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$Ca_V2.1$ channels are crucial players in neurotransmission both in the central and peripheral nervous system. Mutations in the CACNA1A gene, encoding the channel-forming $Ca_V2.1$-α_1 subunit, have been implicated in a number of human neurological diseases, including Familial Hemiplegic Migraine type 1 (FHM1) and Episodic Ataxia type 2 (EA2).

The work presented in this thesis focused on the synaptic consequences of *Cacna1a* mutations on neurotransmitter release at the mouse neuromuscular junction (NMJ). Furthermore, the effects of absence of accessory subunits of Ca_V channels at the NMJ were investigated.

In the following, the main findings will be highlighted and the synaptic phenotypes of the various mutations will be discussed in a neurobiological and disease-oriented context.

Neurobiological Conclusions

Spontaneous acetylcholine release is partly dependent on $Ca_V2.1$ channels

Under basal conditions, single vesicles filled with the neurotransmitter acetylcholine (ACh) are released spontaneously at a frequency of approximately one vesicle per second. To date, there have been conflicting reports on the nature of Ca_V channels mediating spontaneous uniquantal release at the mammalian NMJ (Protti et al., 1991; Protti and Uchitel, 1993; Losavio and Muchnik, 1997; Plomp et al., 2000; Giovannini et al., 2002).

The present thesis demonstrates that a large proportion of spontaneous uniquantal ACh release (~50%) is $Ca_V2.1$ channel-dependent at the adult wild-type mouse NMJ, shown by the sensitivity of miniature endplate potential (MEPP) frequency to the selective $Ca_V2.1$ channel blocker ω-agatoxin-IVA and the insensitivity to blockers of other subtypes (see chapters 2-10). This confirms previous reports by us and others (Plomp et al., 2000; Giovannini et al., 2002). A ~50% reduction in MEPP frequency was found across various genetic backgrounds, including C57/B6J (e.g. *tottering*), C3H (*ducky*), and others (e.g. *rolling Nagoya*). The reasons for discrepancies with other studies may lie in lower concentrations of toxins used (Protti et al., 1991), in species differences (mouse vs. rat NMJ; Losavio and Muchnik, 1997), or in methodology.

$Ca_V2.1$ channels are classified as high voltage-activated (HVA) channels, which are not expected to open at resting membrane potential. The findings presented in this thesis have strengthened the hypothesis that a sub-population of ω-agatoxin-IVA-sensitive $Ca_V2.1$ channels exists, which are low voltage-activated (LVA) and responsible for mediation of spontaneous neurotransmitter release.

Gene-dosage dependent increases in MEPP frequency compared to wild-type have been found in several *Cacna1a* mutants, including FHM1 R192Q KI (chapters 2-3). This further indicates that $Ca_V2.1$ channels underlie spontaneous ACh release. Heterologous expression of R192Q-mutated $Ca_V2.1$ channels has suggested a negative shift in the activation voltage of these channels (Hans et al., 1999; Tottene et al., 2002), which was confirmed in primary cerebellar neurones obtained from FHM1 R192Q KI mice (chapter 2). A similar negative shift in putative LVA $Ca_V2.1$ channels is likely to account for the ω-agatoxin-IVA-sensitive increase in MEPP frequency at FHM1 R192Q KI NMJs, and possibly also those of other *Cacna1a* mutant strains, such as FHM1 S218L KI (chapter 4), *tottering* (chapter 5) and *rolling Nagoya* (chapter 6). An increase in the number of pre-synaptic $Ca_V2.1$ channels at mutant NMJs may also contribute to increases in MEPP frequency. In contrast, the reduced MEPP frequency seen at *leaner* and $Ca_V2.1$ *null*-mutant ($Ca_V2.1$-KO) NMJs may be brought about by a slight positive shift of activation voltage and/or fewer channels at any given nerve terminal.

Only $Ca_V2.1$ channels contribute to ACh release at the adult mouse NMJ

ACh release at neonatal NMJs is mediated largely by $Ca_V2.2$ channels. During the first few postnatal weeks, these are gradually replaced by $Ca_V2.1$ channels (Gray et al., 1992; Breugelmans and Bazzy, 1997; Rosato and Uchitel, 1999; Rosato-Siri et al., 2002). From around postnatal day (P) 15, ACh release is entirely dependent on $Ca_V2.1$ channels, and typically >90% of quantal content can be blocked by application of 200 nM ω-agatoxin-IVA (Uchitel et al., 1992; see also chapters 2-10).

However, some studies report that evoked ACh release at mature NMJs remains sensitive to the selective $Ca_V2.2$ blocker ω-conotoxin-GVIA (Hamilton and Smith, 1992; Rossoni et al., 1994). Studying ACh release in $Ca_V2.1$-deficient mice and using the selective blocker of pre-synaptic K^+ channels, 4-aminopyridine, Uchitel and colleagues have suggested that $Ca_V2.2$ channels are located further away from active zones than $Ca_V2.3$ channels (Urbano et al., 2003). Applying 2.5 μM of the selective $Ca_V2.2$ channel blocker ω-conotoxin-GVIA in the presence of 50 μM 4-aminopyridine to wild-type NMJs, it is demonstrated that $Ca_V2.2$ channels do not contribute to either spontaneous or evoked ACh release at the wild-type NMJ, apparently not even at sites more distant from the active zone (chapter 5). This finding is in accordance with immunohistochemical approaches that identified $Ca_V2.2$ labelling in Schwann cell processes, yet not at motor nerve terminals (Day et al., 1997; Westenbroek et al., 1998; Pagani et al., 2004). It remains elusive why some studies identified a $Ca_V2.2$-sensitive component of ACh release at the NMJ.

Table 1. Effect of *Cacna1a* mutations on spontaneous and nerve stimulation-evoked ACh at the mouse NMJ.

	Spontaneous ACh release	Low-rate evoked ACh release	High-rate EPP rundown level	Compensatory non-$Ca_V2.1$ contribution
FHM1 R192Q KI	↑	=	=	no
FHM1 S218L KI	↑	↑	↓	no
Tottering	↑	=	↓	yes
rolling Nagoya	↑	↓	↑	no
Leaner	↓	↓	↓	yes
$Ca_V2.1$-KO	↓	↓	↓	yes

Spontaneous and evoked ACh release are controlled independently. Inceased MEPP frequency can thus occur in combination with unaltered quantal content (e.g. FHM1 R192Q KI mice), increased quantal content (FHM1 S218L mice) or even reduced quantal content (*rolling Nagoya*). Reduced quantal content is not sufficient for compensatory contributions of non-$Ca_V2.1$ channels to ACh release at the NMJ (*rolling Nagoya*).

Spontaneous uniquantal and evoked ACh release are controlled independently

This thesis provides interesting insights into the control of both spontaneous and evoked ACh release at the murine NMJ. In particular, both these mechanisms appear to be regulated independently from each other (see Table 1).

Opposing effects of a single amino-acid change in $Ca_V2.1$ are particularly baffling in *rolling Nagoya* mice, where a 50% reduction in quantal content is accompanied by a 3-fold increase in spontaneous release (i.e. MEPP frequency). A similar synaptic phenotype has been found at biopsy NMJs of a patient suffering from EA2 (Maselli et al., 2003). As discussed in detail in previous chapters, a possible explanation is that putative LVA $Ca_V2.1$ channels are affected differentially compared with those HVA $Ca_V2.1$ channels that mediate nerve stimulation-evoked quantal release. It remains to be clarified, whether this putative sub-population of LVA $Ca_V2.1$ channels arises from alternative splicing of the *Cacna1a* gene (see e.g. Jurkat-Rott and Lehmann-Horn, 2004) or by mechanisms that may include differential post-translational modification or modulation of HVA $Ca_V2.1$ channels. Furthermore, the localisation of such channels at or near the active zone may be of crucial importance. $Ca_V2.1$

channels have been shown to be associated with lipid rafts, cholesterol-rich plasma membrane microdomains, where they interact with proteins of the exocytotic machinery (Taverna et al., 2004; for review, see Tsui-Pierchala et al., 2002). It is possible that differential compartmentalisation of $Ca_V2.1$ sub-populations in lipid rafts accounts for independently controlled spontaneous and evoked ACh release.

Compensatory expression of other types of Ca_V channels at the $Ca_V2.1$ mutant NMJ

The wild-type NMJ is exclusively dependent on $Ca_V2.1$ channels for ACh release, however, compensatory expression of non-$Ca_V2.1$ channels can occur when $Ca_V2.1$ channels are dysfunctional or absent (Table 2; Uchitel et al., 1992; Urbano et al., 2003; chapters 5 and 7). The exact mechanisms leading to this compensatory contribution remain unknown to date. However, reduced evoked release *per se* is not sufficient to trigger expression of other Ca_V channels, as seen at NMJs of *rolling Nagoya* mutant mice (chapter 6). Neither did increased MEPP frequency in FHM1 R192Q or S218L KI cause other Ca_V channels to contribute to ACh release (chapters 2-4). Recently, the so-called 'slot-hypothesis' has emerged, suggesting that ACh release sites have 'slots', which are preferentially occupied by $Ca_V2.1$ channels, however, can be filled by other Ca_V channels in absence of the former (Cao et al., 2004). Thus, a too small size of the $Ca_V2.1$ channel pool available to occupy these slots may be a necessary signal to induce compensatory recruitment of other Ca_V channels. Arguing against this hypothesis are studies on *leaner* and $Ca_V2.1$-KO mice, which have revealed very different compensatory expression patterns, despite an almost identical neurological phenotype and similarly reduced ACh release in these two mutants (chapter 7). Signalling via protein interaction sites on the $Ca_V2.1$ channel protein are most likely to confer the information required for compensatory expression of other Ca_V channels. The different interaction sites and possible mechanisms have been proposed in chapter 7 of this thesis. It may be that the incapability of certain synapse types to recruit compensatory Ca_V channels is an important determinant of the eventual synaptic phenotype following from different $Ca_V2.1$ channel mutations.

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

Chapter 10 of this thesis describes ACh release at the NMJs of the natural calcium channel mutants *ducky*, *lethargic* and *stargazer*, which lack the functional Ca_V accessory subunits $\alpha_2\delta$-2, β_4 and γ_2, respectively. Interestingly, ACh release at the NMJ is not compromised by loss of any of the three subunits (chapter 10). This suggests that either these three subunits are not present at the NMJ, or if present, they have no functional role. Alternatively, their function might be compensated for by other subunits.

Pharmacological experiments using gabapentin, an anti-epilepsy drug known to modulate $Ca_V2.1$ channel function by acting on the $\alpha_2\delta$ subunit, further suggested that $\alpha_2\delta$ subunits are not present at the mouse NMJ (chapter 10). Immunoblotting assays on NMJ-enriched diaphragm muscle fractions did not detect any $\alpha_2\delta$ signal (A. Davies, personal communication), supporting this hypothesis. The severe phenotype of *ducky* mice is thus the result of central neurological dysfunction alone, without a direct NMJ component.

Similarly, the question remains whether γ_2 (or other γ subunits) are actually present at the mouse NMJ, and what their physiological function is (chapter 10).

The β_4 subunit, in contrast, is thought to be present at the NMJ, as indicated from immunohistochemical analysis (Pagani et al., 2004). Other β subunits can compensate for loss of the β_4 subunit in *lethargic* brain, a process named "subunit reshuffling" (Burgess et al., 1999). It is thus possible that similar mechanisms operate at the *lethargic* NMJ.

The study on the natural mouse mutants *ducky, lethargic* and *stargazer* presented in this thesis is the first to investigate the possible role of Ca_V channel subunits on ACh release at the NMJ. It can be concluded that the $\alpha_2\delta$-2, β_4 and γ_2 subunits do not fulfil crucial physiological roles in neurotransmitter release at the NMJ.

Synaptic Dysfunction in (Familial Hemiplegic) Migraine?

The NMJ studies of FHM1 KI mice carrying the R192Q and S218L mutations (chapters 2-4) provide some scope for speculation on the possible consequences of these $Ca_V2.1$ channel mutations in the central nervous system (CNS). As $Ca_V2.1$ channels are expressed in all regions known to be important in migraine, including the cerebral cortex, trigeminal ganglia, and brainstem nuclei (Goadsby et al., 2002; Pietrobon and Striessnig, 2003; Pietrobon, 2005a), CNS synaptic dysfunction is likely to cause or contribute to the symptoms of (familial hemiplegic) migraine.

What are the potential consequences of $Ca_V2.1$ mutations on central synapses?
Many central synapses are dependent on $Ca_V2.1$ channels for neurotransmitter release, either exclusively or partly (cf. chapter 7; for review, see Snutch et al., 2005). In contrast to the NMJ, central synapses do not possess a safety factor for successful neurotransmission, and the probability and amplitude of evoked release is low at most central synapses (Hessler et al., 1993; Lou et al., 2005) (Stevens, 1993), potentially similar to ACh release at the NMJ in the presence of low extracellular Ca^{2+} concentration (chapter 2-4). Under these conditions, evoked release is increased several-fold at FHM1 R192Q and S218L KI NMJs compared with wild-type. Similar increases in evoked release at central synapses would likely affect neuronal network function by interfering with dendritic signal integration.

Neurotransmitter release in Purkinje cells (PCs) is exclusively mediated by $Ca_V2.1$ channels (Mintz et al., 1992a). Given the NMJ dysfunction in FHM KI mice, it is hypothesised that PC nerve terminals in these mice and in FHM1 patients have increased transmitter release (chapters 2-4). Interestingly, imaging studies showed that the presence of sub-clinical cerebellar lesions correlated with migraine attack frequency in patients with typical migraine (Kruit et al., 2004). Increased neurotransmitter release or chronically elevated pre-synaptic Ca^{2+} influx in PCs may thus contribute to the occurrence of these sub-clinical brain lesions.

Lower thresholds for the initiation of cortical spreading depression (CSD) in FHM1 KI mice (see below) are compatible with the hypothesis that central synapses exhibit increased evoked transmitter output, similar to the situation at the NMJ.

Synapses that rely on multiple types of Ca_V channels for neurotransmitter release, however, may be able to compensate (more effectively) for mutated $Ca_V2.1$-mediated increases in transmitter output by recruiting non-$Ca_V2.1$ channels to the active zone (Cao et al., 2004). The incapability of certain synapse types to target compensatory Ca_V channels to the active zone could underlie the specific neurological symptoms associated with different CACNA1A mutations.

A role of (mutated) $Ca_V2.1$ channels in cortical spreading depression?
It has now been widely accepted that migraine is caused by primary brain dysfunction, which subsequently results in the activation and sensitisation of the trigeminovascular system (Goadsby et al., 2002; Pietrobon and Striessnig, 2003; Silberstein, 2004; Pietrobon, 2005a). However, the triggers for an acute migraine attack have not been identified to date. An early event during the migraine attack is CSD. Electrophysiological and imaging data has correlated CSD with migraine aura (Olesen et al., 1981; Lauritzen, 1994), the mostly visual neu-

rological symptoms migraine patients suffer, and which frequently manifests as scotoma (i.e. an area of loss of vision), usually commencing in the centre of the field of vision and slowly travelling across to the periphery. In animal models, CSD can be triggered by focal stimulation of the cerebral cortex. Being a slowly propagating wave of strong neuronal depolarisation, CSD causes transient intense spike activity that is followed by long-lasting neuronal silence (Lauritzen, 1994; Pietrobon, 2005a).

Glutamate release from cortical synapses is important during CSD, and mediated by $Ca_V2.1$ channels (Turner et al., 1992). Furthermore, FHM1 R192Q and S218L KI mice show a reduced threshold for initiation and a faster velocity of propagation of CSD (chapter 2; Pizzorusso et al., 2006). In contrast, $Ca_V2.1$ *leaner* mice have a significantly increased threshold for initiation of CSD (Ayata et al., 2000). Together with the synaptic effects in these KI mice as presented in this thesis, these findings suggest that increased pre-synaptic Ca^{2+} influx through FHM1-mutated $Ca_V2.1$ channels contributes to both initiation and propagation of CSD (chapter 2; see also Pietrobon, 2005a).

The detailed mechanism underlying the initiation of CSD is still unknown. Most likely, a local build-up of the extracellular K^+ concentration has a pivotal role in triggering CSD (Somjen, 2001). During CSD, extracellular Ca^{2+} concentrations drop to levels around 0.2 mM (Somjen, 2001). Under these conditions (high K^+ and low Ca^{2+}) ACh release at NMJs of R192Q KI and S218L KI mice was significantly increased compared with wild-type (chapters 2-4). It can be hypothesised that glutamate release in the CNS from R192Q or S218L terminals is increased similarly under these conditions and thus facilitates CSD (this thesis; Pietrobon, 2005a; Tottene et al., 2005).

Taken together, it is perceivable how CSD can be triggered more readily in FHM1-mutated cortices: excessive $Ca_V2.1$-mediated glutamate release from K^+-depolarised terminals causes the relief of post-synaptic Mg^{2+} block of NMDA receptors, resulting in NMDA receptor activation, further post-synaptic depolarisation and resultant increase in extracellular K^+ concentration, which by itself causes further pre-synaptic $Ca_V2.1$-mediated glutamate release (Figure 1; see also Pietrobon, 2005a).

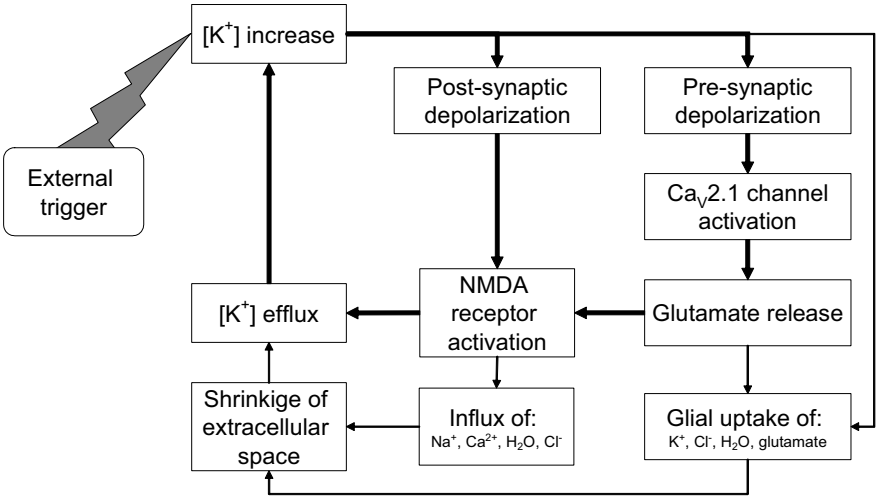

Figure 1. Role of $Ca_V2.1$ channels in Cortical Spreading Depression (CSD).
CSD is initiated and maintained by a positive feedback loop. FHM1-mutated $Ca_V2.1$ channels open more readily under slightly depolarizing conditions and low Ca^{2+}, likely resulting in increased glutamate release during CSD (modified from Pietrobon, 2005a).

$Ca_V2.1$ channel involvement in FHM1 migraine headache?

Migraine headache is caused by the activation of the trigeminovascular system, specifically the trigeminal afferents innervating meningeal blood vessels (Goadsby and Edvinsson, 1993). Whereas the possible direct consequences of $Ca_V2.1$ mutations on trigeminal nociception remain unknown to date, progress has been made in confirming the tentative link between CSD and the activation of trigeminal afferents in animal models (Ebersberger, 2001; Ebersberger et al., 2001; Bolay et al., 2002; Kunkler and Kraig, 2003; Moskowitz et al., 2004).

The activation of trigeminal afferents results in activation of the trigeminal nucleus caudalis and the cervical spinal cord, before the signal is carried through to regions involved in pain reception, including thalamic nuclei and the periaqueductal grey region, which is activated in craniovascular pain. Also, vasoactive neuropeptides such as calcitonin gene-related peptide (CGRP) and substance P are released from perivascular nerve endings following trigeminal afferent activation. CGRP in turn causes the neurogenic vasodilation of pain-sensitive meningeal blood vessels, causing headache. Goadsby and Edvinsson (1993) showed that cranial CGRP levels are high during migraine and normalise when migraine is relieved pharmacologically.

Interestingly, $Ca_V2.1$ channels are involved in CGRP release from perivascular nerve endings (Hong et al., 1999) and control neurogenic vasodilation of meningeal blood vessels (Asakura et al., 2000). Furthermore, $Ca_V2.1$ channels are expressed in the periaqueductal grey region and thus important in the central processing of trigeminal pain (Knight et al., 2002). Mutation-induced $Ca_V2.1$ dysfunction is therefore likely to contribute directly to migraine headache in FHM1.

Is the NMJ a good model synapse to study synaptic pathophysiological mechanisms in $Ca_V2.1$ channelopathies?

FHM1 mutations, or more generally $Ca_V2.1$ channel function, is being studied in a wide variety of model systems, including the NMJ, the calyx of Held synapse, primary cultured (KI) neurones and a wide variety of transfected cells. All these experimental approaches are associated with intrinsic methodological and technical advantages and problems (cf. chapter 2; Pietrobon, 2005a). Of particular relevance, the NMJ is a valuable model due to its relative ease of accessibility and its complete dependence on $Ca_V2.1$ channels for ACh release. Most importantly, it allows for the study of the synaptic effects of mutations in $Ca_V2.1$ channels or accessory subunits in their native pre-synaptic environment. Studies in *lethargic* mice illustrate the possible discrepancies that may evolve from transfection studies and direct synaptic measurement. While heterologous transfection studies predicted severe transmitter release deficits in the absence of the β_4 subunit of Ca_V channels in *lethargic* mice (Burgess et al., 1997; Escayg et al., 1998), the only two direct synaptic studies to date (PC synapses and NMJs) failed to identify any abnormalities (Burgess et al., 1999; chapter 10).

An alternative to the NMJ for the study of $Ca_V2.1$ mutation-induced synaptic effects is the central calyx of Held synapse (for review, see Sakaba et al., 2002). Neurotransmitter release at the calyx of Held, however, is mediated jointly by $Ca_V2.1$ and $Ca_V2.2$ channels (Ishikawa et al., 2005), complicating the characterisation of potential synaptic effects directly induced by $Ca_V2.1$ channel dysfunction.

A unique advantage of heterologous expression systems, such as transfected HEK cells, is the possibility to investigate properties of single channels, their suitability for pharmacological studies and, last but not least, the avoidance of animal experiments (for review, see Thomas and Smart, 2005).

Is $Ca_V2.1$ channel dysfunction involved in typical migraine (with aura)?

In contrast to typical migraine, which is considered a complex multifactorial disease, FHM features a monogenic inheritance pattern. As FHM and migraine with aura (MA) share all clinical features but the hemiplegia characteristic of FHM, FHM can be considered a model for typical migraine (Pietrobon, 2005a), and it may be that the mutated FHM genes are also involved in some way in typical migraine. However, there are contradicting reports regarding the involvement of FHM loci in typical migraine. Whilst some studies identify linkage to the CACNA1A locus in patients with typical migraine or MA (May et al., 1995; Terwindt et al., 1998; Terwindt et al., 2001), others were unable to identify CACNA1A (or ATP1A2) mutations in another subgroup of migraine patients with either typical migraine or inherited MA (Wieser et al., 2003; Jen et al., 2004b; Kirchmann et al., 2006).

In conclusion, $Ca_V2.1$ channels fulfil pivotal roles in the CNS in general, and in regions known to be important in migraine pathophysiology. CNS $Ca_V2.1$ dysfunction resultant from CACNA1A mutations is likely to share features with those observed at the NMJ, and can help us explain the pathophysiological mechanisms leading to migraine. The NMJ is a suitable model system that, together with other approaches, can provide useful insights into possible CNS dysfunction in Ca^{2+} channelopathies.

NMJ Abnormalities in All $Ca_V2.1$ Channelopathies?

Given the prominent role of $Ca_V2.1$ channels in neurotransmitter release at the NMJ, it has been hypothesised that NMJ abnormalities in patients with CACNA1A mutations lead to muscle weakness.

NMJ abnormalities in FHM1?

Schoenen and colleagues published several studies showing single-fibre electromyography (EMG) abnormalities (i.e. increased jitter and occurrence of blocking, indicative of muscle weakness) in patients with migraine with aura, but without identified CACNA1A mutations (Ambrosini et al., 1999; Ambrosini et al., 2001a; Ambrosini et al., 2001b; Ambrosini et al., 2003). Similar findings were recently reported for patients suffering migraine with aura, but not for those with migraine without aura (Domitrz et al., 2005). A single study found dysfunctional neurotransmission in cluster headache (Ertas and Baslo, 2003). However, a detailed clinical electrophysiological analysis of neuromuscular transmission in (CACNA1A-mutated and non-mutated) FHM patients did not indicate neuromuscular transmission failure (Terwindt et al., 2004). This thesis presents a detailed characterisation of neurotransmission in FHM1 R192Q KI mice (chapter 3). Both the results of *in vitro* contraction experiments and *in vivo* repetitive nerve stimulation EMG were normal in R192Q KI mice (chapter 2). Similarly, in the more severely affected FHM1 S218L KI mice, *in vitro* contraction experiments did not reveal any (sub-)clinical effects of the mutation on neuromuscular transmission, neither at two nor twelve months of age (chapter 4, S. Kaja and J.J. Plomp, unpublished observations). These findings are in accordance with those of Van Dijk and colleagues who failed to identify single-fibre EMG abnormalities in FHM1 patients with the I1811L mutation (Terwindt et al., 2004). Although changes in ACh release at the NMJ of FHM1 patients are highly likely, in view of the NMJ studies in KI mice, they are apparently not of such magnitude to cause failure of neuromuscular transmission and ensuing muscle weakness.

NMJ abnormalities in EA2 and SCA6?

Several studies have reported impaired neuromuscular transmission in EA2 patients (Jen et al., 1999; Jen et al., 2001; Maselli et al., 2003), whereas similar defects were not present in spinocerebellar ataxia type 6 sufferers (Jen et al., 2001; Schelhaas et al., 2004). It is to be considered that most EA2 mutations (both mis-sense and non-sense CACNA1A mutations) display dominant-negative effects (Jeng et al., 2006), which may lead to the observed low quantal content and compensatory expression of $Ca_V2.2$ channels (Maselli et al., 2003). In one patient, a concomitant increase in MEPP frequency was found (Maselli et al., 2003). Thus, the picture emerges that FHM1 mutations are associated with changes of evoked quantal ACh release at the NMJ that are not large enough to result in muscle weakness, whereas EA2 mutations reduce release to such an extent that EPPs become subthreshold and muscle weakness ensues.

Is the rolling Nagoya *mouse a genetic model of Lambert-Eaton myasthenic syndrome?*

In Lambert-Eaton myasthenic syndrome (LEMS), auto-antibodies are directed towards $Ca_V2.1$ channels causing muscle weakness and paralysis (Kim and Neher, 1988). Quantal content was reduced by ~60% in biopsies from LEMS patients (Lambert and Elmqvist, 1971; J.J. Plomp, unpublished observations). Interestingly, *Cacna1a* R1261G-mutated *rolling Nagoya* mice exhibit a similar neuromuscular phenotype: reduced quantal content (~50% of wild-type), leading to muscle weakness (as determined by grip strength measurements on the forepaws, and *in vivo* repetitive nerve stimulation EMG on the hind feet; chapter 6). The NMJ electrophysiological phenotype is accompanied by a ~3-fold increase in MEPP frequency (see above; cf. chapter 6). *Rolling Nagoya* mice do not show expression of other types of Ca_V channels to compensate for the reduced quantal content as nerve stimulation-evoked ACh release remains >95% ω-agatoxin-IVA-sensitive (chapter 6). The *rolling Nagoya* mouse, therefore, seems a potentially interesting model for NMJ pharmacological studies with relevance to LEMS.

The Use of the NMJ in the Study of Anti-Migraine and Anti-Ataxia Drugs

The mouse NMJ provides a useful model synapse to test the effects of drugs possibly acting on (mutant) $Ca_V2.1$ channels. In this thesis, the effects of three relevant drugs have been investigated: the anti-ataxia drug acetazolamide, and the two anti-epilepsy drugs gabapentin and topiramate. The latter two have recently been used successfully in migraine prophylaxis (Di Trapani et al., 2000; D'Amico et al., 2005).

Acetazolamide

Acetazolamide is a carbonic anhydrase inhibitor that is widely used in the treatment of glaucoma and epilepsy. Of particular relevance, acetazolamide is commonly used in EA2 (cf. chapter 1). The underlying mutations in CACNA1A in EA2 have led to the suggestion that acetazolamide acts by (partly) restoring dysfunctional $Ca_V2.1$ channel activity. This view has been strengthened by reports that showed that acetazolamide had beneficial effects in familial hemiplegic migraine (Athwal and Lennox, 1996) and in ameliorating NMJ abnormalities in migraineurs (Ambrosini et al., 2003). In contrast, acetazolamide did not have any direct effects on native and EA2 H1736L-mutated $Ca_V2.1$ channels expressed in HEK cells *in vitro* (Spacey et al., 2004).

In chapter 9, the effects of acute application of acetazolamide on ACh release at the NMJ have been investigated. However, no effects on ACh release at wild-type, heterozygous

$Ca_V2.1$-KO or heterozygous *leaner NMJs*. This suggests that the therapeutic effect of acetazolamide is not mediated by any direct effect on (EA2-mutated) $Ca_V2.1$ channels. Instead acetazolamide might act on accessory Ca_V channel subunits. However, to date, there are no studies that have investigated the synaptic effects of accessory Ca_V channel subunits in the CNS. Nevertheless, as acetazolamide ameliorates the NMJ abnormalities found in EA2 patients, and given the redundancy of accessory Ca_V channel subunits at the NMJ (chapter 10), direct effects of acetazolamide on Ca_V channel subunits may seem unlikely. It is possible that the metabolic acidosis effect of acetazolamide, resulting from its carbonic anhydrase activity, indirectly influences (mutant) $Ca_V2.1$ channel function.

Gabapentin

Gabapentin was originally developed as GABA-mimic, but soon shown to possess considerable anti-convulsant effects. Now, gabapentin is used widely as anti-epilepsy drug, in treating neuropathic pain, bipolar disorder and as migraine prophylactic (Di Trapani et al., 2000; Carta et al., 2003; Baillie and Power, 2006). Interaction with the $\alpha_2\delta$ subunit of Ca_V channels is considered one important mechanism of the action of gabapentin (Marais et al., 2001; Qin et al., 2002).

Here, the effect of gabapentin on ACh release at the NMJ was investigated at wild-type and *ducky* NMJs (chapter 10). Gabapentin did not exert any acute effect on ACh release, neither at the wild-type nor *ducky* NMJ, strengthening the hypothesis that $\alpha_2\delta$-2 subunits are not present at the mouse NMJ, supporting preliminary data from immunoblotting on NMJ-enriched diaphragm muscle fractions (cf. chapter 10; A. Davies, University College London, personal communication).

It is concluded that the NMJ is an unsuitable model for studying the synaptic effects of gabapentin, due to the likely absence of Ca_V-$\alpha_2\delta$-2 subunits at the NMJ.

Topiramate

Topiramate is another carbonic anhydrase inhibitor with anti-convulsive effects, and has become frequently used in migraine prophylaxis (for reviews, see Shank et al., 2000; D'Amico et al., 2005; White, 2005). Modulation of Ca_V channels has been proposed to be one of many mechanisms of action of topiramate (cf. chapter 11).

Investigating possible effects of acute topiramate application to nerve/muscle-preparations of both FHM1 R192Q KI and *tottering* mice, topiramate did not exert any effect on ACh release parameters at the NMJ (chapter 11). This study suggests that topiramate is incapable of modulating $Ca_V2.1$ channels directly, as the NMJ is exclusively dependent on $Ca_V2.1$ channels for ACh release. However, long-term modulatory effects such as alterations of the phosphorylation state of $Ca_V2.1$ channels cannot be excluded. Interestingly, previous studies suggested that topiramate can acutely act on $Ca_V2.3$ channels in heterologous expression systems (McNaughton et al., 2004). However, we found no effect of topiramate on ACh release at *tottering* NMJs, where Ca^{2+} influx through $Ca_V2.3$ channels contributes to transmitter release. Possibly, the contribution of $Ca_V2.3$ channels (~15%) is too small to identify modulatory effects of topiramate. It will be interesting to test topiramate at NMJs of $Ca_V2.1$-KO mice, where ~50% of ACh release is reliant on $Ca_V2.3$ channels (chapter 7). Chronic administration of topiramate and subsequent NMJ analysis can potentially provide insights into the mechanism of action of topiramate on (mutant) $Ca_V2.1$ channels.

Future Research

Effects of temperature and membrane micro-environment on mutated $Ca_V2.1$ channels
Studies on frog neuromuscular junctions have shown a temperature-dependency of ACh release at the NMJ (Katz and Miledi, 1965; Bennett et al., 1977). Temperature-dependency could thus be affected by either a direct effect of a $Ca_V2.1$ mutation, or by mutation-specific interactions of $Ca_V2.1$ channels with gangliosides. Gangliosides are potentially important determinants of the temperature-sensitivity of ACh release at the NMJ (e.g. Bullens et al., 2002). Similarly to pre-synaptic $Ca_V2.1$ channels, gangliosides are localised to pre-synaptic lipid microdomains (Kalka et al., 2001; Taverna et al., 2004). It is tentative to suggest that such lipid microdomains are crucial determinants of the specific pre-synaptic environment of $Ca_V2.1$ channels. It is, therefore, interesting to study not only ACh release at FHM1-mutated at different temperatures, but also the effect of different ganglioside(-deficient) backgrounds thereupon.

Investigation of NMJs in muscle biopsies from FHM patients
Neuromuscular abnormalities have been reported for some migraine patients, however, conclusive evidence is missing for FHM1 patients with identified mutations (other than the CACNA1A I1811L mutation (for details, see "NMJ abnormalities in FHM1?", page 179; Terwindt et al., 2004). It is feasible to study ACh release at NMJs in intercostal muscle biopsies from patients, as shown for LEMS and EA2 (Lambert and Elmqvist, 1971; Plomp et al., 1995; Maselli et al., 2003). Measuring ACh release at NMJs in biopsies from FHM patients will provide detailed insights into possible (sub-clinical) neuromuscular transmission deficits in this disease and serve as a potential validation of the findings obtained in animal models and/or from single-fibre EMG studies in patients.

Effects of long-term in vivo administration of anti-migraine drugs on NMJ function
The findings presented in this thesis indicate that the drugs gabapentin, acetazolamide and topiramate have no acute effects on (mutant) $Ca_V2.1$ channels (chapters 9-11). However, given their therapeutic action in Ca_V channelopathies may suggest that Ca_V channels (or even $Ca_V2.1$ channels) may be modulated (possibly indirectly) only in the longer term

Therefore, studying NMJs of wild-type and mutant-$Ca_V2.1$ mice subsequent to chronic *in vivo* administration of gabapentin, acetazolamide or topiramate may be of interest.

Study of conditional $Ca_V2.1$ mutant mice
Chapter 8 of this thesis describes the generation of mice, carrying a floxed *Cacna1a* allele. Cross-breeding these mice with Cre-deleter mice that express the enzyme Cre-recombinase in a time- and site-specific manner, the *Cacna1a* gene can be switched off at any given point during development (given the availability of a suitable promoter with matching properties). Biological processes and plasticity during development of an organism generally differ substantially from those in mature individuals. Switching off a gene during adulthood can, therefore, potentially mimic disease states more realistically. For instance, motor neuron-specific $Ca_V2.1$-KO mice can serve as a model for the loss of functional $Ca_V2.1$ channels in LEMS. Furthermore, the incapability of some synapses to compensate for aberrant $Ca_V2.1$ channel function may underlie the site-specific neurological effects. Conditional ($Ca_V2.1$-) KO mice might provide insights into the capabilities of compensatory expression of other types of Ca_V channels in different cell types.

This ought to potentially help us predict the synapse type-specific effects of a given mutation, and identify novel targets for pharmaceutical intervention.

Using vital stains to monitor NMJ function efficacy

Combining electrophysiological with imaging techniques at NMJs can yield a powerful method to study synaptic efficacy in $Ca_V2.1$ mutant mice under a variety of conditions. For instance, measuring ACh release using the styryl dye FM1-43 at GFP-labelled synapses can reveal information on structure-function relationships. Furthermore, labelling with FM1-43 can serve as a means to determine the dynamics of pre-synaptic vesicle pools.