Validation and characterization of a zebrafish *chw2* mutant:

*A novel model for Lennox-Gastaut Syndrome, photosensitive epilepsy and autism*

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Validation and characterization of the *chas2* zebrafish mutant as a novel model for Lennox-Gastaut Syndrome, photosensitive epilepsy and autism

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Validation and characterization of the zebrafish chd2 mutant

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http://www.duo.uio.no/

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Gezime Seferi
Abstract

Background

Lennox-Gastaut Syndrome (LGS) is one of the most severe childhood epilepsies. The syndrome has an onset between the ages of 3 and 5 years and is characterized by different types of seizures, such as absence, tonic-clonic, myoclonic or even status epilepticus. LGS is also associated with intellectual retardation. The syndrome not only affects the patient’s quality of life but also has an enormous impact on their family, as it stands in the way for the affected to live a normal life. The seizures are very often difficult to control and the epilepsy is in many cases pharmacoresistant. The patients are heavily medicated in the hopes that seizures can somewhat be reduced for LGS patients. Clearly, there is an unmet need for new innovative therapies with better outcomes. The discovery of new therapies with diminished side effects or disease modifying activity to slow or stop the progression of the disorder would be of clear benefit to these patients.

Out of all epilepsy patients, 3-5% have photosensitive epilepsy. This means that their seizures are triggered by visual stimuli, usually flashing lights. These seizures are in most cases difficult to control and the first line drug used in these cases is Valproate. However, Valproate is teratogenic and is not recommended during pregnancy, leaving these patients vulnerable to seizures. An animal model that displays seizures and is photosensitive could therefore aid in the discovery of new innovative therapies towards treating pharmacoresistant photosensitive epilepsy (and perhaps other epilepsies).

The syndrome is linked to the dysfunction of Chromodomain-helicase DNA protein 2 (CHD2). The function of the CHD2 protein is not fully understood and further research on its function is needed as mutations in the CHD2 have been linked to Lennox-Gastaut Syndrome and autism.

Autism is characterized as a developmental disorder with difficulty in social interaction and communication, and by restricted and repetitive behavior. It is estimated that autism affects over 24.8 million people globally. Symptoms are discovered during the early years of a child’s life, usually by ages 2-3 years. There is currently no cure for autism and the mechanisms behind autism are still not fully understood. A better understanding of the underlying mechanisms of the disorder can provide new insights into therapeutic options.
Aim

There is a lack of reliable and validated animal models for LGS, autism and epilepsy. A validated zebrafish model can potentially aid in the discovery of new therapeutic compounds. This new model can also provide the opportunity to discover novel mechanisms underlying epilepsy, LGS and autism. A model with a mutation in the *chd2* gene could therefore shed light on CHD2 protein function and the mechanisms leading to epilepsy, LGS and autism. The aim of this thesis is therefore to develop and validate a new zebrafish model carrying a mutation in *chd2*.

Method

The zebrafish *chd2* mutant was investigated at 3-5 days post fertilization (dpf) by whole-mount imaging to identify phenotypes that correlate with LGS. The brain activity of the *chd2* mutant larvae was also recorded at 4-5 dpf using local field potential (LFP) recordings to detect spontaneous seizures, and then analyzed by computer software to quantify epileptiform discharges. To investigate whether the seizures were photosensitive, a photostimulus consisting of a series of light flashes was also introduced to larvae during the LFP recordings. Mutant larvae were subsequently treated with valproate to investigate whether the observed seizures could be inhibited. Typical seizure-like behavior included hyperactive swimming that could be quantified using automated video tracking. The optokinetic response was also measured to understand whether the mutation might affect visual perception.

Abnormal brain ventricles in the zebrafish have been reported previously as a surrogate phenotype for autism (Gutzman, 2009). To understand whether the *chd2* mutation results in abnormal brain ventricle formation, a fluorescent dye was injected into the forming zebrafish hindbrain at 24 hours post fertilization. The brain ventricles were then imaged with a fluorescent microscope.

Results

Zebrafish *chd2* mutants displayed very similar physical deformities and photosensitive seizures reported for LGS patients. The observed seizures in zebrafish *chd2* mutant larvae could also be suppressed by valproate, similar to patients with photosensitive epilepsy. Automated locomotor activity tracking showed that *chd2* mutants displayed convulsive-like behavior compared to sibling wildtype controls and were equally active during the light and
dark cycles. The optokinetic response however, appeared normal. Lastly, mutant larvae also displayed underdeveloped brain ventricles.

Conclusion

Our findings demonstrate that the zebrafish chd2 mutant phenocopies Lennox-Gastaut Syndrome remarkably well, both with regard to the observed dysmorphologies, seizure types and response to the antiepileptic drug Valproate. We therefore propose this mutant as a validated novel animal model for Lennox-Gastaut Syndrome, photosensitive epilepsy and autism.
# Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AED</td>
<td>Anti-epileptic drug</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ASD</td>
<td>Autism Spectrum Disorder</td>
</tr>
<tr>
<td>CHD</td>
<td>Chromodomain DNA Helicase protein</td>
</tr>
<tr>
<td>CHD2</td>
<td>Chromodomain DNA Helicase protein 2 in humans</td>
</tr>
<tr>
<td>Chd2</td>
<td>Chromodomain DNA Helicase protein 2 in zebrafish</td>
</tr>
<tr>
<td>chd2</td>
<td>Chromodomain DNA Helicase protein 2 gene</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>dNTP</td>
<td>Nucleoside triphosphate</td>
</tr>
<tr>
<td>dof</td>
<td>Day of fertilization</td>
</tr>
<tr>
<td>dpf</td>
<td>Day(s) post fertilization</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EEG</td>
<td>Electroencephalography</td>
</tr>
<tr>
<td>EMA</td>
<td>European Medicines Agency</td>
</tr>
<tr>
<td>ENU</td>
<td>N-ethyl-N-nitrosourea</td>
</tr>
<tr>
<td>FDA</td>
<td>(U.S.) Food and Drug Administration</td>
</tr>
<tr>
<td>g</td>
<td>Gram</td>
</tr>
<tr>
<td>g/l</td>
<td>Grams per liter</td>
</tr>
<tr>
<td>HDAC</td>
<td>Histone deacetylase inhibitor</td>
</tr>
<tr>
<td>hpf</td>
<td>Hour(s) post fertilization</td>
</tr>
<tr>
<td>Hz</td>
<td>Hertz</td>
</tr>
<tr>
<td>ILAE</td>
<td>International League Against Epilepsy</td>
</tr>
<tr>
<td>kb</td>
<td>Kilobases</td>
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<tr>
<td>LFP</td>
<td>Local Field Potential</td>
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<tr>
<td>mg</td>
<td>Milligram</td>
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<tr>
<td>mhb</td>
<td>Mid-hindbrain barrier</td>
</tr>
<tr>
<td>ml</td>
<td>Milliliters</td>
</tr>
<tr>
<td>mM</td>
<td>Millimolar</td>
</tr>
<tr>
<td>MOA</td>
<td>Mechanism of action</td>
</tr>
<tr>
<td>MQ-Water</td>
<td>Milli Q Water</td>
</tr>
<tr>
<td>mV</td>
<td>Millivolt</td>
</tr>
<tr>
<td>NCMM</td>
<td>Center for Molecular Medicine Norway</td>
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<tr>
<td>NEB®</td>
<td>New England Biolabs®</td>
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<tr>
<td>OKR</td>
<td>Optokinetic response</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PFA</td>
<td>Paroxysmal fast activity</td>
</tr>
<tr>
<td>PTZ</td>
<td>Pentylentetrazole</td>
</tr>
<tr>
<td>RBD</td>
<td>Rhodamine B Dextran</td>
</tr>
<tr>
<td>s</td>
<td>Second</td>
</tr>
<tr>
<td>SE</td>
<td>Stauts Epilepticus</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the means</td>
</tr>
<tr>
<td>SSW</td>
<td>Slow Spike Wave(s)</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris base, acetic acid and EDTA</td>
</tr>
<tr>
<td>VHC</td>
<td>Vehicle</td>
</tr>
<tr>
<td>VPA</td>
<td>Valproate/Valproic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<td>--------------</td>
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</tr>
<tr>
<td>WHO</td>
<td>World Health Organization</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>ZMP</td>
<td>The Zebrafish Mutation Project</td>
</tr>
<tr>
<td>µg</td>
<td>Microgram</td>
</tr>
<tr>
<td>µl</td>
<td>Microliter</td>
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<tr>
<td>µM</td>
<td>Micromolar</td>
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1 Introduction

1.1 Lennox Gastaut Syndrome

1.1.1 General introduction

Lennox Gastaut Syndrome (LGS) is one of the more serious childhood epileptic encephalopathies. It is characterized by slow spike wave (SSW) activity in EEG (≤ 2.5 Hz), multiple types of seizures, and slow mental development (Lund, Brodtkorb, & Nakken, 2011). The age onset for LGS is typically 3-5 years, and LGS patients account for about 5-10% of children with seizures (Bourgeois, Douglass, & Sankar, 2014).

The typical EEG pattern observed for LGS patients was first described by Gibbs in 1938. However, it was Lennox who contributed with the description of the clinical correlation to the unusual EEG pattern (Lennox & Davis, 1950). The etiology of LGS can be divided into two groups, *symptomatic* and *cryptogenic*. The *symptomatic* group represents approximately 75% of cases and requires an identifiable cause such as cerebral malformation or hypoxic-ischemic injury. The remaining cases, 25-30%, fall into the *cryptogenic* group where there are no apparent causes and no neurologic precedents (Hancock & Cross, 2013). Little progress in understanding the genetic contributions to the syndrome, lack of animal models and the multitude of etiologies have hindered further understanding of LGS (Blume, 2001; Galizia et al., 2015). A study using a *chd2* zebrafish model has been conducted earlier. However, in the study the *chd2* model was generated microinjecting wildtype zebrafish larvae (Galizia et al., 2015). In this study the *chd2* line was generated at the Sanger Institute giving a model with less of-target effects and we also investigate the correlation between mutation in *chd2* and autism.

1.1.2 Symptoms and manifestations of Lennox Gastaut Syndrome

As mentioned above, patients with LGS have slow mental development leading to moderate to severe intellectual impairment. Prognosis for LGS patients is poor as many of the patients continue to have neurocognitive deficits, medically refractory epilepsy, and frequent episodes of status epilepticus. Almost all patients are dependent on institutional care (Bourgeois et al., 2014).
LGS is typically diagnosed within the first years of a patient’s life usually by the time they turn 5 years old with tonic and atonic seizures. Focal seizures usually occur as the patient ages. LGS is identified by three distinguished electroclinical features (Markand, 2003):

1) Interictal EEG showing generalized SSW, 1.5-2.5 Hz, during wake status and paroxysmal fast activity (PFA) during sleep.

2) Multiple types of epileptic seizures, including tonic, atypical absences, and drop attacks.

3) Slow mental development and/or behavior disturbance.

However, all patients do not have all the core seizure types, especially at onset (Arzimanoglou et al., 2009). Experts agree that the occurrence of tonic seizures during sleep is a criterion required for diagnosis. Since the EEG and the diagnostic clinical features are not present at epilepsy onset, the diagnosis of LGS may occur only over several years (Pr Camfield, 2011).

At the time of diagnosis of LGS, cognitive impairments are apparent in 20-60% of patients. Over time, the cognitive impairments become more apparent and by 5 years of onset, 75-95% of patients have serious intellectual problems (Arzimanoglou et al., 2009).

1.1.3 Underlying Causes and Demographic Picture

Epilepsy has a prevalence of 6 per 1,000 children, 4% of the cases are classified as LGS and affect boys more often than girls (Markand, 2003; Trevathan, Murphy, & Yeargin-Allsopp, 1997). LGS rarely emits and therefore, prevalence studies should find a higher frequency of LGS than incidence studies. 59% of all patients experienced their first seizure during their first year of age (P. Camfield & Camfield, 2007).

As mentioned above, LGS is divided into two subgroups: symptomatic and cryptogenic. The cryptogenic cases have no underlying cause and there is no evidence of brain-imaging abnormalities and the psychomotor development before onset of seizures is normal. In other patients, a usual underlying cause includes hypoxic-ischemic encephalopathy at birth, perinatal and prenatal vascular insults, perinatal meningoencephalitis, various brain malformations and migrational disorders, tuberous sclerosis, Down’s syndrome, head trauma,
hydrocephalus, brain tumor, and radiotherapy. Genetic factors also play a role in LGS and the investigation of genes relevant to LGS is increasing (Markand, 2003).

1.1.4 Social and professional impact

One of the major issues of epilepsy alone is controlling the seizures, although for many, the social stigma and discrimination associated with the condition are often harder to overcome (WHO, 2017). For those with LGS, along with the risk of premature death, reduced life expectancy and numerous co-morbidities, patient families also face many challenges in their work and social life, as they are ostracized from certain parts of society due to their child’s condition. Having LGS may entail limited access to healthcare services, reduced health and life insurance, living self-sufficiently, and getting married and having children (Gaitatzis, Carroll, Majeed, & Sander, 2004) (WHO, 2017). Epilepsy and LGS places a tremendous burden on the individuals as well as their family and even society. LGS is a chronic condition that requires constant regular check-ups, supervision and medical treatment, which takes a toll on the patient as well as the health care system (WHO, 2017). The efforts to improve the quality of life for LGS patients and their families are complex. Attentional problems, aggression, and autistic features can be very prominent in LGS and represents an enormous challenge for the families. “Drop attacks” are especially difficult for the families and represent a “nightmare” for them and for the patient. To prevent facial injuries, helmets with full face mask can be required and these masks are highly stigmatizing and often impractical (Wiebe, Camfield, Jette, & Burneo, 2009).

1.1.5 Challenges and complications of treating LGS

Treating LGS is difficult and usually unsatisfactory. Antiepileptic drugs (AEDs) are in many cases ineffective and do not provide satisfactory control of the seizures. The number of seizures and the multitude of type of seizures require polytherapy, often in high doses. The high doses lead to a high incidence of side effects. Benzodiazepines are highly used in LGS, and the side effects include drowsiness, drooling, incoordination, and hyperactivity are common. In addition, tolerance also becomes a problem. Other traditional AEDs used are phenytoin and carbamazepine, and have proven helpful against tonic and generalized tonic-clonic seizures (Bittencourt & Richens, 1981; Dimario & Clancy, 1988). However, in LGS,
pharmacoresitance is a significant problem that often leads to use of very high doses of AEDs and unpleasant side effects.

1.2 Photosensitive epilepsy

Studies show that up to 70% of epilepsy patients respond well to medical treatment and are able to enter remission (i.e. control their seizure and be seizure free). Meanwhile, an estimated 30% of epilepsy patients are treatment resistant and diagnosed with refractory epilepsy. Patients who fail to respond to available treatments are reported to have a considerably increased risk of injuries, premature death, psychosocial dysfunction, and a reduced quality of life (Kwan, Schachter, & Brodie, 2011). Even for those who attain complete remission, the disease still raises health concerns and often still has unsatisfactory effects on quality of life. The side effects associated with taking antiepileptic medication are most notoriously the negative effects on cognitive function such as mental awareness and concentration. This can be a serious concern as it affects their general wellbeing, function and performance or ability to perform optimally in the workplace, at school or in life in general. Moreover, the medication has to be taken daily for a long period of time and perhaps for the rest of their lives (Karceski, 2007; Kerr, 2012). A family history of epilepsy also indicates a higher risk of LGS, when it comes to the forms of epilepsy that are hereditary (ascribed to genetic factors). This could be a disadvantage when starting a family knowing it could potentially be passed on to their children and could affect their chances at marriage prospects (WHO, 2017).

1.3 Medical treatment and pharmacotherapy

Valproate is the first line drug for many types of epilepsies and is efficient in controlling absences and myoclonic seizures. Drop attacks are most resistant towards valproate treatment. Valproate has fewer side effects compared to the drugs mentioned above, and this is one of the reasons valproate has become the first line drug. The downside of using valproate, especially in polytherapy, is the higher risk of hepatotoxicity (Markand, 2003).

Newer AEDs are already on the market and others under development. Topiramate has a broad spectrum of activity and is used in controlling tonic seizures and drop attacks (Glauser, Levisohn, Ritter, & Sachdeo, 2000). Lamotrigine had been much used together with valproate for controlling any other seizure types in LGS. Felbamate has proven to be very efficient in
controlling drop attacks and tonic seizures in LGS. However, it has also been associated with an increase of incidence of aplastic anemia and acute liver failure (Group, 1993; Markand, 2003).

In the treatment of photosensitive epilepsy, there are more than 20 different AEDs on the market and over 15 new AEDs have been approved more recently. There are nevertheless one third of epilepsy patients that struggle to control their symptoms and continue to have seizures (Galanopoulou et al., 2012). Of these 30%, a subset of patients that suffer from photosensitive epilepsy, where seizures can be triggered by the smallest patterns. Side effects on the central nervous system are still highly prevalent and the negative effects on quality of life are still unsatisfactory (Friedman and Devinsky, 2015). Furthermore, new medications have not significantly reduced the proportion of patients with medically refractory epilepsy (Friedman and Devinsky, 2015). Currently available medications/pharmacotherapeutics act purely at the symptomatic level and merely act to suppress seizures. There has been a lack of success in finding novel targets and medications with mechanisms of action that can prevent the development or progression of epilepsy (disease modifying) or cure the disease (anti-epileptogenic) (Galanopoulou et al., 2012). This means that development of new treatments with reduced side effects and efficacy towards pharmacoresistant epilepsy is of vital importance. However, new and innovative therapies that do not merely suppress and control the symptoms but can actually prevent and cure the disease should be pursued or strived for.

1.3.1 Mechanism of action of current AEDs

AEDs only treat symptoms and, in most patients, rarely lead to seizure freedom. The symptoms of epilepsy that manifest as recurrent seizures is believed to be a manifestation of intermittent hyperexcitability of the neurons, and the AEDs are believed to restore the balance between excitation and inhibition of the nervous system (Brodie & Sills, 2011). The mechanism of action of current AEDs is still not fully understood, but some mechanisms have been recognized as likely modes of actions. The first generation AEDs act by modulating voltage-gated ion channels, either as an antagonist by blocking sodium channels or by enhancing the inhibitory effect of gamma-aminobutyric acid (GABA) on neurotransmission. The newer antiepileptic drugs, encompass some new mechanisms sometimes in addition to the same mechanisms as the older AEDs, including binding to the presynaptic vesicle protein SV2A, causing inhibition of sodium channels, and binding to the calcium channel alpha2delta
subunit, which results in the opening of specific potassium channels. A number of AEDs have multiple mechanisms of action that are yet to be understood (C. Stafstrom, Grippon, & Kirkpatrick, 2011).

1.4 Autism

Autism is classified as a developmental disorder where people affected have trouble in social interaction, communication, and restricted by repetitive behavior. The symptoms for autism are rarely observed before the child is 2-3 years old (Landa, 2008). Autism affects the processing of information in the brain by altering how nerve cells connect and organize. The underlying cause for autism and how symptoms occur is not fully understood (Levy, Mandell, & Schultz, 2009). Several risk factors have been associated with autism, these include; cocaine, alcohol or valproate use under pregnancy as well as infections under pregnancy. However, autism is not only caused by these risk factors. Autism is caused by a combination of risk environmental and genetic factors (Chaste & Leboyer, 2012).

The autism spectrum (ASDs) is a group of autism and autism-like syndromes (e.g Asperger syndrome). About 1.5% of children are diagnosed with ASD, with a higher incidence in boys than girls (Lyall et al., 2017).

1.4.1 Characteristics of Autism

Autism is characterized as a neurodevelopmental disorder with an onset in infancy or childhood. In most patients the disorder follows a steady course throughout life without remission. Symptoms are visible after the first 6 months of the child’s life and become established by age 3. The symptoms continue through adulthood, however, subtler as the patient ages into adulthood (Isabelle & Sylvie, 2008). Autism is characterized by a triad of symptoms of impairments in social interaction, impairments in communication, and restricted interest and repetitive behavior (Filipek et al., 2000). The mechanisms behind autism are not fully understood, but it appear to result from developmental factors that affect many or all functional brain systems (Tuchman, Moshé, & Rapin, 2009).

1.4.2 Management
There is no cure for autism and to date there is no single treatment that is regarded as the first line treatment. Treatment is usually tailored to the patient’s needs and the families, as well as the educational system, are the main resources for treatment (Levy et al., 2009). The goal of the treatment is to ease associated deficits and family distress and also improve the quality of life and functional independence. When behavioral treatment fails, some patients are medically treated. The most common classes of drugs prescribed are antidepressants, stimulants, and antipsychotics. Antipsychotics have shown a positive effect in treating irritability, repetitive behavior, and sleeplessness. When prescribing antipsychotics, the potential benefits need to outweigh the side effects (Levy et al., 2009).

1.4.3 Prognosis and Epidemiology

Most patients with autism experience less severe symptoms as they age, but patients still lack social support and future employment opportunities (Burgess & Gutstein, 2007). Some patients show improvement in communication skills, but they also face significant obstacles in transitioning to adulthood (Hendricks & Wehman, 2009).

1.5 Genetic Epilepsies

For more than three decades have the mechanisms underlying different seizure disorders been studied (Steinlein & Steinlein, 2014).

1.5.1 Ion-channel mutations in epilepsy

The most common cause of epilepsy is mutations in genes coding for voltage- or ligand-gated ion channels (Steinlein & Steinlein, 2014). Genetic and electrophysiological analyses of different ion channels have provided better understanding on the pathophysiological pathways leading from mutation to seizures (3). Mutations in ion channels are known to cause rare monogenic idiopathic epilepsies. These mutations are also suspected to play a role in more common epilepsies such as childhood and juvenile absence epilepsies and juvenile myoclonic epilepsy. Examples in ion channels associated with epilepsy are \textit{SCN1A}, \textit{ADNFLE}, \textit{CHRNA4} and \textit{CACNB4}.

1.5.2 Non-ion channel genes in epilepsy
In 2001 it was demonstrated non-ion channel genes play a role in the etiology of epilepsies. Although these do not play as big of a role as ion channel genes, the discovery of new genes coding for non-ion channels causing epilepsy is increasing. This gives a new view on epilepsy as it before was associated with mutation in only ion channels. Also, genetic disorders can alone cause epilepsy and about 50% of genes coding for proteins crucial to development of embryos can in theory indirectly cause epilepsy (Steinfield & Steinfield, 2014).

1.6 Chromodomain DNA Helicase protein 2 (CHD2)

The function of the CHD2 protein in humans is still not fully understood. The protein is part of the CHD protein family and these proteins participate in the regulation of gene activity (expression) by chromatin remodeling (Galizia et al., 2015). The process of packaging DNA into chromosomes is completed by chromatin complexes. Remodeling of chromatin structure can alter how tightly DNA is packaged. Gene expression is lower when DNA is tightly packed than when DNA is loosely packed.

The CHD family is characterized by the presence of chromo domains and SNF2-related helicase/ATPase domains, the tertiary structure of the protein is shown in figure 1. The mutation used in this study affects the 826\textsuperscript{th} amino acid positioned in the C-terminal helicase domain.

![Figure 1 The chd2 tertiary structure in humans.](image)

One study showed that the CHD2 protein would interact with H3.3 to determine myogenic cell fate. This study indicates that MyoD determines cell fate and facilitated differentiation-dependent gene expression through Chd2-dependent deposition of H3.3 prior to differentiation (Harada et al., 2012).
1.7 Valproate

Valproate, also known as valproic acid (VPA), is a widely used AED and mood stabilizer in the treatment of bipolar disorder (Akimoto, Kusumi, Suzuki, Masui, & Koyama, 2007; Chateauvieux, Morceau, Dicato, & Diederich, 2010). The mechanism of action of valproate is currently unclear, but some of its pharmacological activity has been partially ascribed to the stimulation of glutamate receptors, particularly GABA, inhibition of voltage-gated sodium and calcium channels, and its role as a Histone deacetylase inhibitor (HDAC) (Chateauvieux et al., 2010). The drug has also been reported to influence neurotransmitter systems, increasing levels of dopamine and serotonin in the brain after administration, which might be a contributing factor to how it exerts its neurological effects (Akimoto et al., 2007; Lee et al., 2012).

Valproate is generally regarded as safe and well tolerated, although commonly associated with side-effects including drowsiness, weight gain and nausea (Lagace & Nachtigal, 2004). However, when used during pregnancy, VPA had been associated with severe adverse effects, with a three-fold increase in major anomalies occurring in the offspring (Ornoy, 2009). VPA has thus been determined as a human teratogen (Ornoy, 2009). Adverse effects on fetal development include spina bifida, developmental delay, skeletal and limb defects, decreased intrauterine growth and neurodevelopmental problems, evident by autistic spectrum disorder (ASD) among other neurological abnormalities (Alsdorf & Wyszynski, 2005; Ornoy, 2009). Whether VPA could be associated with the development of other neurological disorders remains to be seen.

1.8 Animal models

Research animals have played a very big role in life science for a very long time and they have given researchers the opportunity to test substances on a complete biological system. Sooner or later the idea will have to be tested on an organism and, even if it looks very promising in vitro there is no guarantee of how the idea will act in a complete biological system. To get the whole picture the disease needs to be produced in a way that allows researchers to study it and, the answer was to use animals that have a close correlation to the human body. Animal models allow closer approximation to a human response. However, the model will never be perfect as different species host different diseases and different
responses. A gene can be present in both a mouse and human but this gene can be a different orthologue than the human one a researcher wants to study. This makes it very important to use the correct animal and to make sure that what is used will be comparable with whatever problem that wants to be solved.

1.8.1 Classic models

Human epilepsy shows a variety of clinical, behavioral and physiological symptoms that has been researched in animal models. Studies have been carried out mainly in mammals on different species, but rats and mice are the primary models of choice and have been preferred in the majority of studies on epilepsy (Brian & Scott, 2015). When discussing models relating to seizures it is important to distinguish between acute seizure models and chronic epilepsy. In the seizure models, the seizures are acute and provoked, whereas in the epilepsy models the seizures are chronic, recurrent, often spontaneous, and more representative of the condition. Despite this, acute seizure models are primarily used to screen for AEDs and to study their effects on epileptic seizures, even though they are not considered models of epilepsy (Kandratavicius, Hallak, Carlotti, Assirati, & Leite, 2014).

Epilepsy and epileptic seizures encompass electrical and behavioral events that are caused by physiological changes in humans that can often be credited to chemical, molecular and anatomical changes in the brain. To generate epilepsy models, these changes in the brain are often induced by the use of chemoconvulsants, genetic modifications, traumatic brain injury, or electrical stimuli (Kandratavicius et al., 2014).

The classical widely used models to study epilepsy are the kindling model and post status-epilepticus model using rats and mice (Gilbert, 2001). The kindling model is created by the process of repeatedly delivering sub-convulsive electrical stimuli, or less frequent chemical stimuli, over a period of time to part of the brain. Due to synaptic plasticity, it is then possible to increase sensitivity to stimuli in the brain and lower the seizure threshold, which results in permanently increased susceptibility to seizures (Gilbert, 2001).

Status epilepticus (SE) is a life-threatening medical emergency that is characterized by The International League against Epilepsy (ILAE) as “continuous seizure activity” (prolonged seizure > 5 minutes) which after an estimated 30 minutes runs the risk for neuronal damage (Trinka, Höfler, Leitinger, & Brigo, 2015). To generate SE models, status epilepticus is either
induced chemically (commonly with pilocarpine or kainite) or by electrical stimulation. This causes neuronal damage and results in spontaneous seizures that appear after a few weeks (Wolfgang Löscher, 2002; Reddy & Kuruba, 2013).

These classical models have served as the cornerstone in fundamental research on epilepsy that have improved our understanding of basic mechanisms and contributed to the development of several antiepileptic drugs. Despite this, the use of these models have not led to the discovery of promising novel compounds, and the new generation of AEDs has made little progress compared to its predecessors. The lack of improvements in AEDs is especially prominent with regard to pharmacoresistant epilepsies as there has been no significant reduction in the number of cases (W. Löscher & Schmidt, 2011).

The mechanisms causing epilepsy still remain elusive, and there is a concern that the traditional classic rodent screening models only reflect a limited part of the pathological mechanisms of epilepsy and exclude novel antiepileptic compounds with other mechanisms. This could hinder the discovery of new mechanisms and compounds that could potentially lead to more efficient treatment options of refractory epilepsy. There is therefore, a pressing need for new and improved animal models that include novel targets (W. Löscher & Schmidt, 2011).

Thus far the main animal model used in the research of Lennox Gastaut Syndrome has been mice (C. E. Stafstrom & Velíšek). There is a need for a model that can provide fast results and can be used in high throughput drug screening, as there is no proper treatment for LGS. The treatment LGS patients receive consists of AEDs already on market. However, many of these patients have epilepsy that has become pharmacoresistant meaning, pharmaceuticals available on the market are not as efficient as they are meant to be.

### 1.8.2 Zebrafish as a model organism

*Danio rerio*, often referred to as “zebrafish” in layman’s terms, are tropical freshwater fish found in fresh water streams and river of South Asia and South East Asia and belong to the Minnow family (Burke, 2016). Zebrafish are popular aquarium fishes but are gaining rapid popularity in science, in particular, biological and medical research to model human diseases (Burke, 2016).
The zebrafish is rapidly emerging as an in vivo model for neurological disorders. Although the adaption of this simple vertebrate as an epileptic model is only in its early stages, the zebrafish has displayed many similar neurobehavioral traits to those found in humans. Behaviors such as diurnal sleep/wake cycles and drug addiction have been observed, demonstrating that they have behavioral traits and endpoints that can be quantified for research (Best & Alderton, 2008).

Viewing the zebrafish from a phylogenetic perspective, they are further removed from humans compared to mammals (Macrae & Peterson, 2015). Nevertheless, a comparison of the zebrafish genome with the human genome reveals that the zebrafish shares many similarities with humans. Approximately 70% of the zebrafish genome shows homology with the human genome, whereas 71.4% of human genes have at least one orthologue in the zebrafish (Howe et al., 2013). In spite of some of the differences between the brain structure of mammals and teleost, the overall organization of the brain is similar in mammals (Best & Alderton, 2008).

Genetic screens in zebrafish have identified several genes relevant to humans, with vital roles in the development of the brain. The function of these genes in the zebrafish may suggest that the orthologous gene(s) in humans are also important for neurological development and may be relevant for the development of some neurological disorders. Further research is necessary to investigate the roles of these specific genes in both humans and zebrafish, and perhaps what influence they may have on the development of neurological disorders, including epilepsy (Parent, 2006).

1.8.3 The advantages of using zebrafish

The zebrafish offers several practical benefits as a model organism allowing for high throughput chemical and behavioral screens. A zebrafish model offers the opportunity to do in vivo studies in a complex system living animal, with the cost, time and test substance quantity efficient benefits of in vitro studies (Truong, Harper, & Tanguay, 2011).

Adult zebrafish are small in size making them easy to house and maintain. The zebrafish also has a short generation time as well as a short maturation period, reaching maturity in a matter of 2-3 months, allowing us to study different life stages in a short time frame. Zebrafish can breed any time of the year and are able to lay up to hundreds of eggs at weekly intervals, making it possible to provide to a large stock of fish in a short period of time for experiments.
The eggs are fertilized externally and the embryos develop *ex utero* in addition to being optically clear, providing advantages in the visualization of neurological development as it can remain living and fully intact without sacrificing the mother.

The rapid development the zebrafish possesses is a great advantage as this makes it possible to perform experiments quickly and still enable experiments to be carried out on a whole biological system (whole organism).

The zebrafish brain is fully developed within 7 days post fertilization and this rapid development makes it suitable for research in neuroscience where neuronal networks, neuronal transmission and psychiatric diseases are very much studied.

### 1.8.4 The challenges of using zebrafish

There are shortcomings and challenges with every model organism used in human disease research. Model organisms are clearly not human, which beg the question of whether or how much of the data gathered can translate to or are predictive for humans. The effects observed in model organisms cannot be directly translated to humans, but has often been able to provide an indication of the effect(s) of a drug in a complex system. In drug development, animal testing is vital for drug screening and preclinical tests, and serves a critical role in determining whether a drug or compound will be developed further. Currently, zebrafish are not accepted as a species for preclinical testing drugs. According to guidelines by the Food and Drug Administration (FDA) and European Medicines Agency (EMA), preclinical testing is required to be performed on two mammalian species (including a rodent and a non-rodent) prior to clinical human trials (Atanasov et al., 2015). Despite zebrafish not being validated as a species, it could still be used as an additional species in preclinical tests. Zebrafish provide many advantaged that could be useful for discovering novel neuroactive compound for further research.

### 1.8.5 Zebrafish in epilepsy

Zebrafish are vertebrates and therefore share many similarities to the human nervous system. Studies of numerous drug (including AEDs) that are known to have an effect on humans have shown similar effect in zebrafish, indicating it as a good model for studying epilepsy (Gupta, Khotragade, & Shingatgeri, 2014; Macrae & Peterson, 2015; Sarah et al., 2012). Their ability
to display seizure-like behavior and epileptiform electrographic discharges that can be quantified, demonstrates that they are suitable models to screen for novel compounds that exert an effect on seizure-like behaviors and events. With the limitations of in vitro systems in mimicking in vivo conditions, the zebrafish offers advantages in neuroscience epilepsy research as it can model intricate cell-to-cell interactions and neurotransmission pathways (Rossignol, Carmant, & Lacaille, 2016).

1.8.6 Zebrafish in autism

Research in the field of autism presents challenges such as the development of assays to mimic and observe the equivalent behaviors displayed by patients in animal models. If one is to look closer into the physical properties of the brain, one can see that patients with autism have abnormal brain ventricles compared to their peers, as mentioned earlier. Abnormalities have also been observed in the zebrafish brain where, the fish has been genetically modified to carry a mutation associated with autism (Stewart, Nguyen, Wong, Poudel, & Kalueff, 2014).

1.9 chd2 mutant

The mutant used in this study was ordered from the Zebrafish Mutation Project (ZMP) at the Sanger Institute. The mutant was generated by ENU mutagenesis, an alkylating agent used for introducing random point mutations into DNA by conversion of the Thymine residue into O4-ethyl thymine and thus allowing the converted Thymine to pair with Cytosine. The ZMP methodology is also shown in Figure 2 below.
The mutation characterized in this study occurs at an intron-exon boundary leading to a splice site mutation. Splice site sequences that drive exon recognition are located at the end of the introns and in this study the splice site mutation leads to a change in one nucleotide. Exon 14 underneath is highlighted in yellow with red font and the mutation is highlighted in red with black font:

5′ GTCTTGTTGCTGTGCTCCAGGGACTGCTGGGGAATATAAACACTGGGCCTTTCCCTGGAGTAGATGAGCTCACCCTTTAAAATAGACGACTGCTGGCTGTTATAAAACTCTGAGTCAGACTTCTGAGATCTTCCGGATCAATCGACGGCTGCTATTACACGAGACTCCTCTGAAAACACTCCCTCAAAGAGCTGTCCTTCTGCTGACTCTTG

3′ TTAGTACTCTAGTTTAGGTTA

The mutant used in this study carried a mutation in the chd2 gene. The mutation denotes as allele s15642, chromosome 18, amino acid 662, exon 14. The point mutation exchanges a guanine base with a thymine base and creating an essential splice site. Where a splice site...
mutation is present, the mRNA transcript has information from introns that normally should not be included. The 5’ end of the intron requires the GT ending (donor site) for the splicing to occur and in this case the guanine is exchanges with a thymine via ENU mutagenesis. When splicing of the introns occurs, the intron will not be fully spliced as the transcription apparatus will move on to the next GT donor site. This leads to inclusion of the intron and aberrant translation, likely rendering the protein nonfunctional.
2 Aim of the study

The aim of this study is to characterize and validate the zebrafish with a mutation in *chd2* as a novel model for LGS, photosensitive epilepsy and autism. The model can aid us in the discovery of novel candidates for treatment of LGS and discovery of alternative candidates for VPA that can also be used by women who wish to conceive. The mechanisms underlying autism are not yet fully understood and, this model could aid us in better understanding the mechanisms behind the disorder better. The model will be validated genotypically and phenotypically including LFP recordings (also referred as EEG) and tracking of movement to observe any spontaneous seizures. In the case of spontaneous seizures, the model will be treated with valproate to investigate whether the seizures are treatable.

To confirm the presence of the mutation adult zebrafish will be finclipped and the samples will be genotyped. After genotyping the samples will also be sent for gene sequencing to confirm that the genotyping protocol is effective and that the mutation is at the assumed position.

Furthermore, the larvae from the genotyped adults will be used in observing phenotypes. Whole mount imaging will give a better understanding of the physical phenotypes of the *chd2* mutant. Here the size of the mutant and morphogenesis of the craniofacial area, brain development, swim bladder inflation and eye development will be investigated. Injections of fluorescent dye will be injected in the hindbrain to better understand the expansion and constriction of the brain ventricles and this will give a better understanding of how the model displays autism.
3 Materials and methods

Substances

Table 1 All substances used in this study listed with their suppliers and storage criteria

<table>
<thead>
<tr>
<th>Substance</th>
<th>Supplier</th>
<th>Formula</th>
<th>Storage criteria*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agarose, low melting</td>
<td>Sigma-Aldrich</td>
<td>Powder</td>
<td>&lt; 25°C, dry</td>
</tr>
<tr>
<td>CutSmart Buffer®</td>
<td>NEB</td>
<td>Solution</td>
<td>-20°C</td>
</tr>
<tr>
<td>dNTPs</td>
<td>Sigma-Aldrich</td>
<td>Solution</td>
<td>-20°C</td>
</tr>
<tr>
<td>Dimethyl Sulfoxide</td>
<td>Sigma-Aldrich</td>
<td>Solution</td>
<td>&lt; 25°C, dry</td>
</tr>
<tr>
<td>DreamTaq Polymerase</td>
<td>NEB</td>
<td>Solution</td>
<td>-20°C</td>
</tr>
<tr>
<td>Ethyl 3-aminobenzoate methansulfonate (also known as Tricaine or MS222)</td>
<td>Sigma-Aldrich</td>
<td>Powder</td>
<td>&lt; 25°C, dry</td>
</tr>
<tr>
<td>Primers</td>
<td>Sigma-Aldrich</td>
<td>Solution</td>
<td>-20°C</td>
</tr>
<tr>
<td>Pentylenetetrazole</td>
<td>Sigma-Aldrich</td>
<td>Powder</td>
<td>&lt; 25°C, dry</td>
</tr>
<tr>
<td>TaqBuffer</td>
<td>NEB</td>
<td>Solution</td>
<td>-20°C</td>
</tr>
<tr>
<td>Valproate</td>
<td>Sigma-Aldrich</td>
<td>Powder</td>
<td>&lt; 25°C, dry</td>
</tr>
</tbody>
</table>

* Dissolved agarose was stored at 54°C. Any other powders dissolved were stored at 4°C.

3.1 Animals

All procedures during this study were carried out at the Centre for Molecular Medicine Norway (NCMM) in accordance with European and National Regulations. The EU Directive 2010/63/EU and the Commission Implementing Decision 2012/707/EU states that law concerning animal experiments until the stage of independent feeding does not protect
animals. Therefore, the limit for zebrafish had been set to be 120 hours post fertilization (hpf) and zebrafish experiments exceeding this time period are to be subjected to animal experiment regulations (Strähle et al., 2012). In this study experiments were not restricted by animal experiment regulation as the experiment ended within the zebrafish reached 120 hpf and, all staging were done according to Kimmel et al, 1995.

3.2 Zebrafish care and maintenance

Zebrafish (Danio rerio) larvae used in this study were obtained from fish housed at NCMM originating from the Zebrafish Mutation Project (ZMP), Sanger Institute. The zebrafish line used in this study were chd2 mutants and AB wild types and all experiments were carried out at NCMM, Oslo Science Park, University of Oslo (see table 2 for water parameters). The zebrafish larvae were studied until they reached 120 hpf.

Table 2 Water parameters for adult zebrafish at the NCMM fish facility.

<table>
<thead>
<tr>
<th>Water Parameters</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>7.0 – 8.0</td>
</tr>
<tr>
<td>Conductivity</td>
<td>900 – 1200</td>
</tr>
<tr>
<td>Temperature</td>
<td>26 to 28.6 °C</td>
</tr>
<tr>
<td>General hardness (GH)</td>
<td>6 – 10 °dH</td>
</tr>
<tr>
<td>Carbonate hardness (KH)</td>
<td>3 – 5 °dH</td>
</tr>
<tr>
<td>Ammonia (NH₃)</td>
<td>≤ 0.25 mg/L</td>
</tr>
<tr>
<td>Nitrate (NO₃)</td>
<td>&lt; 25 mg/L</td>
</tr>
<tr>
<td>Nitrite (NO₂)</td>
<td>&lt; 0.3 mg/L</td>
</tr>
<tr>
<td>Phosphate (PO₄)</td>
<td>&lt; 0.2 mg/L</td>
</tr>
</tbody>
</table>
At the fish facility at NCMM the adult zebrafish are housed in aquatic fish tanks from Tecniplast™ at a 14/10 light/dark cycle at parameters optimal for this fish facility (see Table 1). Adult zebrafish are feed three times daily, once with Artemia (brine shrimp) live feed and twice with commercial dry feed (Gemma Micro 300, Skretting, Norway).

Zebrafish larvae needed for experiments were obtained from chd2 and AB wild type zebrafish housed at the fish facility at NCMM. Zebrafish were set up in breeding tanks in the afternoon before the day of breeding. The females and males are separated from each other by putting them on each side of the tank divider and are left separated overnight. In the morning the divider is removed allowing the zebrafish to spawn. The embryos were collected and transferred to petri dishes marked with day of fertilization (dof), strain, and initials. The embryos were sorted, selecting the good quality embryos. The embryos were incubated in 28.5 °C in an incubator separated from the fish room in 1X E3 medium (see Table 3). To maintain good water quality the medium was changed daily.

*Table 3 Salts and substances included in making the E3 medium.*

<table>
<thead>
<tr>
<th>E3 medium</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>17.4 g/L</td>
</tr>
<tr>
<td>KCl</td>
<td>0.8 g/L</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>2.9 g/L</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>4.89 g/L</td>
</tr>
<tr>
<td>1 % Methylene blue</td>
<td>50 µL/L</td>
</tr>
</tbody>
</table>

### 3.3 Genotyping

When genotyping zebrafish larvae under 120 hpf the whole larvae was used. When genotyping adult zebrafish only a sample from the fish was used and in this study a piece of the fish’ fin was used and obtained by fin clipping. Before using the chd2 larvae in this study, the adult chd2 fish had to be genotyped to distinguish the mutants from their sibling wildtype.
3.3.1 DNA Extraction

The DNA was extracted by incubating the sample in 50 µL 0.05 M NaOH in 95 °C for 20 minutes. For incubation the PCR apparatus was used. For the sample to not be destroyed the incubated sample was then neutralized with 8 µL 1 M TrisCL, 10 mM EDTA solution with pH 8. The amount of neutralizing solution in the protocol was optimized after observation of too much degradation of the DNA product. This method for DNA extraction does not give a very clean extraction, especially considering that the sample consist of a whole zebrafish larva. However, this method was good enough in this study and the method is easy, cheap and quick to use.

3.3.2 Amplification using PCR

For amplification the qualitative polymerase chain reaction (PCR) was used. A PCR master mix was prepared containing (for each sample):

*Table 4 Contents of PCR master mix in each sample.*

<table>
<thead>
<tr>
<th>PCR Master mix</th>
<th>20.0 µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>DreamTaq DNA Polymerase Green buffer</td>
<td>2.0 µL</td>
</tr>
<tr>
<td>dNTP 100 mM</td>
<td>0.4 µL</td>
</tr>
<tr>
<td>DreamTaq DNA Polymerase</td>
<td>0.1 µL</td>
</tr>
<tr>
<td>10 µM primer mix*</td>
<td>0.5 µL</td>
</tr>
<tr>
<td>Target (sample)</td>
<td>2.0 µL</td>
</tr>
<tr>
<td>H₂O (MilliQ water)</td>
<td>15.0 µL</td>
</tr>
<tr>
<td>Total volume</td>
<td>20.0 µL</td>
</tr>
</tbody>
</table>

* Primer mix containing forward and revere primers designed specifically for the chd2 gene

The protocol following the DreamTaq DNA polymerase was used as a starting point.

Primers used for the amplification of the gene were designed and synthesized (Sigma Aldrich).
Forward primer: ‘5 – AGGCCATTTAGAAATGGTC – 3’

Reverse primer: ‘5 – AGATGAAGCTCACCAGCTTA – 3’

Figure 3 The chd2 amplicon with labeled primers, mutation and restriction site. The figure is obtained by using the UGENE software.

The cycle program for amplification was optimized using the following DreamTaq DNA Polymerase protocol as a starting point. The annealing temperature was decided by accounting for the primers and the polymerase used. The final program for amplification was:

Table 5 shows the PCR program

<table>
<thead>
<tr>
<th>Number of Cycles</th>
<th>Temperature</th>
<th>Time</th>
<th>Step</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 cycle</td>
<td>95 °C</td>
<td>1 minute</td>
<td>Initial denaturation</td>
</tr>
<tr>
<td></td>
<td>95 °C</td>
<td>10 seconds</td>
<td>Denaturation</td>
</tr>
<tr>
<td>30 cycles</td>
<td>47 °C</td>
<td>10 seconds</td>
<td>Annealing</td>
</tr>
<tr>
<td></td>
<td>72 °C</td>
<td>15 seconds</td>
<td>Extension</td>
</tr>
<tr>
<td>1 cycle</td>
<td>72 °C</td>
<td>3 minutes</td>
<td>Final extension</td>
</tr>
</tbody>
</table>

To confirm the amplification, the samples were run through gel electrophoresis using a 2% agarose gel. The samples were run on the gel at 120 mV. 500 ml 2% agarose solution was
made by solving 10 g of low melting agarose in a buffer solution containing a mixture of Tris base, acetic acid and EDTA (TAE). The mixture was heated and mixed until all the agarose was solved. The PCR product is 246 bases long and for reference a 1 kb ladder was used.

3.3.3 Restriction reaction

For the restriction reaction, the MluCI enzyme was used. The MluCI enzyme cuts at a site in the sequence where it recognizes the ‘AATT’ sequence. As mentioned before the mutation is a missense mutation where a guanine base is exchanged with a thymine base meaning, the enzyme will only cut where there is a mutation and not if the mutation is not present. The enzyme was ordered from New England Biolabs Inc. (NEB®) and was delivered with the Cutsmart® Buffer. Since the activity of the enzyme is 100% in the Cutsmart® Buffer, this is the buffer used in the restriction reaction.

Table 6 Contents of the restriction digest for each sample.

<table>
<thead>
<tr>
<th>Contents of the restriction reaction</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CutSmart® Buffer</td>
<td>4.0 µL</td>
</tr>
<tr>
<td>MluCI (NEB R0538)</td>
<td>0.4 µL</td>
</tr>
<tr>
<td>PCR product</td>
<td>10 µL</td>
</tr>
<tr>
<td>H₂O</td>
<td>5.6 µL</td>
</tr>
</tbody>
</table>

The samples were incubated in 37 °C for at least one hour, in some cases the samples were left to incubate overnight. See Table 6 for components of the restriction reaction.

3.3.4 Gel electrophoresis

The samples that underwent restriction were loaded on a 2% agarose gel prepared as mentioned before (under “Amplification using PCR”). The DreamTaq DNA Polymerase Green buffer already contains a density reagent and two tracking dyes, therefore, the samples could be loaded directly on the gel. For reference a 1 kb ladder was used and the gel was in this step run at 100 mV.
3.3.5 Gen sequencing

To confirm the genotyping, some samples were also sent for gene sequencing. The DNA extraction was made by using the Invitrogen PureLink™ Quick Gel Extraction Kit and instructions from the kit’s manual were followed using the centrifuge method (see Attachments). Samples were then sent to GATC Biotech for sequencing. After the samples were sequenced they were analyzed using the UGENE software (see figure 5).
Figure 5 Shows the mutation (highlighted in red). The sequencing was done on a sample from fin clipping.

3.4 Fin Clipping

Samples for genotyping adult fish were obtained by fin clipping. In this method, the adult fish is anesthetized and approximately $\frac{1}{4}$ of the fin is clipped and used for genotyping. The procedure is viewed as an experiment and is approved by the Food Safety Authority in Norway (Mattilsynet).

3.4.1 Preparation

The fin clipping took place in the quarantine room. The bench where the fin clipping took place was washed with green soap and disinfected with 70% alcohol before any equipment or tanks were placed on the bench. Equipment needed for the procedure were:

- Sterile scalpel
- Plastic spoon
- Forceps
- Fish net
- Petri dishes
• Stopwatch

All equipment that were not sterile to begin with were disinfected with 70% ethanol included the plastic spoon, forceps, petri dishes and stopwatch. All, except the latter, were disinfected before a new fish was to be fin clipped.

The zebrafish, that was to be fin clipped, was anesthetized in a tricaine solution buffered with sodium bicarbonate.

A 4 g/l stock solution of tricaine was made by solving 200 mg of tricaine in 50 ml system water. A 8 g/l stock solution of sodium bicarbonate was made by solving 400 mg of sodium bicarbonate in 50 ml system water.

100 ml anesthetic solution was prepared by mixing 4.2 ml of tricaine stock solution and 4.2 ml of sodium bicarbonate in 91.6 ml system water.

In addition, a euthanasia solution was made in case euthanasia became necessary during the procedure. The euthanasia solution was made by the same stock solutions as the anesthetic solution. The amounts of the components were, 7.5 ml of tricaine stock solution and 7.5 ml of sodium bicarbonate in 85 ml system water.

3.4.2 The Procedure

An individual fish was picked up with the help of a net and transferred into the anesthetic solution. A stopwatch was started as the fish should not be anesthetized for more than 2 minutes. When the gill movement of the fish was slowed down and the fish did not respond to slight poking of the tail, it was scooped up with the help of a plastic spoon and laid on the petri dish. Not more than ¼ of the tail was cut of with a scalpel, it should not lead to bleeding, and the fish was transferred into fresh system water for recovery. The time for recovery was not supposed to take more than 5 minutes. The sample was picked up with forceps and put in PCR tubes for genotyping. The scalpel, spoon, dish and forceps were disinfected before repeating the procedure.

3.5 Whole mount imaging
Patients with the chd2 mutation have shown to be underdeveloped physically as well as slow brain development. To find a phenotype in the chd2 mutant the VAST Biolmager is used because of its ability to show the zebrafish from different angels. The main angle used was the lateral, but also dorsal view is analyzed for deciding whether hyperpigmentation could be a phenotype.

The zebrafish for inspection was anesthetized by mixing tricaine 4g/l and sodium bicarbonate 8g/l. The zebrafish larvae for inspection was anesthetized in a tricaine solution buffered with sodium bicarbonate.

A stock solution of tricaine was made by dissolving 200 mg of tricaine in 50 ml system water. A stock solution of sodium bicarbonate was made by dissolving 400 mg of sodium bicarbonate in 50 ml system water.

100 ml of anesthetic solution was made by mixing 4.2 ml of tricaine stock solution and 4.2 ml of sodium bicarbonate in 91.6 ml system water.

After imaging the larvae was flushed out to a PCR tube and saved for genotyping.

![Figure 6 5 dpf chd2 mutant](image)

### 3.6 The optokinetic response (OKR)

The optokinetic response (OKR) behavioral screen can be used as a possible strategy for identification of zebrafish with defects in the visual system. The zebrafish retina develops rapidly and the larvae can be analyzed for cone-mediated visual responses by 4 dpf (Easter & Nicola, 1996). The aim of this screen was simply to identify if the chd2 mutation could cause abnormal development of the retina.

#### 3.6.1 Preparing for OKR analysis
The procedure was done on 5 dpf larvae and later on 6 dpf larvae. Due to rapid retinal development the larvae’s OKR can already at this time be analyzed. In this study we opted to use rotating lights to observe the eye movement of the larvae. A band of LED lights was glued to the walls of a petridish and the frequency of the lights was coded to 0.17 Hz with a clockwise movement. The larvae was embedded in 2% agarose and placed on top of an object glass, the larvae was placed in the middle of the object glass. The agarose covering the larvae’s head was removed making it possible for the larvae to move the eyes and some drops of E3 was added to prevent the larvae from drying out. The object glass was placed in the petridish making sure the larvae was positioned in the middle of the dish.

### 3.6.2 Recording OKR

Recording of the eye movements was done under a Leica M205 FA microscope and the Leica microscope’s software was used for the recording. The lights were programmed at a frequency of 0.17 Hz and lights were programmed to have a clockwise direction. The petridish with the embedded larvae and lights was placed on the Leica M205 FA microscope and the lights in the room were turned off. The light sequences was started and the eye movements recorded. Each fish was recorded for 10 minutes.

### 3.6.3 Analyzing the OKR recording

The video of the recording was done in real time and to be able to see if there were any eye movements the video had to be slowed down. The video was analyzed by looking for eye movements that occurred in the direction of light movement, in the opposite direction or any other eye movement.

### 3.7 Tracking locomotor activity

Zebralab® (Viewpoint, Lyon, France) equipment and software were used for recording locomotor activity. The equipment consists of an automated observation and video tracking system and software that assesses the locomotor activity of zebrafish. This equipment was used to detect any spontaneous seizure-like behaviors and to investigate whether the chd2 mutant can be distinguished from AB wild type.
The analysis was conducted with 48-well tissue culture plates, meaning that one can track locomotor activity on 48 larvae at the same time. The 48-well culture plates were chosen over 96-well culture plates to give the larvae more space to move in. Each well contained 400 µl E3 medium and one larva. The 48-well plate was transferred into the ZebraBox® recording system (Viewpoint, Lyon, France). Before starting the recording, the larvae were given a 10 minutes habituation time to allow the larvae to acclimate in the dark before recording. The first time locomotor activity was recorded the experiment was conducted for 60 minutes. However, no significant difference between the chd2 mutants and the AB wildtype was observed. Therefore, this experiment was also conducted for 12 hours of continuous tracking to be able to detect any spontaneous convulsions. The larvae were tracked at 4 dpf after the swim bladder was inflated. The parameters for long distance were set at >8 mm/sec, meaning that when the larvae swim over 8 mm/s it is categorized as long distance. The parameters for short distance were set to <3 mm/s and each time bin (for long- and short distance) was set to 30 seconds. The experiment was conducted with 24 larvae and genotyped.

3.8 EEG recording

Clinically, epilepsy is characterized by events with abnormal electric activity in the brain that results in seizures. The most prominent seizure types in LGS are tonic and atypical absence seizures, with patients showing a slow spike-and-wave pattern (≤2.5 Hz). However, for a patient to be diagnosed with LGS, tonic seizures during sleep must be present (Camfield, 2011). The excessive electrical activity can be measured with using local field potential recordings (also referred to as Electroencephalogram (EEG)) and is an important tool in the identification of epileptic seizures and diagnosis of epilepsy.

3.8.1 Preparing for EEG recording

In this study, EEG recordings were done on zebrafish larvae by embedding the larvae in 2% low-melting-point agarose (Invitrogen) and thus immobilizing it. The excess agarose surrounding the head surface was removed, making the optic tectum visible and accessible for placement of the electrode. A glass electrode connected to a high-impedance amplifier, filled with artificial cerebrospinal fluid (124 mM NaCl, 2 mM KCl, 2 mM MgSO₄, 2 mM CaCl₂, 1.25 mM KH₂PO₄, 26 mM NaHCO₃ and 10 mM glucose) was then inserted into the optic tectum.
3.8.2 Recording EEG

Recordings were performed in current clamp mode, low-pass filter below 2 kHz, high-pass filter above 0.5 Hz, digital gain 10 and a sampling frequency of 2 kHz (MultiClamp 700B amplifier and Digidata 1550B plus HumSilencer, both from Axon Instruments). Single recordings of brain activity were recorded with an interval of 4 minutes without flashing lights and 4 minutes with flashing lights. The brain activity was recorded for a total of 20 minutes.

Figure 7 The setup of the equipment used for recording LFP. The left part of the figure shows how the electrode was positioned.

3.8.3 Analyzing the recordings

Spontaneous events were considered when the amplitude of the spikes exceeded the background noise by three-fold, and the spikes were analyzed with pClamp10 software (Molecular Devices) and coded commands on Rstudio. EEG was performed on larvae between 4 and 5 dpf as seizures can be elicited after 3 dpf, but neuronal discharges become more prominent as the brain develops and matures.

3.9 Brain ventricle imaging

As mentioned before, the chd2 mutation is also associated with autism. Abnormal brain ventricles formation in zebrafish has been proposed as a surrogate phenotype for autism. To further investigate this the ventricles in the zebrafish larvae were injected with a fluorescent dye. In this study Rhodamine B Dextrane 10 000 MW was used. Rhodamine B Dextrane
(RBD) has an excitation/emission of 570/590, respectively. This makes RDB suitable to detect with any fluorescence microscope via the mCherry filter.

The solution to be injected was made by dissolving 2 mg of RBD in 200 µL E3 buffer. To ensure that RBD would solve properly, the solution was vortexed and heated to 45 °C in a hot water bath in 10 minutes and vortexed again. When the solution was clear it was stored at 4 °C.

3.9.1 Preparing for microinjection

Needles were pulled using the Sutter Instruments capillary puller and, the needles were filled with the refrigerated RDB solution. The needle was cut to an appropriate size with an angle to create a beveled tip and to make the puncture of the thin roof plate of the hindbrain easier. The pressure used for injecting could vary depending on how the tip of the needle was cut off but, the drop size used was always between 1.75-2 nl. This was inspected by injecting in a drop of mineral oil and measuring the diameter of the drop.

3.9.2 Preparing the embryos

The ventricles were studied at 24 hpf and the injection took place 30 – 60 minutes before visualization. At 24 hpf, the larvae are still surrounded by the chorion. The chorion was removed carefully by using two forceps. The larvae were then transferred to a plastic dish coated with 1% agarose. The agarose was made using low melting agarose solved in E3 buffer. For the larvae to be positioned correctly there were made wells in the agarose with the tip of a 1 – 200 µl pipette (see figure 8). To avoid movement during the experiment, the larvae were anesthetized using a tricaine solution prepared as mentioned under “Whole Mount Imaging”. The larvae were positioned with their tail in the well and the injection apparatus on the posterior side of the embryo.
3.9.3 Injecting the brain ventricle

The injection was done manually and the needle was carefully put through the thing roof plate of the hindbrain without hitting the brain tissue below. The amount injected was just enough to fill the ventricles and the needle was carefully retracted. In this study only one injection was enough to fill the ventricles.

3.9.4 Imaging

A new plastic plate was covered with 1% agarose (as mentioned under “Preparing for microinjection) and wells were made using a 1 – 200 µl pipette tip. The larvae were anesthetized before transferred to the plate covered with 1% agarose. The larvae were positioned with their tails in the well so they were positioned for a dorsal image. The microscope used for imaging was a Leica M205 FA. The image was taken under transmitted light and with mCherry fluorescent light. The images were processed using Adobe® Photoshop® CS6 where the picture with transmitted light was overlapped with the picture taken with fluorescent light. All settings for every picture was the same.

3.10 Statistical analysis

Data from EEG recording were analyzed using RStudio (R Core Team, Auckland, New Zealand), see attachment 2. The command filters the data from the recording using the
background noise as a standard line. The data is sorted according to when the seizures take place during the recording and their duration. However, the seizures proposed by the command were still manually checked and any activity that was not classified as seizure was excluded. The data was organized and displayed as means ± SEM (Standard error of the means).

The statistical analysis of the EEG recordings were performed using Graphpad Prism (GraphPad Software, Inc., California, USA) and RStudio (R Core Team, Auckland, New Zealand). The data obtained from the EEG recording was analyzed using a paired t-test but, ANOVA was mainly used to determine if the means of two (or more) groups are equal. The ANOVA is more useful than a two-sample t-test since it has a less chance of committing a type I error. The more means used, the higher odds for the t-test of committing an error, which is why ANOVA is used when comparing two or more means. Therefore, the statistical analysis of data obtained from recording brain activity with EEG was analyzed with the use of two-way ANOVA.

Analyzing the data from EEG recording after VPA treatment the ANOVA was used again to compare the different groups (VPA, PTZ and VHC). In addition the different groups were compared to each over setting up VPA versus VHC and PTZ versus VHC.

Statistical analysis was not undertaken for the brain ventricles injection experiments and after wholemount imaging as the differences were clearly observable.
4 Results

4.1 Phenotypic abnormalities displayed by \textit{chd2} mutants

The differences observed between mutant and wildtype larvae were most evident between 4 and 5 dpf. The mutants showed signs of developmental delay compared to their wild type siblings. For example, the lack of a swim bladder in homozygote \textit{chd2} mutants was evident.

By 3 dpf the pectoral fin has normally expanded over the length of the yolk and continues to extend posteriorly. Morphogenesis of most of the larval organs is almost completed and begins to slow down with the exception of the swim bladder, gut and its associated organs.

Before 3 dpf the mouth is open and is located in a midventral position between the eyes. During the last 12 hours of embryogenesis the mouth is dramatically repositioned and jaw morphogenesis moves the mouths position beyond the eye, where it still gapes open. After the hatching period the jaw and mouth morphogenesis will continue rapidly and the jaw should eventually reach the mouth and close it by 5 dpf.

\textit{chd2} mutants have delayed morphogenesis of the pectoral fin as well as delays in craniofacial development. The jaw in the mutants have not reached beyond the mouth so the mouth is still open. By day 4 the swim bladder should be developed, but this is not the case with the \textit{chd2} mutants (see figure 8).

\textit{Figure 9} 5 dpf wild type (A) and \textit{chd2} mutant (B). Arrows show which parts in the mutant are delayed compared to wild type. Scale bar shows 1 millimeter.

In addition to the delays in development pointed out above, the \textit{chd2} mutants displayed a slower touch response than the wild type. The touch response was tested by slightly touching
the larvae on the tail with a 1 – 10 µl pipette tip. For the wild type larvae it was adequate with only one touch for the larvae to swim away, whereas, the chd2 mutant did not respond upon the first touch and only responded after 2nd or 3rd touch. This was tested both before and after the swim bladder was fully developed in both mutants and wild type.

4.2 Absence of optokinetic response

In total, the number of larvae that were recorded for OKR was n = 17 larvae. The first round of recording was performed in a dark room and recorded for 10 minutes with a frequency of 0.17 Hz of flashing lights moving clockwise. During the first round of recordings there was some movement of the eyes but there was no movement that correlated to the direction of light movement. These were therefore categorized as spontaneous eye movements. The recording was also conducted with lights on in the recording room to investigate whether the contrast of the dark room and the lights from the petridish was too strong. The procedure was also repeated on 6 dpf larvae.

For the second round (this time with only 6 dpf larvae) the frequency of the light sequence was lowered to 0.083 Hz to determine whether the frequency was too fast. This time the procedure was also done in a dark room and then with lights on. However, there was also no detectable correlation between the eye movements of the eye and the moving lights.

Larval eyesight was tested by lowering a 1-10 µl pipette tip next to the larval eyes and that resulted in the movement of the larvae away from the tip. However, this is not a clear indication for the larvae being blind, the larvae could have responded to vibrations.

4.3 Difference in locomotor activity during light and dark cycles

Tracking the locomotor activity in the chd2 mutants showed that some of the larvae were almost equally active in the light cycle as in the dark cycle. Figure 10 shows locomotor activity in chd2 mutants and figure 11 shows locomotor activity in wildtype. The tracking was done over a time period of 12 hours to spot any spontaneous seizures. If the larvae have almost the same amount of activity in the dark as in the light cycle, it could be an indication of spontaneous seizures.
For this statistical analysis, the two-way ANOVA test was used. The two-way ANOVA test uses variances to show whether the means are equal or not.

*chd2* mutants traveled a higher distance during lights on compared to the distance traveled when the lights were off (see figure 10).

**Figure 10** Locomotor activity of *chd2* mutants in light and dark cycles. The significant difference between the activity is **p<0.01 (p = 0.0014).**

The distance traveled by the wild type during the light cycle is also significantly higher than the distance traveled in the dark cycle (see figure 11). The significant difference is higher in the wild type compared to the difference in *chd2* mutants. The larvae that had similar locomotor activity during light and dark cycles were taken out and genotyped (1/4 of the samples were taken out). The results from genotyping showed that the larvae with high activity during dark cycles were heterozygous for the mutation.
Figure 11 Locomotor activity of chd2 mutants in light and dark cycles. The significant difference between the activity is ****(p<0.0001).

The data was categorized together to confirm the difference between the locomotor activity in chd2 mutants and wild type (see figure 12). There was a significant difference in distance traveled by chd2 compared to wild type. chd2 mutants did travel a significantly larger distance compared to the wild type both in light cycles and dark cycles. chd2 mutants travel the largest distance during the light cycle. However, the distance traveled in the dark cycle by the chd2 mutant is also larger than the distance traveled by the wild type during the light cycle.
Figure 12 Comparison of the distance traveled in the light and dark cycles. chd2 mutants traveled a significantly larger distance compared to the wild type in both light and dark cycles. The two-way ANOVA test was used to compare the data. ****(p<0.0001).

4.4 chd2 mutants display spontaneous seizures

The chd2 mutants had a higher number of seizures than their wild type siblings (see figure 11). In general, the probability to observe a seizure increases the longer the period of recording (20 minutes or longer). Most larvae displayed seizures over 1200 seconds, likely a result from the trauma experienced due to capillary electrode placement during the recording. The number of seizures per time period is depicted as the mean value of the whole group. The collected data was categorized according to the time the seizure occurred and the results show that the larvae have the highest number of seizures between 720 seconds and 960 seconds, during an interval with flashing light. The number of seizures in the time period between 960 seconds and 1200 seconds is smaller than the latter period with flashing light.

The error bars on the graph are tall and this needs to be taken into consideration. During the experiments the electrode was made sure to be positioned in the optic tectum. However, the
trauma from the way the electrode is positioned can vary. The set-up is programmed so the electrode is positioned at approximately the same depth, however, when puncturing the thin roof plate of the hindbrain. The amount of trauma caused when positioning the electrode can lead to high error bars. However, a two-way ANOVA test showed that the difference in number of seizures between the chd2 mutants and their sibling wild type was significant. The chd2 mutants had a significantly lower number of seizures, with a p-value of 0.0198.

**EEG RECORDING**

![Barplot comparing the number of seizures in chd2 mutants and their wild type siblings. * (p < 0.05).](image)

Figure 13 depicts the number of seizures for each larvae recorded. The data is organized according to lapsed recording time. The highest average number of seizures recorded from larvae is approximately 6 seizures and these occur during the 720 – 960 seconds light period. The mutant larvae showed both low- and high frequency seizures. The wild type sibling used under the LFP recordings also displayed seizures. At the start of the recording, 0 – 240 no light period, most activity was caused by muscle movement in the larvae or by the door being shut after starting recording. Spontaneous seizures at the very end of the recording were
excluded due to the high probability to record seizures from the head trauma induced by the recording electrode in the brain.

Figure 14, 15 and 16 show the different types of seizures observed under the recordings. The seizures are shown in seconds and the amplitude of the spikes is given as mV. In figure 11 the background noise is seen from the beginning in the figure up to 383.8 s. From this point, the seizure starts and lasts until the abnormal brain activity has settled and the background noise is the only activity visible again.

The chd2 mutants displayed low frequency seizures and high frequency seizures. Under the low frequency seizures no eye movement was observed. When comparing the brain activity patterns of those of EEGs from patients, the seizures are reminiscent of absense seizures or a weaker tonic-clonic type of seizure. These seizures usually have a higher frequency at the end of the seizure than in the beginning and the spikes have higher amplitudes at the end than in the beginning (see figure 14 and 15). This is typical for the tonic-clonic kind of seizures where, the tonic part of the seizure is lower in frequency and the spikes have lower amplitude than of those in the clonic part of the seizure (see figure 15).

![Figure 14](image)

*Figure 14 The x-axis shows time in seconds and the y-axis shows the amplitude in millivolt.*
The pattern resembles a tonic-clonic seizure in patients, this could possibly be a tonic-clonic seizure, where the tonic part of the seizure occurs between 924.2 and 926.4 second. The clonic part of the seizure follows the tonic part from 926.4 seconds until 926.9 seconds before the activity goes back to normal. The x-axis shows time in seconds and the y-axis shows the amplitude in millivolts.

Another type of seizure was observed in the chd2 mutants, and this type of seizure look as an atonic type of seizure compared to EEGs from patients, where the spikes are very frequent and of high amplitude (see figure 16). These kinds of seizures were only observed in the chd2 mutants. The longest duration of this kind of seizure was observed as greater than 3 seconds and was observed in the 720 – 960 flashing light period.

The x-axis shows time in seconds and the y-axis shows the amplitude in millivolts.
4.4.1 Valproate reduces seizure frequency in chd2 mutants

VPA is very often used in epilepsies where seizures are difficult to treat and in many cases VPA is also used in combination with other AEDs to manage seizures. The chd2 larvae treated with valproate 2 hours prior to EEG recording still had seizures. However, the total number of seizures was smaller than in the untreated group. For the positive control, PTZ was used at the threshold concentration of 20 mM. The negative control used in the study was embryo medium with 1% DMSO (vehicle) where seizures were also observed with the exception of one negative controle samples, where no seizures were absent. After genotyping, this sample was confirmed to be a wild type sibling. Figure 17 shows the number of seizures in VPA treated chd2 mutants compared to vehicle only, chd2 mutants and PTZ treated wildtypes. The ANOVA one-way statistical test was used to determine if there was an overall difference. The test was also used to compare the VPA group against the VHC group and the PTZ group against the VHC group. The difference between the VPA treated group and the vehicle group was significant. The VPA treated group had a significantly lower number of seizures compared to the untreated group and the test gave a p value of 0.0062.
The graph compares VPA treated, PTZ treated and vehicle chd2 larvae. * (p < 0.05), ** (p < 0.01). Last bar includes data from 960-1200 s, second last bar includes data from 720-959 s and so on.

4.5 chd2 mutants display brain ventricle defects

The ventricles in the chd2 mutant were abnormal or underdeveloped. Figure 18 shows a 24 hpf AB wild type with injected ventricles. The hindbrain had started to expand and the three ventricles are clearly visible and are clearly distinguishable from each other. After 36 hpf, the ventricles would have been completely expanded, but the differences were already clearly evident by 24 hpf. In chd2 mutants the brain ventricles had not expanded and the different brain ventricles were more uniform in shape than in the wild type. In particular, the forebrain- and midbrain ventricles were not expanded and the hindbrain ventricle was not as expanded as in the wild type (see figure 19, 20 and 21). The chd2 mutants were also physically abnormal and looked underdeveloped compared to the wild type embryo.
In the *chd2* mutants, the brain ventricles were abnormal in a way that the brain ventricles had not expanded to the same extent as in the wild type and the different ventricles also not clearly distinguishable (see figure 20 and 21). There were clear malformations in the separation of the hindbrain. The midbrain ventricle and the forebrain ventricle were not fully developed and the ventricles lacked the classical morphology normally displayed by wild type.
Figure 19 Injected ventricles in 24 hpf chd2 mutant, the arrows denote the malformations. The scale bar shows 100 micrometers.

Figure 20 Injected ventricles in 24 hpf chd2 mutant, the arrows denote the malformations. The scale bar shows 100 micrometers.
Figure 21 Injected ventricles in 24 hpf chd2 mutant, the arrows denote the malformations. The scale bar shows 100 micrometers.
5 Discussion

Although marketed AEDs are currently used to treat LGS, these AEDs are not sufficiently effective and many of these patients develop pharmacoresistant seizures. In the last decade, new AEDs have been introduced into the market and with more are being developed or are awaiting regulatory approval (French and Gazzola, 2011). Most of the new pharmaceuticals work through similar mechanisms and have not shown much improvement to their predecessors (Löscher et al., 2013, Löscher, 2011). There reason for this could be the main screening models used for AED discovery. The main screening models that have been used are electroshock seizure (MES) and acute pentylenetetrazole (PTZ) assays. These models have enabled the development of drugs that treat acute symptom (i.e. seizures), but do not actually prevent disease progression (in this case epileptogenesis). In these cases, AEDs with novel mechanisms of action are needed so that epilepsies are not only treated symptomatically but also preventively. In this study, a chd2 model was generated and ordered from ZIRC. The reason for using this kind of mutant instead of knocking down the gene with antisense morpholinos oligomers is that the knockdown effect is transient (morpholinos are injected into the 1-4-cell stage and diluted out over time as cell division continues and new RNA is transcribed) and highly prone to off-target effects. Validating a zebrafish as a novel model for a disease can provide a more efficient way for drug screening can be carried out in a complete biological system, a whole organism rather than simple cell monolayers or protein-proteins.

The results from the LFP recordings show that the chd2 mutants display more seizures than the wild type. Notably however, the wild type siblings also display seizures and in theory, this should not happen. The reason for the seizures is most probably due to cellular damage based on the way the electrode was positioned. Even when the electrode is positioned correctly, the larvae could potentially suffer tissue trauma. Seizures caused by head trauma will happen if the recording period is prolonged, typically past 20 minutes (empirical data). Therefore, each recording was only carried out for maximally 1200 seconds (20 minutes). However, if the electrode is not placed carefully, the larvae could undergo seizures earlier. When analyzing the data any abnormal activity that didn’t classify as a seizure was ruled out, also seizures occurring at the very end of the 20 minutes were ruled out. When treating the larvae with valproate 2 hours prior to recording, the number of seizures decreased.
The statistical analysis of the EEG data shows that in overall, there is a significantly higher number of seizures in the chd2 mutants. However, when comparing each cycle there is not a significant difference observed for the total numbers of seizures in every cycle. In the first cycle, with no lights, there is no significant difference between the wild type and the chd2 mutant. All data was analyzed before the samples were genotyped so there was no knowledge of which samples could were wild type or mutants until after the embryos were genotyped. The categorization of seizure types was made purely by visually comparing EEGs. In the clinic, physical reactions of the patient are also taken into account through video recording during the seizures.

The barplots showing the analyzed LFP data shows large error bars, this can be explained with trauma to the brain when positioning the electrode. The electrode is typically positioned in the optic tectum because this area of the zebrafish brain is easy to identify, relatively large and easy to access. Recordings from the forebrain have also been reported. However, even if the electrode is positioned in the same place every time, the puncture of the skin and the process of positioning the electrode in the optic tectum can potentially cause brain trauma. To minimize trauma, the skin was first slightly scratched to gently produce a small puncture of and the electrode positioned as carefully and smoothly as possible in the right position. We observe a direct correlation to the error bars since the threshold for seizing was lower when the trauma to the brain was larger.

The chd2 mutant was shorter in body length than their wildtype siblings and this correlates with the shorter stature reported for LGS patients. The shortness in body length could be linked to the slow development of the larvae compared to the wildtype that developed at a normal rate. However, shorter stature was observed in subsequent generations of chd2 fish, where approximately one fourth were smaller than the wildtype. Finclipped and genotyping of adult fish confirmed that homozygous chd2 mutants were smaller than their heterozygous and wildtype siblings. The adult homozygous chd2 fish were also more prone to tumors and scoliosis. Some LGS patients display craniofacial malformations and in the chd2 zebrafish model there was craniofacial underdevelopment in the larvae. The jaw was not fully developed leading to an open mouth in the chd2 mutants even at 5 dpf (mouth closure normally occurs by 4 dpf). The underdevelopment/developmental delay in the chd2 mutants also affected the inflation of the swim bladder at 5 dpf wherein the chd2 mutants had not
inflated their swim bladders. However, the swim bladder eventually started inflating at 6 dpf, 3 days later than normal.

The function of the CHD2 protein in humans is not fully understood yet, however, other proteins in the CHD family (e.g CHD5 and CHD7) are known to play an important role in regulating gene transcription and are important for development. Thus, altered CHD2 protein function could also result in developmental defects.

As previously mentioned before, mutation in the chd2 gene is associated with autism. Abnormal brain ventricles formation in zebrafish has been proposed as a surrogate phenotype for autism (McCammon, Blaker-Lee, Chen, & Sive, 2017). The distinguished segment shape of the ventricles in the zebrafish brain is fully formed by 36 hpf, however, one can easily observe the morphology of the brain ventricles as early as 24 hpf. Brain ventricles development begins with the formation of the neural tube followed by segmentation into the forebrain, midbrain and hindbrain. In the middle of the neural tube, a constriction forms that marks the mid-hindbrain barrier (mhb). Posterior and anterior to the mhb, the neural tube expands forming the hindbrain ventricle and the midbrain ventricle. More anterior to this region, another constriction of the neural tube forms the barrier between the midbrain ventricle and the forebrain ventricle. The brain ventricle abnormalities observed in the chd2 mutants appear as if the development of the ventricles was not completed - implicating Chd2 protein function as important for this process during zebrafish brain development.

Autism has also been associated with children whose mothers used VPA during pregnancy. However, whether early VPA treatment would have led to abnormal brain ventricle formation in wild type zebrafish embryos was not studied. Women that wish to conceive are recommended to use folate supplements before conceiving as folate plays an important role in the process of closing the neural tube. The neural tube in humans closes on the 28th day after conception and is has been hypothesized that the embryo at this stage is especially vulnerable to folate deficiency. The vulnerability could be due to differences of the functional enzymes in this pathway during embryogenesis combined with high demand for post translational methylations of the cytoskeleton in neural cells during the process of neural tube closure (Bjorklund & Gordon, 2006). Whether folate could rescue this phenotype was not investigated in this study.
A different part of the zebrafish that undergoes rapid development during larval growth are the retinae. The retinae developed rapidly and within 4 dpf one can investigate the optokinetic response in the larvae. Since the development of other parts of the larvae was delayed, e.g the swim bladder, the OKR was analyzed in 5 and 6 dpf larvae. The movement of the eyes appeared to be spontaneous movements as there was no correlation with the direction or the frequency of eye movements and light movements. The chd2 mutant larvae were not blind as confirmed by the larval escape response to placing a pipette tip close to the eye. In retrospect, the OKR experiment could have been performed differently by using a different setup. One has to take into account that the larvae are not bigger than 5 mm and the lights used in this experiment were too large to be used for this purpose. The high light intensity and speed at which the lights were passing the larvae could have led to an environment of constant brightness where alternating light and dark stimuli were simply not distinct enough. This could have easily been solved by using a black and white striped paper strip instead of a ban of lights. This strip could have been moved by a motor to allow the larvae to follow the alternating black and white pattern instead of light.

The tracking of locomotor activity showed that about $\frac{1}{4}$ of the samples had equal activity in the light as in the dark cycle. However, after genotyping, the samples turned out to be heterozygous mutants. At the time the genotyping protocol was applied, heterozygotes could not be distinguished from homozygotes. The protocol for genotyping was later optimized and new primers were designed to detect also homozygote mutants. However, the samples from the tracking were discarded and could not be analyzed further with new primers. Therefore, the tracking experiments should be repeated in order to determine if differences in behavior may be observable between homozygotes and heterozygotes.
6 Conclusion and future perspectives

*chd2* mutant zebrafish larvae showed spontaneous seizures. There was also no significant
difference in the total number of seizures observed in *chd2* mutants when compared to those
recorded from PTZ treated wild type larvae, implicating a high seizure frequency within the
20 minute recording period. The *chd2* mutants were also responsive to VPA with significant
reduction in total seizure number. The LFP recordings obtained from the *chd2* mutants also
revealed different seizure types, which has also been observed in LGS patients and is a
diagnosis criterion for this syndrome. The light stimulus period lowered the seizure threshold
in the *chd2* mutant indicating potential photosensitivity. Overall the zebrafish *chd2* mutant
phenocopied the LGS patient characteristics of short stature, craniofacial abnormalities,
seizures and slow movements.

The brain ventricles in the *chd2* mutant were proven to be abnormal. The forebrain-,
midbrain- and, hindbrain ventricle was usually not completely inflated as seen in the wild
type and the mid-hindbrain barrier did not constrict completely. The abnormal brain ventricles
are a surrogate phenotype for autism and this correlated well with the *chd2* mutation being
associated with autism. However, the *chd2* mutant’s behavior needs to be further investigated
for autism before the mutant is validated as a model for autism.

The conclusion for this study is that the *chd2* mutant analyzed in this study is a validated
model for Lennox-Gastaut Syndrome and photosensitive epilepsy. The model can for future
projects be used as a model for drug screening and identifying possible hits for the
development of new AEDs and for investigating mechanisms underlying autism.

In future experiments the behavior of the *chd2* mutants will be investigated, specifically
shoaling. Zebrafish larvae establish social behavior early and the first signs of shoaling appear
at 6 dpf. Therefore, disturbances in zebrafish social behavior can be easily detected at early
stages and potentially reinforced the use of the *chd2* mutant as a novel model for autism
further.

By using databases (i.e. Motherrisk or Cochrane) we can find any compound associated with
the syndrome and these can be used in toxicology studies with the *chd2* model to further
investigate whether there are any substances that reinforce the autism phenotype.
Furthermore, compounds (i.e. antipsychotics, antidepressants) can be used to investigate whether the phenotype can be rescued.

A validated novel model for LGS, photosensitive epilepsy and autism can in the future be used for high throughout drug screening. This will give the possibility to have a model with a clear phenotype for testing novel candidates for treatment of the disorders. The model will also aid further research on the underlying mechanisms for the disorders and also help in understanding the function of the CHD2 protein.
References


Markers of Autism Study: A Population-Based Case-Control Study in California. *Environmental health perspectives*, 125(8), 087023. doi:10.1289/EHP1079


Attachments
Purifying DNA Using a Centrifuge, Continued

**Binding, Washing and Eluting DNA**

**Before beginning**, add ethanol to the Wash Buffer (W1, see page 7).

1. Pipet the dissolved gel piece containing the DNA fragment of interest (steps 4–5, page 6) onto the center of a Quick Gel Extraction Column inside a Wash Tube. **Note**: Do not load >400 mg dissolved agarose per Quick Gel Extraction Column.

2. Centrifuge at >12,000 × g for 1 minute. Discard the flow-through and replace the Quick Gel Extraction Column into the Wash Tube.

3. Add 500 μL Wash Buffer (W1), containing ethanol (page 7) to the Quick Gel Extraction Column.

4. Centrifuge at >12,000 × g for 1 minute. Discard the flow-through and replace the column into the Wash Tube.

5. Centrifuge the column again at maximum speed for 1–2 minutes to remove any residual Wash Buffer and ethanol. Discard the Wash Tube and place the Quick Gel Extraction Column into a Recovery Tube.

6. Add 50 μL Elution Buffer (E5) to the center of the Quick Gel Extraction Column.

7. Incubate the column for 1 minute at room temperature.

8. Centrifuge the Column at >12,000 × g for 1 minute. *The Recovery Tube contains the purified DNA.* Discard the Quick Gel Extraction Column.

9. Store the purified DNA (see Storing the Purified DNA), or proceed to your downstream application of choice.

**Storing the Purified DNA**

Store the purified DNA at 4°C for immediate use or aliquot the DNA and store at −20°C for long-term storage. Avoid repeated freezing and thawing of the DNA.
install.packages("signal")
install.packages("abf2")

library(signal)
library(abf2)
rm(list = ls())
direct = "M:/pc/Desktop/090118_chd2/"
nome = "090118_chd2_0000.abf"
ts = 1
dur = 1200

ptm <- proc.time()
track = paste(direct, nome, sep = "")
eeg = abfload(filename = track)
Fs = 1 / eeg$s[2]
a = 100 * 2 / Fs
b = 500 * 2 / Fs
c = 15 * 2 / Fs
bp = fir1(512, c(a, b), "pass")
lp = fir1(128, (c), "low")
#plot(ts(lp))
x = eeg$traces[(ts * Fs):((ts + dur) * Fs)]
z <- filter(bp,x)
#plot(ts(x, start = ts, end = ts+dur, frequency = Fs), ylab="")
#plot(ts(z, start = ts, end = ts+dur, frequency = Fs))
seiz = array(0, length(z))
seiz[which(abs(z) > 7 * mean(abs(z)))] = 1
z2 <- filter(lp, seiz)
#plot(ts(z2, start = ts, end = ts+dur, frequency = Fs))
seiz2 = array(0, length(z2))
seiz2[which(z2 > 20 * mean(z2))] = 1
#plot(ts(seiz2, start = ts, end = ts+dur, frequency = Fs))

z3 = conv(seiz2, rep(10 / Fs, Fs / 10))
seiz3 = array(0, length(z3))
seiz3[which(z3 > 0.4)] = 1
seiz4 = seiz3[1:length(seiz2)]

#find seizures
dati = array(NA, 2)
i = 1
while (i < length(seiz4)) {
  point = 0
  j = 1
  if (seiz4[i] == 1) {
    j = i + 1
    while (seiz4[j] == 1 & j < length(seiz4)) {
      point = point + 1
      j = j + 1
    }
  }
  i = i + 1
}
if (point < 0.100 * Fs) {
    seiz4[i:j] = 0
} else {
    print(c(i / Fs + 1, point / Fs))
    dati = rbind(dati, c(i / Fs + 1, point / Fs))
}
i = i + point + 1

dati2 = na.omit(dati)

# unite seizures that are less than 1 sec apart

dati3 = dati2
i = 1
val = 0

while (i < dim(dati3)[1]) {
    if (dati2[i + 1, 1] - dati2[i, 1] < 1) {
        val = dati2[i, 2]
        j = i
        
        while (dati2[i + 1, 1] - dati2[i, 1] < 1) {
            val = val + dati2[i + 1, 2]
            i = i + 1
            if (i == dim(dati3)[1])
                break
        }
        dati3[i, 2] = val
        dati3[j:(i - 1), 2] = NA
    }
    i = i + 1
}

dati3[, 1] = dati3[, 1] - dati3[, 2]
dati3 = na.omit(dati3)
dati3
proc.time() - ptm

View(dati3)