Gangliosides and anti-ganglioside antibodies in neuromuscular synaptic function

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CHAPTER 2.3

Total ganglioside ablation at mouse motor nerve terminals alters neurotransmitter release level

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Abstract

Neuronal membrane gangliosides, forming a large family of sialylated glycosphingolipids, have been hypothesized to play important roles in synaptic transmission. We studied the *ex vivo* electrophysiological function of neuromuscular junctions of GM2/GD2synthase*GD3synthase compound *null*-mutant mice after acute removal of GM3, the only remaining ganglioside in this mouse, by in vitro treatment with neuraminidase. We found 16% enhancement of the acetylcholine release per nerve impulse at low-rate (0.3 Hz) nerve stimulation. Conversely, the treatment reduced the acetylcholine release evoked by high-rate (40 Hz) nerve stimulation. Also, 25 ms paired-pulse facilitation of endplate potentials was reduced by the neuraminidase-treatment. These effects may indicate a modest modulatory influence of the negative electrical charges carried by the sialic acid molecules of gangliosides on the function of presynaptic $Ca_v2.1$ channels, affecting the magnitude and kinetics of the Ca^{2+} influx that induces neurotransmitter release from the motor nerve terminal. Our results show that gangliosides are to some extent involved in neurotransmission at the neuromuscular junction, but that their presence is not an absolute requirement in this process.

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Gangliosides are glycosphingolipids carrying one or more sialic acid residues. This family of amphipathic molecules resides in the outer layer of cell membranes and is especially enriched in presynaptic nerve endings (Schwarz and Futerman, 1996; for review, see Plomp and Willison, 2009). Hence, gangliosides have been hypothesized to play important roles in synaptic transmission, possibly through affecting presynaptic voltage-gated ion-channels, Ca^{2+} homeostasis and/or surface charge. However, at the neuromuscular junction (NMJ) of transgenic mice lacking major subsets of gangliosides (Figure 2.15), we have previously shown that presence of the *complete* family of gangliosides is not strictly required for functional synaptic transmission. Electrophysiological measurement of acetylcholine (ACh) release at NMJs of GM2synthase knockout (GM2s-KO), GD3synthase knockout (GD3s-KO), or compound *null*-mutant mice lacking both GM2- and GD3synthase (dKO), showed only subtle changes and no failure of synaptic transmission was observed (Bullens et al., 2002; Zitman et al., 2008; Zitman et al., 2009). This suggests a fine-tuning role for gangliosides in transmitter release, rather than their presence being an absolute requirement. Alternatively, the different types of gangliosides might act mutually compensatory in supporting transmitter release. Such a compensatory action of GM3 ganglioside might explain the redundancy we found for the other gangliosides, because in all three investigated transgenic strains at least this ganglioside remains present in the neural membrane (Figure 2.15), most likely at highly accumulated level (Inoue et al., 2002). We here acutely removed the sialic acid component of this only remaining ganglioside at dKO NMJs, thereby creating a ganglioside-free nerve terminal, and studied the effect on transmitter release.

Male and female dKO mice (Inoue et al., 2002; Zitman et al., 2008) were used in the experiments, at ~11 weeks of age and 22.9 ± 1.1 g body weight. Mice were killed by CO_2 asphyxiation. Left and right hemi-diaphragms with phrenic nerves were dissected and pinned out in Ringer's medium (119 mM NaCl, 4.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 1mM NaH_2PO_4, 23 mM $NaHCO_3$, 11 mM glucose, pH 7.4), pre-gassed with 95% O_2/5% CO_2. Animal experiments were carried out according to Dutch law and Leiden University guidelines.

dKO phrenic nerve hemi-diaphragm preparations (6 left and 4 right hemi-diaphragms from n=10 dKO mice) were treated with 2 units neuraminidase type V from *Clostridium perfringens* (Sigma-Aldrich) in 1 ml Ringer's medium for 1 h at 32 °C. This disrupts GM3 by cleaving the α2,3-link between the sialic acid and galactose molecule (Perillo et al., 1994). We used tissue from dKO mice because neuraminidase treatment of wildtype tissue will not induce complete ganglioside ablation due to the inability of the neuraminidase to desialylate GM1 (Perillo et al., 1994). The contralateral hemi-diaphragm from each mouse was incubated as a control in Ringer's medium only. Preparations were then rinsed in Ringer's medium for 10 min. Subsequently, μ-conotoxin-GIIIB (3 μM; Scientific Marketing Associates) was added to block voltage-gated Na^+ channels on the muscle membrane in order to prevent muscle action potentials and contraction. ACh release at NMJs at room temperature (20-22 °C) was assessed using an intracellular electrophysiological method, as described before (Zitman et al., 2008), recording endplate potentials (EPPs, the postsynaptic responses resulting from nerve action potential-evoked ACh release following supramaximal stimulation of the phrenic nerve) and miniature EPPs (MEPPs, the responses resulting from spontaneous uniquantal release). The quantal content (i.e. the number of ACh quanta released per nerve impulse) was calculated from EPP and MEPP amplitudes (Zitman et al., 2008). Ten NMJs were sampled per muscle per experimental condition and at least 30 EPPs and 40 MEPPs were sampled

per NMJ. Data are expressed as group mean ± SEM (n=10 mice) of the mean muscle values calculated from the mean NMJ values. Statistical significance of differences between neuraminidase-treated and control groups was analyzed with paired Student's t-tests.

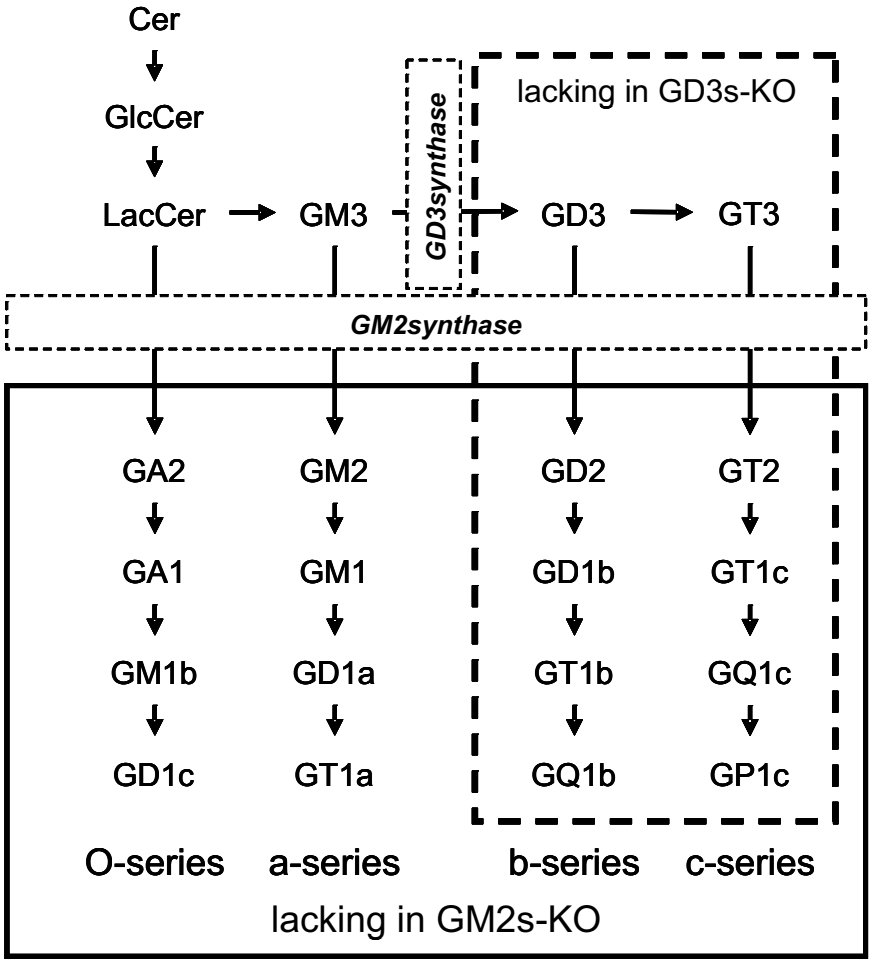

Figure 2.15. Ganglioside synthesis scheme with indication of the subsets lacking in GM2s-KO and GD3s-KO mice. dKO mice only express GM3.

The disruption of GM3 at the dKO NMJ by neuraminidase caused a 16% increase of quantal content at 0.3 Hz nerve stimulation (control 42.3 ± 2.1 and neuraminidase-treated 49.1 ± 1.6; $p<0.05$; Figure 2.16A). Spontaneous uniquantal ACh release, measured as MEPP frequency, showed a tendency of increase in the neuraminidase-treated NMJs, but this was not statistically significant ($p=0.28$, Figure 2.16B). During high rate (40 Hz) nerve stimulation, ACh release at neuraminidase-treated dKO NMJs became more depressed than that at control dKO NMJs. The rundown level of EPPs (calculated as the mean amplitude of the 21^{st} – 35^{th} EPP, expressed as percentage of the first EPP of the train) at NMJs in neuraminidase-treated tissue was 67.3 ± 1.5%, compared to 72.1 ± 0.7% at NMJs of untreated dKO muscles ($p<0.05$; Figure 2.16C-E). For comparison, EPP rundown level at wildtype NMJs under comparable experimental conditions in our previous study was 80.9 ± 1.2% (Zitman et al., 2008). Furthermore, the increase of the second EPP of the 40 Hz evoked train, compared to the first EPP (i.e. 25 ms paired-pulse facilitation), was lower in the neuraminidase-treated group (3.35 ± 0.66% at control vs. 0.32 ± 0.50% at neuraminidase-treated dKO NMJs; $p<0.01$; Figure 2.16F).

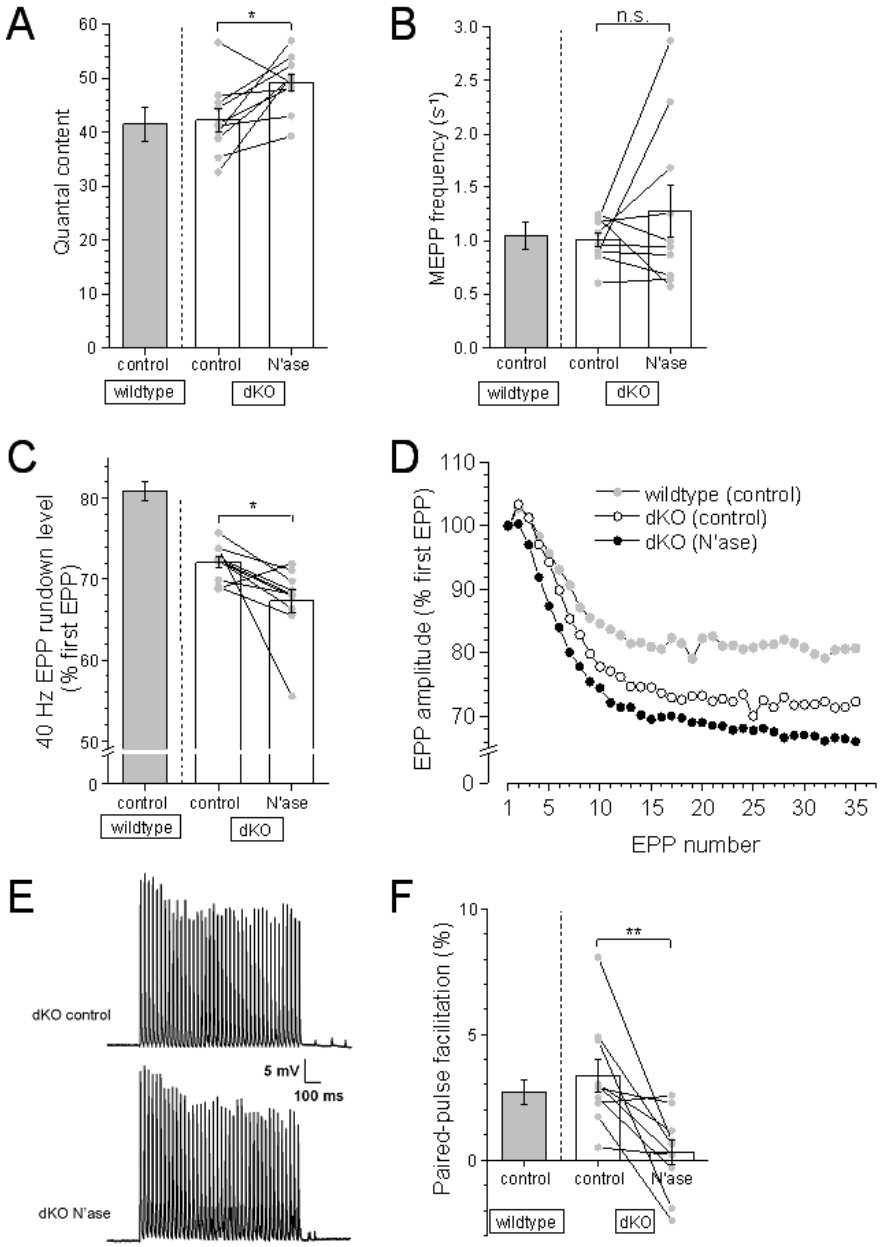

Figure 2.16 Electrophysiological measurement of ACh release at diaphragm NMJs of dKO mice (n=10) to assess the effect of neuraminidase (N'ase). Controls were sham-incubated without neuraminidase. For comparison, the values of synaptic parameters obtained at wildtype NMJ (without neuraminidase treatment) in our previous study (Zitman et al., 2008), have been added to the panels (gray bars, n=4 mice). Data are expressed as group means ± SEM (bars) and hemi-diaphragm pairs from individual mice (gray dots with connecting line). **A.** Quantal content at 0.3 Hz nerve stimulation increased 16% ($p<0.05$) by the neuraminidase treatment. **B.** MEPP frequency showed an increase, but this was not statistically significant ($p=0.28$). **C.** Rundown level of EPPs upon 40 Hz nerve stimulation was more pronounced ($p<0.05$). **D.** Mean EPP amplitude profiles during 40 Hz nerve stimulation. **E.** Representative examples of 40 Hz EPPs at control and neuraminidase-treated NMJs. **F.** Paired-pulse (25 ms) facilitation became smaller ($p<0.01$). *$p<0.05$; **$p<0.01$; n.s.: not statistically significant.

These results show that acute absence of all gangliosides by *in vitro* disruption of GM3, the only remaining type of ganglioside at dKO NMJs, at the one hand *increases* presynaptic neurotransmitter release by enhancing the amount of ACh released per nerve impulse but, on the other hand, *reduces* release by causing a more pronounced rundown during high-rate stimulation. It is yet unclear how gangliosides affect ACh release in this way. Possibly it involves a modulatory effect of the negative electrical

charges carried by sialic acid molecules of gangliosides on the presynaptic $Ca_v2.1$ channels that mediate neurotransmitter release at the NMJ (Kaja et al., 2007b). This type of channel has been shown to co-localize with gangliosides in lipid rafts (Taverna et al., 2004), which may facilitate interaction. Furthermore, experiments using anti-ganglioside antibodies suggest a relationship between gangliosides and $Ca_v2.1$ channels (Nakatani et al., 2009; Ortiz et al., 2001). $Ca_v2.1$ channel behaviour is one important determinant of EPP rundown level at high intensity use of NMJs (Kaja et al., 2005). Another indication for possible modulation of $Ca_v2.1$ channels by gangliosides is the reduction of 25 ms paired-pulse facilitation, because this phenomenon of short term synaptic plasticity is highly dependent on intraterminal accumulation of Ca^{2+}, mainly determined by influx levels and intracellular buffering capacity. Increased Ca^{2+} influx following neuraminidase treatment has also been shown in cardiac myocytes, possibly through opening of "leak" channels (Marengo et al., 1998). Alternatively, the sialic acids of gangliosides adjacent to presynaptic $Ca_v2.1$ channels at the motor nerve terminal might act to locally sequester extracellular Ca^{2+}, making it available upon high intensity use of the synapse in order to enable sustained transmitter release.

Although we are not aware of any sialylated proteins important for presynaptic transmitter release at mouse NMJs, we cannot completely exclude that (part) of the effects of neuraminidase treatment on synaptic transmission at the dKO NMJs was due to desialylation of such putative sialoproteins. Sialic acid removal from hippocampal voltage-gated Na^+ channels has been shown to increase action potential firing threshold (Isaev et al., 2007). However, such an effect occurring in phrenic nerves of our nerve-muscle preparations would be irrelevant because of the supramaximal stimulation we applied.

In view of the rather modest magnitude of the effects of total ganglioside ablation on ACh release, in combination with the increased initial quantal content, compensating for the reduced release at high rate stimulation, it is not to be expected that successful neuromuscular transmission becomes endangered. Synaptic transmission at the NMJ has a large safety factor (Wood and Slater, 2001), and in line with this we observed a sustained tetanic muscle contraction upon 40 Hz nerve stimulation when visually inspecting dKO hemi-diaphragms directly after the neuraminidase treatment.

In conclusion, we show that gangliosides play a role in transmitter release at the NMJ, albeit a rather modest one. Although an indispensable role for gangliosides in neurotransmission was hypothesized previously, they apparently are not absolutely required. The almost normal ACh release levels observed earlier at NMJs of GM2s-KO, GD3s-KO and dKO mice (Bullens et al., 2002; Zitman et al., 2008; Zitman et al., 2009) are likely due to compensatory effects of the remaining subsets of gangliosides, or GM3 ganglioside alone, respectively. It must be noted that we here studied synaptic effects of *acute* ablation of the last remaining ganglioside at dKO NMJs and that it cannot be excluded that *long-term* overall ganglioside deficiency has more severe synaptic effects. Such effects may underlie the severe neurodegenerative symptoms and premature death of transgenic mice lacking both GM2synthase and GM3synthase, which have permanently blocked *in vivo* synthesis of all gangliosides (Yamashita et al., 2005). In humans it was found that a lack of synthesis of the major gangliosides, due to an autosomal recessive truncation mutation in the SIAT9 gene that encodes GM3-synthase, resulted in infantile-onset epilepsy associated with developmental stagnation and blindness (Simpson et al., 2004). The affected children show distorted reflexes and one case of startle myoclonus was observed. Neuromuscular transmission failure has not specifically been reported in these patients.