Severe and Progressive Neurotransmitter Release Aberrations in Familial Hemiplegic Migraine Type 1 Cacna1a S218L Knock-in Mice

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Familial hemiplegic migraine type 1 (FHM1) is caused by mutations in the CACNA1A gene, encoding neuronal presynaptic Ca2+,1 (P/Q-type) Ca2+ channels. These channels mediate neurotransmitter release at many central synapses and at the neuromuscular junction (NMJ). Mutation S218L causes a severe neurological phenotype of FHM and, additionally, ataxia and susceptibility to seizures, delayed brain edema, and fatal coma after minor head trauma. Recently, we generated a Cacna1a S218L knock-in mutant mouse, displaying these features and reduced survival. A first electrophysiological study showed high susceptibility for cortical spreading depression, enhanced neuronal soma Ca2+ influx, and at diaphragm NMJs, a considerable increase of neurotransmitter release. We here assessed the function of S218L knock-in NMJs at several muscle types in great detail. Pharmacological analyses using specific CaV subtype-blocking toxins excluded compensatory contribution of non-CaV2.1 channels. Endplate potentials were considerably broadened at many NMJs. High rate (40 Hz)–evoked acetylcholine release was slightly reduced; however, it was not associated with block of neurotransmission causing weakness, as assessed with grip strength measurements and in vitro muscle contraction experiments. The synaptopathy clearly progressed with age, including development of an increased acetylcholine release at low-rate nerve stimulation at physiological extracellular Ca2+ concentration and further endplate potential broadening. Our results suggest enhanced Ca2+ influx into motor nerve terminals through S218L-mutated presynaptic CaV2.1 channels, likely because of the earlier reported negative shift of activation potential and reduced inactivation. Similar severe aberrations at central synapses of S218L mutant mice and humans may underlie or contribute to the drastic neurological phenotype.

INTRODUCTION

Voltage-gated Ca2+ (CaV) channels are crucial to nervous system function, having roles in many processes, such as neurotransmitter secretion, neuronal firing, and gene expression (Catterall 2000; Snutch et al. 2005). Mutations in CACNA1A, the gene encoding the pore-forming α1 subunit of CaV2.1 (P/Q-type) Ca2+ channels, cause the human neurological diseases familial hemiplegic migraine type 1 (FHM1), episodic ataxia type 2 (EA2), spinocerebellar ataxia type 6 (SCA6), and some forms of epilepsy (Imbrici et al. 2004; Jouveneau et al. 2001; Ophoff et al. 1996; Zhuchenko et al. 1997). In Lambert-Eaton myasthenic syndrome, a neuro-immunological paralytic disorder, auto-antibodies target CaV2.1 channels of the neuromuscular junction (NMJ) (Lennon et al. 1995).

In central synapses, CaV2.1 channels enable the Ca2+ influx required for neurotransmitter release, either exclusively (e.g., at cerebellar Purkinje cells) or jointly with other CaV2 channel subtypes (e.g., at cerebellar granule cells) (Mintz et al. 1992a,b, 1995). At the peripheral NMJ, acetylcholine (ACh) release exclusively depends on CaV2.1 channels (Uchitel et al. 1992). NMJ dysfunction has been identified in some EA2 patients (Jen et al. 2001; Maselli et al. 2003). However, no EMG evidence for NMJ dysfunction was found in SCA6 (Jen et al. 2001; Schelhaas et al. 2004) or in FHM1 with the I1811L mutation (Terwindt et al. 2004).

The S218L mutation of CACNA1A causes FHM with additional susceptibility to delayed brain edema, seizures, and fatal coma after mild head trauma and is associated with altered cerebellar morphology and ataxia (Kors et al. 2001; Stam et al. 2009), forming one of the severest phenotypes within the FHM clinical spectrum. Electrophysiological experiments on cells transfected with S218L-mutated CaV2.1 channels showed a negative shift of the activation potential and changes in inactivation kinetics (Tottene et al. 2005). Recently, we generated a transgenic Cacna1a S218L knock-in (KI) mouse, which replicates this severe phenotype, showing reduced survival (~50% at 5 mo of age), epilepsy, ataxia, enhanced susceptibility for cortical spreading depression, and increased neuronal soma Ca2+ influx (Eikermann-Haerter et al. 2009; van den Maagdenberg et al. 2010). At S218L KI diaphragm NMJs, we showed in a limited first analysis, a profound increase in spontaneous unintegrated ACh release and in action potential–evoked release at low-rate stimulation in the presence of low extracellular Ca2+ concentration, indicating increased Ca2+ influx also at presynaptic nerve terminals. The NMJ is relatively easy accessible for experimental synaptic analyses. It is a model synapse with just one presynaptic input, of which the transmitter release is completely dependent on CaV2.1 channels. It therefore allows for clean and detailed analyses of evoked and spontaneous transmitter release. Here, we further assessed the synaptic defects at S218L KI NMJs in great detail, including age progression of transmitter release aberrations, in electrophysiological and functional experiments at several muscle types.

METHODS

Mice

Generation and genotyping of the S218L KI mice has been described previously (van den Maagdenberg et al. 2010). Animals were
maintained in the Leiden University Medical Centre vivarium on a 12-h light/dark cycle and tested during the light phase. Mice (both female and male animals) were tested at 2, 4.5, and 12 mo of age (as specified in RESULTS), and wherever possible, littermates were used for testing. All experiments were carried out with the investigator blinded for genotype, and genotyping was repeated after the experiments for confirmation. S218L KI mice had similar body weights compared with wild type (data not shown), weighed before experiments. All experiments were carried out according to Dutch law and Leiden University guidelines, including approval by the local Animal Experiments Committee.

**Ex vivo NMJ electrophysiology**

Mice were killed by CO\_2 inhalation. Phrenic nerve-hemidiaphragms and soleus and flexor digitorum brevis (FDB) muscles were dissected and mounted in standard Ringer’s medium containing (in mM) 116 NaCl, 4.5 KCl, 2 CaCl\_2, 1 MgCl\_2, 1 NaH\_2PO\_4, 23 NaHCO\_3, and 11 glucose, pH 7.4, at room temperature. Intracellular recordings of miniature endplate potentials (MEPPs, the spontaneous depolarizing events caused by uniquantal ACh release) and endplate potentials (EPPs, the depolarization resulting from nerve action potential–evoked ACh release) were made at NMJs at 28°C using standard microelectrode equipment, as described previously (Plomp et al. 1992). At least 30 MEPPs and EPPs were recorded at each NMJ, and typically 5–15 NMJs were sampled per experimental condition per mouse. Muscle action potentials were blocked by 3 \mu M of the selective muscle Na\(^+\) channel blocker \(\mu\)-conotoxin GIIB (Scientific Marketing Associates, Barnet, Herts, UK). To record EPPs, the phrenic nerve was stimulated supramaximally at 0.3 and 40 Hz. The amplitudes of EPPs and MEPPs were normalized to –75 mV, assuming 0 mV as stimulated supramaximally (Magleby and Stevens 1972). The normalized EPP amplitudes were corrected for nonlinear summation according to McLachlan and Martin (1981) with an \(f\) value of 0.8. Quantal content, i.e., the number of ACh quanta released per nerve impulse, was calculated by dividing the normalized and corrected mean EPP amplitude by the normalized mean MEPP amplitude at each NMJ.

To assess the possible compensatory contribution of non-CaV2.1 channels to ACh release, EPPs and MEPPs were also measured in the presence of the following specific blockers: 200 nM \(\omega\)-agatoxin-IVA (P/Q-type; Ca\(_{\text{v}}\)2.1), 2.5 \mu M \omega-conotoxin-GVIA (N-type; Ca\(_{\text{v}}\)2.2), and 1 \mu M SNX-482 (R-type; Ca\(_{\text{v}}\)2.3). All toxins were from Scientific Marketing Associates. Measurements were made following a 20–25 min preincubation with the toxin. Because some function alterations in the mutated Ca\(_{\text{v}}\)2.1 channel may only appear under more critical conditions, measurements were made in 0.2 mM Ca\(^{2+}\) and on slight depolarization by high (10 mM) K\(^+\) Ringer’s, as well as in the presence of 200 ng/ml of the K\(^+\) channel blocker 3,4-diaminopyridine (DAP; Sigma, Zwijndrecht, The Netherlands).

**Grip strength assessment**

Muscle strength was measured using a grip strength meter for mice (600g range; Technical and Scientific Equipment, Bad Homburg, Germany), connected to a laptop computer. The test was carried out essentially as originally described for rats (Tilson and Cabe 1978). The peak force of each trial was considered the grip strength. Each mouse performed five trials, each about 30 s apart. The mean value of the five trials was used for statistical analysis.

**Contraction experiments**

Contraction experiments were performed essentially as described previously (Kaja et al. 2005; Plomp et al. 2000). In left phrenic nerve hemidiaphragms of mice, contractions were recorded with a force transducer (type K30, Harvard Apparatus, Hugo Sachs Elektronik, March-Hugstetten, Germany), connected via a transducer amplifier module TAM-A 705/1 (Hugo Sachs Elektronik) and a 1200 series Digitizer (Axon Instruments, Molecular Devices, Union City, CA) to a personal computer running Axoscope 9 (Axon Instruments). The phrenic nerve was stimulated supramaximally once every 5 min with a single stimulus and a train of 120 stimuli at 40 Hz. The muscles were incubated in Ringer’s solution to which \(\alpha\)-tubocurarine (Sigma-Aldrich) was added in increasing concentrations (1 h incubation at each concentration). The amplitude of the recorded contractions was cursor-measured in Axoscope.

**\(\alpha\)-Bungarotoxin staining and image analysis**

NMJ size was determined by staining the area of ACh receptors with fluoroscently labeled \(\alpha\)-bungarotoxin (\(\alpha\)BTX), which irreversibly binds to ACh receptors. Diaphragm preparations were pinned out and fixed in 1% paraformaldehyde (Sigma) in 0.1 M PBS, pH 7.4, for 30 min at room temperature. After a 30 min wash in PBS, diaphragms were incubated in 1 \mu g/ml Alexa Fluor 488–conjugated \(\alpha\)BTX (Molecular Probes/Invitrogen, Breda, The Netherlands) in PBS for 3 h at room temperature. After a final washing step in PBS (30 min), NMJ-containing regions were excised and mounted on microscope slides with Citifluor AF-1 antifadent (Citifluor, London, UK). Sections were viewed under an Axiosplan microscope (Zeiss, Jena, Germany). NMJs were identified on the basis of \(\alpha\)BTX staining, under standardized camera conditions. Images of \(\alpha\)BTX stain were stored on hard disk; quantification was carried out using ImageJ (National Institutes of Health, Bethesda, MD). In every diaphragm, 7–15 NMJs were selected randomly. Length, width, and perimeter of the \(\alpha\)BTX-stained area were measured.

**Statistical analyses**

In the ex vivo electrophysiological and fluorescence microscopical analyses we measured 5–15 NMJs per muscle per experimental condition. The muscle mean value was calculated from the means of all data points obtained at a single NMJ and was subsequently used to determine the grand muscle mean with \(n\) as the number of mice tested. For grip-strength measurements and ex vivo muscle contraction experiments, \(n\) is the number of mice measured.

Data are presented as grand muscle means \(\pm\) SE, with \(n\) indicating the numbers of mice, unless stated otherwise. Possible statistical significances were assessed using paired or unpaired Student’s \(t\)-test, ANOVA (with Tukey’s honestly significant difference post hoc test). \(P < 0.05\) was considered statistically significant in all cases.

**RESULTS**

**Increased spontaneous transmitter release at S218L neuromuscular synapses**

A first series of experiments was performed at diaphragm NMJs from mice of ~2 mo of age. For completeness and comparison, the previously reported increase in spontaneous uniquantal ACh release, measured as MEPP frequency, is given in Fig. 1A. It was increased ~12-fold at homozygous S218L KI NMJs (\(n = 6, P < 0.001\)). Heterozygous KI NMJs showed an intermediate MEPP frequency (\(n = 6, P < 0.01\)). The MEPP amplitude was ~1 mV under all conditions and did not differ between genotypes (\(n = 6, P = 0.17\); Fig. 1C); neither did MEPP rise times, decay times, and half-width values (data not shown).
Occasionally, spontaneous twitches of individual muscle fibers were observed in homozygous S218L KI diaphragm preparations. They could be blocked by 1 μM α-tubocurarine. This suggests that the twitches resulted from occasionally superimposed MEPPs, because of their high frequency, reaching the firing threshold of the muscle fiber.

To assess the contribution of Ca\textsubscript{v}2.1 channels to uniquantal release, we applied 200 nM ω-agatoxin-IVA to the preparation. Whereas MEPP frequency was reduced by ∼60% at wild-type NMJs (n = 3, P < 0.001; Fig. 1A), spontaneous release decreased by ∼90% at heterozygous and homozygous S218L KI NMJs (n = 3, P < 0.05; Fig. 1A), indicating that the increased MEPP frequency in KI mice is almost exclusively mediated by S218L-mutated Ca\textsubscript{v}2.1 channels.

MEPP frequency increased ∼10-fold on slight depolarization of the nerve terminals by raising K\textsuperscript+ to 10 mM (n = 6, P < 0.01; Fig. 1, B and C). The difference of MEPP frequency between genotypes remained similar (n = 6, P = 0.33), however, was abolished on application of ω-agatoxin-IVA (n = 3, P = 0.12).

![Graphs and Diagrams](http://jn.physiology.org/DownloadedFrom)
Quantal content is unaltered at S218L KI NMJs at physiological extracellular Ca\textsuperscript{2+}

When stimulating the phrenic nerve supramaximally at 0.3 Hz in the presence of 2 mM extracellular Ca\textsuperscript{2+}, the EPP amplitude was \(\sim 28\) mV for all genotypes (\(n = 6, P = 0.28\)). The quantal content, calculated from the corrected and normalized EPP and MEPP amplitudes, was \(\sim 40\) in all groups and did not differ between genotypes (\(n = 6, P = 0.82\); Fig. 1D).

No compensatory contribution of non-Ca\textsubscript{v}2.1 channels

We considered the possibility that, as a result of the Ca\textsubscript{v}2.1 mutation, other types of Ca\textsubscript{v} channels may be expressed at the NMJ and contribute to evoked ACh release, as we described previously for the \(\text{t}^\text{ottering}\ P601L\) mutation (Kaja et al. 2005) and has been shown at Ca\textsubscript{v}2.1 KO NMJs (Kaja et al. 2007a; Urbano et al. 2003). However, \(\omega\)-agatoxin-IVA reduced quantal content equally in all genotypes by \(~90\%\) (\(n = 3, P < 0.05\); Fig. 1, D and E). The selective Ca\textsubscript{v}2.2 (N-type) blocking toxin \(\omega\)-conotoxin-GVIA (2.5 \(\mu\)M) as well as the Ca\textsubscript{v}2.2 (R-type) channel blocker SNX-482 did not have any effect on evoked ACh release at homozygous S218L KI synapses (\(n = 2–3\); Fig. 1E). MEPP frequency was not affected by either \(\omega\)-conotoxin-GVIA or SNX-482 (data not shown).

Increased EPP rundown and reduced paired-pulse facilitation at S218L KI NMJs

Some changes of mutant Ca\textsubscript{v}2.1 channel function may only be shown at high-frequency use. We, therefore measured ACh release after a high-frequency stimulus train (35 stimuli at 40 Hz). Normalized EPP amplitudes of wild-type were higher than those of both heterozygous and homozygous S218L KI mice at the end of a stimulus train (Fig. 2A), resulting from an increased rundown of EPP amplitudes at S218L KI synapses compared with wild type. The mean residual EPP amplitude at stimuli 21–35, expressed as percentage of the first EPP in the train, at heterozygote S218L KI NMJs was 79.1 \(\pm 1.9\%\) (\(n = 6, P = 0.08\)) and was only 77.5 \(\pm 0.9\%\) (\(n = 6, P < 0.01\)) at homozygous KI synapses compared with wild-type NMJs, where rundown level was 83.4 \(\pm 0.8\%\) (\(n = 6\); Fig. 2, B and C).

We looked at the first two EPPs of the stimulus train to assess 25 ms paired-pulse facilitation, a process of short-term synaptic plasticity. At the wild-type NMJ, amplitude of the second EPP was 103.7 \(\pm 0.2\%\) of the first EPP. Facilitation was somewhat lower in both heterozygous and homozygous S218L KI mice (101.1 \(\pm 0.4\) and 99.6 \(\pm 0.3\%\), respectively; \(n = 6, P < 0.001\); Fig. 2D).

No (sub-)clinical muscle weakness in S218L mice

Block of transmission at the NMJ will result in muscle weakness. Although our electrophysiological measurements at the NMJ of diaphragms were not indicative of transmission block, the NMJ condition in extirpated muscles might be different. However, when we assessed grip strength of the fore paws in vivo, no differences between genotypes were found. Wild-type, heterozygous, and homozygous S218L KI mice pulled 92.5 \(\pm 1.4\), 93.2 \(\pm 2.5\), and 93.2 \(\pm 1.9\) g, respectively (\(n = 5, P = 0.96\)).

We assessed a possible subclinical muscle weakness by testing the \(\delta\)-tubocurarine sensitivity of the 40 Hz nerve stimulation–evoked tetanic force in ex vivo contraction experiments with diaphragm muscles. However, no differences were observed between S218L and wild-type preparations (Fig. 2E). The absolute twitch and tetanic contraction forces in Ringer’s medium without \(\delta\)-tubocurarine were equal (Fig. 2F).

Higher quantal content at S218L KI NMJs at low extracellular Ca\textsuperscript{2+}

At physiological extracellular Ca\textsuperscript{2+} concentration, Ca\textsuperscript{2+} sensors of the neurotransmitter release machinery may approach saturation during action potential–induced Ca\textsuperscript{2+} influx (Schneggenburger and Neher 2000). Therefore the transmitter release at low extracellular Ca\textsuperscript{2+} concentration may reflect in a more direct way the behavior of Ca\textsubscript{v}2.1 channels and, therefore, possible differences in release between wild-type and mutant NMJs might become more outspoken and easier to detect. At 0.2 mM extracellular Ca\textsuperscript{2+}, the MEPP frequencies were 0.35 \(\pm 0.04\), 0.85 \(\pm 0.16\), and 1.36 \(\pm 0.18\) s\(^{-1}\) at wild-type, homozygous, and heterozygous S218L NMJs, respectively (\(n = 3, P < 0.01\); Fig. 3A). MEPP amplitudes remained unchanged (data not shown). Furthermore, we found a gene dosage–dependent effect on 0.3 Hz–evoked EPP amplitude and quantal content. EPP amplitudes were increased approximately twofold in heterozygous and approximately threefold at homozygous S218L KI NMJs compared with wild type (Fig. 3, B and D). Quantal contents, as reported previously (van den Maagdenberg et al. 2010), were 7.2 \(\pm 1.5\), 13.9 \(\pm 0.7\), and 18.3 \(\pm 1.7\) at wild-type, heterozygous, and homozygous S218L NMJs, respectively (\(n = 3, P < 0.01\); Fig. 3C).

EPP rundown at 40 Hz stimulation differed between genotypes in a gene dosage–dependent fashion (Fig. 3, E and F). The mean amplitude of the last 15 EPPs in the train, expressed as percentage of the first EPP, was 116.4 \(\pm 1.9\%\) at wild-type, heterozygous, and homozygous KI synapses compared with wild-type NMJs, respectively (\(n = 3, P < 0.05\); Fig. 3F).

Synaptic defects at S218L KI NMJs progress with age

Long-term synaptic changes and damage may occur as a result of prolonged presynaptic Ca\textsuperscript{2+} influx. Measuring ACh release at diaphragm NMJs of mice of 4.5 and 12 mo of age, we compared the magnitude of changes compared with age-matched wild-type littermates (Fig. 4A). MEPP frequency at homozygous S218L KI NMJs of mice of 4.5 mo of age was 3-fold higher than at 2 mo of age, corresponding to a >20-fold increase compared with 4.5-mo-old wild-types (\(n = 4, P < 0.001\); Fig. 4, A and B). At NMJs of mice of 12 mo of age, the difference between S218L KI and wild-type mice was similar to that observed at 2 mo of age (\(n = 5, P = 0.32\); Fig. 4, A and B).

Quantal content at S218L NMJs of 4.5-mo-old mice was similar to wild-type, whereas at 12 mo of age, it was \(\sim 50\%\) higher than at age-matched wild-types (\(n = 4, P < 0.01\); Fig. 4C). To test the possibility of long-term compensatory contribution of non-Ca\textsubscript{v}2.1 channels, we applied 200 nM \(\omega\)-agatoxin-IVA to the preparation. However, \(\omega\)-agatoxin-IVA sensitivity of ACh release did not change with increasing age (Fig. 4D).

At 40 Hz stimulation, the difference in EPP amplitude rundown level between S218L and wild-type NMJs remained similar at the different ages (Fig. 4, E–G).
Broadening of EPPs at S218L KI NMJs

At NMJs of homozygous S218L KI mice, we encountered very broad EPPs. Representative traces obtained at 4.5 mo of age are shown in Fig. 5A. There seemed to be variation in EPP broadness during 0.3 Hz stimulation; half-widths generally becoming less pronounced after the first few pulses of the stimulation period (Fig. 5B).

Interestingly, half-width from the first EPP at 0.3 Hz stimulation showed a similar dependence on age as MEPP frequency (Fig. 5C).

The broadening of EPPs observed in S218L KI synapses appeared similar to EPP broadening in the presence of the selective blocker of presynaptic K⁺-channels, 3,4-diaminopyridine (DAP), which causes prolonged opening of Caᵥ2.1 channels because of increased duration of the axonal action potential (Katz and Miledi 1979). We speculated that S218L KI EPP half-widths should be more sensitive to application of DAP than that of wild type. Indeed, 200 ng/ml DAP almost doubled EPP half-width in 2-mo-old S218L KI mice, whereas EPP half-widths at wild-type NMJs were hardly affected (n = 6, P < 0.001; Fig. 5D).

Similar neuromuscular synaptic phenotype in other types of muscles

The diaphragm consists of a mixed population of slow- and fast-twitch muscle fibers, with different NMJ characteristics.
D. KI soleus NMJs increased to 18.70/11006 at 4.5 mo of age. FDB (fast-twitch) muscle preparations of wild-type and S218L NMJs of soleus (exclusively slow-twitch muscle fibers) and the S218L mutation. We therefore also assessed ACh release at (Bewick 2003), and thus may be influenced differentially by the S218L mutation. We therefore also assessed ACh release at NMJs of soleus (exclusively slow-twitch muscle fibers) and FDB (fast-twitch) muscle preparations of wild-type and S218L KI mice at 4.5 mo of age.

MEPP frequency was 1.91 ± 0.21 and 45.30 ± 10.00 s⁻¹ at wild-type and S218L KI soleus NMJs, respectively (n = 4; P < 0.01), and similar to that found in diaphragm (Fig. 4B). Similarly to diaphragm, when subjected to slightly depolarizing medium (10 mM K⁺), MEPP frequency at wild-type and S218L KI soleus NMJs increased to 18.70 ± 3.85 and 188.22 ± 15.81 s⁻¹, respectively (n = 4). Quantal contents were 62.86 ± 3.59 and 75.23 ± 5.84 at wild-type and S218L KI soleus NMJs, respectively (n = 4; P = 0.12). At high-rate (40 Hz) stimulation, EPP amplitude rundown was slightly more pronounced (although not statistically significant) at S218L KI synapses compared with wild-type (EPPs ran down to 73.1 ± 2.7 and 76.9 ± 1.6% of the first EPP, respectively; n = 4; P = 0.27). In contrast to diaphragm, no EPP broadening was found in soleus NMJs from S218L KI mice (half-widths were 2.46 ± 0.16 and 2.48 ± 0.11 ms in WT and S218L KI, respectively, n = 4; P = 0.9).

At S218L FDB NMJs, MEPP frequency was measured and found increased ~20-fold compared with wild type (17.58 ± 5.07 and 0.95 ± 0.20 s⁻¹, respectively; n = 4, P < 0.05).

Unchanged S218L NMJ size

The functional changes at S218L NMJs could possibly also result from changes in morphology and, vice versa, could themselves lead to morphological changes. Because NMJ size is an important determinant in the level of ACh release (Kuno et al. 1971; Smith 1984), we quantified this parameter with the following microscopy in diaphragms of 2-mo-old mice. However, no differences were identified (n = 3–5, P = 0.99; Fig. 6, A and B).

**DISCUSSION**

We studied neurotransmitter release at NMJs of a recently generated KI mice carrying the human S218L mutation in CACNA1A encoded Ca\(_{v}\).2.1 channels (van den Maagdenberg et al. 2010), identified in families suffering from FHM1 with susceptibility for epilepsy, delayed brain edema, and coma after mild head trauma (Kors et al. 2001; Stam et al. 2009). These detailed studies at NMJs of the KI mice showed increased neurotransmitter release at several levels, suggesting that S218L-mutated presynaptic Ca\(_{v}\).2.1 channels mediate increased and prolonged Ca\(^{2+}\) influx at motor nerve terminals. The observed changes were gene dosage-dependent and progressed with age but did not lead to muscle weakness or morphological synaptic abnormalities.

*Increased spontaneous ACh release from S218L KI motor nerve terminals*

S218L KI NMJs showed a very large increase in spontaneous ACh release under resting and mildly depolarizing conditions (i.e., 10 mM extracellular K⁺). This may result from a negative shift in activation voltage of putative Ca\(_{v}\).2.1-enzymes, but further studies are required to understand how such a shift is brought about.
coded low-voltage-activated presynaptic CaV2.1 channels (of which the existence was proposed by us previously; Kaja et al. 2005; Plomp et al. 2000), similar to the 5–10 mV shift observed in S218L-mutated high voltage–activated channels in cell bodies of primary cultured cerebellar granule neurons from S218L KI mice (van den Maagdenberg et al. 2010) and in heterologous expression systems (Tottene et al. 2005; Weiss et al. 2008). Toxin experiments excluded contribution of non-CaV2.1 channels to the increase in spontaneous release.

We have previously reported increased spontaneous ACh release also for a number of other Cacna1a mutants with single amino acid changes, namely the spontaneous mutants tottering (Kaja et al. 2006; Plomp et al. 2000) and rolling Nagoya (Kaja et al. 2007b), and for a transgenic FHM1 R192Q KI strain that carries a missense mutation previously identified in patients with FHM without additional neurological features (Kaja et al. 2005; van den Maagdenberg et al. 2004). All these mutations are localized at or near voltage-sensing segments of CaV2.1.

Apparently, the S218L mutation has the largest impact on presynaptic CaV2.1 channel behavior responsible for spontaneous transmitter release, in view of the relative large increase in MEPP frequency (~12-fold vs. 2–4-fold in other mutants). However, increased MEPP frequency was also observed in biopsy NMJs from an EA2 patient with a CACNA1A truncation mutation (Maselli et al. 2003), showing that other types of mutations can also lead to increased spontaneous transmitter release.

**Increased evoked ACh release at S218L KI synapses**

We observed clear changes in nerve stimulation–evoked ACh release at S218L KI NMJs compared with wild type. Evoked ACh release (0.3 Hz) under physiological conditions was not different between genotypes at NMJs of young mice; however, it reached ~150% of wild type at 12 mo of age. It is not expected that this increase would influence muscle con-
traction because the NMJ is an all-or-none synapse with a firing threshold and EPPs are already suprathreshold at normal extracellular Ca\(^{2+}\). Furthermore, this increase in initial ACh release at S218L KI NMJs is likely canceled out by the extra EPP amplitude rundown at physiological firing frequency of the motor nerve.

In addition, (young) S218L KI NMJs had a threefold higher quantal content than wild type in low (0.2 mM) Ca\(^{2+}\). This condition of low extracellular Ca\(^{2+}\) at the NMJ may model the situation at many central synapses where the release probability and thus the quantal content is generally very low. These changes are compatible with a negative shift in activation potential of presynaptic S218L Ca\(_v\)2.1 channels, as shown for cell soma channels in transfection studies and cultured cerebellar granular cells from S218L KI mice (Tottene et al. 2005; van den Maagdenberg et al. 2010). Other mechanisms may include altered Ca\(^{2+}\)/calmodulin-dependent channel inactivation (Lee et al. 1999) or fast recovery from G protein–mediated

**FIG. 5.** EPP broadening at S218L KI diaphragm NMJs. A: representative EPP recordings obtained at wild-type and S218L KI NMJs from 4.5-mo-old mice (triangles indicate the moment of nerve stimulation). B: development of EPP half-width during 0.3 Hz stimulation at NMJs from 4.5-mo-old mice. EPP half-width is increased ~2-fold compared with wild-type (n = 4, P < 0.05). C: half-widths of the first EPP measured at 0.3 Hz stimulation, plotted as scatter graphs of individual NMJ values at 2, 4.5, and 12 mo of age. D: EPP half-width (mean of 30 EPPs measured at 0.3 Hz stimulation) of 2-mo-old wild-type mice was insensitive to application of 200 ng/ml 3,4-diaminopyridine (DAP; n = 3, P = 0.27). At S218L KI synapses, however, EPP half-width doubled (n = 5, P < 0.05). Representative single EPP recordings are shown for both genotypes before application and in the presence of 200 ng/ml DAP (triangles indicate the moment of nerve stimulation). *P < 0.05.
inhibition, as shown for the S218L mutation in transfected cells (Weiss et al. 2008).

Increased EPP half-width indicates prolonged opening of S218L Ca_{2.1} channels

Broadened EPPs with rather irregular decaying phases were observed frequently in S218L KI mice. EPP broadening is a feature of inhibition or absence of acetylcholinesterase at the NMJ (Burd and Ferry 1987; Hutchinson et al. 1993). If this was the case, because of some indirect effect of the S218L mutation, the half-width of uniquantal responses (MEPPs) should, however, also have been found increased. Because this was not observed, the most likely reason left for EPP broadening is enhanced/prolonged Ca^{2+} influx caused by either reduced inactivation and/or negatively shifted activation of the S218L-mutated Ca_{2.1} channels, as shown in transfection studies (Tottene et al. 2005). The EPP half-widths at S218L KI NMJs being more potentiated by 200 ng/ml DAP than wild types supports this hypothesis. Why the falling phase of the broadened EPPs is of irregular shape (instead of simply decaying exponentially) is unclear. We and others also saw this phenomenon at normal NMJs exposed to high concentrations of DAP (J.J.P., unpublished data; Hong and Chang 1990). Many factors may be of influence, including Ca^{2+} channel behavior, neuro-exocytotic release mechanism (e.g., transmitter vesicle recruitment), and spatial aspects of the synaptic cleft in relation to the diffusion of the high concentration of ACh and the localization and activity of acetylcholinesterase.

There was large inter-NMJ variation in S218L EPP half-widths, a considerable proportion of the NMJs having wild-type values. Changes in EPP half-width may possibly be related to muscle type. The diaphragm consists of a mixed population of slow- and fast-twitch muscle fibers, which may have different NMJ morphology (Sieck and Prakash 1997) but possibly also different NMJ function. The analysis of EPPs at NMJs of soleus muscles, which consist exclusively of slow-twitch fibers, did not show an increase in half-width. This might suggest that EPP broadening is selectively associated with NMJs on fast-twitch fibers.

S218L-Ca_{2.1} channel dysfunction at high-frequency use

We observed a slightly more pronounced rundown of EPP amplitudes on high-frequency stimulation (200 Hz) in S218L than at wild-type NMJs. Physiological EPP rundown is most likely caused by reduced ACh release after a combination of Ca_{2.1} channel inactivation and incomplete replenishment of synaptic ACh vesicles. Possibly, extra rundown at S218L NMJs at this high-rate use of the synapse is caused by an increased fast-component of inactivation, as shown in transfected cells (Tottene et al. 2005; Weiss et al. 2008). The somewhat reduced 25 ms paired-pulse facilitation at S218L KI NMJs may hint toward increased Ca^{2+} influx through mutated Ca_{2.1} channels during the first pulse, causing an increased level of Ca^{2+}/calmodulin-dependent inactivation (a physiological phenomenon described for Ca_{2.1} channels by Lee et al. 1999). A (more distant) possibility is that S218L-mutated Ca_{2.1} channels influence expression and/or behavior of other presynaptic ion channels (K^+, Na^+) in such a way that repetitive activity leads to diminished ACh release.

The slightly increased 40 Hz EPP rundown will reduce the safety factor of neuromuscular transmission, although the extent of it is probably not large enough to cause transmission block. Indeed no muscle weakness was found with in vivo grip-strength testing. However, one would expect increased D-tubocurarine sensitivity of the 40 Hz nerve stimulation–evoked muscle contraction of S218L diaphragm ex vivo, but this was not found. Most likely, the increased EPP widths and slightly increased initial quantal content compensated for the slightly increased amplitude rundown.

Transmitter release abnormalities are gene dosage dependent and progressive

We observed gene dosage effects on several ACh release parameters. This suggests that S218L-mutated Ca_{2.1} channels co-exist with wild-type channels at motor nerve terminals of heterozygous mice. Some S218L NMJ parameters showed age-dependent changes. MEPP frequency and EPP half-width increases were highest at 4.5 mo of age, but again lower at 12 mo. Given the reduced survival rate, the 12-mo age group consists of “survivors,” possibly with a synaptic phenotype biased toward wild type. The 0.3 Hz quantal content, however, became increased at 12-mo-old NMJs. One possible explanation may be a differential response of S218L KI NMJs to maturation. Neurotransmitter release at NMJs increases with age in both humans (Wokke et al. 1990) and rodents (Smith 1984) and is correlated with nerve terminal branching and overall size (Kuno et al. 1971; Smith 1984). However, the fluorescence microscopical quantification of NMJ size of 2-mo-old S218L mice did not show changes compared with wild type.
No functional compensation for S218L-mutated Ca_{2.1} channels

It has been shown that non-Ca_{2.1} channels can compensate for mutated or absent Ca_{2.1} channels, either in the CNS (Cao et al. 2004; Inchauspe et al. 2004) or at the NMJ (Kaja et al. 2006, 2007a; Urbanó et al. 2003). For instance, P601L-mutated tottering mice exhibited reduced Ca_{2.1}-mediated evoked ACh release at the NMJ that was fully compensated for by Ca_{2.3} channels (Kaja et al. 2006). However, no compensatory contribution of non-Ca_{2.1} channels was found at R192Q KI NMJs (Kaja et al. 2005; van den Maagdenberg et al. 2004). Here, at S218L KI NMJs, experiments with selective Ca_{2.1} channel-blocking toxins did not indicate compensatory contribution of non-Ca_{2.1} channels to ACh release.

In conclusion, we showed severe and progressive neurotransmitter release aberrations at Cafcal S218L. Similar defects at central synapses may contribute to the neurological phenotype of S218L mutated mice and humans.

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