# Diseases of the nervous system associated with calcium channelopathies

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# REDUNDANCY OF $CA_V2.1$ CHANNEL ACCESSORY SUBUNITS IN TRANSMITTER RELEASE AT THE MOUSE NEUROMUSCULAR JUNCTION

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## ABSTRACT

$Ca_v2.1$ (P/Q-type) channels possess a voltage-sensitive pore-forming $\alpha_1$ subunit that can associate with the accessory subunits $\alpha_2\delta$, $\beta$, and $\gamma$. The primary role of $Ca_v2.1$ channels is to mediate transmitter release from nerve terminals both in the central and the peripheral nervous systems. Whole-cell voltage-clamp studies in *in vitro* expression systems have indicated that accessory channel subunits can have diverse modulatory effects on membrane expression and biophysical properties of $Ca_v2.1$ channels. However, there is only limited knowledge on whether similar modulation also occurs in the specific presynaptic environment *in vivo* and, hence, whether accessory subunits influence neurotransmitter release. *Ducky*, *lethargic,* and *stargazer* are mutant mice that lack functional $\alpha_2\delta$-2, $\beta_4$, and $\gamma_2$ accessory $Ca_v$ channel subunits, respectively. The neuromuscular junction (NMJ) is a peripheral synapse, where transmitter release is governed exclusively by $Ca_v2.1$ channels, and which can be characterized electrophysiologically with relative experimental ease. In order to investigate a possible synaptic influence of accessory subunits in detail, we electrophysiologically measured acetylcholine (ACh) release at NMJs of these three mutants. Surprisingly, we did not find any changes compared to wild-type littermates, other than a small reduction (25%) of evoked ACh release at *ducky* NMJs. This effect is most likely due to the ~40% reduced synapse size, associated with the reduced size of *ducky* mice, rather than resulting directly from reduced $Ca_v2.1$ channel function due to $\alpha_2\delta$-2 absence. We conclude that $\alpha_2\delta$-2, $\beta_4$, and $\gamma_2$ accessory subunits are redundant for the transmitter release-mediating function of presynaptic $Ca_v2.1$ channels at the mouse NMJ.



*Keywords:* Ca2+ channel subunit, ducky, stargazer, lethargic, synapse, neuromuscular junction, neurotransmitter release

*Abbreviations:*
ACh – acetylcholine
αBTx – α-bungarotoxin
CNS – central nervous system
HVA – high voltage-activated
NMJ – neuromuscular junction
PNS – peripheral nervous system
$Ca_v$ channel – voltage-gated calcium channel

## INTRODUCTION

$Ca_v2.1$ (P/Q-type) voltage-activated $Ca^{2+}$ channels are mediators of synaptic transmission both in the central (CNS) and theperipheral nervous systems (PNS) by conducting the presynaptic $Ca^{2+}$ influx required for neurotransmitter release (*Uchitel et al., 1992*; *Mintz et al., 1995*). As iscommon for all high voltage-activated (HVA) $Ca^{2+}$ channels, $Ca_v2.1$ channels are described to consist of the actual pore-forming channel protein ($Ca_v2.1$-$\alpha_1$) and at least two accessory subunits: $\alpha_2\delta$ and β (for review, see *Catterall, 200*0; *Snutch et al., 2005*). While $\alpha_2\delta$ is a membrane protein, the β subunit is entirely localized in the cytoplasm. To date, four genes encoding $\alpha_2\delta$ ($\alpha_2\delta$-1 to $\alpha_2\delta$-4) and four genes encoding β subunits ($\beta_1$ to $\beta_4$) have been identified (for review see *Arikkath & Campbell, 2003*). Furthermore, eight different γ subunits exist (*Jay et al., 1990*; *Burgess et al., 2001*; *Arikkath & Campbell, 2003*), of which at least $\gamma_2$ can associate with $Ca_v2.1$-α1 (*Kang et al., 2001*). The $Ca_v2.1$-$\alpha_1$ subunit has been shown to co-localize with $\alpha_2\delta$-2 subunits into lipid rafts (*Davies et al., 2006*).

*In vitro* expression system studies have indicated that accessory channel subunits exert specific modulatory actions on $Ca_v$ channels (*Singer et al., 1991*). For example, the $\beta_4$ subunit is known to be responsible for successful channel trafficking to the membrane (*Burgess et al., 1999*; *Brice & Dolphin, 1999*) and to alter activation and inactivation kinetics of the associated pore-forming subunit (*Berrow et al., 1995*). The $\alpha_2\delta$-2 protein increases $Ca^{2+}$ current amplitude and enhances the effects of bound β subunits on channel (in-)activation (*Klugbauer et al., 1999*; *Gao et al., 2000*; *Klugbauer et al., 2003*). Similarly, $\gamma_2$ subunits cause small negative shifts in the activation voltage of $Ca_v2.1$ channels and have increasing or decreasing effects on the amplitude of current mediated by $Ca_v$ channels, depending on the type of co-expressed subunits (for review, see *Black, 2003*). If similar modulation occurs in the nervous system *in vivo*, accessory $Ca_v$ channel subunits would be important regulators of transmitter release. Thus far, just a few studies have investigated this issue of presynaptic function, and only with respect to $\beta_4$ and $\gamma_2$ subunits in (cultured) CNS synapses (*Caddick et al., 1999*; *Hashimoto et al., 1999*; *Qian and Noebels, 2000*; *Wittemann et al., 2000*). To our knowledge, no detailed synaptic studies have been performed on $\alpha_2\delta$-2 subunits and also no studies have been performed on accessory subunit function at the peripheral neuromuscular junction (NMJ), which exclusively relies on $Ca_v2.1$ channels for neurotransmitter release (*Uchitel et al., 1992*). We, therefore, studied neurotransmitter release at the NMJ of the natural mouse mutants *ducky*, *lethargic,* and *stargazer*, which lack functional accessory subunits $\alpha_2\delta$-2, $\beta_4$, and $\gamma_2$, respectively. *Ducky* mice exhibit a wide-open gait, severe ataxia, spike-wave discharges (in humans indicative of absence epilepsy), paroxysmal dyskinesia, and CNS dysgenesis (*Snell, 1955*; *Meier, 1968*; *Barclay et al., 2001*). The mutation in the *Cacna2d2* gene, which encodes the $\alpha_2\delta$-2 subunit (*Barclay et al., 2001*; *Brodbeck et al., 2002*), leads to a much shorter transcript that lacks the transmembrane domain and the binding site for the anti-convulsant drug gabapentin (GBP). The *lethargic* mouse exhibits a phenotype of severe ataxia and slow (lethargic)

movement (*Dickie, 1964*; *Dung & Swigart, 1971*), and carries a mutation in *Cacnb4*, the gene encoding the $\beta_4$ subunit. All studies to date have failed to show any translated $\beta_4$ protein (*Burgess et al., 1997*; *McEnery et al., 1998*; *Burgess et al., 1999*), making *lethargic* a functional $\beta_4$ knockout (KO) model. The s*targazer* mouse displays severe ataxia and typical head-tossing movements (*Noebels et al., 1990*). A transposon insertion in *Cacng2*, the gene encoding the $\gamma_2$ subunit (also known as *stargazin*), has been identified as the underlying mutation (*Letts et al., 1997*; *Letts et al., 1998*). *Stargazer* mice can be regarded as functional $\gamma_2$ KOs, as they do not express any $\gamma_2$ protein (*Sharp et al., 2001*).

Surprisingly, in our present detailed assessment of spontaneous uniquantal ACh release and nerve stimulation-evoked release at the *ex vivo* NMJ of *ducky*, *stargazer*, and *lethargic* mice, we found no changes compared to the wild-type littermates, other than a mild reduction of evoked ACh release at *ducky* NMJs, which is most likely rather due to the smaller synapse size in these mice than the direct consequence of the absence of $\alpha_2\delta$-2. Our studies indicate a functional redundancy of $\alpha_2\delta$-2, $\beta_4$, and $\gamma_2$ subunits at the mouse motor nerve terminal.

## EXPERIMENTAL PROCEDURES

### Mice

All animal experiments were carried out in accordance with national legislation, the European Communities Council Directive of 24 November 1986 (86/609/EEC), and were approved by the Leiden University Animal Experiments Committee. Original breeder pairs of all mouse mutant strains (*ducky*, *stargazer,* and *lethargic*) were obtained from Jackson Laboratories (*Bar Harbor, ME, USA*) and were maintained in the Leiden University Medical Centre vivarium in a 12hrs light/12hrs dark cycle.

The *ducky* strain (*Snell, 1955*) was maintained on C3H background, with the tail-kink (*tk*) mutation segregating with the *ducky* mutation, allowing genetic phenotyping. Homozygous *ducky* mice (identified on the basis of their neurological phenotype) and wild-type controls (littermates wherever possible, otherwise age-matched controls, identified by their tail kink) were used at ~6 weeks of age. Body weights of *ducky* mice were 50% lower than those of the wild-type controls (19.1 ± 0.3 and 9.4 ± 0.3 g for wild-type and *ducky*, respectively, n=8, $p<0.001$).

*Stargazer* and *lethargic* strains were maintained on C57/Bl6J background. Homozygous mice and their wild-type controls (littermates wherever possible, otherwise age-matched controls) were used at ~5 weeks of age. Mice were genotyped by PCR using genomic DNA extracted from tail biopsies. Genotyping was performed as described earlier (*Burgess et al., 1997*; *Letts et al., 1997*). *Stargazer* and *lethargic* mutant mice had a reduced body weight (of ~20 and 50%, respectively), compared to wild-type. Mean body weight was 12.9 ± 1.4 (n=6, $p<0.01$), 8.1 ± 0.5 (n=7, $p<0.001$) and 16.3 ± 0.4 g (n=11), respectively.

### *Ex vivo* electrophysiology

Mice were euthanized by means of carbon dioxide inhalation. Hemi-diaphragms with phrenic nerve were dissected and kept in Ringer's medium (in mM: NaCl 116, KCl 4.5, $CaCl_2$ 2, $MgSO_4$ 1, $NaH_2PO_4$ 1, $NaHCO_3$ 23, glucose 11, pH 7.4) at room temperature (20-22°C) and continuously bubbled with 95% $O_2$ / 5% $CO_2$. Intracellular recordings of both MEPPs and EPPs were made at NMJs at 28°C using standard micro-electrode equipment (as described in detail previously in *Plomp et al., 1992*). At least 30 MEPPs and EPPs were recorded at each NMJ, and 7-15 NMJs were sampled per experimental condition per muscle. Muscle action potentials were blocked by 3 µM µ-conotoxin GIIIB (*Scientific Marketing Associates, Barnet, UK*). For EPP recording, the nerve was stimulated at 0.3 or 40 Hz. Procedures for the analysis of MEPPs and EPPs and the calculation of quantal contents, i.e. the number of ACh quanta released per nerve impulse, have been described before (*Kaja et al., 2005*). EPPs and MEPPs were also measured in the presence of 200 nM of the specific $Ca_v2.1$ channel blocker ω-agatoxin-IVA, following a 20 min pre-incubation period. Toxins were from Scientific Marketing Associates (*Barnet, UK*).

### α-Bungarotoxin staining and image analysis

NMJ size was determined by fluoresence microscopy. Diaphragm preparations were pinned out and fixed in 1% paraformaldehyde (*Sigma-Aldrich, Zwijndrecht, The Netherlands*) in 0.1 M phosphate-buffered saline pH 7.4 (PBS) for 30 min, at room temperature. Following a 30 min wash in PBS, diaphragms were incubated in 1 µg/ml Alexa Fluor 488 conjugated αBTx (*Molecular Probes, Leiden, The Netherlands*) in PBS for 3 h at room temperature, labelling the ACh receptors. After a final washing step in PBS (30 min), NMJ-containing midline regions were excised from the diaphragms and mounted on microscope slides with Citifluor AF-1 antifadent (*Citifluor, London, UK*). Sections were examined with an Axioplan microscope (*Zeiss, Jena, Germany*). NMJs were identified on the basis of αBTx staining, under standardized camera conditions. Images of αBTx stain were stored digitally. The area of the αBTx-staining was measured in ImageJ (*National Institutes of Health, USA*). In total, 3-4 diaphragms per genotype were quantified with the investigator blinded for genotype. In every diaphragm, a minimum of 10 NMJs were selected randomly.

### Muscle fiber diameter analysis

Left hemidiaphragms were pinned out on loose blocks of silicone rubber, snap frozen in liquid nitrogen, and subsequently embedded in TissueTek® (*Bayer BV, Mijdrecht, The Netherlands*). Transversal sections (12-18 µm) were cut on a Microm cryostat (*Adamas Instruments BV, Leersum, The Netherlands*) at –21°C and collected on poly-lysine coated slides. Sections were dried for 1h at room temperature, fixed for 10 s in ice-cold acetone, stained for 10 s in 0.5% alkaline toluidine blue, dehydrated in a graded series of ethanol (50%, 70% 80%, 90%, 96%, 100%, 1 min each), and finally cleared in xylene. Sections

were imbedded in Entellan mounting medium (*Merck, Darmstadt, Germany*) and viewed under a Zeiss Axioplan light microscope (*Zeiss, Jena, Germany*). Photographs were taken with a digital microscope-camera and fiber diameter was estimated using ImageJ (*National Institutes of Health, USA*). Stereological considerations were taken into account by defining the actual diameter of a single muscle fiber by the shortest distance measured. At least ten fibers were measured per muscle.

### Statistical analyses

Possible statistical differences were analyzed with paired or unpaired Student's *t*-tests or analysis of variance (ANOVA) with Tukey's HSD post-hoc test, where appropriate, on grand mean values with *n* as the number of mice tested, and 7-15 NMJs tested per muscle. $p<0.05$ was considered to be statistically significant. Data is presented as mean ± SEM.

## RESULTS

### Synaptic electrophysiology of ducky NMJs

We investigated spontaneous (uniquantal) ACh release at *ducky* NMJs by recording miniature endplate potentials (MEPPs, the postsynaptic membrane depolarizations resulting from the release of a single ACh quantum). MEPP frequency was similar in wild-type and *ducky* mice (1.03 ± 0.13 and 1.21 ± 0.13 $s^{-1}$, respectively; n=9 muscles, 8-15 NMJs per muscle, p=0.45, *Fig. 1A*). MEPP amplitude, in contrast, was increased by ~40% at *ducky* NMJs compared to wild-type (1.46 ± 0.07 and 1.00 ± 0.08 mV, respectively; n=9 muscles, 8-15 NMJs per muscle, $p<0.01$, *Fig. 1B*). Half-width and rise time of MEPPs were unaltered (*data not shown*). Representative MEPP traces are shown in *Figure 1C*.

We then studied low-rate nerve stimulation-evoked ACh release. The quantal content, i.e. the number of quanta released per supramaximal stimulus, was reduced by ~25% at *ducky* NMJs (37.0 ± 2.5 and 26.8 ± 0.4 at wild-type and *ducky* NMJs, respectively; n=9 muscles, 8-15 NMJs per muscle, $p<0.001$, *Fig. 1D*), whereas endplate potential (EPP) amplitudes and kinetics did not differ between genotypes. Normalized EPP amplitudes were 25.1 ± 0.9 and 26.6 ± 0.9 mV at wild-type and *ducky* NMJs, respectively (n=9 muscles, 8-15 NMJs per muscle, p=0.29, *Fig. 1E-F*).

Some types of channel dysfunction may only become apparent upon high-frequency use of the channel. Therefore, we measured ACh release upon 40 Hz stimulation. However, during a 1 s train, rundown of EPP amplitudes was similar in both mutants, reaching a plateau after the $20^{th}$ stimulus of 80.7 ± 0.9 and 81.5 ± 0.6 % at wild-type and *ducky* NMJs, respectively (n=9 muscles, 8-15 NMJs per muscle, p=0.61, *Fig. 1G*).

In order to assess whether the absence of the $\alpha_2\delta$-2 subunit resulted in compensatory expression of non-$Ca_v2.1$ channels, as for instance reported by us for the natural

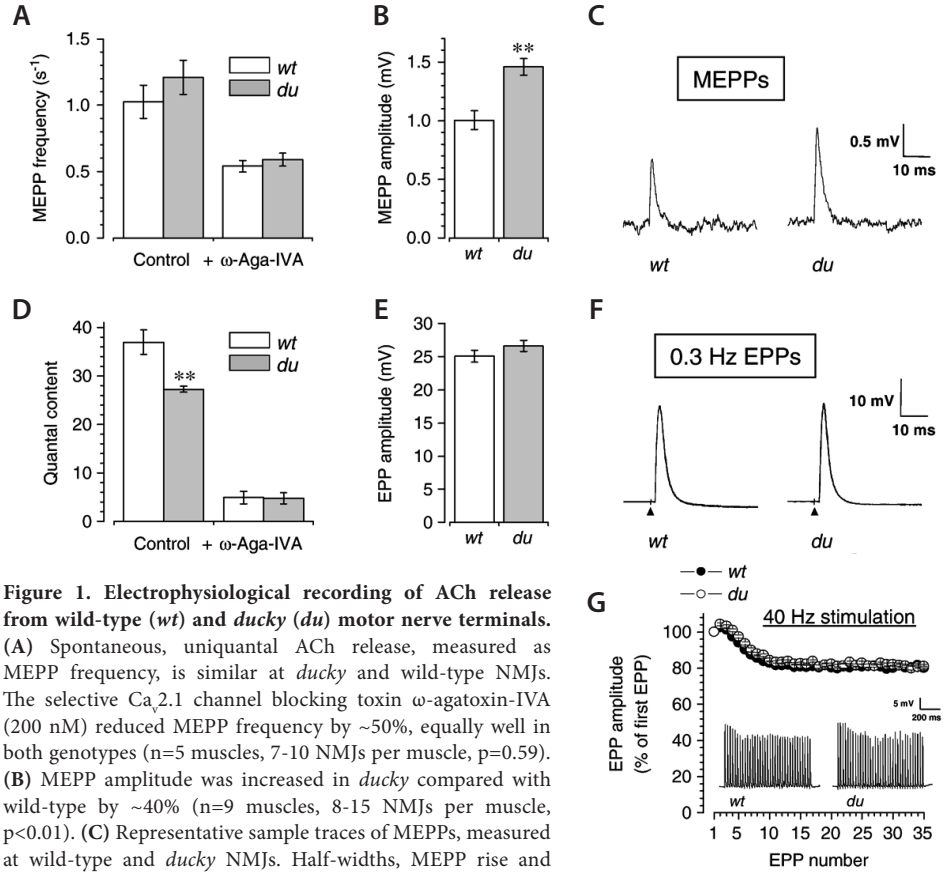


**Figure 1. Electrophysiological recording of ACh release from wild-type (*wt*) and *ducky* (*du*) motor nerve terminals.** **(A)** Spontaneous, uniquantal ACh release, measured as MEPP frequency, is similar at *ducky* and wild-type NMJs. The selective $Ca_v2.1$ channel blocking toxin ω-agatoxin-IVA (200 nM) reduced MEPP frequency by ~50%, equally well in both genotypes (n=5 muscles, 7-10 NMJs per muscle, p=0.59). **(B)** MEPP amplitude was increased in *ducky* compared with wild-type by ~40% (n=9 muscles, 8-15 NMJs per muscle, p<0.01). **(C)** Representative sample traces of MEPPs, measured at wild-type and *ducky* NMJs. Half-widths, MEPP rise and decay times were similar between genotypes. **(D)** Evoked release (quantal content) at 0.3 Hz nerve stimulation was reduced ~25% at *ducky* NMJs (n=9 muscles, 8-15 NMJs per muscle, p<0.01). Quantal content was reduced by 200 nM ω-agatoxin-IVA to similar levels in both genotypes (n=5 muscles, 7-10 NMJs per muscle, p=0.93). **(E)** EPP amplitudes were ~25 mV in both genotypes (n=9 muscles, 8-15 NMJs per muscle, p=0.29). **(F)** Sample traces of six superimposed EPP recordings made at NMJs of wild-type and *ducky* mice at 0.3 Hz stimulation of the phrenic nerve. The moment of nerve stimulation is indicated by a black triangle. **(G)** EPP amplitude rundown at 40 Hz stimulation is not different at *ducky* NMJs, compared with wild-type (n=9 muscles, 8-15 NMJs per muscle, p=0.61). Representative 1 s traces of intracellular recordings are shown for both wild-type and *ducky* (inset). *p<0.05, ** p<0.01.

*Cacna1a* mutant *tottering* (*Kaja et al., 2006*), we applied 200 nM of the selective $Ca_v2.1$ channel blocking toxin ω-agatoxin-IVA to the preparation. It reduced both MEPP frequency (~55%, p<0.05) and quantal content (~90%, p<0.01) to a similar extent in wild-type and *ducky* NMJs. MEPP frequencies in the presence of the toxin were 0.54 ± 0.04 and 0.59 ± 0.05 $s^{-1}$ for wild-type and *ducky*, respectively (n=5 muscles, 10-15 NMJs per muscle, p=0.45, *Fig. 1A*). The quantal contents were reduced to 4.9 ± 1.3 at wild-type and 4.8 ± 1.1 at *ducky* NMJs (n=5 muscles, 10-15 NMJs per muscle, p=0.93, *Fig. 1D*).

## Smaller muscle fiber diameter and reduced NMJ size at *ducky* diaphragms

Transmitter release level at the NMJ is roughly correlated with synapse size (*Kuno et al., 1971*; *Harris & Ribchester, 1979*). Furthermore, muscle fiber diameter is inversely related to electrical input resistance, which, in turn, is a determinant of MEPP amplitude (*Katz & Thesleff, 1957*). Given the severely reduced (~50%) body weight of *ducky* mice, compared to wild-type mice, reduced quantal content and increased MEPP amplitude at *ducky* NMJs may just be the result of thinner fibers and smaller NMJs, respectively, instead of directly being caused by the absence $\alpha_2\delta$-2 subunits. Indeed, fiber diameter at *ducky* NMJs was reduced by ~45% compared with wild-type NMJs ($13.6 \pm 0.2$ and $24.7 \pm 0.5$ µm, respectively, n=4 muscles, 10 fibers per muscle, $p<0.001$, *Fig. 2A*). The NMJ area, defined as the area stained for postsynaptic ACh receptors with fluorescently-labeled α-bungarotoxin (αBTx), were ~40% smaller in *ducky* mice than in wild-type mice ($227 \pm 8$ µm$^2$ in *ducky* and $374 \pm 13$ µm$^2$ in wild-type, n=4 muscles per genotype, 14-31 NMJs per muscle, $p<0.001$, *Fig. 2B*). Representative pictures are shown in *Figure 2C*.

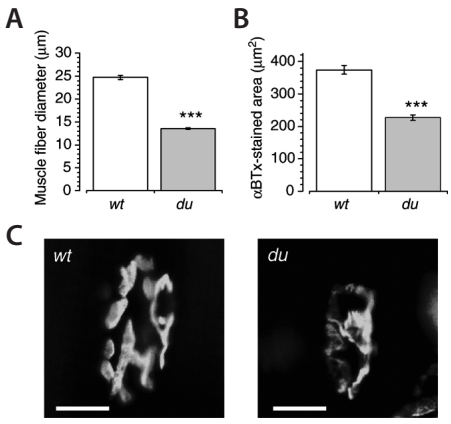


**Figure 2. Quantification of muscle fiber diameter and NMJ size. (A)** Muscle fiber diameter was determined on digital images of toluidine blue stained, transversal diaphragm sections. At least 10 fibers per muscle were quantified. Muscle fiber diameter was ~45% reduced in *ducky* (*du*) diaphragms, compared with wild-type (*wt*) (n=4, 10 fibers per muscle, $p<0.001$). **(B)** NMJ size was determined by measuring αBTx-stained AChR areas at *ducky* and wild-type NMJs. NMJs were ~40% smaller in homozygous *ducky* animals, compared to wild-type (n=4, 14-31 NMJs per muscle, $p<0.001$). **(C)** Fluorescently-labeled NMJs of wild-type and *ducky* mice. Scale bar: 15 µm. ***$p<0.001$

## No effect of gabapentin on ACh release at *ducky* and wild-type NMJs

The lack of effects of the *ducky* truncation on ACh release at the NMJ prompted us to check for the presence of the $\alpha_2\delta$-2 subunit at the wild-type mouse NMJ, using an indirect, pharmacological approach. GBP has been supposed to reduce $Ca^{2+}$ current mainly by binding to the $\alpha_2\delta$-2 subunit of $Ca_v2.1$ channels (*Bayer et al., 2004*), and, in view of the complete dependence on $Ca_v2.1$ channels for ACh release, is likely to have an effect at the wild-type NMJ.

We tested the effect of 300 µM GBP on ACh release at the NMJ in both wild-type and *ducky* mice. None of electrophysiological parameters were different following a 1h incubation with GBP, compared to the control condition without GBP (*Table 1*), further suggesting either redundancy or absence of $\alpha_2\delta$-2 subunits at the mouse NMJ.

## Synaptic electrophysiology of *stargazer* and *lethargic* mice

In a separate series of experiments we compared ACh release at NMJs in *stargazer* and *lethargic* mice to that of their wild-type littermates. Electrophysiological parameters obtained from wild-type mice of both the *stargazer* and *lethargic* mutant breeding lines did not differ significantly. Given that both lines were analyzed at the same age we combined the data from wild-type mice and compared the pooled wild-type data with that of the mutants.

Uniquantal ACh release measured as MEPP frequency did not differ between genotypes (0.93 ± 0.07, 0.99 ± 0.09 and 0.81 ± 0.06 $s^{-1}$ at wild-type, *stargazer,* and *lethargic* NMJs, respectively; n=6-11 muscles, 7-15 NMJs per muscle, p=0.37, *Fig. 3A*). MEPP amplitudes were similar at wild-type and *stargazer* (p=0.54), but statistically significantly increased (31%, p<0.05) at *lethargic* NMJs (1.07 ± 0.07, 1.21 ± 0.06 and 1.40 ± 0.13 mV, respectively, n=6-11 muscles, 7-15 NMJs per muscle, p<0.05, *Fig. 3B*). *Figure 3C* shows representative MEPP traces for all genotypes. Kinetic parameters such as half-widths, and rise and decay times were not different in wild-type mice from either mutant line (*data not shown*).

EPP amplitudes measured following low-rate (0.3 Hz) supramaximal stimulation of the phrenic nerve were not different between genotypes, averaging at 23.1 ± 0.7, 26.1 ± 0.4, and 26.2 ± 1.7 mV at wild-type, *stargazer,* and *lethargic* NMJs, respectively (n=6-11 muscles, 7-15 NMJs per muscle, p=0.15, *Fig. 3C*). Similarly, quantal contents in *stargazer* and *lethargic* mice calculated from the normalized MEPP and normalized and corrected EPP amplitudes were not statistically significantly different from wild-type controls (31.5 ± 1.2, 31.7 ± 1.6 and 28.1 ± 1.3 for wild-type, *stargazer,* and *lethargic*, respectively; n=6-11 muscles, 7-15 NMJs per muscle, p=0.16, *Fig. 3D*). The

**Table 1.** Effect of 300 μM gabapentin on ACh release parameters at wild-type and *ducky* diaphragm NMJs

| Release parameter | Genotype | Control | 300 μM GBP | p |
|---|---|---|---|---|
| MEPP frequency ($s^{-1}$) | *wt* | 0.81 ± 0.03 | 0.81 ± 0.03 | 0.96 |
| | *du* | 0.87 ± 0.11 | 0.90 ± 0.11 | 0.68 |
| MEPP amplitude (mV) | *wt* | 1.04 ± 0.14 | 1.21 ± 0.13 | 0.12 |
| | *du* | 1.54 ± 0.13 | 1.65 ± 0.11 | 0.43 |
| EPP amplitude (mV) | *wt* | 25.8 ± 2.1 | 28.2 ± 1.6 | 0.31 |
| | *du* | 27.9 ± 1.1 | 29.1 ± 0.7 | 0.29 |
| Quantal content (0.3 Hz) | *wt* | 36.7 ± 4.0 | 36.3 ± 2.1 | 0.85 |
| | *du* | 27.3 ± 1.2 | 27.7 ± 1.0 | 0.78 |
| EPP amplitude (40 Hz) (% of first EPP) | *wt* | 78.7 ± 0.8 | 79.4 ± 1.8 | 0.66 |
| | *du* | 81.4 ± 1.0 | 83.9 ± 1.5 | 0.08 |

Gabapentin (GBP, 300 μM) did not affect basic electrophysiological parameters at either wild-type (*wt*) or *ducky* (*du*) NMJs.

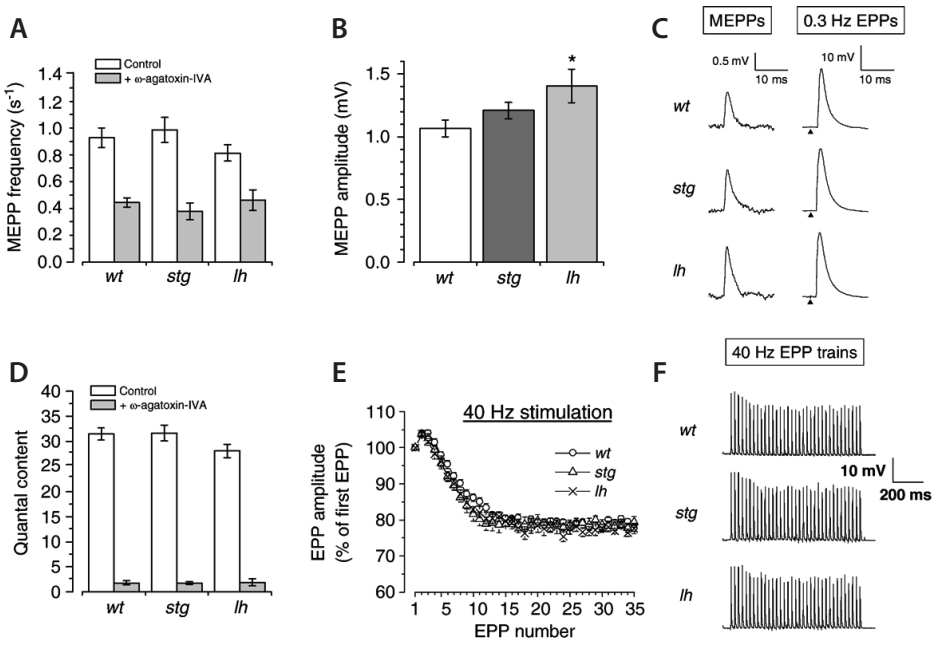


**Figure 3. ACh release from motor-nerve terminals of wild-type (*wt*), *stargazer* (*stg*), and *lethargic* (*lh*) mice. (A)** MEPP frequencies were similar in all three genotypes (n=6-11, ANOVA p=0.37). The selective $Ca_v2.1$ channel blocking toxin, ω-agatoxin-IVA (200 nM), reduced MEPP frequency by ~60%, similarly at wild-type, *stargazer,* and *lethargic* NMJs (n=3-7, 7-15 NMJs per muscle, p=0.18). **(B)** MEPP amplitudes were slightly increased in *lethargic* mice (n=7-11, 7-15 NMJs per muscle, * p<0.05), whereas *stargazer* values were similar to wild-type values (n=6-11, 7-15 NMJs per muscle, p=0.54). **(C)** Representative traces of MEPPs and EPPs (obtained at 0.3 Hz nerve stimulation). MEPP half-widths and rise and decay times were not different between genotypes (data not shown). EPP amplitudes were ~25 mV in all genotypes (p=0.15), and had similar half-widths, and rise and decay times. Black triangle indicates the moment of nerve stimulation. **(D)** Quantal contents were similar for all genotypes (n=6-11, 7-15 NMJs per muscle, p=0.16) and were reduced by 200 nM ω-agatoxin-IVA by ~95% (n=3-7). **(E)** EPP amplitude rundown profiles upon 40 Hz nerve stimulation at *stargazer* and *lethargic* mutant NMJs were comparable to wild-type (n=6-11, 7-15 NMJs per muscle, p=0.42). **(F)** Representative 1 s EPP train traces at 40 Hz stimulation.

quantal content at *lethargic* NMJs showed a trend towards ~10% reduction (p=0.08 with Tukey's HSD post-hoc test).

Again, we tested a possible contribution of non-$Ca_v2.1$ channels to ACh release. ω-Agatoxin-IVA reduced both MEPP frequency and quantal content by ~60% and ~95%, respectively (n=3-7 muscles, 7-10 NMJs per muscle, *Fig. 3A, D*), similarly for all three genotypes.

## Morphological changes in *lethargic* and *stargazer* diaphragms

We measured muscle fiber diameter in wild-type, *stargazer,* and *lethargic* mice. Fiber diameters were 15.8 ± 0.5, 14.2 ± 0.3 and 12.3 ± 1.1 μm, respectively, corresponding to

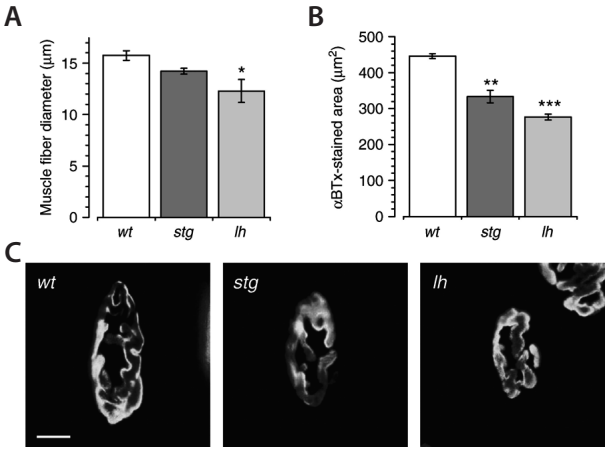


**Figure 4. Quantification of muscle fiber diameter and NMJ size of wild-type (*wt*), *stargazer* (*stg*), and *lethargic* (*lh*) mice.** **(A)** Muscle fiber diameter was reduced by ~20% in *lethargic* diaphragms, compared with wild-type (n=4, 10 fibers per muscle, *p<0.05). Fiber diameters of *stargazer* mice were similar to those obtained from wild-type muscles (n=4, 10 fibers per muscle, p=0.33). **(B)** NMJ size was estimated by measuring the area of fluorescent αBTx-staining and were ~25% smaller in *stargazer* (n=3, 16-25 NMJs per muscle, **p<0.001) and reduced in size by ~40% in *lethargic* mice (n=3, 13-21 NMJs per muscle, p<0.001). **(C)** Representative images of αBTx staining in wild-type, *stargazer,* and *lethargic* mice are shown. Scale bar: 10 µm.

a reduction of 22% at *lethargic* NMJs (n=4 muscles, 10 fibers per muscle, p<0.05, *Fig. 4A*). The αBTx-stained NMJ area in *stargazer* mice was 25% smaller than in wild-type mice (333 ± 18 and 446 ± 7 µm², respectively, n=3 muscles, 16-25 NMJs per muscle, p<0.01, *Fig. 4B*). The NMJ area at the *lethargic* diaphragm was 38% smaller than in wild-type (277 ± 8, n=3 muscles, 13-21 NMJs per muscle, p<0.001, *Fig. 4B*). Representative αBTx-stained NMJs are shown in *Fig. 4C*.

## DISCUSSION

We present the first study on synaptic consequences of functional absence of $Ca_v2.1$ channel accessory subunits at the NMJ. The absence of the $\alpha_2\delta$-2, $\beta_4$ and $\gamma_2$ subunits does not compromise transmitter release at NMJs of the natural mouse mutants *ducky*, *lethargic,* and *stargazer*, respectively. Thus, these accessory channel subunits are functionally redundant at presynaptic release sites of the NMJ.

### *Ducky* synapses

*Ducky* mice, lacking the $\alpha_2\delta$-2 accessory subunit of $Ca_v2.1$ channels, had a ~25% lower quantal content than wild-type mice. This modest reduction is more likely due to the small NMJ size than to the absence of the $\alpha_2\delta$-2 subunit. It is known that ACh release correlates with NMJ size (*Kuno et al., 1971*; *Harris & Ribchester, 1979*), and NMJ size with muscle fiber diameter. *Ducky* mice had a large (~50%) reduction in body weight, with concomitantly reduced muscle fiber diameter and NMJ area. Hence, the reduction in 0.3 Hz nerve stimulation-evoked ACh release at *ducky* NMJs is likely to be the (indirect) result of growth retardation of the mice. Increased input resistance is

the most likely cause of the increase in the MEPP amplitude observed at *ducky* NMJs, in view of the well-known inverse correlation of fiber diameter and input resistance and the positive relationship between input resistance and MEPP amplitude (*Katz & Thesleff, 1957*; *Harris and Ribchester, 1979*). From the data of *Harris & Ribchester* (1979), it can be deduced that the ~40% increase in *ducky* MEPP amplitude, compared to wild-type, corresponds to ~50% increase in input resistance (from ~0.4 to ~0.6 MΩ).

Rundown of EPP amplitudes upon high rate (40 Hz) nerve stimulation at *ducky* NMJs was indistinguishable from that at wild-type NMJs. We have shown previously that some aspects of dysfunction of mutated $Ca_v2.1$ channels can be revealed at high frequency stimulation only (*Kaja et al., 2005*). The normal EPP rundown profile observed here strengthens the hypothesis that $Ca_v2.1$ channel behavior *per se* is not affected at the *ducky* NMJ.

In *ducky* cerebellar Purkinje cells, $Ca_v2.1$-mediated $Ca^{2+}$ current is reduced by ~35%, compared with those of wild-type mice. However, in cerebellar granule cells there was no reduction (*Barclay et al., 2001*). These findings correlate with the expression pattern of the $\alpha_2\delta$-2 subunit, i.e. a very high level in Purkinje cells and a lower level in granule cells (*Barclay et al., 2001*). This raises the question, whether $\alpha_2\delta$-2 subunits are present at all at the wild-type NMJs. We addressed this question using an indirect pharmacological approach testing the effects of GBP. This drug acts by binding to a specific site on $\alpha_2\delta$-1, -2 and -4 subunits (*Marais et al., 2001*; *Qin et al., 2002*). In neocortical brain slices, 100-300 µM GBP significantly reduces $Ca^{2+}$ flux through $Ca_v2.1$ channels (*Fink et al., 2000*; *Dooley et al., 2002*), and in hippocampal synaptosomes it reduces $K^+$-evoked $Ca^{2+}$ influx (*van Hooft et al., 2002*). Here, however, 300 µM GBP had no effect on ACh release at wild-type (or *ducky*) NMJs. This finding contrasts with that of the study by *Bayer et al.* (2004), which showed specific reduction of $Ca_v2.1$-mediated transmitter release at spinal cord synapses by GBP, but agrees with that by *Brown & Randall (2005)* showing the lack of effect of GBP found on $Ca_v2.1$-mediated hippocampal transmission. Thus, our results on the action of GBP indicate that $\alpha_2\delta$-2 subunits are presumably not present or, at least, not physiologically active at the wild-type NMJ. Furthermore, they contribute to the idea that this drug does not seem to act as a general $Ca_v2.1$ channel modulator.

In a very recent paper, *Joshi & Taylor* (2006) showed that pregabalin, a compound related to gabapentin, reduces nerve stimulation-evoked muscle contraction. They hypothesized a reduced presynaptic ACh release through an action of pregabalin on $\alpha_2\delta$-2 subunits. Such an effect is, however, not in agreement with the observed lack of effect of GBP on ACh release at the NMJ found in our present study.

Our experiments with the $Ca_v2.1$ channel blocker ω-agatoxin-IVA show that absence of $\alpha_2\delta$-2 subunits does not lead to compensatory involvement of non-$Ca_v2.1$ channels in ACh release, which could have potentially occurred if $\alpha_2\delta$-2 subunits were involved in $Ca_v2.1$ trafficking or membrane insertion at the NMJ. Taken together, our data suggest that the $\alpha_2\delta$-2 subunit is not associated with presynaptic $Ca_v2.1$ channels at the

NMJ and indicate that it is not likely that GBP has any direct pharmacotherapeutic (or adverse) action on neuromuscular transmission.

### *Lethargic* and *stargazer* synapses

In spite of immunohistochemical data clearly indicating the presynaptic presence of $\beta_4$ subunits at the wild-type mouse NMJs (*Pagani et al., 2004*), we observed unchanged ACh release at the *lethargic* NMJ, whick lack these subunits. This suggests that the $\beta_4$ subunit, although present, does not have any functional role at the NMJ, or alternatively, that other β subunits can compensate for the loss of $\beta_4$. Functional compensation has indeed been demonstrated in *lethargic* brain, where $Ca_v2.1$ and $Ca_v2.2$ channels associate more strongly with other β subunits and are functionally modulated by them, in the absence of $\beta_4$ (*McEnery et al., 1998*; *Burgess et al., 1999*). However, the severe neurological phenotype of *lethargic* mice suggests that not all cell types are able to compensate completely for the loss of the $\beta_4$ subunit. As previously suggested by *Noebels et al.* (1990), the specific $Ca_v2.1$-$\alpha_1/\beta_4$ interaction site may convey a unique signal distinct from that of $Ca_v2.1$-$\alpha_1/\beta_{1\text{-}3}$ interaction (*Walker et al., 1998*; *Burgess et al., 1999*; *Geib et al., 2002*).

Our finding of unchanged transmitter release at *lethargic* NMJ is similar to that at *lethargic* hippocampal synapses, which show normal $Ca_v2.1$-mediated presynaptic $Ca^{2+}$ influx and a normal resulting transmitter release (*Qian & Noebels, 2000*). In contrast, excitatory (glutamatergic) neurotransmission was found to be reduced >60% in *lethargic* ventrobasal thalamic neurons, whereas inhibitory (GABAergic) neurotransmission was unaffected (*Caddick et al., 1999*).

Our detailed characterization of ACh release at the *stargazer* NMJ did not reveal any abnormalities. This suggests that either the $\gamma_2$ subunit is not present at the NMJ, or that it does not modulate presynaptic $Ca_v2.1$ channel function at the NMJ. In a CNS synaptic study on *stargazer* mice, a normal excitatory neurotransmission was found in hippocampal CA1 pyramidal cells (*Hashimoto et al., 1999*). Furthermore, cerebellar excitatory neurotransmission at the mossy fiber-to-granule cell synapse lacked the fast AMPA-mediated component, but this was shown to be due to a loss of postsynaptic receptors rather than reduced transmitter release and suggests a role for $\gamma_2$ subunits in trafficking rather than transmitter release (*Hashimoto et al., 1999*). Recently, $\gamma_2$, $\gamma_3$, $\gamma_4$ and $\gamma_8$ have been shown to represent a family of transmembrane AMPA-receptor regulatory proteins, called TARPs (*Tomita et al., 2003*). Spontaneous uniquantal GABA-ergic responses in the dentate gyrus of the *stargazer* hippocampus were unaltered, consistent with the demonstrated lack of expression of the $\gamma_2$ subunit in the wild-type dentate gyrus (*Payne et al., 2006*). However, the extrasynaptic GABA receptor profile was changed, presumably indirectly through altered dentate gyrus input patterns reducing $\gamma_8$ expression. Our present neuromuscular electrophysiological analyses of *stargazer* mice suggest that a regulatory role of the $\gamma_2$ subunit in postsynaptic NMJ structure or function is unlikely.

### Redundancy of accessory $Ca_v2.1$ channel subunits at the NMJ

The present study suggests that the $Ca_v2.1$ channel subunits $\alpha_2\delta$-2, $\beta_4$, and $\gamma_2$, at least at the NMJ, are not uniquely required for $Ca_v2.1$ channel-mediated neurotransmitter release. These subunits may be absent at presynaptic release sites, or alternatively, their function is fully compensated for by related subunits, as described for Purkinje cells in *lethargic* mice (*McEnery et al., 1998*; *Burgess et al., 1999*).

To date, almost all studies showing modulatory effects of accessory channel subunits on $Ca_v$ function have been performed in heterologous expression systems (for reviews, see *Black, 2003*; *Dolphin, 2003*; *Klugbauer et al., 2003*). However, only a few studies measuring transmitter release at physiological synapses have revealed either an absence of effects, or only cell type-specific effects resulting from the lack of compensating accessory $Ca_v$ channel subunits (*Caddick et al., 1999*; *Hashimoto et al., 1999*; *Qian & Noebels, 2000*). Importantly, the *in vivo* conditions, especially in the specialized presynaptic micro-environment of interacting structural and functional proteins, may differ completely from those at the somata of whole-cell voltage-clamped expression cells *in vitro*. This is illustrated, for instance, by the contradicting results of studies characterizing R192Q-mutated $Ca_v2.1$ channel function either in expression systems or in neurons and synapses isolated from R192Q *Cacna1a* knockin mutant mice (*van den Maagdenberg et al., 2004*). Similarly, despite of the fact that heterologous expression of the $\gamma_2$ subunit has repeatedly shown diverse modulatory effects of this subunit on $Ca_v2.1$ channel function (for review, see *Black, 2003*), $Ca_v2.1$ channel-dependent, $K^+$-evoked glutamate and GABA release from cortical nerve terminals, as determined by *in vivo* microdialysis, was found normal in *stargazer* mice (*Ayata et al., 2000*). Hence, unique modulation by accessory $\alpha_2\delta$-2, $\beta_4$ and $\gamma_2$ subunits of $Ca_v2.1$-$\alpha_1$ subunits that mediate neurotransmitter release seems rather uncommon. This may follow either from absence of modulating properties of these accessory subunits in the specific synaptic environment or, in case of their deficiency, from full functional compensation by other subunit isoforms.



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