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Summary

Samenvatting
(Summary in Dutch)

Zusammenfassung
(Summary in German)

Sommaire
(Summary in French)

Simon Kaja

Synaptic effects of mutations in neuronal $Ca_V2.1$ calcium channels.

Summary

This thesis describes studies on the synaptic effects of neurological disease-associated $Ca_V2.1$ calcium channel mutations, using the neuromuscular junction (NMJ) as experimental model. Voltage-gated calcium (Ca_V) channels are critical to the functioning of the nervous system, where they control neurotransmission, gene expression, long-term potentiation, synaptic plasticity and synchronization of physiological processes. Ca_V channels are subdivided in the classes of high voltage- and low voltage-activated Ca_V channels. Ca_V1 (L-type), $Ca_V2.1$ (P/Q-type), $Ca_V2.2$ (N-type) and $Ca_V2.3$ (R-type) channels form the group of high voltage-activated channels, whereas Ca_V3 (T-type) channels constitute the population of low voltage-activated channels. High voltage-activated Ca_V channels consist of a pore-forming Ca_V-α_1 and the accessory subunits $\alpha_2\delta$ and β. In some instances there is an additional γ subunit. $Ca_V2.1$ channels are localised pre-synaptically and mediate the calcium influx required for neurotransmitter exocytosis. In the central nervous system, neurotransmitter release is mediated either by $Ca_V2.1$ channels alone (e.g. in cerebellar Purkinje cells) or by contributions of several Ca_V channel subtypes. At the peripheral NMJ it is exclusively $Ca_V2.1$ channels, which control the release of the neurotransmitter acetylcholine (ACh).

A number of neurological diseases are associated with $Ca_V2.1$ channel malfunction. Mutations in the CACNA1A gene, which encodes the pore-forming $Ca_V2.1$-α_1 subunit, are known to cause a severe, inherited subtype of migraine (familial hemiplegic migraine type 1; FHM1), episodic ataxia type 2 (EA2), spinocerebellar ataxia type 6 and some forms of epilepsy. In Lambert Eaton myasthenic syndrome (LEMS), auto-antibodies target $Ca_V2.1$ channels at the NMJ causing muscle weakness and even paralysis.

Mouse models exist that carry mutations in the orthologous *Cacna1a* gene. These include the natural mutants *tottering*, *rolling Nagoya* and *leaner*, which display phenotypes of ataxia and/or absence epilepsy, as well as transgenic knock-out ($Ca_V2.1$-KO) and knock-in (KI) mutants. The natural mouse mutants *ducky*, *lethargic* and *stargazer* lack accessory Ca_V channel subunits ($\alpha_2\delta$-2, β_4 and γ_2, respectively) and display neurological phenotypes remarkably similar to *Cacna1a* mutant mice.

Mutations in $Ca_V2.1$ channels are likely to affect neurotransmitter release resulting in central synaptic dysfunction, which may cause or contribute to the neurological phenotype. Dysfunction at the NMJ may lead to (sub-clinical) muscle weakness. Using electrophysiology, ACh release at the mouse NMJ can be measured indirectly. Our laboratory uses the NMJ as model synapse. In 2000, we demonstrated that it is feasible to study the synaptic effects of *Cacna1a* mutations at the mouse NMJ, due to its exclusive reliance on $Ca_V2.1$ channels for neurotransmitter release. Furthermore, study of the NMJ can reveal whether specific CACNA1A mutations are associated with muscle weakness, as previously reported for some migraine and EA2 patients.

This thesis describes an electrophysiological, morphological and functional characterization of neuromuscular synaptic effects of neurological disease-associated $Ca_V2.1$ channel mutations. **Chapter 1** provides a general introduction on neuromuscular synapse structure and function, Ca_V channels and the calcium channelopathies FHM1, EA2 and LEMS. The literature on the calcium channel mutant mice studied in the present thesis is reviewed.

The first part of this thesis investigates the human FHM1 CACNA1A mutations R192Q and S218L. **Chapter 2** describes the generation and characterization of KI mice carrying the *Cacna1a* R192Q mutation. Showing no overt neurological phenotype, homozygous R192Q KI mice had increased spontaneous uniquantal ACh release at the NMJ. Furthermore, low rate nerve-stimulation evoked ACh release in low calcium conditions was elevated several

fold. Cortical spreading depression (CSD), the mechanism generally considered to underlie migraine aura, can result from synaptic dysfunction. In R192Q KI mice, the threshold for the induction of CSD was significantly reduced; once initiated, CSD propagated more rapidly. Measurements in cultured primary cerebellar KI neurones revealed a hyperpolarizing shift of the activation voltage and increased current density of R192Q-mutated $Ca_V2.1$ channels. A more detailed NMJ study (**chapter 3**) revealed gene dosage-dependent effects at the NMJ, and subtle abnormalities of high rate-evoked ACh release. However, R192Q KI mice lacked progression of the neurotransmitter abnormalities at the NMJ. Our light- and electron microscopic analyses showed that the R192Q mutation does neither affect neuromuscular synapse structure nor size. The CACNA1A S218L mutation is associated with a very severe phenotype in patients, including FHM1 with increased susceptibility to brain oedema and fatal coma following mild head trauma. S218L KI mice show increased lethality and a neurological phenotype of mild ataxia. Our electrophysiological analysis revealed severe gene dosage-dependent abnormalities of ACh release at the NMJ (**chapter 4**). Spontaneous uniquantal release was increased nearly fifteen-fold. Low rate nerve stimulation-evoked ACh was similar to wild-type at two months of age, increased, however, to approximately 160% of wild-type levels in mice twelve months of age. Evoked ACh release at S218L KI NMJs was more sensitive to application of the selective K^+ channel blocker 3,4-diaminopyridine, in line with the hypothesis that S218L-mutated $Ca_V2.1$ channels mediate prolonged calcium flux. Our findings are in accordance with the hypothesis that FHM belongs to a spectrum of hyperexcitability disorders.

The natural *Cacna1a* mutants *tottering*, *rolling Nagoya* and *leaner* are the focus in the second part of this thesis. *Tottering* mice carry the P601L mutation in *Cacna1a*-encoded $Ca_V2.1$ channels, resulting in a complex neurological phenotype. Our laboratory had previously published a detailed characterization of the synaptic effects of the *tottering* mutation. Here we investigated sensitivity of ACh release to selective Ca_V2 channel blocking toxins, in order to dissect possible compensatory contributions of non-$Ca_V2.1$ channels to ACh release at the NMJ (**chapter 5**). The wild-type NMJ is exclusively dependent on $Ca_V2.1$ channels for ACh release. However, synapses have the ability to recruit compensatory non-$Ca_V2.1$ channels to the active zone, which can (partly) compensate for dysfunctional (mutated) $Ca_V2.1$ channels. In *tottering* mice, we found that the contribution of $Ca_V2.1$ channels to ACh release at the NMJ, as assessed with ω-agatoxin-IVA, was reduced and compensated for by $Ca_V2.3$ (R-type) calcium channels, which are sensitive to SNX-482. This is the first report of functional compensation by non-$Ca_V2.1$ channels at the *tottering* NMJ. **Chapter 6** investigates the NMJ of *rolling Nagoya* mice, which suffer from severe ataxia and carry a point mutation in *Cacna1a*.We found increased spontaneous uniquantal, but severely reduced nerve stimulation-evoked ACh release. Evoked release was even more severely affected at soleus NMJs and resulted in clinical muscle weakness and impaired neurotransmission, as shown by grip-strength measurements, *in vitro* muscle contraction experiments and *in vivo* electromyography. Interestingly, despite severely reduced evoked ACh release at the *rolling Nagoya* NMJ, we did not identify compensatory non-$Ca_V2.1$ channel contributions. This is the first study describing increases in spontaneous ACh release with a concomitant reduction of nerve stimulation-evoked release. *Leaner* mice carry a *Cacna1a* mutation that leads to a heavily truncated $Ca_V2.1$ protein. This results in early lethality of the animal, typically during the fourth postnatal week, and a severe neurological phenotype of ataxia and dystonia, remarkably similar to that of $Ca_V2.1$-KO mice. In our electrophysiological characterization of both *leaner* and $Ca_V2.1$-KO NMJs we found similar reductions in both spontaneous and evoked

ACh release (approximately 50%). However, the non-$Ca_V2.1$ channel compensation profiles differed significantly between *leaner* and $Ca_V2.1$-KO mice (**chapter 7**). Whereas ACh release at the NMJs of $Ca_V2.1$-KO mice became dependent jointly on Ca_V1 (~25%), $Ca_V2.2$ (~25%) and $Ca_V2.3$ (~50%) channels, ACh release in *leaner* mice remained dependent to a large extent on $Ca_V2.1$ channels (~60%). The remainder of nerve stimulation-evoked ACh release consisted of a $Ca_V2.3$ channel-mediated (~15%) and an unidentified component (~25%).

The experimental ability to switch off the *Cacna1a* gene in a site- and time-specific manner can provide unique insights into synapse function. For instance, $Ca_V2.1$-KO mice show developmental deficits and typically die during the fourth postnatal week. By switching off the gene later, in the mature stage, direct and developmental effects can be discriminated. In **chapter 8**, we describe the generation of transgenic mice that allow for the conditional inactivation of the *Cacna1a* gene. We showed that total ablation of the *Cacna1a* gene early in gestation by using this method resulted in mice that were indistinguishable from $Ca_V2.1$-KO mice generated by a conventional gene-targeting approach, with respect to their ataxic/epileptic phenotype and NMJ electrophysiology (cf. chapter 7). EA2 is an autosomal dominantly inherited disorder caused by CACNA1A mis-sense or non-sense mutations that typically result in dysfunctional, typically truncated $Ca_V2.1$ channels. The situation in EA2 thus shows genetic resemblance to heterozygous *leaner* and $Ca_V2.1$-KO mice. In **chapter 9** we describe ACh release deficits at the heterozygous *leaner* NMJ, including reduced spontaneous and nerve stimulation-evoked release. Heterozygous $Ca_V2.1$-KO mice, in contrast, did not show any abnormalities, suggesting haplosufficiency. The drug acetazolamide, used in the treatment of EA2, did not affect ACh release parameters when directly applied *in vitro*, arguing against a direct effect of acetazolamide on (*leaner*-truncated) $Ca_V2.1$ channels.

Data from studies of heterologous expression systems suggest that accessory subunits of Ca_V channels can also modulate $Ca_V2.1$ channel function *in vivo*. In accordance with this hypothesis, natural mouse mutants lacking Ca_V channel subunits (*ducky*, $\alpha_2\delta$-2; *lethargic*, β_4; *stargazer*, γ_2) show severe neurological phenotypes similar to that of the natural *Cacna1a* mutants. The role of these subunits at the peripheral NMJ, however, is unknown. Our electrophysiological NMJ analysis of these mice (**chapter 10**) revealed no functional abnormalities, suggesting redundancy of these subunits at the mammalian NMJ.

Topiramate is an anti-convulsant and anti-migraine drug. Whilst its detailed mechanism of action is still unknown, it has been suggested that the therapeutic effects of topiramate may result from a direct modulation of (mutated) $Ca_V2.1$ channels. In order to test this hypothesis, we applied topiramate to muscle/nerve-preparations of wild-type, R192Q KI and *tottering* mice (**chapter11**). However, basic ACh release parameters were not affected, suggesting that topiramate does not directly modulate $Ca_V2.1$ channel function.

Chapter 12 highlights the most important findings of the overall experimental work in relation with the published literature. We conclude that the FHM1 mutations R192Q and S218L cause increased calcium influx through $Ca_V2.1$ channels at the NMJ without compensatory contribution of non-$Ca_V2.1$ channels to ACh release. Furthermore, accessory Ca_V channel subunits are (partly) redundant at the mammalian NMJ. Lastly, the drugs acetazolamide and topiramate do not exert their effects via direct acute modulation of $Ca_V2.1$ channels. The NMJ studies presented in this thesis have provided novel insights into the synaptic dysfunction caused by $Ca_V2.1$ channel mutations. The synaptic effects on central synapses are likely to share many features with those observed at the NMJ, and without much doubt underlie (at least partly) the neurological symptoms of human and mouse $Ca_V2.1$ channelopathies.

Samenvatting

Dit proefschrift beschrijft onderzoek naar de synaptische effecten van $Ca_V2.1$ calciumkanaal mutaties die geassocieerd zijn met neurologische ziekten, waarbij de neuromusculaire synaps (NMS) als experimenteel model wordt gebruikt. Calcium kanalen die door veranderingen in membraanpotentialen geactiveerd worden (Ca_V) zijn essentieel voor het functioneren van het zenuwstelsel. Ze zijn verantwoordelijk voor de regulatie van neurotransmissie, genexpressie, lange termijn potentiatie, synaptische plasticiteit en de synchronisatie van fysiologische processen. Ca_V kanalen zijn onder te verdelen in kanalen met een hoog voltage activering en kanalen met een laag voltage activering. Ca_V1 (L-type), $Ca_V2.1$ (P/Q-type), $Ca_V2.2$ (N-type) en $Ca_V2.3$ (R-type) vormen samen de groep van hoog voltage geactiveerde kanalen. Ca_V3 (T-type) kanalen zijn laag voltage geactiveerde kanalen. Hoog voltage geactiveerde Ca_V kanalen bestaan uit verschillende onderdelen: een kanaal-vormende Ca_V-α_1 subunit, de bijbehorende subunits $\alpha_2\delta$ en β en soms een additionele γ subunit. De presynaptische $Ca_V2.1$ kanalen reguleren de calcium influx die nodig is voor de exocytose van neurotransmitter. De afgifte van neurotranmsitter kan in het centraal zenuwstelsel worden gereguleerd door zowel de $Ca_V2.1$ kanalen alleen (bijv. in de Purkinje cellen) als door een combinatie verschillende subtypen van Ca_V kanalen. In de perifere NMS wordt de afgifte van de neurotransmitter acetylcholine (ACh) uitsluitend door $Ca_V2.1$ kanalen gereguleerd.

Een aantal neurologische aandoeningen is geassocieerd met een defect in de $Ca_V2.1$ kanalen. Mutaties in het CACNA1A gen, dat codeert voor de kanaal-vormende Ca_V-α_1 subunit, kunnen een ernstige en erfelijke vorm van migraine (FHM1), episodische ataxie type 2 (EA2), spinocerebellaire ataxie type 6 (SCA6) of bepaalde vormen van epilepsie veroorzaken. In het Lambert Eaton myastheen syndroom (LEMS) grijpen auto-antilichamen aan op $Ca_V2.1$ kanalen in de NMS, hetgeen spierzwakte en soms zelfs verlamming veroorzaakt.

Er bestaan muismodellen met mutaties in het orthologe *Cacna1a* gen. Tot deze modellen behoren de natuurlijke mutanten, *tottering, rolling Nagoya* en *leaner*, die een fenotype laten zien met ataxie en/of epilepsie, en de transgene knock-out ($Ca_V2.1$-KO) en knock-in (KI) mutanten. De spontane muismutanten *ducky, lethargic* en *stargazer* missen de subunits $\alpha_2\delta$-2, β_4 en γ_2 en hebben een neurologisch fenotype dat bijzondere gelijkenis vertoont met dat van de *Cacna1a* mutante muizen.

Mutaties in $Ca_V2.1$ kanalen resulteren hoogstwaarschijnlijk in veranderingen in neurotransmitter afgifte in hersensynapsen, hetgeen leidt tot een synaptische disfunctie, die mogelijk het neurologische fenotype veroorzaakt, of daartoe kan bijdragen. Disfunctie van de NMS kan mogelijk leiden tot (subklinische) spierzwakte. Met behulp van elektrofysiologie kan indirect de ACh afgifte in de NMS gemeten worden. Ons laboratorium gebruikt de NMS van de muis als model synaps. In 2000 hebben we laten zien dat het mogelijk is de synaptische effecten van *Cacna1a* mutaties in de NMS van de muis te bestuderen, omdat deze synaps exclusief afhankelijk is van $Ca_V2.1$ kanalen voor de afgifte van neurotransmitter. Daarnaast kan het bestuderen van de NMS verhelderen welke specifieke CACNA1A mutaties geassocieerd zijn met spierzwakte, zoals eerder beschreven is voor sommige migraine- en EA2-patiënten.

Dit proefschrift beschrijft een elektrofysiologische, morfologische en functionele karakterisering van de neuromusculaire synaptische effecten als gevolg van $Ca_V2.1$ kanaal mutaties die geassocieerd zijn met neurologische ziekten. **Hoofdstuk 1** geeft een algemene introductie over de structuur en de functie van de NMS, de Ca_V kanalen en de calciumkanaal aandoeningen FHM1, EA2 en LEMS. Ook wordt er een overzicht gegeven van de literatuur over de in dit proefschrift gebruikte calciumkanaal mutante muizen.

Het eerste deel van het proefschrift onderzoekt de humane FHM1 CACNA1A mutaties R192Q en S218L. **Hoofdstuk 2** beschrijft de ontwikkeling en karakterisering van de KI muis met de *Cacna1a* R192Q mutatie. Deze muizen vertonen geen duidelijk fenotype, maar de homozygoot R192Q KI muis laat een toegenomen spontane uniquantale ACh afgifte in de NMS zien. Daarnaast is er een verhoging te zien van de ACh afgifte in laag calcium bij een laagfrequente zenuwstimulatie. Cortical spreading depression (CSD), het mechanisme dat wordt gezien als oorzaak van de migraine aura, is mogelijk het resultaat van synaptisch disfunctioneren. In R192Q KI muizen was de stimulusdrempel voor het induceren van CSD significant lager; en een eenmaal geïnitieerde CSD spreidde zich sneller uit. Metingen in primaire gekweekte cerebellum KI neuronen lieten een hyperpolarisatie verschuiving zien van het activeringsvoltage en een toegenomen dichtheid van R192Q-gemuteerde $Ca_V2.1$ kanalen. Een uitgebreidere studie van de NMS (**hoofdstuk 3**) liet gen-dosisafhankelijke effecten in de NMS zien en ook subtiele veranderingen in de hoogfrequent gestimuleerde ACh afgifte. De R192Q KI muizen vertonen echter geen progressie van deze neurotransmitter afgifte veranderingen in de NMS. Met behulp van licht- en elektronen-microscopie hebben we aangetoond dat de R192Q mutatie niet leidt tot veranderingen in de structuur en afmetingen van de NMS. De CACNA1A S218L mutatie is geassocieerd met een ernstig fenotype in patiënten, waaronder FHM1 met gevoeligheid/kwetsbaarheid voor hersenoedeem en dodelijke coma na een mild hoofdtrauma. S218L muizen hebben een verhoogde letaliteit en een neurologisch fenotype bestaande uit milde ataxie. Onze elektrofysiologische analyses hebben uitgesproken gen-dosisafhankelijke veranderingen van de ACh afgifte in de NMS aangetoond (**hoofdstuk 4**). Spontane ACh afgifte was bijna 15 keer verhoogd. Laag frequent gestimuleerde zenuw afgifte van ACh was op een leeftijd van twee maanden gelijk aan die in de wildtype, maar was op een leeftijd van twaalf maanden met bijna 60% toegenomen ten opzichte van het niveau van de wildtype op diezelfde leeftijd. Gestimuleerde ACh afgifte in S218L KI NMSs was gevoeliger voor het selectieve K^+ kanaal blokkerende farmacon 3,4-diaminopyridine, hetgeen de hypothese ondersteunt dat de S218L mutatie van $Ca_V2.1$ kanalen leidt tot langduriger Ca^{2+} flux. Onze bevindingen zijn in overeenstemmingen met de hypothese dat migraine tot een spectrum van stoornissen met hyperexciteerbaarheid behoort.

De nadruk in het tweede deel van het proefschrift ligt op de natuurlijke mutanten *tottering, rolling Nagoya* en *leaner*. *Tottering* muizen dragen de P601L mutatie in de *Cacna1a*-gecodeerde $Ca_V2.1$ kanalen, hetgeen resulteert in een complex neurologisch fenotype. Ons laboratorium heeft eerder een gedetailleerde karakterisering van het synaptische effect van de *tottering* mutatie gepubliceerd. Nu werd de gevoeligheid onderzocht van de ACh afgifte voor selectieve $Ca_V2.1$ kanalen blokkerende toxinen om compensatie van non-$Ca_V2.1$ kanalen in de ACh afgifte te onderzoeken (**hoofdstuk 5**). De wildtype NMS is exclusief afhankelijk van $Ca_V2.1$ kanalen voor de ACh afgifte. Echter, synapsen beschikken soms over de mogelijkheid om non-$Ca_V2.1$ kanalen te rekruteren in de actieve zone, wat (deels) kan compenseren voor de disfunctionele (gemuteerde) $Ca_V2.1$ kanalen. In *tottering* muizen hebben we gevonden dat de bijdrage van $Ca_V2.1$ kanalen aan de ACh afgifte in de NMS, bepaald met behulp van ω-agatoxine-IVA, gereduceerd is en gecompenseerd wordt door SNX-482-gevoelige $Ca_V2.3$ (R-type) calcium kanalen. Het is voor het eerst dat dat functionele compensatie door non-$Ca_V2.1$ kanalen in de *tottering* NMS is waargenomen.

Hoofdstuk 6 onderzoekt de NMS van *rolling Nagoya* muizen, die een puntmutatie hebben in *Cacna1a* en ernstige ataxie vertonen. We hebben gevonden dat er een toename is in de spontane ACh afgifte, maar tegelijkertijd een sterke reductie in de gestimuleerde ACh afgifte in de NMS. Gestimuleerde afgifte was ernstiger gereduceerd in de soleus/tibialis-

zenuw NMS en resulteerde in klinische spierzwakte en verstoorde neurotransmissie, hetgeen werd aangetoond door middel van trekkracht metingen, *in vitro* spiercontractie experimenten en *in vivo* elektromyografie. Opmerkelijk is dat we ondanks de ernstig gereduceerde gestimuleerde ACh afgifte bij de *rolling Nagoya* NMS geen compensatoire non-$Ca_V2.1$ kanalen hebben gevonden die bijdragen aan de ACh afgifte. Dit is de eerste studie die een toename beschrijft van de spontane ACh afgifte in combinatie met een reductie van de gestimuleerde afgifte.

Leaner muizen hebben een *Cacna1a* mutatie, die leidt tot een zeer verkort $Ca_V2.1$ eiwit. Dit resulteert in een vroege letaliteit van het dier rond de vierde week na de geboorte. Tevens leidt de mutatie tot een ernstig neurologisch fenotype van ataxie en dystonie dat opmerkelijk vergelijkbaar is met het fenotype van de $Ca_V2.1$-KO muis. In onze elektrofysiologische karakterisering van de NMS van deze mutanten hebben we in beide mutanten een vergelijkbare reductie gevonden in zowel de spontane als de gestimuleerde ACh afgifte (van ongeveer 50%). De compensatoire profielen van non-$Ca_V2.1$ kanalen waren echter compleet verschillend in de $Ca_V2.1$-KO en de *leaner* muis (**hoofdstuk 7**). In de $Ca_V2.1$-KO muis bleek de ACh afgifte afhankelijk van de Ca_V1 (~25%), $Ca_V2.2$ (~25%) en $Ca_V2.3$ (~50%) kanalen, terwijl dit bij de *leaner* muis voor een groot deel afhankelijk bleef van de $Ca_V2.1$ kanalen (~60%). Het overige deel kwam op conto van $Ca_V2.3$ kanalen (~15%) en een nog ongeïdentificeerd kanaal (~25%).

De experimentele mogelijkheid om het *Cacna1a* gen uit te schakelen op een plaats- en tijdspecifieke manier kan unieke inzichten verschaffen in het functioneren van de synaps. $Ca_V2.1$-KO muizen laten bijvoorbeeld ontwikkelingsproblemen zien en sterven over het algemeen in de vierde week na de geboorte. Door het gen pas in het volwassen stadium uit te schakelen, kunnen directe effecten van ontwikkelingseffecten onderscheiden worden. In **hoofdstuk 8** beschrijven we de experimentele ontwikkeling van transgene muizen waarin een conditionele inactivering van het *Cacna1a* gen kan plaatsvinden. We laten zien dat totale uitschakeling van het *Cacna1a* gen vroeg in de embyonale vorming door bovenstaande methode, resulteert in muizen die niet te onderscheiden zijn van $Ca_V2.1$-KO muizen die op een traditionele manier (*gene-targeting*) zijn gemaakt (cf. hoofdstuk 7).

EA2 is een autosomaal dominante erfelijke ziekte die veroorzaakt wordt door CACNA1A mutaties die resulteren in niet functionele, verkorte $Ca_V2.1$ kanalen. De situatie bij EA2 lijkt genetisch vergelijkbaar met de heterozygote vorm van *leaner* en $Ca_V2.1$-KO muizen. In **hoofdstuk 9** beschrijven we veranderingen in ACh afgifte in de heterozygote *leaner* NMS. Zowel de spontane als de zenuw-gestimuleerde afgifte was gereduceerd. Heterozygote $Ca_V2.1$-KO muizen vertoonden echter geen enkele afwijking, hetgeen haplosufficiëntie suggereert. Het farmacon acetazolamide, dat toegepast wordt in EA2, beïnvloedde de afgifte parameters niet wanneer het direct *in vitro* werd toegediend. Dit is een aanwijzing dat acetazolamide geen direct effect heeft op de (verkorte) $Ca_V2.1$ kanalen.

Data van studies met heterologe expressie systemen suggereren dat accessoire subunits van Ca_V kanalen ook de functie van $Ca_V2.1$ kanalen *in vivo* kunnen moduleren. Het blijkt ook dat spontane muis mutanten die geen accessoire Ca_V subunits bezitten (*ducky*, $\alpha_2\delta$-2; *lethargic*, β_4; *stargazer*, γ_2) ernstige neurologische fenotypes vertonen die vergelijkbaar zijn zijn aan die van de spontane *Cacna1a* mutanten. De rol van de subunits in de perifere NMS is nog niet bekend. Onze elektrofysiologische NMS analyse van deze muizen (**hoofdstuk 10**) toonde geen functionele gebreken en dit suggereert de afwezigheid of complete compensatie van deze subunits in de zoogdier-NMS.

Topiramaat is een anti-convulsie en anti-migraine farmacon. Hoewel het precieze werkings mechanisme nog onbekend is, zou het therapeutische effect van topiramaat het resultaat kunnen zijn van directe modulatie van (gemuteerde) $Ca_V2.1$ kanalen. Tot besluit van het experimentele deel van dit proefschrift, is een studie beschreven die deze hypothese test (**hoofdstuk 11**). We hebben spier-zenuw preparaten van wildtype R192Q KI en *tottering* muizen blootgesteld aan topiramaat. Echter, de basale ACh afgifte parameters werden niet beïnvloed door het farmacon, hetgeen laat zien dat topiramaat geen direct modulerende invloed heeft op de $Ca_V2.1$ calcium kanaalwerking

In **hoofdstuk 12** worden de belangrijkste experimentele vindingen uit dit proefschrift besproken in de context van reeds gepubliceerde relevante literatuur.

We concluderen dat de FHM1 mutaties R192Q en S218L een toegenomen calcium influx veroorzaken door $Ca_V2.1$ kanalen in de NMS, zonder dat er een compensatoire bijdrage optreedt van non-$Ca_V2.1$ kanalen in de ACh afgifte. Accessoire $Ca_V2.1$ kanaal subunits lijken (deels) afwezig zijn in de NMS van zoogdieren. Ten slotte, de farmaca acetazolamide en topiramaat bereiken hun effect niet via directe, acute modulatie van de $Ca_V2.1$ kanalen.

De NMS studies die in dit proefschrift beschreven staan hebben nieuwe inzichten opgeleverd in het synaptisch disfunctioneren dat veroorzaakt wordt door $Ca_V2.1$-kanaal mutaties. Synaptische effecten als waargenomen in de NMS treden hoogstwaarschijnlijk ook op in het centraal zenuwstelsel en liggen daarmee waarschijnlijk (in ieder geval deels) aan de basis van de neurologische symptomen van $Ca_V2.1$-kanaal ziekten van mens en muis.

Zusammenfassung

Die vorliegende Dissertation charakterisiert am Modell der neuromuskulären Endplatte (NME) die Auswirkungen von Mutationen in $Ca_V2.1$-Kalziumkanälen, die mit neurologischen Krankheitsbildern assoziiert sind. Spannungsabhängige Kalziumkanäle spielen eine bedeutende Rolle in der Funktion des Nervensystems, wo sie an der Steuerung von Nervenübertragung, Genexpression, Langzeitpotenzierung (LTP), synaptischer Plastizität und der Synchronisierung physiologischer Prozesse beteiligt sind. Spannungsabhängige Kalziumkanäle werden traditionell in zwei Kategorien unterteilt: Kanäle, die auf eine hohe Membranspannung reagieren („high voltage-activated“), und solche, die bereits auf eine niedrige Membranspannung reagieren („low voltage-activated“). Kanäle vom Typ Ca_V1 (L), $Ca_V2.1$ (P/Q), $Ca_V2.2$ (N) and $Ca_V2.3$ (R) stellen erstere Gruppe, wohingegen Ca_V3 (T-Typ) Kanäle letzterer zugerechnet werden. Jene spannungsabhängigen Kalziumkanäle, die auf eine hohe Membranspannung reagieren, besitzen neben der die Kanalpore bildenden α_1-Untereinheit eine β- und $\alpha_2\delta$-Untereinheit, sowie – in einigen Fällen – eine zusätzliche γ-Untereinheit. Neuronale $Ca_V2.1$ Kanäle befinden sich in der präsynaptischen Membran und vermitteln den Kalziumeinstrom, der für die Exozytose von Neurotransmitter nötig ist. Die Neurotransmitterausschüttung im zentralen Nervensystem (ZNS) wird durch $Ca_V2.1$ Kanäle allein (z.B. in zerebellaren Purkinje-Neuronen), oder durch mehrere verschiedene Kalziumkanalsubtypen geregelt. An der NME bewirken ausschließlich $Ca_V2.1$ Kanäle den für die Freisetzung des Neuro-transmitters Acetylcholin (ACh) benötigten Kalziumeinstrom.

Bedenkt man die wichtige Rolle von $Ca_V2.1$-Kanälen im Nervensystem, so ist es nicht überraschend, daß zahlreiche Erkrankungen auf einer Dysfunktion von Kanälen dieses Typs beruhen. Mutationen im CACNA1A-Gen , das die, die Kanalpore bildende, α_1-Untereinheit kodiert, führen zu einer schweren, erblichen Form der Migräne (Familiäre Hemiplegische

Migräne Typ 1; FHM1), Episodischer Ataxie Typ 2 (EA2), Spinozerebellärer Ataxie Typ 6 (SCA6) und einigen Formen der Epilepsie. Im Lambert-Eaton myasthänischen Syndrom (LEMS) verursachen spezifische, gegen die $Ca_V2.1$-Kanäle an der NME gerichteten Autoantikörper Muskelschwäche und selbst Lähmungserscheinungen.

Zu den Mausmodellen, die Mutationen im orthologen *Cacna1a*-Gen aufweisen, zählen die natürlichen Mutanten *Tottering*, *Rolling Nagoya* und *Leaner*, deren neurologischer Phänotyp durch Ataxie und/oder Absenzepilepsie gekennzeichnet ist. Darüber hinaus bestehen transgene Knockout- ($Ca_V2.1$-KO) und Knockin- (KI) Mutanten, sowie die natürlichen Mausmutanten *Ducky*, *Lethargic* und *Stargazer*, welchen die funktionsfähigen Kalziumkanaluntereinheiten $\alpha_2\delta$-2, β_4 respektive γ_2 fehlen, und die im neurologischen Phänotyp dem von *Cacna1a*-Mutanten ähneln.

Mutationen in $Ca_V2.1$-Kanälen können in vielen Fällen die Neurotransmitterausschüttung beeinträchtigen und zentralsynaptische Funktionsstörungen bewirken, die ursächlich zu dem neurologischen Phänotyp bzw. dem klinischen Krankheitsbild beitragen. An der NME können $Ca_V2.1$-Mutationen zu (subklinischer) Muskelschwäche führen. Die ACh-Ausschüttung an der NME kann mit Hilfe elektrophysiologischer Methoden indirekt gemessen werden. In unserem Labor machen wir von der NME als Modellsynapse Gebrauch. Im Jahr 2000 konnten wir zeigen, daß sich die NME der Maus, aufgrund ihrer ausschließlichen Abhängigkeit von $Ca_V2.1$-Kanälen, ausgezeichnet für die Untersuchung der Auswirkungen von *Cacna1a*-Genmutationen auf die Neurotransmitterausschüttung eignet. Darüber hinaus erlauben die Untersuchungen an der NME Aussagen über das Vorhandensein bzw. den Grad der, bei bestimmten Mutationen vorkommenden, peripheren Muskelschwäche, wie sie bereits bei einigen Migräne- und Ataxiepatienten nachgewiesen werden konnte.

Die vorliegende Dissertation beschreibt die elektrophysiologische, morphologische und funktionelle Charakterisierung der NME bei Mäusen mit Mutationen in $Ca_V2.1$-Kanälen, die mit neurologischen Erkrankungen assoziiert werden. Das **erste Kapitel** gibt eine allgemeine Übersicht über Struktur und Funktion der NME, spannungsabhängige Kalziumkanäle, sowie die $Ca_V2.1$-Kalziumkanalerkrankungen FHM1, EA2, SCA6 und LEMS. Der gegenwärtige Stand der Forschung über die in dieser Dissertation behandelten Kalziumkanal-Mausmutanten wird zusammengefaßt.

Der erste Teil der vorliegenden Dissertation untersucht die humanen FHM1 CACNA1A-Mutationen R192Q und S218L. Das **zweite Kapitel** beschreibt die Erzeugung und Charakterisierung von KI-Mäusen mit einer *Cacna1a*-Mutation. Trotz des Nichtvorhandenseins eines deutlichen, neurologischen Phänotyps, konnte eine Anzahl synaptischer Veränderungen in homozygoten R192Q KI-Mäusen beobachtet werden. Die spontane Ausschüttung einzelner ACh-Quanten war an der R192Q KI-NME drastisch erhöht. Darüber hinaus war in Medium mit verringerter extrazellulärer Kalziumkonzentration, die durch niedrigfrequente Nervenreizung hervorgerufene Quantenzahl um ein vielfaches erhöht. „Cortical spreading depression“ (CSD) wird als physiologisches Korrelat des bei Mirgäne vorkommenden Phänomens der Aura angesehen und kann durch synaptische Funktionsstörungen hervorgerufen werden. Die Schwelle für die Auslösung von CSD war in R192Q KI-Mäusen signifikant reduziert. Außerdem war die Ausbreitungsgeschwindigkeit von CSD in Mutanten erhöht. Messungen an isolierten Kleinhirnneuronen zeigten eine Aktivierung der R192Q-mutierten $Ca_V2.1$-Kanäle bei niedrigeren Membranpotenzialen im Vergleich zum Wildtyp. Außerdem konnte eine Erhöhung der Stromdichte festgestellt werden. Das **dritte Kapitel** zeigt die, in unserer ausführlichen Untersuchung der NME von R192Q KI-Mäusen gefundenen, allelkopieabhängigen Funktionsstörungen in der Transmitterausschüttung auf. Diese wiesen unter anderem

leichte, nicht-progressive Veränderungen in der durch hochfrequente Reizung ausgelösten ACh-Freisetzung auf. In unseren licht- und elektronenmikroskopischen Studien konnten wir ebenfalls keine nachteiligen Auswirkungen auf Struktur oder Größe der NME nachweisen. Dahingegen ist die CACNA1A S218L Mutation mit einem sehr schwerwiegenden klinischen Krankheitsbild behaftet. Patienten leiden unter FHM1, einer Neigung zu Hirnödem und können ein tödlich verlaufendes Koma als Folge eines leichten Kopftraumas erleiden. S218L KI Mäuse haben eine erhöhte Sterblichkeitsrate und das Erscheinungsbild einer milden Ataxie. Unsere elektrophysiologische Analyse zeigte allelkopieabhängige Funktionsstörungen bei der Neurotransmitterausschüttung an der NME (**viertes Kapitel**). Die spontane Freisetzung einzelner ACh-Quanten war beinahe um das Fünfzehnfache erhöht. Die durch niedrigfrequente Nervenreizung hervorgerufene Quantenzahl in S218L KI-Mäusen im Alter von zwei Monaten war vergleichbar mit der des Wildtyps, jedoch stieg die Quantenzahl bei zwölf Monate alten S218L KI-Mäusen auf etwa 160% des Wildtyp-Niveaus an. In S218L KI-Mäusen reagierte die durch niedrigfrequente Nervenreizung hervorgerufene Quantenzahl stärker auf die direkte Applikation des selektiven Kaliumkanalblockers 3,4-Diaminopyridin. Dies stützt die Hypothese, daß S218L-mutierte $Ca_V2.1$-Kanäle einen erhöhten Kalziumeinstrom vermitteln. Unsere Ergebnisse bestätigen die Vermutung, daß es sich bei FHM um eine Übererregbarkeitsstörung handelt.

Die natürlichen *Cacna1a* Mausmutanten *Tottering*, *Rolling Nagoya* und *Leaner* stehen im Mittelpunkt des zweiten Teils dieser Dissertation. *Tottering* Mäuse zeigen einen komplexen neurologischen Phänotyp, der durch eine P601L Mutation in *Cacna1a*-kodierten $Ca_V2.1$-Kanälen verursacht wird. Unser Labor hatte bereits vor einigen Jahren zuvor eine detaillierte Charakterisierung der synaptischen Defizite an der *Tottering* NME publiziert. Im **fünften Kapitel** der vorliegenden Dissertation wird die Wirkung von selektiven Ca_V2-Kanalblockern auf die Neurotransmitterfreisetzung beschrieben. Bei diesen Versuchen wurde der Anteil anderer Kalziumkanäle an der ACh-Ausschüttung gemessen, um einen möglichen kompensatorischen Beitrag dieser Kanäle abschätzen zu können. Neurotransmitterausschüttung an der NME des Wildtyps ist ausschließlich durch $Ca_V2.1$-Kanäle bedingt. Jedoch besitzen Synapsen die Fähigkeit, Kalziumkanäle des nicht-$Ca_V2.1$-Typs zur aktiven Zone zu rekrutieren, um dort den Funktionsverlust von $Ca_V2.1$-Kanälen (zum Teil) auszugleichen. Wir konnten zeigen, daß der ω-Agatoxin-IVA-abhängige Anteil von $Ca_V2.1$-Kanälen an der ACh-Freisetzung bei der *Tottering* NME reduziert war, jedoch vollständig durch SNX-482-abhängige $Ca_V2.3$-Kanäle kompensiert wurde. Dies ist die erste Beschreibung funktioneller Kompensation durch Kanäle des nicht-$Ca_V2.1$ Typs bei der Neurotransmitterausschüttung an der NME der Maus. Das **sechste Kapitel** untersucht die *Rolling Nagoya* Maus Punktmutation im *Cacna1a* Gen. Der neurologische Phänotyp schwerwiegender Ataxie an der *Rolling Nagoya* NME war mit einer vielfachen Erhöhung der spontanen ACh-Quanten Ausschüttung gepaart, die jedoch mit einer deutlichen Reduktion der durch Nervenreizung hervorgerufenen ACh-Quantenzahl einherging. Die Quantenzahl war an der Soleus-Muskel/*Nervus tibialis* NME am stärksten reduziert und verursachte klinisch relevante Muskelschwäche und beeinträchtigte die Nervenübertragung. Dies konnten wir anhand von Muskelkraftmessungen, *in vitro* Muskelkontraktionsexperimenten und *in vivo* Elektromygraphie nachweisen. Interessantweise konnten wir an der *Rolling Nagoya* NME trotz der reduzierten Quantenzahl keine kompensatorischen Einflüsse von anderen Kalziumkanälen nachweisen. Eine reduzierte spontane Freisetzung von ACh-Quanten, einhergehend mit reduzierter Quantenzahl, wird hier zum ersten Mal beschrieben. *Leaner*-Mäuse haben eine Mutation im *Cacna1a*-Gen, die zur Expression eines am C-Ende verkürzten $Ca_V2.1$-Protein führt. Der mit der *Leaner*-

Mutation assoziierte Phänotyp äußert sich in früher Letalität, typischerweise während der vierten postnatalen Woche, und einem schwerwiegenden neurologischen Erscheinungsbild aus Ataxie und Dystonie, das dem von $Ca_V2.1$-KO Mäusen sehr ähnelt. Spontane ACh-Quantenausschüttung sowie Quantenzahl waren im Vergleich zum Wildtyp sowohl in *Leaner* als auch in $Ca_V2.1$-KO Mäusen um ca. 50% reduziert. Die kompensatorischen Anteile anderer, nicht zum $Ca_V2.1$-Typ gehörenden Kalziumkanäle in den beiden Mutanten unterschieden sich dahingegen deutlich voneinander, wie im **siebten Kapitel** gezeigt wird. Während die Neurotransmitterausschüttung an der NME von $Ca_V2.1$-KO Mäusen von Ca_V1 (ca. 25%), $Ca_V2.2$ (ca. 25%) and $Ca_V2.3$ (ca. 50%) gemeinsam vermittelt wurde, blieb der größte Teil (ca. 60%) der ACh-Freisetzung in der *Leaner*-Maus von $Ca_V2.1$-Kanälen abhängig. Der Rest der durch Nervenreizung hervorgerufenen ACh-Freisetzung wurde durch $Ca_V2.3$-Kanäle (ca. 15%), sowie einen bislang unidentifizierten Kanal (ca. 25%) vermittelt.

Die technische Möglichkeit, das *Cacna1a*-Gen sowohl orts- als auch zeitgesteuert auszuschalten, kann einzigartige Einsichten in die Funktion von Synapsen bieten. So weisen $Ca_V2.1$-KO Mäuse zum Beispiel Entwicklungsdefizite auf und sterben normalerweise während der vierten Lebenswoche. Die Möglichkeit ein Gen erst im erwachsenen Tier auszuschalten erlaubte die Möglichkeit, direkte und entwicklungsbiologische Aspekte der Genausschaltung zu differenzieren. Die Erzeugung transgener Mäuse, die die konditionierte Inaktivierung des *Cacna1a*-Gens erlauben, wird im **achten Kapitel** beschrieben. Wir konnten nachweisen, daß Mäuse, bei denen das *Cacna1a*-Gen auf diese Weise und bereits während der Gestation entfernt wurde, im Hinblick auf ihren ataxischen/epileptischen Phänotyp und ihre NME Elektrophysiologie nicht von $Ca_V2.1$-KO Mäusen zu unterscheiden waren, die auf konventionelle Weise erzeugt worden waren (siehe auch siebtes Kapitel).

EA2 ist eine erbliche, über einen autosomal-dominanten Vererbungsweg übertragene Erkrankung, die sich durch CACNA1A missense- oder nonsene-Mutationen äußert. Sie resultiert in dysfunktionellen, meist verkürzten $Ca_V2.1$-Kanälen. Auf diese Weise gleicht die genetische Situation in EA2 mit der in heterozygoten *Leaner* und $Ca_V2.1$-KO Mäusen. Das **neunte Kapitel** enthält eine Beschreibung von Funktionsstörungen bei der ACh-Freisetzung an der NME der heterozygoten *Leaner*-Maus, die sich durch eine Reduktion sowohl der spontanen, als auch der durch niedrig- und hochfrequente Nervenreizung hervorgerufenen Neurotransmitterausschüttung auszeichnen. Die Tatsache, daß heterozygote $Ca_V2.1$-Mäuse dahingegen keine Funktionsstörungen aufwiesen, läßt auf Haplosuffizienz schließen. Direkte *in vitro* Applikation des in der Behandlung von EA2 effizienten Arzneimittels Azetazolamid veränderte die Neurotransmitterausschüttung an der NME nicht signifikant. Dies läßt vermuten, daß der therapeutische Nutzen von Azetazolamid nicht auf einer unmittelbaren Wirkung des Mittels auf (verkürzte) $Ca_V2.1$-Kanäle basiert.

Ergebnisse aus Studien, die sich heterologer Expressionssyteme bedienten, haben die Möglichkeit nahe gelegt, daß akzessorische Kalziumkanaluntereinheiten ebenfalls *in vivo* die Funktion von $Ca_V2.1$-Kanälen modulieren können. Natürliche Kalziumkanalmutanten, denen bestimmte akzessorische Kanaluntereinheiten fehlen (*Ducky*, $\alpha_2\delta$-2; *Lethargic*, β_4; *Stargazer*, γ_2) zeigen neurologische Phänotypen, die denen der beschriebenen *Cacna1a*-Mutanten sehr ähneln, und stützen somit diese Hypothese. Die physiologische Funktion dieser akzessorischen Untereinheiten an der NME ist jedoch weitgehend unbekannt. Unsere elektrophysiologischen Ableitungen, im **zehnten Kapitel** beschrieben, konnten jedoch keine Funktionsstörungen der Neurotransmitterausschüttung nachweisen und deuten somit auf funktionelle Redundanz von Kalziumkanaluntereinheiten an der NME der Säugetiere hin.

Topiramat ist ein Antikonvulsivum und Antimigränepräparat. Die dem therapeutischen Nutzen zugrunde liegenden Wirkungsmechanismen sind im Detail noch nicht entschlüsselt, in der Vergangenheit ist jedoch die Hypothese aufgestellt worden, daß der pharmakologische Effekt auf eine direkte Modulation (mutierter) $Ca_V2.1$-Kanäle zurückzuführen ist. Das **elfte Kapitel** untersucht die Hypothese, daß die Wiederherstellung normaler Kalziumkanalfunktion einer der Wirkungsmechanismen von Topiramat ist. Die direkte Applikation von Topiramat auf Muskel-Nervpräparate von Wildtyp-, R192Q KI- und *Tottering*-Mäusen zeigte keine signifikanten Veränderungen bei der Neurotransmitterausschüttung an der NME, und erlaubt somit die Schlußfolgerung, daß Topiramat die Funktion von $Ca_V2.1$-Kalziumkanälen nicht auf direkte, akute Weise moduliert.

Das **zwölfte Kapitel** hebt die bedeutendsten experimentellen Befunde hervor und vergleicht sie im Kontext der bestehenden Literatur. Wir folgern, daß die FHM1 Mutationen R192Q und S218L einen erhöhten Kalziumeinstrom durch die an der NME befindlichen $Ca_V2.1$-Kanäle vermitteln, ohne daß kompensatorische Kalziumkanäle zur ACh-Ausschüttung herangezogen werden. Darüber hinaus stellen wir fest, daß akzessorische Kalziumkanaluntereinheiten an der NME (zum Teil) redundant sind. Letztlich kann der therapeutische Nutzen der Arzneimittel Azetazolamid und Topiramat nicht auf eine direkte, akute Modulation von $Ca_V2.1$-Kanälen zurückgeführt werden. Die in der vorliegenden Dissertation beschriebenen Studien an der NME bieten neue Einblicke in die synaptischen Funktionsstörungen, die mit $Ca_V2.1$-Kanalerkrankungen verbunden sind. Die synaptischen Funktionsstörungen im ZNS ähneln mit hoher Wahrscheinlichkeit denen an der NME, und liegen ohne großen Zweifel zumindest teilweise dem neurologischen Krankheitsbild von $Ca_V2.1$-Kanalerkrankungen beim Menschen und der Maus zugrunde.

Sommaire

Cette thèse est consacrée à l'étude des effets synaptiques provoqués par des mutations des canaux calciques $Ca_V2.1$ associés aux maladies neurologiques humaines, et ce en utilisant la jonction neuromusculaire (JNM) comme model. Les canaux sensibles au voltage (Ca_V) sont critiques au bon fonctionnement du système nerveux, où ils contrôlent la neurotransmission, l'expression des gènes, la potentiation à long terme, la plasticité synaptique et la synchronisation des processus physiologiques. Les canaux Ca_V se sous divisent en classes de canaux activés à bas voltages et à haut voltages. Le groupe des canaux activés par de hautes tensions est constitués des canaux $Ca_V2.1$ (type L), $Ca_V2.1$ (type P/Q), $Ca_V2.2$ (type N) et $Ca_V2.3$ (type R), alors que les canaux Ca_V3 (type T) sont les seuls constituants faisant partie des canaux activés par des basses tensions. Les canaux Ca_V activés par de hautes tensions sont constitués des Ca_V-$\alpha 1$ formant des pores, ainsi que des sous-unités accessoires $\alpha_2\delta$ et β. Dans certains cas, une sous-unité γ peut s'y ajouter. Les canaux $Ca_V2.1$ sont situés dans la région présynaptique et régulent le flux calcique requis pour l'exocytose des neurotransmetteurs. Dans le système nerveux central, le relargage des neurotransmetteurs est régulé soi par les canaux $Ca_V2.1$ seuls (e.g. les cellules de Purkinje) ou par la contribution de différents sous-types de canaux Ca_V. A la JNM périphérique, ce sont exclusivement les canaux $Ca_V2.1$ qui exercent leur contrôle sur le relargage des neurotransmetteurs acétylcholine (ACh).

Plusieurs maladies neurologiques sont associées avec une dysfonction des canaux $Ca_V2.1$. Les mutations dans le gène CACNA1A, qui code pour la sous-unité formant des pores $Ca_V2.1$-$\alpha 1$, causent un sous-type de migraine héréditaire sévère (migraine hémiplégique de type 1; FHM1), l'ataxie épisodique de type 2 (EA2), l'ataxie spinocérébrale de type 6

et quelques formes d'épilepsie. Dans le cas du syndrome myasthénique de Lambert Eaton (LEMS), une réaction auto-immune vient cibler $Ca_V2.1$ à la JNM causant ainsi des faiblesses ainsi que la paralysie.

Il existe des modèles murins porteurs de mutations dans l'orthologue *Cacnala*. Ceux-ci sont les mutants *tottering*, *rolling Nagoya* et *leaner*, qui démontrent un phénotype d'ataxie et/ou absence d'épilepsie, tout comme le Knock-out transgénique ($Ca_V2.1$-KO) et les modèles transgeniques Knock-in (KI). Les mutants naturels *ducky*, *lethargic* et *stargazer* sont dénués des sous-unités ($\alpha_2\delta$-2, β_4 et γ_2, respectivement) du canal Ca_V et démontrent un phénotype neurologique remarquablement similaire aux souris portant une mutation *Cacna1a*.

Les mutations dans les canaux $Ca_V2.1$ sont susceptibles d'interférer avec le relargage des neurotransmetteurs résultant ainsi en un mauvais fonctionnement du système synaptique central, ce qui peu ensuite causer ou contribuer au développement d'un phénotype neuropathologique. Un mauvais fonctionnement de la JNM peut mener à des faiblesses neuromusculaires. L'électrophysiologie peut nous permettre de mesurer indirectement le relargage de l'ACh à la JNM de la souris. Notre laboratoire utilise la JNM de la souris comme model synaptique. En 2000, nous avons démontré qu'il était possible d'étudier les effets synaptiques des mutations *Cacnala* à la JNM, puisque ils sont strictement dépendants des canaux $Ca_V2.1$ pour le relargage des neurotransmetteurs. De plus, l'étude de la JNM peut aussi révéler si des mutations CACNA1A sont associées avec le développement de faiblesses musculaires, comme on a déjà observé chez des patients EA2 et souffrant de migraines.

Cette thèse se consacre à la caractérisation électrophysiologique, morphologique et fonctionnelle des effets synaptiques des mutations des canaux $Ca_V2.1$ causant une dysfonction neurologique. Le **chapitre 1** introduit la structure et la fonction de la synapse neuromusculaire, des canaux $Ca_V2.1$ ainsi que des maladies des canaux calciques FHM1, EA2 et LEMS. La littérature sur les souris porteuses de mutations des canaux calciques est aussi commentée dans cette thèse.

La première partie de cette thèse se concentre sur les mutations CACNA1A R192Q et S218L menant à la FHM1 chez l'humain. Le **chapitre 2** décrit la génération ainsi que la caractérisation des souris KI portant la mutation *Cacnala* R192Q. Même si elles ne démontrent aucun phénotype neurologique déclaré, les souris KI homozygotes R192Q manifestent un relargage uniquantal augmenté d'ACh à la JNM. De plus, dans des conditions de bas calcium, la stimulation nerveuse de bas niveau était augmentée plusieurs fois. La dépression qui origine du cortex (CSD), le mécanisme généralement associé comme étant à l'origine de l'aura de la migraine, peut résulter d'un mauvais fonctionnement synaptique. Chez la souris mutante R192Q KI, le seuil nécessaire à l'induction de la CSD était réduit de façon significative; mais une fois initiée, la CSD se propageait plus rapidement. Des mesures sur des neurones KI de culture primaire isolés du cerebellum démontrèrent une modification du voltage d'activation de type hyperpolarisation ainsi qu'une intensité de la densité du courant des canaux $Ca_V2.1$ mutant R192Q. Au **chapitre 3**, une étude plus détaillée de la JNM nous révèle que des effets dose dépendants ainsi que de subtiles anomalies du relargage de l'ACh à haut débit. Cependant, la souris KI R192Q ne démontre pas de progression dans les anomalies de neurotransmission à la JNM. Nos analyses en microscopie nous montrent que la mutation R192Q n'affecte ni la taille ni la structure de la synapse neuromusculaire. La mutation CACNA1A S218L est associée avec un phénotype très sévère chez les patients, incluant la FHM1 avec une sensibilité accrue aux oedèmes cérébraux et des comas fatals résultant de traumatismes crâniens. La souris mutante K1 S218L démontrent une morbidité accrue ainsi qu'un phénotype d'ataxie légère. Nos analyses électrophysiologiques de anomalies sévères et dose

dépendantes du relagage d'ACh (**chapitre 4**) montrent que le relargage uniquantal spontané était multiplié par 15. L'ACh résultant de la stimulation nerveuse de bas niveau était la même que chez les individus de normaux à deux mois, mais cependant augmentée de 160% chez les souris de 12 mois. Le relargage d'ACh à la JNM des mutants KI S218L était plus sensible à l'ajout de 3,4-diaminopyridine (bloqueur sélectif des canaux K^+), ceci étant parfaitement compatible avec l'hypothèse voulant que les mutants $Ca_V2.1$ S218L soient caractérisés par un flux calcique d'une durée prolongée. Nos observations sont donc compatibles avec l'hypothèse voulant que la FHM fasse partie des troubles d'hyperexcitabilités.

La deuxième partie de cette thèse se concentre sur les mutants naturels *tottering*, *rolling Nagoya* et *leaner*. La souris *tottering* contient une mutation P601L dans le canal calcique $Ca_V2.1$ *Cacnala*, ceci résultant en un complexe phénotype neurologique. Notre laboratoire avait déjà publié la caractérisation des effets synaptiques de la mutation *tottering*. Pour mieux comprendre la contribution compensatoire des canaux autres que $Ca_V2.1$ dans le relargage d'ACh à la JNM, nous avons étudié la sensibilité à diverses toxines spécifiques aux canaux Ca_V2 (**chapitre 5**). La JNM normale est exclusivement dépendante des canaux $Ca_V2.1$ pour le relargage d'ACh. Cependant, les synapses ont l'habileté de recruter des canaux autres que $Ca_V2.1$ pour compenser dans les zones actives. Ceci peut en partie compenser pour le mauvais fonctionnement des canaux mutés $Ca_V2.1$. En utilisant l'ω-agatoxin-IVA chez la souris *tottering*, nous avons observé que la contribution des canaux $Ca_V2.1$ au relargage d'ACh à la JNM était réduite et compensée par les canaux calciques $Ca_V2.3$ (de type R) qui sont sensible au SNX-482. Ceci est la première étude démontrant une compensation fonctionnelle par des canaux autres que $Ca_V2.1$ à la JNM de la souris *tottering*. Le **chapitre 6** se consacre à la JNM de la souris *rolling Nagoya*, qui souffre d'une ataxie sévère et qui porte une mutation ponctuelle dans *Cacna1a*. Nous avons observé un relargage augmenté du mode uniquantal spontané mais un relargage suscité par la stimulation nerveuse sévèrement réduit. Le relargage suscité par la stimulation nerveuse était encore plus affecté à la JNM du *soleus*. Ceci résulte en une faiblesse musculaire clinique ainsi qu'une détérioration de la neurotransmission comme nous avons démontré par des mesures de force de préhension, des mesures de contraction musculaire *in vitro* et des études d'électromyographie *in vivo*. Malgré la réduction sévère du relargage suscité par la stimulation nerveuse à la JNM de *rolling Nagoya*, nous n'avons pu identifier de compensation de canaux autres que $Ca_V2.1$. Ceci est la première étude décrivant une augmentation spontanée du relargage d'ACh avec une réduction concomitante du relargage suscité par la stimulation nerveuse.

La souris *leaner* porte une mutation *Cacna1a* qui mène à une protéine $Ca_V2.1$ sévèrement tronquée. Ceci résulte en une mortalité accélérée chez l'animal, typiquement durant la quatrième semaine de vie, ainsi qu'en un phénotype neurologique sévère d'ataxie et de dystonie. Ceci ressemble de façon remarquable à la souris KO pour $Ca_V2.1$. Dans notre caractérisation électrophysiologique de la JNM de *leaner* et du KO $Ca_V2.1$, nous avons observé une réduction d'approximativement 50% du relargage suscité par la stimulation nerveuse ainsi que du relargage spontané. Cependant, le profile de compensation impliquant des canaux autres que $Ca_V2.1$ démontre des différences significatives entre *leaner* et la KO $Ca_V2.1$ (**chapitre 7**). Alors que le relargage d'ACh à la JNM du KO $Ca_V2.1$ devenait dépendant à la fois des canaux Ca_V1 (~25%), $Ca_V2.2$ (~25%) et $Ca_V2.3$ (~50%), chez la souris *leaner* le relargage demeurait principalement dépendant des canaux $Ca_V2.1$ (~60%). Le reste du relargage suscité par la stimulation nerveuse provenait des canaux $Ca_V2.3$ ainsi que d'une composante non caractérisée (~25%). La désactivation ponctuelle et localisée du gène *Cacnala* en utilisant des techniques expérimentales peut nous permettre de mieux comprendre la fonction synaptique. Par exemple,

la souris KO $Ca_V2.1$ montre un déficit du développement et meure généralement au cours de la quatrième semaine de vie. En désactivant $Ca_V2.1$ seulement plus tard, à un stade plus mature, les effets directs et développementaaux peuvent être isolés. Au **chapitre 8**, nous décrivons la dérivation d'une souris transgénique qui nous permet de désactiver de façon conditionnelle le gène *Cacna1a*. Nous montrons que l'ablation hâtive et total du gène durant la gestation résulte en un animal semblable aux souris KO pour $Ca_V2.1$ (générées par une méthode conventionnelle) en ce qui concerne leur phénotype ataxique/épileptique ainsi que pour l'électrophysiologie de la JNM.

L'EA2 est une maladie autosomique dominante qui est causée par des mutations non-senses résultant habituellement en des canaux $Ca_V2.1$ non fonctionnels et souvent tronqués. Cette situation chez EA2 manifeste donc une ressemblance génétique avec les formes hétérozygotes de *leaner* et la souris KO pour $Ca_V2.1$. Au **chapitre 9**, nous décrivons un déficit dans le relargage d'ACh à la JNM des hétérozygotes *leaner*, ceci incluant une réduction spontanée du relargage provoqué par la stimulation nerveuse. A l'opposé, les souris hétérozygotes KO $Ca_V2.1$ n'ont pas démontré d'anomalies, ceci suggérant une haplosuffisance. *In vivo*, l'acetazolamide – un médicament utilisé pour le traitement de l'EA2 - n'a pas démontré d'effets sur les paramètres de relargage d'ACh, rejetant la hypothèse voulant que l'acetozalamide exerces des effets directes sur des canaux $Ca_V2.1$ (mutés).

Des études basées sur des systèmes d'expression hétérologues suggèrent que les sous-unités accessoires des canaux $Ca_V2.1$ peuvent aussi moduler la fonction des canaux $Ca_V2.1$ *in vivo*. En accord avec cette hypothèse, les souris mutantes naturelles pour les sous-unités (*ducky*, $\alpha_2\delta$-2; *lethargic*, β_4; *stargazer*, γ_2) des canaux $Ca_V2.1$ montrent des phénotypes neurologiques sévères similaires a ceux que nous observons chez les mutants naturels *Cacna1a*. Le rôle de ces sous-unités à la JNM périphérique est cependant encore inconnu. Nos analyses électrophysiologiques de la JNM de ces souris ne montrent aucune anomalie fonctionnelle **(chapitre 10)**, suggérant ainsi une redondance de ces sous-unités à la JNM des mammifères.

Le Topiramate est un médicament utilisé pour combattre la migraine et un anti-convulsif. Même si son principe actif est encore inconnu, on a suggéré que les effets thérapeutiques observés lors de son utilisation pourraient résulter d'une modulation directe des canaux $Ca_V2.1$ mutés. Pour tester cette hypothèse, nous avons administré du topiramate à des souris normales, des mutants KI R192Q et des souris *tottering* (**chapitre 11**). Les paramètres de base du relargage n'ont cependant pas été affectés, ceci suggérant que le topiramate n'influence pas directement le fonctionnement des canaux $Ca_V2.1$.

Le **chapitre 12** met l'emphase sur les découvertes les plus importantes générées par le présent travail expérimental dans le contexte de la littérature déjà publiée sur le sujet. Nous concluons que les mutations R192Q et S218L de type FMH1 provoquent une augmentation du flux de calcium à travers les canaux $Ca_V2.1$ à la JNM sans contribution compensatoire au relargage d'ACh des canaux autre que $Ca_V2.1$. De plus, à la JNM des mammifères, les sous-unités de canaux CaV sont partiellement redondantes. Finalement, l'acetazolamide et le topiramate n'ont aucun effet direct sur la modulation des canaux $Ca_V2.1$. L'étude de la JNM présentée dans cette thèse nous permet de réexaminer les dysfonctions synaptiques causées par la mutation dans les canaux $Ca_V2.1$. Les effets synaptiques sur les synapses centrales partagent probablement les mêmes caractéristiques que celles observées à la JNM et sont sans doute en partie responsables des symptômes neurologiques observés chez l'humain et chez les souris mutantes dans $Ca_V2.1$.

List of abbreviations

Bibliography

Curriculum Vitae

Simon Kaja

Synaptic effects of mutations in neuronal $Ca_V2.1$ calcium channels.

List of abbreviations

4-AP – 4-aminopyridine
ACh – acetylcholine
AChE – acetylcholinesterase
AChR – acetylcholine receptor
AMPA – α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
AZA – acetazolamide
BTx – α-bungarotoxin, *also:* αBTx
Ca_V channels – voltage-gated Ca^{2+} channels
$Ca_V2.1$-KO – $Ca_V2.1$ *null*-mutant
CGCs – cerebellar granule cells
CGRP – calcitonin gene-rated peptide
CMAP – compound muscle action potential
CNS – central nervous system
CSD – cortical spreading depression
DAP – 3,4-diaminopyridine
d-TC – D-tubocurarine
du – ducky
EA2 – episodic ataxia type 2
EMG – electromyography
EPP – endplate potential
EPSC – excitatory post-synaptic current
FDB – flexor digitorum brevis
FHM – familial hemiplegic migraine
FHM1 – familial hemiplegic migraine type 1
$GABA_A$ – γ-aminobutyric acid type A
$GABA_B$ – γ-aminobutyric acid type B
GBP – gabapentin
HEK – human embryonic kidney
HET – heterozygous *rolling Nagoya*
HVA – high voltage-activated
IPSC – inhibitory post-synaptic current
KI – knock-in
KO – knock-out
KO/wt – heterozygous $Ca_V2.1$-KO
LEMS – Lambert Eaton Myasthenic Syndrome
lh – lethargic
Ln – leaner
Ln/wt – heterozygous *leaner*
LVA – low voltage-activated
MA – migraine with aura
MEPP – miniature endplate potential
MO – migraine without aura
nAChRs – nicotinic acetylcholine receptors
NMJ – neuromuscular junction
P – postnatal day
PBS – phosphate buffered saline
PCs – Purkinje cells
PNS – peripheral nervous system
RN – *rolling Nagoya*
RNS – repetitive nerve stimulation
SCA6 – spinocerebellar ataxia type 6
stg – stargazer
TPM – topiramate
wk – week
wt, wt/wt – wild-type
αBTx – α-bungarotoxin, *also:* BTx
ωAgaIVA – ω-agatoxin-IVA

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Curriculum Vitae

Simon Kaja was born on October 9, 1979 in Düsseldorf, Germany. He received his secondary education at the Städtisches Gymnasium Odenkirchen (Mönchengladbach, Germany), where he passed the German baccalaureate ('Abitur') in spring 1998. In the same year, Simon began his study of Molecular Biology and Biochemistry at Durham University (Durham, United Kingdom). In 1999, he was selected a scholar of the German National Academic Foundation. During his study, Simon was intern at Bayer AG (Uerdingen, Germany), Novo Nordisk A/S (Copenhagen, Denmark), Denmark's Technical University (Lyngby, Denmark) and the University of North Texas Health Science Center at Fort Worth (Fort Worth, Texas, USA). In summer 2002, Simon graduated from Durham University with First Class Honours and was awarded the Boulter Prize for the best degree in Molecular Biology and Biochemistry. For his Bachelor of Science dissertation entitled "Characterization of $GABA_A$ receptors in *Tottering* mutant mice", Simon received the British Neuroscience Association Undergraduate Award 2002/2003.

After his degree, Simon joined the laboratory of Dr. Jaap Plomp at the Leiden University Medical Centre, Leiden, The Netherlands, where he worked towards his doctoral degree.

Simon has been awarded several prestigious post-doctoral fellowships, and since July 2006, he is European Molecular Biology Organization post-doctoral fellow and trainee of the Michael Smith Foundation for Health Research in the laboratory of Prof. Terrance Snutch (University of British Columbia, Vancouver, Canada), where he continues his research into the roles of calcium channels in human neurological disorders.