Diseases of the nervous system associated with calcium channelopathies

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GENERAL DISCUSSION

PREFACE

The ability to move in a coordinated manner is a basic necessity of most living organisms. In higher organisms, it requires adequate processing of information (i.e., *motor control*), which is carried out by a delicate interplay of the peripheral and the central nervous systems (PNS and CNS). Considering the role of the CNS in motor control, the cerebellum plays a key role in the fine coordination of movements, by ensuring precise execution and timing of motor tasks. In the cerebellum, both sensory and cortical information is processed by various neuron types (e.g., Purkinje and granule cells) in the *cerebellar circuitry* (for review, see *D'Angelo & De Zeeuw, 2009*). As a result, motor commands are transmitted downstream from Purkinje cells (PCs) to other brain structures involved in motor control. At the PNS level, muscles contract in response to commands from motor neurons originating in the spinal cord. The transfer of information in the PNS depends on the activity of *neuromuscular junctions* (NMJs), highly specialized synapses that are optimized for transferring neuronal impulses into muscle action.

Key to proper functioning of the motor system is well-controlled neuronal Ca^{2+} influx through voltage-gated Ca^{2+} channels (VGCCs), resulting in presynaptic neurotransmitter release and the generation of postsynaptic currents. The most prominent type of VGCC in this system is the $Ca_V2.1$ channel, which is almost exclusively responsible for neurotransmission in the periphery (i.e., at NMJs) and in signalling to cerebellar PCs[2, 3]. Dysfunction of the motor system compromises fine tuning of motor coordination and results in gait disturbances, also called ataxia.

Many hereditary forms of ataxia are known, however most relevant for this thesis is cerebellar ataxia as a consequence of the deregulation of $Ca_V2.1$ channel function (for review, see *Jen et al, 2007*). One of the genes that causes deregulation of $Ca_V2.1$ function is the *CACNA1A* (or *Cacna1a* in mice) gene, which encodes the pore-forming α_{1A} subunit of the channel. Ataxia associated with *CACNA1A* mutations can present either as *Episodic Ataxia type 2* (EA2), *Spinocerebellar Ataxia type 6* (SCA6), or as chronic or progressive ataxia associated with *Familial Hemiplegic Migraine* (FHM). The precise pathological mechanisms of $Ca_V2.1$-related cerebellar ataxias are not well understood.

Over the past decade, research into motor coordination deficits resulting from $Ca_V2.1$ dysfunction has benefited from studies performed in experimental animal models, mainly naturally occurring mutant mice that carry specific $Ca_V2.1$ gene mutations (for review, see *van de Ven et al., 2007*). The aim of this thesis was to unravel mechanisms of $Ca_V2.1$ channel dysfunction in selected peripheral and central synapses by using $Ca_V2.1$ natural mutants, as well as (newly) generated transgenic mouse models.

Table 1. Overview of major findings in *Cacna1a* transgenic mouse lines relevant for this thesis

	Mice carrying a (recombined) conditional *Cacna1a* allele				**Conventional transgenic mice**	**Knockin (KI) transgenic mice**	
	***Cacna1a*^{FLOX}**	***Cacna1a*$^{\Delta E4}$***	**Granule KO (GCa1KO; $\alpha6^{Cre}$-*Cacna1a*)**	**Purkinje KO (PCa1KO; L7^{Cre}-*Cacna1a*)**	**Tg^{L7CACNA1AT666M}**	**R192Q§**	**S218L**
Viability	Normal	Die ~P21	Normal	Normal	Normal	Normal	Slightly reduced
Cerebellar ataxia present?	No	Yes	No	Yes	No	No	Yes
NMJ neurotransmission	Normal	Decreased	Not tested	Not tested	Not tested	Increased	Increased
Cerebellar (ultra)structure	Normal	Progressive cell loss & ultrastructural abnormalities**	Normal	Progressive cell loss	Normal	Not tested	Ultra-structural abnormalities
PF-PC neurotransmission	Normal	Decreased**	Decreased	Not tested	Not tested	Not tested	Increased
Irregularity of simple spike firing by the PC	Normal	Increased**	Decreased	Not tested	Not tested	Not tested	Increased
Reference in this thesis	Chapters 3, 4, 5	Chapter 3	Chapter 4	Chapter 5	Chapter 6	Chapter 7	Chapters 7, 8

*Phenotypically identical to conventional $Ca_v2.1$ knockout mice (see *Jun et al., 1999; Fletcher et al., 2001; Kaja et al., 2007*); **Studies in conventional CaV2.1 KO mice (see *Miyazaki et al., 2004*); §Previously generated knockin mice (see *van den Maagdenberg et al., 2004*);

GENERATION OF NOVEL TRANSGENIC $CA_V2.1$ MOUSE MODELS

First attempts to unravel the neuronal basis of calcium-dependent motor coordination using natural and transgenic knockout mouse $Ca_V2.1$ mutants were limited by the wide expression of $Ca_V2.1$ channels throughout the brain[5, 6]. Consequently, these mice express mutant $Ca_V2.1$ channels (i.e., natural mutants) or lack $Ca_V2.1$ channels (i.e., knockout) throughout the whole motor system and are not useful to address the question which *specific* neuronal cell types contribute to cerebellar ataxia. For this thesis, we envisaged that expression or ablation of $Ca_V2.1$ channels in *specific cell types* may reveal cell-specific contributions to cerebellar ataxia. Therefore, we generated a mouse model carrying a floxed *Cacna1a* allele, allowing the conditional inactivation of $Ca_V2.1$ channel function. We used the conditional mice to study the contributions of major cell types from the cerebellar circuitry (i.e., Purkinje cells and granule cells) to motor coordination. Next, as such a mouse model was still lacking, we also set out to generate transgenic knockin mice harbouring an FHM mutation (i.e., S218L) that causes cerebellar ataxia in humans[7]. We compared these mice with previously generated knockin mice that harbour another FHM mutation (i.e., R192Q) that is not associated with cerebellar ataxia[8]. A major goal of this thesis was the generation and characterization of, in total, seven novel $Ca_V2.1$ transgenic mouse models to study the role of $Ca_V2.1$ channels in motor coordination (*Table 1*).

A *Cacna1a* conditional mutant mouse model that is instrumental for studying the effects of cell-specific ablation of $Ca_V2.1$ channels

Targeted ablation, in this case of $Ca_V2.1$ channels, can be achieved by crossing so-called conditional mutant mice, in which a (crucial) exon of the *Cacna1a* gene is flanked by loxP sites, with mice that express Cre recombinase in a specific cell type. Only in cells where Cre is expressed, will an intra-strand recombination and genomic deletion occur, resulting in dysfunctional or absent $Ca_V2.1$-α_1 protein (and thereby no functional $Ca_V2.1$ channels). To this end, we generated transgenic mice (i.e., $Cacna1a^{FLOX}$ mice) that carry a conditional allele with a floxed exon 4 of the *Cacna1a* gene (*Chapter 3*). Importantly, in these mice, the expression of the *Cacna1a* gene product seemed unaffected and $Ca_V2.1$ channel function unchanged throughout the CNS and PNS. The conditional *Cacna1a* mutant mice can be used to achieve ablation of $Ca_V2.1$ calcium channels either in *all* cells (using *EIIA-Cre deleter* mice that express Cre recombinase already in the zygote[11]) or in *specific* neuron types (using mice in which Cre recombinase expression is driven by a cell-specific promoter[12]).

As proof of validity, the *Cacna1a* conditional mice were crossed with *EIIA-Cre* deleter mice, to obtain mice that are devoid of $Ca_V2.1$ channels throughout the nervous system (i.e., $Cacna1a^{\Delta E4}$ mice), and were compared with previously generated conventional *Cacna1a* knockout (KO) mice *(Chapter 3*). In the previous conventional KO mice, a disruption of the *Cacna1a* gene was achieved by replacing sequences of exon 4[13] or exons 14 to 17[14] with a *neo* cassette. In $Cacna1a^{\Delta E4}$ mice this was achieved, similar to

one of the earlier mutants, by a deletion of part of exon 4 of the gene. The phenotype of *Cacna1a*$^{\Delta E4}$ mice appeared identical to that of conventional $Ca_V2.1$-deficient mice[13, 14, 15], and was characterized by prominent cerebellar ataxia and early postnatal death.

To dissect the role of $Ca_V2.1$ channels in specific cell types for the disturbance of motor coordination, and to have an opportunity to investigate progression of the phenotype with age, we had to rescue the early postnatal lethality of the conventional $Ca_V2.1$ KOs. To this end, we crossed *Cacna1a* conditional mice with mice that expressed Cre recombinase exclusively in PCs (driven by the *Pcp2* (*L7*) promoter[16]) or exclusively in cerebellar granule cells (driven by the *GABA α6* promoter[17]). Thus, we obtained offspring that lack $Ca_V2.1$ channels only in Purkinje neurons (i.e., L7^{Cre}-*Cacna1a*, Purkinje KO; *Chapter 5*) or in cerebellar granule cell neurons (i.e., α6^{Cre}-*Cacna1a*, Granule cell KO mice; *Chapter 4*), respectively.

Generation of mice selectively overexpressing human mutant $Ca_V2.1$ channels

Using conventional transgenesis, we also generated mice that express human $Ca_V2.1$-α_1 protein that harbors the *T666M* FHM1 mutation (Tg^{L7CACNA1AT666M}; *Chapter 6*). In humans, the *T666M* mutation is associated with hemiplegic migraine and permanent cerebellar ataxia[8, 18]. Expression of the transgene in Tg^{L7CACNA1AT666M} mice was restricted to cerebellar PCs due to the use of the *Pcp2* (*L7)* promoter[19]. To assess the presence of mutant α_{1A} protein against a background of wild-type α_{1A} protein, the transgene also contained an IRES sequence and an *EGFP* reporter[20]. Both T666M-containing α_{1A} protein and EGFP were translated from the same bicistronic messenger. None of the three generated transgenic lines exhibited behavioural deficits as determined by rotarod testing. The expression of EGFP in the transgenic animals indicates that there is little or no doubt that the mutant α_{1A} product is expressed. It is more likely that the lack of a phenotype is due to the relatively low expression levels of the mutant α_{1A} messenger. Apparently, the level of wild-type α_{1A} protein was sufficient to ensure proper $Ca_V2.1$ channel function. It remains to be seen whether transgenic mice would have had a phenotype if for instance another isoform (i.e., splice product) would have been used for making the transgene construct.

Generation of FHM S218L knockin mice

To study an FHM1 mutation that associates with cerebellar ataxia, we generated a transgenic knockin (KI) model in which we introduced the S218L missense mutation into the mouse *Cacna1a* gene using a gene targeting approach (*Chapter 7*). In humans, the S218L mutation is associated with hemiplegic migraine, cerebellar ataxia, epilepsy, and delayed cerebellar edema after mild head trauma[7, 21]. Similar to the phenotype seen in patients, homozygous S218L KI mice exhibit cerebellar ataxia, increased susceptibility to seizures, and an increased mortality rate. Therefore, S218L KI mice are likely to be very useful in investigating the $Ca_V2.1$-dependent mechanisms in cerebellar ataxia and other phenotypes such as seizures.

PERIPHERAL SYNAPTIC DYSFUNCTION IN MICE WITH MUTANT $CA_V2.1$ CHANNELS

In the research conducted for this thesis, we also made use of naturally occurring *Cacna1a* mouse mutants and studied the consequences of the mutations on neuromuscular synaptic transmission that almost entirely depends on $Ca_V2.1$ channels[22]. We envisaged that a dysfunction of these Ca^{2+} channels could affect neurotransmission at NMJs and provide insight in the pathophysiology of Ca 2.1-dependent motor discoordination.

$Ca_V2.1$ channels consist of up to 4 subunits (α_1, $\alpha_2\delta$, β, and γ [for review, see *Catterall, 2000*]). We assessed the relative contribution of the β_4, the $\alpha_2\delta$–2, and γ_2 subunits (for which natural mouse mutants are available) in neuromuscular neurotransmission and compared the results with 3 natural mouse mutants of the α_{1A} subunit that had been investigated earlier (*Table 2*).

Redundancy or absence of the certain accessory subunits of $Ca_V2.1$ channels at the NMJ?

In *Chapters 3* and *3* a detailed analysis is presented of neurotransmission at NMJs in (natural) knockout mouse mutants *Cacna1a*$^{\Delta E4}$, *ducky*, *lethargic,* and *stargazer*, which lack functional $Ca_V2.1$-α_1, $\alpha_2\delta$-2, β_4, and γ_2 subunits, respectively. Only homozygotes of these four strains of mice exhibit a phenotype that is characterized mainly by a prominent ataxic gait starting as early as postnatal day 10 (for review, see *van de Ven et al., 2007*). Interestingly, only the lack of or mutation in the α_{1A} subunit protein resulted in abnormal synaptic transmission at NMJs (*Table 2*). This suggests that, at least at the NMJ synapse, the $\alpha_2\delta$-2, β_4, and γ_2 subunits are dispensable for neurotransmitter release. It is not entirely clear whether these three accessory subunits are not present at the NMJs at all or whether their absence in mutant mice is successfully rescued by other subunit subtypes. Notably, a successful compensation has been demonstrated in for instance, PCs of *lethargic* mice[31]. The fact that all of these natural mutant mice have a severe neurological phenotype indicates that adequate compensation does not occur in all cell types. Indeed, studies of *ducky* cerebellar PCs have shown that their $Ca_V2.1$-mediated Ca^{2+} current is ~35% lower than in wild-type PCs[24]. Similarly, excitatory neurotransmission of *lethargic* thalamic neurons was shown to be reduced by more than 60%[32]. Finally, aberrant cerebellar synaptic activity between mossy fibers and granule cell neurons was demonstrated in *stargazer* mice, although it was suggested that lack of the γ_2 subunit probably affected mechanisms other than neurotransmission[33]. In summary, these findings suggest that cerebellar ataxia in mice that lack certain accessory subunits of $Ca_V2.1$ channels is more likely the result of centrally located neurological dysfunction, rather than dysfunction in peripheral synapses.

Various effects on neurotransmitter release at the NMJ of $Ca_V2.1$ mutant mice with an ataxic phenotype

In homozygous *Cacna1a*$^{\Delta E4}$ mice lacking $Ca_V2.1$ channels, spontaneous and evoked neurotransmitter release at NMJs is reduced by almost 50% (*Chapter 3*). Similar

Table 2. Phenotypes and consequences on neurotransmission at NMJs of relevant natural $Ca_V2.1$ mouse mutants.

	$Ca_V2.1$ $\alpha_2\delta$-2 mutant	**$Ca_V2.1$ γ_2 mutant**	**$Ca_V2.1$ β_4 mutant**	**$Ca_V2.1$ α_1 mutants**		
	***Ducky*[24]**	***Stargazer*[25]**	***Lethargic*[26]**	***Tottering* [27, 28]**	***Leaner* [15, 27]**	***Rolling Nagoya* [29, 30]**
Genetic lesion	Duplication in *Cacna2d2* ($\alpha_2\delta$-2 knockout)	Insertion in *Cacng2* (γ_2 knockout)	Insertion at a slice site in *Cacnb4* (β_4 knockout)	*P601L* missense mutation in *Cacna1a*	Aberrantly spliced C-terminus of *Cacna1a*	*R1262G* missense mutation in *Cacna1a*
Viability	Die at ~P35	Reduced	Reduced	Normal	Die at ~P20	Normal
Motor dysfunction	Severe ataxia, spike-wave discharges & paroxysmal dyskinesia	Ataxia, spike-wave discharges & paroxysmal dyskinesia	Severe ataxia & slow movement	Ataxia, spike-wave discharges & paroxysmal dyskinesia	Progressive ataxia, absence seizures & dystonia	Ataxia & muscle weakness
Spontaneous ACh release	Normal	Normal	Normal	Increased	Reduced	Increased
Evoked ACh release	Normal	Normal	Normal	Normal	Reduced	Reduced
Non-$Ca_V2.1$ contributions	No	No	No	Yes	Yes	No
This thesis	Chapter 2	Chapter 2	Chapter 2	-	-	-

Table 3. Effects of *Cacna1a* mutations on neurotransmitter release at the NMJ.

	Cerebellar ataxia	Spontaneous ACh release	EPP amplitude	Evoked ACh release
***Cacna1a*$^{\Delta E4}$**	Yes	50%	65%*	50%*
S218L KI	Yes	1250%	100%*	240%**
R192Q KI	No	215%	100%*	230%**

The results are presented as percentages of wild type; *measured at 2 mM extracellular Ca2+; **measured at 0.2 mM extracellular Ca^{2+}

findings have been reported for other ataxic natural *Cacna1a* mutants, such as *rolling Nagoya,* and *leaner* mice[15, 30]. *In vitro* electrophysiological studies of missense and nonsense EA2 mutations revealed either no $Ca_V2.1$ channels or $Ca_V2.1$ channels with much diminished activity[34]. In line with the three natural *Cacna1a* mutants, we hypothesized that a changed $Ca_V2.1$ expression level or changed $Ca_V2.1$ activity at NMJs may explain, at least in part, the observed motor behavior deficits (*Table 3*). For this thesis, we investigated neuromuscular synaptic transmission in S218L KI mice that had an ataxic phenotype (*Chapter 7*). These mice exhibit *gain-of-function* effects on both evoked and spontaneous neurotransmitter release at NMJs. Spontaneous release was increased ~12-fold in homozygous mice. This increase is much larger than the ~2-fold increase seen in homozygous R192Q KI mice that also have an FHM1 mutation[9, 35], but do not exhibit signs of motor coordination deficits. Despite this impressive change in neurotransmitter release, the mice do not exhibit motor weakness, making it less likely that the NMJ phenotype is the cause of the motor discoordination. In fact, there is evidence (*see below*) that the motor discoordination has a central, not peripheral, origin.

ROLE OF $CA_V2.1$ CHANNELS IN NEURONS OF THE OLIVOCEREBELLAR SYSTEM

Although $Ca_V2.1$ channels are widely expressed throughout the CNS[6, 36, 37], ataxias associated with *Cacna1a* mutations were thought to be caused by defects in cerebellar motor control[38, 39].

Structural abnormalities in the cerebellar cortex of $Ca_V2.1$ mutant mice

$Ca_V2.1$ channels play an important role in the function of the motor circuitry in the CNS as suggested by, for instance, their high expression level in the cerebellum. Cerebellar abnormalities, such as the loss of PCs and the presence of abnormal neuronal morphology (e.g., axonal torpedos), have been reported in FHM1 S218L patients who exhibit cerebellar ataxia[7]. Although homozygous S218L KI mice were also ataxic, they appeared to have a normal cerebellar structure with no major disruptions in the foliation and/or organization of the molecular, Purkinje, and granular layers (*Chapter 7*). The total estimated volume of the S218L KI cerebellum and the total number of PCs

appeared normal in S218L KI mice. However, the distal ends of PC dendrites showed a decrease in branching. More dramatic changes such as progressive loss of PCs and/or granule neurons as seen in other ataxic $Ca_V2.1$ mutant mice, like *leaner*, *rolling Nagoya*, *tottering*, and $Ca_V2.1$-deficient mice[14, 40, 41, 42], are not observed in S218L KI mice.

The observed reduced branching of the PCs of S218L KI mice prompted further analysis of the afferent innervation of these cells. The density of the parallel fiber varicosities, as well as the density and sizes of PC dendritic spines, did not differ between S218L KI mutants and wild-type mice, indicating that the individual components of the parallel fiber-to-Purkinje cell (PF-PC) synapse remained unaffected (*Chapter 8*). However, a detailed ultrastructural analysis of these synapses revealed frequent, multiple dendrite spines making contacts with a single parallel fiber varicosity in S218L KI mice. Similar findings have also been reported for the naturally occurring $Ca_V2.1$ mutants *tottering*, *leaner,* and *rolling Nagoya*[43, 44]. Notably, an increase in multiple synaptic indexes in the cerebellar cortex have also been observed in juvenile *tottering* mice, before the onset of neurological symptoms, suggesting that cerebellar ataxia may result rather from abnormal $Ca_V2.1$ synaptic formation, rather then from the loss of cells.

Miyazaki and colleagues have demonstrated that $Ca_V2.1$ channels are crucial for synapse formation in dendrites of PCs in $Ca_V2.1$-deficient mice[10]. In these mice, there is a persistent innervation by proximal parallel fibers and surplus climbing fibers, which should normally be expelled from the compartment during development. As $Ca_V2.1$ channels are highly expressed at both parallel fiber terminals and the soma of PCs, it remains unclear which of the two was responsible for the observed defects in synaptic formation. To answer this question, $Ca_V2.1$ channels were specifically ablated either in parallel fiber terminals (i.e., granule cell neurons) or in PCs. While the lack of $Ca_V2.1$ channels in PCs (i.e., using *Purkinje KO* mice) resulted in a progressive loss of PCs starting around P45 (*Chapter 5*), no such structural abnormalities were observed when $Ca_V2.1$ channels were ablated in cerebellar granule cell neurons (i.e., using *Granule KO* mice) (*Chapter 4*). In fact, granule cell morphology and connectivity were seemingly normal in *Granule KO* mice. Considering that *Purkinje KO* mice, unlike *Granule KO* mice, exhibited severe cerebellar ataxia, starting around P12, it could be concluded that $Ca_V2.1$-mediated PC dysfunction, rather than granule cell dysfunction, is the cause of the abnormal cerebellar morphology and ataxia seen in some of the other $Ca_V2.1$ mutant mice.

Changes in the efficacy of PF-PC synapse in $Ca_V2.1$ mutant mice with cerebellar ataxia

PCs fire so-called simple spikes with a frequency of between 50 and 150 Hz, depending on the strength of parallel fiber input that is related to $Ca_V2.1$ channel functioning[45, 46]. Most natural $Ca_V2.1$ mouse mutants show either an absence or a strong reduction of the function of $Ca_V2.1$ channels[5]. Consequently, it is not so surprising that a decrease in neurotransmission at PF-PC synapses was observed for *tottering*, *leaner,* and *rolling Nagoya* mice[47, 48]. Interestingly, an apparently "normal" neurotransmitter release

(albeit compensated by $Ca_V2.2$ calcium channels) from the parallel fibers in *tottering* mice was reported in another study[49]. The shift in channel-type dependence facilitated an increase in the modulatory effect of inhibitory neurons in the molecular layer, thus resulting in an overall decrease of the neurotransmission at the PF-PC synapse in these $Ca_V2.1$ mutant mice. Together these results suggest a link between decreased neurotransmission at PF-PC synapses and motor coordination deficits as observed in $Ca_V2.1$ mutants.

In this thesis, I showed that a decrease in PF-PC neurotransmission *alone* is not sufficient to cause motor coordination deficits. After all, no signs of cerebellar ataxia were noticed in *Granule KO* mice, in which the neurotransmission between the PF and PCs is severely reduced (*Chapter 4*). Furthermore, paradoxically, an increase in synaptic transmission at PF-PC synapses (as evidenced by the increased amplitude of the PF-EPSCs) was shown in ataxic S218L KI mice (*Chapter 8*). Similarly, *Walter and colleagues*, have recently shown normal PF-EPSCs in ataxic *ducky* mice[50]. Together these results indicate that alterations of neurotransmission at PF-PC synapses alone cannot account for the occurrence of motor coordination deficits in the $Ca_V2.1$ mutants, therefore suggesting another mechanism, likely one that alters the excitability of the PCs.

An increased irregularity of Purkinje cell firing in ataxic $Ca_V2.1$ mutant mice

Electrophysiological studies of PCs of ataxic $Ca_V2.1$ mutants *tottering*, *rolling Nagoya*, and *leaner* have shown a reduction in Ca^{2+}-current density[29, 51]. Consistently, the PCs of *tottering* and *leaner* mice have been shown to fire simple spikes with increased irregularity[50, 52]. As bursts of spiking and pauses in activity determine the signal-to-noise ratio, an increased irregularity of simple spiking patterns of PCs may lead to the loss of essential information for controlling motor behavior. Indeed, in *tottering* mice, the activity of the cerebellar nuclei neurons was abnormal with an enhanced frequency and irregularity of their spiking patterns, suggesting deficient inhibitory input by PCs in this mutant[53].

In this thesis, we showed that the gain of function of the $Ca_V2.1$ S218L mutation in KI mice leads to similar changes in the regularity of simple spiking activity of PCs, albeit via a different mechanism (*Chapter 8*). We demonstrated that $Ca_V2.1$-mediated Ca^{2+}-influx in PCs of S218L KI mice is increased, resulting in their hyperexcitability. S218L PCs have a reduced threshold for generating dendritic Ca^{2+}-spikes, which contributes to the increase of the irregularity in the firing pattern of these mutant neurons. Based on this observation, it can be concluded that bidirectional disturbances of the cerebellar Ca^{2+}-influx could alter the regularity of PCs simple spike firing and therefore decrease the information content in the cerebellar output, resulting in ataxia (*Table 4*).

Table 4. Effects of $Ca_V2.1$ mutations on synaptic input and electrophysiological properties of Purkinje cells in ataxic $Ca_V2.1$ mutant mice.

	$Ca_V2.1$ loss-of-function (*tottering* mice)	**$Ca_V2.1$ gain-of-function** (*S218L KI* mice, *Chapter 8*)
Amplitude of PF-EPSCs (i.e., efficacy of PF-PC transmission)	Reduced[47]	Increased
Paired-pulse facilitation of PF-EPSCs	Increased[47]	Reduced
Dependence on $Ca_V2.2$ channels for F neurotransmitter release	Increased[47, 49]	Reduced
Amplitude of CF-EPSCs (i.e.,efficacy of CF-PC transmission)	Normal[47]	Normal
Threshold for PC action potential	-	Reduced*
PC firing frequency	Slightly lower[52]	Lower**
Irregularity of PC simple spiking	Increased[52]	Increased

*the left-shift of the $Ca2^+$-current activation curve in Purkinje cells leads to burst-like activity with lower current injections; **frequent burst-like activity, which includes a pause in the action potential firing, caused a significant decrease in the average firing frequency

Decrease in the irregularity of the Purkinje cell simple spiking does not result in ataxia, but affects motor learning

Traditionally, the role of cerebellar granule cells has been thought to convey mossy fiber input to the PCs, thereby regulating their firing frequency[54]. Mutations with either gain or loss of $Ca_V2.1$ function have been shown to alter PCs firing frequency, thereby suggesting a possible role of cerebellar granule cell neurons. Here, we studied the firing behavior of PCs in *Granule KO* mice that lack functional $Ca_V2.1$ channels in their granule cell neurons. We showed that despite a nearly 65% decrease in synaptic transmission at the PF-PC synapse of *Granule* KO mice, the firing frequency of their Purkinje cells remained unchanged (*Chapter 4*). The resulting firing patterns of the *Granule* KO PCs were, however, significantly more regular than those of the wild-type PCs. Strikingly, this change in firing pattern was not accompanied by disturbances of the motor coordination of the *Granule* KO mice. Thus, it seems when the irregularity of PC firing decreases, motor behavior remains unaffected, whereas an increase affects motor coordination. The lack of $Ca_V2.1$ channels in *Granule KO* mice did, however, affect the consolidation of motor learning in these mice, but it did not affect their ability to learn new motor tasks. Therefore, our results imply that the consolidation of motor learning itself happens downstream of the cerebellar cortex and is therefore dependent on the output of the cerebellar cortex.

FUTURE RESEARCH

Using various mouse models, it was shown that cerebellar ataxia can be associated with an increased irregularity in the firing of PCs. Evidence came from, (1) natural $Ca_V2.1$ mouse mutants *tottering* and *leaner* that have a loss of $Ca_V2.1$ channel function in both cerebellar granule cell neurons and PCs[50, 52]; (2) transgenic S218L KI mice showing gain of $Ca_V2.1$ channel functioning in both cell types (*Chapter 8*); and (3) *ducky* mice that show *loss* of $Ca_V2.1$ channel function only in Purkinje cells[50]. However, when $Ca_V2.1$ channels are absent only in cerebellar granule cell neurons, the irregular firing of PCs decreases and does not result in cerebellar ataxia (*Chapter 4*).

Generation of cell-specific, $Ca_V2.1$ gain-of-function, double transgenic mice

At this moment, it is unclear whether an increase in neurotransmission from the parallel fiber alone, for example in granule cell-specific $Ca_V2.1$ *gain-of-function* mutants, will increase the irregularity of Purkinje cell firing, thereby causing cerebellar ataxia. This hypothesis could be tested by studying the double transgenic offspring of heterozygous *Granule KO* mice and heterozygous S218L KI mice.

It also remains to be seen whether a $Ca_V2.1$ *gain-of-function* mutation, specifically expressed in PCs, will be as effective as a *loss-of-function* mutation (*Chapter 5*) in eliciting motor coordination deficits. This hypothesis could be tested by studying the double transgenic offspring of heterozygous *Purkinje KO* mice and heterozygous S218L KI mice. Furthermore, as S218L KI mice do not show signs of degeneration in their cerebellum (*Chapter 8*), it could be expected that the same will hold true for these double transgenics. The survival of PCs in these cell-specific mutants would allow electrophysiological analyses of the effects of PC-specific $Ca_V2.1$ mutation on motor coordination.

What role do molecular layer interneurons play in coordinating movement?

One factor that might affect simple spike activity, and which we were unable to address, is the modulating role of the inhibitory interneurons for the PC firing[55, 56]. Experiments that are aimed at analyzing synaptic transmission from granule cells upon stellate and basket cells, as well as their firing patterns, could further elucidate the potential role of inhibitory interneurons on Purkinje cell firing patterns and motor behavior.

Do $Ca_V2.1$ channels upstream and/or downstream of the cerebellar cortex also contribute to the observed ataxic phenotype?

In this thesis, we used conditional *Cacna1a* mice to assess the role of PCs and granule cell neurons in motor coordination. However, crossing these conditional mice with other Cre mice can provide insight into a possible role of $Ca_V2.1$ channels in other cell types of the motor system that are located either upstream or downstream of the cerebellar cortex. For example, selective ablation of $Ca_V2.1$ channels in olivary neurons

(i.e., in climbing fibers) could be achieved by using a parvalbumin promotor to drive Cre expression[57]. Similarly, ablations in deep cerebellar nuclei or thalamic neurons are also interesting, and require proper Cre-expressing transgenic mice, or another Cre delivery mechanism such as stereotactic injections.

Is it possible to alleviate the cerebellar ataxia of $Ca_V2.1$ mouse mutants by restoring normal $Ca_V2.1$ function?

Several drugs, such as acetazolamide, flunarizine, and aminopyridines, can be used to prevent (or attenuate) ataxic episodes in patients with $Ca_V2.1$-mediated neurological disorders[58]. However, the mode of action of these drugs, and how they may prevent ataxia, is not fully understood. It has been suggested that aminopyridines, for example, restore the firing patterns of cerebellar PCs by prolonging depolarization and reducing the latency for Ca^{2+} spikes[59], whereas the carbonic anhydrase inhibitor acetazolamide probably acts by introducing intracellular pH changes, thus interfering with the Ca^{2+}-dependent activities of PCs[59]. A better understanding of the pathophysiology of motor discoordination is expected to yield pharmacological targets that enable the development of novel and better treatments that cure or prevent ataxia.

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