with a characteristic set of craniofacial features that include a pair of small, low-set, and deformed ears, a square face, facial palsy, broad neck, and a broad nasal root (Figure 1). The disorder occurs over a range of combinatorial defects that have varying degrees of severity. In severe cases, individuals with CHARGE syndrome rarely survive beyond 5 years of age. Yet, unfortunately, there is no known treatment for the disorder beyond behavioural therapy and need based surgical interventions to manage the symptoms.

CHARGE syndrome is majorly caused due to pathogenic mutations in the *CHD7* gene (Vissers et al., 2004). 70-90% of individuals clinically diagnosed with CHARGE syndrome have a pathogenic *CHD7* variant (Bergman, Janssen, et al., 2011). A small portion (5-10%) of CHARGE syndrome cases however, have no known aetiology. The gene *CHD7* (Chromodomain Helicase DNA binding protein 7) is an ATP dependent chromatin remodeller, that belongs to the Chromodomain Helicase DNA binding (CHD) family of chromatin remodellers. It is widely expressed in the human body throughout early stages of development and is involved in the dynamic and tissue specific regulation of gene expression through maintaining an open chromatin structure via ATP dependent nucleosome translocation (Bouazoune & Kingston, 2012; W. Feng, Kawauchi, et al., 2017; Layman, Hurd, & Martin, 2010; Reddy et al., 2021; Sanlaville et al., 2006).

In most cases, CHARGE syndrome is a sporadic disorder caused due to *de novo* pathogenic variations in *CHD7*. A few familial hereditary cases have been reported with high intra-familial variability in transmission and severity (Bergman, Janssen, et al., 2011; Legendre et al., 2017). There is a 50% chance of heritability to the next generation via probands with mild presentations of CHARGE syndrome. In cases where there is no pathogenic variant of *CHD7* detected in the parents of the proband, there is still 1-2% risk of recurrence in siblings due to possible germline mosaicism (Jongmans et al., 2008; C. van Ravenswaaij-Arts & Martin, 2017).

Figure 1 CHARGE syndrome
The above set of pictures represents individuals with CHARGE syndrome. We can observe a square face presentation (females in 2a and 2d, male in 2c), unilateral facial palsy (female in 2a), bilateral facial palsy (female in 2d), round eyes (female in 2a), broad necks and sloping shoulders (males in 2c, 2e and 2f and female in 2d), prominent ears (males in 2c and 2f) and mild presentation of CHARGE syndrome (female in 2b) often observed in CHARGE syndrome manifestations.
Adapted from (C. van Ravenswaaij-Arts & Martin, 2017; C. M. van Ravenswaaij-Arts et al., 1993)

CHARGE syndrome has an occurrence of one in 10,000-15,000 live births, with minor variations in frequency depending on the country of assessment (Blake et al., 1998; Issekutz, Graham, Prasad, Smith, & Blake, 2005; Janssen et al., 2012; Sanlaville & Verloes, 2007). Male and female patients are equally likely to be affected by CHARGE syndrome. However, in genetically confirmed *CHD7* mutant cohorts there appears to be a female predominance (Lalani et al., 2006).

1.1.1 The discovery of CHARGE syndrome

In 1979, Bryan Hall, a dysmorphologist and Helen Hittner, an ophthalmologist, independently described an association of congenital malformations that they had consistently observed in many children (Hall, 1979; Hittner, Hirsch, Kreh, & Rudolph, 1979). They described children that were predominantly ascertained to be having either choanal atresia or coloboma, as also having other developmental defects. These defects included characteristic small, low-set, and deformed ears, cardiac defects, deafness, hypogenitalism, facial palsy, and postnatal growth problems with developmental delay. In 1981, Pagon et al. first coined the acronym CHARGE to together describe this typical co-occurrence of a certain set of developmental defects in individuals (Pagon, Graham, Zonana, & Yong, 1981). The acronym was meant to highlight the most consistent features that occurred together - as a non-random

pattern of congenital anomalies - more frequently than one would expect based on chance. This combination of multiple developmental anomalies was at first considered an association rather than a syndrome because the symptoms were not delineated enough to represent a common aetiologic identity.

Much later, in 2001 it came to be believed that the organ systems affected in CHARGE could be traced back to a specific window of foetal development and were largely stemming from neural crest cells' developmental defects (J. M. Graham, Jr., 2001). This provided a degree of confidence that this non-random occurrence of consistently recognizable pattern of congenital defects would have a common aetiologic identity. Graham et al, suggested that CHARGE should be called as the Hall-Hittner "syndrome", and not an association (J. M. Graham, Jr., 2001). In 2004, pathogenic variants of the *CHD7* gene were identified in individuals described as having CHARGE syndrome, providing the required genetic aetiology that underlined the multiple developmental defects, qualifying CHARGE as a syndrome (Vissers et al., 2004).

CHARGE syndrome remains a predominantly clinically diagnosed disorder. The term CHARGE was coined close to 40 years ago providing a simple rule of thumb for making or rejecting a diagnosis through the symptoms covered in the acronym. However, over the years, multiple additional features have been described and added to the diagnostic features associated to CHARGE syndrome (Bergman, Janssen, et al., 2011; Blake et al., 1998; Oley, Baraitser, & Grant, 1988; Pagon et al., 1981; Tellier et al., 1998; Verloes, 2005; Zentner, Layman, Martin, & Scacheri, 2010). A set of major and minor criteria were first described by Blake et al., and later refined by Verloes et al., as a structure for the diagnosis of CHARGE syndrome (Blake et al., 1998; Verloes, 2005). Table 1 describes the occurrence rate of each of the documented phenotypes described in CHARGE patients. Recently, testing for pathogenic variants for CHARGE syndrome was added as an important criterion to make the diagnosis (Hale, Niederriter, Green, & Martin, 2016). An updated report on the range of phenotypes observed and the available methods of diagnosis was recently published (Hale et al., 2016; C. van Ravenswaaij-Arts & Martin, 2017; C. M. van Ravenswaaij-Arts et al., 1993).

Table 1: Symptoms of CHARGE syndrome
The table below lists all the symptoms presented by individuals with CHARGE syndrome along with their frequencies of occurrence. These symptoms appear in CHARGE syndrome over a range of different combinations and severities.
Adapted from (Balasubramanian & Crowley, 2017; C. van Ravenswaaij-Arts & Martin, 2017; C. M. van Ravenswaaij-Arts, Hefner, Blake, & Martin, 1993)

Features		Frequency (%)
Ocular coloboma		80%
Choanal atresia/stenosis		45%
Cranial nerve dysfunction/ anomaly	I: hyposmia or anosmia	90%
	VI: facial palsy	40%
	VIII: SNHL &/or vestibular dysfunction	>95%
	IX/X: suck & swallow, abnormal GI motility	60%-80%
Ear malformations	Abnormal auricle	90%
	Ossicular malformations	80%
	Mondini defect	90%
	Semicircular canal defect	94%
Cleft lip and/or palate		25%-50%
Endocrine	Hypogonadotropic hypogonadism	50%-70%
	Growth deficiency	70%
	Hypothyroidism	15%-20%
Developmental delay / Intellectual disability		>90% / 60%
Cardiovascular defects		74%
Tracheoesophageal anomalies		20%
Brain	Clivus hypoplasia Hypoplasia/J-shaped sella	95%
	Other	50%
Seizures		30%
Renal anomalies		30%

1.1.2 *CHD7* and CHARGE syndrome

A majority of the individuals described as having CHARGE syndrome have been identified with a pathogenic *CHD7* variant (Bergman, Janssen, et al., 2011; Janssen et al., 2012; Jongmans et al., 2006; Jongmans et al., 2009; Lalani et al., 2006; Legendre et al., 2017; Vissers et al., 2004; Zentner et al., 2010). Over 500 different human pathogenic variants of *CHD7* have been identified so far (www.chd7.org). In most incidents the pathogenic variants are *de novo* and autosomal dominant with no known genotype- phenotype correlation (Bergman et al., 2012; Jongmans et al., 2006). The pathogenesis is assumed to be due to the haploinsufficiency of the *CHD7* gene (Zentner et al., 2010).

The pathogenic mutations in the *CHD7* gene are equally scattered over the entire coding region and in some intronic sequences (Janssen et al., 2012; Martin, Salem-Hartshorne, Hartshorne, Scacheri, & Hefner, 2016; Zentner et al., 2010). Majority (~73%) of the mutations are either nonsense or frameshift mutations. The other less frequent mutations include- slice variants (~11%), and missense (~8%) (Jongmans et al., 2006; Lalani et al., 2006; Sanlaville et al., 2006; Zentner et al., 2010). Typically, missense *CHD7* mutations have been noted to cause a reduction or loss of the ATPase and remodelling activity *in vitro.* Further, missense mutations have been often reported with milder clinical presentations (Bouazoune & Kingston, 2012; Sanlaville & Verloes, 2007). Whole-exon deletions in *CHD7* (~1%) and translocations that disrupt *CHD7* have been identified in very few cases so far (Bergman et al., 2008), (rarediseases.org).

In the small percentage of individuals with clinically typical CHARGE syndrome that have an apparent lack of *CHD7* pathogenic variants, the disorder is supposed to be because of other alterations in *CHD7* that are not detected with routine genotyping/sequencing strategies. These alterations may include intragenic rearrangements, mutations in intronic/promoter/untranslated regions, or whole gene/exon deletions or duplications. Other assessment techniques like whole-genome sequencing in combination with analysis for deletions/duplications, employment of chromosomal microarray or single gene/multigene arrays may help to find the cause for the proportion of patients with an unexplained CHARGE syndrome (updated(C. M. van Ravenswaaij-Arts et al., 1993)).

1.1.3 Other proposed aetiologies for CHARGE syndrome

Recent studies have discovered that there is a possibility that pathogenic variations in genes other than *CHD7* could cause CHARGE syndrome (Belanger et al., 2018; Schulz et al., 2014). One such gene, *FAM172a* (family with sequence similarity 172, member A) plays a key role in the regulation of co-transcriptional alternative splicing, by interacting with Ago2

(Argonaute-2) and Chd7. Mutations in *Fam172a* were identified as a potential cause for CHARGE syndrome upon characterization of mouse models that were *CHD7* mutation-negative representations of CHARGE syndrome and were generated by insertional mutagenesis of *Fam172a* (Belanger et al., 2018). Validation in LCLs acquired from human CHARGE syndrome patients suggested that dysregulation of co-transcriptional alternative splicing could be a unifying pathogenic mechanism for both *CHD7* mutation-positive and *CHD7* mutation-negative cases (Belanger et al., 2018). In a different study, non-synonymous *SEMA3A* variations in 3 out of 45 *CHD7*-negative CHARGE patients were detected. However, the authors strongly suggest that non-synonymous *SEMA3A* variants are not sufficient to produce the phenotype but could play an important role in the pathogenesis of a multiple malformation syndrome like CHARGE (Schulz et al., 2014).

1.1.4 Overlap of CHARGE syndrome with other developmental disorders

CHD7 is a chromatin remodeller that regulates the expression of a wide range of genes in a dynamic, developmental stage and tissue specific manner (W. Feng, Shao, & Liu, 2017; Sanlaville et al., 2006). It can therefore be assumed that a disruption in *CHD7* would lead to a variety of developmental or functional defects in different organ systems. Other than CHARGE syndrome, studies have reported that pathogenic variations in *CHD7* also cause autism spectrum disorder (ASD) (Y. H. Jiang et al., 2013; O'Roak, Vives, Girirajan, et al., 2012). *CHD7* has been reported to be mutated in cases with gonadotropin-releasing hormone deficiency (Balasubramanian et al., 2014). *CHD7* pathogenic variants cause up to 6% of Kallmann Syndrome cases (H. G. Kim et al., 2008). Kallmann syndrome is a genetic disorder marked by hypogonadotropic hypogonadism and anosmia (Balasubramanian et al., 2014; Marcos et al., 2014). *CHD7* polymorphisms have been linked to idiopathic scoliosis (X. Gao et al., 2007). Further, a set of common target genes that are mutated in Alagille, Pallister-Hall, and Feingold syndromes are known to be regulated by CHD7 in conjunction with the Sox2 transcription factor- a CHD7 binding partner(Engelen et al., 2011).

Some of the CHARGE syndrome symptoms also overlap with the primary manifestations of other developmental disorders like 22q11.2 deletion syndrome, DiGeorge syndrome and Kabuki syndrome (Butcher et al., 2017; de Lonlay-Debeney et al., 1997; Jyonouchi, McDonald-McGinn, Bale, Zackai, & Sullivan, 2009). The 22q11.2 deletion syndrome includes congenital heart disease, learning difficulties, immune deficiency, hearing loss, renal problems, feeding problems, growth hormone deficiency and skeletal abnormalities that are all very prominently noticed in CHARGE syndrome as well (McDonald-McGinn, Hain, Emanuel, & Zackai, 1993). DiGeorge syndrome consists of complex heart defects, immunodeficiency, and abnormalities of the thyroid and parathyroid glands – all symptoms

potentially overlapping with CHARGE syndrome(de Lonlay-Debeney et al., 1997). Kabuki syndrome presents with facial features like widely spaced eye, a low nasal bridge, prominent low-set ears, intellectual disability, skeletal deformities like scoliosis associated with other health difficulties like heart problems, kidney abnormalities, hearing loss and eye problems. Some of the Kabuki syndrome cases are in fact also caused by pathogenic variants of *CHD7* (Butcher et al., 2017). A possibility of Kabuki syndrome is also recommended in *CHD7* mutation-negative cases of CHARGE syndrome. Some of the other disorders that show overlapping symptom with CHARGE are Rubinstein-Taybi syndrome (caused mainly by *EP300 mutation*), Verheji syndrome (caused by *PUF60 mutation*), and *RERE*-associated developmental disorder (caused by *RERE mutation*) (Moccia et al., 2018).

A recent paper demonstrated an interplay between CHD7, KMT2D, KDM6A and EP300 thus suggesting a regulatory link between CHARGE, Kabuki, and Rubinstien-Taybi syndromes (Ufartes et al., 2021). Some of the other less common albeit overlapping disorders include the Sox2 disorder, Pax2 disorder, Joubert's syndrome, syndromic microphthalmia 5 and 6, branchiootorenal spectrum disorder and Teacher Collins syndrome among a few others (Ufartes et al., 2021).

1.1.5 Neurodevelopmental features presented in CHARGE syndrome

Depending on the mode of investigation, 58-80% of individuals with CHARGE syndrome have definite CNS anomalies (Lin, Siebert, & Graham, 1990; Sanlaville & Verloes, 2007). In fact, recently brain structural defects were proposed to be included as minor diagnostic criteria for the diagnosis of CHARGE syndrome (Hale et al., 2016). Studies from normal human embryos and foetal tissues obtained from elective termination of pregnancies have shown that CHD7 is well expressed in the human brain and depicts a spatial- and temporal-specific expression pattern during early development (Sanlaville et al., 2006). CHD7 is expressed in the undifferentiated neuroepithelium, in the mesenchyme of the neural crest origin and in fact in the later stages of foetal development it is expressed in the dorsal root ganglia, cranial nerves, auditory tissues, pituitary tissues, nasal tissues and the retina (Sanlaville et al., 2006). This suggests an important role for CHD7 in brain development. In CHARGE syndrome the defects in brain development include hypoplasia of olfactory bulb and cerebellum, agenesis of the corpus callosum, microcephaly and atrophy of the cerebral cortex (R. Becker, Stiemer, Neumann, & Entezami, 2001; Hale et al., 2016; Johansson et al., 2006; Legendre et al., 2012; Lin et al., 1990; Sanlaville et al., 2006; Tellier et al., 1998; T. Yu et al., 2013). Deficiency of olfactory bulb and sulci is the most frequently occurring defect in CHARGE syndrome (Blustajn, Kirsch, Panigrahy, & Netchine, 2008; Chalouhi et al., 2005; Pinto et al., 2005). Rhinencephaly, holoprosencephaly, hydrocephalus, and cerebral

dysgenesis are some of the other reported anomalies (Sanlaville & Verloes, 2007). A retrospective analysis of the radiology data base from 2005 to 2015 of individuals with CHARGE showed additional unreported anomalies of CHARGE syndrome (Hoch et al., 2017). They included vestibular dysplasia, cochlear dysplasia, cochlear nerve deficiency, olfactory sulcus and groove dysplasia and brain stem hypoplasia among many other features (Hoch et al., 2017). There appears to be a clear correlation between the expression patterns of CHD7 and the developmental defects observed in CHARGE syndrome (updated in 2020 (C. M. van Ravenswaaij-Arts et al., 1993)

Cranial nerve developmental defects occur in about 70-90% of CHARGE syndrome cases and are now included among the major features for the diagnosis of CHARGE syndrome (Blake, Hartshorne, Lawand, Dailor, & Thelin, 2008). The defects in cranial nerves development affects ears and vestibular organs, eyes, facial characteristics, and the endocrine system with remarkable variability. The cranial nerves typically affected in CHARGE syndrome include I (olfactory), II (optic), VII (causes facial palsy), VIII (acoustic defects) and most commonly IX,X (glossopharyngeal/vagus defects). The cranial nerves III, IV, V, VI, XI and XII are less frequently affected in CHARGE syndrome (Zentner et al., 2010).

Pathogenic variants of *CHD7* have also been identified to cause autism spectrum disorder (ASD) and Kallmann syndrome (Y. H. Jiang et al., 2013; H. G. Kim et al., 2008; O'Roak, Vives, Fu, et al., 2012). Anosmia and hypogonadotropic hypogonadism as presented in Kallmann syndrome is highly corelated with CHARGE syndrome (Bergman, Bocca, Hoefsloot, Meiners, & van Ravenswaaij-Arts, 2011). Autism spectrum disorders are neurodevelopmental disorders typically characterized by difficulties in social interaction and communication associated with repetitive behaviour. Individuals with CHARGE syndrome have also been described to be having intellectual disability, social immaturity and autistic behavioural phenotypes (Bergman, Janssen, et al., 2011). Further, individuals with CHARGE syndrome have been observed with behaviours that overlap with hyperactivity, obsessive-compulsive disorder (OCD), Attention Deficit Hyperactive Disorder (ADHD), Tourette syndrome and often have a high pain threshold (Hartshorne, Hefner, & Davenport, 2005). The shared behavioural manifestations between CHARGE and other disorders suggests there could be some unifying mechanistic link that underlines these symptoms. The extent of CNS anomalies observed in CHARGE syndrome strongly suggests an important role for CHD7 in brain development and functioning. Exploring the role of *CHD7* particularly in the development and functioning of the brain could shed light on the potential ways in which these shared behaviours occur and therefore offer therapeutic targets.

1.2 Chromodomain Helicase DNA binding family and *CHD7*

Regulation of gene expression during development and homeostasis is a delicately concerted process that involves ordered recruitment of transcriptional machinery and alterations to the chromatin structure. Chromatin remodelling enzymes play critical roles in maintaining and altering chromatin structures and are thereby indispensable for normal development (Clapier & Cairns, 2009; Ho & Crabtree, 2010). CHD7 (Gene ID: 55636) belongs to the CHD (Chromodomain Helicase DNA binding protein) family of chromatin remodelling proteins. The CHD family consists of nine member proteins that are divided into three groups based on the similarities in their protein domains and in some cases their time of discoveries (Marfella & Imbalzano, 2007). The CHD member proteins are conserved among a range of eukaryotic organisms including yeast, drosophila, C. *elegans*, xenopus, zebrafish, mice and humans. Despite a high level of structural homology among the member proteins, functionally, the CHD family of proteins have been involved in transcriptional regulation as activators, repressors, or both (Stokes, Tartof, & Perry, 1996). The wide spectrum of developmental defects presented in CHARGE syndrome upon mutations in *CHD7*, suggests that the genes regulated by *CHD7* play crucial roles in vertebrate development and functioning. Elucidating the role that *CHD7* plays via various gene regulatory networks in the organ specific development will help in identifying potential therapeutic targets that can be employed to alleviate symptoms of CHARGE syndrome.

1.2.1 The *CHD7* Gene

Chromodomain helicase DNA binding protein 7(CHD7) belongs to the subgroup III of the CHD family of proteins which also includes CHD6, CHD7, CHD8 and CHD9. *CHD7* regulates gene expression by ATP dependent chromatin remodelling via nucleosome translocation(Reddy et al., 2021). In vitro studies using reconstituted chromatin have shown that CHD7 is able to slide nucleosomes on DNA in an ATP dependent manner (Bouazoune & Kingston, 2012). CHD7 maintains an open and accessible chromatin state at the promoters of *CHD7* target genes (W. Feng, Shao, et al., 2017; E. Lee et al., 2021; Reddy et al., 2021). Further, CHD7 has been reported to be a positive and negative regulator of gene expression in ESCs (Schnetz et al., 2010).

In humans the *CHD7* gene is located on chromosome 8, location 8q12 (Gene ID: 55636). RNA-seq of tissue samples from 95 human individuals representing 27 different tissue types shows that CHD7 is well expressed in most of them including a ubiquitous expression in the bone marrow and a high expression in the brain (Fagerberg et al., 2014). NCBI reports that *CHD7* gene is well conserved across vertebrate species. 402 species have a *CHD7* gene

ortholog with conserved homologs in chimpanzee, Rhesus monkey, dog, cow, mouse, rat, chicken, zebrafish, and frog. In Zebrafish, the *chd7* gene is located on the chromosome 2 (ID: ZDB-GENE-070912-179). Like the human counterpart the zebrafish *chd7* homolog is composed of 38 coding exons and is well expressed throughout early stages of development (Patten et al., 2012). Suggesting, it must play similar roles in regulating gene expression that contributes to the early vertebrate development in zebrafish as in higher vertebrates.

1.2.2 Protein Structure and function

The CHD7 protein consists of multiple conserved functional domains that include the two chromodomains, N and C terminal helicase domains, a SANT-like domain, and two BRK (Brahma and Kismet) domain (Figure 2) (Vissers et al., 2004). There is varying degree of information known about the specific functions of each of these domains in the context of their presence and role in chromatin remodelling and chromatin remodelling proteins. Typically, for the CHD7 protein, the chromodomain is involved in identifying appropriate regions of the chromatin – particularly methylated histone tails and recruiting enzymes to bind to distinct DNA locations and initiate the remodelling process. The Helicase domain is involved in unwinding and separating the double stranded DNA in an ATP dependent manner during the remodelling process. There is an additional DEXDc domain or DEAD-like helicases superfamily domain that contains the ATP-binding region and is also involved in ATP-dependent DNA unwinding. The SANT-like domain is suggested to combine the histone tail binding to the ATPase enzymatic activity (Allen, Bycroft, & Zinzalla, 2020; Boyer, Latek, & Peterson, 2004). There is very little understood about the BRK domain over and above its involvement in protein complexes that affect chromatin remodelling. It is believed that it could play a role in protein-protein interactions. Each of these domains is extremely important for the nucleosome sliding activity in the process of chromatin remodelling and is conserved to varying degrees across the vertebrate organisms.

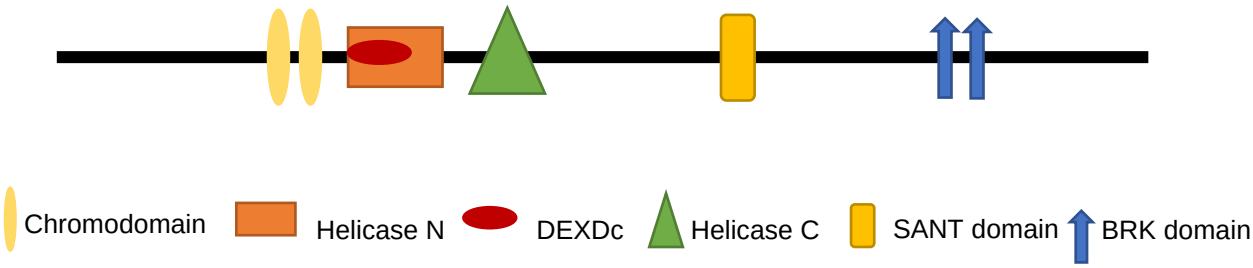

Figure 2 Major Functional domains of CHD7 protein.
The above schematic shows the major functional domains of the CHD7 protein.

1.3 Current understanding about the role of CHD7 in CHARGE syndrome

Multiple efforts have been made to elucidate the function and role of *CHD7* in the pathogenesis of CHARGE syndrome using model systems ranging from human and mouse embryonic stem cells lines, neural stem cells, cultured neural crest cells *in vitro*, to *C*. *elegans*, drosophila, xenopus, zebrafish and mice *in vivo*. The studies have discovered important insights into the regulatory role of CHD7 in an organ/cell type specific and developmental stage specific manner. Employing some of the simple organisms (like zebrafish) has helped in not just modelling CHARGE syndrome but also in identifying small molecules through drug screenings that can act as potential therapies. In the following sections, I detail some of the most notable reports across the different animal systems that have added to the knowledge of *CHD7's* function and *CHD7* caused pathogenesis in CHARGE syndrome.

1.3.1 CHD7: Binding site, interacting partners and genes regulated

CHD7 binds to discrete locations along the chromatin that are specific to each cell type. The CHD7 binding regions on the chromatin have been found to correlate with subsets of methylated lysine 4 at histone 3(H3K4me), mostly mono/demethylated and acetylated lysine 27 on histone 3 (H3K27ac) (W. Feng & Liu, 2013; Schnetz et al., 2009). These epigenetic signatures typically associate with an open chromatin suggesting that CHD7 in a positive regulator of gene expression. CHD7 typically associates with components of the BAF- (Brahma associated factor complex) and PBAF - (polybromo- and BRG1-associated factor-containing complex) complexes that include subunits like BRG1, BAF170, BAF155, BAF57, PB1, ARID2, and BRD7 (Bajpai et al., 2010)**.** BAF and PBAF both belong to the SWI/SNF-family of ATP-dependent chromatin remodelling complexes and can act as transcriptional activators or repressors via both nucleosome sliding and eviction (Bajpai et al., 2010).

A CHD7 containing PBAF complex was shown to occupy a neural crest cell specific distal SOX9 enhancer, a conserved genomic element located upstream of the TWIST1 gene marked by H3K4me1 and to regulate SLUG (Bajpai et al., 2010; W. Feng et al., 2013). In neural crest cell derived cardiomyocytes, CHD7 was shown to form complexes with R-SMAD proteins SMAD1/5/8, (components of the BMP signalling pathway) which then regulated the expression of the cardiac transcription factor gene *Nkx2.5* (Y. Liu et al., 2014). Further, Brg1-CHD7-containing PBAF complex was reported to facilitate the function of the master transcription factor MITF (Microphthalmia-associated transcription factor)(Laurette et al., 2015).

In mesenchymal stem cells CHD7 forms a complex with SETDB1, NLK, and PPAR-gamma that binds to DNA in a Wnt-5a responsive manner and promotes Runx2 transcription (Takada, Suzawa, Matsumoto, & Kato, 2007). Among the CHD family proteins, CHD8 was identified as an interacting partner of CHD7 (Batsukh et al., 2010). The interaction of CHD7 with BRG1 and CHD8 was confirmed in HEK293T cells (W. Feng, Kawauchi, et al., 2017). FAM124b has also been identified as a potential interacting partner of a CHD7 and CHD8 containing complex (Batsukh et al., 2012).

In human embryonic stem cells (hESCs) and mouse granule progenitor cells (GNPs), CHD7 has been shown to be associated with super-enhancers in the genome (W. Feng, Kawauchi, et al., 2017; Hnisz et al., 2013). In mouse derived neural stem cells (NSCs), knock down of *Sox2* impairs the binding of Chd7 to its targets, indicating that Sox2 is involved in the recruitment of Chd7(Engelen et al., 2011). Chd7 is also enriched at the *Sema3a* promotor in neural crest cells and loss of function of *Chd7* inhibits *Sema3a* expression (Ufartes et al., 2018). In differentiating oligodendrocytes (OLs), CHD7 has been shown to interact with the SoxE family transcription factor SOX10 and colocalizes with SOX10 genome-wide(He et al., 2016). In murine embryonic stem cells (ESCs), genomic approaches like chromatin immunoprecipitation followed by next generation sequencing showed mostly enhancer regions as a part of over 10,000 CHD7 interacting chromatin sites. At most of these sites, CHD7 localized with a unique group of DNA binding proteins like p300, Oct4, Sox2, Nanog, Smad1, and STAT3 (Schnetz et al., 2010). A cooperative function of CHD7 and DNA topoisomerase TOP2B also plays a role in transcriptional regulation (W. Feng, Shao, et al., 2017). CHD7 has been reported to regulate ribosomal RNA biogenesis as well (Zentner et al., 2010).

The epigenetic effects of CHD7 on chromatin and gene regulation seem to depend upon the precise composition(s) of the various protein complexes with which it interacts and is specific to the developmental stage, tissue, and cell types. The congenital anomalies in CHARGE syndrome are most likely due to alterations in transcription of tissue-specific genes normally regulated by CHD7 during development.

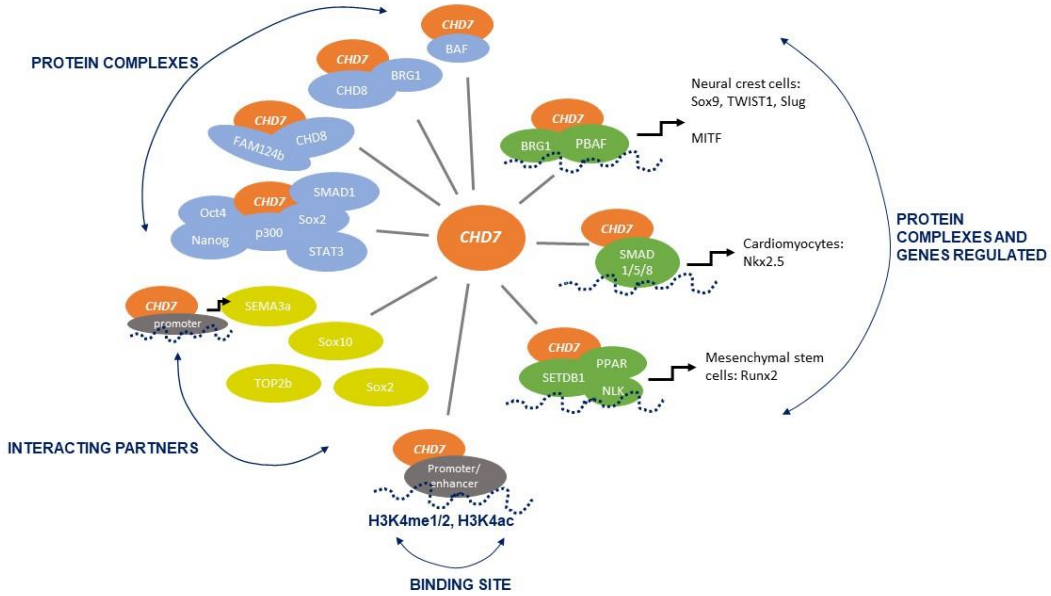

Figure 3 Binding site, Interacting Partner and genes regulated by CHD7
The above figure depicts some of the notable interacting partners and the genes regulated by CHD7. It must be noted that CHD7 forms protein complexes and regulates genes in a developmental stage specific and tissue specific manner. The dotted line in the above figure represents a region of the DNA.

1.3.2 Models for studying CHARGE syndrome

Neural Crest Cell Studies

Neural crest cells' abnormalities have been long suggested to contribute to the pathophysiology of CHARGE syndrome. Neural crest cells (NCCs) are a transient cell population that are ectodermal in origin but undergo a major transcriptional reprogramming event to acquire a broad differentiation potential and ability to migrate throughout the body. They give rise to craniofacial bones and cartilages, the peripheral nervous system, pigmentation, and cardiac structure. CHD7 containing PBAF complexes synergistically activate neural crest cells' gene expression and migration. Further, CHD7 is essential for the formation of these multipotent migratory neural crest cells (NCCs) (Bajpai et al., 2010). Characterization of neural crest cells differentiated from iPSCs derived from two patients with typical CHARGE manifestations showed that expression of genes associated with cell migration was altered in CHARGE iPSC-NCCs compared to control iPSC-NCCs. CHARGE iPSC-NCCs showed defective delamination, migration, and motility in vitro, and their transplantation *in ovo* revealed overall defective migratory activity in the chick embryo (Okuno et al., 2017).

Drosophila:

Kismet is the Drosophila *melanogaster* orthologue for CHD7 and CHD8. It is expressed in both motor neuron nuclei and postsynaptic muscle nuclei of the drosophila larvae. Kismet is important for the motor neuron synaptic morphology, localization and clustering of postsynaptic glutamate receptors, larval motor behaviour, and synaptic transmission (Ghosh et al., 2014). Kismet has been reported to be required for proper axonal pruning, guidance, and extension in the developing fly's central nervous system (Melicharek, Ramirez, Singh, Thompson, & Marenda, 2010). Along with defects in neuroanatomy, flies with reduced *kismet* expression show defects in memory and motor function, which are phenotypes consistent with symptoms observed in individuals with CHARGE syndrome (Melicharek et al., 2010).

Kismet mutant flies also present with an abnormal RNA polymerase II transcriptional elongation, suggesting that kismet promotes early elongation (Srinivasan, Dorighi, & Tamkun, 2008). It was shown to positively influences transcript levels and to bind to transcription start sites and promoters in the nervous system (Latcheva et al., 2019). Kismet regulates endocytosis in neuronal axons and influences synaptic localization of Dynamin/Shibire. Further, *kismet* mutants exhibit reduction in VGLUT (a synaptic vesicle marker) and along with reduced levels of synaptic Rab11 at stimulated synapses (Latcheva et al., 2019).

According to a report, pharmacological inhibition of HDAC function reversed the loss of function phenotypes associated with Kismet. The pharmacological inhibition of HDAC suppressed motor deficits, overgrowth of the neuromuscular junction, and defective neurotransmission associated with loss of Kismet (Latcheva et al., 2018). Kismet and HDACs may therefore potentially converge on a similar set of target genes in the nervous system.

C. elegans:

In *C. elegans*, a screening for ASD-associated missense variants in their C. elegans orthologs was conducted using a CRISPR/Cas9-mediated homology-directed knock-in strategy to generate missense mutants. The analysis of the Chd7 missense variants showed the Chd7 missense variant impacted the behaviour and development via several broad-spectrum assays. The tested missense variant showed detectable phenotypic changes in morphology, locomotion, fecundity and impacted neurodevelopment and movement functions (Wong, Gau, & Chou, 2019)(Wong 2019). Another study reported that *chd-7* in *C. elegans* is required for dauer morphogenesis, lifespan determination, and stress response that were further validated in the xenopus model (Jofré et al., 2021).

Xenopus:

In Xenopus embryos, knockdown of *Chd7* or overexpression of its catalytically inactive form recapitulates all major features of CHARGE syndrome, in a dosage sensitive response. The morphant tadpoles presented with missing or malformed otolith, coloboma of the eye with or without microphthalmia, malformations of craniofacial cartilage, including compression of ceratohyal cartilage, malformation of Meckel's cartilage, and collapsed branchial pouches, and heart defects, including abnormal positioning of the *truncus arteriosus* and cardiac outflow tract, heart structures that receive developmental contribution from the neural crest. The studies showed that intact ATPase domain is important for the role of CHD7 in neural crest migration. Thus, providing yet another potential route for early developmental intervention (Bajpai et al., 2010). Another Xenopus CHARGE model also showed that human *SEMA3A* rescued *Chd7* loss of function (Ufartes et al., 2018). Further, knockdown experiments for *Chd7* in Xenopus laevis embryos, showed abnormalities in the expression pattern of *Sema3a* (Schulz et al., 2014). Taking forward from a c. elegans based study, another morpholino mediated knockdown of Chd7 led to a reduction in *col2a1* mRNA levels and embryonic lethality and craniofacial defects. These defects in Chd7-depleted tadpoles were partially rescued by over-expression of *col2a1* mRNA suggesting the pathogenic features of CHARGE syndrome like craniofacial malformations, result from the reduction of collagen levels denoting a new target of chd7 in CHARGE syndrome (Jofré et al., 2021).

Zebrafish:

The *chd7* morphant zebrafish present with developmental defects that are characteristic of CHARGE features. The morphants showed smaller eyes, heart development defects, asymmetric otoliths, defects in axis development and vertebral mineralization(Asad et al., 2016; Balow et al., 2013; Cloney et al., 2018; Jacobs-McDaniels & Albertson, 2011; C. Liu, Li, Xiao, Gong, & Kang, 2020; H. Liu & Liu, 2020; Z. Z. Liu et al., 2018; Patten et al., 2012). Defects in retinal organization and the development of photoreceptors were also observed (Patten et al., 2012). *chd7* knockdown also presented with cranial nerve developmental defects like deformations, improper number, or complete absence of cranial nerves (Patten et al., 2012). A separate study showed that chd7 deficiency led to asymmetric segmentation of the presomitic mesoderm, suggesting abrogation of *chd7* results in defects in left-right asymmetry thus making it a good model of study for human spinal deformities linked to *chd7* (Jacobs-McDaniels & Albertson, 2011).

Another set of zebrafish morphants showed elevated expression of several cell-cycle inhibitors like ink4ab (p16/p15), p21 and p27, and had reduced cell proliferation. A MO-mediated knockdown of histone demethylase fbxl10/ kdm2bb, a repressor of ribosomal RNA genes, rescued zebrafish embryos from patterning defects of craniofacial cartilage suggesting Chd7 is required for proper organization of neural crest-derived craniofacial cartilage structures (Balow et al., 2013).

A MO-mediated knockdown of *sox10*, a gene important for neural crest development, was shown to rescue zebrafish *chd7* morphant embryos from defects in craniofacial cartilage and peripheral myelination (Asad et al., 2016). Later in 2020 Asad et al conducted a selective chemical screen with epigenetic and signalling modulators that identified 4 chemical molecules: DAPT, M344, DNMT1 and CHIC-35 that focused on rescuing the defects in neural crest-derived tissues such as craniofacial cartilage, peripheral neurons in cranial and trunk region and myelinating Schwann cells in their zebrafish model for CHARGE Syndrome.

In 2018, another *chd7* morphant zebrafish model showed that loss of *chd7* resulted in physically smaller GI tracts with decreased and disorganized vagal projections, particularly in the foregut but normal epithelial and muscular histology which was minimally improved by the prokinetic agents, domperidone and erythromycin, mimicking the mixed responses to these agents in individuals with CHARGE syndrome as well (Cloney et al., 2018).

chd7 knockdown and knockouts in zebrafish presented a dramatic decrease of T cells with a seriously impaired organogenesis and homing function of the thymus (Z. Z. Liu et al., 2018). Separately, a *SEMA3E* mutant and morphant model presented with craniofacial malformations, including small eyes, defective cartilage and an abnormal number of otoliths in zebrafish embryos, which resemble the major features of CHARGE syndrome. Further, an overexpression of sema3E rescued the phenotype of scattered cranial neural crest cells in *chd7* morphants, indicating that *chd7* may control the expression of sema3E to regulate cranial neural crest cell migration (Z. Z. Liu et al., 2018).

Most recently, heterozygous *chd7* mutant adults, showed anxious-like and aggressive-like behaviour in the open-field test and in the mirror-induced attack test, mimicking the reported behavioural abnormalities in CHARGE syndrome in humans(H. Liu & Liu, 2020). The expression of glycine transporters was dramatically increased in the heterozygous mutant brains and a glycine and D-cycloserine treatment rescued the aggressive behaviour of *chd7* heterozygous zebrafish mutants (H. Liu & Liu, 2020).

Mouse:

The mouse models created to study CHARGE syndrome range from homozygous and heterozygous *Chd7* gene trap models, ENU knockout models and cell type specific conditional knockout models. Specific phenotypic and molecular studies have uncovered novel interacting partners and regulatory roles for Chd7 in inner ear, eyes, heart, brain development, genital development among other CHARGE affected organ systems (Hurd et al., 2011) (Adams et al., 2007; Bosman et al., 2005; Gage, Hurd, & Martin, 2015) (Schulz et al., 2014).

Homozygous *Chd7* mutant mouse embryos die shortly after embryonic day 10.5 (E10.5)(Bosman et al., 2005; Hurd et al., 2007). However, heterozygous, and conditional knock out mutant mice revealed a range of defects with reduced penetrance, such as cleft palate, choanal atresia, septal defects of the heart, haemorrhages, prenatal death, vulva and clitoral defects and keratoconjunctivitis sicca (Bosman et al., 2005). *Chd7* heterozygous mutant mice also show decrease in circulating levels of luteinizing hormone and follicle-stimulating hormone and females particularly showed delays in vaginal opening and estrus onset, and erratic estrus cycles (Layman, Hurd, & Martin, 2011). A loss of *Chd7* from the neural ectoderm and surface ectoderm results in severely dysmorphic eyes generally lacking recognizable optic cup structures and small lenses. A genome-wide microarray expression analysis on wild-type and *Chd7* deficient mouse embryos identified 98 differentially expressed genes, many of which were found to be involved in neural crest cell and axon guidance like semaphorins and ephrin receptors (Schulz et al., 2014). Further, a genome-wide expression profiling revealed downregulated expression of the gene encoding the glycoprotein reelin (Reln) in *Chd7*-deficient GCps (Whittaker, Kasah, et al., 2017).

Chd7 null embryonic stem cells derived from *Chd7* mutant mouse blastocysts showed significantly reduced neuronal and glial differentiation. Sholl analysis showed that loss of *Chd7* impaired neuronal complexity and neurite length in differentiated neurons (Yao et al., 2020). Chd7 seems to be dispensable for both the generation and the proliferation of neural progenitor cells but ablation of *Chd7* in adult NSCs led to a mild increase of cell proliferation in the SGZ, a loss of stem cell quiescence in the hippocampus, a transient increase in cell divisions, followed by a significant decline in neurogenesis (W. Feng & Liu, 2013; Jones et al., 2015). Loss of *Chd7* in neural progenitor cells led to increased cell death in both adult neurogenic regions and cerebellum and to a defect in terminal differentiation of mouse neural progenitor cells. (W. Feng, Kawauchi, et al., 2017; W. Feng & Liu, 2013; Whittaker, Riegman, Kasah, Mohan, Yu, Pijuan-Sala, et al., 2017). Further, a loss of *Chd7* also results in cell-autonomous proliferative, neurogenic, and self-renewal defects in the perinatal and mature mouse SVZ stem cell niche (Micucci et al., 2014; Yao et al., 2018).

Deletion/genetic inactivation of *Chd7* from cerebellar granule cell progenitors (GCPs) resulted in reduced GCP proliferation, impaired terminal differentiation of granule neurons in DG and cerebellum, and cerebellar hypoplasia- possibly due to the impairment of granule neuron differentiation, induction of apoptosis and abnormal localization of Purkinje cells. An ablation of *Chd7* in mouse OPCs led to defects in the differentiation of OLs but did not affect the generation and proliferation of OPCs or GNPs. Further, genetic inactivation of *Chd7* from cerebellar granule cell progenitors also led to developmental delay, and motor deficits in mice (W. Feng, Kawauchi, et al., 2017; He et al., 2016; Whittaker, Kasah, et al., 2017). Haploinsufficiency of Chd7 leads to a mild cerebellar hypoplasia and distinct cerebellar foliation and resultes in reduced Fgf8 expression in the isthmus organiser (IsO), an embryonic signalling centre that directs early cerebellar development (Whittaker, Kasah, et al., 2017; T. Yu et al., 2013). Chd7 and Top2b have been shown to be necessary for the transcription of neuronal genes in cerebellar granule neurons (W. Feng, Kawauchi, et al., 2017).

Chd7 protects non-proliferative OPCs from apoptosis by closing the chromatin and repressing the transcription of *p53.* Chd7 controls OPC differentiation by activating the transcription of key regulators, including *Sox10*, *Nkx2.2*, and *Gpr1* (Marie et al., 2018). Independently, *Chd7* mutant mice presented smaller olfactory bulbs, reduced olfactory sensory neurons, and disorganized epithelial ultrastructure despite the seemingly normal functional cilia and sustentacular cells. Significant reductions in the proliferation of neural stem cells and regeneration of olfactory sensory neurons in the mature Chd7(Gt/+) olfactory epithelium indicate critical roles for Chd7 in regulating neurogenesis (Layman et al., 2009).

Modulation of retinoic acid (RA) signalling prevented in vivo inner ear and in vitro neural stem cell defects caused by Chd7 deficiency, and CHD7 and RA may have common target genes or signalling pathways (Micucci et al., 2014; Yao et al., 2018). Through a range of cKO studies specifically in the otic mesenchyme, hair cells, developing neuroblast and spiral ganglion neurons it was found that pan otic deletion of Chd7 shortened cochleae with aberrant projections and axonal looping, disorganized, supernumerary hair cells at the apical turn and a narrowed epithelium with missing hair cells in the middle region. However, none of the other cell specific deletions lead to any significant alterations in the integrity of the auditory neurons (Balendran et al., 2021). Analysis of single-cell multiplex qRT-PCR derived 192 genes showed that upon chd7 haploinsufficiency in heterozygous mutants, there was an enrichment of cells adopting neuroblast vs otic identity in the otocysts. Further, disruptions to prosensory and pro-neurogenic genes were noted suggesting chd7 is required for cell fate determination in the developing ear (Durruthy-Durruthy et al., 2018).

Further, a Chd7 conditional knockout study, using Foxg1-Cre to delete Chd7 in the developing eye, ear, nose, pharyngeal pouch, forebrain, and gut and Wnt1-Cre to delete Chd7 in migrating neural crest cells indicate that CHD7 has an important, dosage-dependent role in development of several different craniofacial tissues (Sperry et al., 2014). The two cKO mice driven by Foxg1-Cre and Wnt1-Cre presented with postnatal respiratory distress and death, dysplasia of the eye, concha, and frontal bone, hypoplastic maxillary shelves and nasal epithelia, reduced tracheal rings and frontal and occipital bone dysplasia, hypoplasia of the maxillary shelves and mandible, and cleft palate, respectively (Sperry et al., 2014). Foxg1-Cre mediated loss of *Chd7* resulted in cochlear hypoplasia, complete absence of the semicircular canals and cristae along with reductions in vestibulo-cochlear ganglion size and neuron number. There was a reduced expression of Ngn1, Otx2 and Fgf10, with expansion of the neural fate suppressor Tbx1 and reduced cellular proliferation (Hurd, Poucher, Cheng, Raphael, & Martin, 2010).

Genome-wide studies demonstrated that loss of *Chd7* led to modified chromatin accessibility (ATAC-seq) and differential nascent expression (Bru-Seq) of neural-specific genes suggesting that Chd7 acts preferentially to alter chromatin accessibility of key genes during the transition of NPCs to neurons to promote differentiation (Yao et al., 2020). Further altered expression of the homeobox genes *Otx2* and *Gbx2* in the developing neural tube of *Chd7* mutant embryos was identified. Expression of the Fgf8 gene is sensitive to *Chd7* gene dosage and they have an epistatic relationship during cerebellar vermis development (Basson, 2014). *In vitro* studies suggested that CHD7 may directly regulate Bmp4 expression by binding with an enhancer element downstream of the Bmp4 locus (X. Jiang et al., 2012).

It is clear from the studies conducted across the different model organisms that CHD7 plays an important role in early embryonic and specific organ system development. CHD7 is globally expressed in the mouse ESCs and hESCs and gets restricted to specific organ systems along development. CHD7 regulates developmental stage specific and organ specific gene expression by binding and altering the chromatin accessibility of enhancers and promoters, thereby positively and negatively altering target gene transcription in specific organ systems.

1.3.3 A focus on the role of *CHD7* in neurodevelopment

CHD7 is well expressed throughout early stages of development, particularly in the undifferentiated neuroepithelium, NCCs, foetal brain and cranial nerves during development (in humans), NCCs, NSPC during early brain development and SVZ derived Sox2+ and GFAP+ and SGZ regions of neurogenesis of adult brain (in mice) and throughout early brain

development (in zebrafish) (Patten et al., 2012; Sanlaville et al., 2006; Yao et al., 2018). Through the studies particularly in mouse, zebrafish, hESC derived NSCs, there have been interesting revelations about the role of CHD7 in CNS development. hESC carrying intronic variant of CHD7 showed development delay and maturity defects in the forebrain neuronal lineage. There was a delay in the differentiation of neurons supported by increased Sox2+ cells and a reduced TUJ+ mature neurons (R. Zhang et al., 2021). Loss of CHD7 affects differentiation of mouse derived ESCs into NSCs derived neurons and glial cells. Further, loss of CHD7 also affected the number, length, and complexity of neurites in the neurons differentiated from the NPCs (Yao et al., 2020). shRNA derived knockdown of CHD7 in iPSC derived neuroepithelium cells causes loss of neuroepithelial identity (Chai et al., 2018). cKO of CHD7 in the SVZ, SGZ, and Nestin+ cells show neurogenesis defects that include decrease in proliferating cells and an increase in the GFAP+ cells in the SGZ and SVZ. Cultured neurospheres of neural progenitors derived from adult and CHD7 embryonically cKO mice showed defects in proliferation, self-renewal and differentiation that was rescued by dosage dependent RA treatment (Micucci et al., 2014). In another study, a cKO of CHD7 from SGZ derived neural culture showed that CHD7 is involved in maintaining NSPC quiescence and differentiation and maintaining NSCs (Jones et al., 2015). cKO of CHD7 in GCPs has been shown to cause a decrease in GCP proliferation and reduction in differentiation leading to cerebellar hypoplasia(Whittaker, Kasah, et al., 2017). CHD7 regulates GCPs genes in conjunction with TOP2b and an overexpression of Reelin rescues the cerebellar hypoplasia (W. Feng, Kawauchi, et al., 2017). Further, CHD7 is also involved in the differentiation of OPC into OLs, and in the myelination and remyelination in the CNS (He et al., 2016). CHD7, in conjunction with Sox2, is important in initiating OPCs into remyelination after a spinal cord injury (Doi et al., 2017).

Studies from mouse, drosophila and zebrafish have particularly shown an important role of CHD7 in brain development and behaviour. CHD7 plays a role in forebrain, cerebellum, cranial nerve, retina, inner ear and synaptic development and in maintaining synaptic integrity at the NMJ. Knockdown of CHD7 in drosophila presented with motor and memory deficits, loss of CHD7 in mice leads to a head-bobbing and circling behaviour. While in zebrafish, heterozygous loss of CHD7 leads to anxious like and aggressive like behaviour contributed by abnormal glycine receptors. However, little is still known about the specific molecular and cellular substrates in the brain that underline the behavioural manifestations observed in the models for CHARGE syndrome. Further characterization of the brain developmental aspects that may underline behaviour is necessary to identify potential therapeutic targets.

1.4 Zebrafish as a Model organism

1.4.1 Benefits of Zebrafish

Zebrafish (*Danio rerio*) are a small tropical fish species endemic to south asia. They were first used as a model organism by George Streisinger in the 1980s for their ease in manipulation. Since then, zebrafish have been extensively used as a model organism for developmental, genetic, molecular, environmental and toxicology studies (Langheinrich, 2003; McGrath & Li, 2008; Scholz et al., 2008). They are highly convenient to rear and can be housed at relatively higher densities, in aquariums, reducing the animal husbandry time and costs. The adult fish breed often (close to once every 10 days), gain sexual maturity within 2.5-3 months from fertilization and the female fish can lay up to 200 eggs in each round of mating. The eggs undergo external fertilization, and the larvae develop in near transparent embryos making them perfect for observing and studying early vertebrate development (Kimmel, Ballard, Kimmel, Ullmann, & Schilling, 1995; McGrath & Li, 2008). The larvae have a fast developmental cycle, and the fish have a short generation time (Kimmel et al., 1995) Figure 4.

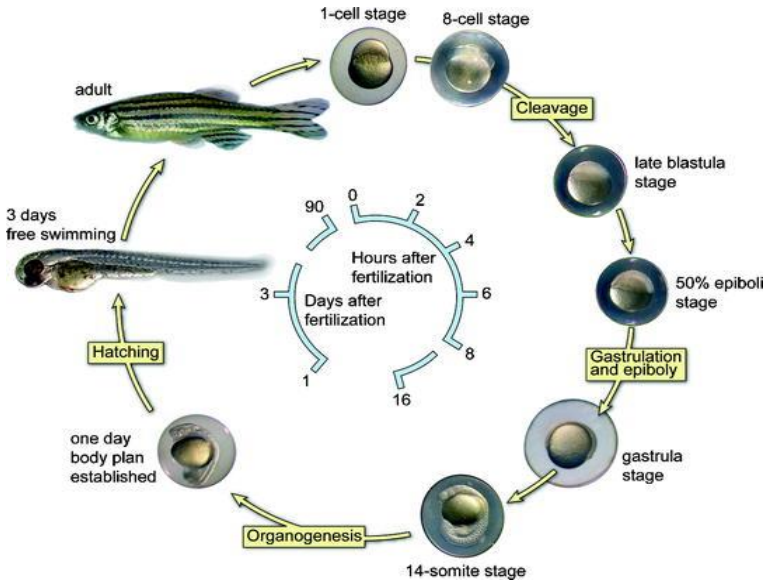

Figure 4 Zebrafish Developmental cycle
The above schematic marks the major developmental stages during the development of the zebrafish from a single celled embryo to an adult fish.
Adapted from (Willemsen, Padje, van Swieten, & Oostra, 2011)

The zebrafish genome is fully sequenced, has about 70% homology to the human genome and most importantly hosts counterparts for about 80% of the human disease-causing genes (Howe et al., 2013). A point of consideration in employing zebrafish for genetic manipulations is that the *Danio rerio* genome went through a genomic duplication event during the teleost evolution. This event caused an initial increase in the number of protein coding genes, that later led to the loss of many ohnologs over the period of further evolution. Thus, many but not all human genes have two zebrafish counterparts. Systematic exposure to large-scale forward genetic screens focusing on embryonic and larval stages have identified over 400 different mutations in the genome affecting development and physiological processes, with most of them having higher vertebrate or human orthologs reinforcing the suitability of the zebrafish as a highly useful model for studying vertebrate gene functions.

Zebrafish are highly amenable to forward and reverse genetic approaches alike. The external fertilization makes it easy to access and inject the single cell embryo with engineered DNA and RNA to effect genetic mutations/alterations. Multiple gene editing tools have been easily and successfully employed in Zebrafish like – Zinc finger nucleases (ZFNs), TALENs, Morpholinos, CRISPR/Cas9, Gal4/UAS systems, TILLING, microRNA and RNAi techniques. The ease of manipulation has also led to the creation of a vast library of cell specific transgenic fluorescent lines. Zebrafish is a powerful system that can be further employed to elucidate gene functions in context of a lot of complex vertebrate developmental processes.

After fertilization, the zebrafish body structural layouts develop rapidly over the first 24 hours post fertilization (hpf) and most of the organs are fully developed by 120 hpf (~5 days post fertilization) (Kimmel et al., 1995). The preliminary yet functional central nervous system structures form within 3 days post-fertilization (dpf) with majority of the brain regions and networks developed by 5 dpf. In the brain, zebrafish have evolutionarily conserved neuronal populations with functional homology to mammals. (Ganz et al., 2014; Lal et al., 2018; Mueller, Dong, Berberoglu, & Guo, 2011; Nieuwenhuys, 2011; Stednitz et al., 2018). The external fertilization, fast development in transparent embryos, and larvae, along with the availability of a wide range fluorescent genetic lines, make in vivo studies on brain development, direct manipulations, and live observations of development extremely convenient.

Behaviourally, the zebrafish larvae develop motility phenotypes in the form of spontaneous bursts as the early neuron connections form as early as 20 hpf. The larvae start to respond to the touch stimuli by 2 dpf and gain the ability to swim independently by 4/5 dpf. Their swimming behaviour can be used as a direct readout of accurate development and can be easily recorded and assessed in response to genetic manipulations or exposure to various drugs and small molecules. The transparent larvae and neuron specific fluorescent lines can

be employed here as well in studying the effect of small molecules on a distinct, relevant population of neurons via accessible in vivo and live imaging. Further, along with developmental studies, the fast development, high clutch size, small size and early developed swimming behaviours make zebrafish a very useful model for thigh throughput phenotypic drug screenings as well (Asad et al., 2016; Babu et al., 2018; Basu et al., 2018; P. Y. Lam & Peterson, 2019).

1.4.2 Early brain development

The central nervous system (CNS) of vertebrate organisms develops from a specialized region of the ectoderm called the neuroectoderm or neural plate that is specified during gastrulation. This neural plate transforms into a neural tube in a process known as neurulation. In most vertebrate organisms, neurulation occurs via a folding mechanism in which the lateral edges of the neural plate come together and fuse at the dorsal midline of the tube. The zebrafish neural tube formation is initiated as early as 10–11 hpf. However, in contrast to other vertebrates, neural plate is converted initially into a solid structure first- the neural keel, which then develops a central canal by detachment of cells, by 18hpf. This canal then subsequently forms the ventricular system of the brain and spinal cord (Ciruna, Jenny, Lee, Mlodzik, & Schier, 2006; Hong & Brewster, 2006; Lowery & Sive, 2005; Papan & Campos-Ortega, 1997; Schmitz & Campos-Ortega, 1994) (Figure 5, A).

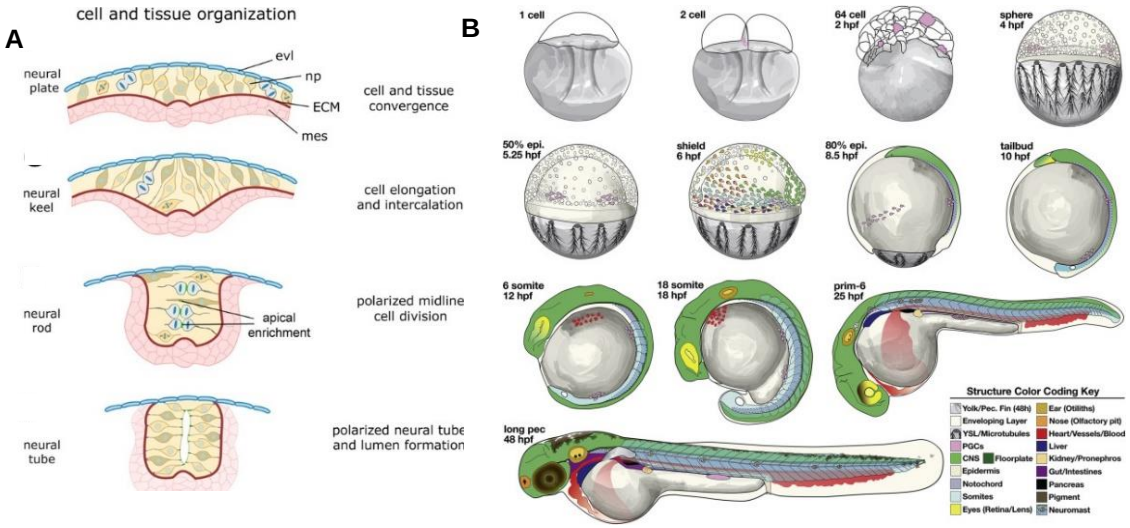

Figure 5 Zebrafish development.
A**:** Describes the process of zebrafish neurulation. B: Describes the development of the major Zebrafish organ system development upto 48 hours post fertilization. All the major organ systems are in place the larva by 48 hours post fertilization.
Adapted from: (Araya, Ward, Girdler, & Miranda, 2016; Pathak & Barresi, 2020)

Although neurulation in the zebrafish starts differently compared to other vertebrates, the eventual result is the generation of a conventional neural tube composed of a polarized epithelium that forms the brain and spinal cord ventricular system comparable to other vertebrate organisms (Clarke, 2009; Harrington, Chalasani, & Brewster, 2010; Lowery & Sive, 2004). By 24 hpf, most CNS structures can be identified, and the forebrain, midbrain and hindbrain structures can be distinguished. Focusing on the brain - the structures form along the ventricles of the neural tube in a rostro-caudal manner – forebrain, midbrain, and hindbrain. The midbrain – hindbrain boundary is strongly corroborated as the first singularly definable boundary and signalling centre in the brain with another centre at the forebrain-midbrain boundary. The forebrain consists of the telencephalon and diencephalon. Most prominently, the telencephalon consists of the pallium, subpallium and the olfactory bulb. The diencephalon mainly consists of the thalamus, prethalamus, hypothalamus, posterior tuberculum, habenulae, pretectum and pre-optic area. The midbrain consists of the optic tectum, torus semicircularis, torus longitudinalis and the midbrain tegmentum. And finally, the hindbrain consists of the cerebellum and the medulla oblongata (Zebrafish UCL).

Neurogenesis in zebrafish consists of two stages - primary and secondary neurogenesis. Primary neurogenesis of the central nervous system, particularly the brain is mostly complete by 2 dpf. Starting 3 dpf the post embryonic secondary neurogenesis starts which in part carries on till about 10 dpf. The neurons from primary neurogenesis differentiate and establish many of the initial projections and neuronal scaffolds within the first 24hpf. The first neurons become post-mitotic in the neural plate shortly after gastrulation and contribute to the spontaneous bursts of the larval motility. The secondary neurogenesis basically heavily adds neurons, derives mature brain structures and builds on the primary scaffold of the neural circuits, thereby ensuring basic functions of the autonomous zebrafish larvae are established. Neuromodulatory monoaminergic circuits, neurohormones, and other regulatory systems involving excitatory and inhibitory neurotransmitters (glutamate, aspartate, GABA, glycine) are mostly established during the secondary phase of neurogenesis. Fate maps have been generated to define domains that will become part of the eyes, forebrain, midbrain, hindbrain, and spinal cord (Figure 5, B) (Kimmel, Warga, & Schilling, 1990; Woo & Fraser, 1995).

Dynamic expression patterns of several morphogens, pro-neural genes, neurogenic genes, and developmental pathways orchestrate the proper genesis, migration, and organization of neurons and neuronal circuits in the zebrafish brain. The major contributors to this phenomenon have been well conserved in zebrafish and include the BMP morphogens, Wnt signalling pathway, Fgfs, Delta/Notch signalling molecules, basic helix-loop-helix (bHlH) genes like NeuroD1, Neurogenin1, Ascl1a/b among others (Atlas of early zebrafish brain

development by Muller and Wulliman 2015, Book Chapter by Alunni et al 2020). Each of these genes have a spatio-temporal dynamic expression pattern, during primary and secondary neurogenesis that guides the rapid development of the larval zebrafish brain. Once the neurons begin to be generated from neuroepithelial cells they get assembled into a primary neuronal network. The neural tube becomes multi-layered, and a centrifugal cellular organization becomes visible. The layer of cells located around the ventricular zone, are the dividing neural progenitors and the newly generated neurons populate more basal layers (Mueller & Wullimann, 2003; Wullimann & Knipp, 2000). The expression of stem cell markers, such as nestin, musashi1&2, and sox2, is restricted to the ventricular cells (Dirian et al., 2014; Mahler & Driever, 2007; Okuda et al., 2006; Shibata et al., 2012). In most parts of the brain, ventricular cells, while retaining some epithelial characters, also adopt new cellular phenotype, characterized most notably by a radial process reaching the pial surface called radial glia cells (RGCs). RGCs express astroglial markers that include the glial fibrillary acidic protein (GFAP), the brain lipid binding protein BLBP (fabp7a), and glutamine synthetase GS (Bernardos & Raymond, 2006; Dirian et al., 2014; Esain, Postlethwait, Charnay, & Ghislain, 2010; Johnson et al., 2016; H. G. Kim et al., 2008; C. S. Lam, Marz, & Strahle, 2009; Lyons, Guy, & Clarke, 2003). In the second phase of embryonic neurogenesis, RGCs are responsible for the generation of large number of neurons and glia, that increase the complexity of the primary neuronal network (Dirian et al., 2014; Duncan, Lowe, Dalton, & Valenzuela, 2016; Esain et al., 2010; Galant et al., 2016; Johnson et al., 2016; Lyons et al., 2003). Observing the expression of these markers in correlation to the ventricular neuronal proliferation, neuronal migration and differentiation can help ascertain brain development, neurogenesis, and differentiation patterns in the zebrafish brain (Book Chapter by (Alunni, Coolen, Foucher, & Bally-Cuif, 2020)).

Early forebrains of mice (E12.5/13.5) and zebrafish (3 dpf) show highly comparable expression patten of pro-neural and neural genes, and even the expression patterns of specified GABA/GAD positive neurons (Wullimann, 2009).The zebrafish brain shows great similarity to humans in containing all the major structures and their derived functions (Figure 6). Many regions of the zebrafish brain have been identified as neuroanatomical homologues of the mammalian counterparts, except the

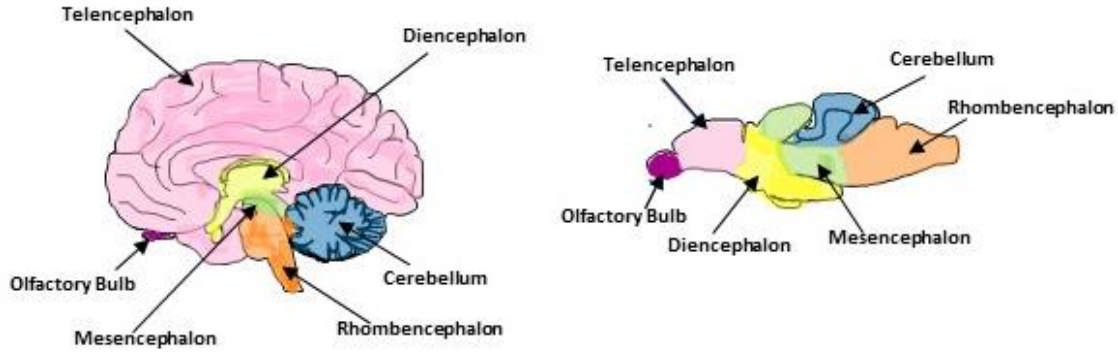

Figure 6 Structural homology between the human and zebrafish brains.
The Zebrafish brain represents structural and functional homologs of the human brain as described in the in the figure above

telencephalon structure, which differs from mammals due to early developmental differences (Rink & Wullimann, 2002a). Although simpler in form, the conserved brain structures in zebrafish are composed of the same cell types that follow similar specification and differentiation pathways as in mammals and perform similar functions, thus supporting the use of zebrafish as a model to study neurodevelopmental disorders (Vaz, Hofmeister, & Lindstrand, 2019). The zebrafish neuronal subtypes are similar to the mice and higher mammalian counterparts and are also marked by the same proteins - Glutamatergic excitatory neurons expressing glutamate transporter (Vglut); glycinergic inhibitory neurons expressing glycine transporter (Glyt), GABAergic neurons expressing GAD/GABA; cholinergic neurons expressing acetylcholine, typically excitatory; and aminergic neurons expressing other neuromodulatory molecules like dopamine (expressing TH), noradrenergic, serotonin (5-HT), and histamine. Neurotransmitter systems of the CNS relevant to development, functioning and neuropsychiatric diseases and disorders are also well conserved in the zebrafish brain and have been well characterized, including neurotransmitters like GABA, glutamate, dopamine, noradrenaline, adrenaline and serotonin (Panula et al., 2006). All the neuronal subtypes are present in most parts of the CNS and can generate a wide range of zebrafish behaviours upon defects in development or functioning (McLean & Fetcho, 2004; Pilorge et al., 2016; Rink & Wullimann, 2002b; Robles, Smith, & Baier, 2011).

1.4.3 The zebrafish midbrain

The midbrain consists of the optic tectum, torus semicircularis, torus longitudinalis and the midbrain tegmentum. One of the central processing centres of the teleost brain, the optic tectum, forms a big part of the midbrain. In vertebrates, the superior colliculus, or optic tectum, is a highly laminated structure located in the midbrain. It receives afferent inputs from multiple sensory regions of the brain, and contains intricate and overlapping topographic maps of the

sensory world (Chabot, Mellott, Hall, Tichenoff, & Lomber, 2013; Drager & Hubel, 1976; Druga & Syka, 1984; Jay & Sparks, 1987; King, Schnupp, Carlile, Smith, & Thompson, 1996; Krauzlis, Lovejoy, & Zenon, 2013; Lane, Allman, Kaas, & Miezin, 1973; May & Gaser, 2006; Meek, 1983; Robinson & McClurkin, 1989; Sparks, 1988; Sparks & Hartwich-Young, 1989). In contrast to mammals, amphibians and fish lack a visual cortex (Lazar, 1973) but have a proportionally larger tectum that carries out most of the visual processing that the cortex performs in mammals (Nevin, Robles, Baier, & Scott, 2010; Orger, 2016). In zebrafish, the tectal afferents arrive in the tectal neuropil, which comprises of (from dorsal to ventral): the stratum fibrosum marginale (SM), the stratum opticum (SO), stratum fibrosum et griseum superficiale (SFGS), stratum griseum centrale (SGC) and the stratum album centrale and stratum griseum periventriculare (SAC/SPV) layers (Meek, 1983; Sas & Maler, 1986; Vanegas, Amat, & Essayag-Millan, 1974). The neuropil receives robust innervation from the axons of retinal ganglion cells (rGCs), which convey visual information to the tectum that it processes and further passes on to the rest of the brain for relevant action (Corbo, Othman, Gutkin, Alonso Adel, & Fulop, 2012; Fiebig, Ebbesson, & Meyer, 1983; Niell & Smith, 2005; Stuermer, 1988).The optic tectum cell bodies are majorly located in the SPV zones with few scattered cell bodies in the neuropil and consists majorly of GABAergic and glutamatergic neurons with rare occurrence of cholinergic neurons(Robles et al., 2011) .

1.5 GABAergic neurons in the brain

Gamma-aminobutyric acid (GABA) and glutamate neurotransmitter secreting neurons form the main inhibitory and excitatory neurons of the mammalian brain, respectively. GABA is also an excitatory neurotrophic factor during early brain development until the initial cortical network is established, and influences early proliferation, neuronal migration, synapse formation and neurite growth (Ben-Ari, 2002; Peerboom & Wierenga, 2021). There occurs a postnatal switch in the later parts of development where GABA shifts to its hyperpolarizing role. In zebrafish as well, GABA acts as an excitatory neurotrophic factor and makes the shift to hyperpolarization around 60 hpf (~3 dpf) (Reynolds et al., 2008; R. W. Zhang, Wei, Xia, & Du, 2010).

GABA is an amino acid neurotransmitter synthetized from glutamate in a reaction catalysed by glutamate decarboxylase (GAD 65/67). Firing of GABAergic synapses takes place mainly through two kinds of receptors, GABA A and GABA B. The GABA A receptors are fast selective ion channels (ionotropic receptors), mainly chloride ion channel receptors that enable flow of chloride into the cytoplasm setting up the gradient for an inhibitory firing potential. GABA A receptors are known to act along with chloride – cation co-transporters (CCCs – NKCCs and KCCs) during early development. The relative increase in the number of NKCC transporters (Sodium-Potassium and Chloride co-transporters, intakes chloride into the cytoplasm) as compared to KCC transporters (Potassium and double Chloride co-transporters, that extrude chloride out of the cytoplasm) sets up a reverse chloride gradient thereby contributing to the early excitatory roles of GABAergic neurons. Later in development, an increase in the KCC transporters reverses the chloride gradients turning GABAergic neurons into inhibitory neurons (Bormann, Hamill, & Sakmann, 1987). The second kind of receptors are the slower G-protein coupled GABA receptors (metabotropic receptors) – GABA B. Activated GABA B receptors decrease adenylate cyclase activity (Wojcik & Neff, 1984), calcium membrane conductance, and increase potassium ions flow (Bowery et al., 2002).

In zebrafish, GABA, and glutamate decarboxylase (GAD) have been well characterized in the central nervous system (Delgado & Schmachtenberg, 2008; Kim, Wan, Mathers, & Puil, 2004). Zebrafish have three isoforms of GAD of which, two- gad1a and gad1b, resemble the mammalian GAD67 and the third- gad2, is homologous to GAD65. There are 22 genes encoding for GABA A receptor subunits and 7 subunit-like genes in zebrafish. And there are three zebrafish genes for the GABA B receptor- two, gabbr1a and gabbr1b, are homologous to the human gene for subunit B1 and a third one, gabbr2, to human B2 (Delgado & Schmachtenberg, 2008; Kim et al., 2004).

GABAergic neurons are morphologically, chemically, electrically and connectionally a heterogenous group of inhibitory neurons. They can be further classified into various subtypes based on the expression of calcium binding proteins like calbindin (CB), calretinin (CR), parvalbumin (PV), and/or neuropeptides like somatostatin (SOM), neuropeptide Y (NPY), vasoactive intestinal peptide (VIP), cholecystokinin (CCK) and combinations of these subtypes. Although, unlike the mouse and higher mammalian organisms, the different GABAergic neuronal subtypes have not yet been well characterized in the zebrafish brain. Retrograde tracing studies have however shown the distribution of calcium binding calbindin, calretinin and parvalbumin neurons in the adult zebrafish spinal cord and descending neurons of the hindbrain region (Berg, Bertuzzi, & Ampatzis, 2018).

1.5.1 GABA neuron development in the brain

During vertebrate neurodevelopment, the appropriate migration and positioning of interneurons plays an essential role in the proper establishment and functioning of the CNS. Common developmental programs give rise to GABAergic neurons in zebrafish and mouse (Wullimann, 2009). In zebrafish telencephalon, GABAergic neurons are generated near the medial subpallial ventricular wall as early as 24 hours post fertilization and during the larval stage they migrate from the ventral telencephalon to the dorsal telencephalon where they become post-mitotic (Mueller, Vernier, & Wullimann, 2006). Much has been studied about the general neurogenesis and the development of the GABAergic neurons in the telencephalon, diencephalon, and hindbrain regions of the zebrafish brain (MacDonald et al., 2013; Mack-Bucher, Li, & Friedrich, 2007; Miyake et al., 2014; Wullimann, 2009). However, precise migratory patterns of GABAergic neurons in the other parts of developing larval zebrafish brain (Solek, Feng, Perin, Weinschutz Mendes, & Ekker, 2017) and their development in the midbrain region of the zebrafish brain is very little understood. Functional studies show that visual information principally enters the neuropil in the optic tectum, and is progressively filtered by GABA superficial inhibitory neurons (SINs) and the GABA periventricular layer (PVL) interneurons, before being relayed to other regions of the brain by the PVL projection neurons (Barker & Baier, 2015; Del Bene et al., 2010; Gabriel, Trivedi, Maurer, Ryu, & Bollmann, 2012; Robles et al., 2011; Scott & Baier, 2009; Semmelhack et al., 2014; Temizer, Donovan, Baier, & Semmelhack, 2015).

Genes of the transcription factor encoding Dlx family have been shown to play essential roles in GABAergic neuron development and differentiation(Anderson et al., 1997). Studies using triple fluorescent hybridization have shown overlapping expressions of dlx1a and dlx5a and gad1b in the zebrafish forebrain at 24 hours post fertilization, indicating that the dlx genes could also be used as markers for GABAergic neurons (MacDonald, Debiais-

Thibaud, Talbot, & Ekker, 2010). The dlx genes function in bigene clusters as dlx1a/2a, and dlx5a/6a. The intergenic sequence of the zebrafish dlx5a/6a cluster is sufficient to drive reporter gene expression effectively mimicking endogenous expression of dlx5a/6a (M. Yu et al., 2011). The intergenic sequence driven transgenic line Tg(dlx5a/dlx6a:GFP) in fact successfully expresses GFP in all of the GABAergic neurons of the zebrafish brain and can thus be effectively used to track GABAergic neuron development (Figure 7).

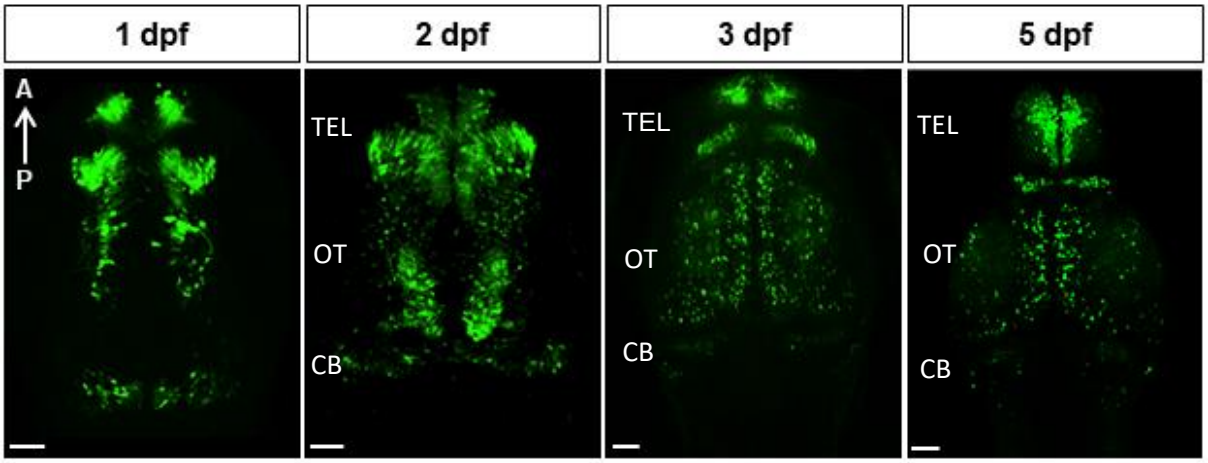

Figure 7 GABAergic neurons in the larval zebrafish brain.
The figure shows Dlx5a/6a:GFP marked GABAergic neurons in the zebrafish brain. The above panel represent dorsal zebrafish brains through stages 1 dpf to 5 dpf. The specific regions of the brain get easier to identify post 2 dpf during the larval development
A: anterior of the brain, B: posterior of the brain, TEL: telencephalon, OT: optic tectum, CB: cerebellum
Adapted from (Jamadagni et al., 2021)
Scale bar: 50µm

1.5.2 GABAergic network dysfunction in Neurodevelopmental Disorders

GABAergic neuron network defects have been identified to underline behavioural hyperactivity and seizures, among many other symptoms, in autism, epilepsy and fragile-x syndromes (Bozzi, Provenzano, & Casarosa, 2018; Cea-Del Rio & Huntsman, 2014; Pizzarelli & Cherubini, 2011; M. Y. Xu & Wong, 2018). GABAergic network defects in the form of defects/mutations in specific GABA A/B receptor subunits, mutations in GAD proteins, GABA neurotransmitter release, synaptic integrity/formation and lower number of GABAergic neurons have been reported as neuropathogenesis mechanisms and in some cases have contributed to the E/I (excitatory/inhibitor) imbalance as well (Cea-Del Rio & Huntsman, 2014; Coghlan et al., 2012; Liao et al., 2019; Pizzarelli & Cherubini, 2011; Pocklington et al., 2015; Rubenstein & Merzenich, 2003). An E/I imbalance has been reported as a pathogenic mechanism underlining multiple neurodevelopmental disorders like autism, epilepsy, fragile x syndrome, rett syndrome, Tourette syndrome, schizophrenia and bipolar depression (Chattopadhyaya & Cristo, 2012; Jankovic & Kurlan, 2011; Kalanithi et al., 2005; Volk, Edelson, & Lewis, 2016)

Autism spectrum disorders are neurodevelopmental disorders that are typically characterized by challenges in social and communication skills, repetitive and restrictive behaviours, and intellectual disability. Involvement of GABAergic neurons has been well reported in the pathogenesis of ASD (Pizzarelli & Cherubini, 2011). Alterations in levels of glutamate and glutamine in the cortex and basal ganglia of children, and adults with ASD has been revealed in vivo (Horder et al., 2018). GABA synthesizing enzymes GAD65 and GAD67 are significantly reduced in post mortem tissues of individuals with autism (Fatemi et al., 2002). Variations in the number of GABA neurons' subtypes in specific regions of the brain like the cerebral cortex and hippocampus have been reported to cause an E/I imbalance in the context of autism and epilepsy (Sgado et al., 2013). Reduced GAD65/67 expression and abnormalities in genes coding for subunits of the GABA A receptors like *GABRB3, GABRA5* and *GABRG3*, due to microdeletion / microduplication CNVs in the chromosome 15q11-q13 region causes ASD (Coghlan et al., 2012). The absence of contactin associated protein-like 2 (CNTNAP2), an autism-related gene, leads to a decrease in the number of parvalbumin and calretinin interneurons and thereby to autism-related deficits in mice (Abraham, Bai, & Leube, 2011). Haploinsufficiency of SYNGAP1- an autism risk gene, has been reported to cause a decrease in perisomatic innervations in parvalbumin neurons in a cell autonomous manner. Further, a MGE GABAergic specific haploinsufficiency impairs their connectivity, reduces inhibitory synaptic activity of GABAergic neurons and cortical gamma oscillations (Berryer et al., 2016). A deficit specifically in the forebrain GABAergic neurons, with potential

functional dysregulation of GABAergic and glutamatergic systems was tested via pharmacological treatments in a zebrafish model for the CNTNAP2 autism risk gene (Hoffman et al., 2016).

In mouse models as well as in human temporal lobe Epilepsy (TLE) a loss of the GABAergic neurons has been reported (Bernard, Cossart, Hirsch, Esclapez, & Ben-Ari, 2000). Post-mortem brain neuropathological studies have demonstrated an association of Tourette syndrome (TS) to possible defects in the migration of GABAergic neurons. A profound parvalbumin-positive neuronal imbalance in the basal ganglia of individuals with TS has been reported indicating a defect in the migration of GABAergic neurons (Kalanithi et al., 2005). Defects and alterations in both presynaptic and postsynaptic components of GABA neurotransmission with a particular involvement of the parvalbumin-positive neurons plays an important role in the pathophysiology of schizophrenia and its cognitive deficits (de Jonge, Vinkers, Hulshoff Pol, & Marsman, 2017; M. Y. Xu & Wong, 2018). A functional GABA A signalling deficit has been reported in several regions of the Fragile X syndrome mouse brain (El Idrissi et al., 2005). GABAergic abnormalities in the thalamus and medulla develop in *Mecp2*-null Rett syndrome mice even prior to the onset of symptoms (Medrihan et al., 2008; Z. W. Zhang, Zak, & Liu, 2010).

CHARGE syndrome shares overlapping behavioural symptoms with hyperactivity and ASD (Hartshorne, Hefner, et al., 2005) and there could be converging neuropathogenesis mechanisms that underline these behaviours. Elucidating molecular and neurogenesis mechanisms involved in GABAergic neurons' development and functioning in the context of CHARGE syndrome could shed light on other potential neuropathogenesis mechanisms upon mutations in CHD7 and add to potential therapeutic targets.

2 Overview and Hypothesis.

Pathogenic variants of *CHD7*, an ATP-dependent chromatin remodeller have been identified as a major cause of CHARGE syndrome. Individuals with CHARGE consistently present with brain developmental defects and behavioural anomalies. The brain developmental defects typically include microcephaly, cerebral atrophy, cerebellar and olfactory bulb hypoplasia among other varying defects (Bergman, Janssen, et al., 2011; T. Yu et al., 2013). Behaviourally, individuals with CHARGE often present with cognitive and intellectual disability, social immaturity, and have symptoms overlapping with hyperactivity disorder, ADHD, OCD and ASD (Hartshorne, Grialou, & Parker, 2005; Hartshorne, Hefner, et al., 2005; O'Roak, Vives, Fu, et al., 2012; Smith, Nichols, Issekutz, Blake, & Canadian Paediatric Surveillance, 2005). In fact, pathogenic variants in *CHD7* have also been associated with autism spectrum disorder (ASD) (O'Roak, Vives, Fu, et al., 2012). Evidently *CHD7* plays an important role in the development and functioning of the human brain. However, there is still no effective treatment or cure for the neurological deficits in CHD7-related CHARGE syndrome and ASD. Studies have shown that CHD7 is well expressed throughout early stages of development, with high expression in the brain (Sanlaville et al., 2006). There could be common neurological mechanisms that underline the shared phenotypes between CHARGE syndrome and the other neurodevelopmental disorders like ASD. Uncovering pathogenic mechanisms occurring in the central nervous system (CNS) will be key to understanding and treating autistic features as well as other neurological deficits due to CHD7 deficiency. Being a transcription regulator, *CHD7* potentially influences the expression of genes and regulatory networks that may play crucial roles in brain developmental processes and functioning. The overall goal is to understand the function of CHD7 in the CNS and the consequences of its loss to provide mechanistic insights and potential therapeutic targets for neurological symptoms of CS and perhaps ASD.

I, **hypothesize** that *CHD7* regulates the expression of genes that are crucial for proper neural network development, input balance and maintenance in the brain. Dysregulation of these genes upon mutations in *CHD7* contributes to the neuropathogenesis in CHARGE syndrome. Studying the brain developmental defects upon mutations in *CHD7* may add important knowledge toward understanding the mechanisms that underline the shared symptomology of CHARGE with other neurodevelopmental disorders like ASD.

Chd7-/- mice die at embryonic day 10.5 and is a roadblock for studying the role of *CHD7* in neurodevelopment *in vivo*. However, *Chd7+/-* mice are viable and phenocopy some aspects of CS features, often to a lesser severity. In addition to *Chd7+/-* mice, *Chd7* conditional

knockout mice and other models such as cell culture, drosophila, zebrafish and xenopus have provided insights on the function of CHD7. Loss of *Chd7* in neural progenitor cells led to increased cell death in both adult neurogenic regions and cerebellum and to a defect in terminal differentiation of mouse neural progenitor cells. (W. Feng, Kawauchi, et al., 2017; W. Feng & Liu, 2013; Whittaker, Riegman, Kasah, Mohan, Yu, Pijuan-Sala, et al., 2017). *CHD7* facilitates neural stem cell maintenance and proliferation in the developing brain, it is required for the formation of migratory neural crest cells (Schulz et al., 2014). Chd7 coordinates with Sox10 to regulate the initiation of myelination and is required for oligodendrocyte precursor cell survival (He et al., 2016). Genetic inactivation of Chd7 in cerebellar granule neuron (GN) progenitors leads to cerebellar hypoplasia in mice, due to impairment of GN differentiation (W. Feng, Kawauchi, et al., 2017; He et al., 2016; Whittaker, Kasah, et al., 2017). Although these are interesting findings, there has been limited focus on the inhibitory neuronal networks in the brain of these animals. Alterations in neural circuits development and function that may underlie the behavioural deficits in CHARGE syndrome upon loss-of-function of *CHD7* remain to be investigated. To explore this further, we will be utilizing the zebrafish as a model organism to study CHARGE syndrome. Zebrafish is an extremely favorable model organism to study of early development owing to its fast external fertilization in transparent embryos. It is amenable to genetic manipulations and large-scale studies because of its large clutch size and relatively short life cycle. The fish has a fully sequenced genome with nearly 70% homology to the human genome and representing more than 80% of the disease-causing genes (Howe et al., 2013; Kimmel et al., 1995).

Patten et al. (2012), previously showed that *CHD7* is conserved in zebrafish and is widely expressed throughout the early stages of development. In this study, Patten et al created a morpholino mediated knockdown of the *chd7* gene, and the morphant fish presented with characteristic CHARGE syndrome features along with nervous system defects, being the first to show that zebrafish can be a very useful model to understand the function of CHD7 in CHARGE syndrome pathogenesis (Patten et al., 2012). Subsequent studies in zebrafish by other groups have provided important insights into the pathogenesis of CHARGE syndrome (Asad et al., 2016; Balow et al., 2013; Cloney et al., 2018; Jacobs-McDaniels & Albertson, 2011; C. Liu et al., 2020; H. Liu & Liu, 2020; Z. Z. Liu et al., 2018; Patten et al., 2012). However, given the limitations of the morpholino models in sustaining effects into later stages of development and their transmissibility, it was important to create a more stable *chd7-/-* mutant model to characterize the complete effects of *chd7* loss of function, particularly on the brain, to investigate for a mechanism for the neuropathogenesis. To address this, the Patten lab created a zebrafish *chd7* knockout model using CRISPR/Cas9 gene editing approach.

2.1 Objectives and aims.

A zebrafish CRISPR/Cas9 mediated *chd7* knockout model was created in the lab and primary characterization of the *chd7-/-* mutant line was conducted in collaboration with master's student, Betelhem Kassa. We observed that *chd7*-/- mutants presented with some of the most characteristic features of CHARGE syndrome. The *chd7*-/- mutant fish particularly presented with a sharp decline in the survival 10 days post fertilization, heart developmental defects, craniofacial defects, cranial nerve defects along with consistent small head phenotype. Interestingly, the *chd7*-/- mutant fish exhibited a hyperactive swimming behaviour. Previously, such a hyperactive swimming behaviour has been reported to be underlined by defects in the development and functioning of the GABA neuron network in a zebrafish model for an ASD risk gene *CNTNAP2* (Hoffman et al., 2016). The development of GABAergic neurons upon loss-of-function of CHD7 has not yet been explored. Moreover, defects in the mechanisms by which GABAergic neurons develop have likewise not been analysed but may be of critical importance for understanding the cellular mechanisms that result in autistic-like behaviour and other neurological symptoms in CHARGE syndrome. Thus, the **objective** of my PhD project was to first characterize GABAergic network development in the zebrafish brain in our zebrafish *chd7-/-* mutant model for CHARGE syndrome and then investigate if a GABA neuron network defect could also potentially contribute to the behavioural deficits observed upon mutations in *chd7*. More importantly, another objective was to investigate the molecular mechanisms underlying the brain and behavioural defects upon loss of *chd7* function in zebrafish.

My specific aims during the PhD are:

Aim 1: Characterize the neurodevelopmental features in our zebrafish model of CHARGE syndrome.

Aim 2: To identify the roles of the downstream targets of *chd7* in brain development and behaviour.

Aim 3: To perform a phenotypic drug screen in our zebrafish model of CHARGE syndrome

Note: Experimental approach and findings of Aims 1, 2 and 3 are described in Section II, chapter 3 and 4, and forms part of a published article (Jamadagni et al., 2021 EMBO reports) and will compose a part of a paper in preparation.

SECTION II: RESULTS

3 Chromatin remodeller CHD7 is required for GABAergic neuron development by promoting PAQR3 expression

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Author Contributions:

SAP conceived this work. PJ designed, collected, analysed and interpreted the results from studies related to GABAergic neurons and behaviour in zebrafish. ES, MB collected and analysed the results of RNAseq. KS collected, analysed and interpreted the results from studies related to *C. elegans* experiments. BK, SAP generated and characterized the CRISPR *chd7-/-* mutant line. PJ and BK performed the drug analyses in zebrafish. TC performed the ChIP analyses. PJ, MB, KS, JAP, ES, TC, NP and SAP interpreted the results. JAP, NP and SAP secured the research funding. PJ, KS, TC, NP and SAP drafted the manuscript. PJ and SAP with contributions from all authors prepared the final version of the manuscript. All authors read the final version of this manuscript.

2.1 Abstract:

Mutations in the chromatin remodeller-coding gene *CHD7* cause CHARGE syndrome (CS). CS features include moderate to severe neurological and behavioural problems, clinically characterized by intellectual disability, attention-deficit/hyperactivity disorder, and autism spectrum disorder. To investigate the poorly characterized neurobiological role of *CHD7*, we here generate a zebrafish *chd7*−/− model. *chd7*−/− mutants have less GABAergic neurons and exhibit a hyperactivity behavioural phenotype. The GABAergic neuron defect is at least in part due to downregulation of the CHD7 direct target gene *paqr3b*, and subsequent upregulation of MAPK/ERK signalling, which is also dysregulated in *CHD7* mutant human cells. Through a phenotype-based screen in *chd7*−/− zebrafish and *Caenorhabditis elegans*, we show that the small molecule ephedrine restores normal levels of MAPK/ERK signalling and improves both GABAergic defects and behavioural anomalies. We conclude that *chd7* promotes *paqr3b* expression and that this is required for normal GABAergic network development. This work provides insight into the neuropathogenesis associated with CHD7 deficiency and identifies a promising compound for further preclinical studies.

Keywords: GABA, neurodevelopment, behaviour, CHD7, zebrafish

2.2 Introduction

Heterozygous loss-of-function pathogenic variants in *CHD7* are the major cause of a rare congenital disorder termed CHARGE syndrome (CS), which stands for Coloboma of the eye, Heart defects, Atresia of the choanae, Retardation of growth and/or development, Genital abnormalities, and Ear abnormalities (Pagon et al., 1981) (Janssen et al., 2012; Zentner et al., 2010). The mutations are equally distributed along the coding region of *CHD7* and the most prevalent types are nonsense mutations (44%), followed by frameshift deletions or insertions (34%) (Janssen et al., 2012). Although neurological abnormalities are not considered for clinical diagnosis of CS, many individuals with CS display moderate to severe neurological deficits, which include autism-like behaviour, obsessive-compulsive disorder, attention-deficit/hyperactivity disorder, anxiety, aggressivity and seizures (Bergman, Janssen, et al., 2011; Hartshorne, Grialou, et al., 2005; Hartshorne, Stratton, Brown, Madhavan-Brown, & Schmittel, 2017; Johansson et al., 2006; Souriau et al., 2005). Along these lines, *CHD7* mutations have been identified in individuals with autism spectrum disorder (ASD) (O'Roak, Vives, Girirajan, et al., 2012; Takata et al., 2018). These reports strongly suggest an important role for CHD7 in the development and functioning of the central nervous system. However, the precise mechanisms underlying the neurological deficits in CS remain poorly understood. A recent study reported anxious- and aggressive-like behaviours with increased expression of glycine transporters in adult *chd7* heterozygous mutant zebrafish, leaving however unexplored the molecular and cellular mechanisms of brain circuitry (H. Liu & Liu, 2020). Also, noteworthy, there are no pharmacological and/or genetic treatments to ameliorate/rescue CS-related neurological features. Current treatment options primarily focus on behavioural management as well as educational and physical therapies. Development of successful therapeutic strategies would benefit from the identification and targeting of causative factors.

Chd7-/- mice die *in utero* around embryonic day 10.5 (Van Nostrand et al., 2014), a stage incompatible for studying the role of CHD7 in the neuropathogenesis of CS. Additionally, *Chd7+/-* mice are viable and phenocopy a number of aspects of CS, but the full spectrum and severity of certain CS malformations are not seen (Payne et al., 2015). Yet, *Chd7+/-* mice, *Chd7* conditional knockout mice and other cellular and animal (*Drosophila*, zebrafish and *Xenopus*) models have provided insights on the general function of CHD7 (Belanger et al., 2018; W. Feng, Kawauchi, et al., 2017; Ohta et al., 2016; Patten et al., 2012; Schnetz et al., 2010; Whittaker, Riegman, Kasah, Mohan, Yu, Sala, et al., 2017). For instance, it has been shown that CHD7 is capable of both enhancing and inhibiting expression of embryonic stem cell genes (Schnetz et al., 2010). In that respect, CHD7 facilitates neural stem cell maintenance and proliferation in the developing brain (Ohta et al., 2016) and quiescence in

the adult (Jones et al., 2015). It is also required for the formation of migratory neural crest cells (Bajpai et al., 2010; Okuno et al., 2017). CHD7 coordinates with the transcription factor SOX10 to regulate the initiation of myelinogenesis (He et al., 2016) and is required for oligodendrocyte precursor cell survival (Marie et al., 2018). Genetic inactivation of *Chd7* in cerebellar granule neuron (GN) progenitors leads to cerebellar hypoplasia in mice, due to impairment of GN differentiation (W. Feng, Kawauchi, et al., 2017; Whittaker, Riegman, Kasah, Mohan, Yu, Sala, et al., 2017) but these cerebellar defects did not alter the social behaviour in these mice (Whittaker, Riegman, Kasah, Mohan, Yu, Sala, et al., 2017). Although these recent findings point to an important role of CHD7 in brain development, the precise neural substrates that may contribute to CS-associated neurological deficits such as autistic traits and/or hyperactivity disorder remain poorly understood. Emerging evidence suggests that abnormalities in inhibitory GABAergic neurons development / function in the context of neurodevelopmental disorders are characterized by a shared symptomatology of ASD symptoms (Coghlan et al., 2012; Rubenstein & Merzenich, 2003). Whether such alterations in brain inhibitory neural networks underlie the neurological deficits in *CHD7* mutation-positive cases of CS is currently unknown.

The zebrafish is a powerful tool for studying neurological diseases including ASD (Meshalkina et al., 2018; Stewart, Nguyen, Wong, Poudel, & Kalueff, 2014). Here, we report the generation of a *chd7-/-* mutant zebrafish line and show that these animals exhibit altered number and positioning of GABAergic neurons in the brain and display a hyperactive behaviour phenotype. Using genetic, pharmacological and biochemical approaches, we unravel the molecular mechanisms by which *chd7* regulates GABAergic network development and behaviour in zebrafish. Finally, through a chemical-genetic screen, we identified ephedrine that effectively ameliorates behavioural anomalies as well as the GABAergic defects in *chd7-/-* mutants. This study provides novel insights into the role of CHD7 in brain development and disease and has important translational implications.

2.3 Results

2.3.1 Zebrafish *chd7-/-* mutants display phenotypic characteristics of CHARGE syndrome

To investigate the neurobiological function of CHD7, we generated a *chd7* knockout zebrafish line using CRISPR/Cas9 to target the helicase domain of the *chd7* gene (17^{th} exon) for disruption. A positive founder transmitting a single nucleotide insertion causing a frame-shifting mutation was selected (Fig. 1A). This mutation causes a premature stop codon 8 amino acids after the mutation site (Fig EV1A). To assess whether the mutant *chd7* transcript

underwent nonsense-mediated decay upon that mutation, we performed qPCR. The relative abundance of *chd7* mRNA in mutant zebrafish was significantly decreased, suggesting a loss of mutant transcript via nonsense-mediated decay (Fig EV1B). While no major morphological differences were observed between wild-type (*chd7+/+)* and heterozygous (*chd7+/-*) fish (Fig. 1B), the survival rate of homozygous (*chd7-/-*) larvae sharply declined after 10 days postfertilization (dpf) (Fig. 1C). Remarkably, *chd7-/-* zebrafish larvae displayed a small head phenotype (Fig. 1D) compared to controls but nevertheless all the brain regions were fairly well-preserved in mutant fish (Fig EV1C). Additionally, *chd7-/-* zebrafish larvae exhibited a low frequency of pericardial edema (20 %) (Fig EV1D), cranial cartilage malformations (Fig EV1E) and cranial nerve defects (Fig. 1E). Notably, there were less arborizations of peripheral projections from the V^{th} cranial nerve in *chd7-/-* fish. Precisely, *chd7-/-* fish had reduced growth and branching of the peripheral axons, resulting in a significant decrease in the mean total length of the axon projections as compared to controls (Fig. 1E). Strikingly, these phenotypic characteristics are hallmarks of CS (Hsu et al., 2014). Additionally, this new *chd7-/-* mutant fish recapitulates other anomalies previously reported in *chd7* morphants (Patten et al., 2012) and other *chd7-/-* mutant zebrafish lines (Cloney et al., 2018; Z. Z. Liu et al., 2018), but with less pronounced cardiac defects and no apparent blindness, thereby making it an ideal model to investigate the pathogenic mechanisms underlying CS-associated neurological deficits. Behaviourally, *chd7-/-* larvae were significantly hyperactive compared to wild-type and *chd7+/-* fish larvae at 5dpf (Fig. 1F,G). This hyperactive phenotype was particularly prominent and persistent during the dark cycles (Fig. 1G).

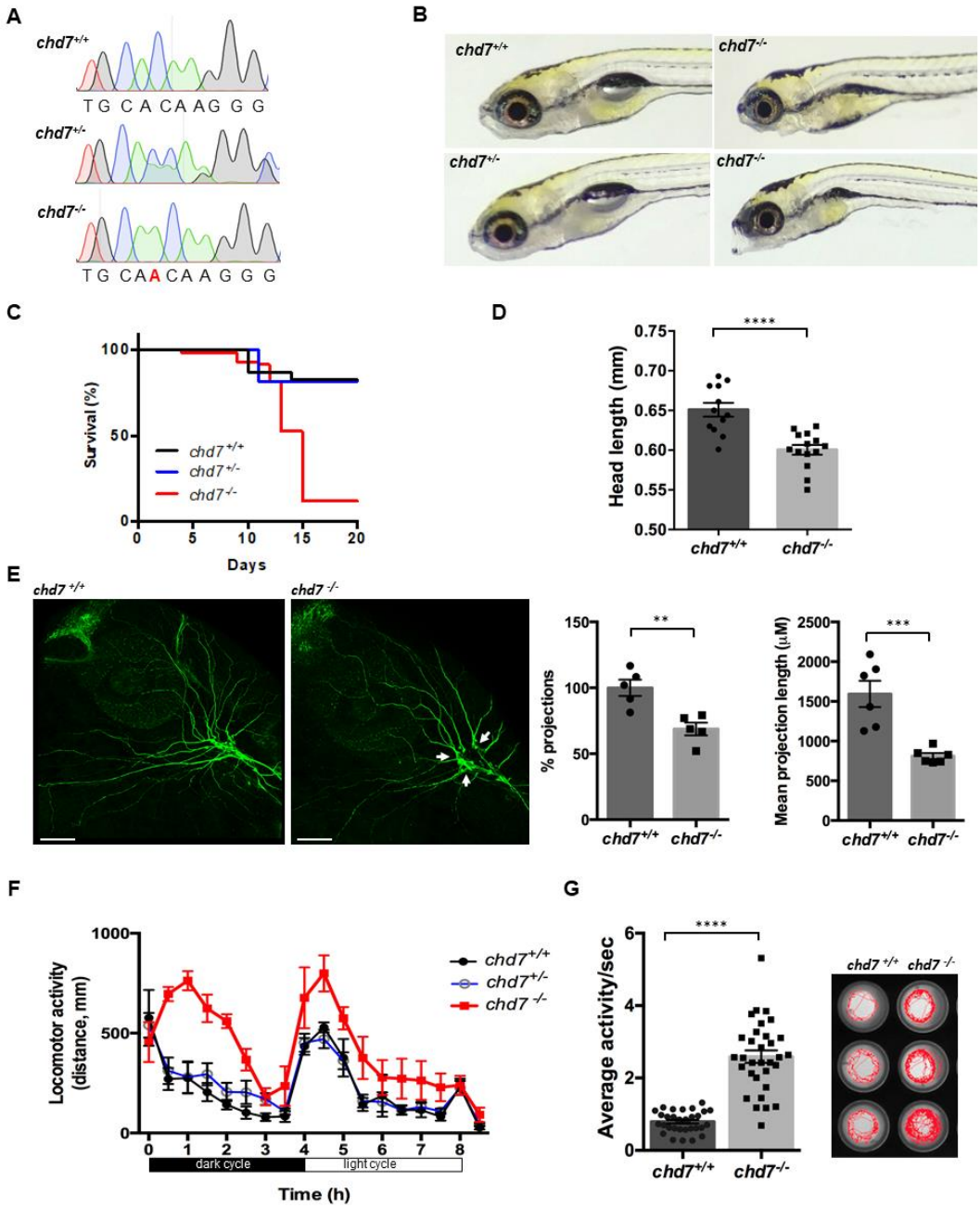

Figure 1 . Generation of a zebrafish *chd7-/-* mutant using CRISPR/Cas9

A Chromatograms showing the confirmation a 1-nucleotide insertion mutation by Sanger sequencing.
B Gross morphological analyses of control (*chd7*+/+; top left image), heterozygous (*chd7*+/−; bottom left image) and knockout mutants (*chd7*−/−; images in right panel).
C Kaplan–Meier survival plot showing low survival of *chd7-/-* mutants after 12 dpf (*N* = 5).
D Measurement of head size of control (*n* = 12) and mutants (*n* = 14) showing significantly smaller head size in *chd7*−/− fish (****$P < 0.0001$, Student's *t*-test).
E Acetylated tubulin staining in 28 hpf controls (left) and mutants (right) showing severely affected outbranching of the trigeminal nerve (Vth cranial nerve).
Notably, *chd7*−/− display reduced branching of the Vth cranial nerve (arrows) and axonal arborization in the tectal area. Graphs showing quantitative analyses of percentage (*n* = 5) and mean total length of peripheral projections (*n* = 6) per zebrafish in controls and mutants (***$P < 0.001$; **$P < 0.005$, Student's *t*-test).
F Locomotor activity of control (black), heterozygous (blue) and mutants (red) showing significant hyperactivity of mutants in dark and light cycles (*N* = 3, *n* = 48).

G Average activity per second during dark cycle (left) is significantly increased in *chd7*−/− mutants compared with *chd7*+/+ (*n* = 32; ****$P < 0.0001$, Student's *t*-test).
Representative swimming tracks during dark cycle of control and mutant fish (right). Mutant *chd7*−/− larvae displayed hyperactive swimming.
Data information: ****$P < 0.0001$; ***$P < 0.001$; **$P < 0.005$, Student's *t*-test. Data are presented as mean ± SEM. Scale bar = 50 μm. *n* represents number of fish. *N* represents number of experimental repeats.

2.3.2 GABAergic neuron differentiation is defective in chd7-/- mutants

A hyperactivity behavioural phenotype in ASD mouse (S. Lee et al., 2018) and zebrafish (Hoffman et al., 2016) models has been reported to be due to alterations in GABAergic interneuron development. To test if similar alterations in GABAergic neuron development occur in *chd7-/-* mutants, we analysed the inhibitory GABAergic neuronal populations in wild-type controls and *chd7-/-* mutants during early brain development (Fig. 2), using a transgenic line that labels GABAergic interneurons (Tg(*dlx5a/6a: GFP)*). Compared to controls, *chd7-/-* larvae had a significant reduction in the density of GFP-positive GABAergic cells in the brain at 5 dpf (Fig. 2A,B). Particularly, we observed a highly significant decrease in the number of GABAergic neurons in the optic tectum (OT) (Fig. 2C,D) and a near complete loss of GFP positive cells in the cerebellum (CB) compared to the controls. Reduced number and malpositionning of GABAergic cells were also observed in the hypothalamus (HYP), (Fig. 2E,F) and in the telencephalon (TEL) (Fig. 2G,H).

We also examined the development of GABAergic neurons in *chd7-/-* brain throughout major developmental phases between 1 to 5 dpf (Fig EV2A). The reduced number of GFP positive GABAergic neurons occurs very early in *chd7-/-* embryos, with a striking decrease of GABAergic neurons posteriorly between 1 and 2 dpf (Fig EV2A). We next tested whether the reduced number of GABAergic neurons in *chd7-/-* fish could be due to reduced proliferation, defects in neuronal differentiation and/or enhanced cell death. The zebrafish CNS proliferative profile is still very high at 2 dpf and is rapidly downregulated up to 5 dpf(Wullimann & Knipp, 2000). At 2 dpf, we did not observe a change in either the proliferation marker pH3 (Fig EV2B,C) or differences in the number of apoptotic cells (Fig EV2D). Additionally, we did not notice differences in the number of double-positive cells in pH3 and NeuroD1 (neuronal progenitor marker) co-staining (Fig EV2E). However, at 5 dpf, while no apoptosis was observed, a significant increase in pH3 positive cells was detected (Fig EV2F-H), suggesting a failure in differentiation of progenitor cells into GABAergic neurons.

We, thus, next sought to evaluate further neurogenesis in *chd7-/-* mutants during brain development, with a focus on the midbrain region - the brain region where the reduced number of GABAergic neurons was more prominent in 5 dpf *chd7-/-* fish. Zebrafish larvae

(4 dpf) were exposed to BrdU-containing media for 24h and fixed. In both *chd7*+/+ and *chd7*-/- fish, BrdU-labelled cells were noted in the medial tectal proliferation zone (m), dorsal thalamus (DT), posterior tuberculum (PT) and the lateral tectal proliferation zone (l) of the midbrain (Fig EV3A,B). Interestingly, compared to *chd7*+/+, BrdU-labelled cells in *chd7-/-* did not migrate over long distances to reach the early migrated region of pretectum and proglomerular (Fig EV3A; asterisks), which are regions involved with visual and other sensorimotor circuits (Yamamoto & Ito, 2008). We also found an increased in the number of BrdU-labelled cells in *chd7-/- mutant* fish compared to wild-type controls (Fig EV3C).

In order to determine the phenotype of BrdU-positive cells after the 24h incubation period, brain sections were double-labelled to detect the colocalization of BrdU with HuC/D (a neuronal marker; Fig EV3A,B,D,E) , *dlx5a/6a*-GFP (a GABAergic neuron marker; Fig EV3F,G) or NeuroD1 (a neuronal progenitor marker; Fig EV4A-D) in the midbrain area of the 5 dpf zebrafish larvae. The number of cells positive for both BrdU and HuC/D (Fig EV3E), BrdU and *dlx5a/6a*-GFP (Fig EV3G) or BrdU and NeuroD1 (Fig EV4D) in the midbrain area, surrounding the proliferation zone m, of the 5 dpf larvae was significantly lower in the *chd7*$^{-/-}$ larvae, indicating suppressed neurogenesis and impaired GABAergic neuronal differentiation in *chd7-/-* mutants. Noteworthy, we found no change in the expression of the early glial marker *scl1a3* (*Glast* in mammals) between *chd7-/-* and control fish (Fig EV4E), suggesting no alterations in gliogenesis upon loss of *chd7*.

2.3.3 GABAergic dysfunction is the underlying cause of behavioural defects

The altered number of GABAergic neurons in the *chd7-/-* mutant zebrafish brain suggests that these fish may have abnormal neural network that subsequently gives rise to abnormal behavioural outputs. Indeed, a hyperactive behavioural phenotype during dark cycles in zebrafish has been closely linked to disturbances in GABAergic signalling (Hoffman et al., 2016). We thus attempted to rescue/ameliorate the nighttime hyperactive phenotype by targeting GABA receptors with two agonists, muscimol and baclofen (targeting GABA-A and GABA-B receptors respectively). Both agonists rescued the constitutive hyperactivity observed in *chd7-/-* mutants (Fig. 2I). We further reasoned that if GABAergic signalling was perturbed in *chd7-/-* mutants, these animals would be more susceptible to show signs of seizure upon treatment with pentylenetetrazol (PTZ), a GABA-A receptor antagonist that induces seizures in rodents and zebrafish (Baraban, Taylor, Castro, & Baier, 2005; Watanabe, Takechi, Fujiwara, & Kamei, 2010). As expected, PTZ treatment induced more severe seizures in *chd7-/- mutant*s compared to wild-type controls, for both the time of onset and overall locomotor activity during the 20-min test period (Fig. 2J,K). Altogether, our findings

provide strong evidences that neurodevelopmental GABAergic signalling deficits underlie behavioural defects in *chd7-/-* mutants.

2.3.4 Brain gene expression profile is altered in chd7-/- zebrafish

CHD7 controls DNA accessibility by remodeling chromatin via translocating nucleosomes, thereby influencing gene transcription of many genes in both a positive and negative manner (Bouazoune & Kingston, 2012; Martin, 2010; Schnetz et al., 2010). To identify the molecular pathways underlying the neurodevelopmental defects in *chd7-/-* mutants, we thus performed an unbiased transcriptomic (RNA-seq) analysis on zebrafish *chd7-/-* larval brains (5dpf) compared to wild-type controls. This age was chosen to perform an exhaustive analysis of the molecular phenotype because it corresponds to a stage when the behavioural phenotype is distinct. We identified 1251 genes expressed in the *chd7-/-* larval brain that were significantly up- (677 genes) or down-regulated (574 genes) ($p<0.05$) (Fig. 3A, Dataset EV1). Gene ontology analysis revealed that several biological processes (Fig. 3B) and molecular functions (Fig. 3C) are enriched in the differentially expressed genes such as binding, signalling, catalytic activity, cellular process, metabolic process and biological adhesion. Using pathway analysis, the expression of the significantly dysregulated genes was mainly assigned to the following pathways: MAPK signalling, cell adhesion, calcium ion signalling, lipid transport, heme binding, tryptophan metabolism and sterol synthesis (Table EV1). Analysis of the chromatin immunoprecipitation-sequencing (ChIP-seq) datasets from the ENCODE Transcription Factor Targets project (Rouillard et al., 2016), revealed that many of the dysregulated genes within the above listed pathways are likely direct targets of CHD7 in murine and human cell lines (Table EV1).

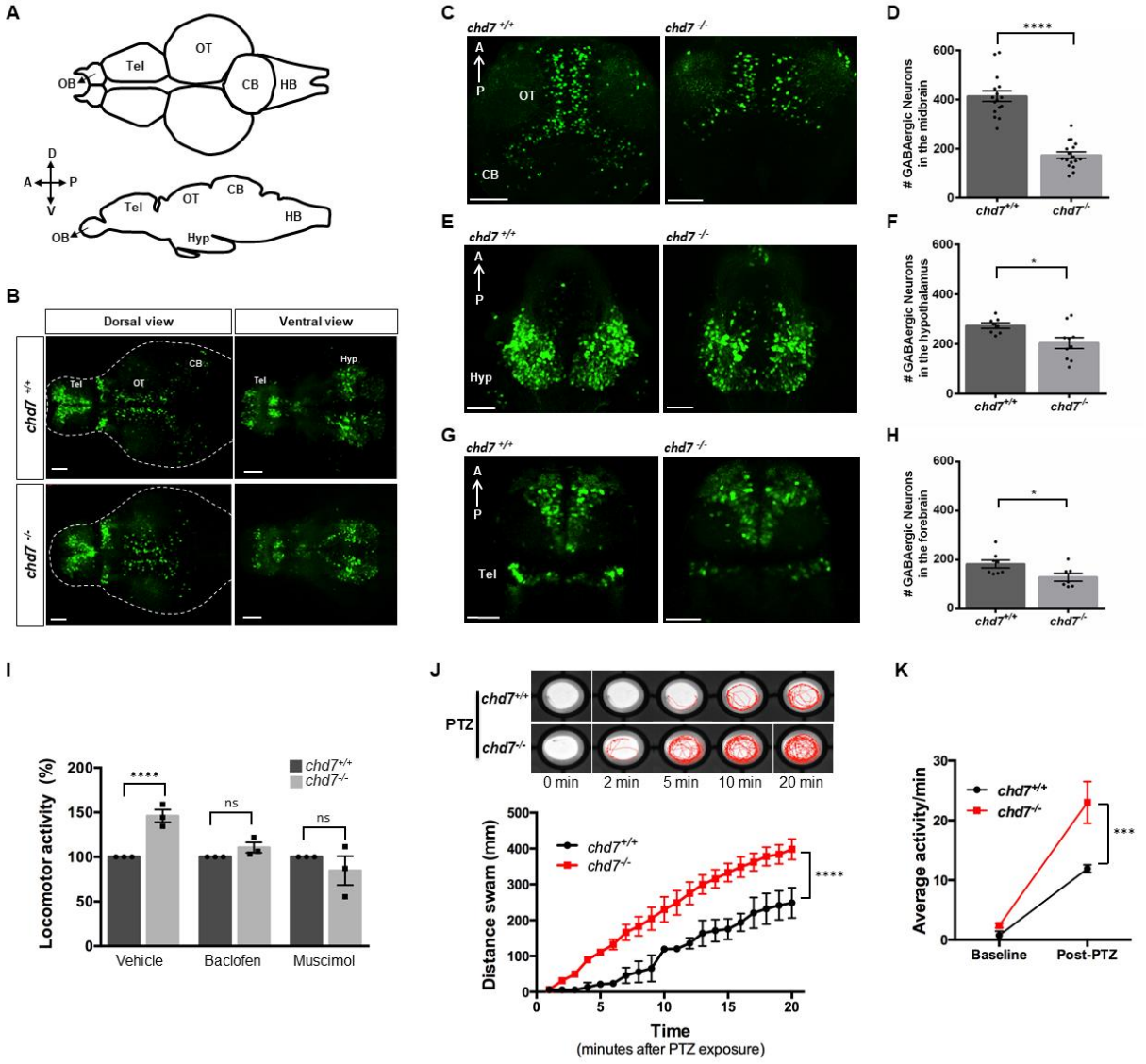

Figure 2 GABAergic neuron defects in zebrafish *chd7-/-* mutant brain

A. Structural illustration of 5 dpf zebrafish brain from dorsal (top) and lateral (bottom) view (OB: Olfactory bulb, Tel: Telencephalon, OT: Optic tectum, CB: Cerebellum, HB: Hindbrain).
B. 5 dpf *dlx5a/6a* transgenic line showing GFP⁺ GABAergic neurons are reduced in *chd7*−/− mutants (bottom) in comparison with controls (top) in both dorsal (left) and ventral (right) view.
C–H. Total number of GABAergic neurons (GFP⁺ cells) in (C, D) the optic tectum (OT) and cerebellum (CB) regions of 5 dpf wild-type and *chd7-/-* mutant fish (n = 16; ****P < 0.0001; Student's unpaired t-test), (E, F) the hypothalamus (hyp) region (n = 10; *P = 0.0182; Student's t-test) and (G, H) the telencephalon (tel) (n = 7; *P = 0.0347; Student's t-test).
I. Treatment of control (dark grey) and mutants (light grey) with GABA agonists Baclofen (N = 3, n = 24; ns, P = 0.1427; Student's t-test) and Muscimol (N = 3, n = 24; ns, P = 0.3987; Student's t-test) showing recovery of hyperactive locomotor activity in *chd7*−/− mutants (vehicle: N = 3, n = 24, ****P < 0.0001; Student's t-test).
J. Functional analysis of GABAergic signalling shows increased responsiveness to GABA antagonist PTZ in both onset and overall locomotor activity (n = 24; ****P < 0.0001; one-way ANOVA).
K. Average locomotor activity between 2 dpf controls (black) and *chd7*−/− mutants (red) shows increased activity after 3 mM PTZ exposure (n = 24; ***P < 0.001; two-way ANOVA).
Data information: Data are presented as mean ± SEM. Scale bar = 50 µm. n represents number of fish used. N represents number of experimental repeats.

2.3.5 Downregulation of paqr3b in chd7-/- zebrafish contributes to GABAergic defects via MAPK/ERK signalling

Among the identified dysregulated pathways, MAPK signalling caught our attention the most based on its well-known role in the pathogenesis of a wide range of neurodevelopmental disorders, including autism (Vithayathil, Pucilowska, & Landreth, 2018). To complement our transcriptomic analysis, we thus decided to evaluate the status of MAPK/ERK signalling in *chd7-/-* mutant brains and determine whether alteration of this pathway contributes to the observed GABAergic and behavioural defects. We detected a significant increase of phospho-Erk1/2 (p-ERK) in 5dpf *chd7-/-* mutant brains compared to wild-type controls, using both western blotting (Fig. 3D) and immunostaining (Fig. 3E). Additionally, treatment with a specific MEK/ERK inhibitor that prevents ERK phosphorylation (U0126) significantly increased the number of GABAergic neurons in *chd7-/-* brains (Fig. 3F,G) and reduced the hyperactive locomotor phenotype in *chd7-/-* larvae (Fig. 3H). Altogether, these data strongly suggest that CHD7 regulates GABAergic neuron development and behaviour via MAPK signalling.

Amongst the dysregulated genes in *chd7-/-* brains that were assigned to the MAPK signalling pathway, we noted that 6 of them (*paqr3b*, *flnb*, *nr4a1*, *dusp2, hspa8* and *dusp16*) were also identified as direct targets of CHD7 in murine and human cell lines tested in the ENCODE project (Table EV1). We further noted that *paqr3b*, an inhibitor of the MAPK/ERK signalling (L. Feng et al., 2007; Y. Zhang et al., 2010), is normally highly expressed in the zebrafish brain (Fig EV5A,B), and the most dysregulated gene in our RNA-seq dataset (Table EV1). Using RT-qPCR, we confirmed the strong downregulation of *paqr3b* in *chd7-/-* mutant brains (Fig EV5C). Importantly, we also validated that this finding was relevant for human CS using previously described lymphoblastoid cell lines (LCLs) derived from a *CHD7* mutation-positive child and its unaffected parents (Bélanger et al., 2018). LCLs are especially useful for analysing molecular mechanisms relevant for CS (Bélanger et al., 2018). Accordingly, RT-qPCR analysis showed that *PAQR3* gene expression was robustly decreased in *CHD7* mutation-positive LCLs compared to parental control LCLs (Fig. 4A), while ChIP-qPCR revealed that this decrease was associated with markedly reduced occupation of the *PAQR3* proximal promoter by CHD7 (Fig. 4B,C). These observations in human LCLs thus confirm that CHD7 directly regulates the expression of *PAQR3*.

Human *PAQR3* is a regulator of ER (endoplasmic reticulum)-to-Golgi transport (Cao et al., 2018) that is essential for maintaining cellular and physiological homeostasis (L. Feng et al., 2007). To indirectly test this key role of PAQR3 in the context of GABAergic neuron development, we treated wild-type zebrafish with the ER-to-Golgi transport blocker brefeldin A (BFA) (Donaldson, Finazzi, & Klausner, 1992). Using a low dose of BFA to affect ER-to-

Golgi trafficking without inducing cell stress and death in wild-type zebrafish (Le Corre, Eyre, & Drummond, 2014), we found that BFA treatment was sufficient to recapitulate the decreased number and malpositioning of GABAergic neurons observed in *chd7-/-* mutant brains (Fig. 5A,B). Importantly, we found that overexpression of *paqr3b* mRNA in *chd7-/-* mutants partially rescued the GABAergic neuron development defects at 3 dpf (Fig. 5C,D) as well as restored pERK level to basal wild-type level (Fig. 5E). Of note, overexpression of *paqr3b* mRNA in zebrafish embryos did not alter their gross morphology and viability (Fig EV5D). Altogether, these findings strongly suggest that a CHD7-PAQR3-MAPK/ERK regulatory axis is especially important for proper development of GABAergic neurons.

2.3.6 Ephedrine restores MAPK/ERK signalling and rescues GABAergic defects and associated behavioural anomalies

We recently demonstrated the power of combining simple genetic models such as worm (*C. elegans*) and zebrafish (*D. rerio*) for identifying neuroprotective compounds that can rapidly be translated into preclinical and clinical testing (Patten et al., 2017). In *C. elegans*, loss-of-function of *chd-7* (Fig EV6A) leads to significant impairment of swimming locomotion when compared to wild-type animals (Fig. 6A), and reduced lifespan (Fig. 6B). Locomotion of *C. elegans* is controlled by inhibitory GABAergic and excitatory cholinergic motor neurons (Zhen & Samuel, 2015). To visualize neurodevelopmental problems in these neurons, *unc-47*p::mCherry and *unc-17*p::GFP reporters for GABAergic and cholinergic motor neurons, respectively, were crossed into *chd-7* mutant animals (referred to *chd-7* mut hereafter). We found that GABAergic neurodevelopment is significantly impaired in *chd-7* mut when compared to wild-type animals at the L4 stage, whereas the cholinergic motor system seems not to be affected by a mutation in the *chd-7* gene (Fig. 6C). Particularly, a major neurodevelopmental problem in *chd-7* mut consists of interrupted connections in the GABAergic neuronal network, as indicated by significantly more axonal gaps and axonal loss/breaks larger than 50 µM (Fig. 6D-G). The numbers of GABAergic cell bodies (Fig. 6H) and commissures (Fig. 6I) are slightly but significantly decreased to 22 and 12, respectively, compared to 26 and 16 in healthy worms. These observed phenotypes in *chd-7* mut worms are strikingly similar to those we previously reported in several *C. elegans* genetic models for ASD (K. Schmeisser, Fardghassemi, & Parker, 2017).

We next exploited the impaired locomotion phenotype in *chd-7* mut worms to perform a comprehensive drug screen with 3850 compounds (Fig. 6J). We identified 49 compounds with beneficial effects that partially corrected the impaired locomotion of *chd-7* mut worms (Table EV2). Based on their suggested function, these compounds could be clustered into 6 main categories (Fig. 6K). From all positive substances, three that improved swimming movement

particularly well were chosen for further investigation: fisetin, meloxicam, and ephedrine. The compounds were retested in worms and validated in our zebrafish model (Fig. 6J) at various concentrations ranging from 1-50 µM. We confirmed two active compounds in zebrafish and identified ephedrine as the most potent lead compound. Ephedrine was found to significantly improve behavioural and GABAergic defects in both *C. elegans* (Fig. 6L,M) and zebrafish (Fig. 6N,O). Ephedrine also improved the V^{th} cranial nerve branching defects in zebrafish (Fig EV6).

To test whether ephedrine is exerting its beneficial effects by correcting the aberrant MAPK/ERK signalling in *chd7-/-* mutants, we examined the level of p-ERK in these fish at 5dpf and found a significant reduction upon ephedrine treatment (Fig. 6P,Q). Our findings thus support a model whereby development of GABAergic neurons in zebrafish is regulated by *chd7* via MAPK/ERK signalling. However, ephedrine treatment did not affect the level of *paqr3b* expression in *chd7-/-* mutants (Fig. 6R), suggesting that ephedrine is acting downstream in the MAPK/ERK signalling cascade.

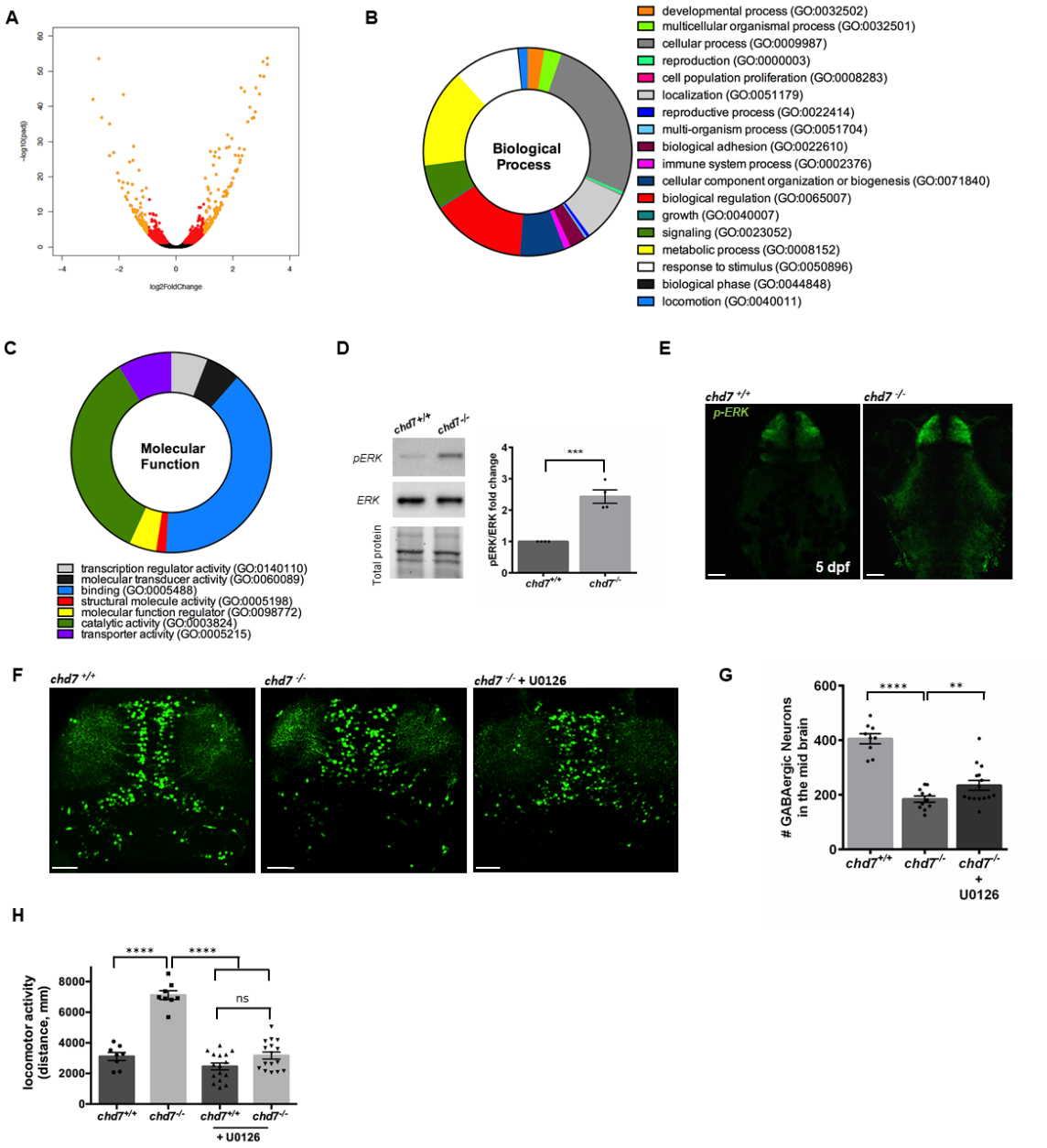

Figure 3 *chd7* regulates GABAergic neuron development via MAPK/ERK signalling
Transcriptomic analysis was performed on dissected 5 dpf brains.
A. Volcano plot showing each gene plotted according to its $\log_2$ fold change. All highly differentially expressed genes with $P < 0.05$ are in orange with fold change > 1.5.
B, C. Biological processes (B) and molecular function (C) that are enriched in the differentially expressed genes.
D. ERK activation by phosphorylation is increased in mutants as shown by Western blot quantification ($N = 4$; ***$P < 0.005$; Student's *t*-test).
E. pERK immunohistochemistry showing increased ERK activation in the mutant brain ($N = 3$, $n = 8$).
F, G. Treatment with the ERK signalling inhibitor U0126 ameliorated the number of GABAergic neuron ($n = 10–15$; **$P < 0.05$; ****$P < 0.0001$, one-way ANOVA).
H. Treatment with the ERK signalling inhibitor U0126 rescues locomotor hyperactivity phenotype in *chd7-/-* mutant fish ($n = 8$ for *chd7*$^{+/+}$ and *chd7*$^{-/-}$; $n = 16$ for U0126-treated fish; ****$P < 0.0001$; ns, not significant, one-way ANOVA).
Data information: Data are presented as mean ± SEM. Scale bar = 50 μm. *n* represents number of fish used. *N* represents number of experimental repeats.

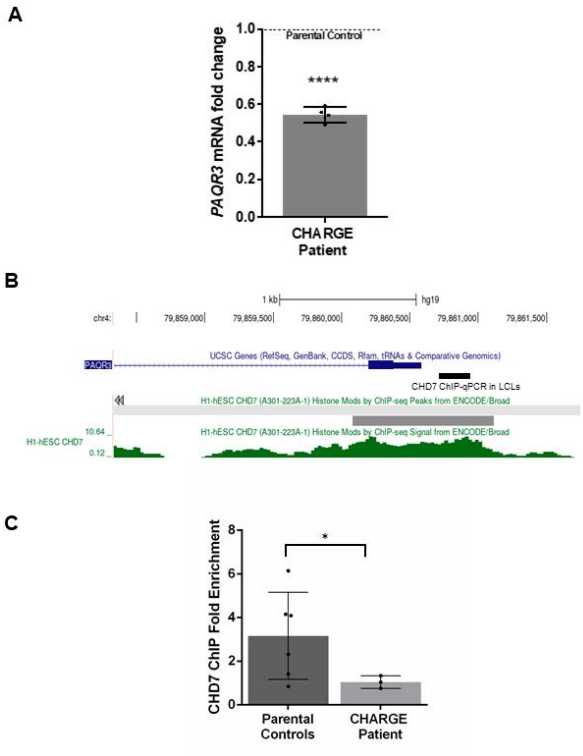

Figure 4 CHD7 regulates PAQR3 expression in human cells
A. qPCR analysis of PAQR3 expression in a *CHD7* mutation-positive patient compared with parental controls set ($N = 4$; ****$P < 0.0001$; Student's *t*-test). Fold change was calculated according to the 2−ΔΔCt2-ΔΔCt method, using *HPRT1* and *RPS1* as housekeeping genes for normalization. All data were expressed as mean fold change ± SD across replicates, relative to control parents set to 1 (dotted line). *N* is the number of experimental repeats.
B. Schematic view of *PAQR3* exon 1 and proximal promoter on chromosome 4 (hg19 assembly), obtained with the UCSC genome browser (https://genome.ucsc.edu/) and showing the sequence amplified in ChIP-qPCR assays in LCLs (thick black line) along with a previously described CHD7 ChIP-seq peak (thick grey line) and signal (green) in H1-hESC (ENCODE3).
C. ChIP-qPCR assays in LCLs showing decreased occupation of CHD7 on the *PAQR3* proximal promoter in a *CHD7* mutation-positive patient ($N = 3$) compared with parental controls ($N = 6$); *$P < 0.05$; Student's *t*-test. All data were expressed as mean fold change ± SD. *N* is the number of experimental repeats.

2.4 Discussion

Despite advances in genetic studies of CS, the underlying mechanisms for the neurological deficits in this disease remain poorly understood. In this study, we showed that loss-of-function of the chromatin remodeller-coding gene *chd7* disrupts normal number of the inhibitory GABAergic neurons in the zebrafish brain. Importantly, we also discovered that these GABAergic neuron defects result in behavioural anomalies that occur via an ERK-dependent mechanism. Furthermore, through a phenotype-based drug screening strategy, we identified a clinically approved drug, ephedrine, which proved to be very effective at correcting increased

MAPK/ERK signalling, GABAergic defects and behaviour deficits caused by *chd7* loss-of-function.

We observed a significant reduction (close to 50% in the optic tectum, $p<0.05$) in the number of GABAergic interneurons in the zebrafish *chd7-/-* brain during development. GABAergic interneurons play an essential role in neural circuitry and in behaviour. Proper differentiation of GABAergic neurons during brain development is important for establishing anatomical and functional circuitry. We found that the hyperactivity behavioural phenotype of *chd7-/-* mutant fish could be suppressed by modulating GABAergic signalling with agonists. These findings suggest that the abnormal behaviour due to loss of *chd7* in our mutants is likely in part due to defects in GABAergic network development. Interestingly, our observations are consistent with an ASD-related phenotype recently described in zebrafish mutants of the ASD risk gene, *CNTNAP2* (Hoffman et al., 2016). Individuals with CHARGE syndrome frequently exhibit autism-like behaviour (Hartshorne, Grialou, et al., 2005; Smith et al., 2005). Several studies have shown that GABAergic neurons and circuits are altered in ASD (Coghlan et al., 2012; Rubenstein & Merzenich, 2003) and the dysfunction of inhibitory GABAergic circuits has been proposed as a cause for ASD (Pizzarelli & Cherubini, 2011). Our work suggests that the overlapping symptom of autistic features in CS and ASD may share a common neurobiological pathway implicating improper GABAergic development and function.

Noteworthy, we did not observe any abnormalities in *chd7* heterozygous mutants at the larval developmental stages that we studied. This is likely due to some teleost-specific genetic compensation mechanisms occurring in heterozygous background. Such a lack of phenotype in the heterozygous mutant population has also been observed while modelling several autosomal dominant diseases in zebrafish such as Dravet syndrome, hyperexplexia and juvenile mycolonic epilepsy, where disease-related phenotypes are only recapitulated in a homozygous mutant (-/-) background (Samarut et al., 2019; Samarut et al., 2018; Sourbron et al., 2019). It is, however, plausible that CHARGE-related behavioural changes may occur in juvenile or adult *chd7* heterozygous mutants and this warrants further studies.

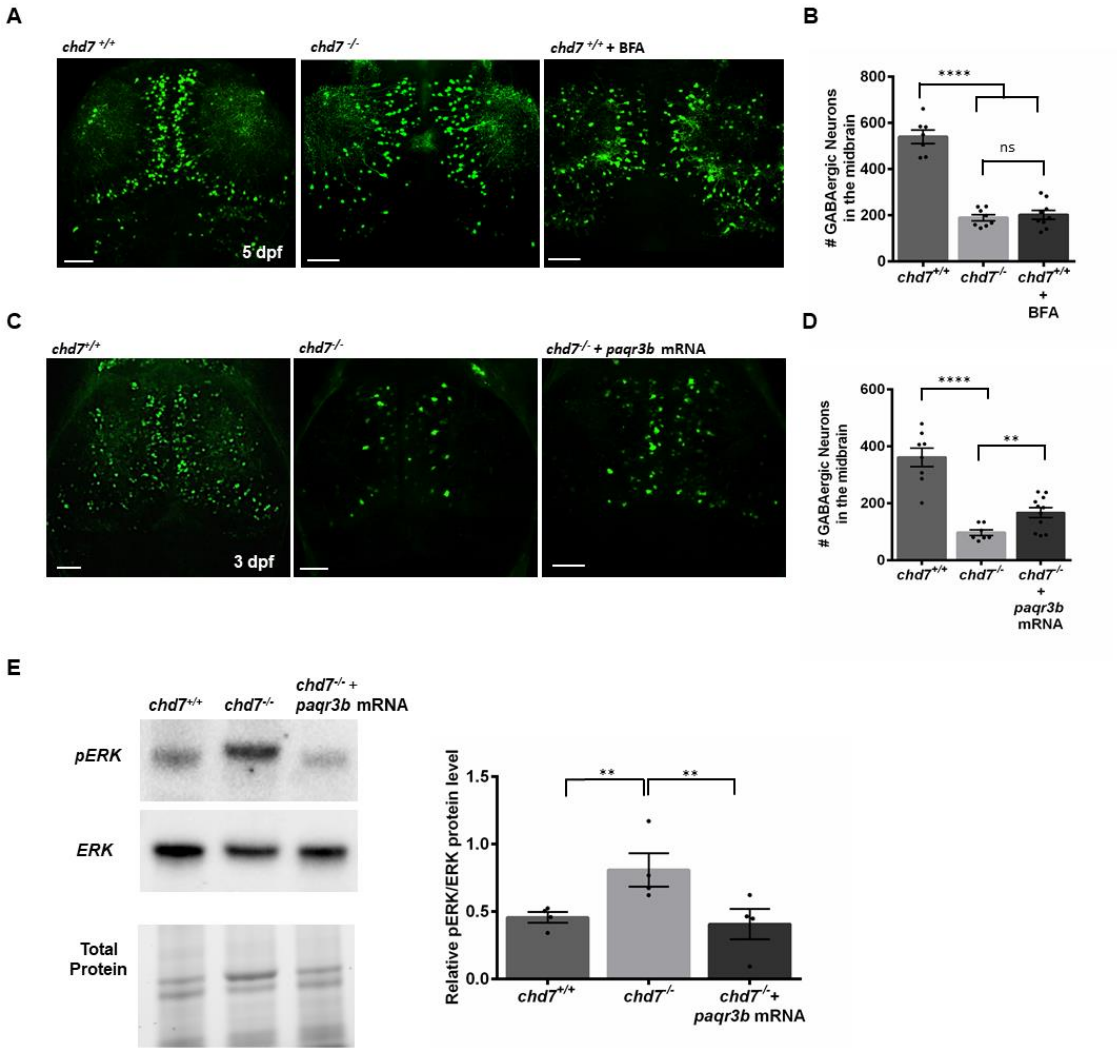

Figure 5 Zebrafish *paqr3b* regulates GABAergic neuron development

A, B. GABAergic neuron defects in wild-type fish treated with ER/Golgi traffic inhibitor BFA (n = 9; ****P < 0.0001; ns, not significant; one-way ANOVA).

C, D. Overexpression of *paqr3b* mRNA improve the number of GABAergic neurons in 3dpf *chd7-/-* mutant fish (n = 7 for *chd7*−/− and n = 11 for *chd7*−/− + *paqr3b* mRNA; ****P < 0.0001; **P < 0.05; One-way ANOVA). Of note, the rescue experiment was performed at 3 dpf given the transient nature of mRNA.

E. ERK activation by phosphorylation in *chd7*−/− mutants was restored to normal levels upon overexpression of *paqr3b* mRNA shown by Western blot quantification (N = 4; **P < 0.05; One-way ANOVA, N is the number of experimental repeats).

Data information: Data are presented as mean ± SEM. Scale bar = 50 μm.

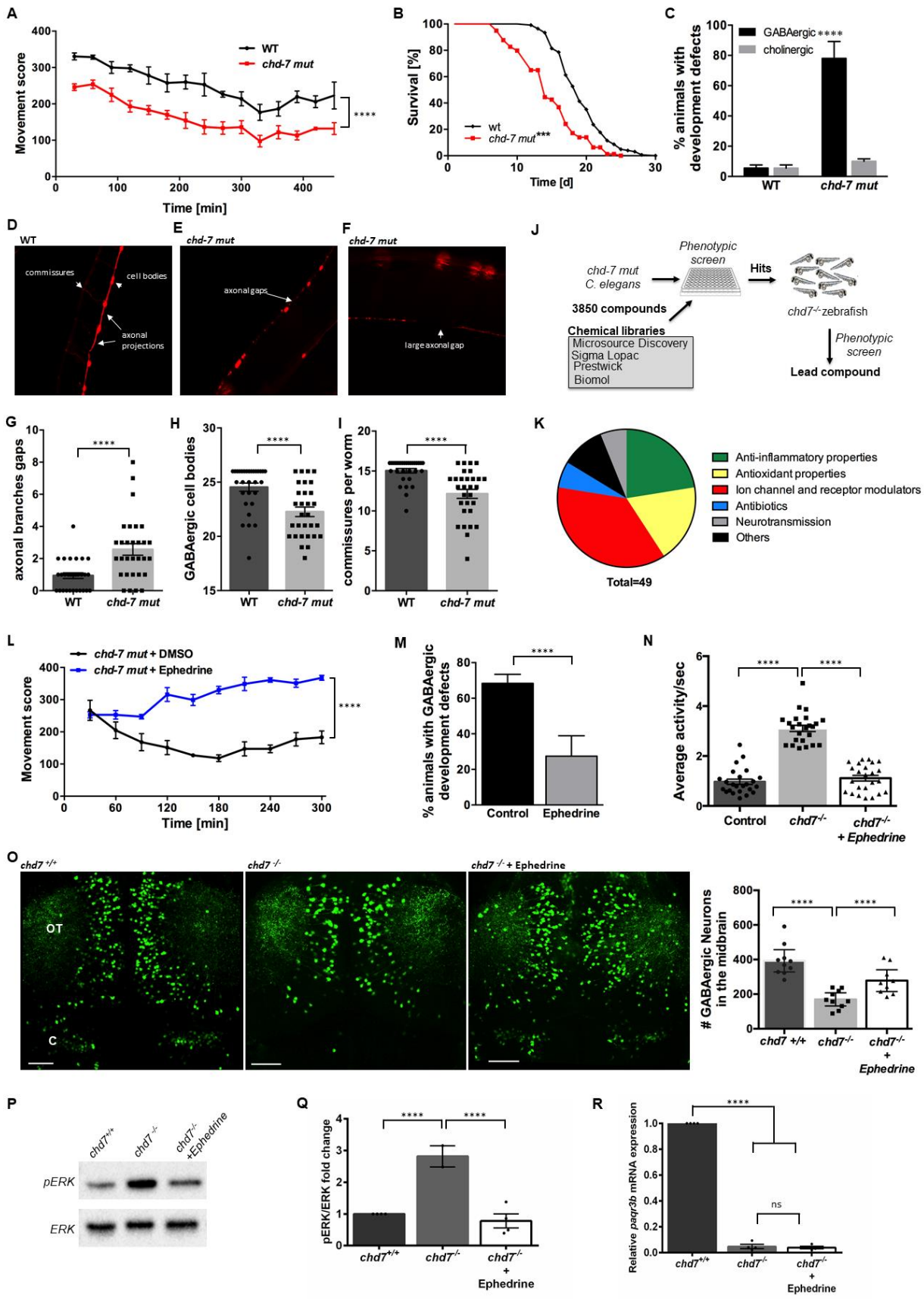
A
Movement score
WT
chd-7 mut
****
Time [min]
B
Survival [%]
wt
chd-7 mut***
Time [d]
C
% animals with development defects
GABAergic ****
cholinergic
WT
chd-7 mut
D
WT
commissures
cell bodies
axonal projections
E
chd-7 mut
axonal gaps
F
chd-7 mut
large axonal gap
J
chd-7 mut
C. elegans
Phenotypic screen
Hits
chd7-/- zebrafish
3850 compounds
Chemical libraries
Microsource Discovery
Sigma Lopac
Prestwick
Biomol
Phenotypic screen
Lead compound
G
axonal branches gaps
H
GABAergic cell bodies
I
commissures per worm
K
Anti-inflammatory properties
Antioxidant properties
Ion channel and receptor modulators
Antibiotics
Neurotransmission
Others
Total=49
L
chd-7 mut + DMSO
chd-7 mut + Ephedrine
Movement score
Time [min]
M
% animals with GABAergic development defects
Control
Ephedrine
N
Average activity/sec
Control
chd7-/-
chd7-/- + Ephedrine
O
chd7 +/+
chd7 -/-
chd7 -/- + Ephedrine
OT
C
GABAergic Neurons in the midbrain
chd7 +/+
chd7-/-
chd7-/- + Ephedrine
P
chd7+/+
chd7-/-
chd7-/- +Ephedrine
pERK
ERK
Q
pERK/ERK fold change
chd7+/+
chd7-/-
chd7-/- + Ephedrine
R
Relative paqr3b mRNA expression
ns
chd7+/+
chd7-/-
chd7-/- + Ephedrine

Figure 6 GABAergic defects and behavioural anomalies are suppressed by ephedrine treatment in mut-*chd7 Caenorhabditis elegans*

A. Movement scores of *chd-7(gk290)* mutants (red) compared with N2 wildtype (WT; black). Two-tailed *t*-test was performed for statistical analyses, and movement score was considered different to WT (*N* = 3, *n* = 30; ****$P < 0.0001$, Student's *t*-test).

B. Lifespan analyses of *chd-7(gk290)* mutants (*n* = 339; red) compared with WT (*n* = 444; black). Log-rank test was performed for statistical analyses. ***$P < 0.001$.

C. WT and *chd-7(gk290)* animals with defects in the GABAergic (black) and cholinergic nervous system (grey) at the L4 stage (*N* = 4, *n* = 100; ****$P < 0.0001$; Student's *t*-test).

D–F. Example pictures of the GABAergic nervous system cell bodies, commissures, axonal gaps and axonal breaks larger than 50 µm per worm in *chd-7(gk290)* mutants expressing *unc-47*p::mCherry.

G–I. GABAergic neurodevelopmental defects of WT (black) and *chd-7(gk290)* mutants (grey) at the L4 stage classified in axonal gaps per worm (G), number of GABAergic cell bodies (H) and number of commissures (I). *N* = 4, *n* = 100; ****$P < 0.0001$, Student's *t*-test.

J. Chemical libraries (3,850 compounds) were first screen in *chd-7(gk290) C. elegans* mutants and positive hits were tested on *chd7* zebrafish mutants.

K. 49 compounds that improved motility phenotypes in *chd-7(gk290)* mutants were identified. These compounds were clustered in 6 main functional categories.

L. Movement scores of *chd-7(gk290)* mutants treated with ephedrine (blue) compared with the solvent DMSO (black). *N* = 3, *n* > 100; ****$P < 0.0001$, Student's *t*-test.

M. Defects of the GABAergic nervous system at the L4 stage in *chd-7(gk290)* mutants treated with ephedrine (grey) compared with DMSO (black) (*N* = 3, *n* > 100). ****$P < 0.0001$, Student's *t*-test.

N. Average activity per second is wild-type and mutants treated with ephedrine compared with non-treated mutant (wild-type treated with ephedrine is referred to as Control). Ephedrine suppresses hyperactivity in *chd7-/-* mutant fish. *n* = 24; ****$P < 0.0001$, one-way ANOVA.

O. Representative images of GABAergic neurons and total number of GABAergic neurons (GFP^+ cells) in the optic tectum (OT) and cerebellum (CB) regions of wild-type, *chd7-/-* mutant and ephedrine-treated fish (*n* = 9; ****$P < 0.0001$; One-way ANOVA). Treatment with ephedrine ameliorated the number of GABAergic neurons.

P, Q. pERK is reduced in mutants following treatment with ephedrine as shown by Western blot quantification (*N* = 4). ****$P < 0.0001$, one-way ANOVA.

R. qPCR analysis of *paqr3b* expression in $chd7^{+/+}$, $chd7^{-/-}$ and $chd7^{-/-}$ treated with ephedrine (*N* = 4; ****$P < 0.0001$; ns, not significant, one-way ANOVA).

Data information: Data are presented as mean ± SEM. Scale bar = 50 µm. *n* is the number of fish or worms used. *N* is the number of experimental repeats.

PAQR3 negatively regulates Raf/MEK/ERK signalling (L. Feng et al., 2007; Y. Zhang et al., 2010). The downregulation of *paqr3b* expression in *chd7-/-* fish likely results in overactive Raf/MEK/ERK signalling and hyperphosphorylation of ERK subsequently has detrimental consequences on neuronal network development. Xu and colleagues showed PAQR3-deleted mice display motor and behavioural abnormalities (D. Q. Xu et al., 2016). It is thus possible that *paqr3b* is important for neural connectivity and motor function in vertebrates. Aberrant signalling through the MAPK/ERK pathway is involved in the pathogenesis of neurodevelopmental syndromes that involve autism, intellectual disability, neurodevelopmental delay, and seizures (Vithayathil et al., 2018). Perturbation in MAPK/ERK activity in *chd7-/-* mutants is consistent with findings observed in genetic models of ASD. Recent work by Shah et al., revealed that hyperactive ERK signalling during mouse development affects the number of GABAergic interneurons (Shah S, 2017). In our study, we similarly observed that with inhibition of ERK phosphorylation ameliorated the defective behaviour and number of GABAergic neurons. However, other mechanistic pathways under

the control of CHD7 may also be important in GABAergic network development and this warrants further investigation. Indeed, we showed that the number of GABAergic neurons in *chd7-/-* fish can be rescued by overexpressing *paqr3b* mRNA. PAQR3 is a key player in regulating ER-to-Golgi transport (Cao et al., 2018) and perturbing the secretory pathway may affect neurogenesis.

Ephedrine is both an α- and β-adrenergic agonist (Ma et al., 2007; Vansal & Feller, 1999). Our findings showed that this adrenergic receptor agonist significantly reverses GABAergic defects as well as abnormal behaviour in our models. Adrenergic receptors (ARs), α1-AR, have also been shown to play a critical role in regulation of neurogenesis (Gupta et al., 2009). For instance, in neonates, α1-ARs are important for the differentiation of neural progenitors into catecholaminergic neurons and GABAergic interneurons (Gupta et al., 2009). It has been suggested that α1-AR is required in the neuronal maturation stages of neurogenesis by regulating the levels of the *Dlx2*, *Mash1*, *NeuroD* and *bHlH* mRNA (Gupta et al., 2009). Importantly, α1-ARs colocalize with both GABAergic and NMDA receptor-containing neurons and are likely to be involved in their regulation (Papay et al., 2006). As previously shown (Ferraro et al., 1993), α_1-ARs can modulate GABA release in the human cerebral cortex and they have also been shown to regulate CA1 GABAergic interneurons in the rat hippocampus (Bergles, Doze, Madison, & Smith, 1996). Interestingly, AR agonist treatment can significantly increase the excitability of GABAergic interneurons (Bergles et al., 1996; Kawaguchi & Shindou, 1998; Marek & Aghajanian, 1996; Papay et al., 2006) while having a contrasting effect in pyramidal cells (Papay et al., 2006). An α_1-AR-mediated facilitation of spontaneous GABA release from interneurons has been observed in several brain regions (Bergles et al., 1996; Kawaguchi & Shindou, 1998). It is possible that the increased in GABAergic signalling by ephedrine in our model is likely one of the underlying mechanisms in ameliorating the phenotypic anomalies in *chd7-/-* mutants. Ephedrine acts as both a direct and indirect sympathomimetic. Its primary mode of action is achieved indirectly, by inhibiting neuronal norepinephrine reuptake and by displacing more norepinephrine from storage vesicles (D. E. Becker, 2012; Wellman, Miller, & Ho, 2003). In addition to norepinephrine, detection of dopamine has also been reported under ephedrine-stimulated conditions in the CNS (Ruwe, Naylor, Bauce, & Veale, 1985). Interestingly, dopaminergic signalling has been shown to regulate GABAergic neuron development and motor behaviour in zebrafish (Souza, Romano-Silva, & Tropepe, 2011). The next step will be to unravel ephedrine's exact target and mechanism of action in our *chd7* models to increase the number of GABAergic neurons and to ameliorate behaviour.

In conclusion, we show for the first time that *chd7* controls GABAergic network development in zebrafish brain via regulating *paqr3b*. We also provide novel insight on the pathogenic

mechanisms -from molecular pathway to brain circuits and behaviour- associated with *chd7* loss-of-function with relevance to CS and ASD. Five of nine members of the CHD family proteins have been implicated in neurodevelopmental disorders(Goodman & Bonni, 2019). Our findings may additionally be relevant to other neurodevelopmental diseases such as autism and epilepsy with which mutations in chromatin remodellers such as *CHD8* and *CHD2* have been associated. In this study, we also identify a clinically approved compound and its action on MAPK/ERK signalling that may be therapeutic avenues to be further explored in CHARGE syndrome. Overall, our work suggests that *in vivo* drug screening and experimental analysis of simple genetic models could prove extremely valuable in understanding and perhaps ultimately aid in developing treatments for certain neurological features associated with CHD7 deficiency.

2.5 Material and methods

Zebrafish

Fish husbandry

Adult zebrafish (*Danio rerio*) were maintained at 28°C at a light/dark cycle of 12/12 h in accordance with Westerfield zebrafish book(Westerfield, 1993). Embryos were raised at 28.5 °C, and collected and staged as previously described(Kimmel et al., 1995). The zebrafish lines used in this study were: wild-type, *chd7-/-* mutants, Tg(*dlx5a/6a*:GFP) which was obtained from the laboratory of Marc Ekker and used to generate Tg(*dlx5a/6a*:GFP;*chd7*$^{-/-}$) fish. All experiments were performed in compliance with the guidelines of the Canadian Council for Animal Care and the local ethics committee of INRS. For imaging studies, pigment formation was blocked by adding 0.003% phenylthiourea (PTU) dissolved in egg water at 24 hours after fertilization (hpf).

CRISPR/Cas9 generated mutagenesis

A guide RNA (gRNA) targeting the helicase domain of the *chd7* gene was designed using the online tool CRISPRscan (TGTATTCCTGCTGTGCACAAGGG; PAM site underlined). Synthesis of gRNA and of Cas9 mRNA was performed as previously described(Swaminathan et al., 2018). *Cas9* mRNA was synthesized using the mMESSAGE mMACHINE T3 kit from pT3TS-nCas9n plasmid (Addgene #46757) linearized with Xba1. A volume of 1nl containing a mix of 100 ng/µl *Cas9* mRNA and 30 ng/µl gRNA was injected into one-cell stage embryos using the Picospritzer III pressure ejector. Genotyping of *chd7*+/+ (wild-type), +/−

(heterozygous) and −/− (homozygous) fish was performed by high resolution melting (HRM) analysis using genomic DNA extracted by boiling larva/clipped caudal fin in 50 mM NaOH for 10 minutes and then neutralized in 0.1 M Tris HCl (pH8). Rescue experiment was performed using *paqr3b* (NM_001030148.2) zebrafish open reading frame cloned into a pCS2^{+} expression vector. *In vitro* transcription was done using the SP6 message machine kit (Ambion) and 1nl of *paqr3b* mRNA (40 ng/µl) was injected into the 1-cell stage embryos.

Gross morphology and survival assessment

Larvae (*chd7*+/+, *chd7*+/- and *chd7*-/-) were assessed for their survival rate and morphological phenotypes. The sample sizes for the different genotypes were as follows: three different batches (N=5) each batch containing 30 larvae (n=30) for *chd7*+/+, *chd7*-/- larvae and N=3, n=18-19 for *chd7*+/- larvae. Gross morphology was observed under a stereomicroscope (Leica S6E). The head size at 3 dpf was measured using the software, ImageJ. Briefly, a straight line was drawn between the lowest point of the otolith and the anterior end of the brain, around the upper jaw area.

Neuronal network analysis

To visualize the axonal tracts, fluorescent immunohistochemistry was performed using the marker acetylated α-tubulin (Sigma-Aldrich; Cat# T7451). 28 hpf fish were fixed in Dent's fixation (80% methanol and 20% DMSO) overnight (O/N) at 4 °C. Samples were rehydrated in 75 %, 50 % and 25 % Methanol in PBST for 30 minutes each. They were then washed 4 times in PBST for 30 minutes (twice under agitation and twice without followed by blocking in 10 % normal goat serum (NGS) and 2 % bovine serum albumin (BSA) in PBST for 1 hour at room temperature (RT), under agitation. Primary acetyl-tubulin monoclonal mouse antibody was added to the blocking (1:500) and incubated overnight at 4 °C. After washing the primary at least 6 times 30 minutes in PBST and blocking for 1 hour, the secondary antibody Alexa 488 goat anti-mouse (Sigma-Aldrich; Cat# SAB4600387) was added O/N at a ratio of 1:1000. Secondary anti-body was washed and samples are mounted laterally for 28hpf. Imaging was done on confocal microscope (Zeiss LSM780). The projection images were semi-automatically traced with NIH ImageJ using the NeuronJ plugin. The total length of processes in each individual embryo were subsequently measured and analysed.

Craniofacial Cartilage staining

To visualize the cranio-pharyngeal cartilage, Alcian blue staining was applied. Larvae were fixed in 4% paraformaldehyde (PFA) in PBST (1 pellet in 200ml dH2O and 0.1 % Tween). Fish were fixed at 6dpf for 5 hours at RT. Then the samples were dehydrated in methanol solution with increasing concentration; 25 %, 50 % and 75 % in PBST (10-15 minutes each) and stored

at -20 °C until use. Before the staining was started, the samples were rehydrated in the reverse order of methanol concentrations for 15 minutes each. After 3 quick washes in PBST, the samples were incubated in 0.1 % Alcian blue solution with 70 % ethanol (EtOH) and 0.37 % HCl for two hours, under agitation. Then they were washed in EtOH and HCl solution; 3 quick washes and then twice 15 minutes. The larvae were digested in 10 % trypsin in 30 % saturated borax water which was pre-warmed a 42 °C. At the end, the samples were washed overnight at 4 °C in 0.1 % KOH in H2O. The following day, they were washed in 0.25 % KOH for 1h and stored in 80 % glycerol and 0.25 % KOH solution at 4 °C until being imaged. Imaging was done on the Leica stereomicroscope (Leica S6E).

Behavioural Analysis

Larvae (5 dpf) were separated into single wells of a 96-well plate containing 200µl of E3 media and habituated in the the Daniovision® recording chamber (Noldus) for 1 hour before start of experiment. Larval locomotor activity was monitored over light-dark cycles using the Daniovision® apparatus. Analysis was performed using the Ethovision XT12 software (Noldus) to quantify the total swimming distance in given hours and the locomotor activity per second.

GABAergic neurons: Confocal Imaging and data analysis

To image and quantify GABAergic neurons (*dlx5a/6a*:GFP^{+} neurons), zebrafish larvae (1, 2, 3 and 5 dpf) of Tg(*dlx5a/6a*:GFP;*chd7*+/+) and Tg(*dlx5a/6a*:GFP;*chd7*-/-) were fixed in 4% PFA for 2 hours. Zebrafish larvae (1, 2 and 3 dpf) were ventrally mounted for brain imaging and in case of 5 dpf larvae, the brains were first dissected and then dorsally or ventrally mounted for imaging. Z-stack images were taken using a Zeiss LSM780 confocal microscope (Carl Zeiss, Germany). GABAergic neurons (*dlx5a/6a*:GFP^{+} neurons) were counted in the brain regions of wild-type and mutant larvae manually and blindly using the Cell Counter plug-in for Fiji/Image J (NIH) Imaging software.

Bromodeoxyuridine (BrdU) Labelling

Zebrafish larvae at 4 dpf were incubated in a 10 mM solution of BrdU (BD Biosciences; Cat# 550891) in fish water at a temperature of 28.5°C for 24h. All of the larvae were then fixed at 5 dpf in 4% PFA O/N at 4°C and used to perform cryo-sections of the brain.

Zebrafish brain cryo-sections and double-immunohistochemistry

Fixed larvae (5 dpf) were given serial sucrose treatment with 15% and 30% sucrose in 1X PBS, till the larvae sank to the bottom. The fish brains were then cryo-sectioned (transverse

section) in 10-micron-thick sections and dried at room temperature for 20 min, and frozen to store.

For the immunostaining, the 5 dpf zebrafish embryo brain sections, were first post-fixed in acetone at -20°C for 20 min. The sections were then washed with PBS for 15 min, and processed for epitope retrieval with Tris-HCl (pH 8.2, 50 mM) at 85°C for 6 min treatment (for HuC/D, NeuroD1) and additionally with HCl (4N) at 37°C for 10 min followed by Sodium borate (0.1 M) washes for 20 min (for BrdU). Sections were then washed in 0.5% PBS-Triton for 30 min , blocked in 10% NGS for 1 hr at room temperature and then incubated in primary antibodies: HuC/D at 1:50 (Invitrogen; A21271); NeuroD1 at 1:500 (Abcam; ab60704), BrdU at 1:250 (Abcam; ab152095) and anti-GFP at 1:250 (Invitrogen; GF28R) diluted in 5% NGS, 1% BSA in 0.1% PBS-Triton, O/N at 4°C. The following day sections were washed in 0.3% PBS-Triton and incubated with species-specific secondary antibodies coupled to Alexa Fluor 488 or 555 (Invitrogen) diluted in 0.1% PBS-Triton for 2-3h at room temperature, followed by washes with 0.3% PBS-Triton and mounted in DAPI glue (Invitrogen; Cat# P36941).

For the quantitative analyses, cells stained for BrdU, NeuroD1, GFP (for Dlx5/6) or HuC in the zebrafish midbrain area were taken under 40× magnification with an oil immersion lens using a Zeiss confocal microscope (LSM780) (Carl Zeiss, Germany). The images were then processed with ZEN software (Carl Zeiss). Stained cells in consecutive sections from 3 brains per genotype (N=3) were counted using ImageJ (NIH) and used to calculate the total number of double positive stained cells relative to BrdU positive cells in each larval brain section. Of note, the total number of sections (n) used varied between experiments. Neuroanatomical designations are taken from the Atlas of Early Zebrafish Brain Development(Mueller & Wullimann, 2015).

Hematoxylin and Eosin staining

For Hematoxylin and Eosin staining, brain sections were post fixed in 10% formol (Chaptec) for 5 minutes and rinsed with tap water. The sections were stained with Hematoxylin (Statlab) for 4 min, and washed with alcohol-acid, and were rinsed with tap water. The sections were then soaked in saturated Lithium Carbonate solution for 10 sec, and then rinsed it with tap water. Finally, staining was performed with Eosin Y (Statlab) for 2 min, and mounted under coverslip with permount mounting media. Neuroanatomical designations are taken from the Atlas of Early Zebrafish Brain Development(Mueller & Wullimann, 2015).

Drug treatment

Drugs were purchased from Sigma-Aldrich and stock solutions were prepared: Pentylenetetrazol (PTZ) – Muscimol-30 mM, Baclofen-15 mM, U0126-10 mg/ml and Brefeldin A(BFA)- 10 mg/ml. Embryos were treated from 8 hpf for GABA agonists Muscimol and

Baclofen, and at 2 dpf for 20 min with 3 mM PTZ (Sigma-Aldrich). For treatment with BFA, an established low dose concentration 3.56 µM (1 µg/ml; Sigma-Aldrich) without inducing cell stress and death in zebrafish was used and added at 24 hpf (Le Corre et al., 2014). For treatment with U0126, a non-toxic low-dose of 4µM (1.7 µg/ml; Sigma-Aldrich) concentration was used and added at 8 hpf (Guo et al., 2015; Hawkins, Cavodeassi, Erdelyi, Szabo, & Lele, 2008). The water was replaced every day with fresh water containing final concentration of the drug, until the activity measurement and/or brain imaging. For the chemical genetic screens, zebrafish embryos from 8 hpf were treated with fisetin, ephedrine and meloxicam (1-50 µM; all purchased from Sigma-Aldrich) in E3 medium. The medium was replaced every day with fresh solution containing final concentration of the drug, until the locomotor activity measurement and/or imaging.

Western Blotting

Larvae were collected at 5 dpf and placed in the dark for at least 1 hr, following which lysates were rapidly prepared by homogenization in high salt lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl pH 7.5, 1% triton, 0.1% SDS, 1% sodium deoxycolate, and protease inhibitors cocktail (1:10, Sigma-Aldrich). The lysates were centrifuged at 13,000 rpm for 10 minutes at 4 degrees. The supernatant was collected, and protein concentration was estimated using Bradford assay (Biorad). Western blotting was performed using 20 µg lysate per sample which were resolved on a 7.5% SDS-polyacrylamide gel (BioRad). After electrophoresis, proteins on the gel were electro transferred onto PVDF mini membranes (BioRad). The membranes were blocked with 5% non-fat milk solution in 1X phosphate buffered saline or with 5% bovine serum albumin (Sigma) in 1X Tris buffered saline for immunoblotting with anti-ERK (1: 2000, Cell Signalling Technologies; Cat# 9102) and anti-pERK (1: 2000, Cell Signalling Technologies; Cat# 9101) respectively. Detection was performed using goat anti-mouse and goat anti-rabbit antibodies respectively conjugated with horse radish peroxidase. Bands were visualized with ECL and imaged using ChemiDoc (Biorad).

TUNEL assay

Whole mount TUNEL staining to determine apoptosis was performed on 2 and 5 dpf larvae as previously described(Jamadagni & Patten, 2019). Briefly, larvae were fixed in 4% PFA and then serially dehydrated and rehydrated with 25, 50 and 75% MeOH in PBST (0.1% Tween) and rinsed with PBST several times. The embryos were then digested with Proteinase K (10 µg/ml) for 20 min., followed by rinses with PBST and re-fixed with 4% PFA for 20 min. This was followed by 2 quick washes and 3 long washes of 20 min. in PBST (1% Triton-X), then were rinsed again with PBS and incubated in TUNEL reaction mix (as directed by the

manufacturers; Roche/Sigma-Aldrich) for 1 h at 37 °C. The larvae were then mounted and imaged under a Zeiss LSM 780 confocal microscope.

Whole-mount fluorescence immunohistochemistry

Whole mount fluorescence immunohistochemistry for proliferation (pH3 marker) was performed as previously described (Verduzco & Amatruda, 2011). Briefly, 2 and 5 dpf zebrafish larvae were fixed in 4% PFA overnight at 4 °C. After fixation, the embryos were rinsed two times for ten minutes each with PBST (0.1% Tween). They were then incubated acetone (100%) at – 20 °C for 7 min. Following this the larvae were rinsed with PBST (0.3% Triton-X) and PBS-DT (1% BSA, 1% DMSO, 1% Triton-X) and further blocked in 5% NGS in PBS-DT for 1 h. Primary antibody pAb Rabbit Anti-phospho-Histone H3 (Ser10) (1:250; Millipore, Cat# 06–570) was added to the blocking solution and incubated overnight at 4 °C. The next day, larvae were rinsed with PBS-DT and secondary antibody (Alexa fluor 488, 1:1000; Invitrogen) was then added and incubated overnight at 4 °C. Larvae were rinsed and imaged with a Ziess LSM780 confocal microscope.

For pERK staining, zebrafish larvae (5 dpf) were placed in the dark for 1.5–2 hours prior to rapid fixation in 4% PFA O/N. The larvae were then rinsed multiple times with PBST (0.1% Tween) followed by incubation in acetone (100%) at – 20 °C for 15 min. The larvae were then rinsed with PBST (0.3% Triton-X) and PBS-DT (1% BSA, 1% DMSO, 1% Triton-X) and further blocked in 5% NGS in PBS-DT for 1 h. Primary antibody anti-phospho-ERK (1:500, Cell Signalling Technologies; Cat# 9101) was added to the blocking solution and incubated overnight at 4 °C. The following day, larvae were rinsed with PBS-DT and incubated with secondary antibody (Alexa fluor 488, 1:1000; Invitrogen) overnight at 4 °C. Larvae were rinsed and imaged with a Ziess LSM780 confocal microscope.

Transcriptomic, differential expression and pathway analyses

Three independent batches of 5 dpf *chd7*+/+ and -/- larvae were dissected to extract the whole brain, by each of two experimenters, corresponding to experimental triplicates. Total RNA was extracted from these flash frozen brains using PicoPure RNA extraction kit (Thermo Fisher Scientific) following the manufacturer's standard protocol. For each sample, RNA extraction was made from 5 whole brains. Absence of contamination was assessed by Nanodrop using 260/280 and 260/230 ratios. Quality of total RNA was assessed with the BioAnalyser Nano (Agilent) and all samples had a RIN above 8.3. Library preparation was performed using the Truseq RNA (Illumina). 8 PCR cycles were required to amplify cDNA libraries. Libraries were quantified by Nanodrop and BioAnalyser. All libraries were diluted to 10 nM and normalized with the Miseq SR50 v2. Libraries were pooled to equimolar concentration and multiplexed by

6 samples per lane. Sequencing was performed with the Illumina Hiseq2000 using the SBS Reagent Kit v3 (100 cycles, paired-end) with 1.6 nM of the pooled library. Cluster density was targeted at around 800k clusters/mm^2. Between 75 and 140 million reads were generated for each sample. Library preparation and sequencing was done at the genomics platform of the Institute for Research in Immunology and Cancer (University of Montreal). More than 93% of high quality reads were mappable onto the zv9 version of the zebrafish genome (ensemble release 77) using TopHat version 2.0.10. Differential gene expression analysis was assessed by DeSeq2 package using R software. Genes showing an absolute fold-change>1.2 and an adjusted p value (false discovery rate) <0.05 were considered to be significantly differentially expressed. Gene enrichment and pathway analysis were performed using PANTHER and DAVID bioinformatics resources(Huang da, Sherman, & Lempicki, 2009; Mi, Muruganujan, Ebert, Huang, & Thomas, 2019). The RNA-seq has been deposited to the GEO database (GSE139623).

RT-qPCR

RT-qPCR was performed as previously described in Breuer et al. (2019)(Breuer et al., 2019). In short, RNA was isolated from ~30 embryos using TriReagent® (Sigma) according to manufacturer's protocol. 1µg of RNA was used for cDNA synthesis by SuperScript®Vilo™ kit (Invitrogen). RT-qPCR was run with SYBR Green Master Mix (Bioline) using the LightCycler® 96 (Roche). *ef1a* was used as the reference gene for normalization and following primers were used for *paqr3b*: FW: 5' – CGCTGGCTTGCTCTGGATTA – 3'; RV:5' – CCTGCCTCCAAAAACTGTTGC – 3'.

Lymphoblastoid cell lines

Cell culture

Lymphoblastoid cell lines (LCLs) from a *CHD7* mutation-positive (c.5050+1G > T) CHARGE syndrome patient and its unaffected parents were maintained in RPMI medium as described previously (Belanger C. et al. 2018). Families provided informed consent on studies approved by the respective institutional review board of the Baylor College of Medicine (experimental cohort for this study).

RT-qPCR (LCLs)

Relative transcript levels of *PAQR3* mRNA in LCLs were analysed by RTqPCR. The RTqPCR analyses were performed as described above. The following primers were used for *PAQR3:* (Forward) 5'- CCAATTACCTCACGCAGCAA- 3' and (Reverse) 5'- GGAGCACCAATTCCTCCATT- 3'. Fold change was calculated according to the 2(−ΔΔCt) method, using *HPRT1* and *RPS1* as housekeeping genes for normalization. All data were expressed as mean fold change ± SD across replicates, relative to control parents set to 1.

ChIP assays

ChIP-qPCR assays in 2X10^8 LCLs were performed as previously described (Belanger et al. 2018), using 2µg of rabbit anti-CHD7 antibody (Cell Signalling; Cat# 6505) and 2µg of rabbit anti-HA antibody (Abcam; Cat# ab9110) as negative control. Target sequence (196 bp) at the *PAQR3* proximal promoter was determined based on a previously described ChIP-seq peak of CHD7 in H1-hESC (Sethi et al., 2020) that was downloaded from the ENCODE portal (Sloan et al., 2016) (https://www.encodeproject.org/); the relevant track (#ENCFF628RLE) was visualized on the UCSC genome browser using the GRCh37/hg19 human assembly (https://genome.ucsc.edu/). Primer sequences were: (Forward) 5'- GCT ACA GGC GAA TAC AAG TGG - 3' and (Reverse) 5'- CTG ACT TCA GCT TAG AAA TCC TC - 3'). ChIP-qPCR efficiency was calculated in % of CHD7 IP relative to input and expressed in fold enrichment relative to HA negative control.

C. elegans

C. elegans strains and maintenance

C. elegans was handled applying standard conditions (Stiernagle, 2006). Worms were kept on NGM agar with an *E. coli* OP50 lawn at 15°C for maintenance and 20°C for assays. The mutant strain *chd-7(gk290)* (VC606) was provided by the Caenorhabditis Genetics Center at the University of Minnesota and backcrossed four times to N2 wildtype (WT).

C. elegans locomotion assay

A worm tracking machine (*Wmicrotracker*, Phylum Tech) was used to track the swimming locomotion of *C. elegans*. Assays were performed in 96-well-microtiter plates with approximately 30 worms and 100 µl M9 buffer per well. *E. coli* OP50 were added to the wells to prevent worms from starving. Each microtiter well was crossed by two infrared light rays from top to bottom and a detector determined interruptions of these light rays by worms moving in the well. From the signal a movement score was calculated, which is defined as animal movement in a fixed time period. Movement was tracked for 10 hours. The assay was

performed in triplicates and movement scores of *chd-7(gk290)* over time were compared to WT in three independent experiments using two-tailed t-test to determine significance.

Drug libraries

Natural and FDA-approved compounds were provided by Sigma-Adrich (Sigma Aldrich's Library of Pharmacologically Active Compounds (Lopac) 1280 library, containing compounds as of July 2015, Oakville/Canada), Prestwick Chemicals (Prestwick Chemical Library; containing compounds as of March 2015, Illkirch/France), Microsource (880 compounds, Gaylordsville, CT/USA), Enzo Life Science, Inc. (BML-2865 Natural Products Library, containing compounds as of April 2015, Farmingdale, NY/USA). All compounds were dissolved in DMSO and tested at a concentration of 20 µM.

Drugs screen in C. elegans

Chd-7(gk290) nematodes were exposed to the drugs from the libraries in microtiter wells at a concentration of 20 µM in M9 buffer with OP50 and movement was tracked for at least 4 hours. The average movement score of *C. elegans* treated with a specific drug was compared to the DMSO control and the average movement score of the whole plate. If values for a drug were higher than the respective controls, a second screen to validate the increased movement due to the specific drug was performed. Compounds that increased locomotion of *chd-7(gk290)* significantly according to a two-tailed t-test in the second screen were counted as positive. A complete list of all drugs tested can be found in reference (K. F. Y. Schmeisser, Parker, JA, 2017).

Assessment of neuronal integrity of GABAergic and cholinergic motor neurons in C. elegans

An *unc-47*p::mCherry and *unc-17*p::GFP reporter for GABAergic and cholinergic motor neurons was crossed into *chd-7*(*gk290*) to analyse motor neurodevelopment at the L4 stage *in vivo*. Worms were put on microscopy slides with 2% agarose pads, paralysed with 5 mM levamisole and covered with a cover slip. Neuronal examinations were performed with a Zeiss Axio Imager M2 microscope (*unc-47*p::mCherry) and a Leica DM6000 microscope (*unc-17*p::GFP). About 100 worms were analysed in four independent experiments and compared to WT.

For a more detailed neuronal status in *chd-7*(*gk290*), neuronal morphology was categorized into the following groups: Gaps in the axonal branches per animal, number of axon commissures per animal, number of GABAergic and cholinergic cell bodies, and frequency of breaks > 50µm in the nerve cords, which goes along with massive loss of neuronal tissue.

About 30 *chd-7*(*gk290*) worms with dysmorphic neuronal morphology were scored and compared to the neuronal morphology of WT worms.

Testing of beneficial compounds identified in the drug screen was performed as follows: NGM agar plates containing 20 µM of the respective substance were freshly prepared prior to each assay and seeded with OP50. Young adult *C. elegans* were allowed to lay eggs for about 4 h, and assays for neuronal morphology were performed in the progeny at the L4 stage as described above. Mean +/- standard deviation of three independent experiments were calculated and two-tailed t-tests determined significance. Gaps in axonal branches, number of axon commissures, cell bodies, and breaks > 50µm in the nerve chords where determined in three independent experiments. Average and standard deviation of all tested animals was calculated and two-tailed t-tests were performed.

C. elegans lifespan assay

Lifespan assays were performed as previously described(K. Schmeisser et al., 2013). Briefly, worms were synchronized at the egg stage (day 0) and about 50 nematodes were transferred to each of 3 fresh lifespan plates per condition at the L4 stage. Worms were transferred on plates containing 10 µM FUDR (solved in water; applied on top of the grown bacteria lawn) after 24 – 48 hours to prevent contamination with progeny generations. Nematodes that did not react to repeated gentle stimulation were scored as dead. Non-natural deaths (bagging, protrusive vulvae) and lost animals were censored. JMP 11.0.0 (SAS institute Inc.) was used for statistical analyses, and the log-rang test determined p-value and significance.

Statistical analysis

All zebrafish experiments were performed on at least three replicates (N) and each consisted of a sample size (n) of 8-30 fish. All *C. elegans* experiments have been performed for a minimum of three biological replicates. The number of samples was determined empirically. Data are presented as Mean±SEM. Significance was determined using either Student's t-test, One-way ANOVA or two-way ANOVA using Graphpad PRISM software. All graphs were plotted using the Graphpad PRISM software.

Data availability

The RNA-seq has been deposited to the GEO database (GSE139623). (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE139623).

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Author Contributions

SP conceived this work. PJ designed, collected, analysed and interpreted the results from studies related to GABAergic neurons and behaviour in zebrafish. ES, MB collected and analysed the results of RNAseq. KS collected, analysed and interpreted the results from studies related to *C. elegans* experiments. BK, SP generated and characterized the CRISPR *chd7-/- mutant* line. PJ and BK performed the drug analyses in zebrafish. TC performed the ChIP analyses. PJ, MB, KS, JAP, ES, TC, NP and SP interpreted the results. JAP, NP and SP secured the research funding. PJ, KS, TC, NP and SP drafted the manuscript. PJ and SP with contributions from all authors prepared the final version of the manuscript. All authors read the final version of this manuscript.

Conflict of interests

The authors declare no competing interests. ES and JAP are co-founders of Modelis Inc.
The commercial affiliations did not play any role in this study; in particular, they did not have any additional role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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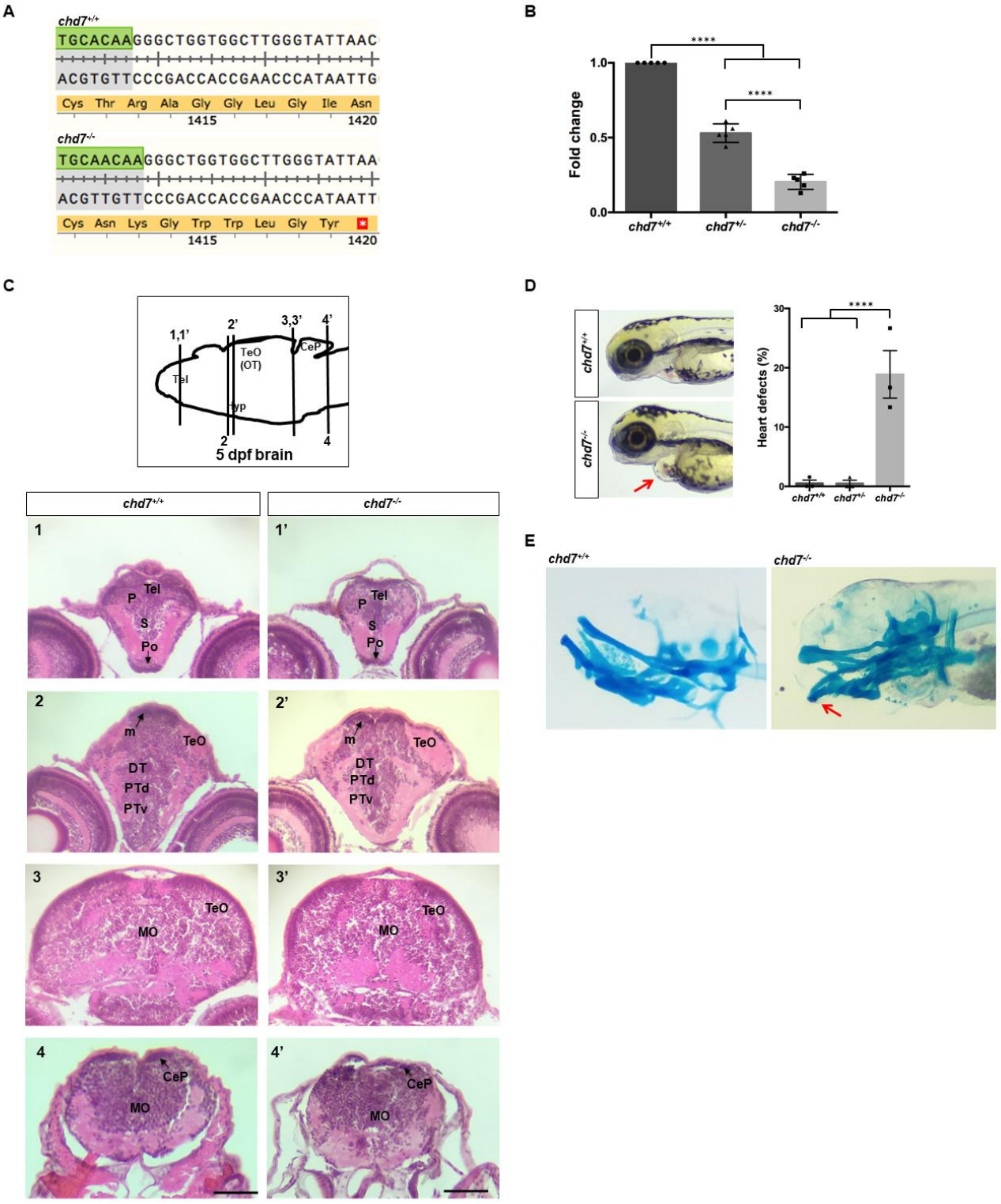
Figure EV1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chd7+/+
TGCACAAGGGCTGGTGGCTTGGGTATTAAC
ACGTGTTCCCGACCACCGAACCCATAATTG
Cys Thr Arg Ala Gly Gly Leu Gly Ile Asn
1415
1420
chd7-/-
TGCAACAAGGGCTGGTGGCTTGGGTATTAA
ACGTTGTTCCCGACCACCGAACCCATAATT
Cys Asn Lys Gly Trp Trp Leu Gly Tyr
1415
1420
B
Fold change
1.0
0.5
0.0
****
****
chd7+/+
chd7+/-
chd7-/-
C
1,1'
2'
3,3'
4'
TeO (OT)
CeP
Tel
Typ
2
4
5 dpf brain
chd7+/+
chd7-/-
1
1'
Tel
P
S
Po
2
2'
m
TeO
DT
PTd
PTv
3
3'
TeO
MO
4
4'
CeP
MO
D
chd7+/+
chd7-/-
Heart defects (%)
30
20
10
0
****
chd7+/+
chd7+/-
chd7-/-
E
chd7+/+
chd7-/-

Figure EV2

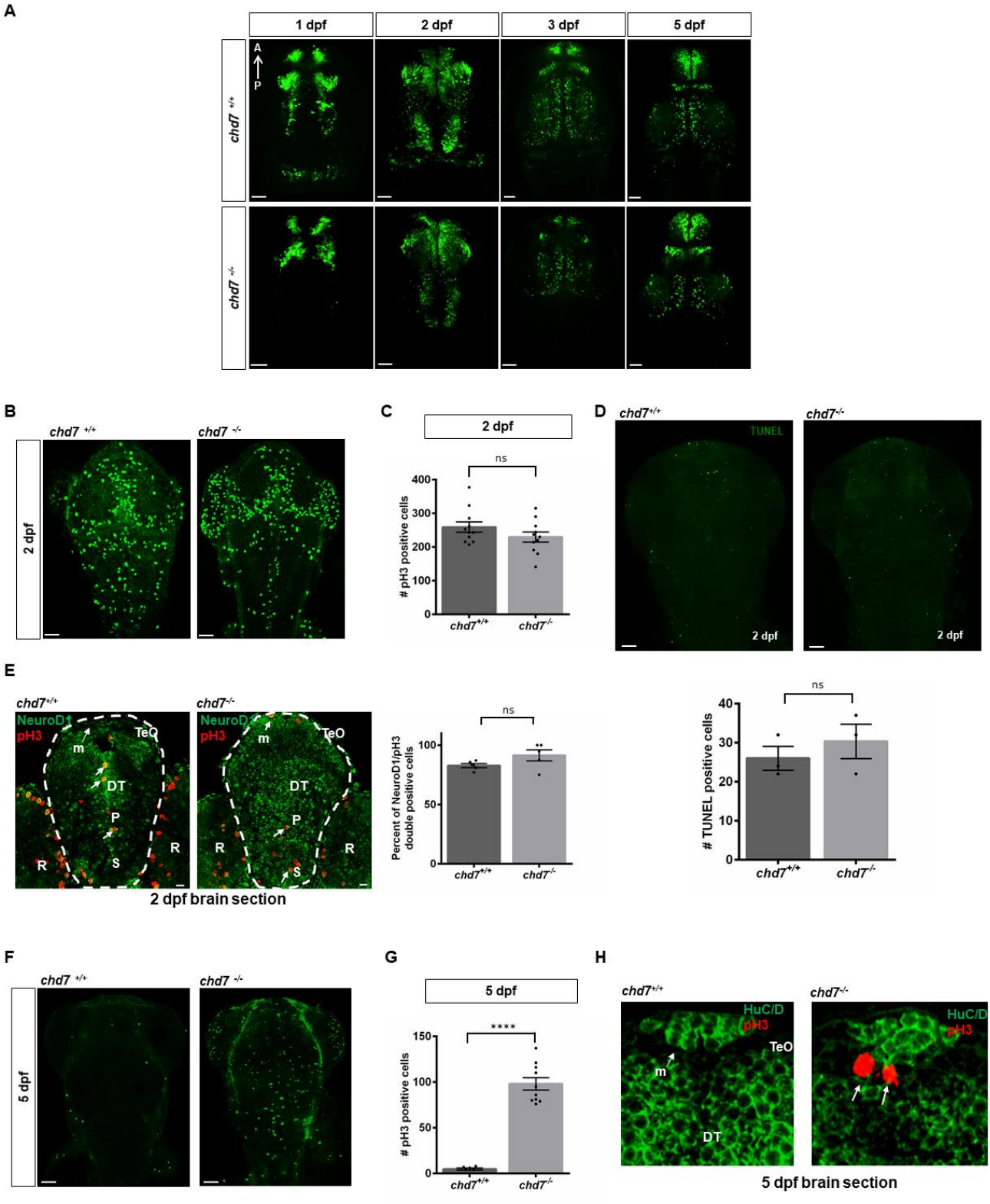

A
1 dpf
2 dpf
3 dpf
5 dpf
A
P
chd7 +/+
chd7 -/-
B
chd7 +/+
chd7 -/-
2 dpf
C
2 dpf
ns
pH3 positive cells
400
300
200
100
0
chd7+/+
chd7-/-
D
chd7+/+
chd7-/-
TUNEL
2 dpf
2 dpf
E
chd7+/+
chd7-/-
NeuroD1
pH3
m
TeO
DT
P
R
R
S
R
R
S
2 dpf brain section
ns
Percent of NeuroD1/pH3 double positive cells
100
50
0
chd7+/+
chd7-/-
ns
TUNEL positive cells
40
30
20
10
0
chd7+/+
chd7-/-
F
chd7 +/+
chd7 -/-
5 dpf
G
5 dpf
****
pH3 positive cells
150
100
50
0
chd7+/+
chd7-/-
H
chd7+/+
chd7-/-
HuC/D
pH3
TeO
m
DT
HuC/D
pH3
5 dpf brain section

Figure EV3

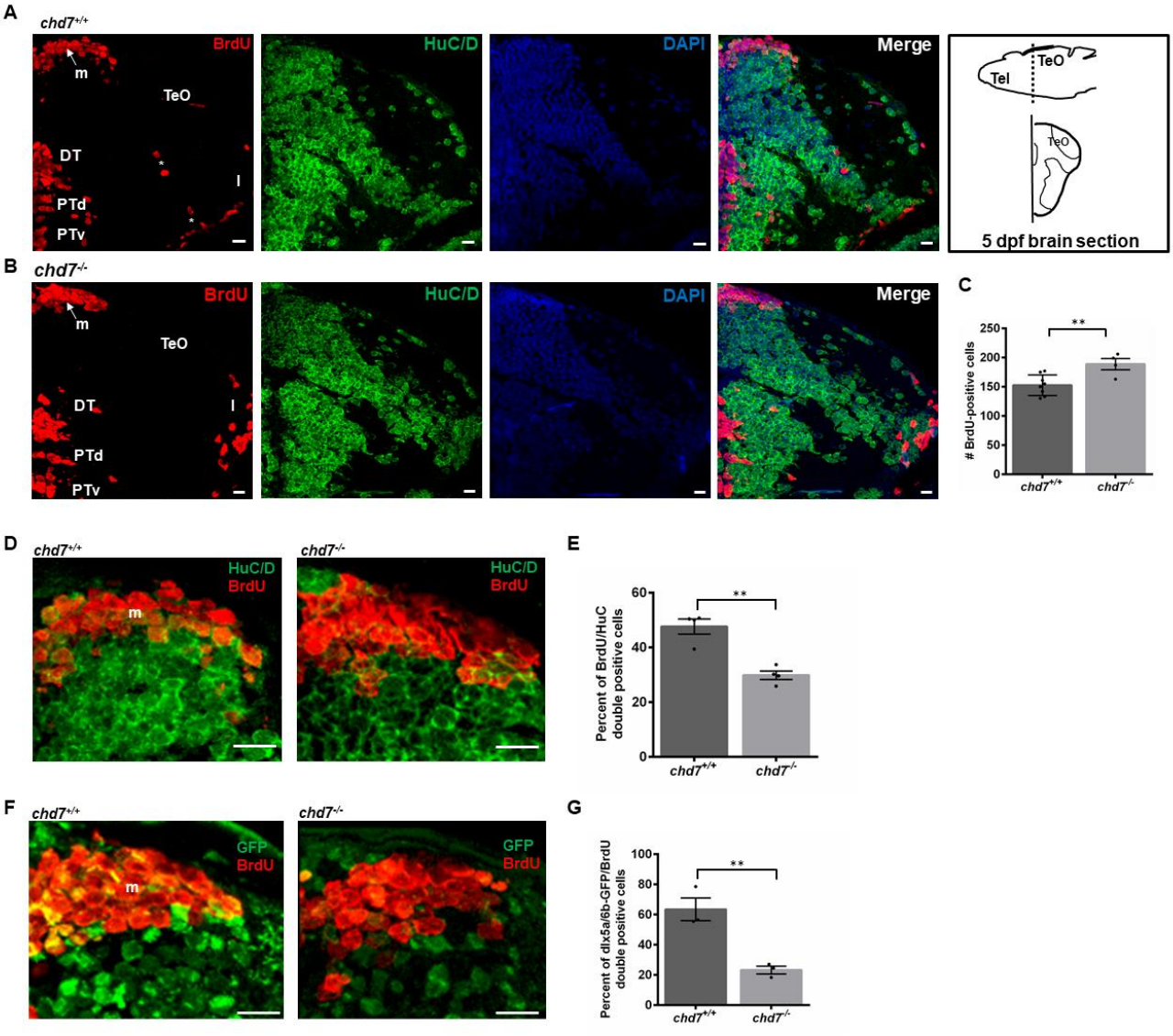

A
chd7+/+
BrdU
HuC/D
DAPI
Merge
m
TeO
DT
l
PTd
PTv
Tel
5 dpf brain section
B
chd7-/-
C
BrdU-positive cells
**
D
HuC/D
BrdU
E
Percent of BrdU/HuC double positive cells
F
GFP
BrdU
G
Percent of dlx5a/6b-GFP/BrdU double positive cells

Figure EV4

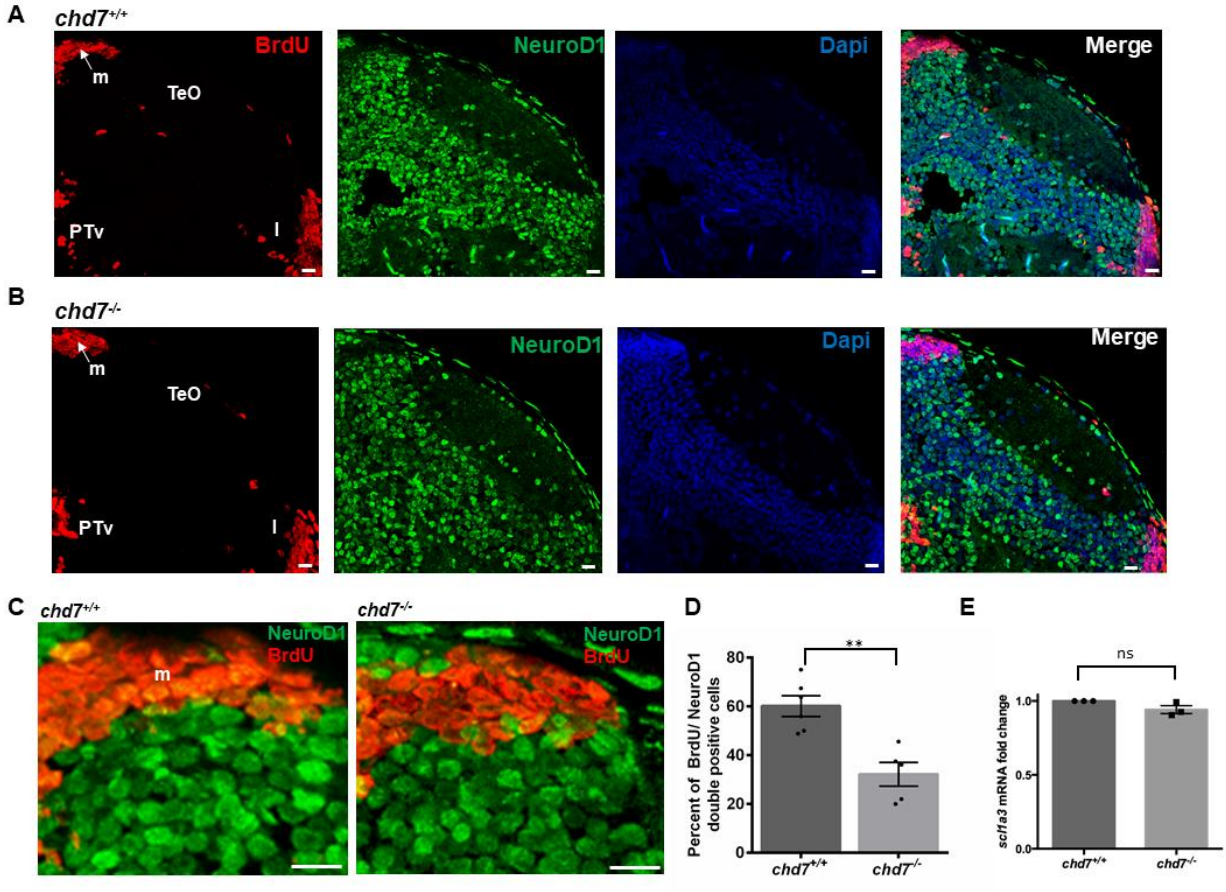

A
chd7+/+
BrdU
NeuroD1
Dapi
Merge
m
TeO
PTv
l
B
chd7-/-
C
chd7+/+
chd7-/-
NeuroD1
BrdU
D
Percent of BrdU/ NeuroD1 double positive cells
**
E
scl1a3 mRNA fold change
ns

Figure EV5

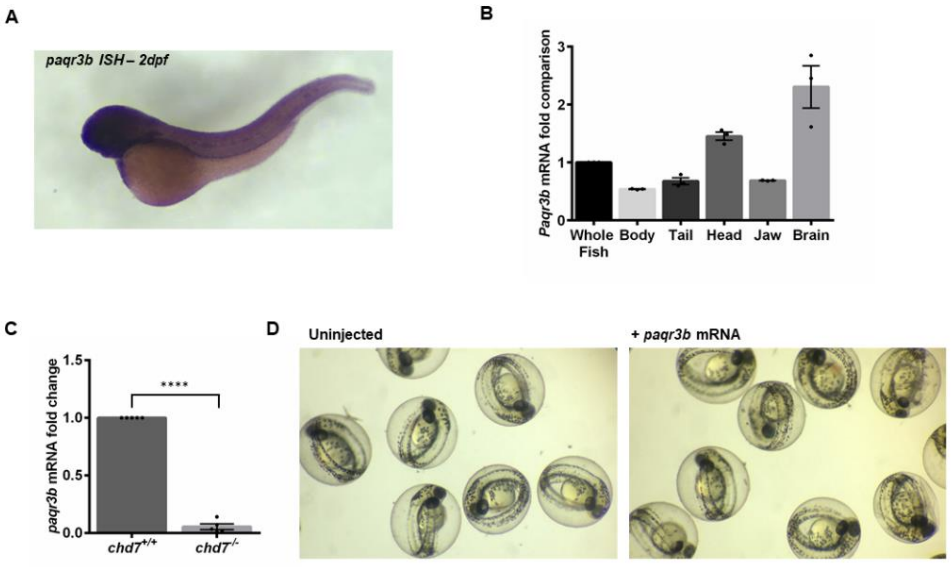
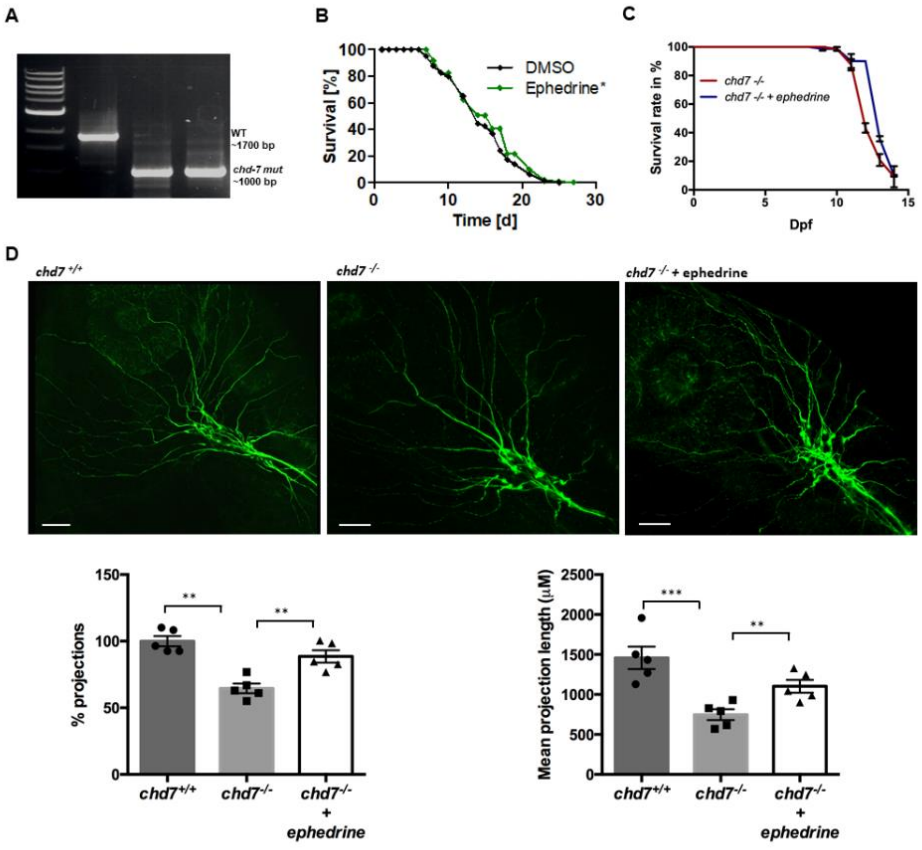


Figure EV6



Expanded View Figure Legends

Figure EV1 Generation and characterization of *chd7-/-* mutants

A Translation following genome editing resulted in a premature stop codon (*) in *chd7*−/− fish.

B qPCR analysis of RNAs from 3dpf larvae shows a significant reduction of *chd7* mRNA expression in both *chd7*+/− and *chd7*−/− compared with wild-type ($N = 5$). ****$P < 0.0001$, one-way ANOVA.

C Examination of brain tissues in *chd7*+/+ (left images) and *chd7*−/− (right images) by H&E staining at 5 dpf. Zebrafish brains were sectioned at telencephalic (1,1'), diencephalic (2, 2'), mesencephalic (3,3') and rhombencephalic levels (4,4'). Levels of sections are indicated in the sketch of a sagittal view of a 5 dpf zebrafish brain (top image). The scale bar is 0.12 mm. P: pallium, S: subpallium, Po:preoptic region, Tel: Telencephalon; TeO: tectum opticum (or OT: optic tectum), m: medial tectal proliferating zone, DT: dorsal thalamus, PTd: dorsal part of posterior tuberculum, PTv: ventral part of posterior tuberculum, MO: medulla oblongata, Hyp: hypothalamus, CeP: cerebellar plate.

D *chd7*−/− mutant fish displayed features of CS such as heart defects (red arrow) at a low penetrance ($N = 3$). ****$P < 0.0001$; Student's *t*-test.

E Alcian blue staining of 6dpf larvae showing craniofacial defects at Meckel's cartilage (red arrow).

Data information: Data are presented as mean ± SEM. *n* is the number of fish used. *N* is the number of experimental repeats.

Figure EV2 Proliferation and apoptosis analyses in wild-type and *chd7-/-* mutant zebrafish

A Analysis of GABAergic neurons network development between 1 dpf and 5 dpf between control (top row) and *chd7-/-* mutants (bottom row) ($N = 3$).

B Proliferation analysis by pH3 staining at 2 dpf in control and *chd7-/-* mutants.

C Bar graph showing no difference in pH3-positive cells in zebrafish brain at 2 dpf between control and mutants ($n = 11$; ns, $P = 0.1816$; Student's *t*-test).

D Cell death analysis by TUNEL assay in *chd7*−/− and *chd7*+/+ brains shows no change in apoptotic cells at 2 dpf ($N = 3$, $n = 8$; ns, $P = 0.464$; Student's *t*-test).

E Transverse sections of 2 dpf larvae after immunostaining with pH3 (red) and NeuroD1 (green). Bar graph showing no difference in pH3 and NeuroD1 double-positive cells (arrows) in zebrafish brain at 2 dpf between control and mutants ($N = 3$, $n = 5$; ns, $P = 0.124$; Student's *t*-test). P: pallium, S: subpallium, TeO: tectum opticum, m: medial tectal proliferating zone, DT: dorsal thalamus R: retina.

F Proliferation analysis by pH3 staining at 5 dpf in control and *chd7-/-* mutants.

G An increase in pH3-positive cells was noted in brains of mutant fish compared with controls at 5 dpf ($N = 4$, $n = 10$; ****$P < 0.0001$; Student's *t*-test).

H Transverse sections of 5 dpf larvae after immunostaining with pH3 (red) and HuC/D (green). pH3-positive cells (arrows) were observed at 5 dpf in the medial tectal proliferating zone of mutant fish brains but none in controls ($N = 3$). *P*: pallium, S: subpallium, TeO: tectum opticum, m: medial tectal proliferating zone, DT: dorsal thalamus.

Data information: Data are presented as mean ± SEM. Scale bar = 50 μm and 10 μm for 2 dpf NeuroD1 and pH3 co-stain.

Figure EV 3 Aberrant GABAergic neuronal differentiation in *chd7-/-* mutant zebrafish

A, B. Immunostaining with BrdU and HuC/D in brain sections of the zebrafish tectal region in *chd7*+/+ (A) and *chd7*−/− (B). Level of the sections is indicated in the sketch of a 5 dpf zebrafish brain (top right image in (A)). The scale bar is 10 μm. Tel: Telencephalon; TeO: tectum opticum, m: medial tectal proliferating zone, DT: dorsal thalamus, PTd: dorsal part of posterior tuberculum, PTv: ventral part of posterior tuberculum, l: lateral tectal proliferation zone. Asterisks (*) marks early migrated region of pretectum and proglomerular.

C. The number of BrdU-positive cells in transverse sections of the zebrafish brain in *chd7*+/+ and *chd7*−/− (*N* = 3, $chd7^{+/+}$: *n* = 8; $chd7^{-/-}$: *n* = 4; ***P* < 0.05; Student's *t*-test).

D. Immunostaining with BrdU and HuC/D in brain sections of the zebrafish medial tectal region. Scale bar = 10 μm. m: medial tectal.

E. The percentage of BrdU and HuC/D-double positive cells among the BrdU-positive cells in the medial tectal zone (*N* = 3, *n* = 4; ***P* < 0.05; Student's *t*-test).

F. Immunostaining with BrdU and GFP (to label *dlx5a/6a-GFP* + GABAergic neurons) in brain sections of the zebrafish medial tectal region. Scale bar = 10 μm. m: medial tectal.

G. The percentage of BrdU and *dlx5a/6a-GFP*-double positive cells among the BrdU-positive cells in the medial tectal zone (*N* = 3, *n* = 3; ***P* < 0.05; Student's *t*-test).

Data information: Data are presented as mean ± SEM. *n* is the number of fish used. *N* is the number of experimental repeats.

Figure EV 4 Impaired neurogenesis in *chd7-/-* mutant zebrafish

A, B. Immunostaining with BrdU and NeuroD1 in brain sections of the zebrafish tectal region in *chd7*+/+ (A) and *chd7*−/− (B). The scale bar is 10 μm. Tel: Telencephalon; TeO: tectum opticum, m: medial tectal, DT: dorsal thalamus, PTd: dorsal part of posterior tuberculum, PTv: ventral part of posterior tuberculum, l: lateral tectal proliferation zone.

C. Immunostaining with BrdU and NeuroD1 in brain sections of the zebrafish medial tectal region. Scale bar = 10 μm. m: medial tectal.

D. The percentage of BrdU and NeuroD1-double positive cells among BrdU-positive cells in the medial tectal zone (*N* = 3, *chd7*+/+: *n* = 6; *chd7*−/−: *n* = 6; ***P* < 0.05; Student's *t*-test).

E. Expression level of *scl1a3* mRNA in *chd7*−/− relative to *chd7*+/+ (*N* = 4). ns, not significant; Student's *t*-test.

Data information: Data are presented as mean ± SEM. *n* is the number of fish used. *N* is the number of experimental repeats.

Figure EV5 Expression of *paqr3b* in wild-type and *chd7-/-* mutant zebrafish

A, B. Expression profile of *paqr3b* in whole-mount zebrafish by *in situ* hybridization (A) and in tissues by qRT–PCR (B). *N* = 4.

C. qRT–PCR validation of the downregulation of *paqr3b* (*N* = 4; *****P* < 0.0001, Student's *t*-test).

D. Images of gross morphology of 2 dpf zebrafish embryos with or without overexpression of *paqr3b* mRNA. Of note, neither abnormalities nor death were observed in zebrafish embryos upon overexpression of *paqr3b* mRNA.

Data information: Data are presented as mean ± SEM. *n* is the number of fish used. *N* is the number of experimental repeats.

Figure EV6 Pharmacological responses of *chd7-/-* mutants and amelioration of neuronal network development by ephedrine

A PCR proof of a 700 bp deletion in the *chd-7* gene in *chd-7(gk290)* mutant worms.

B Lifespan analyses of *chd-7(gk290) Caenorhabditis elegans* mutants treated with ephedrine (green) compared with control DMSO (black). Log-rank test was performed for statistical analyses. (N = 3, n = 50; *P < 0.05).

C Survival rate of *chd7*−/− zebrafish mutants treated with ephedrine (blue) compared with untreated mutants (red). N = 3, n = 60.

D Acetylated tubulin staining in non-treated and ephedrine-treated *chd7*−/− zebrafish mutants showing rescue of the severely affected outbranching structure of V^{th} cranial nerves. Graphs showing quantitative analyses of percentage and mean total length of peripheral projections per zebrafish in controls and mutants without and with ephedrine treatment (n = 5; ***P < 0.001; **P < 0.005; one-way ANOVA).

Data information: Data are presented as mean ± SEM. Scale bar = 50 µm. n is the number of fish or worms used. N is the number of experimental repeats.

4 Chd7-Paqr3b axis regulates neural precursor proliferation and differentiation in the developing zebrafish brain

This chapter is composed of a paper in preparation:

Priyanka Jamadagni and Shunmoogum A. Patten. Chd7-Paqr3b axis regulates neural precursor proliferation and differentiation in the developing zebrafish brain.

Contribution: SAP and PJ conceived the work. PJ designed, collected, analysed and interpreted the results. PJ and SAP drafted and prepared the final version of the manuscript.

4.1 Summary

Mutations in the ATP-dependent chromatin remodeller chromodomain, helicase, DNA binding (CHD) 7 are the primary cause of CHARGE syndrome and have been associated with autism spectrum disorder (ASD). CHARGE syndrome individuals often present brain developmental defects like microcephaly, cerebral atrophy and cerebellar and olfactory bulb hypoplasia among others and behavioural problems such as hyperactivity, seizure, intellectual disability and autism. However, little is known about the molecular mechanisms that underlie these brain developmental deficits. In our previous work we have showed that *chd7*$^{-/-}$ mutants have reduced number of GABAergic neurons, exhibit a hyperactivity behavioural phenotype. Overexpression of *paqr3b* – a gene that is downregulated upon loss of *chd7*, partially rescues the number of GABAergic neurons and the hyperactivity in the mutants. This suggests that *chd7* along with *paqr3b* potentially play a role in neurogenesis. To test this, we conducted histological analyses of the *chd7-/-* mutant zebrafish brain through early stages of development in comparison to the wildtypes. Here, we report that the *chd7-/-* mutant zebrafish brains retain a higher number of Sox2+ Neural Stem/Progenitor cells (NSPCs) in the midbrain at 5 dpf. Furthermore, there is also an abnormal number of radial glial cells (GFAP+) and they develop fewer radial processes in the optic tectum region of the *chd7-/-* mutant larval midbrain. The defects in neural precursor cells occur early in zebrafish embryonic development and are sustained at 5 dpf larval stage. Interestingly, overexpression of the *paqr3b* mRNA in the *chd7-/-* mutants significantly rescues the number of Sox+ and GFAP+ NSPCs, and the number of GABAergic-Sox2 co-labelled neurons. Thus, we report a novel role for the *chd7-paqr3b* regulatory link in NSPCs regulation and their differentiation into GABAergic neurons.

4.2 Introduction

CHARGE syndrome is a rare congenital disorder caused by mutations in the *CHD7* gene. It is a multisystem disorder characterized by **C**oloboma of the eye, **H**eart defects, **A**tresia choanae, **R**etarded growth and development, **G**enital abnormalities, and **E**ar defects. Individuals with CHARGE consistently present with defects in brain development like microcephaly, cerebral atrophy and cerebellar and olfactory bulb hypoplasia among others (R. Becker et al., 2001; Hale et al., 2016; Johansson et al., 2006; Legendre et al., 2012; Lin et al., 1990; Sanlaville et al., 2006; Tellier et al., 1998; T. Yu et al., 2013). Additionally, CHARGE syndrome individuals often present with behavioural anomalies such as hyperactivity, cognitive and intellectual disability and social behavioural deficits that have overlapping symptoms with other neurodevelopmental disorders like autism spectrum disorders, ADHD, hyperactivity disorder and OCD (Bergman, Janssen, et al., 2011; Hartshorne, Hefner, et al., 2005). Evidently, CHD7 plays an important role in neurodevelopment and behaviour (W. Feng, Kawauchi, et al., 2017; W. Feng & Liu, 2013; Whittaker, Riegman, Kasah, Mohan, Yu, Pijuan-Sala, et al., 2017) (Schulz et al., 2014) (He et al., 2016).

We have been investigating the role of CHD7 in brain development in zebrafish. In our previous work, we made two striking observations (Jamadagni et al., 2021): (i) the number of GABAergic neurons is significantly reduced in 5 days postfertilization (dpf) zebrafish *chd7-/-* mutant brain and (ii) *chd7* promotes *paqr3b* expression, and that this is required for normal GABAergic neuron development in the zebrafish brain. Several studies suggest that CHD7 regulates neural progenitor differentiation. For instance, loss of *Chd7* in embryonic stem cells has been reported to affect neuronal and glial differentiation in mouse embryos and leads to an impaired neuronal complexity and neurite length in differentiated neurons (Yao et al., 2020). *Chd7* also affects terminal differentiation upon haploinsufficiency or inactivation from progenitor cells like the granule neuron progenitor cells (GNPs), oligodendrocyte precursor cells (OLPs) without affecting the generation or proliferation of the GNPs and OLPs (Cao et al., 2018; W. Feng, Kawauchi, et al., 2017; W. Feng & Liu, 2013; He et al., 2016; Whittaker, Kasah, et al., 2017). In *chd7-/-* zebrafish larvae, we observed a failure in differentiation of progenitor cells into GABAergic neurons (Jamadagni et al., 2021). Importantly, overexpression of *paqr3b* mRNA in *chd7-/-* mutants significantly rescued the GABAergic neuron development defects. However, the mechanistic basis behind this rescue remains to be determined. Human *PAQR3* is a regulator of ER (endoplasmic reticulum)-to-Golgi transport(Cao et al., 2018) that is essential for maintaining cellular and physiological homeostasis (L. Feng et al., 2007). It has also been demonstrated to be a spatial regulator of Raf-1 by sequestrating Raf-1 to the Golgi apparatus, thereby inhibiting the MAPK/ERK signalling(L. Feng et al., 2007; Y. Zhang et al., 2010). ERK signalling plays a key role in early neuronal differentiation (Lei, Ling, Chen, Hong,

& Ling, 2020; X. Yu, Li, Chan, & Wu, 2015). Therefore, we hypothesized that *chd7* through its target gene *paqr3b* might play an important role in the neurogenesis in the zebrafish brain.

In this study, we show that the zebrafish brain retains a high number of Neural Stem/Progenitor cells (NSPCs; Sox2+) upon loss-of-function of *chd7*. Furthermore, radial glial cells (GFAP+) are abnormal in number and develop fewer radial processes in *chd7-/-* mutant larval brain. These defects in neural precursor cells occur early in zebrafish embryonic development and are sustained at larval stage (5 dpf). and is rescued to near normal levels upon overexpression of the *paqr3b* mRNA in the *chd7-/-* mutants. Overexpression of *paqr3b* in *chd7-/-* mutants rescues the number of Sox2+-NSPCs and radial glial cells. Altogether, our study demonstrates a novel molecular mechanism implicating the *chd7-paqr3b* axis regulating neural precursor cell number in zebrafish brain development.

4.3 Results

4.3.1 Abnormal NSPC number in *chd7-/-* mutant zebrafish developing brain

We previously reported a decrease in the number of GABAergic neurons upon loss-of-function of *chd7* in the 5 dpf zebrafish brain (Jamadagni et al., 2021). The marked reduction in the number of GABAergic neurons was particularly more prominent in the midbrain/optic tectum region (Figure 1A-C). A tight regulation of the neural stem/progenitor cells, neuroepithelial, radial glial cells and their differentiation controls the precise number and type of the final neuronal and glial types in the brain and spinal cord (Johnson et al., 2016; Paridaen & Huttner, 2014). Therefore, we asked whether changes in the nature of the neural stem/progenitor cells' (NSPCs) population in *chd7-/-* fish resulted in the reduction of GABAergic neurons in *chd7-/-* fish. To test this, we first examined the expression of the pluripotency marker Sox2 in coronal sections of 5dpf *chd7-/-* midbrain. The number of Sox2-positive cells was significantly higher in the *chd7-/-* fish compared to *chd7+/+* control (Figure 1D, E). This marked increased in the number of Sox2-positive NSPCs was very prominent along the dorsal midbrain ventricles in the *chd7-/-* mutants compared to controls. The ventricle lining proliferative cells forms the consistent neural progenitor zone in the zebrafish brain (Grandel, Kaslin, Ganz, Wenzel, & Brand, 2006). Thus loss-of-function of *chd7* likely increases the neural progenitor pools in zebrafish brain.

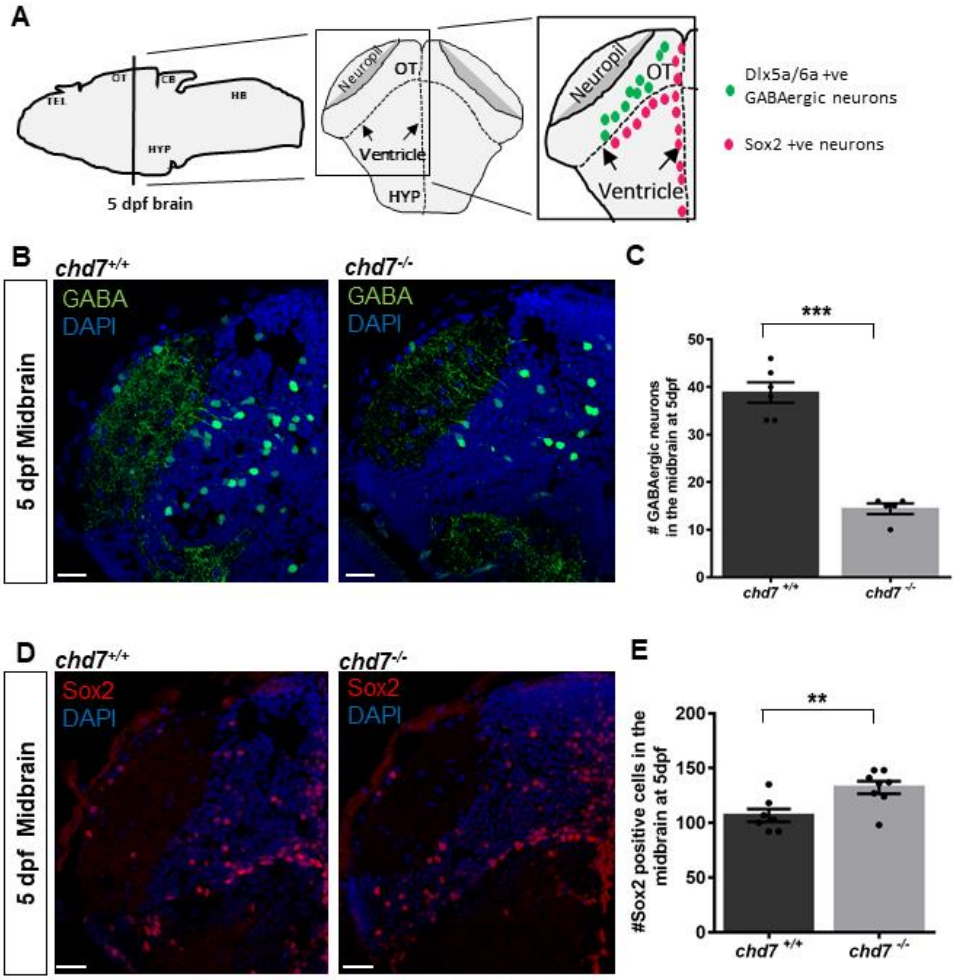


Figure 1 Abnormal GABAergic neuron and Sox2+ neural stem/progenitor cells' numbers in zebrafish *chd7-/-* mutant brain

A A schematic representation of the 5 dpf midbrain (left most panel) and a midbrain coronal brain section of the zebrafish brain at 5 dpf (middle panel). The right most panel shows a representative distribution of sox2+ and GABAergic neurons in the midbrain at 5 dpf. The dotted line marks the midbrain ventricle at 5 dpf.

B,C Represents the distribution of Dlx5a/6a:GFP positive GABAergic neurons in the wildtype and *chd7-/-* mutant brains in the midbrain region at 5 dpf (B). There is significantly lower number of GABAergic neurons in the optic tectum/midbrain region of the *chd7-/-* mutant zebrafish brains compared to the controls (C).

D,E Represents the distribution of the Sox2+ progenitor cells in the wildtype and *chd7-/-* mutant midbrain at 5 dpf (D). There is a significantly higher number of Sox2+ neural stem/progenitor (NSPCs) cells in the midbrain region of the mutant brains (E).

OT: optic tectum, HYP: hypothalamus, HB: hindbrain, TEL: telencephalon, CB: cerebellum

Scale: 20µm, n=6-9, **p=0.008, ***p=0.0001, *student's t test*.

The zebrafish CNS proliferative profile is very high at 2 dpf and is rapidly downregulated up to 5 dpf (Wullimann & Knipp, 2000). We next examined and compared the total number of Sox2-positive cells in *chd7-/-* and control zebrafish over the course of embryonic development at 2 (Figure 2A, B) and 3 dpf (Figure 2C,D). At 2 dpf, we did not observe any differences in the number of Sox2-positive cells ((Figure 2A, B). However, at 3 dpf, a significant increase in Sox2-positive cells in *chd7-/-* mutant brain was observed compared to controls (Figure 2C,D). Altogether, our findings suggest that an abnormal number of NSPCs in *chd7-/-* mutant brain occur early in development as of 3 dpf (Figure 2C, D), and is sustained at larval stages (5 dpf; Figure 1D,E).

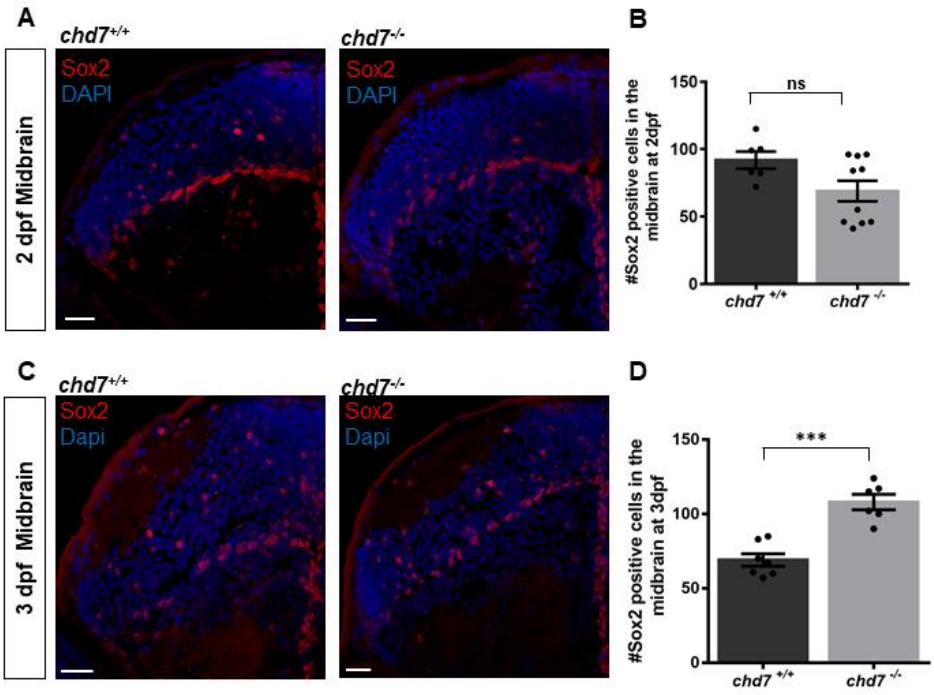
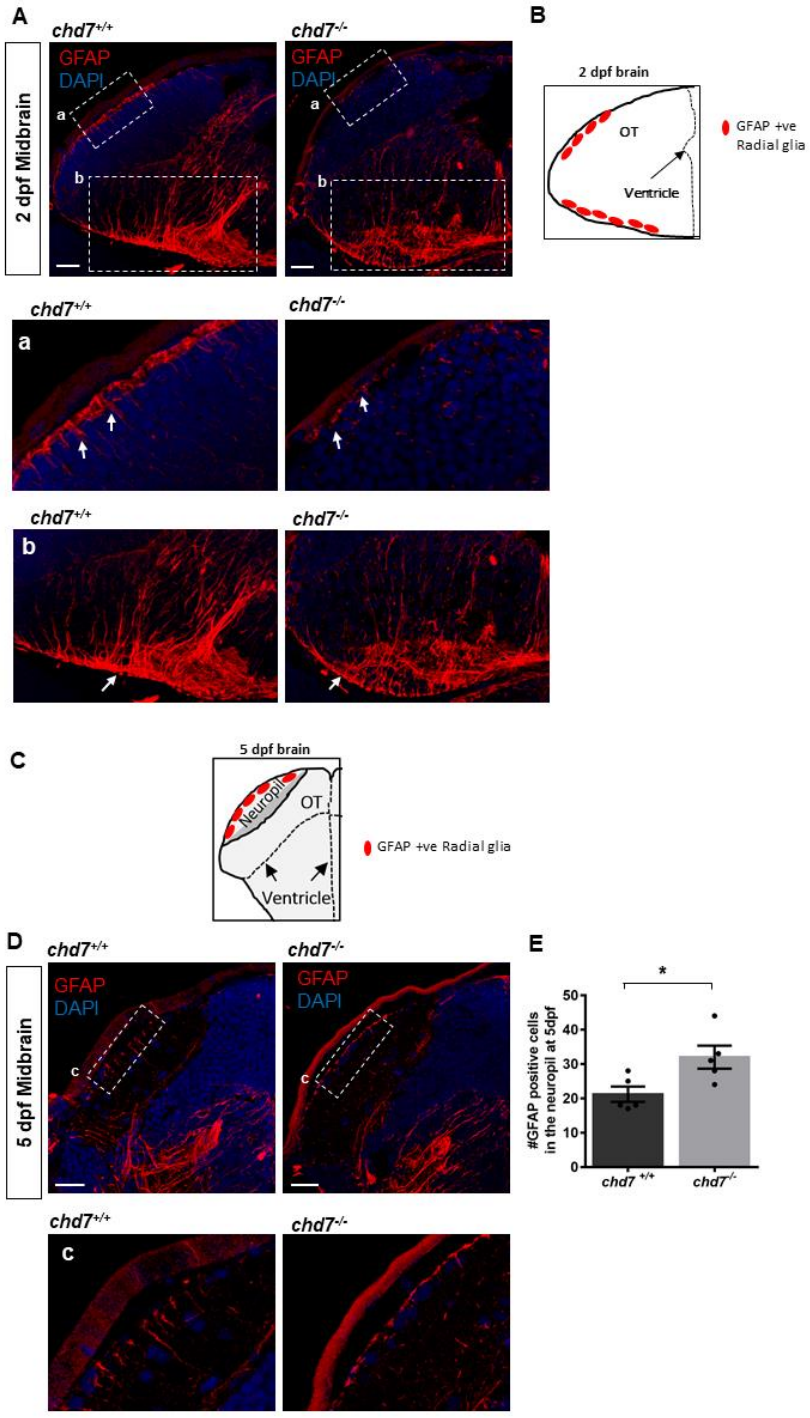


Figure 2 Number of Sox2+ NSPCs at early stages of development in mutant zebrafish midbrain.
A,B Presents the distribution of Sox2+ cells in the coronal section of wildtype and *chd7-/- mutant* midbrains at 2 dpf (A). There is no difference in the number of Sox+ NSPCs in the midbrain of mutant and control brains at 2 dpf (B).
C,D Presents the distribution of Sox2+ cells in the coronal section of wildtype and *chd7-/-* mutant midbrains at 3 dpf (C). There is significant increase in the number of Sox2+ NSPCs in the midbrain of the mutant brains compared to the controls starting 3 dpf (D).
Scale 20µm, n=6-10, ***p=0.0001, student's t test

4.3.2 Alterations in the number of GFAP+ radial glial cells and their radial processes in *chd7-/-* mutant zebrafish developing brain

We next sought to examine the GFAP+ radial neural stem cells in *chd7-/-* mutant and control brain. GFAP+ radial glial cells are located along the edge (Figure 3A) and they extend radial fibers towards the surface of the optic tectum. At 2 dpf, we observed a marked reduction in the density of radial fibers which is sustained at 5 dpf. Radial glial cells are formed in zebrafish at 10 hours post fertilization (H. Kim et al., 2008).They can both self-renew and give rise to



differentiated cell types early during development, first to neurons as of 1 dpf, oligodendrocytes progenitor cells (1-2 dpf) and astrocytes (2 dpf) (Neely & Lyons, 2021). Surprisingly, we observed a significantly higher number of GFAP+ radial glial cells in the

Figure 3 Defects in GFAP+ radial glia/NSPCs in the mutant brains at embryonic and larval stages of brain development

A Presents the GFAP positive radial glia cells in the 2dpf coronal section of the midbrain for wildtype and *chd7-/-* mutant brains. The cells line the edges of the optic tectum and extend process inward. **a** and **b** show the enlarged regions of the 2 dpf midbrain that host GFAP+ cells. There is an abnormal number of radial glia in the optic tectum region, and the arrows show the defects in their processes in the mutant brains compared to controls.
B,C A schematic representation of the 2 dpf (B) and 5 dpf (C) zebrafish midbrain with a representative distribution of the GRAP+ radial glia/NSPCs. The dotted line marks the ventricle in the 2dpf and 5dpf midbrain.
D,E; Presents the GFAP+ cells in the coronal section of the 5 dpf midbrain for wildtype and *chd7-/-* mutant brains (D). The abnormal number of GFAP+ radial glia continues till 5dpf. At 5 dpf there is significantly higher number of GFAP+ cells, with defects in the extension of their processes. **c** shows the enlarged neuropil region of the optic tectum in the 5 dpf brain where the GFAP+ cells are present at the edges and the arrows show their processes extend internally.
OT: optic tectum Scale: 20µm n= 5-7, *p=0.02

ventricular zone around the neutrophil region in 5 dpf *chd7-/-* mutant compared to control (Figure 3C-E).

4.3.3 Overexpression of *paqr3b* restores the number of NSPC in *chd7-/-* mutant brain

We previously showed that the overexpression of the *chd7* direct target gene *paqr3b* rescues the number of GABA neurons in the 5dpf *chd7-/-* mutant brain (Jamadagni et al., 2021). To test for changes in neurogenesis upon overexpression of *paqr3b* in *chd7-/-* fish, we performed rescue experiments and examined the proportion of Sox2-positive cells in the midbrain of 5 dpf larval zebrafish. Overexpression of *paqr3b* significantly reduced the number of Sox2-positive cells in the in *chd7-/-* midbrain (Figure 4A, D). As Sox2-positive neural progenitors are determined into mature neurons and differentiate, there is a progressive decrease in its expression during differentiation (Barbosa, Di Giaimo, Gotz, & Ninkovic, 2016). However, Sox2 expression is retained in several neurons and Sox2 protein has been shown to play an important role at early differentiation stages, in particular, for maturation of a subsets of GABAergic neurons (Cavallaro et al., 2008). Interestingly, we found that *paqr3b* overexpression in *chd7-/-* zebrafish brain restores the number of Sox2-positive GABAergic

neurons (Figure 4B, E). Altogether, our findings suggest the *paqr3b* regulates Sox2-positive neural progenitor number and differentiation into GABAergic neurons.

To test whether *paqr3b* also regulates GFAP+ radial neural stem cells, we examined changes in GFAP+ radial glial defects in *chd7-/-* mutant brain. Overexpression of *paqr3b* restores the density of radial fibers in the ventricular zone around the neutrophil region in *chd7-/-* mutant (Figure 4C,F).

4.3.4 NSPC defects in the hypothalamic region of *chd7* zebrafish mutant brain

Although, the GABAergic neuron defects were very prominent in the *chd7-/-* midbrain, a significant reduction of GABAergic neurons was also observed in the hypothalamus (Jamadagni et al., 2021). The hypothalamus is another major proliferative and neural stem cell zone during the early vertebrate brain development. We, thus, next sought to assess whether the observed NPSC defects is restricted to the midbrain or extends to other brain regions such as the hypothalamus. The number of Sox2-positive NSPCs was found to be significantly higher in proportion *chd7-/-* hypothalamic brain region compared to control (Figure5A,B).Overexpression of *paqr3b* rescued the abnormal number of NPSC in the *chd7-/-* hypothalamus (Figure 5A,B). These data suggest that the regulation of NSPC number by the *chd7-paqr3b* axis is not restricted to a specific brain region.

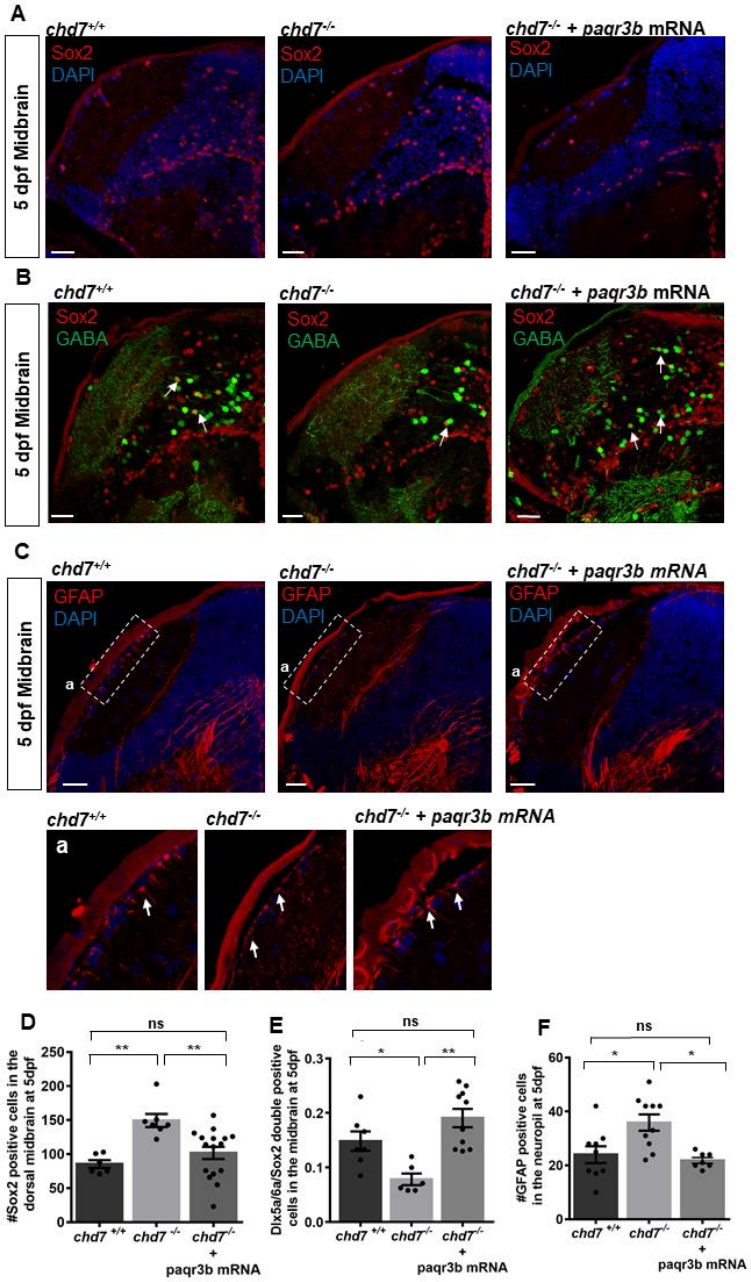


Figure 4 paqr3b restores the NSPCs defects and the number of GABAergic neurons in the mutant zebrafish brain

A,D Presents the distribution of Sox2+ cells in the 5 dpf midbrain for wildtype, *chd7-/-* mutant and *paqr3b* mRNA injected mutant brains (A). Overexpression of *paqr3b* mRNA in the mutants significantly decreases the number of Sox2+ NSPCs in midbrain compared to the non-injected mutants and restores them to almost wildtype brain numbers at 5 dpf (D).

B,E Presents the distribution of Sox2+-Dlx5a/6a:GFP positive GABAergic cells in the 5 dpf midbrain for wildtype, *chd7-/-* mutant and *paqr3b* mRNA injected mutant brains (B). There is a significant increase in the number of co-labelled Sox2+ and Dlx5a/6a GABA neurons upon overexpression of *paqr3b* in the mutants compared to the non-injected mutants at 5 dpf (E).

C,F Presents the distribution of GFAP+ cells in the 5 dpf midbrain for wildtype, *chd7-/-* mutant and *paqr3b* mRNA injected mutant brains (C). Overexpression of *paqr3b* mRNA in the mutants restores the number of GFAP+ radial glia/NSPCs in the midbrain/optic tectum region of the mutant brains (F). **a** shows the enlarged neuropil region of the optic tectum in the 5dpf midbrain. There is a significant decrease in the number of GFAP+ positive radial glia, and the arrows show that *paqr3b* overexpression also restores the elongation of their processes.
Scale: 20µm, n=10, *p<0.03, **p<0.005, one-way ANOVA

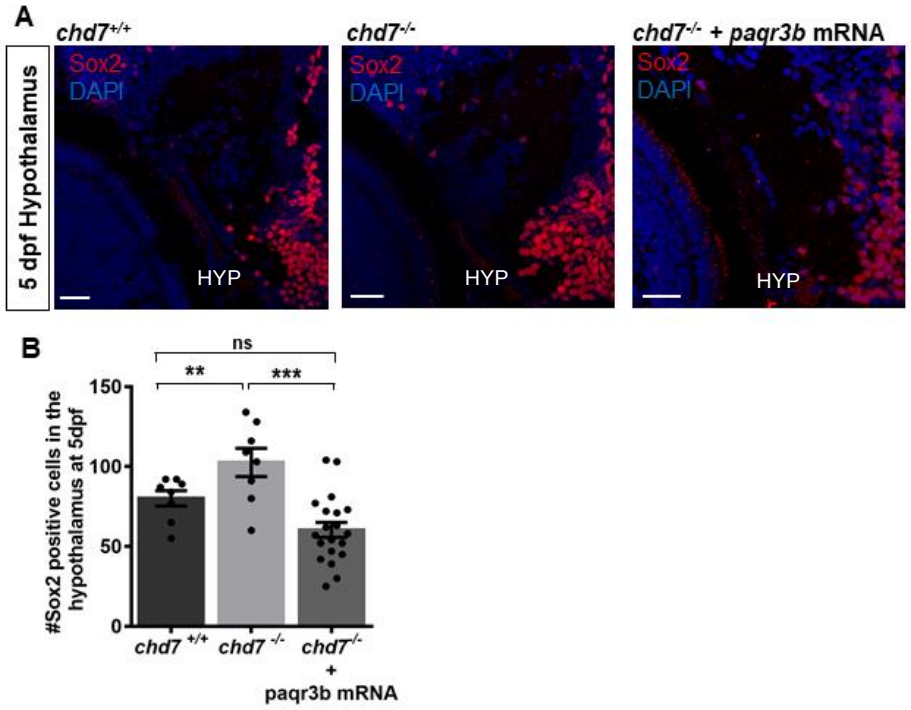


Figure 5 Loss of *chd7* affects number of Sox2+ NSPCs in the hypothalamus that is also restored by *paqr3b*

A,B Presents the distribution of Sox2+ cells in the 5 dpf hypothalamus region for wildtype, *chd7-/-* mutant and *paqr3b* mRNA injected mutant brains There is a significant increase in the number of Sox2+ NSPCs in the hypothalamus region of the mutant zebrafish brain at 5 dpf. This increase is decreased to near normal levels after overexpression of paqr3b mRNA in the mutant brains.
Scale: 20µm, n=10, **p<0.005, ***p<0.0001, one-way ANOVA, HYP: hypothalamus

4.4 Discussion

During early zebrafish embryonic development (2 dpf), proliferation is very high in the zebrafish brain and is rapidly downregulated up to 5 dpf, with less NPSCs being present (Wullimann & Knipp, 2000). In this study, we demonstrate that there is an increase in the number of Sox2+ and GFAP+ NSPCs upon loss-of-function of *chd7* in the zebrafish midbrain at late larval developmental stage (5 dpf). Additionally, we show a reduction in GFAP+ radial glial cell processes in *chd7-/-* mutant brain during development. Importantly, these defects were rescued with the overexpression of *paqr3b*, a gene that is highly downregulated in *chd7-*

/- mutant fish (Jamadagni et al., 2021). We report a novel molecular mechanism whereby *chd7* and its downstream target *paqr3b* regulate neural precursor cell number and differentiation in zebrafish brain development.

Sox2 positive cells are predominantly restricted along the ventricle zones of the brain, which are typically the dividing neural progenitors while the newly generated neurons populate the remote, more basal layers (Mueller & Wullimann, 2003; Wullimann & Knipp, 2000). We also observed the expression of Sox2 in the neurogenic ventricles' zones of the brain in *chd7-/-* mutant fish suggesting appropriate regional development despite loss-of-function of *chd7*. Sox2 has been known known for maintaining pluripotent properties of the stem cells(V. Graham, Khudyakov, Ellis, & Pevny, 2003; Nakatake et al., 2006) and differentiation (Gong et al., 2020). A deficiency of Sox2 promotes neural progenitor cells from the ventricular zone to exit from the cell cycle (V. Graham et al., 2003). The expression of Sox2 is high in undifferentiated cells, declines with differentiation and remains visible in some of the mature neurons in later stages of development (Cavallaro et al., 2008). A high level of Sox2 is expressed in specific neuronal and glial types even after maturation, like GABAergic neurons. and has been reported to be involved in GABAergic neuron development (Cavallaro et al., 2008; Ferri et al., 2004; Mercurio, Serra, & Nicolis, 2019). Interestingly, we noted an increase in the number of Sox2+ cells in the midbrain, associated with a decrease in the number of Sox2-GABA co-labelled neurons at 5dpf in *chd7-/-* mutant fish. It is therefore plausible that the chd7 may play a role in the differentiation of the Sox2+ into GABAergic neurons in zebrafish as well.

The ventricular progenitor cells also adopt another phenotype characterized by radial processes that reach the basal surface called radial glia cells expressing GFAP. These cells are responsible for the generation of large number of neurons, that increase the complexity of the primary neuronal network in the second phase of embryonic neurogenesis – the equivalent of post embryonic CNS development in higher vertebrate organisms (Galant et al., 2016; Johnson et al., 2016; Mueller & Wullimann, 2003; Wullimann & Knipp, 2000). Particularly, subsets of interneurons, secondary motor neurons, and oligodendrocytes originate from the GFAP positive radial glial cells during CNS development (Johnson et al., 2016). We noticed that the *chd7-/-* mutant brains had an increased number of GFAP+ radial glia with a defect in the elongation of their processes at 5 dpf. An abnormal number of GFAP+ radial glia with defective processes was observed to set in as early as 2 dpf in the mutant brains. Interestingly, increased GFAP levels have also been associated to autistic individuals (Singh, Warren, Averett, & Ghaziuddin, 1997). Further, increased plasma GFAP levels have been reported as markers for abnormal neuropsychological development (Vergine et al., 2021). Radial glial processes help to direct the migration of young neurons (Jinnou et al., 2018; Parnavelas &

Nadarajah, 2001). We previously reported mispositioning of GABAergic neurons in the zebrafish *chd7-/-* mutant brain (Jamadagni et al., 2021). It is therefore possible that GABAergic neurons are not migrating properly in the midbrain region due to aberrant radial glial processes in mutant *chd7* fish.

Previous reports have demonstrated that CHD7 plays an essential role in neuronal differentiation(W. Feng, Kawauchi, et al., 2017; W. Feng & Liu, 2013; Hurd et al., 2010; Jamadagni et al., 2021; Yao et al., 2020). We demonstrate in this study that chd7 regulates the NPSCs function and their differentiation into GABAergic neurons via *paqr3b*. Human Progestin and adipoQ receptor family member III (PAQR3) is a regulator that negatively regulates the MAPK/ERK signalling cascade(Y. Jiang et al., 2011). It has been shown to play an essential role in cell proliferation, migration, sprouting and angiogenesis of endothelial cells by suppressing MAPK signalling(Wu et al., 2014). PAQR3-deleted mice display motor and behavioural abnormalities (D. Q. Xu et al., 2016). However, the precise role of PAQR3 in the nervous system has received little attention. ERK signalling plays an essential role in NPC function and early neuronal differentiation (Chan et al., 2013; Hamilton & Brickman, 2014; Z. Li, Theus, & Wei, 2006; Rhim et al., 2016). In zebrafish *chd7-/-* mutant, we showed that an aberrant ERK signalling in the brain that is rescued by overexpression of *paqr3b (Jamadagni et al., 2021).* It is thus likely that the role of *paqr3b* in neurogenesis as per our findings occurs via its regulation of the ERK signalling. Additionally, it will be interesting in future studies to evaluate whether this role of PAQR3 in regulating NPSC number and neuronal differentiation is conserved across vertebrates.

In conclusion, this study provides the first evidence for chd7 and its target gene *paqr3b* as an essential genetic axis for regulating NPSCs number and differentiation into GABAergic neurons in zebrafish. We also show an important role of chd7 in the development of radial glial processes that may be crucial for neuronal migration. Overall, our study provides insights into the role of *chd7* and *paqr3b* in zebrafish brain development.

4.5 Materials and Methods

Fish husbandry

Adult zebrafish (*D. rerio*) were maintained at 28°C at a light/dark cycle of 12/12 h according to the Westerfield zebrafish book (Westerfield, 1993). Embryos were raised at 28.5°C and collected and staged as previously described (Kimmel et al, 1995). The zebrafish lines used in this study were wild-type, *chd7-/-* mutants, *Tg(dlx5a/6a: GFP)* which was obtained from the laboratory of Marc Ekker and used to generate *Tg(dlx5a/6a:GFP;chd7−/−)* fish. All

experiments were performed in compliance with the guidelines of the Canadian Council for Animal Care and the local ethics committee of INRS.

Bromodeoxyuridine (BrdU) labelling and mRNA injections

Zebrafish larvae at 1, and 2 dpf were incubated in a 10 mM solution of BrdU (BD Biosciences; Cat# 550891) in fish water at a temperature of 28.5°C for 24 h. All the larvae were then fixed at 2 and 3 dpf respectively, in 4% PFA O/N at 4°C and used to perform cryo-sections of the brain. *Paqr3b* mRNA was injected at the one cell stage and the fish were raised till 5 dpf at 28.5°C and then fixed in 4% PFA O/N at 4°C and used to perform cryo-sections of the brain.

Zebrafish brain cryo-sections and double-immunohistochemistry

Fixed larvae (2, 3 and 5 dpf) were given serial sucrose treatment with 15 and 30% sucrose in 1X PBS, till the larvae sank to the bottom. The fish brains were then cryo-sectioned (transverse section) in 10-micron-thick sections and dried at room temperature for 20 min, and frozen to store. For the immunostaining, the zebrafish embryo brain sections were first post-fixed in acetone at −20°C for 20 min. The sections were then washed with PBS for 15 min and processed for epitope retrieval with Tris–HCl (pH 8.2, 50 mM) at 85°C for 6-min treatment (for HuC/D, NeuroD1, Sox2, GFAP) and additionally with HCl (4 N) at 37°C for 10 min followed by Sodium borate (0.1 M) washes for 20 min (for BrdU). Sections were then washed in 0.5% PBS-Triton for 30 min, blocked in 10% NGS for 1 h at room temperature and then incubated in primary antibodies: HuC/D at 1:50 (Invitrogen; A21271); NeuroD1 at 1:500 (Abcam; ab60704), Sox2 at 1:200 (Abcam; ab5603), GFAP at 1:200 (Abcam, ab7260), BrdU at 1:250 (Abcam; ab152095) diluted in 5% NGS, 1% BSA in 0.1% PBS-Triton, O/N at 4°C. The following day sections were washed in 0.3% PBS-Triton and incubated with species-specific secondary antibodies coupled to Alexa Fluor 488 or 555 (Invitrogen) diluted in 0.1% PBS-Triton for 2–3 h at room temperature, followed by washes with 0.3% PBSTriton and mounted in DAPI glue (Invitrogen; Cat# P36941).

Confocal Imaging and data analysis

For the quantitative analyses, cells stained for BrdU, Sox2, NeuroD1, GFP (for Dlx5/6), HuC, GFAP in the zebrafish midbrain area were taken under 40× magnification with an oil immersion lens using a Zeiss confocal microscope (LSM780; Carl Zeiss, Germany). The images were then processed with ZEN software (Carl Zeiss).

Stained cells in consecutive sections from five/six brains per genotype (N = 3) were counted using ImageJ (NIH) and used to calculate the total number of double-positive stained cells in the following scheme: Sox2-HuC/D double positive cells, Sox2-GFP (Dlx5a/6a:GFP positive

GABAergic neurons) double positive cells, BrdU-NeuroD1 double positive cells, BrdU-HuC/D double positive cells; and only GFAP, Sox2 and BrdU positive cells in each larval brain section. The total number of sections (n) used varied between experiments. Neuroanatomical designations are taken from the Atlas of Early Zebrafish Brain Development (Mueller & Wullimann, 2015).

Statistical Analysis

All data is represented as mean ± SEM. Two tailed- *t* test and one-way ANOVA was conducted as applicable using GraphPad Prism software. ns = non-significant, *p<0.05, **p<0.005, ***p<0.0001; n represents the number of sections analysed, and N is the number of trials.

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SECTION III: DISCUSSION, PERSPECTIVES AND CONCLUSION

5 DISCUSSION

5.1 General Discussion

Through this thesis we have, (i) created a CRISPR-Cas9 *chd7* knockout zebrafish model that presents with characteristic CHARGE syndrome features, (ii) identified brain developmental defects in the form of GABAergic network defects that underlie the hyperactive behaviour in the mutant fish, (iii) identified *paqr3b* regulated MEK/ERK pathway as a mechanistic link that contributes to the hyperactivity and GABA network defect. Reduced levels of PAQR3 were also noted in LCLs derived from a CHD7 mutation positive CHARGE syndrome individual in comparison to parental controls. This reduction was because of the reduced occupation of *PAQR3's* proximal promoters by *CHD7*. Further, we report that *paqr3b* also plays a role in neurogenesis in the zebrafish midbrain. Finally, (iv) we identified a small neuroprotective molecule – Ephedrine via drug screening studies employing chd7-mut *C. elegans* and zebrafish. Ephedrine ameliorated the observed GABA network defects and the hyperactivity through the MEK/ERK pathway.

5.1.1 A CRISPR-Cas9 mediated *chd7-/- mutant* zebrafish model for CHARGE syndrome

CHARGE syndrome was first modeled through ENU mediated mutations in mice (Bosman et al., 2005). Since then, multiple organisms have been employed to model CHARGE syndrome and study *Chd7's* mechanisms of action. In the context of zebrafish as a model for CHARGE syndrome, morpholinos have been the common choice for gene knockdown (Asad et al., 2016; Balow et al., 2013; Cloney et al., 2018; Jacobs-McDaniels & Albertson, 2011; H. Liu & Liu, 2020; Z. Z. Liu et al., 2018; Patten et al., 2012). The morpholino mediated knockdowns of *chd7* in zebrafish successfully produced a combination of phenotypes that mimic those presented in CHARGE syndrome individuals, like, smaller eyes, heart development defects, asymmetric otoliths, defects in axis development and vertebral mineralization, and cranial nerve defects, thereby affirming the suitability of zebrafish to model the disorder. However, morpholinos typically cause knockdown of genes that can be sustained effectively only for a short duration of time, usually only up to a few days post fertilization in the case of zebrafish (Varshney et al., 2015). This restricts the scope of study to early developmental stages, and one may not be able to follow the effects into the later stages of development. Further, there can be significant off target effects with morpholinos, and variations with every injection that may not assure consistent knockdown and therefore further limit the range of effects and their uniform utilization for experiments. These effects can be only mitigated but never completely avoided (Varshney et al., 2015).

The CRISPR-cas9 method of gene editing helps circumvent these shortcomings and complements the morpholino studies. Although it may take a bit longer to establish a mutant line in comparison to morpholino studies, CRISPR-cas9 assures the establishment of a stable genetic mutant line that can be used over generations to study the consequential phenotypes. The off – target effects and variability in transmission of the created mutation can be circumvented via out-crossing and genetic screening of the mutant fish (Varshney et al., 2015). Other methods of creating stable homozygous and heterozygous mutations like gene-trap mediated knockouts, Cre- mediated conditional knockouts, and knockdowns have been previously also employed in mice. However, the *in-utero* development of the mouse embryos limits the access to observing *in vivo* early brain development thereby also limiting the understanding of the effects of loss of *chd7* on early brain development. Employing zebrafish as a model helps to circumvent this limitation. Zebrafish undergo rapid ex-utero fertilization and development in transparent embryos with easy access to observing early vertebrate brain development. There have so far been two attempts at studying the *chd7-/- mutant* effects, via zinc finger nucleases in larvae (Z. Z. Liu et al., 2018) and in heterozygous adult mutant fish (H. Liu & Liu, 2020). Our model adds a stable CRISPR-cas9 *chd7* homozygous knockout line created in zebrafish viable for study and characterization. The isolated mutation was an insertion in the exon 17, that aimed at targeting the functional helicase domain of the normal chd7 protein, creating a premature stop codon that led to reduced levels of chd7.

In humans, CHARGE syndrome is caused by autosomal dominant pathogenic variants in *CHD7* that are mostly sporadic and *de novo* with no specific genotype to phenotype co-relations. Interestingly, the heterozygous mutant larval zebrafish did not present with any significant phenotypes (unpublished). It is believed that this may be because of a genetic compensatory mechanism in larval zebrafish. A similar scenario has been observed while modeling other human autosomal dominant disorders in zebrafish, like Dravet syndrome, hyperekplexia and myocionic epilepsy where it was the homozygous mutants that displayed the full extent of phenotypes in the fish model and not the heterozygous mutants (Samarut et al., 2019; Samarut et al., 2018; Sourbron et al., 2019). It however warrants further studies to assess if the heterozygous mutation presents with any effects at later stages of development in juvenile and adult fish.

In keeping with the type of symptoms observed in the human phenotypic presentation of CHARGE syndrome, about 20% of the *chd7-/-* mutant fish had heart developmental anomalies, particularly a pericardial edema. A quarter of the mutant fish presented with defective inflation of the swim bladder by 5 dpf. The swim bladder is an inflated air sac in the fish that helps them to swim and float. It has long been believed that the lungs in humans have

functions that can be extrapolated from the role of the air sacs in the fish (Daniels et al., 2004). CHARGE syndrome is often known to present respiratory and lung defects as well (Asher, McGill, Kaplan, Friedman, & Healy, 1990). In addition, the mutant larvae consistently presented with craniofacial developmental defects with a visible defect in the proper development of the jaw. The craniofacial defects were underlined by cranial nerve developmental defects as well. Particularly the 5th cranial nerve or the trigeminal nerve. Motor efferents of the trigeminal nerve innervate the muscles of the mandibular arch which is a major jaw arch (Higashijima, Hotta, & Okamoto, 2000). Cranial nerve underlined craniofacial developmental defects are the characteristic features of CHARGE syndrome. Most importantly, mimicking the microcephaly in the CHARGE syndrome individuals, the mutant fish consistently presented with a small head phenotype suggesting a developmental effect on the brain.

Based on the combination of features presented by our mutant fish larvae, our *chd7-/-* mutant model successfully represents the human CHARGE syndrome. Given the ease in manipulating larval zebrafish, this model could be used to further elucidate the role of *chd7* in brain development along its various regulatory axes that potentially govern the symptoms observed in CHARGE syndrome.

5.1.2 Role of the GABAergic network defects in hyperactivity and other CHARGE syndrome behaviours.

Intellectual disability, social immaturity, anosmia due to defects in the olfactory bulb, hyperactivity and behaviours overlapping with OCD, ASD, and ADHD are some of the common presentations in CHARGE syndrome (C. M. van Ravenswaaij-Arts et al., 1993). Hyperactivity, in addition to CHARGE syndrome has also been associated with autism spectrum disorder (ASD) and ADHD (Hartshorne, Grialou, et al., 2005; Luffe et al., 2021; Murray, 2010). The *chd7-/-* mutant zebrafish presented with a hyperactive behavioural phenotype as well. A GABAergic network defect contributing to hyperactivity has been previously observed in zebrafish models for an autism risk gene and Rett syndrome (Hoffman et al., 2016; Luffe et al., 2021). Interestingly, we also noted that a defect in the GABA network development and functioning underlined the hyperactivity phenotype in our mutants.

The *chd7-/-* mutants had a lower number of GABA neurons in most regions of the mutant brain where GABA neurons are normally present- the telencephalon, optic tectum, cerebellum and the hypothalamus (Jamadagni et al., 2021; Kim et al., 2004). The reduction was most apparent in the optic tectum, and the cerebellum region missed most of its GABA neurons. The optic tectum is a major and crucial processing center of the teleost brain. It

receives afferents from retinal ganglion cells, forming a processing link between the sensory input and motility output for the fish (Heap et al., 2017). The cerebellum as well is extremely crucial for the sensory perception and motor control (Bae et al., 2009). The telencephalon consists of centers that are involved in the processing of learning, sensory, motor and cognitive behaviours (Vaz et al., 2019). Further, the *chd7-/-* mutant zebrafish larvae were noted to be highly active and particularly hyperactive during the dark phase of the light and dark cycle. The dark phase in our motility assessment assay for the mutant zebrafish corresponds to the night phase in the day and night cycle. Calming and sleep induction has been reported to be controlled to a great extent by the GABA neurotransmitter releasing neurons particularly in the hypothalamus region of the brain (Siegel, 2004). The *chd7-/-* mutant zebrafish present with a reduced number of GABAergic neurons in the hypothalamus region of the brain as well. The loss of GABA neurons from such crucial centers of processing suggests an effect on the other aspects of behaviour like learning, sensory perception, cognitive behaviours, motility and motor control which are all behaviours also often presented in CHARGE syndrome (C. M. van Ravenswaaij-Arts et al., 1993).

The mutant fish were severely affected by PTZ induced seizures – a GABA receptor antagonist treatment, and the hyperactivity could be partially rescued after a treatment with GABA A and B receptor agonists respectively, like Muscimol and Baclofen, suggesting a GABA network functioning defect in the mutants. Independently, both A and B subtypes of the GABA receptor have been reported to play important roles in the normal functioning and development of the brain networks and have also been implied in neurodevelopmental disorders like ASD and epilepsy (Chandra, Korpi, Miralles, De Blas, & Homanics, 2005; Liao et al., 2019; Spigelman et al., 2002). Further GABA receptor-based experiments are required to assess for their role in the GABAergic network defects upon mutations in *chd7*. The functioning of the GABA network is influenced by several other factors like the appropriate positioning of the neurons to their designated locations, the amount and release of the neurotransmitters at the synapse, the reuptake/metabolism of the neurotransmitter, and even the presence and function of the receptor channels of the synaptic neurons (Cellot & Cherubini, 2014; Purkayastha, Malapati, Yogeeswari, & Sriram, 2015; X. Zhang et al., 2021). In fact, compared to the controls there was an improper positioning of the GABA neurons in the mutant brain, particularly in the optic tectum region of the midbrain. However, analysis focused more on the molecular aspects of the existing GABA neuron network is required to gain a more refined perspective.

5.1.3 *PAQR3* is a novel regulator of the GABA network development and neurogenesis.

The unbiased whole transcriptomic analysis on the mutant zebrafish brain showed *paqr3b* as the most downregulated gene. *paqr3b* – progestin and adiposeQ receptor protein 3b is a direct target gene of *chd7* (Jamadagni et al., 2021) and belongs to the highly conserved PAQR family of 'G-protein coupled receptor like' family of transmembrane receptor proteins. The human PAQR3 is a highly conserved gene with orthologs in 405 organisms including chimpanzee, mice, rat, zebrafish, fruit fly, frog, chicken and C.*elegans.* The zebrafish *paqr3b* is a conserved ortholog of the human *PAQR3* gene.

PAQR3 has been previously known to regulate ER-Golgi traffic via interaction with the Sec13/Sec31A coat protein complexes of the COPII vesicles (Cao et al., 2018). It particularly functions as a RKTG (Raf kinase trapping to the Golgi) where it anchors Raf to the Golgi membrane thereby inhibiting the Ras/Raf/MEK/ERK signalling pathway (Lei et al., 2020; X. Yu et al., 2015). We reported an increase in the levels of phosphorylated-ERK possibly caused due the downregulation of *paqr3b* leading to hyperphosphorylation of ERK in the mutant fish.

The *PAQR3* gene is also known to affect multiple physiological processes, like cholesterol metabolism, insulin metabolism, H3K4 methylation, GPCR activity, cell homeostasis and cell survival through autophagy (Huang et al., 2019; Lei et al., 2020; L. Wang et al., 2013). It is well expressed in the human body with relatively high expressions in the brain, small intestine, and the testis (Fagerberg et al., 2014). *PAQR3* has also been widely reported to inhibit uncontrolled cell division as a tumor suppressor in a range of cancers including gliomas, breast cancer, prostate cancer among others (Bai, Chu, Eli, Bao, & Wen, 2017; Cao, You, Xu, Wang, & Chen, 2020; Tang, Gao, & Hu, 2017; X. Wang et al., 2012; Zhou, Wang, & Wang, 2017). In fact, independently, cholesterol metabolism is extremely important for the proper functioning of the brain and defects in cholesterol metabolism and turnover have been previously reported to cause neurodegenerative disease and cognitive defects (Petrov, Kasimov, & Zefirov, 2016). A downregulation of the *paqr3b* gene upon mutations in *chd7*, could be potentially affecting many of these processes, possibly in conjunction with some of the other dysregulated genes, thereby contributing to the pathogenesis in brain development. This warrants more experiments to understand the full scope of the role it plays in the neuropathogenesis in CHARGE syndrome.

Knock-out of *PAQR3* in mice has been reported to cause motility and behavioural defects in them (D. Q. Xu et al., 2016). Possibly these motor deficits are underlined by defects in neuron network development or functioning. However, the neuron networks and possible

molecular mechanisms that underline the behavioural defects upon loss of PAQR3 haven't been further explored yet. Over expression of *paqr3b* via mRNA injections in our studies could modulate the levels of p- ERK and at the same time partially improve the reduction in the number of GABA neurons. PAQR3 contributes to the reduction in GABAergic neurons via the MEK/ERK pathway.

The eventual reduction in the number of GABAergic neurons in the mutant brains could potentially be because of errors in a single / a combination of brain neurogenesis processes that include proliferation, cell death, differentiation, presence and nature of the neuronal progenitor populations and migration. In the overall brain, we report that the *chd7-/-* mutants showed an increase in the number of proliferating cells at 5 dpf. A co-stain among the proliferating cells in the midbrain showed that there were a significantly lesser number of BrdU positive proliferating cells that had specified into transient neurons (NeuroD1+ cells), mature neurons (as HuC/D+ cells) and differentiated GABAergic neurons (as Dlx5a/6a+ cells) (Jamadagni et al., 2021). Further we show that the mutant zebrafish brain retains a high number of neural Stem/Progenitor cells (NSPCs; Sox2+). There are also an abnormal number of radial glial cells (GFAP+) that develop fewer radial processes in *chd7-/-* mutant larval brain. These defects in neural precursor cells occur early in zebrafish embryonic development and are sustained at larval stage 5 dpf. *Chd7* acts preferentially to alter chromatin accessibility of key genes during the transition of neural progenitor cells (NPCs) to neurons to promote differentiation (Yao et al., 2020). Although, little is known about its effect on GABAergic neurons. In the developing brain, Sox2 expression is retained in several neurons even after differentiation. In fact, Sox2 protein has been shown to play an important role at early differentiation stages and is particularly important for the maturation of subsets of GABAergic neurons (Cavallaro et al., 2008). Interestingly we noted that overexpression of *paqr3b* in the mutant fish rescued the number of Sox2+ NSPCs, the number and nature of GFAP+ radial glial cells, and lead to a near normal number of differentiated GABAergic neurons in the mutant midbrain. Further, we also found that *paqr3b* overexpression in *chd7*$^{-/-}$ zebrafish brain increases the number of Sox2-positive GABAergic neurons suggesting that *paqr3b* regulates Sox2-positive neural progenitor number and differentiation into GABAergic neurons. A role for *PAQR3* in neurogenesis has not been previously studied. Our work suggests an important role for *chd7- paqr3b* regulatory link in zebrafish brain neurogenesis (Fig 1) and GABAergic neurons development in the midbrain.

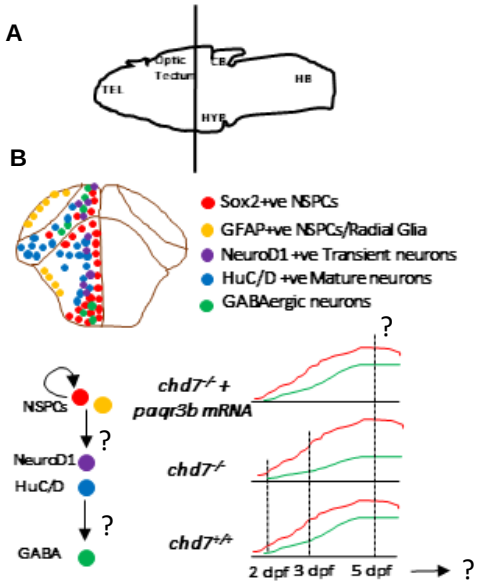

Figure 1 A dysregulation in the NSPC population influenced by PAQR3 could provide a possible explanation to the reduced number of GABAergic neurons upon mutations in CHD7

A A coronal section of the 5 dpf zebrafish midbrain with a representative depiction of the different neuronal and progenitor subtypes.
TEL: telencephalon, CB: cerebellum, HB: hindbrain, HYP: hypothalamus
B Each line on the graph represents the colour of the cell type as described in (A). In the mutant brains, there is lower number of Sox2 and GFAP positive NSPCs at 2 dpf compared to the wild type brains and a higher number of NSPCs at 5 dpf. There is a reduced number of GABAergic neurons at 5 dpf in the mutants compared to the wild type. An overexpression of paqr3b mRNA partly rescued the number of Sox2 positive neurons and the eventual number of GABAergic neurons at 5 dpf. However, the destiny of the increased Sox2 positive cells that still leads to a reduced number of GABAergic neurons still needs to be explored.
Red line: represents the projected curve of increase in number of Sox2+ cells through early brain development.
Green line: represents the projected curve of increase in number of GABAergic neurons through early brain development.

5.1.4 MEK/ERK pathway's role in neuropathogenesis and GABAergic development.

ERK and MAPK are highly expressed in the developing nervous system. Dysregulation of the ERK pathway as a part of 'Rasopathies' has been well reported to contribute to the neuropathogenesis of a range of neurodevelopmental disorder like autism spectrum disorders, Fragile-X syndrome, Rett syndrome, intellectual disability and epilepsy and even craniofacial orofacial and dental disorders (Motta et al., 2020; Vithayathil et al., 2018). Particularly an enhanced/over activation of the ERK pathway has been reported to lead to cancers and developmental disorders like Noonan syndrome, LEOPARD (multiple lentigines,

electrocardiographic conduction abnormalities, ocular hypertelorism, pulmonic stenosis, abnormal genitalia, retardation of growth and sensorineural deafness), cardio-facio-cutaneous (CFC) and Costello syndrome(Schubbert, Shannon, & Bollag, 2007). Hyperactive MEK has been reported to cause loss of mature parvalbumin expressing cortical interneurons in the mouse subpallium (Holter et al., 2019). Hyperactive MEK1 mice exhibit seizure-like phenotypes with a reduction in inhibitory neurons (Holter et al., 2019). A conditional deletion of ERK5 in mouse neural stem cells leads to loss of GABAergic neurons in the olfactory bulb (Zou et al., 2012). Anxiety like behaviours have been associated to neuropathogenesis by ERK1/2-GAD1-GABA cascade where a reduction in GABA was caused after hyperphosphorylation of ERK1/2, in the rat hippocampus upon RtPA administration(Dong et al., 2018). In the *chd7-/-* mutant zebrafish, we report hyperphosphorylation of ERK. Inhibition of phosphorylation of ERK via a specific ERK pathway inhibitor in the mutants restored the number of GABAergic neurons in the mutant brains and rescued the hyperactivity behaviour. Further, inhibition of the ERK pathway via overexpression of *paqr3b* also partially rescued the number of GABAergic neurons in the mutant brains. We report a new role for the MEK/ERK pathway via *paqr3b* in the GABAergic network development and defects that underlie hyperactivity. However, not enough is understood about the exact subcellular mechanisms by which the ERK pathway affects GABAergic network's development or contributes to the various developmental defects.

The MEK/ERK pathway is well known to govern many cellular processes such as proliferation, differentiation, cell fate, homeostasis, and survival in all eukaryotes via a range of interacting subunits of the pathway (Chambard, Lefloch, Pouyssegur, & Lenormand, 2007; Thiel, Ekici, & Rossler, 2009). In the developing vertebrate brain, the MEK/ERK signalling pathway is activated in the proliferative zones of the forebrain where the cortical and hippocampal neurons are generated (Pucilowska, Puzerey, Karlo, Galan, & Landreth, 2012). ERK signalling has also been reported to be required in a cell autonomous manner to drive proliferation and maintenance of the neural stem/progenitor cell population (Pergola et al., 2008; Pucilowska et al., 2012). It is specifically involved in the GABAergic neuron development, as in the case of hippocampal and cortical GABAergic neurons (S. Li et al., 2014; Z. J. Zhang, Guo, & Xing, 2015). We report a role for the PAQR3 gene in the regulation of NSPCs and their differentiation, however further investigation is required to deduce how PAQR3 linked MEK/ERK pathway is involved in the effect on neurogenesis upon mutations in *chd7.*

The canonical MAPK pathway is activated by Ras proteins. On the cell surface, ligand binding activates receptor tyrosine kinases (RTK), RTKs activate Ras GTPases which then

drive the phosphorylation of the Raf proteins. Raf in turn phosphorylates and activates MEK1/2, MEK1/2 then activates ERK1/2 by phosphorylation (Ahn, Seger, Bratlien, & Krebs, 1992; Kuo et al., 1996; Pearson, Bumeister, Henry, Cobb, & White, 2000; Seger et al., 1992). Phosphorylated ERK1/2 then either activate substrates in the cytoplasm or translocate to the nucleus where it activates transcription factors (Chen, Sarnecki, & Blenis, 1992; Deak, Clifton, Lucocq, & Alessi, 1998). In addition to Ras/Raf proteins, the MEK/ERK pathway is activated and regulated in a cell specific way via other scaffolding/ adapter proteins, positive and negative feedback regulations that control not just the amplitude or strength of the signal, but also their spatial and temporal specificity (Kolch, 2005; Roskoski, 2012). Cues other than ras, could also lead to the activation of Raf proteins thereby activating the ERK1/2 pathway (Adachi et al., 2010; Feig, 2011; Gureasko et al., 2008).

Typically, Ras proteins are transported to the cell membrane where they cause local activation of the ERK/MAPK pathway (Downward, 1996; Gureasko et al., 2008; Overbeck et al., 1995). However, Ras has also been identified in Golgi membranes (Chiu et al., 2002), suggesting that the localization of the Ras GTPase can vary depending on interactions with other proteins (Vithayathil et al., 2018). PAQR3 is known to be an inhibitor of the Ras/Raf/MEK/ERK signalling pathway by anchoring Raf to the golgi membrane (Lei et al., 2020; X. Yu et al., 2015) and to regulate ER-Golgi traffic via interaction with the Sec13/Sec31A coat protein complexes of the COPII vesicles (Cao et al., 2018). Further, Raf-MEK-ERK pathway has been reported to control the COPII vesicle budding, the ER-Golgi trafficking and the formation of the ER-exit sites (ERES) (Farhan et al., 2010). This indicates a potential role for the PAQR3-MEK/ERK pathway -ER/Golgi trafficking axis in neuropathogenesis. Further characterization is however required to identify the exact link of regulation between PAQR3 and the MEK/ERK pathway and how PAQR3's role in anchoring certain Raf proteins affects MEK and the downstream signalling.

5.1.5 Ephedrine and its potential mode of action

The high throughput drug screen employing C.*elegans* and zebrafish *chd7-/-* mutant models identified Ephedrine as the most effective neuroprotective agent that could improve the number of GABAergic neurons and also the hyperactivity phenotype. Ephedrine is a synthetic, non-catecholamine, sympathomimetic amine, that is typically used to prevent low blood pressure (hypotension) during anesthesia, or less frequently as a nasal decongestant. It is a small molecule with an ability to cross the blood brain barriers, and therefore is an effective nervous system stimulant.

Ephedrine is known to stimulate both α- and β -adrenergic receptors by the release of norepinephrine from storage vesicles in the sympathetic neurons. α- and β -adrenergic receptors have been reported to affect defense-like and escape behaviours in mice(Uribe-Marino et al., 2019). Blockade of the α2-adrenergic receptors increases anxiety like behaviours in mice, α2- adrenergic receptor agonists are often used in treatments for ADHD and are known to affect sleep and arousal (Broese, Riemann, Hein, & Nissen, 2012; Cinnamon Bidwell, Dew, & Kollins, 2010). α- adrenergic receptor agonists improve hyperactivity, impulsivity, hyperarousal and social relationships in individuals with ASD (Beversdorf, 2020). β -adrenergic receptor antagonists also have been shown to have effects on social and language domains in ASD (Beversdorf, 2020). Further, an overstimulation of the β -adrenergic receptors via drugs to arrest preterm labour have been reported to cause autism (Connors et al., 2005), and leads to decreased functional connectivity in the human dorsal medial prefrontal cortex leading to processing difficulties that lead to behaviours observed in ASD (Hegarty et al., 2017). However, there is not much understood about the exact role and mechanism of action of adrenergic receptors in the pathophysiology of CHARGE syndrome.

The α-adrenergic receptors are involved in neurotransmission, activate the sympathetic nervous system and play a role in neurogenesis and maturation of neurons. Particularly, α1-ARs are important for the differentiation of neural progenitors into catecholaminergic neurons and GABAergic interneurons (Gupta et al., 2009). They can modulate GABA release in the human cerebral cortex and have been shown to regulate the CA1 GABAergic interneurons in the rat hippocampus (Bergles et al., 1996). Spontaneous GABA release from interneurons mediated by α1-ARs has been observed in several brain regions (Bergles et al., 1996; Kawaguchi & Shindou, 1998). It is possible that upon Ephedrine treatment, there occurs an α-adrenergic receptor mediated release of GABA influencing the GABA network functioning. β -adrenergic receptors as well have been reported to co-localize with GABAergic neurons and play an important role in the presentation of autism-like behaviours in zebrafish (Perdikaris & Dermon, 2022).

In the ventral tegmentum, both dopaminergic and GABAergic neurons express adrenergic receptors and respond to adrenergic receptor stimulation via α1-, α2-, and β-adrenergic receptors (Pradel, Blasiak, & Solecki, 2018). In certain cases, Ephedrine also causes the release of dopamine. Dopaminergic signalling has also been shown to regulate GABAergic neuron development and motor behaviour in zebrafish (Souza & Tropepe, 2011). The increase in GABAergic signalling upon treatment by ephedrine in our mutant model could possibly be one of the underlying mechanisms in ameliorating the phenotypes observed in mutants. It would however be important to investigate further the exact mechanism of action

of Ephedrine that eventually results in the rescue in the number of GABAergic neurons and the hyperactive phenotype.

6 Future Perspectives

Through this work, we created a homozygous *chd7-/-* mutant zebrafish, with some larvae that were viable throughout early stages into adulthood, present characteristic CHARGE syndrome features and can be employed for developmental studies. The heterozygous larval *chd7* mutants did not present with significant phenotypes, additional experiments would be required to observe if they develop phenotypic manifestations at later stages of development. We reported a GABA network defect that underlines the hyperactivity behaviour contributed by the *paqr3b* linked MEK/ERK pathway. However, glutamatergic and glycinergic inputs also form important connections in the networks that underline behaviour. Further, other regulators of the MEK/ERK pathway could also be contributing to the neuropathogenesis in the mutant brain. Further molecular and neural network characterization is important to elucidate a more detailed picture of the neuropathogenesis upon pathogenic variations in *chd7*. In the following sections, I detail future directions for the work reported in this thesis.

6.1 Characterizing GABA and other neuronal networks in CHARGE syndrome

Neurodevelopmental defects and behavioural anomalies form an important aspect of CHARGE syndrome (Lin et al., 1990; Sanlaville & Verloes, 2007). Our study focused on quantifying GABAergic neurons in the optic tectum and in part the cerebellum region of the zebrafish brain in response to *paqr3b* overexpression, effects of the MEK/ERK pathway inhibition and the neuroprotective effects of Ephedrine. However, other GABA neurons containing regions like the cerebellum, hypothalamus and telencephalon constitute important links in sensory processing, response to external cues, learning, cognition and regulation of motility and behavioural output. Each of these regions present with a reduction in GABAergic neurons upon mutation in *chd7*. The Tg(*Dlx5a/6a:GFP*) transgenic line could be further used to study the effect of the chd7-paqr3b-ERK/MEK pathway regulatory link on the GABAergic neurons in the other regions of the brain. Further, a molecular and pharmacological analysis of the existing GABAergic network via inhibition/overexpression studies is required to elucidate in detail the role of the neurotransmitter release, reuptake/metabolism at the inhibitory synapses, the density and function of the receptors at the synaptic junctions in the neuropathogenesis upon mutations in *chd7*.

GABA and glutamate neurons are the major inhibitory and excitatory neuron types and the most important inputs that maintain the E/I balance in the brain. An E/I imbalance has been identified as a mechanism of pathogenesis in the context of neurodevelopmental disorders like autism spectrum disorders and schizophrenia (Culotta & Penzes, 2020; R. Gao

& Penzes, 2015; E. Lee, Lee, & Kim, 2017; Rubenstein & Merzenich, 2003). We report a developmental and functional defect in the GABA neurons upon mutations in *chd7*. However, the effect on E/I balance needs to be further explored in the *chd7-/-* mutants. Hyperexcitability at glutamatergic synapse due to overstimulation of the receptors have been implicated as a contributor to the E/I imbalance in neurodevelopmental disorders like Autism, Fragile X syndrome, Rett syndrome, intellectual disability, ADHD, epilepsy and schizophrenia (Catania et al., 2007; Moretto, Murru, Martano, Sassone, & Passafaro, 2018; Uzunova, Hollander, & Shepherd, 2014). However, there is not much known about the role of glutamatergic neurons development or functioning in CHARGE syndrome. Characterization of the integrity of glutamatergic neurons and their functioning in the brain upon mutations in *chd7* is important to better understand neuropathogenesis in CHARGE syndrome. We conducted preliminary characterization of the glutamatergic neurons using a Tg(*Vglut2a: RFP*) zebrafish line. The Tg(*Vglut2a:RFP*) is a stable fluorescence line that expresses RFP specifically in the glutamatergic neurons in zebrafish. We created Tg(*Vglut2a:RFP*) *chd7-/-* fish for experiments and performed *in vivo* fluorescence imaging of the glutamatergic network in the brain (unpublished, Annex I, Supplementary Figure 2). Overall, we could notice lesser density of glutamatergic neurons in the mutant brain compared to the controls. However, the high density of glutamatergic neurons in the brain rendered conducting histological quantification analysis difficult. To effectively quantify the glutamatergic neurons, the transgenic line could be employed to conduct cell sorting experiments (FACS) in the mutant brains and conduct further characterizations.

Glycinergic neurons are the other major inhibitory neuron population in the brain. Glycinergic neurons have been recently linked to social and cognitive impairments in a mouse and zebrafish models for non-syndromic autism (Pilorge et al., 2016). In another study, aggressive like behaviours observed in heterozygous *chd7* mutant fish could be partially rescued by treatment with glycine transporter inhibitor, suggesting a role for the glycinergic neurons in regulating CHARGE related behaviours (H. Liu & Liu, 2020). A characterization of the glycinergic inhibitory neurons is important to completely describe the role of inhibitory inputs in the CHARGE syndrome behaviours and neuropathogenesis. The ease in conducting immunofluorescence imaging and histology studies in zebrafish could be employed to further explore the role of the glycinergic neurons in the *chd7-/-* mutant brains.

6.2 Neurogenesis defects in the mutant brain.

We reported an increase in the NSPCs and proliferative cells with a reduced number of GABAergic neurons in the *chd7-/-* mutant brains. Interestingly, an overexpression of *Paqr3b* rescued the number of Sox+ and GFAP+ NSPCs and the number of GABAergic neurons in

the mutant brain. This suggests a role for the chd7-paqr3b regulatory link in the NSPCs regulation and differentiation. Additional characterisation is required to explore the exact role *paqr3b* plays through the different stages of neurogenesis and to explore the fate of the increased NSPCs. Sox2-BrdU and Sox2-cleaved Caspase 3 co-labelling experiments would help to ascertain if the NSPCs remain in a proliferative state or undergo cell death. Since neurogenesis in the zebrafish brain occurs in concerted manner with different regions of the brain generating distinct neuronal subtypes along the ventricles of the brain. A characterization of neurogenesis via histology studies in the telencephalon, and hindbrain regions of the mutant brains could also be performed to test the effect on other regions of the brain. This will help to establish the overall effect on neurogenesis in the mutant brain but will also aid in following the effect on the birth of excitatory and inhibitory neurons in the brain as a neuropathogenesis mechanism in the CHARGE syndrome brain. Further, to fully examine a role of *paqr3b* in neurogenesis, a genetic loss-of-function paqr3b zebrafish model can be created using CRISPR-cas9 genome editing approach and NPSCs proliferation and differentiation can be assessed.

6.3 Additional potential neuropathogenesis mechanisms from the transcriptomic analysis.

Although the focus of this dissertation has been on the *paqr3b* gene linked regulation of the ERK signalling pathway, several other genes from the transcriptomic analysis were also enriched to the ERK pathway. Five other genes were dysregulated upon mutation in *chd7* that were enriched to the ERK pathway- *flnb, nr4a1, dusp2, hspa8* and *dusp16*. The *flnb* (Ensembl id: ENSG00000136068) gene codes for a protein Flamin b that is involved in connecting the cell membrane to the actin cytoskeleton. It potentially promotes branching of actin filaments and thus may play important role in cell migrations in the brain. *Nr4a1* (nuclear receptor subfamily 4 group A number 1) belongs to the family of intracellular transcription factor proteins that plays an important role in mediating inflammatory responses, and in the survival and death of cells (ENSG00000123358). *Dusp2* (ENSG00000158050) and 12 (ENSG0000081721) belong to the dual specificity protein phosphatase subfamily of proteins. They both perform phosphatase functions to negatively affect members of ERK/MAPK pathway in context of differentiation and proliferation of cells. And finally, the *hspa8* (ENSG00000109971) (heat shock protein family a member 8) belongs to the constitutively active member of the heat shock protein family, and potentially contributes to cell trafficking. There is not enough known about where in the signalling cascade of the MEK/ERK pathway the enriched genes act and function. It would be interesting to further explore the exact mechanism of action between *paqr3b*, the other enriched genes and the ERK pathway. The regulatory link between *chd7*

and the above-mentioned genes also remains to be conclusively established. Preliminary validation of the dysregulated genes in the mutant fish via rt-PCR analysis followed by knockdown or overexpression experiments is required to elucidate their role in the observed GABA network defects or modulating the hyperphosphorylated- ERK in the mutant brains.

Beyond the ERK pathway, some of the other major molecular processes that appeared in the pathways analysis included cell adhesion, calcium signalling, lipid transport, tryptophan metabolism and sterol metabolism. Interestingly, in conjunction to the cell adhesion process, an important group of genes - the gamma protocadherins (*Pcdh1g-1, 22, 26, 29, b6* and *c9*), were also significantly dysregulated in the mutant brains. Protocadherins are a set of clustered genes predominantly involved in cell adhesion, migration, and positioning (Bass, Ebert, Hammerschmidt, & Frank, 2007; Biswas et al., 2014). Some of the gamma protocadherins have been reported to be enriched intracellularly with the Golgi or COPII vesicles, suggesting that they play a role in intracellular membrane trafficking (Fernandez-Monreal, Kang, & Phillips, 2009; Phillips et al., 2003). Preliminary experiments showed that morphant and F0 crispant models for gamma protocadherins affects the patterning of the GABAergic network in wildtype zebrafish larvae (unpublished; Annex Supplementary Figure 1). However, more work is required to further elucidate the exact mechanism of the *chd7* linked protocadherin function and the specific way in which this regulator axis contributes to the GABAergic network development in the zebrafish brain.

Sterol metabolism defects and calcium channel signalling defects have been previously associated to neurodevelopmental disorders like autism and fragile-x syndromes (Kanungo, Soares, He, & Steiner, 2013; Nguyen, Medvedeva, Ayyagari, Schmunk, & Gargus, 2018; Schmunk et al., 2017). More specifically, both processes have been known to potentially affect different aspects of GABA network functioning or the GABA/glutamate joint functioning. (Berridge, 2013; Maguire & Mody, 2009). Elucidating each of them in further detail could provide a more refined perspective of the exact malfunctioning in the processes of the neuronal cells that eventually lead to network level defects. This may further add to potential avenues that could be tested as new therapeutic targets for CHARGE syndrome.

6.4 Implications for the application of Ephedrine

There is currently no known treatment or cure for CHARGE syndrome beyond behavioural therapy and occasional surgical interventions. Therefore, it is important to invest in studies that could identify therapeutic approaches that could prevent or at least arrest the development of the CHARGE syndrome malformations. Using the simple models like zebrafish and *C. elegans*, we screened thousands of FDA approved compounds and identified

a small molecule – Ephedrine that could most effectively rescue the GABA network defects and the hyperactivity phenotype. Although Ephedrine is the most potent drug in our zebrafish model, it may have possible off-target effects upon its treatment, particularly cardiovascular effects, that may prove to be disadvantageous in employing it as a neuroprotective molecule. It is therefore important to further assess to find the right form and way for its administration so that it can be used to improve the neuropathogenesis in CHARGE syndrome. To this end the future plan is to test commercially available derivatives of ephedrine with greater selectivity/specificity for their ability to improve the neurological phenotypes in our zebrafish and a mouse model. The mouse model studies would be employing a recently characterized heterozygous *Chd7* gene trap mouse that presented with CHARGE syndrome like hyperactive behaviour associated with cerebellar foliation defects. Defects in cerebellar foliation have been previously reported in heterozygous *Chd7* gene trap mice, and in mice with cKO of *Chd7* from cerebellar granule cells along with mispositioned Purkinje cells (Whittaker, Kasah, et al., 2017). Thus, establishing the suitability of the mouse model to mimic CHARGE syndrome features and provide a higher vertebrate model to study the effects of Ephedrine. Ephedrine is known to function through α- and beta-adrenergic receptors. Employing our *chd7-/-* mutant zebrafish, the further plan is to confirm the mechanism of action through those pathways for Ephedrine by using traditional genetic and chemical targeting approaches. It would also be interesting to seek for different drugs through the screening of derivatives that could target the same pathway(s) thus adding to the available potential therapeutic options and opening wider avenues for research in CHARGE syndrome.

7 Conclusion

In this thesis, I report the creation of a stable homozygous *chd7-/-* mutant zebrafish model for CHARGE syndrome and report important insights into the neuropathogenesis mechanisms that underlie the hyperactivity behaviour observed in CHARGE syndrome. In chapter 3, I describe the characteristic CHARGE syndrome features, particularly, the brain developmental features and the hyperactivity behaviour presented in the *chd7-/-* mutant zebrafish (Figure 1). The hyperactive behaviour upon *chd7* mutations is underlined by a reduction in the number of GABAergic neurons and a functional defect in the existing GABA neuron network of the mutant brain. There is a significant reduction in the overall number of GABAergic neurons in all regions of the mutant brains compared to the wild type brains, along with an error in their positioning in the network. The unbiased transcriptomic analysis in the mutant brains provided a wide range of dysregulated genes upon loss of function mutation in *chd7*. We identified *paqr3b* -a direct *chd7* regulated gene – as a link that at least in part contributes to hyperactive behaviour and reduced number of GABA neurons. CHIP-PCR showed that *PAQR3* levels are also reduced in LCLs derived from a human *CHD7* mutantion-positive CHARGE syndrome individual in comparison to parental controls. This reduction is because of reduced occupation of *PAQR3's* proximal promoters by *CHD7*. Further, by employing the chd7-mutant *C. elegans* and zebrafish, we identified a small neuroprotective molecule – Ephedrine. Ephedrine ameliorates the GABA network defect and the hyperactivity phenotype and restores the hyperphosphorylated-ERK to near normal levels in the mutant brains upon treatment.

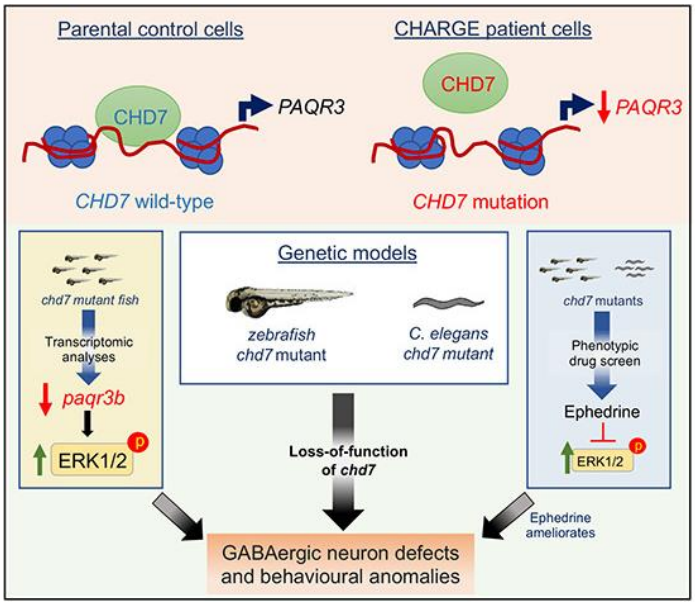

Figure 1 Chromatin remodeller CHD7 is required for GABAergic neuron development by promoting PAQR3 expression
The schematic describes the role CHD7 plays in the reduction in the number of GABAergic and hyperactive behaviours in CHARGE syndrome. Adapted from (Jamadagni et al., 2021)

In chapter 4, I report a dysregulation in the number of NSPCs through early stages of brain development starting 2 dpf until 5 dpf that potentially underlines the reduction in the number GABAergic neurons in the *chd7-/-* mutant brain. At 5 dpf there is an increase in the Sox2+ NSPCs in the midbrain/optic tectum region and the hypothalamus. There is also an increase in the number of GFAP+ positive radial glia/NSPCs that have abnormal processes into the brain. Overexpression of *paqr3b,* could rescue the number of Sox2+ and GFAP+ NSPCs in the mutant brains and increased the number of GABAergic neurons. This suggests an important role for the *chd7-paqr3b* regulatory link in the regulation of NSPCs and their differentiation.

Thus, in conclusion, we have been able to ascertain a neurogenesis and molecular basis for the GABA network defect underlined hyperactive behaviour in CHARGE syndrome. This work provides an initiation point for future research into the role of *chd7* and *paqr3b* in neuropathogenesis and brain development. Employing our *chd7-/-* mutant model will greatly help in furthering the knowledge about the neuropathogenesis mechanisms in CHARGE syndrome.

SECTION IV: REFERENCES

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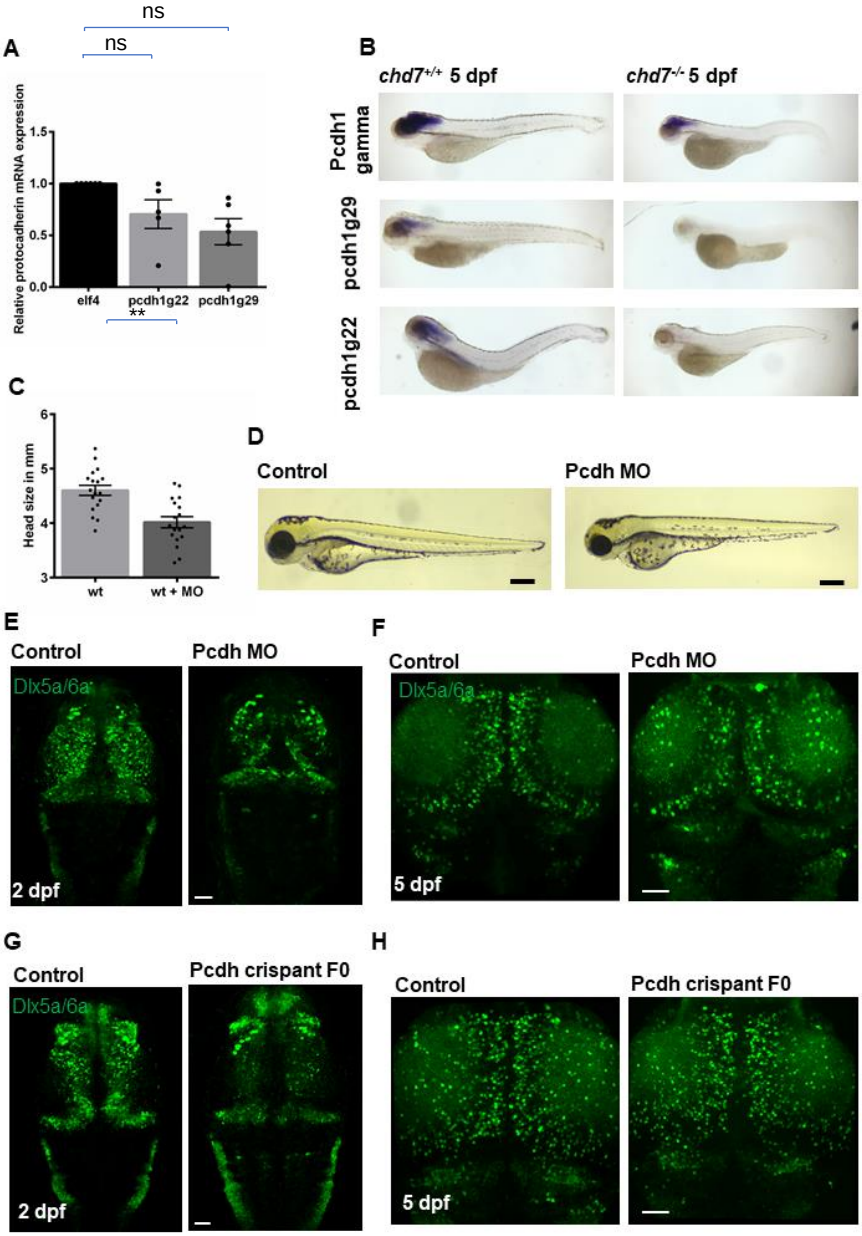
SECTION V: ANNEXES

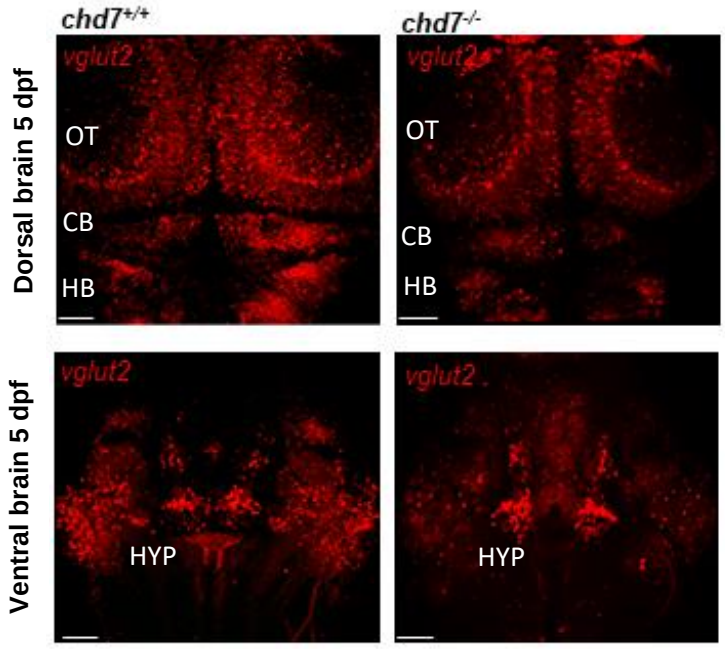
Annex I

Supplementary Figures

Supplementary Figure 1 Gamma protocadherins have effects on the GABA neuron Network in the brain



A,B rt-PCR and *insitu* validation of the reduction in protocadherins -pcdh1g29 and pcdh1g22
C,D Small head presentation in morpholino mediated knockdown of total gamma protocadherins
E,F Effect of morpholino mediated knockdown of gamma protocadherins at 2 dpf (E) and 5 dpf (F) in the zebrafish midbrain.
G,H GABAergic neuron networks in gamma protocadherin crispants at 2 dpf (G) and 5 dpf (H) in the zebrafish midbrain.
Scale: D: 5 µm; E,G: 10 µm; F,H: 50 µm



Supplementary Figure 2 Glutamatergic neurons in the *chd7-/-* mutant zebrafish brains
The above panel shows glutamatergic neurons in the dorsal and ventral brain at 5dpf in wild type and *chd7-/-* mutant zebrafish brains. There seems to be a reduced density in the Glutamatergic neurons overall in the mutant brain compared to wild-type brain.
OT: optic tectum, CB: cerebellum, HB: hindbrain, HYP: hypothalamus; Scale: 50µm

Annex II

List of Additional Publications

1. **Jamadagni P** and Patten S, *'25-hydroxycholesterol impairs neuronal and muscular development in zebrafish'* NeuroToxicology (2019), ISSN: 0161-813X

   DOI 10.1016/j.neuro.2019.08.007

   Contribuition:

   PJ designed, collected, analysed and interpreted the results. PJ and SAP drafted and prepared the final version of the manuscript.

2. Poujol de Molliens M**, Jamadagni P**, Létourneau M, Devost D, Hébert TE, Patten S, Fournier A, Chatenet D '*Design of membrane-tethering peptides derived from the pituitary adenylate cyclase-activating polypeptide receptor 1 and characterization of their neuroprotective properties in neurodegenerative disease models',* Biochimica et Biophysica Acta (2019), Volume 1863, 129398

   DOI 10.1016/j.bbagen.2019.07.007

   Contribuition:

   PJ helped to design, collect, analyse and interpret the results involving the zebrafish experiments.