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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 peripheral nervous system. 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.



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*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

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## 1. Introduction

$Ca_v2.1$ (P/Q-type) voltage-activated $Ca^{2+}$ channels are mediators of synaptic transmission both in the central (CNS) and peripheral nervous system (PNS) by conducting the presynaptic $Ca^{2+}$ influx required for neurotransmitter release (Uchitel et al., 1992; Mintz et al., 1995). As common 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 $\beta$ (for review, see Catterall, 2000; Snutch et al., 2005). Whereas $\alpha_2\delta$ is a membrane protein, the $\beta$ 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 $\beta$-subunits ($\beta_1$ to $\beta_4$) have been identified (for review, see Arikkath and Campbell, 2003). Furthermore, eight different $\gamma$ subunits exist (Jay et al., 1990; Burgess et al., 2001; Arikkath and Campbell, 2003), of which at least $\gamma_2$ can associate with $Ca_v2.1$-$\alpha_1$ (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 and 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 $\beta$ 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 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 occurred 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 regarding $\alpha_2\delta$-2 subunits and also no studies were 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 here, 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, encoding the $\alpha_2\delta$-2 subunit (Barclay et al., 2001; Brodbeck et al., 2002), leads to a much shorter transcript which 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 and Swigart, 1971) and carries a mutation in *Cacnb4*, the gene encoding the $\beta_4$ subunit. All studies to date failed to show any translated $\beta_4$ protein (Burgess et al., 1997; McEnery et al., 1998; Burgess et al., 1999), making *lethargic* a functional $\beta_4$ knock-out model. The *stargazer* 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 $\gamma_2$ knock-outs, 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 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.

## 2. Results

### 2.1. *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 Fig. 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, $p=0.29$, Figs. 1E–F).

Some types of channel dysfunction may only become apparent upon high-frequency use of the channel. We, therefore, 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 20th 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 *Cacna1a* mutant *tottering* (Kaja et al., 2006), we applied 200 nM of the selective