UNIVERSITÄTSKLINIKUM HAMBURG-EPPENDORF

Zentrum für Geburtshilfe, Kinder- und Jugendmedizin

Klinik und Poliklinik für Kinder- und Jugendmedizin

Arbeitsgruppe Experimentelle Neuropädiatrie

Prof. Dr. Dirk Isbrandt

Characterization of hippocampal network activity and pharmacological pilot study in a *Scn2a* epilepsy mouse model

Dissertation

zur Erlangung des Grades eines Doktors der Medizin an der Medizinischen Fakultät der Universität Hamburg.

vorgelegt von:

Sarah-Christina Schöb

aus München

Hamburg 2018

Angenommen von der
Medizinischen Fakultät der Universität Hamburg am:
01.03.2019

Veröffentlicht mit Genehmigung der
Medizinischen Fakultät der Universität Hamburg

Prüfungsausschuss, der/die Vorsitzende:

Prof. Dr. med. Dirk Isbrandt

Prüfungsausschuss, zweite/r Gutachter/in:

PD Dr. med. Axel Neu

Table of content

1 Introduction

1.1 Epilepsy

1.1.1 Definition of Epilepsy

Epilepsy is one of the most common neurological diseases worldwide (e.g. Fisher et al., 2014). It affects people of all ages, both sexes and all ethnical backgrounds. Epilepsy and seizures have been described and recorded throughout the entire medical history. Babylonian texts from about 1000 before Christ (BC) already mention seizure like states in humans (WHO, 2005). In earliest times, seizures were thought to be of divine or demonic origin and were treated religiously or spiritually. It was Hippocrates who was the first to state: “The brain is the seat of this disease, as it is of other very violent diseases.” (cited after WHO, 2005) Nevertheless, the view of supernatural causes prevailed until the 18th and 19th century when Hippocrates’ view of a brain disorder became more and more popular. Michael Faraday’s studies of electrical discharges and the introduction of Robert Bentley Todd’s theory of seizures being a result of electric discharges in the brain changed the perception of epilepsy in the medical world. The first human electroencephalogram (EEG) that allowed scientists to measure such electric discharges in the brain was performed by the German psychiatrist and physiologist Hans Berger in 1924. In 1935 Lennox then presented the electroencephalographic proof of seizures (Haas, 2003; WHO 2005). With the growing knowledge and continuing research regarding the origin of epileptic seizures, research spread out to treatment possibilities, and the first antiepileptic drugs (AEDs) were developed. Bromide was the first known AED discovered in 1856, followed by phenobarbital in 1912 and phenytoin discovered in 1938. Phenobarbital and phenytoin are still in broad use today.

The development and further research on AEDs is essential. Today, about 50 million people worldwide suffer from epilepsy. The lifetime incidence is up to 3% (WHO, 2012). The highest incidence of epileptic seizures is during the first year of life (Freitag et al., 2001), and 40% of all epileptic syndromes manifest during childhood and adolescence (Neubauer & Hahn, 2014).

The International League Against Epilepsy (ILAE) and the International Bureau of Epilepsy (IBE) are two prominent organizations representing medical and nonmedical

aspects of epilepsy and providing information about diagnostics, treatments, research or prevention for clinicians and patients worldwide. In 2005, the ILAE and the IBE proposed a definition of epilepsy, differentiating between epilepsy and seizures. Seizures are described as “a transient occurrence of signs and/or symptoms due to abnormal excessive or synchronous neuronal activity in the brain” and epilepsy was defined as “a disorder of the brain characterized by an enduring predisposition to generate epileptic seizures and by the neurobiologic [sic], cognitive, psychological, and social consequences of this condition (Trinka et al., 2015). The definition of epilepsy requires the occurrence of at least two unprovoked epileptic seizures, one unprovoked seizure with a high risk of recurrence or the diagnosis of an epilepsy syndrome (Fisher et al., 2014). Seizures can be described depending on their localization in the brain, their etiology or their ictal electroencephalogram (EEG) presentation (Berg et al., 2010).

Depending on the localization of the seizure onset in the brain, seizures are described as either focal or generalized. Focal seizures affect one region of the brain and may have specific clinical signs depending on the affected brain region. During simple focal seizures, the patient is fully aware of the situation while consciousness is impaired during complex focal seizures. When seizure activity spreads to multiple other brain regions focal seizures can generalize secondarily (Fisher et al., 2014). Generalized seizures appear to affect all parts of the brain. These seizures present themselves in divergent ways: a) as atonic seizures, i.e., a sudden loss of muscle tonus at full consciousness, b) as tonic seizures, i.e., symmetric or asymmetric constant contractions of muscles, c) as myoclonic seizures, consisting of single or multiple rapid repetitive jerks, d) as clonic seizures which show rhythmic alternations between contraction and relaxation of muscles, e) as tonic-clonic seizures, which are also called Grand Mal, f) as absence seizures, which are also called Petit Mal, i.e. sudden and brief periods of “staring into space”, or g) as epileptic spasms, defined as sudden bilateral tonic contractions which appear mostly in young children and infants (Duncan 2003; Fisher et al., 2014).

The underlying causes of epileptic seizures are categorized in three etiological groups: genetic causes, structural/metabolic causes and a group consisting of unknown causes. Genetic causes of epileptic seizures are mostly channelopathies, i.e., mutations which lead to changes in ion flux and excitability (Weber & Lerche, 2008). Structural and metabolic epilepsies comprise diseases or brain dysfunctions

which lead to seizures, such as cortical malformations, hypoxic-ischemic encephalopathy, stroke, hemorrhage, infection, tumor or trauma, as well as metabolic disorders (Berg et al., 2010). The third etiological group describes all epileptic phenotypes of unknown cause (idiopathic seizures). They may be due to genetic defects or other disorders that are still unidentified today (Berg et al., 2010).

Seizures may also be categorized as isolated electrographic or electroclinical seizures based on their ictal EEG activity. The clinical diagnosis of seizures may be very difficult, as clinical symptoms accompanying electrical ictal EEG patterns can be very subtle or even absent. Only one third of epileptic EEG patterns manifest clinically (electroclinical seizures), the other two thirds show pathological EEG patterns without clinical equivalent (Murray et al., 2008). This phenomenon is called electroclinical dissociation and is especially common in premature infants, neonates and in infants after antiepileptic treatment (Duncan, 2003) (this will be further discussed in later chapters).

1.1.2 Neonatal seizures

The neonatal period, which describes the first 28 days of life, shows a particularly high susceptibility to seizures with an incidence of about three per 1000 live births (Freitag et al., 2001; Pressler & Mangum, 2013). In preterm neonates, i.e., infants born before the end of the 36th week of pregnancy, the incidence is even higher with about 57-132 cases per 1000 live births (Panayiotopoulos, 2005). These seizures are often consequences of peri- or early postnatal complications such as hypoxic-ischemic encephalopathy (probably 80% of neonatal seizures), intracerebral hemorrhage, stroke, or cerebral infection (Panayiotopoulos, 2010). Other causes of neonatal seizures are traumata, metabolic dysregulations, cerebral malformations, drug withdrawal, or genetic causes (Panayiotopoulos, 2010). Contrary to seizures in adults, neonatal and infantile seizures seldom manifest as generalized or tonic-clonic seizures (Korff & Nordli, 2005). Subtle seizures are the most common seizure type in neonates. They may be difficult to identify clinically as they seem to immitate normal behaviour such as ocular movement (like eye roling), oral-buccal-lingual movements (like sucking or chewing), progression movements (pedalling or swimming), or complexe purposeless movements (like hyperactivity or crying) (Panayiotopoulos, 2010).

In order to treat epileptic patients it is essential to study and identify the underlying causes. Therefore, the following chapter will focus on basic mechansims and brain structures essential in physiologic brain activity as well as in epileptogenesis.

1.2 Neuronal networks

1.2.1 Oscillations in the brain

Oscillations are created by “periodic fluctuations of excitability in groups of neurons” (see Buzsáki & Watson, 2012 for the following paragraph). These periodic fluctuations are generated by synchronous changes of membrane potentials in neurons, which lead to changes in the transmembrane currents and the extracellular field. These currents can be measured with the EEG, which is one of the most important diagnostic methods in clinical neurology. The EEG, as well as the electrocorticogram (ECoG) measure electrical activity of the superficial layers of the cortex. The local field potential may be measured to observe events in deeper locations. The summation of excitatory and inhibitory potentials from a vast number of neighboring neurons is called local field potential. It is measured in the extracellular space via depth electrodes (Schaffelhofer & Scherberger, 2016).

The oscillations observed in the EEG or local field potential are categorized by their frequency range in Hertz (Hz = one wave-cycle per second). The main frequency bands of oscillations described in the brain are: delta (0.5 – 3.5 Hz), theta (4 – 8 Hz), alpha (8 – 12 Hz), beta (13 – 30 Hz), and gamma (30 – 80 Hz) (see Figure 1). They are correlated with different states of behavior and consciousness (Buzsáki & Watson, 2012). In general, high-frequency ranges with low amplitudes represent alert and awake states of consciousness or dreaming states during sleep. Low frequencies with high-amplitude waves are correlated with non-dreaming sleep or pathological sleep such as coma (Bear et al., 2015 p. 645ff). These brain oscillations can be recorded via electroencephalographic recordings, which is also how they have been discovered. Deep sleep is represented by delta oscillations with a frequency range of 0.5 to 3.5 Hz being associated with learning processes and the brain reward system (Engel & Fries, 2010). Theta oscillations range from 4 to 8 Hz and are linked to some sleeping stages and especially with working memory functions and emotional arousal or fear conditioning (Engel & Fries, 2010).

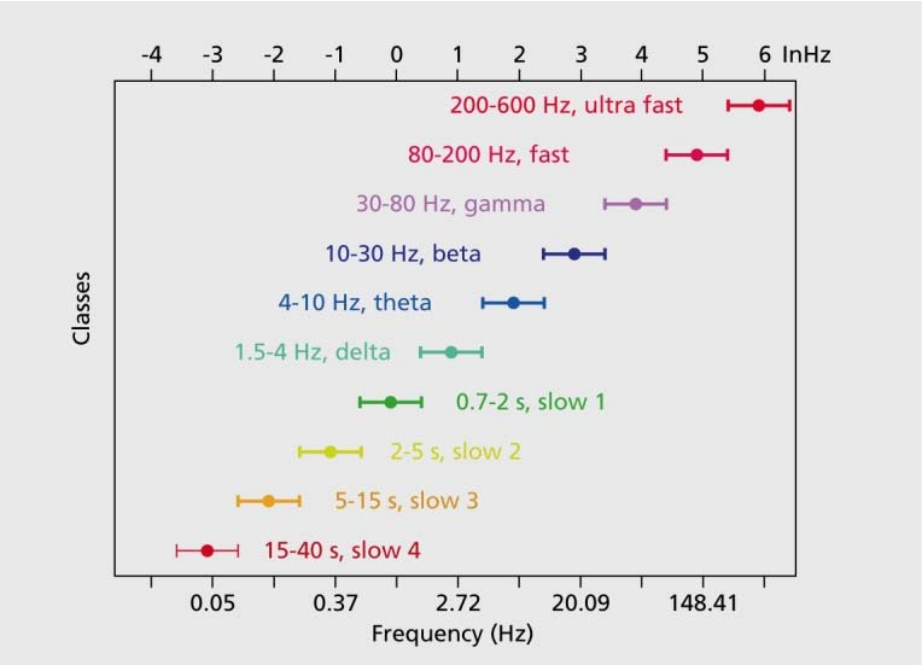

Figure 1: Oscillation classes in the cortex and their frequencies (Buzsaki & Draguhn, 2004), see text for details.

Hans Berger was the first to describe alpha oscillations in 1929 (Berger, 1929). Alpha waves range from 8 to 12 Hz and are present during quiet waking stages in the absence of sensory input (Bear, Connors, & Paradiso, 2006) (Engel & Fries, 2010). Beta oscillations range from 13 to 30 Hz and are probably the oscillations least understood today. Until today, the beta activity in the adult brain is associated with sensorimotor functions (Engel & Fries, 2010). Engel & Fries 2010 proposed the theory of maintenance of the status quo. Beta oscillations are prominent during steady concentrations and after voluntary movements during holding periods. Engel and Fries hypothesize that an intended or predicted maintenance of an individual's status quo is correlated with increased beta oscillation expression. Beta oscillations are also supposed to reflect anticipatory processes. Their theory could be supported by the fact that enhanced beta activity has been found in patients suffering from Parkinson's disease, who find it difficult to initiate changes in movement (Davis et al., 2012; Engel & Fries, 2010). High-frequency oscillations between 30 and 80 Hz are called gamma oscillations. In the awake brain both the sensory and the motor system produce synchronous neuronal activity resulting in the gamma rhythm. This multisensory and sensorimotor integration has been linked to attention, movement preparation, memory formation and conscious awareness (Butler & Paulsen, 2015; Engel & Fries, 2010; Haggerty et al., 2013).

As described above, oscillations represent a synchronous activity of neuronal function in the brain, which is important to carry out various physiological cognitive, sensory and motor functions. The most severe form of synchronous activity in the brain is an epileptic seizure. Therefore, the detection and the analysis of brain oscillations play an important role in epilepsy diagnosis and description of seizure events.

1.2.2 The hippocampus

The hippocampus is a formation in the brain playing an important role in epileptogenesis. The following paragraph will therefore give a quick overview on this formation and is based on Taupin (2007).

The hippocampus is a bilateral structure located at the floor of the temporal horn and belongs to the lymbic system. It plays an important role in learning and memory. The hippocampus and the subiculum together form the hippocampal formation. It consists of the dentate gyrus (DG) and the cornu ammonis (CA) and receives its main afferences via the entorhinal cortex. The main output of the hippocampus is from the CA1 pyramidal cells to the subiculum and deep layers of the entorhinal cortex. Other efferences go to other limbic structures such as the hypothalamus and also via the alveaus to the fornix and the temporal neocortex. The principal pathway of information passing through the hippocampus is shown in Figure 2.

The hippocampus has been associated with many neurological diseases such as Alzheimer's disease and epilepsy. Temporal lobe epilepsy may be treated with surgical resection of parts of the hippocampus when pharmacological treatment fails (Taupin, 2007). The hippocampal formation is assumed to be the generator of many more epileptic syndroms and therefore a focus in epilepsy research. Depth electrodes measuring the local field potentials in the CA1 or CA3 regions are used to investigate oscillations and epileptiform discharges up close.

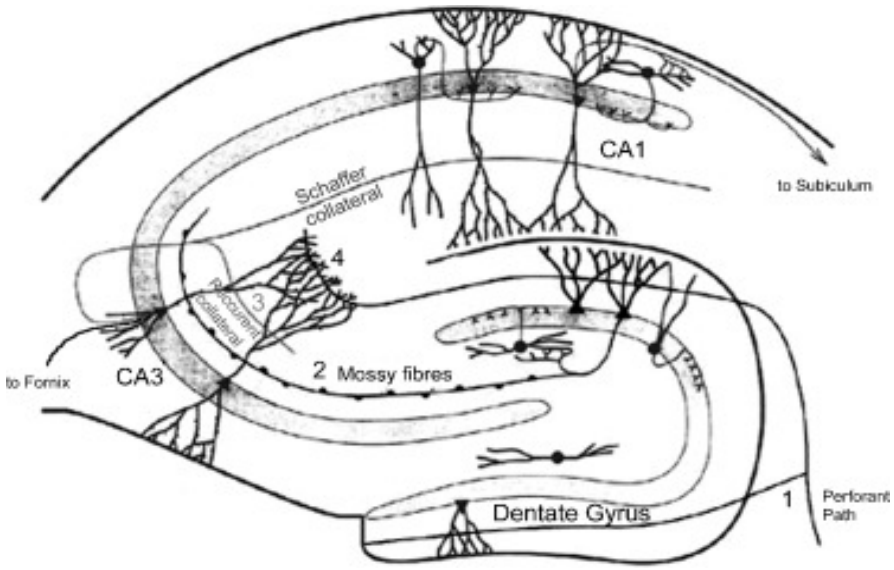

Figure 2: Architecture of the hippocampus (Daumas et al., 2004)

The afferences of the entorhinal cortex arrive by the perforant path. It projects either directly to CA3 pyramidal neurons or to granule cells of the DG, which then project mossy fibers (2) to the CA3 neurons. The Schaffer collaterals project from CA3 pyramidal cells to the CA1 pyramidal cells, which then project the efferent output of the hippocampus to the fornix and to other limbic or cortical structures. The neurons of the hippocampus are not only linked together in a unilateral way: CA3 pyramidal cells also project to the entorhinal cortex, other CA3 neurons or the subiculum. CA1 also projects to the entorhinal cortex. (Daumas et al., 2004, Taupin, 2007).

1.2.3 Seizure susceptibility of the immature brain

Why is the seizure incidence highest in the neonatal period? Increased excitability in the premature brain is physiological and thought to be essential for synapse formation and to ensure brain growth and development. Not all mechanisms leading to increased excitability in the neonatal brain are fully understood so far, but it is clear that these processes are age dependent (Ben-Ari & Holmes, 2006). Actions of neurotransmitter and neurotransmitter receptors as well as ion channel composition are highly developmentally regulated and may all lead to the enhanced excitatory neuronal activity early in life (Ben-Ari et al., 2012; Jensen, 2009; Rakhade & Jensen, 2009) (see Figure 3).

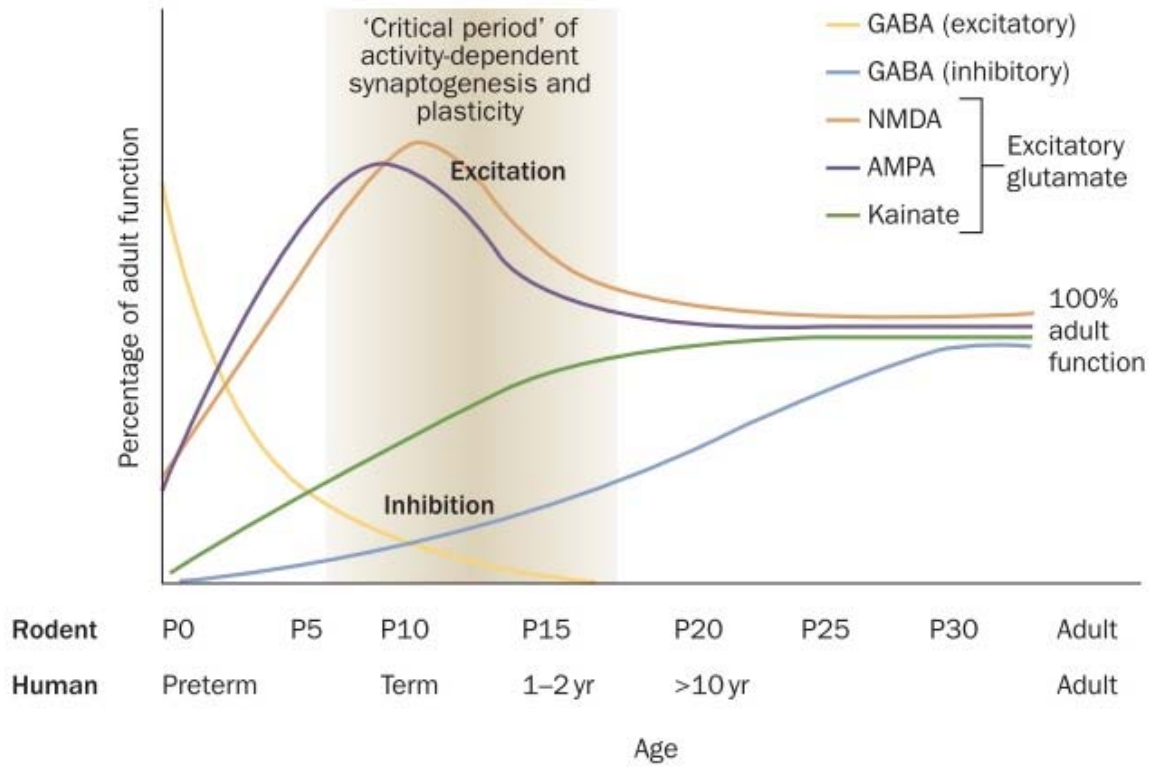

Figure 3: Changes in receptor function of the developing brain (Rakhade & Jensen, 2009). The x-axis displays equivalent developmental time periods for rats and humans. The y-axis displays the excitatory and inhibitory function in percentage of the receptor function during development in adults. GABA receptor action in the postnatal age of rodents and perinatal age of humans can lead to depolarization thus potentially having an excitatory function. Gradually the inhibitory function of GABA receptors is reached during development. Glutamate receptors show an excitatory function both in neonates and in adults. NMDA and AMPA receptors, subtypes of glutamate receptors, show a peak of action percentage in the postnatal age of rodents and perinatal age of humans which is significantly higher than the excitatory function in the adult age. Kainate receptors gradually rise to the adult level of excitatory function.

The most prominent change in transmitter action is the so-called GABAergic switch. GABAergic action changes from excitatory in neonates to inhibitory signaling in adults and may provide one explanation for electroclinical dissociation, as described in the following paragraph (see Ben-Ari et al., 2012 for the following).

The neurotransmitter GABA is known for its important inhibitory role in adult brains. Two major classes of GABA receptors exist: GABA-A receptors, which are ionotropic ligand-gated chloride channels, and GABA-B receptors, which are metabotropic, G-protein coupled receptors signaling to calcium and potassium ion channels.

The following paragraph will focus on GABA-A receptors and their role in the GABAergic switch. The action of GABA on GABA-A receptors depends on intra- and

extracellular chloride concentrations (see Figure 4). Contrary to the adult brain, neurons of the developing brain show higher intracellular chloride concentrations.

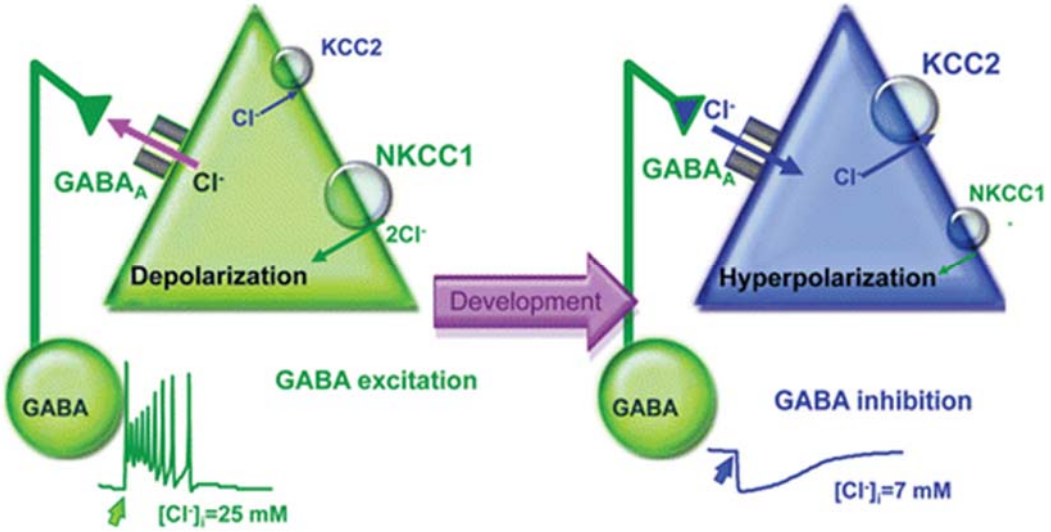

Figure 4: GABAergic shift: GABAergic action is dependent on the intracellular concentration of chloride. The concentration is regulated by the transporters NKCC1 and KCC2. NKCC1 is a inward cotransporter of sodium, potassium and chloride. KCC2 is a outward cotransporter of potassium and chloride. In the adult brain KCC2 is more active and leads to a low intracellular chloride concentration. GABAergic action then leads to chloride influx and hyperpolarization. GABA acts inhibitory in adult cells. In the neonatal brain NKCC1 is more active and leads to a higher intracellular chloride concentration. This leads to Hyperpolarization in neonatal cells when GABA A receptors are activated. GABA acts excitatory (Ben-Ari et al., 2012).

This can lead to a positive equilibrium potential of chloride relative to the resting membrane potential of the neuron. When GABA activates the $GABA_A$ receptor, an efflux of chloride ions and hence a depolarization of the cell is induced. Mature neurons have low intracellular chloride concentrations. In consequence, the activation of $GABA_A$ receptors generates hyperpolarization as it leads to an influx of chloride ions following the chloride gradient. Therefore, GABA, which is a potent inhibitory neurotransmitter in the mature brain, may act as an excitatory transmitter in the peri- and neonatal brain. However, as opening of GABA-A receptors also decreases the input resistance and thus causes shunting inhibition, the net effect of GABA action in the immature brain might still be inhibitory (Kirmse et al., 2018). It has been proposed that the GABAergic switch along with the development of the hippocampus has been conserved throughout the evolution as it is present in rodents as well as in primates (Khazipov et al., 2001).

The different concentrations of chloride in neurons at different developmental stages are most likely due to altered expression and activity of the sodium-potassium-chloride cotransporter NKCC1 and the potassium-chloride cotransporter KCC2 during maturation. NKCC1 uses the sodium gradient across cell membranes which is due to the different ion concentrations intra- and extracellular, and leads to a chloride influx. KCC2 uses the outward potassium current to co transport chloride and therefore generate a chloride efflux. During early brain development NKCC1 shows a much higher activity than KCC2 in hippocampal neurons (Ben-Ari et al., 2012), which could explain the higher intracellular chloride level in immature neurons. The GABAergic switch from an excitatory to an inhibitory action in rodents follows a caudal-to-rostral pattern and takes place around birth and during the neonatal period.
In addition to the GABAergic switch, neurotransmitter receptors show different expressions in the neonatal period. Glutamate, the most prominent excitatory transmitter in adults, also plays an import role in the increased excitability of the neonatal brain. During the neonatal period, NMDA- and AMPA-receptors express increased amounts of specific subunits which lead to a lower seizure threshold via higher calcium influx, reduced desensitization and prolonged current duration (Rakhade & Jensen, 2009)
Ion channel composition in the immature brain also favors excitability. Potassium channels such as $K_V7.2$, $K_V7.3$ or HCN1, as well as voltage-gated sodium channels show different quantity in their expression comparing immature and mature neurons. During the neonatal period these channels express subunits or splice variants that lead to higher neuronal excitability and less inhibition (Coppola & Moshé 2009; Rakhade & Jensen 2009; Velísková et al. 2004). But increased excitability not only contributes to brain maturation, it also leads to a lower seizure threshold. Mutations in ion channel-subunits have been linked to neurological diseases. Slight alterations in neurotransmitter receptor actions or in ion channel expression can lead to an increased risk of neonatal convulsions or epileptic syndromes (Velísková et al. 2004). The role of ion channels in physiologic brain function and their role in epileptogenesis are described in the following chapter.

1.3 Ion channels

Ion channels are transmembrane proteins that conduct or transport ions across the cell membrane. They have multiple functions such as establishing a resting membrane potential or contributing to the initiation of action potentials (Speckmann et al., 2008, p.14-34 also for the following). They are ubiquitously expressed, especially in the cardiac and nervous system, and divide into two groups: ligand-gated and voltage gated ion channels. Ligand-gated channels, also known as ionotropic receptors, change their conformational structure upon binding of its specific ligand. This leads to an ion flux across the membrane, which can induce or result in excitatory or inhibitory postsynaptic potentials (EPSPs or IPSPs). Acetylcholine, glutamate, serotonin and GABA are transmitters binding to ligand-gated ion channels.

Voltage gated ion channels open and close in response to changes of the membrane potential. They play a very important role in neurons as they take part in the integration and propagation of electrical signals. There are voltage-dependent ion channels for calcium, potassium, chloride and sodium ions.

1.3.1 Voltage-gated sodium channels

Voltage-gated sodium channels (VGSC) are transmembrane channels, which are essential for the initiation and the propagation of action potentials. VGSCs can be found in central and peripheral neurons, spinal ganglia, cardiac myocytes and skeletal muscle cells (Gazina et al., 2010; Oliva et al., 2012). They consist of one α subunit, which is pore forming, and multiple β subunits. β subunits are supposed to play an important role in trafficking and gating of the VGSCs. It is suggested that β subunits also establish cell adhesion and contacts with the cytoskeleton (Oliva et al., 2012).

There are nine known α subunit genes, which are each specific for their location and function. *SCN1A, SCN2A, SCN3A* and *SCN8A* are the four subunits that are highly expressed in the brain (Raymond et al. 2004). They each code for one channel protein, i.e. $Na_v1.1$, $Na_v1.2$, $Na_v1.3$ and $Na_v1.6$, respectively, which have all been associated with epilepsy (Oliva et al., 2012). Each α subunit consists of four domains (D I-IV), each formed by six transmembrane segments (S1-S6). S4 of each domain contains the voltage sensing helix and is responsible for activating and opening the

channel (Oliva et al., 2012). The channels are closed at resting membrane potential, which is about −70 mV (Ogiwara et al., 2009). Synaptic activity leads to mild depolarization, which triggers the S4 domain and opens the channel for sodium influx. The extracellular concentration of sodium is about 145 mmol/l, while the intracellular concentration is about 12 mmol/l. The influx of positively charged ions along the electrochemical gradient leads to a rapid depolarization of the cell and initiation of an action potential (AP). During this rapid depolarization, the VGSC already inactivates and no more sodium ions pass through these channels, the influx is stopped. Meanwhile voltage-gated potassium channels open, leading to membrane repolarization, which can be very fast, particularly in interneurons. As the extracellular concentration of potassium is 4 mmol/l and the intracellular concentration is approximately 140 mmol/l, potassium ions follow their electrochemical gradient out of the cell into the extracellular compartment. During this slow repolarization the sodium channels recover from inactivation and are now closed but can be reactivated (Buzsaki & Draguhn, 2004; Oliva et al., 2012; Speckmann et al., 2008).

1.3.2 SCN2A

This thesis concentrates on one particular VGSC, the voltage-gated sodium channel $Na_v1.2$ encoded by the *SCN2A* gene. *SCN2A* has recently been identified as an important gene in neonatal and infantile seizures (see Chapter 1.4). It has been localized by Litt et al. (1989) on chromosome 2q21.21-q33. $Na_v1.2$ is primarily expressed in the brain and predominantly located in unmyelinated/immature axons, in the Ranvier nodes of myelinated axons and at the axon initial segment (AIS) (Gazina et al., 2015). The AIS, located between the soma and the proximal end of an axon, shows a high density of sodium and potassium channels. Action potentials are initiated at the AIS based on the sum of EPSPs and IPSPs (Hu et al. 2009; Kole et al. 2008; Oliva et al. 2012).

The expression of *SCN2A* is age- and location-dependent. In rodent experiments it has been shown that $Na_v1.2$ is highly expressed during the neonatal period and gradually replaced by the sodium channel $Na_v1.6$ during maturation (Liao et al., 2010). During this neonatal period *Scn2a* is abundantly expressed in hippocampal areas Cornu Ammonis (CA)1, CA2, CA3, the dentate gyrus (DG) and also in the cortex from postnatal day 5 to 15 (P5-P15), showing diminishment, but no total loss afterwards (Liao et al., 2010). In mossy fibers, which connect the dentate gyrus with

the CA3 region of the hippocampus, however, *SCN2A* is expressed from P14/15 to adulthood (Liao et al., 2010). The expression of *SCN2A* is also subdivided into two splice variants: the neonatal 5N variant and the adult 5A variant, due to alternative splicing of the coding exon 5. Neonatal sodium channels show a lower excitability compared to the adult channels (Gazina et al., 2015). This may be a mechanism to counteract the higher excitability of neurons during the neonatal period and to reduce seizure susceptibility in healthy neonates and children (Xu et al., 2007).

1.3.3 Channelopathies

Channelopathies describe a group of disorders caused by ion channel dysfunctions. They may be acquired, e.g., by drugs or toxins, but genetic mutations are the most common cause for channelopathies (see Kim, 2014 for the following paragraph). Ion channels are present in every cell but play a particularly important role in the nervous as well as the cardiac system, they are active in the respiratory system as well as in the endocrine and urinary systems. Therefore, the range of ion channel disorders is broad. Skeletal muscle disorders have early on been discovered as often having channelopathies as underlying pathomechanism, e.g. myotonia congenita or flaccid paralysis. A loss of function mutation in the *CLCN1*-gene, a chloride channel in the skeletal muscle, leads to attacks of extreme stiffness in the patients. Other diseases that can be caused by alterations of ion channel function in the nervous system are epilepsy, ataxia, migraine, blindness, deafness, or periphery pain syndrome. The most prominent disorder in the cardiovascular system associated with an ion channel defect is the congenital long QT syndrome (LQT). A number of potassium gene mutations such as in *KCNQ1* or *KCNH2* lead to prolonged ventricular repolarization that increases the risk of tachyarrhythmia, syncope or sudden cardiac death. In the respiratory system a channelopathy leads to the most common genetic disorder in the Caucasian population: the mutation in the CFTR-gene. Cystic fibrosis (CF) is caused by a defect in the chloride channel gene CFTR. This leads to hyperviscosity that impairs the mucociliary clearance in the respiratory system, resulting in recurrent pulmonary infections. In the endocrine system an increased activity of the inward rectifying potassium channel K_{ATP} (encode by *KCNJ11*) may lead to neonatal diabetes mellitus, while its decreased activity leads to congenital hyperinsulinism and chloride channel mutations may lead to osteopetroesis (Kim, 2014).

In epilepsy, channelopathies show a broad clinical spectrum. The *SCN1A*-associated Dravet Syndrome was described in 1978 by Charlotte Dravet. It was the

first syndrome due to genetic mutation in an ion channel to be described. Mutations in potassium channel genes *KCNQ2* and *KCNQ3* have been associated with benign familial neonatal seizures (BFNS) (Singh et al., 1998). Mutations in sodium channel genes *SCN1A*, *SCN1B* and *SCN8A* have been associated with generalized epilepsy with febrile seizure plus (GEFS+) (Lossin et al. 2002), while mutations of the $GABA_A$ receptor are associated with the Dravet syndrome, as well as with childhood absence epilepsy (Kim, 2014; Oliva et al., 2012).

Over the last decade the role of *SCN2A* mutations as underlying mechanism in infantile neurological disorders has gained more and more attention. *SCN2A* mutations have not only been associated with a diversity of epileptic phenotypes but also with severe intellectual disability and autism (Rauch et al., 2012; Sanders et al., 2012), which shows the ample variety of brain functions in which $Na_v1.2$ seems to play a role. As this thesis concentrates on neonatal epilepsy, the following chapter will focus on the role of *SCN2A* mutations in epilepsy.

1.4 Clinical spectrum of *SCN2A* mutations in epilepsy.

The first mutation of clinical relevance in an *SCN2A* gene was reported by Sugawara et al. (2001) and associated with febrile seizures during infancy. It was then suggested that mutations in $Na_v1.2$ may play an important role in epileptogenesis. Until today numerous cases with mutations in the *SCN2A* gene have been reported with a broad variety of phenotypic expression (see Figure 5). The following paragraph will give a short overview on the epileptic syndromes *SCN2A* mutations have so far been associated with. It has been associated with benign familial neonatal infantile seizures (BFNIS), generalized epilepsy with febrile seizures plus (GEFS+), Dravet syndrome (DS), Ohtahara Syndrome (OS) and other intractable childhood epilepsies, showing that $Na_v1.2$ indeed plays an important role in epileptic channelopathies (Shi et al. 2012; Touma et al. 2013).

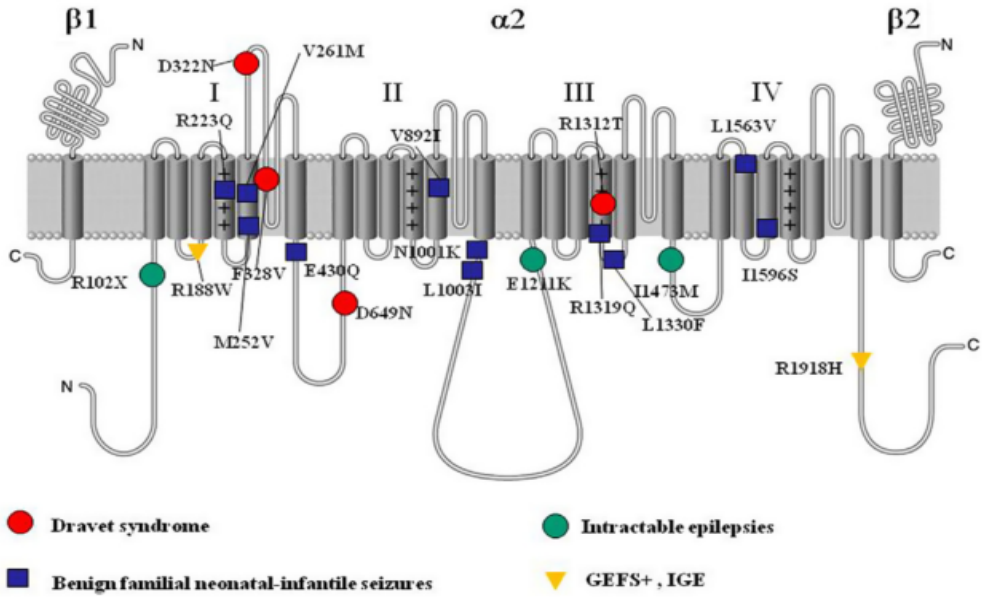

Figure 5: *SCN2A* mutations identified in epilepsy patients (Shi et al., 2012)
The blue square demonstrates published missense mutations causing BFNIS or benign familial infantile seizure; the green circles identify published de novo mutations causing intractable epileptic encephalopathies; the red circles show mutations identified in Dravet syndrome; the yellow triangle presents missense mutations associated with febrile and afebrile seizures (Shi et al., 2012).

1.4.1 Clinical manifestations of the *SCN2A* - A263V mutation

The mouse model used for my thesis is based on a mutation found in a patient and described by Liao et al. in 2010. The following chapter refers to the paper from (Liao et al., 2010). The patient was born in 1999 and suffered from tonic-clonic seizures starting on the first day of life. Bilateral seizures continued weekly to monthly until the age of 15 months. Interictal EEG and MRI results were normal at this age. From 18 months on, the patient suffered from episodes of ataxia, myoclonus, severe distress with headache, back pain and hyperventilation, occurring one to three times a month. At the age of 11 years he was seizure free but had problems with visual processing, fine motor function and tactile sensation. From 1 month to 2.8 years of age, he was treated with phenytoin. Other antiepileptic drugs were ineffective. The mutation found in this patient was a *de novo* mutation in the transmembrane segment D1/S5 of the *SCN2A* gene. This missense mutation exchanged the amino acid alanine versus valine at position 263 (pAla263Val) and was neither found in the parents nor in 93 healthy controls indicating that it is a *de-novo* mutation. In *in vitro* patch-clamp recordings, Liao et al. (2010) showed that this mutation leads to a gain-of-function of both splice variants of $Na_v1.2$, with a three-fold increase of the persistent sodium

current. The fast inactivation was slowed, whereas the recovery from slow inactivation was accelerated in the neonatal splice variant.

As *SCN2A* is highly expressed in neonatal hippocampal and cortical neurons, as described above (see Chapter 1.3.2), seizures at this age could be explained by the altered biophysical properties caused by this mutation. According to Liao et al. (2010) the late onset of ataxia and pain may be explained by the increased expression of *SCN2A* in unmyelinated mossy fibers or unmyelinated spinal cord fibers. Touma et al., (2013) identified the same *de novo* mutation in monozygotic twins suffering from Ohtahara syndrome with severe developmental delay. Both boys suffered from 50 – 60 seizures a day starting on day one of life. The first twin died of cardiorespiratory arrest of iatrogenic cause on day 19. The second twin was seizure free at 8 months on topiramate, levetiracetam, phenobarbital, vigabatrin and lamotrigine. Without medication he was seizure free at two years of age but suffered from severe developmental delay.

These findings suggest that *SCN2A* is not only associated with benign seizure phenotypes, but can also lead to severe consequences. It is therefore important to pursue and continue the study of *SCN2A* mutations and their role in epileptogenesis.

1.4.2 Pharmacologic treatment

The newest German guideline on cerebral seizures in newborns was presented by the AWMF (Arbeitsgemeinschaft der wissenschaftlichen medizinischen Fach-gesellschaft) in 2012 and states that the recommendation of pharmaceuticals for seizing newborns is founded on no evidence-based trials, since there are none (Roll et al., 2012). The common consent lies in the first-line treatment with phenobarbital. If this does not lead to sufficient seizure reduction add-on pharmaceuticals may be given: benzodiazepines, phenytoin, lidocaine or levetiracetam are potential alternatives. Experimental approaches may be possible with bumetanide and topiramate (AWMF, 2012).

In the following, four pharmaceuticals, which are relevant to this thesis, will be further described: bumetanide, phenobarbital, phenytoin and retigabine.

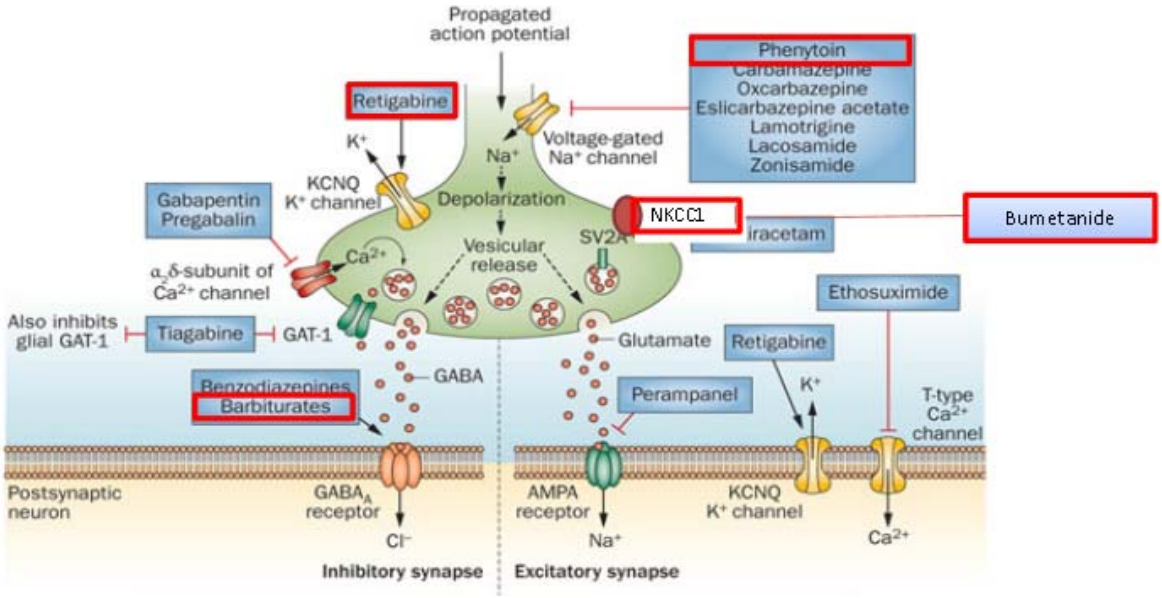

Figure 6: mechanism of action of different antiepileptic drugs (Schmidt et al., 2012).

Red boxes indicate thesis relevant drugs, bumetanide and NKCC1 are added to the figure.

Bumetanide

Bumetanide is an inhibitor of the cation-chloride co-transporters, which is used as a diuretic and currently also discussed as a new antiepileptic drug (AED). It specifically blocks the cation-chloride co-transporters NKCC1 and NKCC2 (sodium-potassium-calcium co-transporter 1 and 2) (Kahle & Staley, 2008). NKCC1 is found throughout the body and expressed in central as well as peripheral neurons and glial cells, NKCC2 is expressed in the kidney (Löscher, Puskarjov, & Kaila, 2013). In the brain, NKCC1 plays a significant role during early brain development. It is an inwardly-directed co-transporter of sodium, potassium and chloride. As described above, NKCC1 leads to a higher intracellular concentration of chloride in the neonatal period, which results in a chloride equilibrium potential, which is positive compared to the resting membrane potential. Activation of GABA-A receptors leads to chloride efflux and depolarization of the cell (see Chapter 1.2.3 on the GABAergic switch). Blocking NKCC1 in immature neurons leads to a decrease in intracellular chloride concentrations, which reduces or reverses the action of GABA. With lower intracellular chloride concentration the activation of GABA A receptors leads to chloride influx and to hyperpolarization (see Figure 7; Dzhala et al., 2005; Löscher et al., 2013).

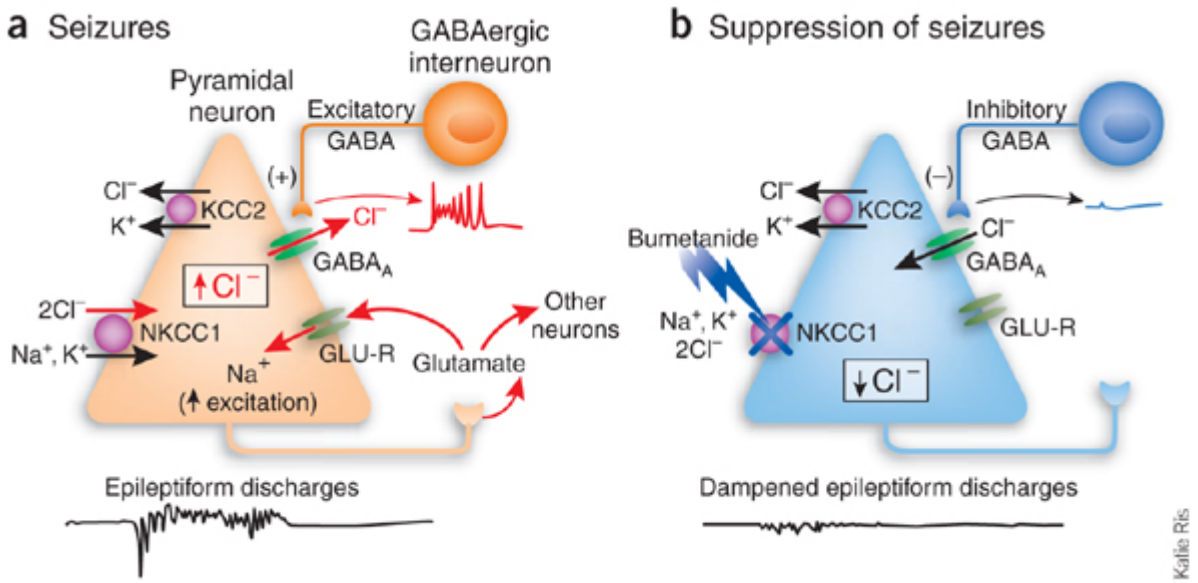

Figure 7: The proposed mechanism of action of bumetanide in the neonatal brain (Fukuda, 2005 p. 1153)

Immature pyramidal neuron with high intracellular chloride level. NKCC1 uses the sodium and potassium gradient created by the Na^+/K^+-ATP-pump to cotransport chloride into the cell leading to a positive chloride equilibrium potential compared to the resting membrane potential. The activation of $GABA_A$ receptor-channels results in chloride efflux and depolarization. The depolarization may cause the release of glutamate, which further induces excitation by activation of the glutamate receptors, rendering the neonatal cortex highly susceptible to seizures (Dzhala et al., 2005; Fukuda, 2005).
Bumetanide blocks NKCC1 and reduces the intracellular chloride concentration. This leads to influx of chloride after GABA A receptor activation and may then lead to a hyperpolarizing GABA response and in the following may prevent glutamate release (see p. 13). This mechanism could therefore be a way to suppress seizure discharges (Dzhala et al., 2005; Fukuda, 2005).

Phenobarbital

Phenobarbital belongs to the group of barbiturates and has sedative, anesthetic and anticonvulsant properties. Barbiturates bind to the $GABA_A$ receptor and increase the duration of the channel opening. Prolonged channel opening leads to more chloride influx, hyperpolarization of the cell and therefore an enhancement of the GABAergic inhibition (Kwan, Sills, & Brodie, 2001). A higher intracellular chloride concentration, like present in immature neurons, barbiturates may further increase the depolarizing action of GABA. This mechanism may be one cause for the treatments lack of efficacy, particularly in pre-term children. In the absence of viable alternatives, barbiturates are still broadly used in long-term treatment of children and adults suffering from focal or generalized seizures and especially used in anticonvulsive therapy of neonates and infants (Neubauer & Hahn, 2014).

Phenytoin

Phenytoin (PHT) is a selective blocker of voltage gated sodium channels (VGSCs). This block is voltage and frequency dependent, meaning that the drug binds to the inactivated state of the channel (Davies, 1995), and blocks most effectively during high-frequency repetitive firing of action potentials (Kwan et al., 2001). Phenytoin also acts as an inhibitor of the persistent sodium current (Colombo, Franceschetti, Avanzini, & Mantegazza, 2013). It is a potent antiepileptic drug used for the treatment of focal or generalized seizures, status epilepticus and neonatal seizures (Neubauer & Hahn, 2014). (Colombo et al., 2013)

Retigabine

Retigabine (RTG) is an antiepileptic drug, which acts as a neuronal potassium channel opener. It is selective for the potassium channels *KCNQ2*, *KCNQ3* and *KCNQ5,* which are expressed in neurons and play an important role in the control of cellular excitability. Retigabine also acts as a positive allosteric modulator on $GABA_A$ receptors enhancing GABAergic inhibition (Gunthorpe, Large, & Sankar, 2012).

Retigabine was used as an add-on therapy for long-term treatment of focal and primary or secondary generalized seizures in adults (Neubauer & Hahn, 2014, Arzneimittelkommission der deutschen Ärzteschaft AkdÄ, 2011). In 2012, retigabine was withdrawn from the German market by the manufacturing company after the Federal Joint Committee (Gemeinsamer Bundesausschuss G-BA) decided that the drug showed no added value in comparison to the reference substances (G-BA 2012).

1.5 Aim of this work

The introduction so far has described the clinical symptoms and genetic causes of neonatal seizures, as well as the important role of treatment options for the patients. A focus in neuronal network activity was laid on brain oscillations and excitatory and inhibitory network activity in the immature brain, since this thesis will concentrate on these topics.

The aim of this thesis is to investigate whether the *Scn2a* (p.A236V) mouse model presents a valid model for neonatal epilepsy and possible treatment trials. Therefore, the electrophysiological characteristics of this mouse model were investigated and

pharmaceutical treatment was given. These investigations are based on two hypotheses:

1. The *Scn2a*(p.A236V) mutation leads to altered (epileptic) brain activity, in neonatal mice compared to wildtype mice.

If the first hypothesis proves to be true, the second hypothesis has to be tested:

2. Epileptic patterns in *Scn2a* mutant neonatal mice can be prevented via prophylactic pharmaceutical treatment.

1.6 Preexisting data

In vitro acute slice patch-clamp recordings from CA1 pyramidal neurons of *Scn2a*(p.A263V) *mice* revealed increased excitability both in cells from heterozygous (Schattling et al., 2016) and homozygous animals (H. Lerche, personal communication). These results confirm the gain-of-function nature of the *Scn2a* mutation suggested by the biophysical properties of mutant channels in heterologous expression experiments (Y Liao et al., 2010).

Analysis of the survival of heterozygous and homozygous mice showed a significantly lower survival rate of homozygous animals compared to heterozygous and wildtype mice. As tonic-clonic seizures had been observed and recorded in adult mice carrying the *Scn2a*(p.A263V) mutation, early death was likely attributable to lethal seizures (W. Fazeli, personal communication). Dr. Fabio Morellini and Francesca Xompero, PhD, did weight measurement in *Scn2a* homozygous and heterozygous pups from P1 to P7 compared to wildtype pups (personal communication). The results showed significant difference of weight in mut/mut pups to wild type pups from P4 to P48 and between heterozygous and homozygous pups from P30 to P48. Later in life the weight discrepancy grows between the genotype groups. These data are part of the foundation on which the following thesis bases.

2 Materials and Methods

2.1 Materials

2.1.1 Animals

The **C57BL/6J-*SCN2A*^{A263V}**, transgenic mouse line with a knock-in mutation of the *SCN2A* gene was generated through homologous recombination in embryonic stem cells (Schattling et al., 2016) by Dr. Birgit Engeland, Experimental Neuropediatrics, Center for Molecular Neurobiology Hamburg (ZMNH). Mice with a homozygouse knock-in mutation will be called mut/mut in the following, while heterozygous mice will be called wt/mut compared to wild type animals without any *SNC2A* mutation which will be called wt/wt.

2.1.2 Chemicals and substances

Bumetanid, Burinex 2mg/4ml; LEO Pharmaceutical Products, Ballerup, Denmark
Bupivacain, Bucain-Actavis 0,5%; Actavis Group, Hafnarfjödur, Island
Buprenorphine, Temgesic; Essex Pharma GmbH, Munich, Germany
Carbon dioxide (CO_2); Wonsak Kohlensäureservice GmbH, Hamburg, Germany
Dental cement, Tetric EvoFlow; Ivoclar Vivadent Corporate, Salerno, Italy
Dental adhesive, OptiBond All-In-One; Kerr Italia, Salerno, Italy
Fluorescent nucleic stain, Dapi; Sigma Aldrich Chemie GmbH, Steinheim, Germany
Roti -**Histofix**, 4% Paraformaldehyde-solution; Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Hydrogen peroxide (H_2O_2) solution, 30%; Sigma Aldrich Chemie GmbH, Steinheim, Germany
Isoflurane, Florene; AbbVie, Ludwigshafen, Germany
Natriumchlorid, Braun injection solution 0,9%; B.Braun Melsungen AG, Melsungen, Germany
Oxygen, medical; TMG GmbH, Krefeld, Germany
Phenobarbital, Luminal 200mg/1ml; Desitin GmbH, Hamburg, Germany
Phenytoin, Phenhydan injection solution 271,8mg/5ml; Desitin, Hamburg, Germany
Retigabine, ChemPacific; Baltimore, USA
Tattoo ink, Ketchum Animal Tattoo Ink; Ontario, Canada

2.1.3 Consumables

24 well plates; Sarstedt AG & Co; Nümbrecht, Germany

Cannula, Sterican, 40x20mm, Braun Melsung AG, Melsung, Germany

Cotton swab, sterile, Raucotupf; Lohmann & Rauscher GmbH & Co. KG, Neuwied GmbH, Braunschweig, Germany

Microhematocrit capillary tubes, heparinized, Brand GmbH, Wertheim, Germany

Microscope cover glasses, 24x50mm; Carl Roth GmbH & Co. KG, Karlsruhe, Germany

Microscope object slides, Menzel Gläser Superfrost Ultra Plus, Thermo Scientific; Gerhard Menzel, Braunschweig, Germany

Mikrotube 1,5ml; Sarstedt AG & Co., Nümbrecht, Germany

Mikrotube 15ml; Sarstedt AG & Co., Nümbrecht, Germany

Pipette tips; Sarstedt AG & Co., Nümbrecht, Germany

Razor blade, Croma, Eisfeld, Germany

Swabs, non sterile, Sugi; Kettenbach GmbH, Eschenburg, Germany

Tape, Leukosilk; Hamburg, Germany

Superglue, Pattex Ultra Gel; Henkel AG & Co. KGaA, Düsseldorf, Germany

Welted glasses with snap on lids, 5ml; Carl Roth GmbH & Co. KG, Karlsruhe, Germany

2.1.4 Equipment

3-axis Manipulator; Märzhäuser Wetzlar GmbH &Co. KG, Germany

Digital data acquisition system, Digital Lynx SX; Neuralynx, Bozeman, USA

Balance, CP4202S-OCE; Sartorius AG, Göttingen, Germany

Balance, TP200, Dipse, Oldenburg, Germany

Camera, PEN E-P1, Olympus Deutschland GmbH, Hamburg, Germany

Centrifuge, 5424; Eppendorf AG, Hamburg, Germany

Cork tile, Ikea, Hamburg, Germany

Curing light, Woodpecker, Guangxi, China

Dentist drill; Emax Electer, NSK Europe GmbH, Eschborn, Germany

Dust off, Falcon Safety Products Inc., Branchburg, USA

***In vivo* depth-electrodes**, A1x16-3mm-50-703-A16; NeuroNexus Technologies Inc., Ann Arbor, USA

Light, TH4-200; Olympus Deutschland GmbH, Hamburg, Germany

Microscope; fl 70, Carl Zeiss AG, Oberkochen, Germany

Mikroscope, BX51WI, Olympus Deutschland GmbH, Hamburg, Germany

Operating instruments; World Precision Instruments, Sarasota, USA

Pipettes; Eppendorf AG, Hamburg, Germany

Soldering iron, Weller; Cooper Tools, Besigheim, Germany

Stereotaxis system; Model 940, small animal stereotaxic instrument with digital display console, David Kopf Instruments, Tujunga, USA

Stereotaxis system; Model 942, dual small animal stereotaxic instrument with digital display console, David Kopf Instruments, Tujunga, USA

Temperatur Controller, ATC1000; World Precision Instruments, Sarasota, USA

Thermomixer, HTM 130; HLC by Ditabis AG, Pforzheim, Germany

Ultrasonic cleaner, Elma, Elma Schmidbauer GmbH, Singen, Germany

UV-Lamp, X-Cite, Series 120 Q; EXFO, Hampshire, UK

Vaporizer for Isoflurane, NarkoVet; Eickemeyer, Tuttlingen, Germany

Vibratome, VT 1200S; Leica Microsystems GmbH, Wetzlar, Germany

Water bath, Haake DC10 mit Haake 6P, Thermo Fisher Scientific Inc., Waltham, USA

2.1.5 Software

Matlab; MathWorks, Ismaningen, Germany

The Observer; Noldus Information Technology B.V., Wageningen, The Netherlands

Prism5.02; GraphPad Software Inc., La Jolla, USA

2.2 Methods

All treatments and surgical procedures were done in line with the German Animal Welfare Act. This project was approved by the Ministry of Science and Public Health of the City of Hamburg, Germany (G09/068, G13/006, G13/053).

Breeding was done with the kind help of Birgit Engeland, PhD, Dr. Fabio Morellini and Dr. Walid Fazeli. Genotyping and *in vivo* electrophysiology was partly done

together with Dr. Walid Fazeli. DNA analysis was done by Julia Mienert and Janis Szeremeta. Statistical analysis was done with the help of Dr. Fabio Morellini.

2.2.1 Breeding

The breeding of the mouse line was done in the central breeding facility and the experimental mouse facility of the Center for Molecular Neurobiology Hamburg (ZMNH). The animals were kept in a pathogen free environment at 23 ± 1°C and 50% humidity with food and water *ad libidum*. The experimental mouse facility had an inverted day-night rhythm, meaning that the lights were turned on at 8 pm and turned off at 8 am. As mice are nocturnal animals, all experiments were done during the night-phase of the animals to ensure that the wake-sleep cycle of the mice was not disturbed.

2.2.2 Genotyping

2.2.2.1 Marking and tissue extraction

Pregnant mice were checked upon twice a day to determine the time of birth of the pups. On postnatal day 1 (P1) the number of animals per litter was counted. Litters were included into the trial if the litter size was between six and eight pups. Considering only approximately equal litter sizes, litter effects such as differences in birth weights or maternal care (e.g., feeding of the pups) were minimized. If a litter contained more than eight pups, the litter size was reduced to the number of eight pups. The surplus animals were taken from this litter and added to a different litter of the same birth date with less than eight pups until a maximum of eight pups in this litter was reached. The pups added to a different litter were randomly taken from their birth litter. Before being added to a new litter, the pups were covered with urine of the new dam. No problems in accepting the new pups could be observed. All adopted pups were accepted and nursed. Never- the- less litter effects could not be prevented entirely and are therefore discussed in Chapter 4.4 of the Discussion.

On P1, the pups were marked and a tissue sample was taken in order to determine the genotype. The marking was done with green tattoo ink. A cannula was used to place a small amount of color right under the skin of the pup's paws. To distinguish the pups each animal received an individual tattoo, such as one on the front right paw, front left paw etc. To obtain a tissue sample a disinfected small cutaneous scissor was used and a small piece of the pup's tail was cut off. The tissue was put in

a sterile test tube and further examined. Each animal's weight was measured and written down before marking.
After marking and tissue extraction, the animals were put back into their home cage to their respective nests and mothers.

2.2.2.2 DNA analysis

The DNA analysis of the tissue samples was done with the polymerase chain reaction with *Scn2a*-specific primers.

2.2.2.3 Subcutaneous injections

To investigate pharmacological effects on the epileptic phenotype of neonatal *Scn2a* mice, pups were injected with different pharmaceuticals, which are also described above: bumetanide, phenobarbital, phenytoin, retigabine, and saline.
Litter characteristics necessary for the inclusion of pups into the trial were determined as described in Chapter 2.2.2.1. The litters were randomly assigned to the different treatment groups. To prevent confusing pups and treatments, all pups of one litter were injected with the same treatment. This grouping was susceptible to litter effects such as different caretaking of the mother. Therefore, one treatment group consisted of at least two different litters and pups were taken randomly from both litters for electrophysiological recordings.
The mice were injected from P1 to P7 subcutaneously twice a day, at about 9am and 6pm. All animals received 5 µl/g body weight of treatment solution, so that possible effects due to different volume injections should be excluded.
The dosages used were based on pediatric dosages used in the clinic:

- The vehicle group received 5 µl/g body weight of saline.
- For retigabine treatment, the pups received 20 µg/g body weight per day. 2 µg/µl retigabine were dissolved 2:1 in 10% of cyclodextrane solution.
- The bumetanide group received 0.4 µg/g body weight per day. The stock solution of 2 mg/4 ml bumetanide was diluted 1:25 in saline.
- Pups in the phenobarbital group received 10 µg/g body weight per day, for which the stock solution of 200 mg/1 ml was diluted 1:200 in saline.
- The phenytoin treatment was done with a dosage of 60 µg/g body weight per day. The stock solution of 750 mg/50 ml solution was diluted 1:2.5 in saline.

For subcutaneous injections the neonates were transiently separated from the mother, the weight of each pup was measured and the respective amount of the drug given. To inject the mouse, the skin at the neck of the animal was lifted and the cannula was inserted parallel to the back of the mouse into the subcutaneous space where the drug was injected. The injection was considered to be correct when no bleeding was observed at the injection site and a subcutaneous bleb of fluid could be detected. Afterwards all littler mates were returned to their nest and their mother.
Subcutaneous treatment with phenytoin frequently caused skin lesions at the injection site after a few days of treatment, likely due to the basic pH of the phenytoin solution. When this was discovered, the treatment and the trial were stopped and the phenytoin treatment was continued via drinking water of pregnant or nursing females.

2.2.2.4 Treatment via drinking water

After discovering the severe side effects of the subcutaneous phenytoin injection, the way of treatment application was changed. The dam received phenytoin treatment via the drinking water. As soon as pups were one day old, treatment was started. Because phenytoin is excreted in breast milk, pups received the treatment via nursing. The calculation of the dosage of phenytoin was based on a daily drinking amount of 5 ml for an adult mouse and on dosages that are commonly used in the antiepileptic treatment of humans.

- The first group received phenytoin with 30 µg/g body weight per day, diluted in drinking water from P1 to P7. Therefore, the stock solution of 750 mg/50 ml was diluted 1:75 in drinking water.
- The second group received the doubled dosage, 60 µg/g body weight per day, diluted in drinking water from P1 to P7. The stock solution was then diluted 1:37.5 in drinking water.
- The third group received 60 µg/g body weight per day, diluted in drinking water from E14 (day 14 after conception) to P7. The stock solution was then diluted 1:37.5 in drinking water.

2.2.2.5 Blood sampling

To investigate the serum levels of phenytoin treatment, the blood was sampled from the treated pups and analyzed. The pups were treated from P1 to the day of the sampling, which was between P10 and P12.

The treatments and the concentrations used are described in 1.4.2 and 2.2.2.3. The blood samples were taken in the early afternoon. This time span was chosen to obtain comparable serum concentrations between the subcutaneous and the drinking water treatment trials. As the subcutaneous injections were given in the morning and evening, the serum concentration in the early afternoon was supposed to be close to a steady state serum concentration. The pups treated via drinking water had two different treatment time spans: they did not ingest any treatment at night, as mice mostly do not drink during their sleeping period, but they received constant treatment via steady ingestion over the course of the day. A steady state like serum concentration was therefore also supposed to be reached in the early afternoon. Taking all blood samples at this time was supposed to prevent incomparable results in serum concentrations due to different treatment methods.

For one serum level concentration analysis a minimum of 80 µl blood serum was considered to be necessary by the central laboratory of the Universitätsklinikum Hamburg Eppendorf (UKE). At the age of 12 days the pups had an average weight of 3 g and a total blood volume of about 210 µl to 240 µl. As the pups had such a small blood volume, a normal blood withdrawal via arterial or venous puncture was not possible. In consequence, the method used was terminal blood sampling, which is a method of blood sampling by puncture of the heart. With this method about 80 µl of whole blood could be obtained from one pup. Therefore the whole blood of three to four pups of the same treatment group was pooled together, to be sure to obtain enough blood serum for the drug analysis.

Starting the procedure, the pups were anesthetized with 4% isoflurane and injected with 0.05 µg/g body weight buprenorphine. Isoflurane is a volatile anesthetic of the halogenated ether group, used in veterinary medicine and buprenorphine is a highly potent opioid. The anesthesia was used during the whole procedure with 1 to 1.5% isoflurane and reflexes tested repeatedly to ensure a deep narcosis and sufficient analgesia. When no reflexes could be detected any more, the pup was fixated with leukotape on its back on a cork plate. With a small cutaneous scissor the skin was cut transversally under the ribs and the xiphoid. Then the thorax was opened by cutting through the diaphragm and the parietal pleura and the heart was carefully fixated with an anatomical forceps. With a cannula the left ventricle of the heart was punctuated. Once in the ventricle, the blood was aspirated slowly, put in a test tube and cooled right away. The pup was then sacrificed under narcosis, by decapitation.

The blood from the different treatment groups was pooled separately into test tubes. The blood serum was obtained by centrifugation of the whole blood at 2000 rounds per minute for 10 minutes. With a pipette the serum was transferred into a new test tube and brought to the central laboratory of the UKE where it was analyzed via PETINIA immunoassay right away.

2.2.3 Electrophysiology

2.2.3.1 *In vivo* electrophysiology

In vivo depth recording was used to measure brain and seizure activity in the living animal. Multichannel depth electrodes were used and inserted in the hippocampus (HC) in mice at the age of P6/7 and P15.

To begin the procedure, the mice were anesthetized with 4% isoflurane. During the whole surgical procedure the mice were anaesthetized with 1.5% isoflurane in 100% O_2 and kept warm with a heat plate at 34°C. The reflexes were tested repeatedly to ensure a deep analgesia. As soon as the pup was fully anaesthetized, 0.05 µg/g body weight buprenorphine were injected. The injection was done 30 minutes before the operation started to ensure its full effect. If no reflexes could be triggered after those 30 minutes, a small scissor was used to make the first cutaneous cut parallel to the sagittal suture of the skull. The dorsal part of the skull was exposed, revealing lambda, the intersection of the sagittal and the lambdoid suture, and bregma, the intersection of the sagittal and coronal suture. The edges of the wound were anaesthetized with bubivacain, which is a long-acting local anesthetic belonging to the amino amide group. The periosteum was detached using a cotton swap saturated with hydrogen peroxide, H_2O_2, which was neutralized with 0.9% sodium chloride, NaCl 0.9% a few seconds later. With a dentist's drill a hole was made in the calvarium over the cerebellum and the ground and reference electrode was inserted and fixed with dental cement. The skull was then fixated with a small metal bar on the stereotaxis table. The body of the mouse was also fixed on the stereotaxis table with leukotape and a piezo element as movement sensor was attached to the thorax of the mouse.

The location of the hippocampus in the brain is dependent on the size of the brain. With a microscope and a micromanipulator (Kopf digital stereotaxic instrument) the distance between lambda and bregma, the meeting points of the coronal suture with the sagittal suture and the sagittal suture with the lambdoid suture, was measured. Depending on the lambda-bregma distance, the positions for the electrode holes were adapted as follows:

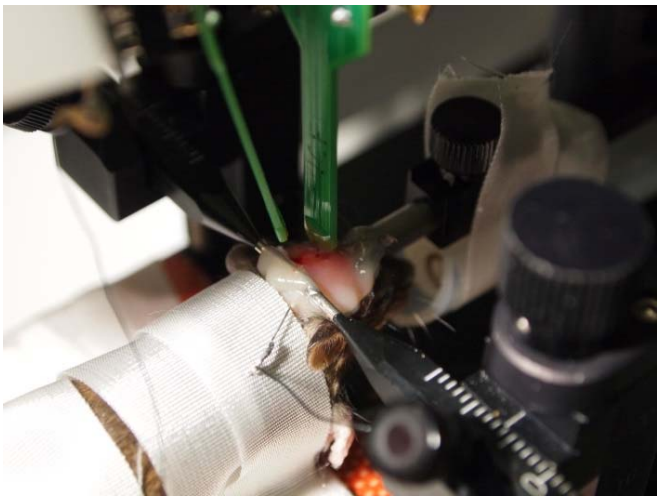

Figure 8: Operation

P7 pup fixated in the stereotact with hippocampal and cortical electrodes inserted.

The position of the hippocampal electrode was between 1.5 and 1.7 mm lateral to the sagittal suture and 1.4 to 1.6 mm posterior to the bregma suture. The positions were measured with the digital stereotactic instrument (Kopf Instruments) and a hole was drilled at the designated position with a dental drill. The electrodes used, were 16-channel-silicon probes with a 50 µm distance between electrode site (A1x16-5mm-50-703, NeuroNexus) and were inserted in the CA1 region of the hippocampus (see Figure 8: Operation). To record the LFP a preamplifier of an acquisition system with a sampling rate of 32 kHz and a digital bandpass filter of 0.5-9000 Hz. Before the recording started, the isoflurane anesthesia was stopped and the pup woke up. To measure the physiological network patterns pathological in neonatal mouse brain and seizure activity, it was necessary to do the recording on awake animals (See Chapter 4.4). To ensure that no isoflurane residuals affected the brain activity 15 to 20 minutes were waited before the start of the recording.

The duration of the recording was a minimum of 60 minutes to a maximum of 100 minutes. After the recording, isoflurane anesthesia of 4% was given and kept this high, in preparation of the elimination of the pup later on. The electrode positions were marked in the brain using the colorant DiI on a color electrode (2.2.4). Afterwards the mouse, in deep narcosis, was beheaded and the brain was taken out to be fixed in paraformaldehyde solution. Later on, brain slices were done to check

the exact position of the electrode (see 2.2.5). A tissue probe of the mouse's tail was taken and put in a test tube to double check the genotype (see 2.2.2)

2.2.4 Histology of brain slices

After every recording session the electrode positions were marked with a fluorescent dye. The fluorescent stain of the electrode in brain slices was then observed to ensure that the electrode positions had been correct. The tip of an electrode was covered with the dye DiI and afterwards inserted into the brain of the recorded pup at the same position where the hippocampal or the cortical electrodes were located before. Each brain therefore showed two electrode positions marked in the brain slices.

During this procedure the pup was under deep narcosis with 4% isoflurane anesthesia. When the electrode positions had been marked, the pup was beheaded and the brain was taken out. Therefore, the skull of the pup was cut open with a small scissor from the foramen magnum to the coronal suture parallel to the sagittal suture. The skull was carefully dissected from the brain. Then the brain was taken out with a spatula and put into a welted glass where they were fixed with paraformaldehyde solution for a few weeks. When the brain was fixed, brain slices could be done with a vibratome to determine the exact electrode position. Therefore, the brain was fixed on a small plate with super glue and positioned in the water tank of the vibratome. At a thickness of 50 µm, brain slices were cut off and fixed on microscope object slides. On the object glasses the slices were saturated by Dapi, a fluorescent nucleic acid stain, and a cover glass was put on top. After one or two days of incubation time, the slices could be examined under the microscope and the exact electrode position could be determined (see Figure 9).

Figure 9: brain slice of a P7 pup with DiI marked electrode position of the hippocampal electrode.

2.2.5 Analysis and statistics

Analysis and statistics of the data derived from *in vivo* electrophysiological recordings was done with Matlab and Prism 5. The statistical tests used were nonparametric

tests due to small group numbers, which do not represent normal distribution of variables. The Mann Whitney test was used for two groups. For more than two groups analysis was done with the Kruskal-Wallis test. The significance level was set at $p = 0.05$ as significant (*), $p \leq 0.01$ as very significant (**) and $p \leq 0.001$ as highly significant (***).

3 Results

3.1 Breeding of *Scn2a*(p.A263V) mice

Spontaneously occurring epileptic seizures were frequently observed in adult homozygous *Scn2a* mice. Seizures could often be seen while handling the cages or the mice. The seizures showed a clonic manifestation of the entire body, lasting for a few seconds up to approximately one minute. This was followed by a static tonic episode, where mice sat “frozen” for up to eight minutes. Afterwards the mice slowly regenerated, going back to grooming and eating. Occasionally, homozygous animals also suffered from lethal seizures. In contrast to homozygous mice, heterozygous ones did not show any seizures during handling. Homozygous female mice displayed severe behavioral abnormalities during pregnancy and lactation, which included impaired nesting behavior, and neglecting or killing their own offspring. Therefore, all our breeding was done with heterozygous dams, which did not show abnormal behaviors during nursing.

The first paragraph will give an overview of the breeding statistics of the *Scn2a* (p.A263V) mouse line. Litters of prenatally treated pups are excluded from this analysis. The litter sizes did not vary greatly and gender distribution was homogeneous.

In the breeding for postnatal treatment two different breeding experiments were done. In the first one heterozygous female and heterozygous male mice were paired, while breeding of heterozygous female and homozygous male mice was used for the second experiment. The first litter analysis includes both breeding experiments (a total of 23 litters) and demonstrates litter distribution in general. Afterwards the two different breeding experiments will be separately observed and analyzed.

For the postnatal treatment analysis litters with six to eight pups were included (see Chapter 2.2.2.1). If containing more than eight pups, the litter was reduced to a number of eight pups. The surplus animals were randomly taken from the litter and given to a different nursing dam with less than eight pups. The following analysis of breeding includes all bred litters and their distribution before artificial alternation of the pup size.

When comparing the total of 23 litters the mean litter size was 7.8 ± 1.9 pups per litter, with a range of five to 13 pups per litter.

The distribution between female and male pups showed equal distribution with a mean number of 3.7 ± 1.4 females and 4.2 ± 1.5 males per litter (see Figure 10). For all experiments breeding was done as described in Chapter 2.2.1. Mice with a homozygous knock-in mutation are called mut/mut, while heterozygous mice are marked as wt/mut and wild type animals without any *SNC2A* mutation are called wt/wt.

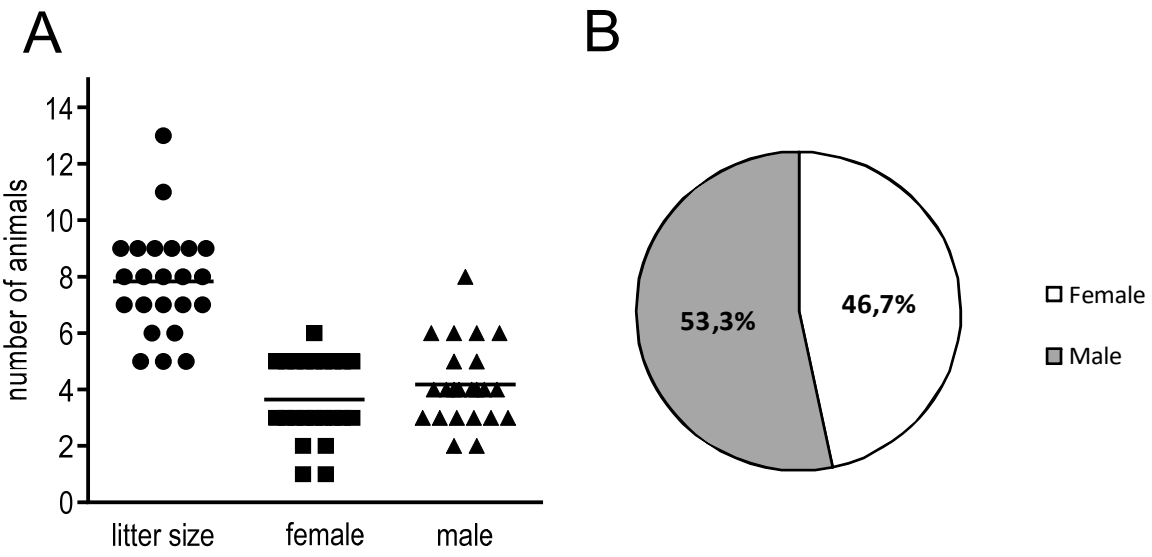

Figure 10: Characteristics of a total of 23 litters

Breeding of 23 litters with wt/mut female mice and wt/mut or mut/mut male mice. A: the scatter plot shows the mean litter size and mean sex distribution of litters in numbers of animals. B: The pie chart shows sex percentage over all litters.

For the first experiments, heterozygous female and male mice were used for breeding. According to the Mendelian genetics (Witkowski, 1976) breeding of two heterozygous mice produces three genotypes in the following ratio: 25% wildtype

(wt/wt), 50% heterozygous mutant (wt/mut) and 25% homozygous mutant (mut/mut). During the first experiment three litters were produced. The distribution of sex in these litters was 46.7% female to 53.3% male (see Figure 10). The genotypes were distributed as follows: 30.4% wildtype (wt/wt), 47.8% heterozygous (wt/mut) and 21.7% homozygous mutant (mut/mut) (see Figure 11).

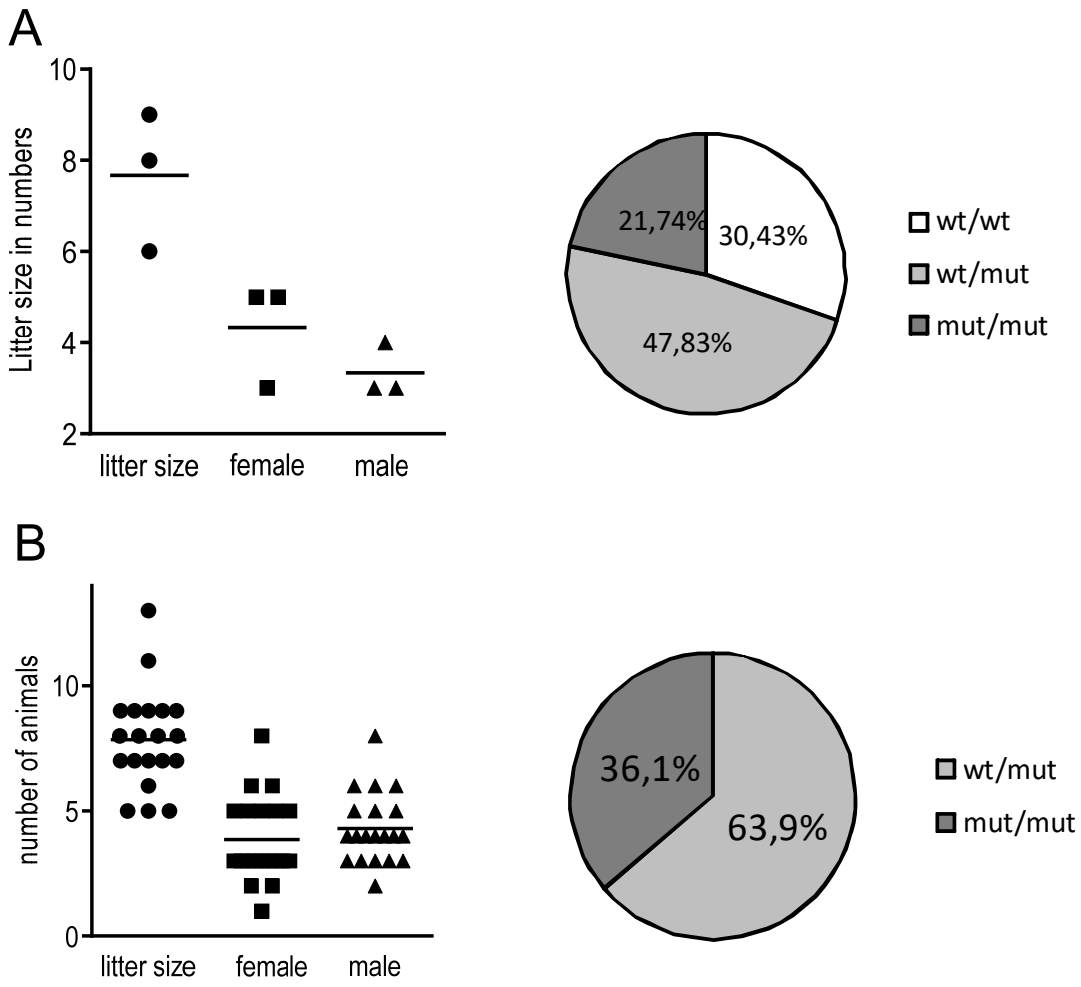

Figure 11: Litter distribution in different breeding groups

A: breeding of wt/mut females and wt/mut males showing the distributions of sexes and genotypes. *n* (litters)=3 B: breeding of wt/mut females and mut/mut males showing the distribution of sexes and genotypes. *n* (litters)=20

The breeding of heterozygous female with homozygous male mice resulted in a sex distribution of 45.2% female to 54.8% male, and the genotype distribution in the litters was 36.1% homozygous and 63.9% heterozygous *Scn2a* pups. Following the Mendelian genetics, such a breeding scheme should lead to heterozygous and homozygous offspring in a ratio of 1:1 (Witkowski, 1976).

The weight of the pups was measured at different time points: the weight on the first day of life (P1), weight before *in vivo* depth recordings at postnatal days six (P6) and postnatal days 14 or 15 (P14/15). Weight measurement was done to examine P1 weight differences between genotypes, which could indicate consequences of the genotype already being present *in utero* (see Figure 12). The mean P1 weight of heterozygous pups was 1.5 g ± 0.19 g and of homozygous pups 1.3 g ± 0.17 g. No data was obtained for wildtype animals at P1 because of the breeding scheme, which did not produce wildtype offspring.

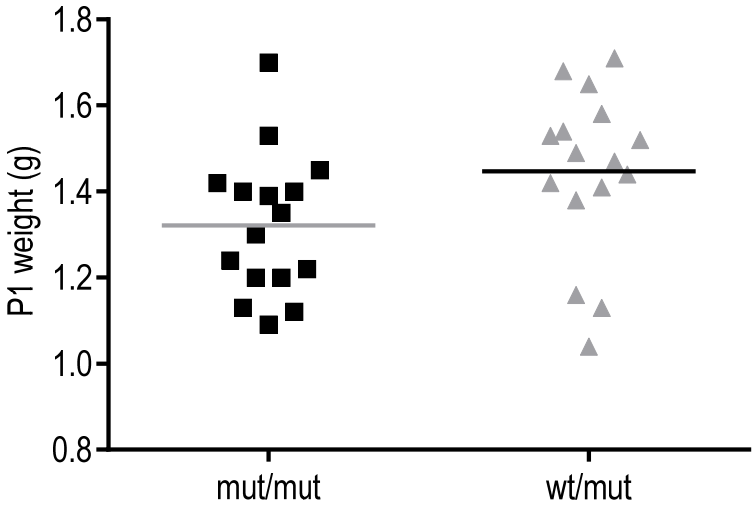

Figure 12: Weight comparison on P1 between the genotypes

32 animals were measured at the first day of birth. The weight distribution is shown in the scatterplot. The mean weight of wt/mut pups shows 1.5 g ± 0.19 g and of mut/mut pups 1.3 g ± 0.17 g. The minimum weight in wt/mut pups is 1.04 g compared to 1.09 g in mut/mut animals. The wt/mut same as the mut/mut pups show a maximum weight of 1.7 g. No statistical significance can be detected.

As the observation of the pups gave the impression of differences in size between homozygous pups and their littermates at around one week of age, weight was also measured at P6. Comparing the sex and the genotypes for differences in weight presented a mean weight at P6 of 4.1 g ± 0.63 g in wildtype, 3.4 g ± 0.26 g in heterozygous and 3.3 g ± 0.27 g in homozygous mutant pups. No significant difference could be detected but a trend towards lower body weights in hetero- and homozygous mutant mice was observed. Significant results in weight differences between the genotypes were seen in another dataset generated by Dr. Fabio Morellini and Francesca Xompero, PhD (see Chapter 1.6)

To investigate the phenotype further, brain activity was measured and compared between the genotypes, and is described in the following chapter.

3.2 Analysis of hippocampal network activity in *Scn2a*(p.A263V) neonates

To investigate the hippocampal network activity in the *SCN2A* mouse model *in vivo*, depth-profile recordings along the CA1-dentate gyrus axis were performed as described above (see Chapter 2.2.3) in 15 pups of three different genotypes (four wt/wt, five wt/mut, six mut/mut).

Investigating the brain activity involved screening all animals for physiological hippocampal network activity (Marguet *et al.* 2015) as well as for seizure-like events. The analysis of the *in vivo* depth recordings showed that seizures occurred in both heterozygous and homozygous mutant mice but not in wildtype control mice. The seizures were then analyzed to investigate the electrophysiological as well as the clinical manifestation of the seizure, and to determine an exemplary pattern with which all seizures in further experiments could be marked and analyzed. To quantify the synaptic inputs the neonatal CA1 region receives form EC and CA3, all recording files were screened for oscillatory activity, e.g., hippocampal beta activity (HCBeta, see Figure 13) and LFP patterns that differed from the physiological HCBeta activity, such as seizures. All eletrographic seizure events were compared and similarities used to determine the characteristics of these events, we then categorized as seizures.

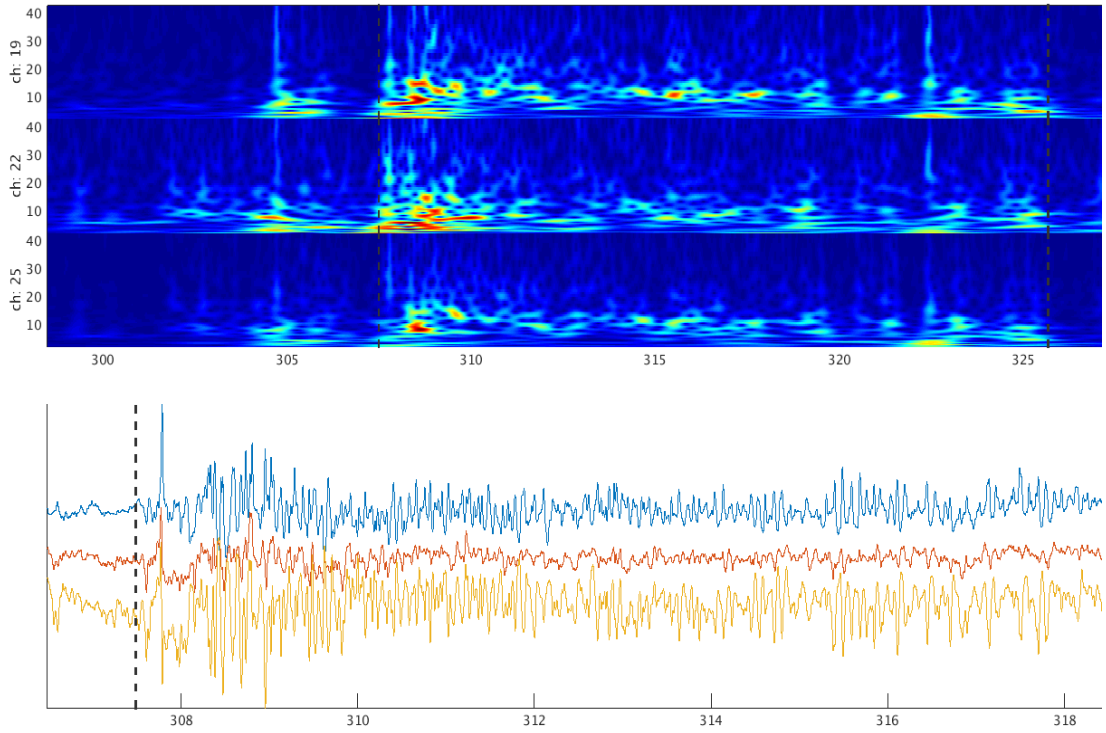

Figure 13: Spectrogram of an *in vivo* depth recording of hippocampal Beta activity (HCBeta) in a P7 *Scn2a* homozygous mutant mouse

HCBeta is shown as current source density (CSD) in the spectrogram and below as local field potential. The spectrogram shows three channels of the 16-channel electrode. Channel 22 represents the 6th channel of this electrode and the stratum pyramidale of the CA1 hippocampal region. (Spectrograms kindly generated by Stephan Marguet, PhD)

All seizures showed a stereotypic local field potential (LFP) pattern. Every event showed an increasing activity wave complex in the beginning, repetitive bursting in the middle of the seizure and a short period of reduced activity in the end. A seizure was characterized as a period consisting of the wave complex and the burst activity. The beginning and the end of a seizure could be well determined by the beginning of the wave complex and the short non-active, time after the event (see Figure 14). Using this classification all following analyses and calculations were produced.

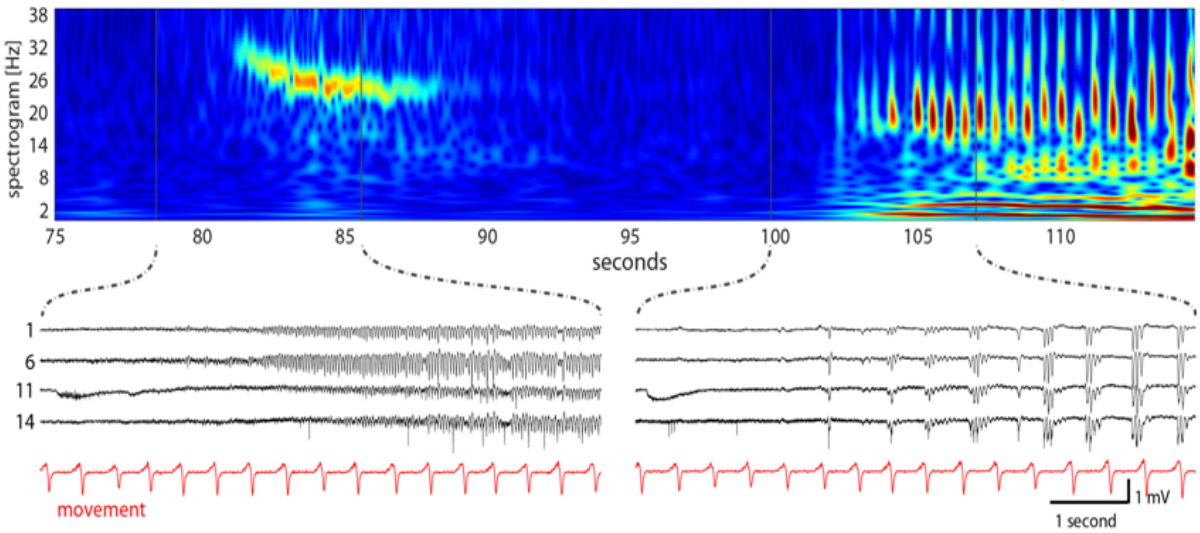

Figure 14: Spectrogram of an *in vivo* depth recording in the dorsal hippocampus CA1 of a P7 homozygous mutant mouse.

Spectrogram showing the frequency range of the seizure activity ranging mostly between 15 and 35 Hz. Below the spectrogram the csd. The red movement channel recorded the breathing rhythm of the mouse. The first part shows the wave complex followed by a short pause and then by the bursting period

The wave complex has a specific form of a curve (see Figure 15), during which the frequency increased from a beta activity of an observed mean frequency of about 20 Hz to gamma frequency of about 40 Hz. The mean duration of this activity was 17.4 seconds ranging from 14.5 to 31.3 seconds. This complex could be observed before each bursting activity. No seizure like episode was observed without a wave complex announcing the following bursts.

Following the wave complex the seizures continue with high-amplitude slow sharp-wave activity with superimposed repetitive bursts of oscillations at a mean frequency of about 30 Hz (see Figure 16). The interval between the sharp wave/burst activity is becoming increasingly longer towards the end of the seizure until the seizure stops.

The seizure activity was usually followed by a short period of no activity – a so-called postictal state. Then the physiological hippocampal beta activity reappeared and continued until interrupted by the next seizure.

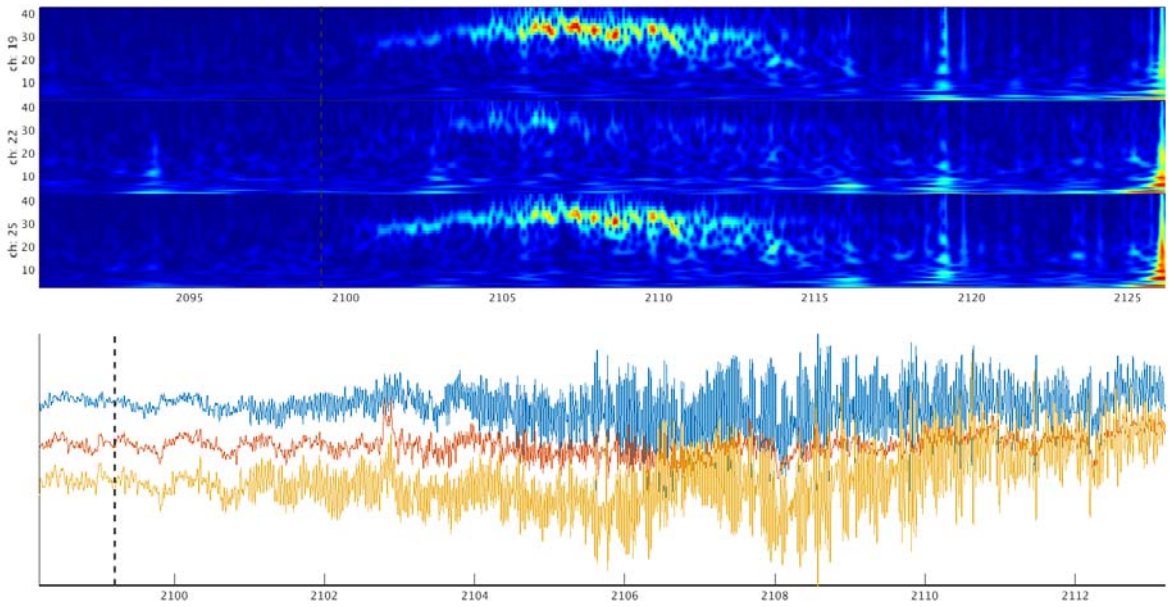

Figure 15: Spectrogram of an *in vivo* LFP recording from the hippocampal region of a *Scn2a* (p.A263V) homozygous mutant pup at P7 at the beginning of a seizure

Spectrogram of the beginning of a seizure in a mut/mut pup at P7 showing three channels of a 16-channel recording in the hippocampus. The spectrogram with the CSD and LFP below shows the typical curve form at the beginning of a seizure.

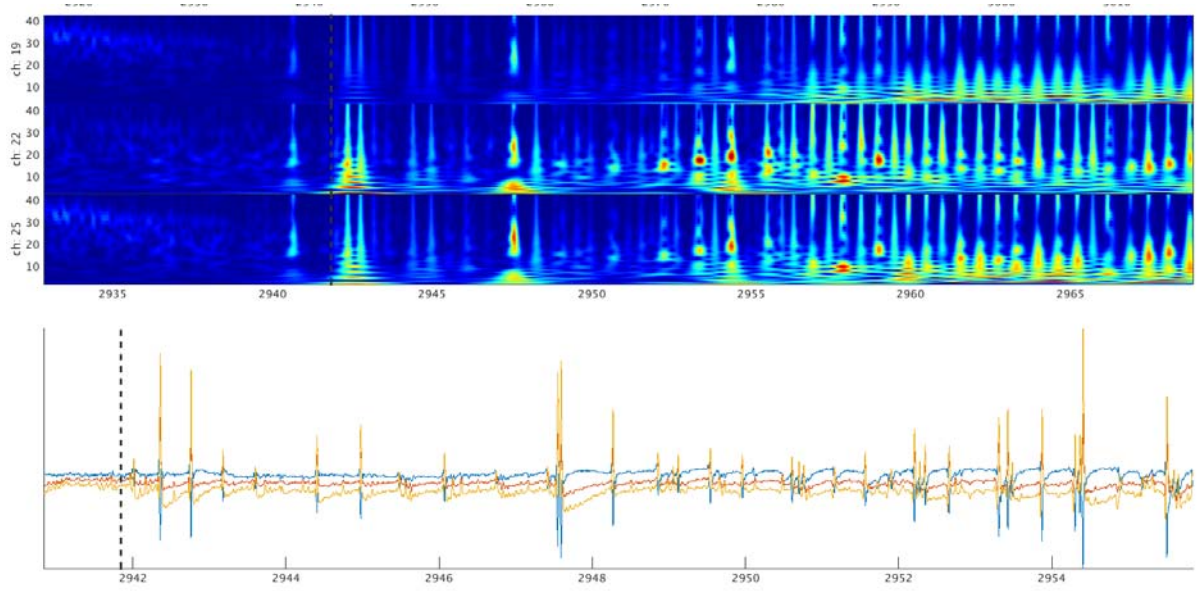

Figure 16: Spectrogram of *in vivo* LFP recording from the hippocampal region of a *Scn2a* (p.A263V) homozygous mutant pup at P7 during a seizure

Spectrogram of the bursting phase in a mut/mut pup at P7 showing three channels of a 16 channel recording in the hippocampus. The spectrogram with the csd and LFP below shows the single bursts in the middle of a seizure.

Once the seizures were identified, the seizure rate and seizure duration of the three different genotypes were determined and compared. The control group (wt/wt) consisted of four wild type animals, two females and two males. The heterozygous group consisted of five pups, three females and two males. The homozygous mut/mut group consisted of six female pups. One wt/mut and one mut/mut pup died during recording. Before and after each recording, the genotypes were double-checked as described in chapter 2.2.2.

The wild type animals showed no seizure activity at all. One of five heterozygous pups showed seizure activity and all six animals from the homozygous group showed seizure activity during the recordings. The seizure rate in homozygous animals was significantly higher than in the control group with $p < 0.01$ and in the heterozygous group with $p < 0.05$ (see Figure 17).

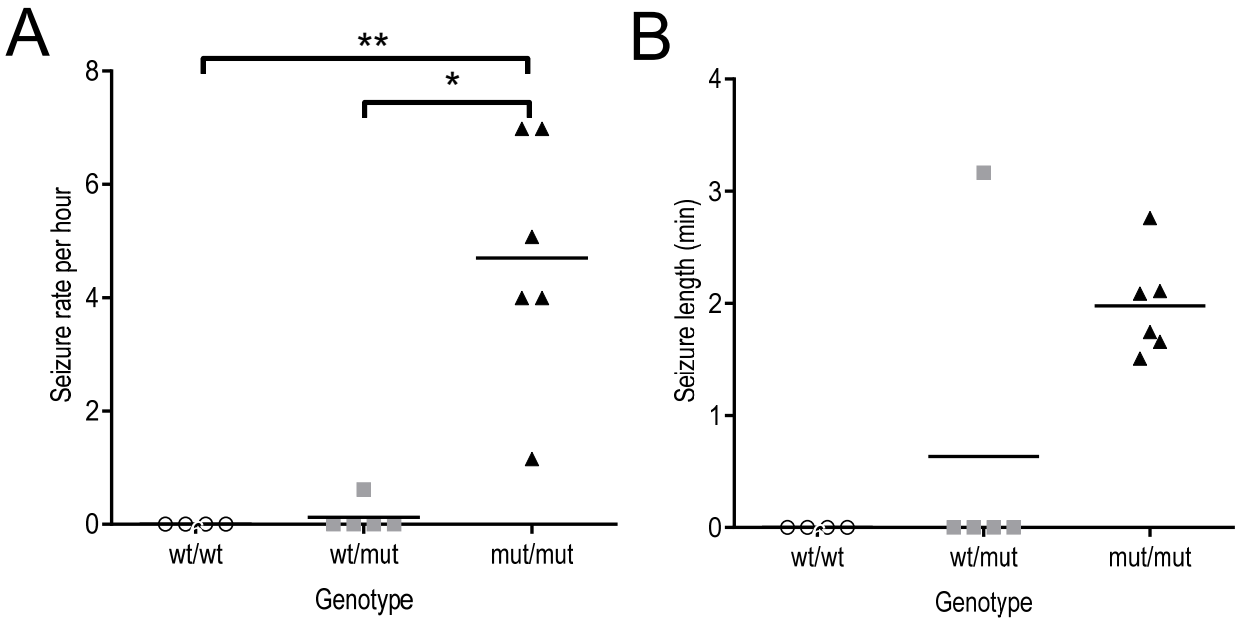

Figure 17: Hippocampal brain activity in untreated *Scn2a* pups at the age of P6/P7

A: Seizure rate per hour in comparison of the three genotypes: The amount of seizures in the mut/mut group was ranging from 1.15 seizures to 6.98 seizures per hour, the mean seizure rate being 4.7 ± 2.20 seizures per hour. The only heterozygous animal in which seizure activity was detected had a seizure rate of 0.61 per hour.

B: Seizure length in minutes in comparison of the three genotypes: The seizure length varied substantially between 1.51 minutes and 3.17 minutes.

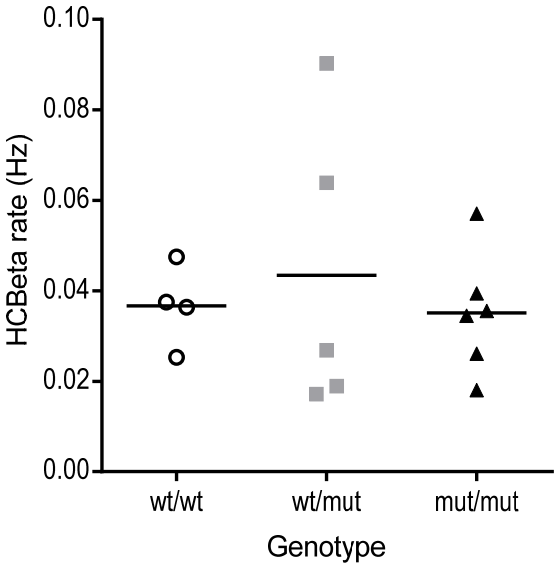

Figure 18: Hippocampal beta rate (HCBeta) in Hz compared between three genotypes.

Scatterplot of the HCBeta rate in Hz in comparison of the three genotypes: the mean HCBeta rate in wildtype pups is 0.037 ± 0.010 Hz compared to 0.043 ± 0.032 Hz in heterozygous pups and 0.035 ± 0.013 Hz in homozygous pups. HCBeta activity shows no significant difference between the genotypes.

No significant difference in HCBeta rate between the genotypes could be detected (see Figure 18). The comparison between the three genotypes showed a strong seizure phenotype in the homozygous mice and a possible intermediate phenotype in the heterozygous mice. The next question addressed was whether the seizure frequency could be altered by antiepileptic treatment.

3.3 Analysis of pharmacological treatments of *Scn2a* pups

3.3.1 Litter analysis

For the pharmacological pilot experiments we focused on the heterozygous and homozygous genotypes. In the treatment trial, the primary outcome measure was whether a clear reduction in seizure rates could be achieved. Specifically, as every naïve mut/mut mouse had seizures, we asked whether prophylactic pharmacological treatment could prevent mut/mut mice from developing a seizure phenotype.

At the first day of life the weight of the pups was measured and compared. The weight was compared between the two genotypes wt/mut and mut/mut as well as

between the treatment groups: bumetanide, retigabine, phenobarbital and vehicle. There was no difference between the treatment groups (see Figure 19). At P1 there seemed to be a trend towards a reduced body weight in homozygous mutant pups without any statistical significance.

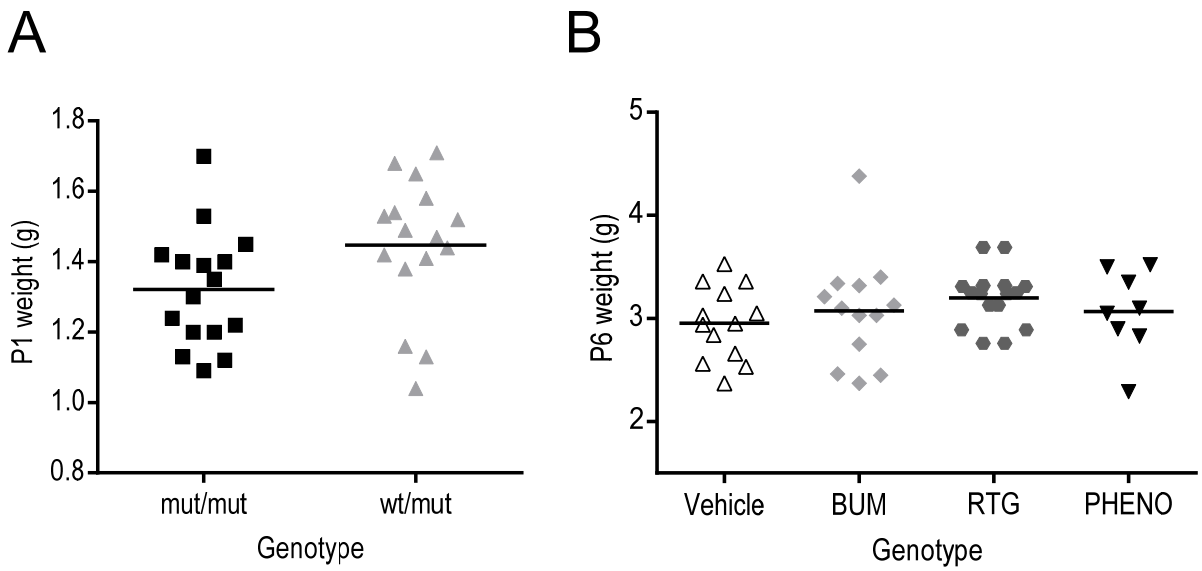

Figure 19: Weight analysis of treated *Scn2a* pups

A: Mean weight at P1: comparison between the genotypes without statistical significance. The mean weight of heterozygous pups was 1.5 ± 0.19 g, while the mean weight of homozygous pups was 1.3 ± 0.17 g.

B: Mean weight at P6: comparison between the treatment groups showed no significant difference. The mean weight at P6 of the vehicle treated pups was 2.96 ± 0.36 g compared to 3.08 ± 0.53 g in the bumetanide treated group, 3.20 ± 0.27 g of the retigabine treated pups and 3.07 ± 0.41 g of the phenytoin treatment group. No significant difference between the treatment groups concerning weight at P6 could be observed.

3.3.2 Treatment: Phenobarbital

As described above, phenobarbital is an AED commonly still used in the clinic today although its inefficacy in improving the long-term outcome in epileptic children. We investigated its effect on *Scn2a* mutant and heterozygous pups treated from P1 to P7 compared to the control group. Three mut/mut pups (one female, two male) and one wt/mut (female) were treated with phenobarbital. All mut/mut mice, both in the control and the treatment group showed seizures (100%) (see Figure 20).

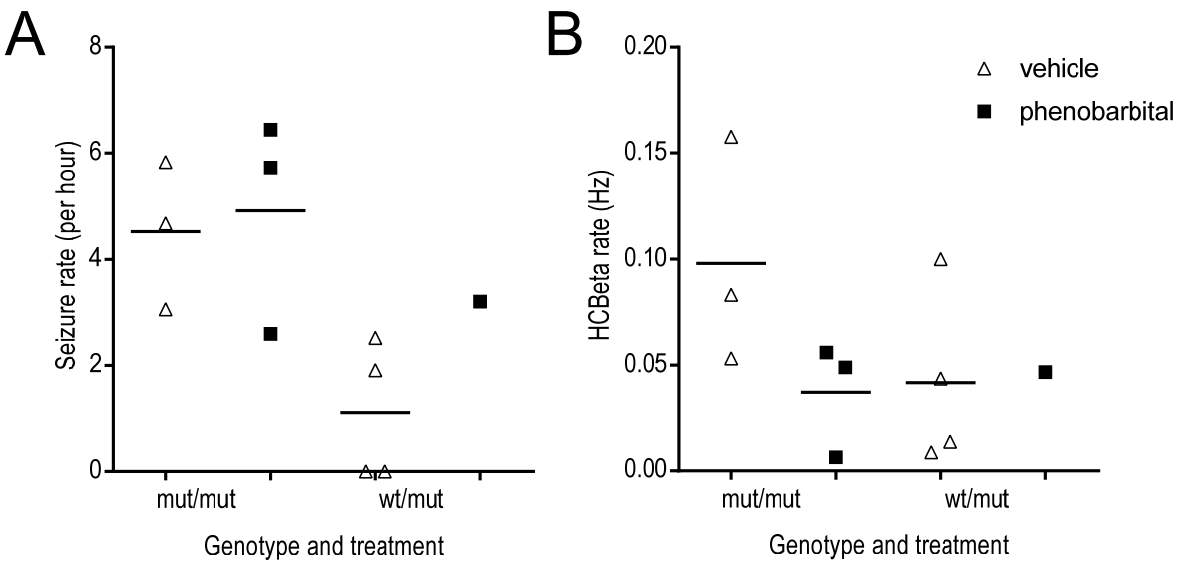

Figure 20: Phenobarbital treatment versus vehicle treatment in *Scn2a* pups

A: Seizure rate per hour in vehicle and bumetanide treated pups at P6/P7. The one treated heterozygous pup showed a seizure rate of 3.2 seizures per hour, compared to 50% of the heterozygous untreated pups showing seizure activity with a mean seizure rate of 1.1 ± 1.3 seizures per hour. The mean seizure rate of the homozygous treated mice was 4.9 ± 2.05 seizures per hour compared to the seizure rate of 4.5 ± 1.39 seizure per hour of the control group.
B: HCBeta rate in Hz in vehicle and bumetanide treated pups at P6/P7. The heterozygous mouse treated showed a HCBeta rate of 0.046 Hz compared to the control wt/mut mice with a HCBeta rate of 0.042 ± 0.042 Hz. The mean HCBeta rate in mut/mut treated mice was 0.037 ± 0.027 Hz compared to 0.098 ± 0.045 Hz in mut/mut untreated mice.

3.3.3 Treatment: Bumetanide

Bumetanide was tested in five wt/mut animals (four male and one female pup) and three mut/mut animals (one female and two male), and then compared to the control group described above. The seizure rate, determined by acute *in-vivo* depth recordings from the dorsal hippocampus at P7, showed no significant difference between the treated and the control group. 100% of the mut/mut untreated pups and 100% of the mut/mut treated pups showed seizure activity. 50% of the heterozygous untreated pups presented seizures as well as 40% of the wt/mut treated pups (see Figure 21). The HCBeta rate was normalized to the seizure free time and showed, no significant difference between the treatment and the genotypes.

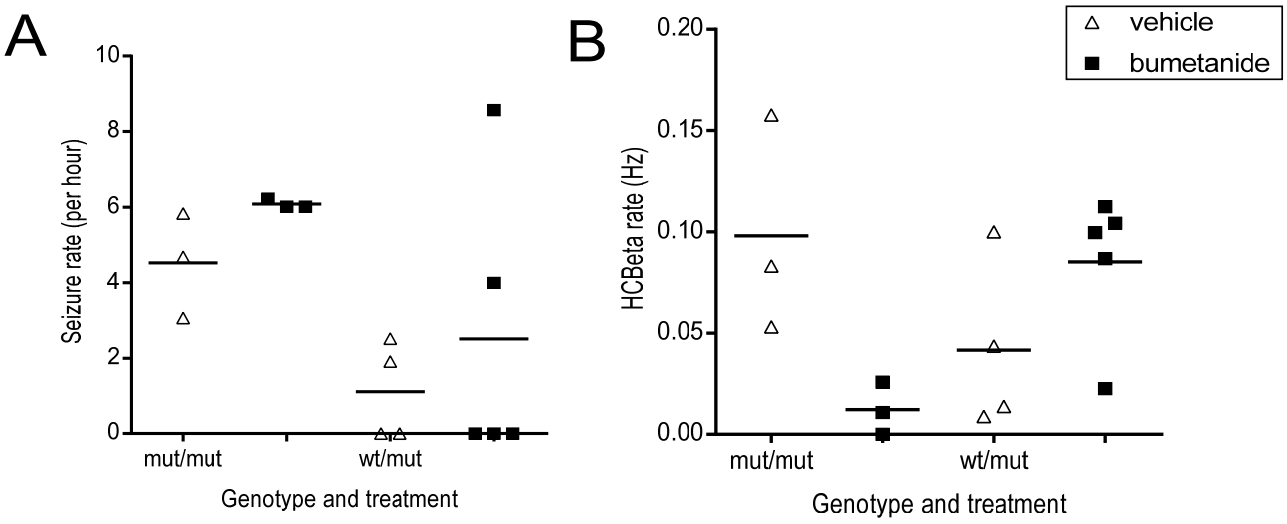

Figure 21: Bumetanide treatment versus vehicle treatment in *Scn2a* pups

A: Seizure rate per hour in vehicle and bumetanide treated pups at P6/P7: the homozygous (mut/mut) treated pups showed an average of 6.1 ± 0.12 seizures per hour compared to the untreated mut/mut mice with a mean seizure rate of 4.5 ± 1.39 seizures per hour. The mean seizure rate of the heterozygous (wt/mut) pups treated with bumetanide was 2.5 ± 3.8 seizures per hour compared to 1.1 ± 1.3 seizures in the wt/mut control group. There was no statistical significance observed.
B: HCBeta rate in Hz in vehicle and bumetanide treated pups at P6/P7: the mean HCBeta rate of the homozygous treated animals was 0.012 ± 0.013 Hz compared to 0.098 ± 0.045 Hz in the control group. The wt/mut pups under bumetanide treatment showed a mean HCBeta rate of 0.085 ± 0.036 Hz compared to 0.042 ± 0.042 Hz in the vehicle-treated wt/mut pups. No statistical significance was obtained.

3.3.4 Treatment: Retigabine

The subcutaneous treatment with retigabine was done with six wt/mut pups (two female and four male) and three mut/mut pups (two female and one male). 100% of the untreated homozygous mutant pups showed seizures, while 33.33% of the mut/mut pups treated with retigabine showed no seizures at all (2 out of 3 animals presented seizures). 50% of the wt/mut untreated mice showed seizures and 16.67% of the treated wt/mut pups (one out of six pups) presented seizures (see Figure 22). No significant difference could be observed in the HCBeta rate between the treatment groups.

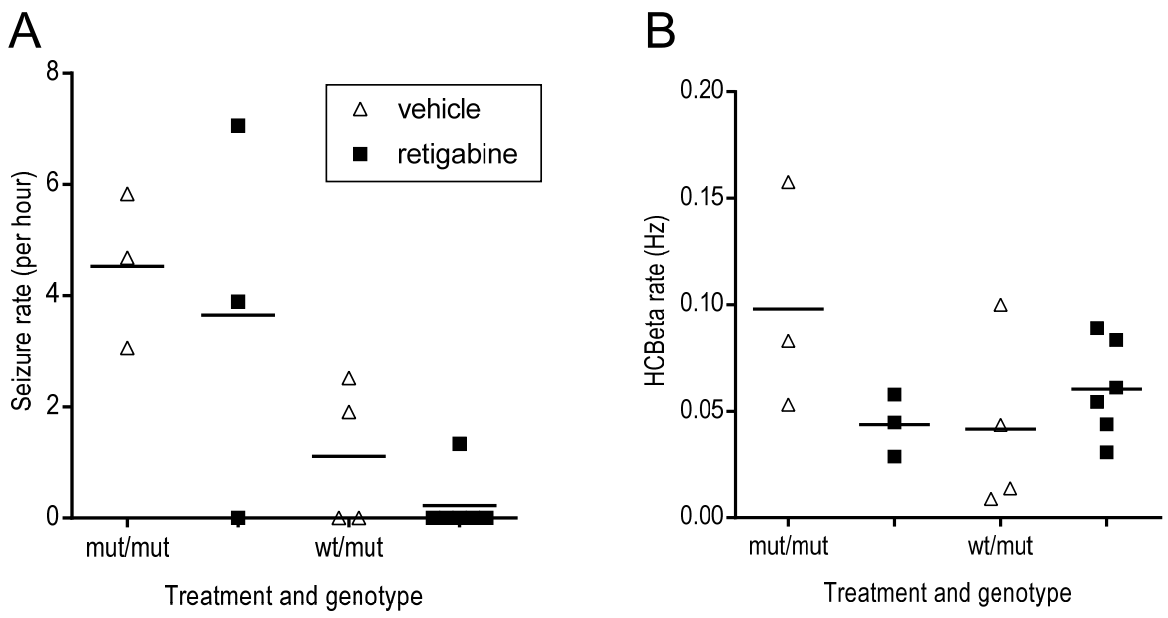

Figure 22: Retigabine treatment versus vehicle treatment in *Scn2a* pups

A: Seizure rate per hour in vehicle and bumetanide treated pups at P6/P7. The mean seizure rate of the mut/mut retigabine-treated pups was 3.7 ± 3.53 seizures per hour compared to the control group with 4.5 ± 1.39 seizures per hour. The heterozygous mice treated showed a mean seizure rate of 0.2 ± 0.54 seizures per hour while the control group showed an average of 1.1 ± 1.30 seizures per hour. No statistical significance was obtained.
B: HCBeta rate in Hz in vehicle and bumetanide treated pups at P6/P7. The mut/mut mice treated with retigabine showed a mean HCBeta rate of 0.044 ± 0.015 Hz compared to 0.098 ± 0.054 Hz in the control group. In the heterozygous treated mice the mean HCBeta rate was 0.06 ± 0.023 Hz compared to 0.042 ± 0.042 Hz in the untreated pups. There was no statistical significance observed.

3.3.5 Treatment: Phenytoin

Subcutaneous treatment

The number of homozygous mutant pups in the vehicle group as well as in the treatment group consisted of two pups per group. The heterozygous vehicle group contained four pups and the heterozygous treatment group consisted of two pups. The distribution among the litters concerning genotype and sex was comparable to the litter analysis described above. The weight of the pups in the phenytoin treatment group showed no difference to the weights measured in the previous treatment groups. Since the subcutaneous treatment showed severe side effects (severe skin necrosis (Chapter 2.2.3.2) the trial had to be stopped and only a few animals had

been measured until this point. Compared to the control group none of the phenytoin-treated mut/mut mice showed seizure activity, while all of the vehicle-treated mut/mut mice presented seizures. The treated heterozygous pups showed no seizures as well, compared to 50% of the untreated wt/mut pups showing seizures (see Figure 23).

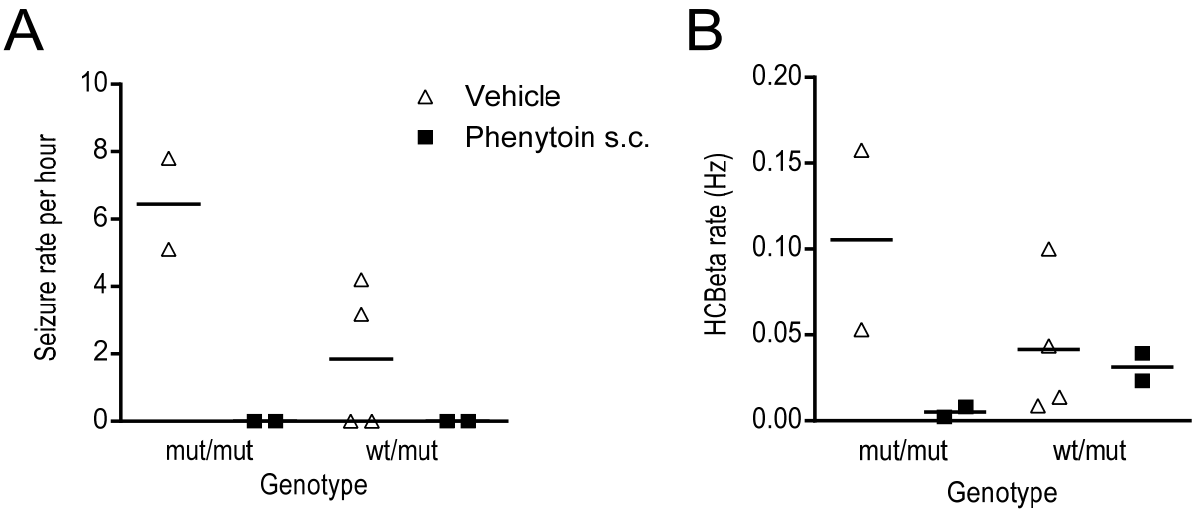

Figure 23: Phenytoin subcutaneous treatment

Phenytoin presented an observable effect in seizure rate comparing the mutant treated versus mutant vehicle group, as well as the heterozygous treated against the heterozygous untreated group. No statistical significance was observed.
A: Mean seizure rate per hour measured in pups at P6/P7. The mut/mut pups treated with phenytoin presented no seizures at all. (Seizure rate 0 ± 0 seizures per hour). The homozygous pups in the vehicle group presented a mean of 6.45 ± 1.91 seizures per hour. The heterozygous treated pups had no seizures during the recording period while the heterozygous pups of the vehicle group presented a mean of 1.85 ± 2.17 seizures per hour.
B: Mean HCBeta rate in Hertz measured in pups at P6/P7. The mut/mut pups in the treatment group had a mean HCBeta rate of 0.005 ± 0.004 Hz and in the vehicle group 0.105 ± 0.073 Hz. Heterozygous pups of the treatment group showed a mean rate of 0.031 ± 0.011 Hz and the vehicle wt/mut pups showed a mean rate of 0.042 ± 0.042 Hz.

As the side effects of s.c. phenytoin injection were not acceptable, we decided to continue phenytoin treatment via drinking water application, since the subcutaneous treatment showed a treatment trend in both heterozygous and homozygous mutant pups.

Treatment via drinking water

To avoid the severe side effects in subcutaneous treatment with phenytoin we treated a new group of pups with phenytoin via the drinking water of the nursing dams (see chapter material and methods). The treatment group consisted of eight wt/mut and seven mut/mut pups. The control group contained seven wt/mut and nine mut/mut pups. No difference in litter distribution of genotype or sex could be observed compared to the previous litter analysis.

All mutant control mice experienced seizures. In the mutant treatment group one of the seven pups showed no seizure activity. In the control group of the heterozygous pups one out of seven, and none of the eight treated wt/mut pups showed seizure activity. The mean seizure rates compared between the treatment and control group showed no significant difference (see Figure 24). The HCBeta rate showed a similar distribution of activity between the treatment and genotype groups and no significant difference could be observed.

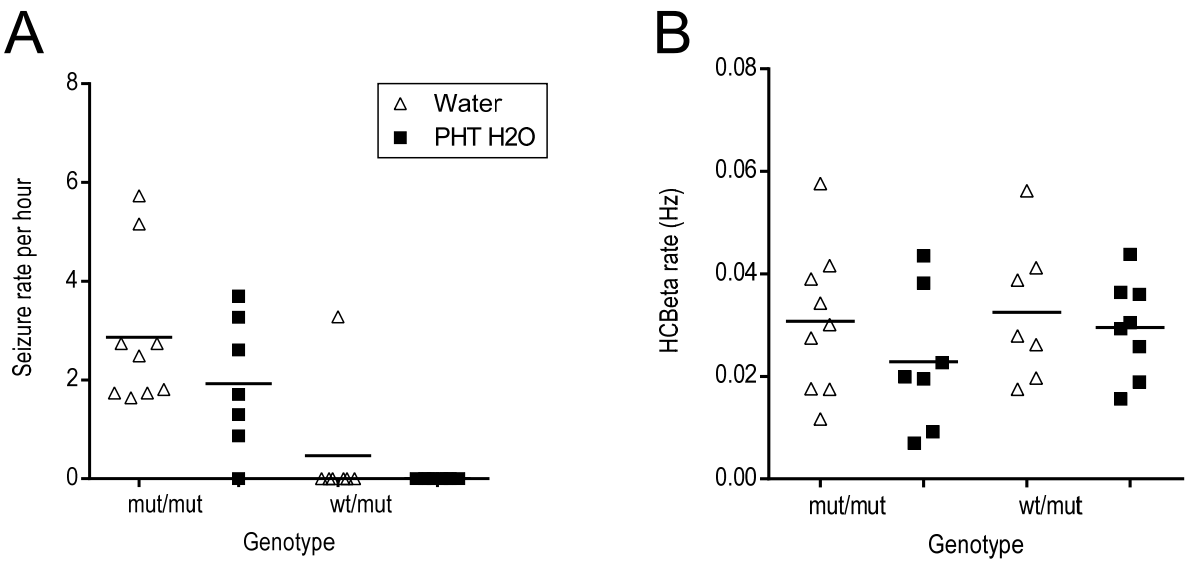

Figure 24: Phenytoin treatment via drinking water from P1 to P7

Phenytoin treatment showed no significant reduction of seizure rate comparing the treatment groups.
A: Mean seizure rate per hour measured in pups at P6/P7. The mean seizure rate was 1.92 ± 1.33 seizures per hour in the mutant treatment group compared to 2.9 ± 1.53 seizures per hour in the mutant control mice. The treated heterozygous mice showed a mean seizure rate of 0 ± 0 seizures per hour, compared to the untreated heterozygous pups which showed a mean seizure rate of 0.47 ± 1.24 seizures per hour.

B: Mean HCBeta rate in hertz measured in pups at P6/P7. The mean HCBeta rate in the mutant pups who received phenytoin via drinking water is 0.023 ± 0.014 Hz compared to the mean rate of 0.031 ± 0.014 Hz in mutant pups receiving water. In the heterozygous phenytoin treated pups the mean HCBeta rate was 0.030 ± 0.010Hz compared to 0.033 ± 0.014 Hz in the heterozygous control group

Double-dosage phenytoin treatment via drinking water

Since two of the mutant pups in the subcutaneous and one mutant pup in the drinking water treatment group showed no seizure activity, which could not be observed in any mutant control group, we decided to do a second treatment trial with phenytoin in a doubled concentration in drinking water of the nursing dams. Nine pups formed the mutant vehicle group and seven pups the heterozygous vehicle group. The treatment group consisted of six mut/mut and three wt/mut pups. The distribution of genotype and sex in the litters showed no significant difference to the previously analyzed litters.

All nine mut/mut pups of the control group showed seizure activity (100%). Five of the six treated mutant mice showed seizure activity (83.34%). Statistically no significance could be observed (see Figure 25). One of the seven heterozygous pups in the control group presented seizures compared to none of the three pups in the treated heterozygous group. The HCBeta activity showed a slightly higher rate in treated heterozygous pups but in total no significant difference could be observed.

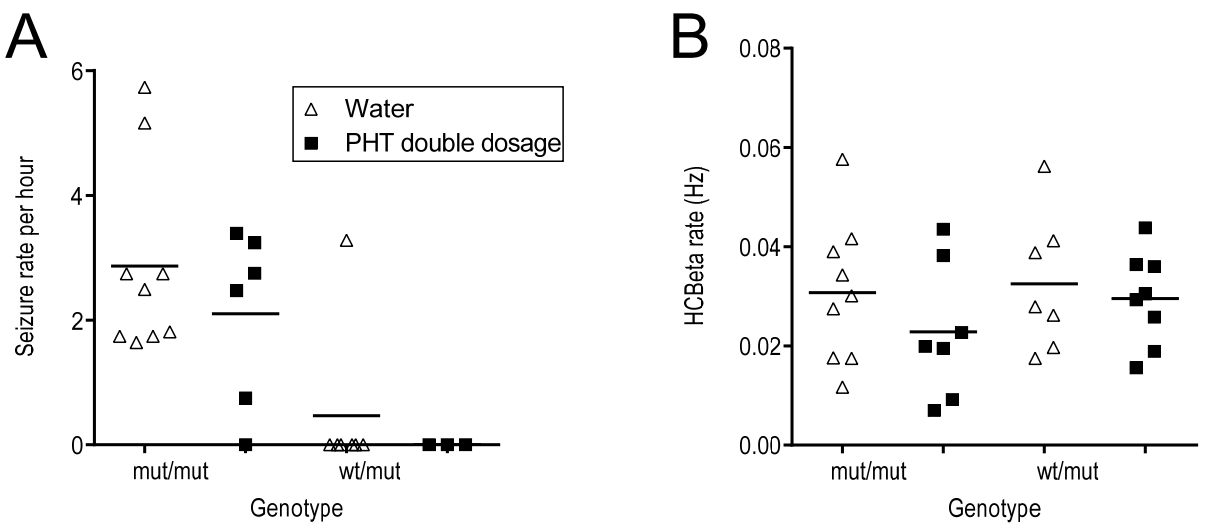

Figure 25: Double dosage phenytoin treatment via drinking water from P1 to P7

No statistical significance between treatment groups of each genotype.
A: Mean seizure rate per hour of phenytoin treated pups at P6/P7. The mean seizure rate of mut/mut untreated pups was 2.87 ± 1.53 seizures per hour compared to the mean seizure rate of 2.10 ± 1.40 seizures per hour in the mut/mut treated pups. The heterozygous control group showed a mean seizure rate of 0.47 ± 1.24 seizures per hour and the heterozygous treatment group a mean seizure rate of 0 ± 0 seizures per hour.
B: Mean HCBeta rate in Hertz of phenytoin treated pups at P6/P7. The mutant water receiving group showed a mean HCBeta rate of 0.03 ± 0.014Hz compared to the mutant treated pups with a mean rate of 0.03 ± 0.010 Hz. The heterozygous water treated group had a mean HCBeta rate of 0.03 ± 0.014 Hz compared to a mean HCBeta rate of 0.06 ± 0.017 Hz in the wt/mut pups phenytoin.

Prenatal phenytoin treatment via drinking water

The breeding and the litter analysis of litter size, sex and genotype distribution showed no deviation to the previous litter analysis results. The weight at P6 showed differences between the prenatal treatment group and the control group. The mean weight at P6 in untreated pups was 3.3 g (mut/mut) and 3.3 g (wt/mut) compared to the treatment group with a mean weight of 2.8 g (mut/mut) and 2.9 g (wt/mut). The difference between the heterozygous control and treatment group was very significant with a *p*-value of 0.0079. At P7 the difference between the mutant vehicle and the mutant prenatal treatment group varied very significantly with a mean weight of 3.7 g (vehicle) to 2.9 g (PHT) and a *p-value* of 0.0016. The heterozygous mean

weight at P7 also differed significantly between the control pups (4.5 g) and the treatment pups (3.4 g) (*p-value* = 0.0179) (see Figure 26).

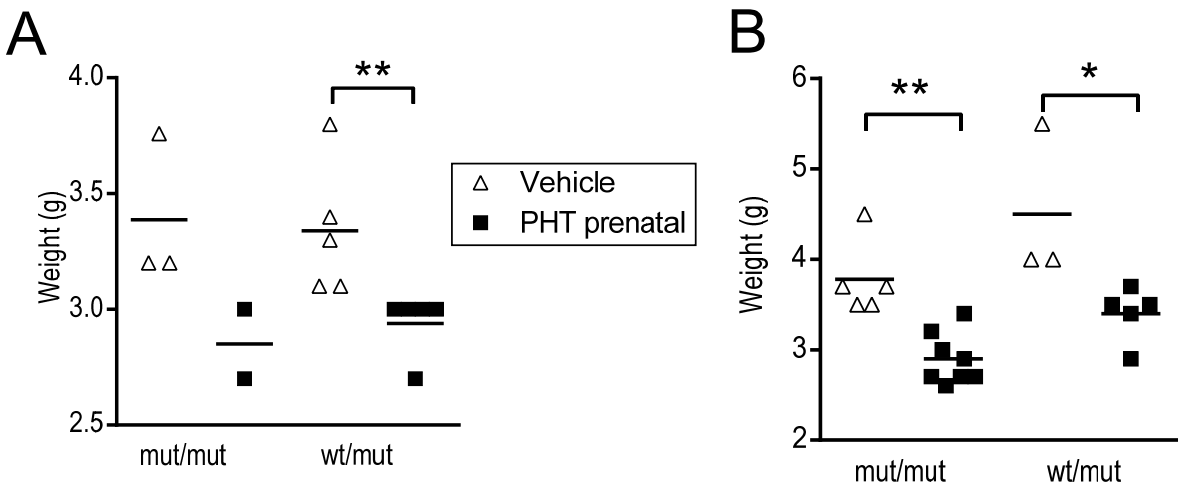

Figure 26: Prenatal phenytoin treatment: weight at P6/ P7

A: Weight at P6 in comparison between the genotypes and treatment groups. The mut/mut water treated pups showed a mean P6 weight of 3.39 ± 0.32 g compared to the phenytoin treated mut/mut pups with 2.85 ± 0.21 g. The weight presented a significant difference between the wt/mut control group and the phenytoin wt/mut group with 3.34 ± 0.29 g compared to 2.94 ± 0.13 g with $p = 0.0079$.
B: Weight at P7 in comparison between the genotypes and treatment groups. The mut/mut pups receiving water showed a mean P7 weight of 3.78 ± 0.41g, the phenytoin treated mut/mut pups showed a mean weight of 2.90 ± 0.28g, the difference being significant with a *p*-value of 0.0016. The wt/mut control group presented a mean weight of 4.50 ± 0.87 g compared to the phenytoin wt/mut group with 3.4 ± 0.30 g, which shows a statistical significance with $p = 0.0179$.

The seizure rate between the treatment group and the vehicle group showed no significant difference. In comparison to the postnatal treatment no significance could be detected (see Figure 27). The HCBeta rate in prenatal treatment showed a significant difference in both genotypes. Compared to the postnatal treatment the prenatal HCBeta rate shows a lower rate.

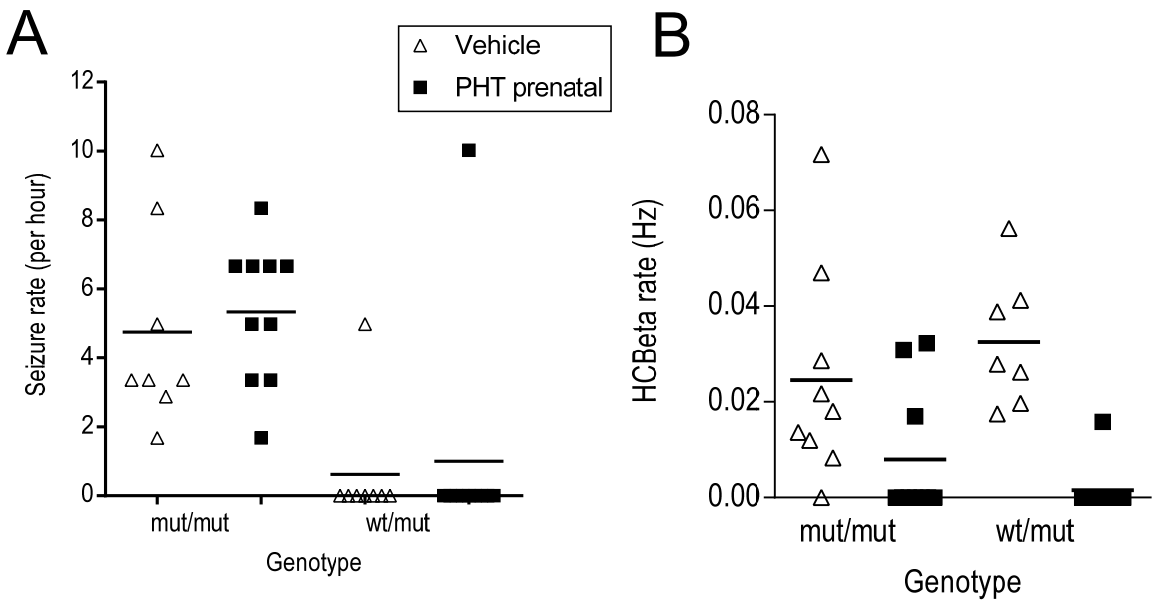

Figure 27: Prenatal phenytoin treatment: seizure rate and HCBeta rate

Prenatal treatment with phenytoin showed no effect on seizure rate or HCBeta rate compared between the treatment groups in neither heterozygous nor mutant pups.
A: Mean seizure rate per hour of prenatally treated pups measured at P6/P7. The treated mutant pups showed a mean seizure rate of 5.3 ± 2.04 seizures per hour, while the untreated mut/mut pups presented a mean seizure rate of 4.7 ± 2.91 seizures per hour. The mean seizure rate of the heterozygous treated pups is 1.0 ± 3.17 seizures per hour compared to 0.62 ± 1.67 seizures per hour in untreated heterozygous pups.
B: Mean HCBeta rate in Hertz of prenatally treated pups measured at P6/P7. 0.008 ± 0.013 Hz is the mean HCBeta rate in treated mutant pups compared to 0.025 ± 0.022 Hz in untreated homozygous pups. The heterozygous pups in the treatment group presented a mean rate of 0.002 ± 0.005 Hz and the untreated heterozygous pups showed a mean HCBeta rate of 0.033 ± 0.014 Hz.

4 Discussion

4.1 Summary of results

At the age of six days, homozygous mice showed a lower body weight than heterozygous - or wildtype litter mates. *In vivo* electrophysiological recordings of neonatal *Scn2a*(p.A236V) mice enabled us to detect a strong epileptic phenotype in this genetic mouse model. A difference in body weight gain of the pups could also be shown. Homozygous pups showed a complete penetrance of the epilepsy phenotype with recurring seizures in 100% of the animals. The heterozygous genotype presented itself with an intermediate phenotype and incomplete penetrance. During the recording periods of up to one and a half hours, about 20% of the heterozygous animals presented seizures at the neonatal age of seven days. Thus, compared to the wildtype control mice, which did not show any signs of electrographic seizures at P7, the homozygocitiy for the *Scn2a*(p.A236V) allele had a significant effect on seizure rates in these pups. Furthermore, a significant difference in seizure occurrence between homozygous and heterozygous *Scn2a* pups could also be shown. The temporal structure of a seizure was very stereotyped in naïve animals and throughout all treatment trials, which allowed reliable detection and classification of the seizures.

We could demonstrate that the *Scn2a* mice show a strong and highly penetrating epileptic phenotype and therefore present a valid mouse model for neonatal epilepsy. These findings confirm the first hypothesis posed in my thesis:

> “The *Scn2a*(p.A236V) mutation leads to altered brain activity in neonatal mice compared to wildtype mice.”

The second hypothesis stated:

> “Epileptic patterns in *Scn2a* mutant neonatal mice can be prevented via pharmaceutical treatment.”

Pharmacological treatment proved to be difficult. In this pilot study with a limited number of treated animals, we could not show any statistically significant effect of the treatments used. No effect of bumetanide or phenobarbital subcutaneous postnatal treatment could be detected. In retigabine postnatal subcutaneous treatment we could see a slight trend of seizure rate reduction in homozygous mice although without any statistical significance. In subcutaneous postnatal phenytoin treatment,

both homozygous treated mice did not present any seizure activity while all other homozygous control mice showed seizures (100%). This could be an indicator for a treatment effect. In addition, also none of the two heterozygous treated mice showed any seizure activity. Since the number of animals in this treatment trial was very low (two mice per genotype in the phenytoin treatment group), there was no statistical significance. Since the severe side effects led to an early stop of the trial, we continued phenytoin treatment via drinking water. We could show a slight reduction of seizure rate as sign of a treatment effect in the postnatal phenytoin administration via drinking water, though no significance was obtained. Since subcutaneous administration seemed to promise a strong effect, it is likely that the dosage of phenytoin the pups received via the maternal drinking water did not suffice for a steady treatment effect. Therefore we doubled the concentration of phenytoin in the drinking water. Also, the doubling of the phenytoin concentration in the water did not achieve any significant effect. For the potential limitations of the data obtained via drinking water experiments see chapter 4.4. Prenatal phenytoin treatment of the pups did not show any effect on seizure activity at all.

4.2 Pharmacological treatment

4.2.1 Pharmacological treatment in the *Scn2a*(p.A263V) mouse model

As described above, we observed a strong trend of seizure reduction in the subcutaneous phenytoin treatment. Since 100% of the untreated homozygous pups produced seizures, the fact of two homozygous pups showing no seizure activity under phenytoin subcutaneous treatment was impressive. The small number of two animals in this trial presents a problem for statistical evaluation. A larger number of animals in the subcutaneous phenytoin trial could possibly have produced a significant treatment effect. A possible explanation for the reduced treatment effect of phenytoin administration via the drinking water trial could be that phenytoin dosage *in utero* could not reach a level high enough to be effective. The dam received diluted phenytoin via drinking water, and the pups hence received the drug via nursing. Phenytoin is passed into the breast milk at low quantity (Steen et al., 1982), and therefore it may be possible that the drug dosage in the milk could not reach levels high enough to show a treatment effect in the mice.

Phenytoin is the only drug of the four drugs tested in our trials to affect voltage-gated sodium channels directly. Since our *Scn2a* mutation leads to a gain of-function with increased activity of the Nav1.2 sodium channel, a blockage of this channel should lead to decreased activity and less firing of action potentials. We expected phenytoin to show the strongest effect on seizure rate in mice compared to the other treatments. It may be an interesting future experiment to investigate different ways of the phenytoin application and different dosages in *Scn2a* pups, to observe the effect on the seizure rate and to determine whether this effect may be reproducible.

Bumetanide, retigabine and phenobarbital are drugs that do not affect the sodium channel activity. As described above, there are several causes for neonatal epilepsy. We tested drugs with different target mechanisms (i.e., potassium channel activation or enhanced GABAergic inhibition) in the same mouse model to investigate whether alterations in the epileptic neuronal network may reduce seizure occurrences, even though the drugs do not directly target the source of the seizure, the gain of function mutation in the sodium channel $Na_V1.2$.

Insufficient serum levels might also play a role in the minimal seizure reduction observed in the retigabine treatment trial. Higher dosages might lead to more seizure reduction but might also increase the risk of side effects in the neonatal mice.

Our electrophysiological analyses revealed that two genotypes of our mouse model showed electrographic seizures. In humans, most described cases show a heterozygous carrier status for mutations in sodium channel or other channel genes. Homozygous mutations in voltage-gated sodium channels have not been reported so far. It is possible that homozygous human fetuses do not survive *in utero* due to severe fetal seizures or serious impairment of the brain development.

4.2.2 Antiepileptic pharmacologic treatment in clinical use and research today

Especially in neonatal epilepsy, many problems arise in treatment and therapy. First, not all seizures and epilepsy syndromes are yet treatable (Gunthorpe et al., 2012). In children, almost one third of the patients show refractory seizures, which may lead to impaired cognitive development and persistent seizures during adulthood (Gunthorpe et al., 2012; Rakhade & Jensen, 2009). Despite the known severity of neonatal epilepsy and the possible consequences, there is currently no evidence-based guideline for the treatment of neonatal seizures (Glass et al., 2009). It is therefore crucial to study and identify novel treatment methods to develop guidelines and safe treatment plans for clinical use.

This discussion focuses on the prophylactic treatment with the two drugs bumetanide and retigabine, which could be new potential antiepileptic drugs in neonates. The thesis also investigated the effects of phenobarbital and phenytoin, which are commonly used AEDs in neonatal epilepsy, but will not focus on these drugs in this discussion.

GABA-modulating drugs like phenobarbital and benzodiazepines are well established AEDs in adults. In neonates however, seizures show limited response to these AEDs (Löscher et al., 2013). This may be explained by the depolarizing action of GABA in neonates in contrast to the hyperpolarizing GABA action in adults (see Chapter 1.2.3 about the “GABA switch”). The strong inhibitory role of the neurotransmitter GABA in adults presents a valid drug target. Phenobarbital, as described above (see Chapter 1.4.2), enhances the GABAergic inhibition by modulating the $GABA_A$ receptor, thus acting anticonvulsant (Kwan et al., 2001). In the immature brain GABA may act depolarizing (Ben-Ari et al., 2012). This age-specific characteristic may, therefore, present a possible drug target in neonates. In contrast to phenobarbital, reducing the GABAergic action in immature neurons should hence lead to decreased excitability in the neuronal network and fewer epileptic discharges (Löscher et al., 2013).
The excitatory function of GABA in neonates is assumed to be due to the increased intracellular chloride concentration (Ben-Ari et al., 2012). As the intracellular chloride concentration is generated by the action of *NKCC1* and *KCC2* (Ben-Ari et al., 2012; Löscher et al., 2013), research has focused on this mechanism (see Chapter 1.2.3). Dzhala et al. (2005) showed that *NKCC1* was present in the rodent as well as in the human neonatal cortex. Dzhala et al. therefore deduced that bumetanide might be a potent anticonvulsive drug. In *in vitro* whole-cell voltage-clamp recordings of adult CA3 pyramidal cells they could show an effective blockage of *NKCC1* and suppressed epileptiform activity after bumetanide administration. *In vivo* application of bumetanide also showed a therapeutic effect in the kainic acid-induced seizure model. Dzhala et al. also described the NKCC1 expression in humans and could show that expression levels peak at postconceptional week 35 and decrease rapidly during the first year of life. Hence, they concluded that bumetanide might be a useful treatment option for seizures in the neonatal period.
Wang & Kriegstein (2011) discussed the possible long-term consequences of bumetanide treatment. They investigated two treatment periods in rodents. During

the first trial bumetanide was intraperitonealy injected into the pregnant dam from E17 (day 17 after conception) to P7, during the second trial from P7 to P14. During both trials the bumetanide-treated pups presented lower body weight during the treatment period compared to the control mice. The pups treated prenatally presented a deficit in cortical synapse formation. This did not occur in the pups treated from P7 to P14. P7 in mice is estimated to correspond with the 36th week of pregnancy in humans (Workman et al., 2013). Therefore, a bumetanide administration from E17 to P7 in rodents would correspond to prenatal treatment in the second and third trimester in humans. Treatment of preterm neonates corresponds to a prenatal treatment period and may therefore be an indicator for early antiepileptic treatment, which is already in use today. Due to their findings, Wang and Kriegstein argue to show a cautionary approach in bumetanide treatment of neonates. Today preterm and term neonatal antiepileptic treatment does not differ. Glass et al. (2012) show that phenobarbital is the most commonly used first-line AED in preterm and term neonates followed by phenytoin as second-line treatment. With continuing research and establishing critical treatment periods there may be different antiepileptic approaches in preterm and term neonatal treatment in the future, which may use the differing mechanisms of seizures in the maturing brain.

A Europe-wide trial was started in 2011 called NEMO. The NEMO Europe project is a study to develop effective antiepileptic treatment for neonates by medication of patent (nemo-europe, 2017)**.** The study was a phase I/II trial to test bumetanide administration in newborns. The neonates tested had to be full-term infants (gestational age of 37 weeks to 42 weeks), with a postnatal age not older than 48 hours. Inclusion criteria was also the evidence of perinatal asphyxia, as the study based on infants with hypoxic ischemic encephalopathy (Pressler et al., 2015). The trial has now been stopped due to hearing loss observed in three out of eleven neonates (Ben-Ari et al., 2016; Pressler et al., 2015). In this context, it should also be noted that all but one neonate also received antibiotic treatment with aminoglycosides, which is also an ototoxic drug. No improvement of seizure control could be observed (Pressler et al., 2015). We tested bumetanide in our mouse model where no effect on seizure rate could be detected. We did not test mice for side effects such as hearing loss. As Ben-Ari et al. argued that even though bumetanide seems to have failed in anticonvulsive treatment of neonates, it may still prove to be a potent drug in prevention of epileptogenesis or in other neurological disorders such

as autism spectrum disorders, schizophrenia or Parkinson's disease, as they have been associated with abnormal high intracellular chloride concentrations and could therefore profit of a NKCC1 blockage.

Today's AED are based on symptomatic treatment of the seizures and do not focus on prevention of epileptogenesis. Seizure syndromes in neonates differ from those in adults, since the brain is still evolving and maturing (see Chapter 1.2.3). The reasons for enhanced seizure susceptibility in neonates are still a target of research, as well as the age-dependent mechanisms of epileptogenesis. Learning more about these mechanisms should present potential therapeutic targets to prevent epileptogenesis at an early time point (Rakhade & Jensen, 2009).

Results from our laboratory (Marguet et al., 2015) indeed presented an epilepsy mouse model in which the development of a seizure syndrome could be prevented successfully by the prophylactic administration of bumetanide during a crucial time period. The mice expressed a dysfunctional potassium channel (K_v7) resulting in increased neuronal and network activity at P7 and epileptic encephalopathy in adult life. The administration of bumetanide in K_v7-deficient mice during the first two weeks of life led to a significant normalization of cortical and hippocampal neuronal activity. This prevention of seizure occurrence continued into adult life, even though bumetanide administration was stopped at P14. Behavioral deficits in untreated adult K_v7 mice were also prevented by bumetanide administration. In addition, structural changes of the hippocampus due to long-term Kv7 insufficiency could also be prevented, and at around ten weeks of age, the bumetanide treated mutants presented nearly no difference to the control mice in the hippocampal structure. Marguet et al. (2015 p.1442) conclude that a "prophylactic pharmacological treatment during a vulnerable time period can prevent a channelopathy-induced encephalopathy".

Considering the conclusion of a crucial treatment period in neonates, we also decided to investigate whether treatment at an earlier point in time as P1 could reduce seizure occurrence in *Scn2a* mice. Since P1 to P7 bumetanide or retigabine treatment did not show effects on the seizure rate in our mouse model, we did not treat with these two pharmaceuticals during other time periods. The mutant pups treated with phenytoin showed more effect in seizure reduction compared to the other drugs tested, therefore, we tested phenytoin during the last third of pregnancy

until P7. At P7 the treated mutant mice did not show any change in seizure rate compared to the mutant vehicle treated mice. It should be considered that the phenytoin concentration in the P1-P7 treatment group seems to have been too low, meaning that prenatal phenytoin concentration should be adapted as well. Different concentrations of phenytoin in early treatment, as well as long-term follow-up in seizure occurrence of prenatally treated adult mice would be interesting experiments for the future, to examine the long-term seizure-reducing effect. Nevertheless, it has to be considered that the prenatally treated pups presented a significantly lower birth weight than the untreated animals, which may be a contraindication for the dosage increase.

Retigabine has been tested in several *in vitro* electrophysiological studies on *KCNQ2* mutated cells, a mutation which is known to present seizure activity in neonates (Biervert et al., 1998; Scheffer et al., 2005). These *in vitro* experiments indicate a possible effect of retigabine on seizure occurrence in cells with voltage-gated potassium channel mutations. Ihara et al. 2016 presented the first *in vivo* experiments in mice harboring *KCNQ2* mutations. In humans, this dysfunction leads to benign neonatal epilepsy and epileptic encephalopathy. In this mouse model retigabine has shown to be an effective antiepileptic treatment reducing kainic acid-induced seizure rate significantly compared to the saline control group and compared to mice treated with phenobarbital. In a different paper, Frankel et al. (2016) describe the long-term side-effects of retigabine compared to phenobarbital in neonatally treated rats. The rats were treated from P7 to P14 and behavior was tested in the adult mice for deficits due to treatment side effects. Retigabine as well as phenobarbital showed increased alteration in anxiety-like behavior in adult rats, but did not show an effect on learning and memory function, whereas phenobarbital impaired both these functions (Frankel et al., 2016). In 2012, retigabine was withdrawn from the German market by the manufacturing company after the Federal Joint Committee (Gemeinsamer Bundesausschuss G-BA) decided that the drug showed no added value in comparison to the reference substances (G-BA 2012). While, retigabine will probably not play an important role in antiepileptic treatment, novel Kv7.2-enhancing compounds with higher specificity and fewer side effects may still be valuable treatment options in the future.

4.3 *Scn2a* Mouse model and electroclinical dissociation

My thesis is based on a clinical case of the *Scn2a* mutation as described in Chapter 1.4.1. The role of sodium channel mutations and especially the role of *Scn2a* is important in the clinic, as it has been shown that these mutations lead to a broad spectrum of seizure phenotypes such as BFNIS or Dravet syndrome. Establishing different genetic mouse models also creates the opportunity to compare epileptogenic pathomechanisms and to work on general treatment standards for the patients.

Kearney et al. (2001) described a similar genetic *SCN2A* mouse model (Q54). These mice suffered from a mutation in domain 2 (p.GAL879-881QQQ) which led to a gain of function in the sodium channel, similar to our mouse model. Seizures in adult heterozygous mice were not observed or recorded by Kearney and her colleagues. The homozygous mice presented seizures starting at the age of two months and detected via EEG-recordings. The seizures were associated with behavioral arrest and stereotypic, repetitive behavior. Kearney et al. described the seizures as tonic-clonic convulsions with frozen posture and occasionally combined with face twitching, which could be spontaneous or induced e.g. through handling. In neonatal Q54 mice no seizures could be observed clinically and no EEG recordings were done to investigate whether the neonates presented electrographic seizures.

These findings coincide with the findings discussed in this thesis. Chronic telemetric electrocorticogram (ECoG) recordings have been obtained from *Scn2a*(p.A263V) adult mice by Dr. Walid Fazeli (personal communication) and revealed spontaneous, sometimes even lethal seizures. In this study, we could observe behavioral seizures of adult mice during handling and breeding. The homozygous adult mice of our mouse line presented clinical seizures at about one month of age and appear to display similar epileptic symptoms and potential lethal outcome as did the Q54 mice. We did not observe seizures in heterozygous adult animals. Kearney et al. described a mouse model of SCN2A focusing on seizure expression at adult age in mice. In our mouse model we focused on the neonatal expression of the phenotype. In the electrophysiological recordings, we could detect neonatal seizures in both hetero – and homozygous pups at the age of seven days, though no clinical manifestations in neonates were observed during all trials or handling. The presence of electrographic seizures in the absence of clinical manifestations is a phenomenon called electroclinical dissociation (see Chapter 1.1.1) and 80% of seizures which manifest in

the EEG do not show any clinical correlation (Jensen, 2009). Jensen claimed that electroclinical dissociation may be due to immature interhemispheric and corticospinal interconnectivity in neonates: the myelination of white matter at an early age of the brain is still incomplete and may be an explanation why seizures occurring e.g. only in the hippocampus do not manifest clinically as they do not show cortical involvement. Another possible explanation, according to Pressler & Mangum (2013) is the GABAergic switch taking place at different time points in maturation, occurring in subcortical regions prior to cortical regions. This may lead to less expansion of hyperexcitability from subcortical to cortical regions.

Electroclinical dissociation is an important phenomenon to consider in the clinic as it makes seizure diagnosis complex and difficult. Especially in preterm and term neonates, when screening and monitoring are conducted, electrographic seizures without clinical correlation can be observed.

Children may not manifest overt seizure symptoms but may still suffer from seizure activity in the brain. The question is whether these “silent seizures” lead to consequences in the infant’s maturation and if treatment should be proposed or not (Glass et al., 2012; Wilmshurst et al., 2015).

Besides the fact that the neonatal brain is more susceptible to seizures, due to its more excitatory than inhibitory GABAergic action in this period, Gazina et al. (2010) proposed another possible explanation for seizure diminishment at a later age. Voltage-gated sodium channels are expressed during all developmental stages in the brain. But expression of the gene varies due to different splice variants. All VGSCs show a neonatal (N5) and an adult (A5) splice variant.

Liao et al. (2010) proposed another explanation to the transient seizure occurrence in *Scn2a* mutation based seizure syndromes. The expression of *Scn2a* is dependent on the maturation of the brain. Liao and his colleges showed that $Na_v1.2$ was highly expressed during early development in the axon initial segments of the hippocampus and the cortex. The expression gradually decreased over time and was replaced by Nav1.6. The highest expression of $Na_v1.2$ in the CA1 region for example was between P5 and P15. Afterwards the expression diminished, got replaced by $Na_v1.6$ but still minimally visible in the adult brain. The occurrence of seizures in early life in BFNIS and the lack of seizures later on in life may be explained by this development-regulated expression.

4.4 Limitations of the experiments

There are several limitations to these experimental pilot studies, which need to be considered when interpreting the results. Differences in breeding and nursing of the pups may befactors contributing to the biological variance observed. Litter effects such as different litter size, maternal care, or possible variation in handling may lead to unwanted side effects in the trial. Different litter sizes (varying from five to 12 pups in a litter) can lead to different distribution of nourishment of single pups *in utero* or during nursing. A systematic characterization of the effects, which variable litter sizes may have on the development of individual neonatal mice *in utero* was beyond the scope of this study. However, birth weight was routinely measured to investigate whether embryonic and fetal development had been affected by genotype or treatment. To minimize unequal nursing, pups of big litters were taken and put to litters with a small number of pups. A mean litter size of six to eight pups was aimed for. If bad maternal care, such as no nesting or no feeding of the neonates, was observed, the pups were not included into the trial. Pups that experienced lower levels of maternal care may suffer from malnutrition and stress, which could interfere with the experimental outcome, since stress is a well-known trigger of epileptic seizures.

During the surgery, the pups were anesthetized with isoflurane (see Chapter 2.2.3.1). Isoflurane suppresses brain activity. Since during deep anesthesia loss of consciousness is wanted, the brain function needs to be reduced. In neonatal rats the administration of isoflurane led to a total suppression of cortical activity (Sitdikova et al., 2014). Before recording the hippocampal and cortical brain activity, we waited at least 15 – 20 minutes for isoflurane effects on network activity to be minimal or not present at all. Nevertheless, we cannot rule out, that isoflurane may have had some effects on the recordings, specifically during the first recording minutes. Since all animal recordings were done with the same protocol, potential isoflurane effects, if present, should similarly affect all recordings. The necessary head fixation during the electrophysiological recordings, although generally well tolerated, maybe another stressor for neonatal mice. Furthermore, it may be that slight variations in body temperature, and light and noise conditions in the recording room may have affected seizure occurrence as well.

The treatment of pups via the drinking water of the mother also presents a limitating factor. The water volume each nursing mouse drank each day was not measured.

Therefore the exact dosage of phenytoin the dam obtained is unclear. Furthermore, after doubling the concentration of phenytoin in the drinking water we assumed that the mice drank approximately the same amount of water, but it may also be possible that significantly less or more water was consumed. To ensure accurate data, the drinking volume should be carefully monitored and documented.
To minimize these potential perturbation factors, recording conditions were kept as constant as possible throughout all recordings.

4.5 Outlook

Establishing a stable *Scn2a* mouse model and discovering electrographic neonatal seizures in this model has answered several questions and also given rise to new questions to be answered in the future.
Electroclinical dissociation is not only a phenomenon seen in our mouse model but also, and most importantly, in the clinic. Hence, this should be a focus of future work. Investigating the earliest point in time when neonatal seizures start developing and whether seizures in the hippocampus are already present during pregnancy can be important to establish early diagnostics and treatment protocols. And, the second point in time to take interest in, should be when seizures stop being electrographic and start being electroclinical with behavioral symptoms. How does this interaction take place? To answer this question it would be interesting to investigate cortical brain function during different time points in the neonatal period and to correlate this data with hippocampal brain function. When does seizure activity spread to the cortex? Is this time point constant in the mouse model or does it vary? These findings will help to understand when and how seizures manifest. This may also prove to be a crucial time period in which interfering with AEDs could prove most effective.
Based on the pilot studies of this thesis, treatment studies should also be continued, as it would be interesting to see whether the application form for phenytoin with cyclodextrin, which is compatible with repeated s.c. injections, can be found to achieve significant treatment effects. Since retigabine also showed a trend of seizure frequency reduction, a higher dosage trial with more animals could show if retigabine could represent a treatment option in *SCN2A*-channelopathies.

5 Summary

Epilepsy is one of the most common neurological diseases worldwide with about 50 million people suffering from this disease according to the World Health Organization. In infancy, seizures and epileptic syndromes are the most common neurological disorders with the neonatal period showing a particularly high susceptibility to seizures. During maturation, the brain is more vulnerable to seizures as the GABAergic inhibitory system is not yet fully functional. This mechanism contributes to synaptogenesis and brain development, but contributes to higher seizure susceptibility. Idiopathic epilepsies are assumed to comprise 47% of all epilepsies and to be mainly due to genetic causes.

My thesis focuses on a genetic mouse model with a mutation in the voltage-gated sodium channel *Scn2a*. Voltage-gated sodium channels (VGSC) are transmembrane channels that are essential for the initiation and the propagation of action potentials. Channelopathies, diseases based on mutations in ion channels, show a broad clinical spectrum of epileptic syndromes. Based on this mouse model two hypotheses are discussed in this thesis:

1. The *Scn2a*(p.A236V) mutation leads to altered (seizure like) brain activity in neonatal mice compared to wildtype mice.
2. Epileptic patterns in *Scn2a* mutant neonatal mice can be prevented via prophylactic pharmaceutical treatment.

In vivo electrophysiological recordings of neonatal *Scn2a* mice enabled us to detect spontaneous electrographic seizures indicative of a marked epileptic phenotype in this genetic mouse model. Furthermore, we could demonstrate that the incidence of seizures was dependent on the *Scn2a* genotype, and particularly homozygous mice consistently developed an epileptic phenotype during the first postnatal week and, therefore present a valuable mouse model for the study of neonatal epilepsy. The pharmacological pilot treatment studies proved to be difficult due to the limited number of treated animals and treatment side effects. However, the trend of a reduced seizure frequency in retigabine and phenytoin treated neonates will lead to further trials.

This thesis contributes to the understanding of epileptogenesis and especially to the understanding of the electrophysiological phenotype of a sodium channel gain-of-function mutation.

6 Zusammenfassung

Epilepsie ist mit 50 Millionen Betroffenen eine der häufigsten neurologischen Erkrankungen weltweit. Bei Kindern sind Epilepsie und Krampfanfälle die häufigsten neurologischen Erkrankungen. Dabei zeigen Kinder besonders in der neonatalen Periode eine hohe Anfallsbereitschaft. Das Gehirn ist während der Entwicklung und Reifung anfälliger für Krampfanfälle, da das inhibitorische GABAerge System des Gehirns noch nicht ausgereift ist. Dabei trägt das GABAerge System in dieser Zeit zur Synaptogenese und Entwicklung des Gehirns bei, jedoch ebenso zu einer höheren Anfallsbereitschaft. 47% aller Epilepsien werden als idiopathisch klassifiziert, und es wird vermutet, dass ihnen eine genetische Ursache zu Grunde liegt.

Die vorliegende Arbeit konzentriert sich auf ein genetisches Mausmodel für Epilepsie mit einer Mutation in dem spannungsabhängigen Natriumkanal Scn2a. Spannungsabhängige Natriumkanäle sind Transmembrankanäle, die für die Auslösung und die Weiterleitung von Aktionspotentialen eine bedeutende Rolle spielen. Kanalopathien, Erkrankungen die auf Mutationen solcher Kanäle beruhen, zeigen ein großes Spektrum an epileptischen Syndromen. Basierend auf diesem Mausmodel werden zwei Hypothesen in dieser Arbeit diskutiert:

1. Die Scn2a(p.A236V) Mutation führt zu veränderter (anfallsähnlicher) Hirnaktivität in neonatalen Mäusen im Vergleich zu Wildtypmäusen.
2. Epileptische Hirnaktivität in Scn2a mutierten Mäusen kann durch eine prophylaktische medikamentöse Therapie verhindert werden.

Die durchgeführten in vivo elektrophysiologischen Messungen ermöglichten es spontane elektrographische Anfälle zu erkennen, die deutlich auf den epileptischen Phänotyp unseres genetischen Mausmodels hinweisen. Die Anfallsinzidenz zeigte sich abhängig von dem Scn2a Genotyp. Besonders bei homozygoten Mäusen konnte die Entwicklung eines konstanten epileptischen Phänotyps in der ersten postnatalen Woche gezeigt werden, wodurch sich dieses genetische Mausmodel als valides Mausmodel für die weitere Erforschung neonataler Epilepsien erweist. Die pharmakologische Pilotstudie erwies sich als schwierig auf Grund der niedrigen Fallzahl sowie auf Grund der beobachteten, teils schweren Nebenwirkungen. Der positive Trend von Anfallsreduktion bei der Therapie mit Phenytoin und Retigabin spricht jedoch dafür, weitere Versuche anzuschließen.

7 Abbreviations

AED	Antiepileptic drugs
AP	Action potential
AWMF	Arbeitsgemeinschaft der Wissenschaftlichen Medizinischen Fachgesellschaften
BFNIS	Benign familial neonatal infantile seizures
BFNS	Benign familial neonatal seizures
CA	Cornu ammonis
CF	Cystic fibrosis
CFTR	Cystic fibrosis transmembrane conductance regulator
DG	Dentate gyrus
DS	Dravet Syndrome
EEG	Electroencephalogram
EOEE	Early onset epileptic encephalopathy
EPSP	Excitatory postsynaptic potential
GABA	gamma-aminobutyric acid
GEFS+	Generalized epilepsy with febrile seizures plus
HC	Hippocampus
HCBeta	hippocampal beta activity
HCN1	gene for the hyperpolarization activated cyclic nucleotide gated potassium channel 1
IBE	International Bureau of Epilepsy
ILAE	International League Against Epilepsy
IPSP	Inhibitory postsynaptic potential
KCC2	Potassium-chloride-cotransporter 2
KCNH2	gene for the potassium-voltage gated channel subfamily h member 2
KCNQ1	gene for the potassium-voltage gated channel K_v7
LFP	Local field potential
MRI	Magnetic resonance imaging
$Na_v1.2$	Voltage-gated sodium channel type II
NEMO	Study: treatment of neontatal epilepsy with medication off-patent
NKCC1	Sodium potassium chloride cotransporter
OS	Ohtahara Syndrome

P1-P14	Postnatal day 1 – 14
SCN2A	Gene for the alpha unit of the voltage gated sodium channel $Na_V1.2$
SMEB	Severe myoclonic epilepsy borderline
UKE	Universitätsklinikum Hamburg Eppendorf
VGSC	Voltage gated sodium channels
WHO	World Health Organization

8 Bibliography

(AkdÄ). (2011). Trobalt® (Retigabin). *Arzneimittelkommission Der Deutschen Ärzteschaft*, 64–65.

Bear, M., Connors, B., & Paradiso, M. (2015). *Neuosience - Exploring the brain* (4th ed.). Wolters Kluwer Health.

Ben-Ari, Y., Damier, P., & Lemonnier, E. (2016). Failure of the nemo trial: Bumetanide is a promising agent to treat many brain disorders but not newborn seizures. *Frontiers in Cellular Neuroscience*, *10*(APR), 1–6. https://doi.org/10.3389/fncel.2016.00090

Ben-ari, Y., & Holmes, G. L. (2006). Effects of seizures on developmental processes in the immature brain. *Lancet Neurology*, (5).

Ben-ari, Y., Khalilov, I., Kahle, K. T., & Cherubini, E. (2012). The GABA Excitatory / Inhibitory Shift in Brain Maturation and Neurological Disorders. *The Neuroscientist*, *18*(5), 467–486. https://doi.org/10.1177/1073858412438697

Berg, A. T., Berkovic, S. F., Brodie, M. J., Buchhalter, J., Cross, J. H., Van Emde Boas, W., … Scheffer, I. E. (2010). Revised terminology and concepts for organization of seizures and epilepsies: Report of the ILAE Commission on Classification and Terminology, 2005-2009. *Epilepsia*, *51*(4), 676–685. https://doi.org/10.1111/j.1528-1167.2010.02522.x

Berger, H. (1929). Über das Elektroenkephalogramm des Menschen. *Archiv F. Psychatrie*, *87*(1), 527–570. https://doi.org/https://doi.org/10.1007/BF01797193

Biervert, C., Schroeder, B. C., Kubisch, C., Berkovic, S. F., Propping, P., Jentsch, T. J., & Steinlein, O. K. (1998). A Potassium Channel Mutation in Neonatal Human Epilepsy. *Science*, *279*(5349), 403 LP-406. Retrieved from

http://science.sciencemag.org/content/279/5349/403.abstract

Butler, J. L., & Paulsen, O. (2015). Hippocampal network oscillations — recent insights from in vitro experiments. *Current Opinion in Neurobiology*, *31*, 40–44. https://doi.org/10.1016/j.conb.2014.07.025

Buzsaki, G., & Draguhn, A. (2004). Neuronal Oscillations in Cortical Networks. *Science*, *304*(June), 1926–1930.

Buzsáki, G., & Watson, B. O. (2012). Brain rhythms and neural syntax: implications for efficient coding of cognitive content and neuropsychatric disease. *Dialogues in Clinical Neurosience*, 345–367.

Colombo, E., Franceschetti, S., Avanzini, G., & Mantegazza, M. (2013). Phenytoin inhibits the persistent sodium current in neocortical neurons by modifying its inactivation properties. *PloS One*, *8*(1), e55329. https://doi.org/10.1371/journal.pone.0055329

Coppola, A., & Moshé, S. L. (2009). Why is the developing brain more susceptible to status epilepticus? *Epilepsia*, *50 Suppl 1*, 25–26. https://doi.org/10.1111/j.1528-1167.2009.02371.x

Daumas, S., Halley, H., & Lassalle, J.-M. (2004). Disruption of hippocampal CA3 network : effects on episodic-like memory processing in C57BL／6J mice. *Eur. J. Neurosci.*, *20*, 597–600. https://doi.org/10.1111/j.1460-9568.2004.03484.x

Davies, J. a. (1995). Mechanisms of action of antiepileptic drugs. *Seizure*, *4*(4), 267–271. https://doi.org/10.1016/S1059-1311(95)80003-4

Davis, N. J., Tomlinson, S. P., & Morgan, H. M. (2012). The Role of Beta-Frequency Neural Oscillations in Motor Control, *32*(2), 403–404. https://doi.org/10.1523/JNEUROSCI.5106-11.2012

Duncan, J. (2003). *A clinical guide to epileptic syndromes and their treatment*. *Journal of neurology, neurosurgery, and psychiatry* (Vol. 74). https://doi.org/10.1136/jnnp.74.9.1355-b

Dzhala, V. I., Talos, D. M., Sdrulla, D. A., Brumback, A. C., Mathews, G. C., Benke, T. A., … Staley, K. J. (2005). NKCC1 transporter facilitates seizures in the developing brain, *11*(11), 1205–1213. https://doi.org/10.1038/nm1301

Engel, A. K., & Fries, P. (2010). Beta-band oscillations — signalling the status quo ?, 156–165. https://doi.org/10.1016/j.conb.2010.02.015

Fisher, R. S., Acevedo, C., Arzimanoglou, A., Bogacz, A., Cross, J. H., Elger, C. E., … Wiebe, S. (2014). ILAE official report: a practical clinical definition of epilepsy. *Epilepsia*, *55*(4), 475–482. https://doi.org/10.1111/epi.12550

Frankel, S., Medvedeva, N., Gutherz, S., Kulick, C., Kondratyev, A., & Forcelli, P. a. (2016). Comparison of the long-term behavioral effects of neonatal exposure to retigabine or phenobarbital in rats. *Epilepsy & Behavior : E&B*, *57*(Pt A), 34–40. https://doi.org/10.1016/j.yebeh.2016.01.018

Freitag, C. M., May, T. W., Pfäfflin, M., & König, S. (2001). Incidence of Epilepsies and Epileptic Syndromes in Children and Adolescents : A Population-Based Prospective Study in Germany. *Epilepsia*, *42*(8), 979–985.

Fukuda, A. (2005). Diuretic soothes seizures in newborns, *11*(11), 1153–1154.

Gazina, E. V, Leaw, B. T. W., Richards, K. L., Wimmer, V. C., Kim, T. H., Aumann, T. D., … Petrou, S. (2015). Neonatal Nav1.2 reduces neuronal excitability and affects seizure susceptibility and behaviour, *24*(5), 1457–1468. https://doi.org/10.1093/hmg/ddu562

Gazina, E. V, Richards, K. L., Mokhtar, M. B. C., Thomas, E. a, Reid, C. a, & Petrou, S. (2010). Differential expression of exon 5 splice variants of sodium channel alpha subunit mRNAs in the developing mouse brain. *Neuroscience*, *166*(1), 195–200. https://doi.org/10.1016/j.neuroscience.2009.12.011

Glass, H. C., Kan, J., Bonifacio, S. L., & Ferriiero, D. M. (2012). Neonatal seizures. Treatment practices among term and preterm. *NIH Public Access*, *46*(2), 111–115. https://doi.org/10.1016/j.pediatrneurol.2011.11.006.Neonatal

Glass, H. C., MDCM, MAS, FRCPC, Wirrell, E., MD, & FRCPC. (2009). Controversies in Neonatal Seizure Management. *Journal of Child Neurology*, *24*(5), 591–599.

Gunthorpe, M. J., Large, C. H., & Sankar, R. (2012). The mechanism of action of retigabine (ezogabine), a first-in-class K+ channel opener for the treatment of epilepsy. *Epilepsia*, *53*(3), 412–424. https://doi.org/10.1111/j.1528-1167.2011.03365.x

Haas, L. (2003). Hans Berger (1873-1941), Richard Caton (1842-1926), and electroencephalography. *Journal of Neurology, Neurosurgery, and Psychiatry*, *74*, 9.

Haggerty, D. C., Glykos, V., Adams, N. E., & Lebeau, F. E. N. (2013). Bidirectional modulation of hippocampal gamma (20-80 Hz) frequency activity in vitro via alpha(α)- and beta(β)-adrenergic receptors (AR). *Neuroscience*, *253*, 142–154. https://doi.org/10.1016/j.neuroscience.2013.08.028

Hu, W., Tian, C., Li, T., Yang, M., Hou, H., & Shu, Y. (2009). Distinct contributions of Na(v)1.6 and Na(v)1.2 in action potential initiation and backpropagation. *Nature Neuroscience*, *12*(8), 996–1002. https://doi.org/10.1038/nn.2359

Jensen, F. E. (2009). Neonatal Seizures: An Update on Mechanisms and Management. *Clinics in Perinatology*, *36*(4), 1–20. https://doi.org/10.1016/j.clp.2009.08.001.Neonatal

Kahle, K. T., & Staley, K. J. (2008). The bumetanide-sensitive Na-K-2Cl cotransporter NKCC1 as a potential target of a novel mechanism-based treatment strategy for neonatal seizures. *Journal of Neurosurgery*, *25*(September), 1–8.

Kearney, J. a, Plummer, N. W., Smith, M. R., Kapur, J., Cummins, T. R., Waxman, S. G., … Meisler, M. H. (2001). A gain-of-function mutation in the sodium channel gene Scn2a results in seizures and behavioral abnormalities. *Neuroscience*, *102*(2), 307–317. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11166117

Khazipov, R., Esclapez, M., Caillard, O., Bernard, C., Khalilov, I., Tyzio, R., … Ben-ari, Y. (2001). Early Development of Neuronal Activity in the Primate Hippocampus In Utero. *The Journal of Neuroscience*, *21*(24), 9770–9781.

Kim, J.-B. (2014). Channelopathies. *Korean Journal of Pediatrics*, *57*(1), 1–18. https://doi.org/10.3345/kjp.2014.57.1.1

Kirmse, K., Hübner, C. A., Isbrandt, D., & Witte, O. W. (2018). GABAergic Transmission during Brain Development : Multiple Effects at Multiple Stages. https://doi.org/10.1177/1073858417701382

Kole, M. H. P., Ilschner, S. U., Kampa, B. M., Williams, S. R., Ruben, P. C., & Stuart, G. J. (2008). Action potential generation requires a high sodium channel density in the axon initial segment. *Nature Neuroscience*, *11*(2), 178–186. https://doi.org/10.1038/nn2040

Korff, C., & Nordli, D. R. (2005). Do generalized tonic-clonic seizures in infancy exist? *Neurology*, *65*(11), 1750–1753.

https://doi.org/10.1212/01.wnl.0000187125.87414.f3

Kwan, P., Sills, G. J., & Brodie, M. J. (2001). The mechanisms of action of commonly used antiepileptic drugs. *Pharmacology & Therapeutics*, *90*(1), 21–34. https://doi.org/10.1016/S0163-7258(01)00122-X

Liao, Y., Anttonen, A., Liukkonen, E., & Gaily, E. (2010). SCN2A mutation associated with neonatal epilepsy, late-onset episodic ataxia, myoclonus, and pain. *Neurology*. Retrieved from http://www.neurology.org/content/75/16/1454.full

Liao, Y., Deprez, L., Maljevic, S., Pitsch, J., Claes, L., Hristova, D., … Lerche, H. (2010). Molecular correlates of age-dependent seizures in an inherited neonatal-infantile epilepsy. *Brain : A Journal of Neurology*, *133*(Pt 5), 1403–1414. https://doi.org/10.1093/brain/awq057

Litt, M., Luty, J., Kwak, M., Allen, L., Magenis, R. E., & Mandel, G. (1989). Localization of a human brain sodium channel gene (SCN2A) to chromosome 2. *Genomics*, *5*(2), 204–208. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/2571571

Löscher, W., Puskarjov, M., & Kaila, K. (2013). Cation-chloride cotransporters NKCC1 and KCC2 as potential targets for novel antiepileptic and antiepileptogenic treatments. *Neuropharmacology*, *69*, 62–74. https://doi.org/10.1016/j.neuropharm.2012.05.045

Lossin, C., Wang, D. W., Rhodes, T. H., Vanoye, C. G., & George, A. L. (2002). Molecular basis of an inherited epilepsy. *Neuron*, *34*(6), 877–884. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12086636

Marguet, S. L., Le-schulte, V. T. Q., Merseburg, A., Neu, A., Eichler, R., Jakovcevski, I., … Isbrandt, D. (2015). Articles Treatment during a vulnerable developmental period rescues a genetic epilepsy. *Nature Medicine*, *21*(12), 1436–1444. https://doi.org/10.1038/nm.3987

Murray, D. M., Boylan, G. B., Ali, I., Ryan, C. a, Murphy, B. P., & Connolly, S. (2008). Defining the gap between electrographic seizure burden, clinical expression and staff recognition of neonatal seizures. *Archives of Disease in Childhood. Fetal and Neonatal Edition*, *93*(3), F187-91. https://doi.org/10.1136/adc.2005.086314

Neubauer, B. A., & Hahn, A. (2014). *Dooses Epilepsien im Kindes- und Jugendalter* (14th ed.). Springer.

Ogiwara, I., Ito, K., Sawaishi, Y., Osaka, H., Mazaki, E., Inoue, I., … Yamakawa, K. (2009). De novo mutations of voltage-gated sodium channel alphaII gene SCN2A in intractable epilepsies. *Neurology*, *73*(13), 1046–1053. https://doi.org/10.1212/WNL.0b013e3181b9cebc

Oliva, M., Berkovic, S. F., & Petrou, S. (2012). Sodium channels and the neurobiology of epilepsy. *Epilepsia*, *53*(11), 1849–1859. https://doi.org/10.1111/j.1528-1167.2012.03631.x

Panayiotopoulos, C. (2010). Neonatal seizures and neonatal epileptic syndromes. In *c* (Vol. 2, pp. 237–258). https://doi.org/10.1016/S0733-8619(05)70025-6

Pressler, R. M., Boylan, G. B., Marlow, N., Blennow, M., Chiron, C., Cross, J. H., … Rennie, J. (2015). Bumetanide for the treatment of seizures in newborn babies with hypoxic ischaemic encephalopathy (NEMO): an open-label , dose fi nding , and feasibility phase 1 / 2 trial. *Lancet Neurology*, (14), 469–477. https://doi.org/10.1016/S1474-4422(14)70303-5

Pressler, R. M., & Mangum, B. (2013). Newly emerging therapies for neonatal seizures. *Seminars in Fetal & Neonatal Medicine*. https://doi.org/10.1016/j.siny.2013.04.005

Rakhade, S. N., & Jensen, F. E. (2009). Epileptogenesis in the immature brain : emerging mechanisms. *Nature Reviews. Neurology*, *5*(7). https://doi.org/10.1038/nrneurol.2009.80.Epileptogenesis

Rauch, A., Wieczorek, D., Graf, E., Wieland, T., Endele, S., Schwarzmayr, T., … Strom, T. M. (2012). Range of genetic mutations associated with severe non-syndromic sporadic intellectual disability: an exome sequencing study. *Lancet*, *380*(9854), 1674–1682. https://doi.org/10.1016/S0140-6736(12)61480-9

Raymond, C. K., Castle, J., Garrett-Engele, P., Armour, C. D., Kan, Z., Tsinoremas, N., & Johnson, J. M. (2004). Expression of alternatively spliced sodium channel ??-subunit genes. Unique splicing patterns are observed in dorsal root ganglia. *Journal of Biological Chemistry*, *279*(44), 46234–46241. https://doi.org/10.1074/jbc.M406387200

Roll, C., Korinthenberg, R., Merkenschlager, A., & Jorch, G. (2012). Zerebrale Anfälle beim Neugeborenen. *AWMF Leitlinie*, 1–14.

Sanders, S. J., Murtha, M. T., Gupta, A. R., Murdoch, J. D., Raubeson, M. J., Willsey,

a J., … State, M. W. (2012). De novo mutations revealed by whole-exome sequencing are strongly associated with autism. *Nature*, *485*(7397), 237–241. https://doi.org/10.1038/nature10945

Schaffelhofer, S., & Scherberger, H. (2016). Object vision to hand action in macaque parietal, premotor, and motor cortices. *eLife*, *5*. Retrieved from ttp://doi.org/10.7554/eLife.15278

Schattling, B., Fazeli, W., Engeland, B., Liu, Y., Lerche, H., Isbrandt, D., & Friese, M. A. (2016). Activity of NaV1.2 promotes neurodegeneration in an animal model of multiple sclerosis. *JCI Insight*, *1*(19), 1–9. https://doi.org/10.1172/jci.insight.89810

Scheffer, I. E., Harkin, L. A., Dibbens, L. M., Mulley, J. C., & Berkovic, S. F. (2005). Neonatal Epilepsy Syndromes and Generalized Epilepsy with Febrile Seizures Plus (GEFS +), *46*(3), 41–47.

Schmidt, D., Löscher, W., & Schmidt, D. (2012). Epilepsy: Perampanel - New promise for refractory epilepsy? *Nature Reviews Neurology*, (November), 10–12. https://doi.org/10.1038/nrneurol.2012.222

Shi, X., Yasumoto, S., Kurahashi, H., Nakagawa, E., Fukasawa, T., Uchiya, S., & Hirose, S. (2012). Clinical spectrum of SCN2A mutations. *Brain & Development*, *34*(7), 541–545. https://doi.org/10.1016/j.braindev.2011.09.016

Singh, N. a, Cherlier, C., Stauffer, D., DuPont, B., Leach, R., Melis, R., … Leppert, M. F. (1998). A novel potassium channel gene, KCNQ2, is mutated in inherited epilepsy of newborns. *Nature Genetics*, *18*, 25–29.

Sitdikova, G., Zakharov, A., Janackova, S., Gerasimova, E., Lebedeva, J., & Khazipov, R. (2014). Isoflurane suppresses early cortical activity. *Annals of Clinical and Translational Neurology*, 15–26. https://doi.org/10.1002/acn3.16

Speckmann, E.-J., Hescheler, J., & Köhling, R. (2008). *Physiologie* (8th ed.). München: Elsevier.

Steen, B., Rane, A., Lönnerholm, G., Falk, O., Elwin, C., & Sjörgvist, F. (1982). Phenytoin excretion in human breast milk and plasma levels in nursed infants. *Therautic Drug Monitoring*, *4*(4), 331–334.

Sugawara, T., Tsurubuchi, Y., Agarwala, K. L., & ... (2001). A missense mutation of the Na ⁄. channel ⎵ II subunit gene Na v 1 . 2 in a patient with febrile and

afebrile, *98*(18).

Touma, M., Joshi, M., Connolly, M. C., Grant, P. E., Hansen, A. R., Khwaja, O., … Agrawal, P. B. (2013). Whole genome sequencing identifies SCN2A mutation in monozygotic twins with Ohtahara syndrome and unique neuropathologic findings. *Epilepsia*, *54*(5), e81-5. https://doi.org/10.1111/epi.12137

Trinka, E., Cock, H., Hesdorffer, D., Rossetti, A. O., Scheffer, I. E., Shinnar, S., … Lowenstein, D. H. (2015). A definition and classification of status epilepticus - Report of the ILAE Task Force on Classification of Status Epilepticus. *Epilepsia*, *56*(10), 1515–1523. https://doi.org/10.1111/epi.13121

Velísková, J., Claudio, O., Galanopoulou, A., Lado, F., Ravizza, T., Velísek, L., & Moshé, S. (2004). Seizures in the Developing Brain. *Epilepsia*, 6–12. https://doi.org/10.1016/S0896-6273(00)80642-X

Wang, D. D., & Kriegstein, A. R. (2011). Blocking early GABA depolarization with bumetanide results in permanent alterations in cortical circuits and sensorimotor gating deficits. *Cerebral Cortex*, *21*(3), 574–587. https://doi.org/10.1093/cercor/bhq124

Weber, Y. G., & Lerche, H. (2008). Genetic mechanisms in idiopathic epilepsies. *Developmental Medicine and Child Neurology*, *50*(9), 648–654. https://doi.org/10.1111/j.1469-8749.2008.03058.x

WHO, International League Against Epilepsy, & International Bureau for Epilesy. (2005). *Atlas Epilepsy Care in the World 2005*. Geneva: WHO Press.

Wilmshurst, J. M., Burman, R., Gaillard, W. D., & Cross, J. H. (2015). Treatment of infants with epilepsy : Common practices around the world, *56*(7), 1033–1046. https://doi.org/10.1111/epi.13003

Workman, A. D., Charvet, C. J., Clancy, B., Darlington, R. B., & Finlay, B. L. (2013). Modeling Transformations of Neurodevelopmental Sequences across Mammalian Species. *The Journal of Neuroscience*, *33*(17), 7368–7383. https://doi.org/10.1523/JNEUROSCI.5746-12.2013

Xu, R., Thomas, E. a, Jenkins, M., Gazina, E. V, Chiu, C., Heron, S. E., … Petrou, S. (2007). A childhood epilepsy mutation reveals a role for developmentally regulated splicing of a sodium channel. *Molecular and Cellular Neurosciences*, *35*(2), 292–301. https://doi.org/10.1016/j.mcn.2007.03.003

9 Acknowledgements

Foremost I would sincerely like to thank Prof. Dirk Isbrandt for his continuous support, his long lasting patience and answer to every question asked. His great passion for science is impressive and has thought me so much, it has been a great pleasure to be able to work with him.

I deeply thank my supervisor Dr. Walid Fazeli with whom the long hours of surgery, the presentations, the multiple hours of brainstorming and even the days of proofreading were not only very instructive but also always a lot of fun.

My earnest thanks go to Stephan Marguet, PhD, for his enormous patience with me and his great help in programming and processing data figures, as well as Dr. Fabio Morellini for his continuous help in all mouse handling questions and his knowledge of statistics.
I thank Birgit Engeland, PhD, so much for all her help in breeding the mice, genotyping and also brainstorming, and I deeply thank Dr. Axel Neu, who has supported me and my work so kindly as well as helped me of to my work in the clinic.
Thank you to my colleges Robin and Kolja for all the supporting talks and thoughts and all the honestly needed help in IT problems. A huge thank you to the whole ENP Team, it has been such a pleasure working with those awesome colleagues.

My very special thanks goes to my parents, Christine and Ronnie, who may now know this thesis by heart, as they have read it more often than one should think possible. Thank you for all your endless support, your time, your care, your advice, your motivation, your “Knödel”, sometimes your “kick in the but” and thank you so much for your love.
A heartfelt thank you also goes to my grandparents, Elisabeth and Manfred, who are always there for me, no matter what; and to Johann: thank you for always being by my side, for all your support, sometimes also for bearing with me.
And: Thank you for making me so happy.

And last but not least to Flocke, who has kept me company during so many all-nighters.

10 Curriculum vitae

Name:	Sarah-Christina Schöb
Geburtsdatum:	05. April 1990
Geburtsort:	München
Anschrift:	Höxterstraße 11 22529 Hamburg

Schule (Auslandsaufenthalte und Abschlüsse):

1994 – 1995	Millfields School, Wivenhoe Colchester, England
2000 – 2001	Tecumseh Public School, London, Ontario, Canada
2006 – 2007	Lycée Gustave Jaume, Pierrelatte, Frankreich Abschluss Baccalauréat Géneral (Französischer Hochschulabschluss mit Anerkennung der Deutschen Hochschulreife)
2009	Bilinguales Abitur (Deutsch-Englisch; Note 1,7), Dreilinden Oberschule, Berlin mit Certilingua

Studium:

Seit 10/2009	Humanmedizin am Universitätsklinikum Hamburg Eppendorf
08/2011	1. Ärztliche Prüfung
10/2015	2. Ärztliche Prüfung (schriftlicher Teil)
12/2016	2. Ärztliche Prüfung (mündlicher Teil) und Abschluss des Medizinstudiums (Note 2)

Promotion:

Seit 01/2013	Doktorandin der Arbeitsgruppe Experimentelle Neuropädiatrie Kinderklinik des Universitätsklinikum Hamburg Eppendorf, Zentrum für Molekulare Neurobiologie Hamburg
2013 – 2014	SFB Stipendium für die Promotion im Bereich „SFB 936 Multi-Site Communication in the Brain“ Bereichsleiter: Dr. Fabio Morellini

Arbeit:

Seit 04/2017	Assistenzärztin am Zentrum für Geburtshilfe, Kinder- und Jugendmedizin in der Sektion Neonatologie und Pädiatrische Intensivmedizin am Universitätsklinikum Hamburg Eppendorf

1 Eidesstattliche Versicherung

Ich versichere ausdrücklich, dass ich die Arbeit selbständig und ohne fremde Hilfe verfasst, andere als die von mir angegebenen Quellen und Hilfsmittel nicht benutzt und die aus den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen einzeln nach Ausgabe (Auflage und Jahr des Erscheinens), Band und Seite des benutzten Werkes kenntlich gemacht habe.
Ferner versichere ich, dass ich die Dissertation bisher nicht einem Fachvertreter an einer anderen Hochschule zur Überprüfung vorgelegt oder mich anderweitig um Zulassung zur Promotion beworben habe.
Ich erkläre mich einverstanden, dass meine Dissertation vom Dekanat der Medizinischen Fakultät mit einer gängigen Software zur Erkennung von Plagiaten überprüft werden kann.

.....