9

General Discussion

This dissertation primarily focusses on the neural mechanisms underlying cerebellar impact on thalamocortical networks, particular during pathological oscillations. We have in detail investigated how the cerebellum and its up- and downstream targets are and can be modulated during epilepsy. This was not possible without the preexisting evidence regarding functional research mostly performed in the 20th century about the anatomical organization of cerebello-thalamo-cortical and cortico-ponto-cerebellar projections. We focused upon cerebellar's primary output, i.e. cerebellar nuclei, and combined this with the use of different (epilepsy) mouse models. These models are well-characterized, reliable and 'easy to use'. The combination of the wealth of knowledge about the cerebellum and the existence of different well-known epilepsy models created close to ideal circumstances to understand the functioning and potency of this remote structure of control. In this general discussion I will only briefly touch upon the main findings of this thesis and further discuss and coordinate the findings to suggest future lines of animal and clinical research.

9.1 Seizures are hard-wired but also tend to dial in

In **Chapter 5** we demonstrate a limited occurrence of phase-locked modulation of Cerebellar Nuclei (CN) firing during GSWDs which is in line with previous work [104]. Modulation of CN activity during GSWDs has previously been suggested as a potential brain node essential for maintaining cortical GSWD activity [104], which was supported by anatomical connectivity in cerebello-thalamo-cortical networks of various mammalian species [89, 103, 104, 475]. Question remains why some CN neurons do modulate and some don't.

In addition to the discussion of Chapter 5 I came to think about a brain which is not as black and white as often suggested. We show significant interictal firing differences between modulatory and non-modulatory CN neurons [46] while supervised machine-learning algorithms could not clearly identify particular cell types [346]. These results were somewhat surprising to us, since previous in vitro data showed clear differences in activity patterns between CN cell-types [243]; and reviewed by [112, 242]. One of the reasons for interictal firing activity differences might be due to the variance in afferent connections. In other words, axonal projections ('hard-wiring') predefine partially if a cell 'participates' in a seizure or not. Furthermore it is likely that seizures differ in the number of participating cells. It has been suggested that there is always a most-committed group of cells which modulate back and forth other groups of cells within a seizure [476]. This so called 'waxing and waning' phenotype is also resembled in our data where we see differences in the maximal amplitude of GSWDs within a seizure and a large group of CN cells which has Z-scores not clearly indicative of being 'modulatory' although they fall in that category because of values slightly above significance (**Chapter 5**). Therefore it wouldn't surprise me if CN neurons which have a high Z-score (and thus are likely to modulate very reliably) are directly connected in a core-network of SWD occurrence while others are not. These show per GSWD much less reliable modulation or are not modulated during every seizure like the most-committed group.

These results are in line with our results from **Chapter 4** where we show that modulating Purkinje cells occur more often in lateral parts of the cerebellum compared to medial. As with CN neurons they also exhibit a wide range of Z-scores. The representation of modulating Purkinje and CN cells from medial to lateral is in line with data evidencing that cerebello-cerebral communication is represented by fundamental architectural closed-loop circuits [383, 477, 478]. The value of these closed-loop circuits for GSWD generation remains to be seen but it does raise the question if the amount and likelihood of CN neurons being modulated determines or is contributory to the spread and/or length of GSWDs.

This is shown in other brain areas such as the robust correlation between the participation probability of Reticular thalamic neurons at the first cycle and the length and spread of sleep spindles [465]. A similar although much weaker relationship existed for thalamocortical relay cells and spindle duration [465]. In **Chapter 7** we show that some GSWD-modulated thalamocortical relay neurons have a low but still significant Z-score. Seeing the weak relation between thalamocortical relay neurons and spindle-activity and the fact that unnatural spindle-activity is believed to underlie absence epilepsy and thus GSWDs [328, 479-481] I think it is hard to expect a strong correlation between thalamocortical relay neurons and GSWD duration. This suggests that low-Z-score modulated thalamocortical relay neurons are relatively unimportant for seizure maintenance.

9.2 The impact of enhancing cerebellar output

In **Chapter 5** we show that enhancing cerebellar output reduces GSWD activity either directly by optogenetic activation of CN or chronically by pharmacologic modulation. It is interesting to know if on-demand interruption of CN activity is also capable of stopping GSWDs. In that case it would hint towards a mechanism which is about breaking the closed-loop circuit like earlier discussed. For this reason I performed unpublished experiments in which I tested on-demand optogenetic interruption of CN activity using Archaerhodopsin during GSWDs. This was achieved by injecting AAV2.2-hSyn-Arch(eArch3.0)-EYFP bilaterally in cerebellar nuclei basically according to the same protocols and stereotactic locations as used in **Chapter 5 and 7** (Figure 1A, 1B)**.** Inhibition stopped only 21 out of 286 seizures (7.3%) (Fig 1C, 1D). Of 47 thalamic neurons was not capable of stopping GSWDs. (please note that I used 590nm ligt pulses whereas the ideal stimulation of Archaerhodopsin is reached with 540 nm light pulses).

Because of the purely excitatory nature of cerebellar output these results are in line with the suggested mechanism of action in **Chapter 7** where our thalamic recordings hint to signs of desynchronization upon enhancing cerebellar output.

The profound impact of CN activity raises to me the question if there are thalamic cells which are purely driven by excitatory CN input. I think it is likely that many thalamic cells have differential tasks processing different input but regarding cerebellar's motor task it must be that the cerebellum needs to rely on a 1:1 feedforward messaging system to the cortex which means that some thalamic cells will be purely driven by cerebellar input. Evidence for this is given in **Chapters 6 en 7** where we show both for *in vitro* and *in vivo* experiments the existence of cells which show a very reliable and high (amplitude) response. Although the evidence is meager I found one thalamic cell during my Archaerhodopsin

experiment which shows a near complete collapse of firing upon inhibition of CN firing. If we extrapolate this as a given fact this suggests there are thalamic cells which significantly need CN input to overcome either their own action potential generation threshold or other (inhibitory) input to fire action potentials. The function of these cells might be that the cerebellum is involved in preparing future motor actions for which it needs such a reliable feedforward system as was shown for inhibition of thalamic motor nuclei neurons causing a near complete stop of firing in anterior lateral motor cortex [388]. The specifics about this will need further research before even thinking about implementation in a broader line of perspective in epilepsy.

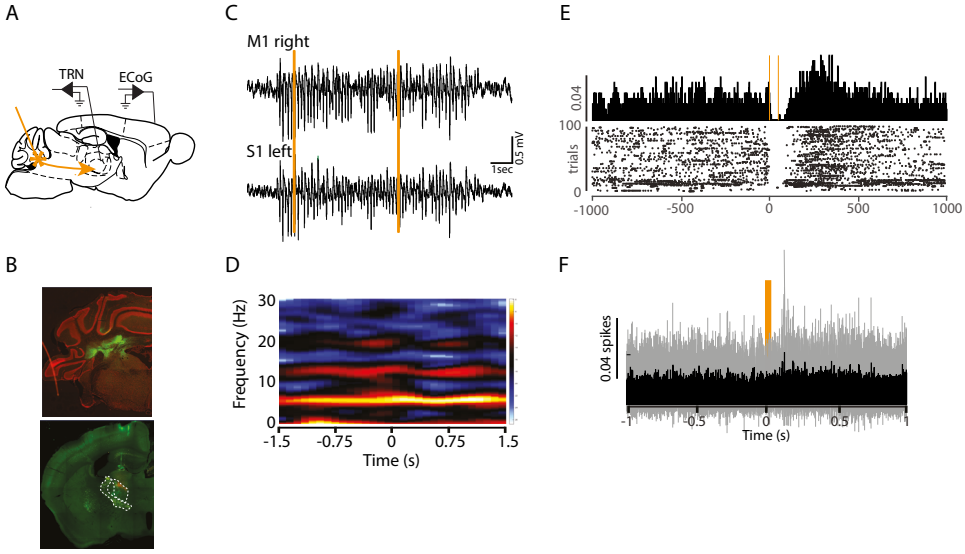

Figure 1. Optogenetic inhibition of cerebellar nuclei (CN) does not stop generalized spike-and-wave discharges (GSWDs).

(A) Schematic of sites for CN inhibition and recording for thalamic relay neurons and electroccorticogram (ECoG) from primary motor and sensory cortices. (B) Confocal image of coronal brain section showing Archaerhodopsin expression in CN (top panel) with projections to thalamus (bottom panel) with a biocytin mark (red; bottom panel) after recording thalamic single units. (C) Representative ECoG of primary motor and sensosry cortices which exemplifies how bilateral optogenetic inhibition (590nm ligt pulse of 50 milliseconds indicated by the vertical orange bar) is not capable of stopping GSWDs. (D) Mean ECoG wavelet spectrogram for all bilateral stimuli (n = 286 seizures; n = 2 animals). (E) Peri-stimulus time histograms (PSTH) and raster plot indicating decreased action potential (AP) firing for one individual thalamic neuron evoked by 590 nm light pulse in CN. (F) Average PSTH (n = 47 thalamic neurons) with in black mean and in gray SD action potential firing upon 590 nm inhibition in CN

9.3 The likelihood of cerebral desynchronization

One of the general objectives of this thesis was to establish a first view on cerebellar impact on the thalamus in the epilepsy-prone brain. In particular my thesis shows different types of responses in thalamic cells upon cerebellar stimulation in the epileptic brain (**Chapter 7**). This was in part evidenced by our results in **Chapter 6** where we found variable amplitudes in thalamic neurons upon cerebellar pulse and train stimulation. Although our results fuels further research of cerebellar communication with thalamocortical networks the polysynaptic individuality of cerebellar projections remains far from elucidated. Experiments in cats and monkeys have identified functional anatomical projections to thalamic motor nuclei and motor cortex in concordance with activation which was depended upon microstimulation in cerebellar nuclei [399, 401, 482-485]. Over the past years evidence accumulated indicating that the cerebellum is also considerably important for neurological non-motor disorders such as autism and schizophrenia [486]. This is supported by anatomical evidence regarding projections extending to premotor and non-motor parietal regions [487, 488]. Nevertheless there are relatively few studies investigating cerebellar effect on cerebral cortices regarding epilepsy (see **Chapter 2** of this thesis for a review of studies) and other neurological diseases [384].

The way cerebral cortices respond to cerebellar modulation remains to be elucidated. Our data using chronic (gabazine) or optogenetic activation of CN neurons shows enhancement of its output to frequency rates > 50 Hz (gabazine) and > 100 Hz (optogenetic). It has been suggested that increasing cerebellar output enhances cortical excitability by activation of the ascending di-synaptic glutamatergic excitatory dentate-thalamo-cortical pathway [489]. This however seems in sharp contrast with the anti-seizure effect proposed in this thesis and the multi-observed increased cortical excitability in juvenile myoclonic, idiopathic generalized and focal epilepsy in humans [490, 491]. It is believed however that increased sensorimotor cortex excitability and desynchronization go hand in hand [492-495].

Another question which stands out is how this desynchronization comes about. Major CN projections towards subcortical nuclei as superior colliculus, anterior pretectal nucleus and Zona Incerta (ZI) have been shown [84-86, 88, 89]. Since cerebellothalamic output is thought to be purely excitatory, the feed-forward excitation-inhibitory responses as recorded in neurons of group 2 in **Chapter 7** might originate from the non-thalamic innervation of extra-thalamic centers of inhibition (see also [442]) by CN axons. The ZI is one of these centers and receives cerebellar projections [89]. The ZI is related to motor-sensory integration and provides massive GABAergic feedback to virtually the entire neocortex [496, 497]. During my research I tried to bidirectionally control cerebellar

injected channelrhodospin and zona incertal injected halorhodopsin in an effort to find dorsal thalamic cells which would be alleviated from inhibition upon optogenetic activation of both opsins. Although my recordings and reconstructions of recording locations did not yet provide unequivocal evidence for this, I strongly believe zona incerta is capable of providing dorsal thalamus with gabaergic inhibition mediated by cerebellum [498].

9.4 Cerebellar control of human refractory epilepsy

An emanating question for the near future regarding CN stimulation in refractory epilepsy is whether or not different CN are capable of influencing various cortical areas involved in contrasting seizure types (Figure 2). In principle the cerebellar nuclei are perfectly suited for different epileptic phenotypes.

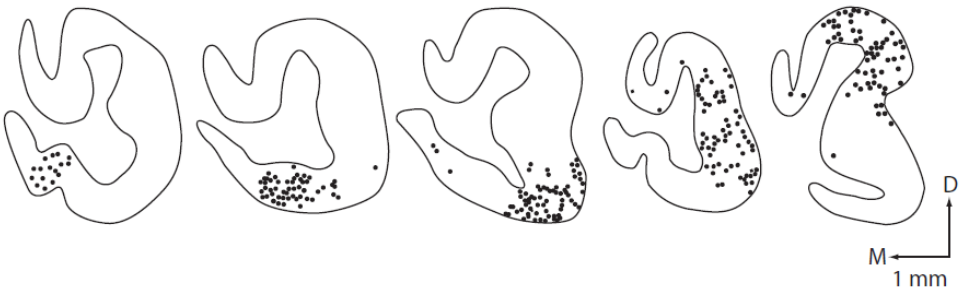

Figure 2. Output Channels in the dentate.

The dots on representative coronal sections show the location of dentate neurons that project to a specific area of the cerebral cortex in the cebus monkey. The cortical target is indicated above each section. (Abbreviations: M1, primary motor cortex; PMv, ventral premotor area) (Taken from Strick 2009).

The impact of cerebellar output changed over the last years significantly from being predominantly thought to influence motor coordination and control to also affecting cortical nonmotor areas important for visuospatial, cognitive and affective processes [499]. In **Chapter 3** we suggested that medial CN project more densely to the limbic system and thus in principle can be effective in stopping temporal seizures. Thus the possibilities of reaching several brain areas via different CN (medial to lateral) indicates that differently positioned stimulation is effective against several types of seizures, as is not uncommonly occurring in epilepsy patients [45, 46, 48, 89]. However, will medial CN be targeted? Seeing the relative small size of more medial CN compared to dentate nucleus and the

vicinity of brainstem structures I think changes are small [127, 128]. A good start in general would be to reestablish recent results [48] regarding CN efficacy in humans. That is why in combination with this thesis' results we started to work towards a clinical trial regarding DBS modulation of CN in pediatric refractory epilepsy patients, regardless of the numerous (ethical) considerations for experimental, invasive treatment options in pediatric patients. Still, given the long-term effects of refractory pediatric epilepsy on cognition, psychological health in adulthood and quality of life in general [500] I feel that efforts to push for CN DBS as a potential novel treatment is justified. However, physicians (and researchers!) must never forget that patients and parents feel undertaking DBS is a life altering decision. The perceived risks, uncertainty of neurosurgical intervention and its outcome, personal fears and the hope for a better life is an unimaginable delicate balance [501].

9.5 Cerebellar induced structural network changes

Structural network changes can occur after epilepsy surgery which not only controls seizures, but also substantially improves patients' behavior, quality of life and cognition [502]. Furthermore it is known that functional and structural brain abnormalities in epilepsy patients are not restricted to the epilepsy onset zone but reach out to other regions of the brain whichever are connected to this zone [503, 504]. This suggests that if focal lesions can give downstream changes in a neuronal system it seems not farfetched to think that a neuronal intervention, such as cerebellar stimulation, is also capable of inducing structural network changes in downstream targets. Any estimates on the most beneficial cerebellar stimulation is difficult seeing the knowledge gaps regarding which cerebellar cell subtypes project to which areas and the many differential non-pathological operating firing frequencies between cell subtypes (let alone seeing this in perspective of the many behavioral states; sleep, drowsy, awake etc) in combination with the problem of a neuromodulation probe being not capable of targeting a specific cell type but only an area. To achieve long lasting changes in downstream targets like the thalamus and further I believe open-loop chronic burst firing superimposed on high frequency (50-80 Hz) activity might be most beneficial.

The thalamus was recently shown to have subdivisions contributing to thalamocortical network abnormalities [505] which makes the CN with its widespread and divergent thalamic projections an ideal candidate for implementing structural cortical network changes as is evidenced over the last years by Machado and colleagues [152, 489, 506-508]. Besides from this evidence that structural changes can occur after lesion like damage from both epilepsy surgery and artificial infarcts, long-term follow-up of studies which were

initially started to evidence efficacy of neurostimulation show that outcome improves over time to over 60% when looking at median seizure reduction compared to baseline [15, 56]. To my knowledge there are no studies which investigate properties of cortical phase amplitude coupling or structural dynamics, e.g. by DTI imaging, several years after the start of neuromodulation. Nevertheless, all this evidence is suggestive for longlasting changes in network activity as a consequence of neuromodulation. In general I think that adding cerebellar output stimulation to the line of possibilities for treating refractory epilepsy is a step towards unraveling the best possible treatment. Among others future research goals need to seek for targets which can cause interictal changes to network activity for reducing the chance on an epileptic attack. Achieving this by implementing one of the most controversial aspects of DBS, i.e. its mechanism of action, will be the most challenging task for the near future.

It has been proposed that AN thalamic stimulation reduces seizure susceptibility because of increasing short-interval intracortical inhibition and increasing motor thresholds (and thus decreasing motor cortex excitability) [509]. This same group showed that stimulating cerebello-thalamo-cortical pathway using DBS VIM activates cortical M1 [510]. However, it remains highly speculative what the exact mechanism is of CN stimulation. Most likely it causes predominantly excitatory effects on thalamic neurons causing widespread desynchronization in thalamus and cortex. Pre and or postsynaptic neurotransmitter depletion or depolarization blockade of the CN soma because of high-frequency stimulation is unlikely when taking into account the natural high firing rate of CN neurons. Our **Chapter 6** results indicate however that optogenetic trains of high frequency stimulation lead to paired pulse depression in VL, VM and CL when measured *in vitro* at 32 degrees. Other unpublished observations from our group indicate that short interval pauses using electrical stimulation is sufficient to regain neurotransmitters in the synapse. Something which seems in concordance with the burst-like firing behavior of natural firing CN neurons. Another option for DBS to fail is somatic hyperpolarization which also seems unlikely because of mounting evidence that DBS is enhancing target output instead of causing lesions. Regarding plasticity it has been shown that upon subthalamic nucleus stimulation potentiation of field potentials did occur in substantia nigra but only following oral L-dopa administration [511]. Direct evidence regarding cerebellar induced LTP or LTD in dorsal thalamic neurons is very scarce [512]. Although evidence is mostly lacking regarding cerebellar induced LTP or LTD in thalamic neurons both in animals and in humans it is hard to come with any arguments why this will not be possible [512]. Furthermore I would like to point out that any ideas regarding downstream thalamic effects upon CN stimulation lacks information regarding the effects of dorsal thalamic

interneurons. Until date GABAergic interneurons have not been recognized in the dorsal thalamus in rodents apart from the lateral geniculate nuclei where they represent 20-25% of the total neurons [513, 514]. On the contrary primates and humans have neuronal thalamic populations containing between 20 and 30% interneurons [86, 515]. Apart from all these considerations future research can guide ideal stimulation parameters and whether or not crucial windows of opportunity exist to intervene in epileptic networks and inducing above described changes.

9.6 How to translate cerebellar epilepsy research?

Irving Cooper (1922-1985) set the stage by pioneering with functional neurosurgery. He aimed to manage neurological disorders such as cerebral palsy and epilepsy. Techniques under his command evolved into scientific reports where he described cerebellar stimulation for spasticity, cerebral palsy and epilepsy and deep brain stimulation for managing tremor and dystonia [69, 516-518]. I think it is safe to assume that his clinical research was at least in part initiated due to the work of Horsley (see the introduction of this thesis) and Sherrington who independently reported after anterior cerebellar lobe stimulation the release of decerebrate rigidity [47, 519, 520]. Over the years many more evidence about the cerebello-cerebral connection revealed first starting with primarily physiology related cerebellar cortex papers which subsequently were followed by reports about cerebello-thalamic connectivity [92, 307, 380, 401, 440, 441, 521, 522]. These results might have even further fueled Cooper's efforts. However, because of his anecdotal style of reports, lack of notion for the need of scientific validated reports of his results, lack of effort to collaborate and lack of criticism on his own work, his work on cerebellar stimulation was met with a lot of skepticism. Over the last years I have met that skepticism. Cerebellar stimulation for epilepsy was, according to many 'established scientists' at international conferences, already investigated and proved not to work. Case closed. Even referring to the work of Dr. Sozari Chkhenkeli [48] was put down as 'work performed in Georgia, so do the math'. Looking at all the work in this thesis performed in the experimental setting, using optogenetics and electrophysiological recording techniques, I would like to state that some of our findings confirm previous findings, but also allowed us to improve the understanding of the cerebellar impact on thalamo-cortical network activity in health and disease. Confirming previous results is very important in today's scientific environment since the human brain is skilled in self-deception which creates a neglect for our own biases. This has been evidenced multiple times over the last years and the fast rising pile of research results inevitably leads to more bias and incorrect results [523-525]. I feel that now the time has come to

re-introduce cerebellar stimulation into the clinic and establish the therapeutic value for patients of refractory seizures.

In that perspective our results in the Dravet Mouse model are difficult to interpret. On one hand we have results in different benign epileptic mouse models showing that cerebellar stimulation can be efficient stopping attacks using on-demand and chronic activation [46]. Furthermore others have shown that cerebellar cortex stimulation is capable of decreasing seizure duration in a temporal lobe seizure mouse model [45]. On the other hand our **Chapter 7** results clearly show that on-demand electrical stimulation of several brain targets, among which the cerebellar, cannot stop generalized epileptic attacks in the Dravet mouse model. It is a possibility that cerebellar projections do not reach all necessary brain areas to stop ongoing severe epileptic attacks. Motor and sensory cerebellothalamic nuclei projections have been shown but cerebellar projections to brain stem nuclei are far from evident [89] while explicitly the combination of stimulation of centrolateral thalamic nucleus and pontine nucleus oralis was shown to be crucial for seizure control in a focal limbic seizure model [433]. Whether or not this was limiting in our experiments is not clear. Furthermore ECoG characteristics and behavior during epileptic attacks in the Dravet mouse model were much more severe compared to our absence epilepsy models. This however does not need to be limiting since human experience of (sub)thalamic stimulation is considerably meager but results were presented as a moderate to significant seizure frequency reduction in a very low number of patients [526, 527]. This is a soft argument that although we were not able to stop epileptic attacks this does not exclude the possibility of reducing seizure frequency for which our research was not setup for [48].

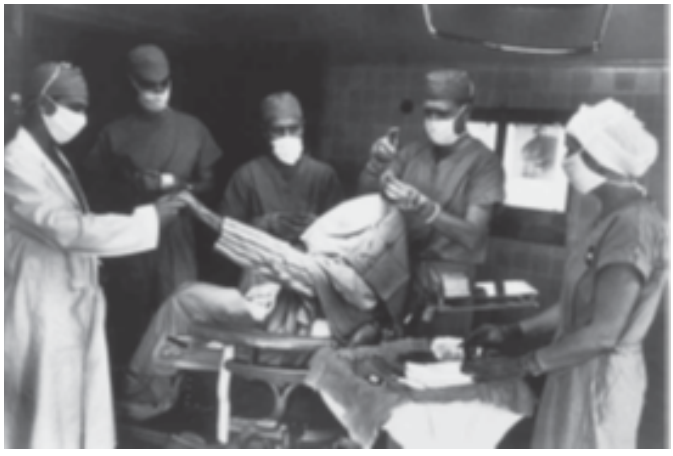

Figure 3. Irving Cooper placing a probe into the thalamus with patient awake in a seated position (Picture taken from [528])

To conclude, whether or not CN neurons are capable of suppressing pathological oscillations remains an intriguing open one. Mouse transgenics is a very promising approach to examine this but we need more specific methods to target specific subtypes of CN neurons and a better understanding of their impact on thalamus. In this way, decades later after Irving Cooper started pioneering with functional neuromodulation we can further identify the potential benefit of cerebellar impact on thalamocortical networks in epilepsy (Figure 3).