Neuromuscular synaptic transmission in aged ganglioside-deficient mice

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Abstract

Gangliosides are sialylated glycosphingolipids that are present in high density on neuronal membranes, especially at synapses, where they are assumed to play functional or modulating roles. Mice lacking GM2/GD2-synthase express only the simple gangliosides GD3 and GM3 and develop progressive motor behaviour deficits upon ageing, apparently due to failing complex ganglioside-dependent maintenance and/or repair processes or, alternatively, toxic GM3/GD3 accumulation. We investigated the function of neuromuscular junctions (NMJs) of aged (>9 months-old) GM2/GD2-synthase *null*-mutant mice, because synaptic dysfunction might develop with age and could potentially contribute to the late-onset motor phenotype. In addition, we studied NMJs of old mice lacking GD3-synthase (expressing only O- and a-series gangliosides), which do not show an overt neurological phenotype but may develop subclinical synaptic deficits. Detailed electrophysiological analyses showed subtle changes in presynaptic neurotransmitter release. Acetylcholine release at 40 Hz nerve stimulation at aged GM2/GD2-synthase *null*-mutant NMJs ran down slightly more pronounced than at wild-type NMJs, and spontaneous acetylcholine release rate at GD3-synthase *null*-mutant NMJs was somewhat higher than at wild-type, selectively at 25 °C bath temperature. Interestingly, we observed faster kinetics of postsynaptic electrophysiological responses at aged GD3-synthase *null*-mutant NMJs, not previously seen by us at NMJs of young GD3-synthase *null*-mutants or other types of (aged or young) ganglioside-deficient mice. These kinetic changes might reflect a change in postsynaptic acetylcholine receptor behaviour. Our data indicate that it is highly unlikely that transmission failure at NMJs contributes to the progressive motor defects of aged GM2/GD2-synthase *null*-mutants and that, despite some kinetic changes of synaptic signals, neuromuscular transmission remains successful in aged GD3-synthase *null*-mutant mice. Apparently, mutual redundancy of the different gangliosides in supporting presynaptic function, as observed previously by us in young mice, remains adequate upon ageing or, alternatively, gangliosides have only relatively little direct impact on neuromuscular synaptic function, even in aged mice.

Abbreviations

ACh, acetylcholine; AChR, acetylcholine receptor; EPP, endplate potential; GD3s-KO, α2,8-sialyltransferase knockout; GM2s-KO, β1,4-GalNAc-transferase knockout; MEPP, miniature endplate potential; NMJ, neuromuscular junction; WT, wild-type.

1. Introduction

Gangliosides are sialic acid-containing glycosphingolipids (Ledeen, 1985) that are enriched in neuronal membranes and, therefore, are thought to have neuron-specific functions, e.g. in modulation of membrane-bound enzymes and ion-channels, cell-adhesion, neuritogenesis and membrane stability (Hakomori, 2003; Hashiramoto et al., 2006; Ledeen and Wu, 2006; Salazar et al., 2004; Sohn et al., 2006; Susuki et al., 2007; Wu et al., 2007; Yates and Rampersaud, 1998). Furthermore, anti-ganglioside autoimmunity and ganglioside turnover deficits cause neurological disorders (Ang et al., 2004; Maegawa et al., 2006; Simpson et al., 2004; Willison and Yuki, 2002).

Gangliosides may be particularly important in synaptic function because they 1) show high expression at synapses (Ando et al., 2004; Waki et al., 1994); 2) co-localize with neuroexocytotic proteins (Chamberlain et al., 2001; Lang et al., 2001; Salaun et al., 2004; Taverna et al., 2004) and 3) influence neurotransmitter release and synaptic plasticity when applied exogenously (Ando et al., 1998; Egorushkina et al., 1993; Furuse et al., 1998; Ramirez et al., 1990; Tanaka et al., 1997; Wieraszko and Seifert, 1985). In spite of this, we recently showed near-normal synapse function at neuromuscular junctions (NMJs) of mice lacking either complex gangliosides, b/c-series gangliosides or all gangliosides except GM3 (Fig.1) (Bullens et al., 2002; Zitman et al., 2008), suggesting neurotransmission was supported by GM3 alone or, alternatively, is ganglioside-independent.

One reason for this ganglioside redundancy could be the relatively young age (5-15 weeks) of the mice we investigated. Specific neuronal ganglioside levels vary during embryogenesis but also in postnatal phases, especially during ageing (Ando, 1983; Aydin et al., 2000; Malisan and Testi, 2002; Ngamukote et al., 2007; Svennerholm et al., 1989; Yamamoto et al., 2008). Gangliosides seem to become increasingly important upon ageing, as late-onset and progressive histological and behavioural abnormalities develop in both GM2/GD2-synthase knockout (GM2s-KO) mice and compound *null*-mutant mice lacking GM2/GD2- and GD3-synthase. Young GM2s-KO mice (~3 months) show no phenotype (although they have histological signs of peripheral nerve degeneration (Sheikh et al., 1999), worsening upon ageing (Sugiura et al., 2005)). Eventually, starting at 8-16 months of age, these mice develop motor deficits, i.e. abnormalities in gait (Chiavegatto et al., 2000; Sugiura et al., 2005), balance, coordination, rearing and locomotion, whole-body tremor and catalepsy (Chiavegatto et al., 2000). The exact sequence of the development of these disturbances and the potential correlated neurodegenerative sites (i.e. sensory nerves, motor nerves or CNS) are not known yet. Spinal cord synaptic rearrangements occur in >10 weeks-old mice

(Sugiura et al., 2005). Young GD3-synthase knockout (GD3s-KO) mice have no phenotype (Kawai et al., 2001; Okada et al., 2002) but show impaired nerve regeneration (Okada et al., 2002). To our knowledge, they have not been studied at old age. Compound *null*-mutant mice are born normal but show sudden death (~50% survival at three (Kawai et al., 2001) or six months (Inoue et al., 2002), depending on strain) and get skin lesions at >6 months of age, presumably due to sensory nerve impairment (Inoue et al., 2002). Thus, these late-onset and progressive phenotypes may be due to a lack of the particular ganglioside sub-sets in these *null*-mutants. Alternatively, they might be caused by accumulation of the remaining ganglioside sub-sets, amounting to neurotoxic levels during ageing. Brains of GM2s-KO mice accumulate GM3 and GD3 ganglioside (Kawai et al., 2001; Takamiya et al., 1996), while those of GD3s-KO mice accumulate a-series gangliosides (Handa et al., 2005; Okada et al., 2002) and possibly also O-series gangliosides. Brains of compound *null*-mutants have increased GM3 expression (Inoue et al., 2002; Kawai et al., 2001). Our previous studies on pathophysiological effects of anti-ganglioside antibodies suggest that similar ganglioside accumulations also take place at NMJ motor nerve terminals of these mutants (Bullens et al., 2002; Goodfellow et al., 2005). The exact time course of these ganglioside accumulations in brain and NMJ are not known.

In view of these progressive behavioural and histological deficits, possibly involving progressive synaptic dysfunction due to either chronic lack of ganglioside subsets or toxic overexpression of the remaining sub-sets, we here investigated neurotransmission at NMJs of aged (i.e. >9 months-old) GM2s-KO and GD3s-KO mice.

2. Methods

2.1. Mice

GM2s-KO and GDs3-KO mice were bred and genotyped as described by Inoue *et al.* (Inoue et al., 2002). Both male and female homozygous KO and wild-type (WT) littermates were used at ages older than 9 months (range 9.5 to 17 months). Upon visual inspection, aged homozygous GM2s-KO mice showed impaired motor behavior (see Introduction), as described (Chiavegatto et al., 2000; Sugiura et al., 2005). The aged GD3s-KO mice showed no overt neurological phenotype. The body weight of the aged GD3s-KO mice was higher than that of the aged WT and GM2s-KO mice (47.1 ± 1.8, 41.5 ± 1.5 and 36.6 ± 1.4 g, respectively; $p<0.01$). Muscle strength was assessed with a grip strength meter as described before (Zitman et al., 2008). All animal experiments were carried out according to Dutch law and Leiden University guidelines.

2.2. In vitro electrophysiology

Mice were killed by CO_2 asphyxiation. Left and right hemi-diaphragms were dissected with their phrenic nerve attached and mounted in standard Ringer's medium (119 mM NaCl, 4.5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgSO_4$, 1 mM NaH_2PO_4, 23 mM $NaHCO_3$, 11 mM glucose, pH 7.4) at room temperature, pre-gassed with 95% O_2 / 5% CO_2.

Intracellular recordings of miniature endplate potentials (MEPPs) and endplate potentials (EPPs) in the NMJ were made using a glass micro-electrode (10-20 MΩ, filled with 3 M KCl) connected to a Geneclamp 500B (Axon Instruments / Molecular Devices, Union City, USA) for amplifying and filtering (10 kHz low-pass, 1 Hz high-pass) of the signal. Signals were digitized and analyzed using a Digidata 1322A digitizer, Clampex 9, Clampfit 9 (Axon instruments) and Mini Analysis 6.0.3 (Synaptosoft, Fort Lee, USA). At least 10 NMJs were sampled per muscle per experimental condition and at least 30 EPPs and/or 40 MEPPs were sampled per NMJ.

Muscle action potentials were eliminated by incubation with 3 μM of the selective skeletal muscle Na^+-channel blocker, μ-Conotoxin GIIIB (Scientific Marketing Associates, Barnet, Herts, UK). For recording of EPPs, the phrenic nerve was stimulated with supramaximal rectangular pulses of 0.1 ms width at 0.3 and 40 Hz, using a bipolar stimulation electrode. The mean EPP and MEPP amplitudes at each NMJ were normalized to a standard resting membrane of -75 mV, with the reversal potential for acetylcholine (ACh)-induced current assumed to be 0 mV (Magleby and Stevens, 1972) and using the following

formula: $(M)EPP_{normalized} = (M)EPP \times ((-75-V_{reversal})/V_m)$, where $V_{reversal}$ is the reversal potential for ACh and V_m is the measured resting membrane potential. In order to calculate the quantal content (i.e. the number of ACh quanta that are released upon a single nerve impulse), the normalized mean amplitude of EPPs evoked at low rate (0.3 Hz) were corrected for non-linear summation using the following formula: $EPP_{corrected} = EPP/(1-f(EPP/75))$, with f set to 0.8 (McLachlan and Martin, 1980). The normalized and corrected mean EPP amplitude was divided by the normalized mean MEPP amplitude. Temperature of the bath medium was controlled using a Peltier device placed around the recording bath and adjusted by varying the DC output of a power supply (Delta Elektronika, Zierikzee, The Netherlands). A miniature probe connected to a digital thermometer was used to monitor temperature, which was held at 24-26 °C, unless stated otherwise.

MEPPs were also recorded after addition of hypertonic medium (0.5 M sucrose Ringer's medium), in order to estimate the pool of ACh vesicles ready for immediate release (Stevens and Tsujimoto, 1995; Varoqueaux et al., 2005).

2.3. Statistical analysis

Data is presented as group mean ± SEM of the mean muscle values calculated from the mean NMJ values. Statistical differences were analyzed with an unpaired Student's *t*-test or an analysis of variance (ANOVA) with Bonferroni's post-hoc test.

3. Results

3.1. In vivo assessment of muscle strength

Grip strength testing showed similar muscle power of WT, GM2s-KO and GD3s-KO mice, when normalized for body weight (~3 g/g; p=0.13 ; Fig. 2A). The absolute force delivered by GD3s-KO mice was ~20% higher than that delivered by WT mice ($p<0.05$; Fig. 2B), paralleling the higher body weight of this mutant.

3.2. Spontaneous and evoked ACh release parameters

First we measured synaptic signals at NMJs under our standard conditions (2 mM extracellular Ca^{2+}, 25 °C). Each genotype group consisted of 6-11 mice. Spontaneous uniquantal transmitter release, measured as MEPP frequency, was 43% larger in GD3s-KO than in WT NMJs (1.91 ± 0.23 and 1.34 ± 0.09 s^{-1}, respectively, $p<0.05$; Fig. 3A). GM2s-KO MEPP frequency was 1.42 ± 0.11 s^{-1} and did not differ statistically significantly from the other genotype groups. MEPP amplitude in the GD3s-KO NMJ was ~25% larger than in WT or GM2s-KO (0.79 ± 0.05; 0.63 ± 0.03; 0.65 ± 0.02 mV, respectively; $p<0.05$; Fig. 3B, C). In addition, we found a ~30% shorter 10-90% rise time of MEPPs in the GD3s-KO mice, as compared to GM2s-KO and WT (0.72 ± 0.04, 1.01 ± 0.07 and 1.06 ± 0.05 ms, respectively, $p<0.01$) as well as a ~25% smaller halfwidth (2.29 ± 0.08, 2.92 ± 0.23 and 3.06 ± 0.15 ms, respectively, $p<0.05$). The amplitudes of EPPs evoked by 0.3 Hz stimulation were 22-24 mV for all groups (p=0.22; Fig 3D, E). The calculated quantal contents of 0.3 Hz EPPs were of similar value (~48) at all three genotype NMJs (p=0.23; Fig. 3F). However, the 10-90% rise time of GD3s-KO EPPs was shorter than that of WT and GM2s-KO (0.81 ± 0.05, 1.13 ± 0.07 and 1.11 ± 0.05 ms, respectively; $p<0.01$; Fig. 3G). Similarly, the width of the EPPs at 50% of their amplitude was smaller in GD3s-KO than WT, but not GM2s-KO NMJs (2.93 ± 0.11, 3.89 ± 0.14 and 3.49 ± 0.28 ms, respectively; $p<0.05$ GD3s-KO vs. WT; Fig. 3H). Example traces are shown in Fig. 3E. These kinetic differences were not observed previously in NMJs of younger GD3s-KO mice (Zitman et al., 2008). Upon high rate (40 Hz) stimulation, GM2s-KO EPPs ran down to a slightly, but statistically significantly ($p<0.05$), lower plateau level than the WT EPPs did (79.5 ± 1.0 and 83.2 ± 0.9 % of the first EPP in the train, respectively; Fig. 4). GD3s-KO EPPs ran down to 82.1 ± 0.8%.

Within the tested groups of old GM2s- and GD3s-KO mice we observed no clear correlation between the age of individual mice (which ranged from 9.5-17 months) and the

parameters that had shown statistically significant change compared with the WT group on group-average level (data not shown).

3.3. Hypertonic shock- and K^+-induced ACh release

Upon incubation of nerve-muscle preparations in hypertonic (0.5 M sucrose) Ringer's medium, MEPP frequency increased to similar levels of 50-60 s^{-1} for all groups (n=6 mice; p=0.16; Fig. 5A).

Cultured GM2s-KO neurons have been shown to degenerate upon K^+-induced depolarization, caused by an underlying impairment of cellular Ca^{2+} regulation (Wu et al., 2001). In order to investigate possible effects at the GM2s-KO NMJ, we measured MEPP frequency in extracellular K^+ concentrations varying from 4.5-30 mM and compared it to WT NMJs (n=3-7 mice per condition per genotype). As expected, the MEPP frequency steeply rose with increasing K^+ concentration, but we found no statistically significant differences between the genotypes (Fig. 5B).

3.4. Temperature-dependency of ACh release parameters

Gangliosides may be involved in thermal adaptation of neuronal membrane function (Rahmann et al., 1998). We, therefore, performed synaptic electrophysiological measurements in a further series of experiments at 17, 20, 30, and 35 °C (Fig. 6). Each aged genotype group consisted of 4-5 mice. At all temperatures tested, the MEPP amplitude at GD3s-KO NMJs tended to be somewhat higher at GD3s-KO NMJs than at GM2s-KO and WT NMJs, although this was only statistically significantly at 25 °C, as described above (Fig. 6A; $p<0.01$). The temperature dependence of spontaneous ACh release rate, i.e. MEPP frequency, seemed roughly similar at WT, GM2s-KO and GD3s-KO NMJs, although at the low end of the tested temperature range the MEPP frequency at WT NMJs was somewhat higher, compared to GM2s-KO (Fig. 6B; $p<0.05$). The amplitudes of EPPs evoked by 0.3 Hz nerve stimulation amplitudes tended to be somewhat higher at GD3s-KO NMJs at every temperature tested, although this reached no statistical significance (Fig. 6C). No differences in the calculated quantal contents were observed at any temperature tested (Fig. 6D). Rise times and halfwidths of GD3s-KO EPPs were consistently shorter than that of WT and GM2s-KO EPPs, but showed no different temperature dependence profile (Fig. 6E, F). No differences were observed between genotypes in rundown of EPPs at 3 Hz stimulation rate (Fig. 6G), while at 40 Hz the WT EPPs ran down slightly less pronounced than at

ganglioside-deficient NMJs, albeit only statistically significantly at 25 °C ($p<0.05$, vs. GM2s-KO) and 35 °C ($p<0.05$, vs. both GD3s-KO and GM2s-KO; Fig. 6H).

3.5. Ca^{2+}-dependency of ACh release parameters

Gangliosides have been suggested to play a role in the membrane flux of Ca^{2+} and its cellular homeostasis (Ledeen and Wu, 2006; Rahmann et al., 1998; Wu et al., 2001). Therefore, we explored synaptic transmission at NMJs from muscle preparations incubated at low (0.2 mM) and high (5 mM) extracellular Ca^{2+}, at 25 °C bath temperature. Genotypes showed no differences in MEPP amplitude at either 0.2 or 5 mM Ca^{2+} concentration (Fig. 7A), whereas at 2 mM Ca^{2+} it was somewhat higher at the GD3s-KO NMJ (see above). As expected, in view of the well-known dependence of neurotransmitter release on extracellular Ca^{2+}, MEPP frequency, EPP amplitude and quantal content of WT NMJs steeply depended on Ca^{2+}. No different Ca^{2+}-dependency of these parameters was observed at GM2s-KO NMJs. However, the dependency of GD3s-KO NMJs seemed less steep, as we found increased ACh release at 0.2 mM Ca^{2+}, compared to WT and GM2s-KO NMJs (Fig. 7B-D). EPP rundown levels at 40 Hz stimulation were not statistically significantly different between genotypes at 0.2 and 5 mM Ca^{2+} (Fig. 7E), whereas they differed somewhat at 2 mM Ca^{2+}, see above. Paired-pulse facilitation at 25 ms stimulation interval was not different between genotypes at any of the Ca^{2+} concentrations tested. Its values, expressed as percentage increase of the second EPP, compared to the first, were about 25, 2.5 and -0.5% at 0.2, 2, and 5 mM Ca^{2+}, respectively, for all genotypes.

As observed at 2 mM Ca^{2+} (see above), the rise time and halfwidth of EPPs were shorter at GD3s-KO than at WT and GM2s-KO NMJs at both 0.2 and 5 mM Ca^{2+} (although only statistically significantly at 5 mM Ca^{2+}, Fig. 7F,G). The extent of this difference, however, was similar at all Ca^{2+} concentrations.

4. Discussion

We performed a detailed electrophysiological characterization of ACh release parameters at NMJs of aged GM2s-KO and GD3s-KO mice. This included high and low-rate nerve stimulation-evoked release, spontaneous release and the release evoked by hypertonic medium or elevated extracellular K^+. However, neuromuscular synaptic transmission of both the aged *null*-mutant mice showed remarkably little change compared to the WT controls. From the evoked release parameters under standard conditions (25 °C, 2 mM extracellular Ca^{2+}), only the ACh release at 40 Hz nerve stimulation at aged GM2s-KO NMJs was found to be running down more pronouncedly than at WT NMJs. However, the extent of this difference was only small (EPPs ran down to a plateau level of 79.5% of the first EPP, compared to 83.2% at WT NMJs). In view of the presence of a large safety factor in neuromuscular transmission (Wood and Slater, 2001), such a small reduction will most likely not threat successful transmission and cause muscle weakness. This was confirmed in testing the aged GM2s-KO mice with a grip strength meter, where they pulled equal forces as WT controls. Thus, the motor coordination defects observed in aged GM2s-KO mice (Chiavegatto et al., 2000; Sugiura et al., 2005), and also observed by us in the mice used here, do not contain a muscle weakness component due to neuromuscular transmission failure. Therefore, they are likely attributable to progressive dysfunction of the peripheral sensory or proximal motor nerves and/or possibly the CNS, caused by chronic lack of complex gangliosides or, alternatively, by upregulation of GM3 and GD3 ganglioside to toxic levels. Neurotransmission at central synapses has no safety factor and, consequently, small changes in transmitter release as observed here at the NMJ already may have pronounced effects on neuronal network function in the CNS.

Earlier we have also observed some extra rundown of high-rate EPPs at NMJs of young GM2s-KO mice, but only at higher bath temperatures (30-35°C) tested and at high (5 mM) extracellular Ca^{2+} concentration (Bullens et al., 2002). Furthermore, GD3s-GM2s compound *null*-mutant NMJs showed some degree of extra EPP rundown, at all temperature and Ca^{2+} conditions tested (Zitman et al., 2008). However, at GD3s-KO NMJs, either from young or aged mice, we never observed extra EPP rundown compared to WT, except for the 35 °C temperature condition at aged NMJs. Together these data suggest that O- and/or a-series gangliosides may support the fine tuning of high-rate transmitter release. In particular, GM1 ganglioside may be important, because it has been shown to influence cellular Ca^{2+} membrane flux and homeostasis (Wu et al., 2004; Wu et al., 1990). Perhaps this involves

inhibition of voltage-gated Ca^{2+}-channel inactivation, one important factor in high-rate transmitter release. Another relevant factor, the size of the pool of synaptic vesicles ready for release, is not likely to be influenced by gangliosides, because we did not find differences between genotypes when we assessed for this parameter with hypertonic stimulation (Stevens and Tsujimoto, 1995; Varoqueaux et al., 2005), either here at aged NMJs or previously in younger ones (Zitman et al., 2008). The less steep relationship between extracellular Ca^{2+} and evoked ACh release observed at NMJs of old GD3s-KO mice (and seen previously in young ones) may also be due to altered Ca^{2+} buffering or membrane flux, possibly resulting from a progressive increase in GM1 density due to accumulation of O- and/or a-series gangliosides (Handa et al., 2005; Okada et al., 2002). This may also explain the higher MEPP frequency at GD3s-KO NMJs in 0.2 and 2 mM extracellular Ca^{2+}.

At aged GD3s-KO NMJs we observed decreased rise times and halfwidths of both EPPs and MEPPs, not previously seen at NMJs of young GD3s-KO or any of the other ganglioside-deficient mice. These kinetic changes may reflect a change in the behaviour of postsynaptic ACh receptors (AChRs). Gangliosides are present in the vicinity of AChRs (Pato et al., 2008) and can interact with the protein, as shown for AChR-rich membrane from *Torpedo marmorata* (Mantipragada et al., 2003). Accumulation of O- and/or a-series gangliosides may occur at muscle membranes of GD3s-KO mice, as it does at neuronal membranes (Handa et al., 2005; Okada et al., 2002), and progressiveness of this process may lead to the observed age-specific changes in EPP kinetics. EPP rise time and halfwidth are highly temperature-dependent parameters (Fig. 6E, F) and the progressive accumulation of O- and/or a-series gangliosides may induce a shift in this dependency by progressively altering the membrane fluidity and charge density in the vicinity of the AChRs in a similar way that gangliosides have been shown to influence artificial voltage-gated ion channels in lipid bilayers in a temperature-dependent fashion (Kappel et al., 2000). The small increase in MEPP amplitude at aged GD3s-KO, specifically at 25 °C and not observed at young GD3s-KO NMJs, may possibly have followed from an influence by gangliosides on ion permeation through AChRs (Barrantes, 1989), progressively changing due to O- and/or a-series gangliosides accumulation. Alternatively, the increased MEPP amplitude may be due to reduced activity of cholinesterase in the synaptic cleft, inhibiting ACh breakdown. However, in that case one would expect an accompanying MEPP and EPP broadening (due to a prolonged action of ACh, as shown in many electrophysiological experiments using cholinesterase inhibitors, see e.g. (Fiekers, 1985; Maselli and Leung, 1993)), but this was not found here. Instead, we

observed faster MEPPs and EPPs. These might have resulted from *increased* cholinesterase activity, but this would in turn be expected to be associated with reduced amplitude, which was not the case. Furthermore, to our knowledge, no direct relationship between gangliosides and cholinesterase has been reported in the literature. These considerations make changes in cholinesterase activity a more distant possibility. A further alternative explanation for changes of (M)EPP kinetics may be that O- and/or a-series accumulation influenced the passive electrical characteristics of muscle membrane (i.e. reduced capacitance and increased input resistance). A presynaptic explanation for the changed EPP and MEPP kinetics and quantal size (e.g. increased filling of ACh vesicles) is less likely because gangliosides have been reported to lack effect on ACh synthesis (Tanaka et al., 1997). On the basis of the only relatively small magnitude of the observed kinetic changes of the EPPs and MEPPs in aged GD3s-KO mice, and the EPP amplitude being somewhat increased compared to wild-type, it is not to be expected that successful neuromuscular transmission is endangered. Absence of muscle weakness in grip strength measurements and the observed absence of any overt neurological symptoms in the aged GD3s-KO mice is in agreement with this view.

GM2s-KO cerebellar neurons have been shown to degenerate when cultured under K^+-induced depolarization, caused by an underlying impairment of cellular Ca^{2+} regulation (Wu et al., 2001). We tested in acute experiments whether a similar effect occurs at aged GM2s-KO motor nerve terminals by measuring the increase in MEPP frequency evoked by increased extracellular K^+ concentrations. However, no differences were observed between GM2s-KO and WT NMJs, indicating no major differences between aged GM2s-KO and WT motor nerve terminals in influx or intracellular buffering of Ca^{2+}.

In conclusion, the investigated aged ganglioside-deficient mice show remarkable little change in presynaptic neuromuscular function, compared to age-matched WT controls. Apparently, the mutual redundancy of the different gangliosides in supporting synaptic function, as observed in young mice (Zitman et al., 2008), remains adequate even upon ageing, when there is likely an increased demand for (gangliosides-dependent) maintenance and repair processes. Alternatively, gangliosides may have relatively little direct impact on neuromuscular synaptic function, even at old age. The observation of fast EPP and MEPP kinetics at GD3s-KO NMJs is interesting and indicates a role for O- and/or a-series gangliosides in AChR function, which deserves more detailed study. Finally, in view of the only slightly reduced 40 Hz nerve stimulation-evoked ACh release at NMJs of aged GM2s-KO mice, it is highly unlikely that NMJ dysfunction contributes to the age-related motor defects of GM2s-KO mice.

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Disclosure statement

The authors reported no conflicts of interest.

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Figure legends

Figure 1. Gangliosides synthesis scheme.

Ganglioside nomenclature is according to Svennerholm (Svennerholm, 1994). Membranes of WT mice contain all gangliosides. GM2s-KO mice lack complex gangliosides (light gray rectangle); GD3s-KO mice lack b- and c-series gangliosides (dark grey rectangle).

Figure 2. Grip strength measurements.

Grip strength was averaged for ten trials (n=6-8 mice). **A.** Pulling force normalized to the body weight of each mice; all groups pulled ~3 g per g body weight. **B.** Absolute pulling values show increased grip strength in GD3s-KO vs. WT mice ($p<0.05$), paralleling their higher body weight.

Figure 3. Basic synaptic parameters at the NMJ.

In vitro intracellular electrophysiological measurements at 25°C in diaphragm-phrenic nerve preparations. Each group consisted of at least 6 mice; at least 10 NMJs were sampled per muscle. **A.** MEPP frequency was higher in the GD3s-KO NMJs compared to WT. **B.** MEPP amplitude was larger at GD3s-KO NMJs. **C.** Typical traces of MEPPs. **D.** EPP amplitude was similar at each genotype. **E.** EPP example traces for WT, GM2s-KO and GD3s-KO mice. Black triangles indicate moment of phrenic nerve stimulation. **F.** Calculated quantal content at 0.3 Hz nerve stimulation was similar across genotypes. **G.** GD3s-KO mice exhibit significantly shorter 10-90% rise time. **H.** EPP halfwidth at GD3s-KO NMJs was shorter than WT.

*$p<0.05$, **$p<0.01$

Figure 4. High-rate nerve stimulation-evoked ACh release is reduced at GM2s-KO NMJs.

A. EPP amplitude rundown profiles at 40 Hz nerve stimulation (n=6-11 mice per group). Each EPP amplitude is expressed as percentage of the amplitude of the first EPP. EPPs at GM2s-KO NMJs showed increased rundown, compared to WT ($^{\#}p<0.05$). **B.** EPP rundown level (the mean amplitude of the 21^{st}-35^{th} EPP, expressed as percentage of the amplitude of the first EPP in the train, was lower at GM2s-KO NMJs, compared to WT (* $p<0.05$). **C.** Typical 40 Hz nerve stimulation EPP trains.

Figure 5. Hypertonic shock- and K^+-induced ACh release is unchanged.
A. The levels of ACh release induced by hypertonic medium (0.5 mM sucrose-Ringer's) were not different between genotypes (n=6 mice). **B.** No differences in K^+-MEPP frequency concentration-effect relationship between genotypes (n=3-7 mice).

Figure 6. Effect of temperature on synaptic transmission.
Electrophysiological analysis of ACh release at diaphragm NMJs at different bath temperatures. Each genotype group consisted of 4-5 mice. **A.** MEPP amplitude at GD3s-KO mice was larger than at GM2s-KO and WT NMJs, only at 25 °C. **B.** Spontaneous ACh release, measured as MEPP frequency, was lower at GM2s-KO than WT NMJs at 17°C ($p<0.05$), and higher at GD3s-KO compared to WT NMJs at 25°C. **C.** No differences in temperature dependency between genotypes of EPP amplitude at 0.3 Hz nerve stimulation. **D.** The temperature dependency of quantal content at 0.3 Hz nerve stimulation was similar across genotypes. **E.** GD3s-KO NMJs showed equal reductions of rise time at all temperatures tested. **F.** Halfwidths of EPPs at GD3s-KO NMJs were shorter than at WT and GM2s-KO NMJs at temperatures >20 °C. **G.** No changes in temperature dependency of EPP amplitude rundown level at 3 Hz stimulation. **H.** EPP amplitude rundown level at 40Hz nerve stimulation. GM2s-KO rundown was somewhat more pronounced that WT at 25 °C and GD3s-KO rundown was more pronounced than at WT at 35 °C.
* $p<0.05$, ** $p<0.01$, WT vs. GM2s-KO and GD3s-KO; † $p<0.05$, GD3s-KO vs .GM2s-KO; # $p<0.05$, GD3s-KO vs. WT; ‡ $p<0.05$, GM2s-KO vs. WT.

Figure 7. Effect of extracellular Ca^{2+} concentration on synaptic transmission.
Electrophysiological measurements were performed in Ringer's medium at 25°C containing either 0.2, 2 or 5 mM Ca^{2+}. N=6-11 mice per group. **A.** MEPP amplitude at GD3s-KO NMJs was larger than at GD3s-KO and WT NMJs at 2 mM Ca^{2+}. **B.** Spontaneous ACh release, measured as MEPP frequency, was higher at GD3s-KO NMJs compared to the other genotypes at 0.2 mM Ca^{2+} and higher than WT at 2 mM. **C.** EPP amplitude was increased at GD3s-KO NMJs at low Ca^{2+} conditions and was lower than WT in high Ca^{2+}. **D.** Quantal content at 0.3 Hz nerve stimulation in 0.2 mM Ca^{2+} was higher at GD3s-KO NMJs. **E.** EPP rundown level was only somewhat lower for GM2s-KO in 2 mM Ca^{2+}. **F.** GD3s-KO mice exhibit smaller EPP rise times at 2 and 5 mM Ca^{2+}. **G.** Similarly, halfwidths of GD3s-KO EPPs were reduced

* p<0.05, ** p<0.01, WT vs. GM2s-KO and GD3s-KO; † p<0.05, †† p<0.01, GD3s-KO vs. GM2s-KO; # p<0.05, GD3s-KO vs. WT; ‡ p<0.05, GM2s-KO vs. WT.

Figure 1, Zitman et al.

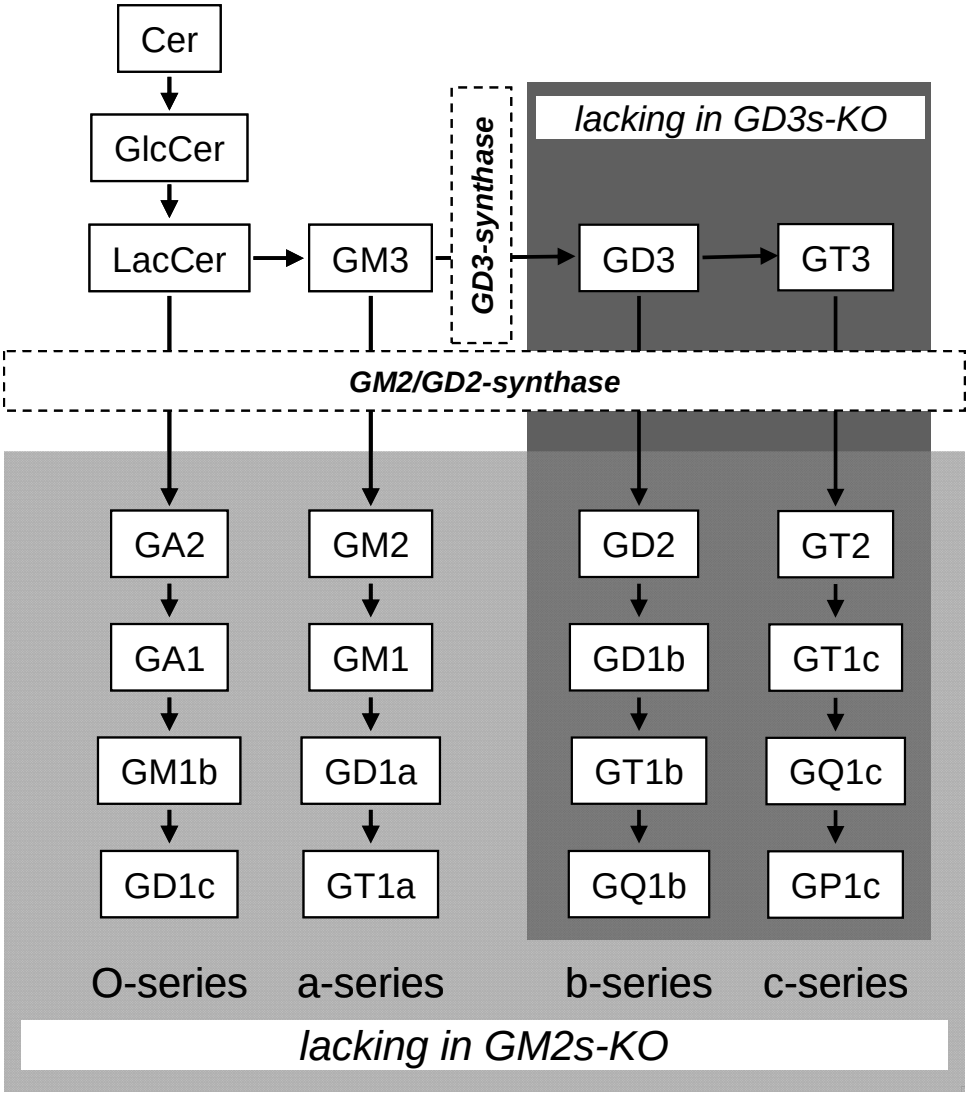

Figure 2, Zitman et al.

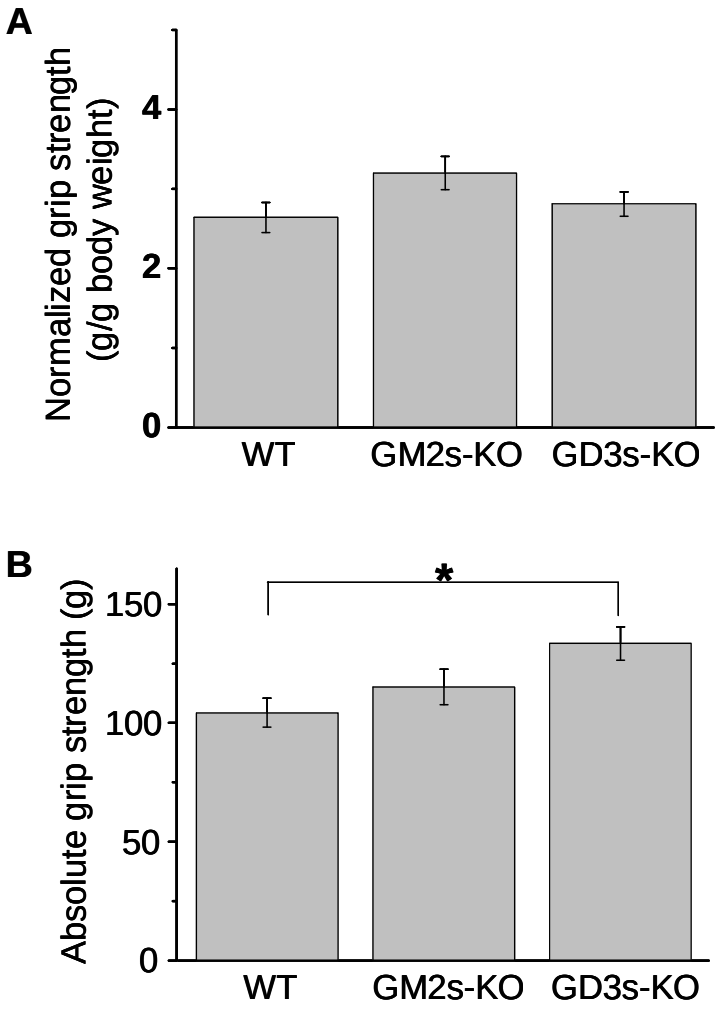

Figure 3, Zitman et al.

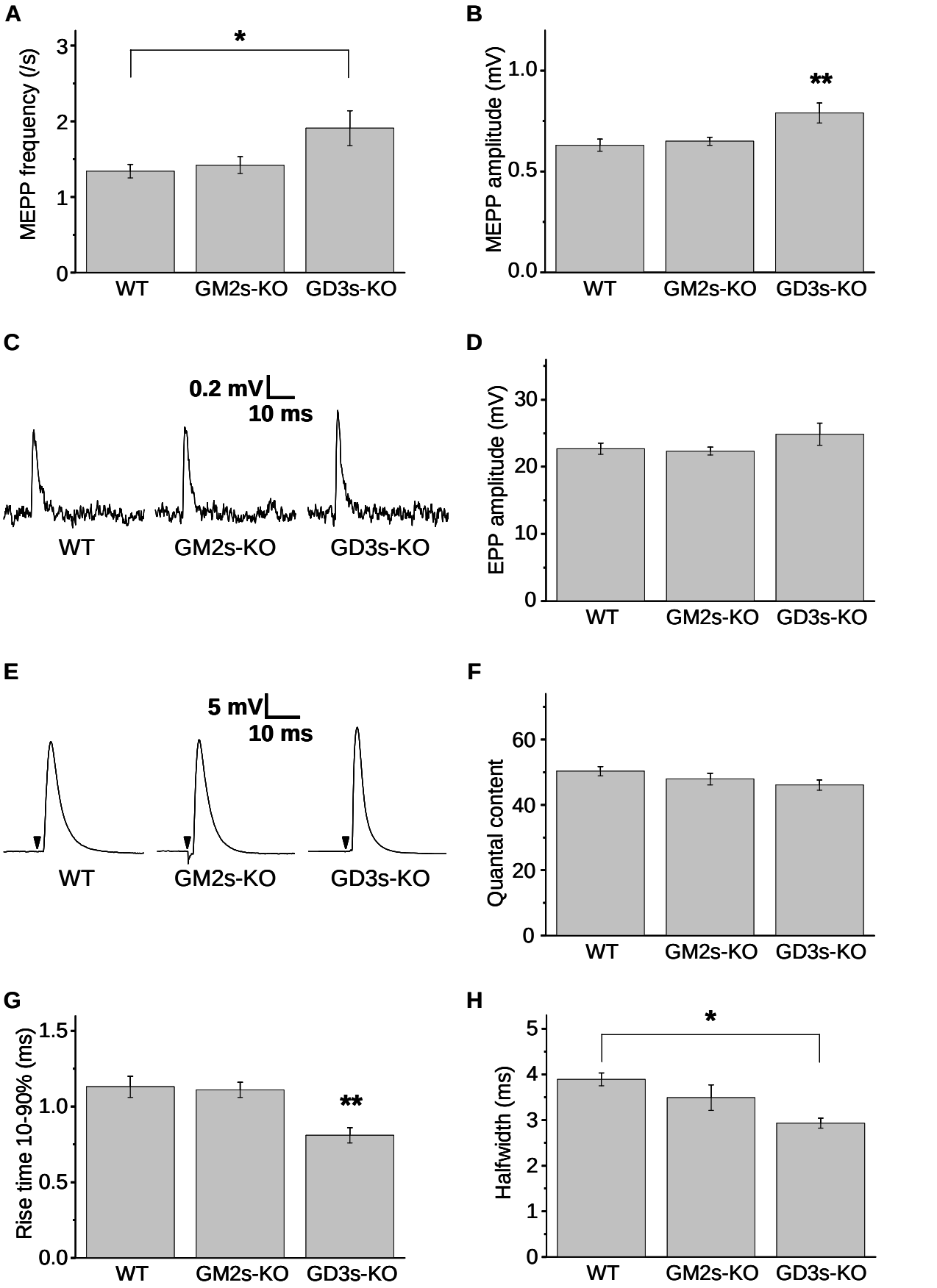

Figure 4, Zitman et al.

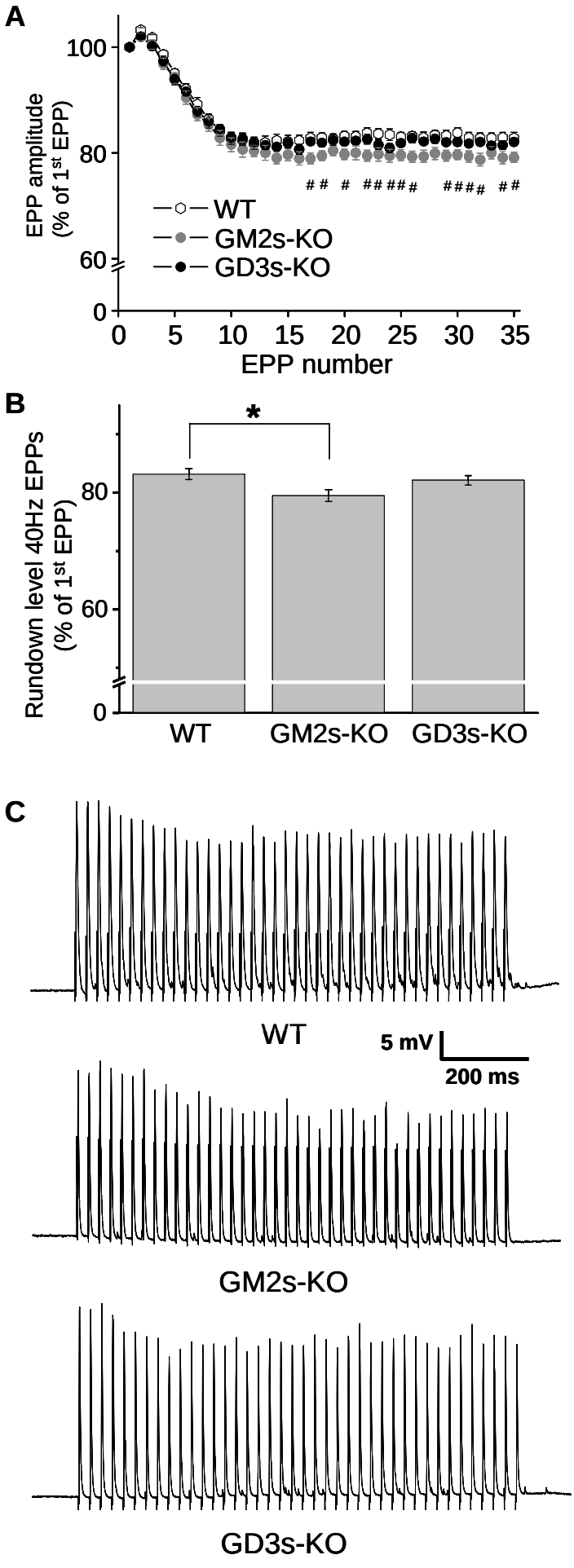

Figure 5, Zitman et al.

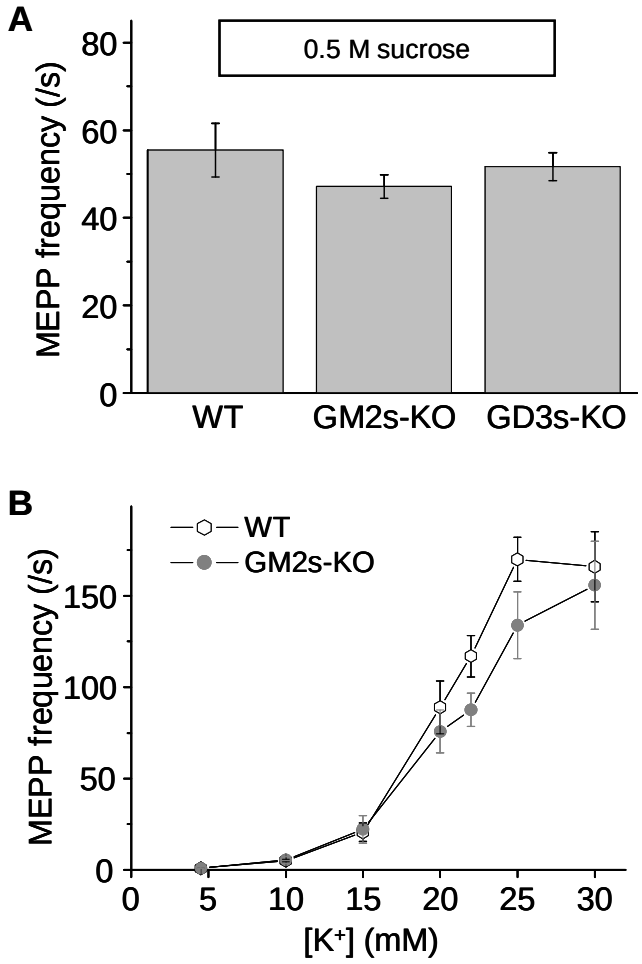

Figure 6, Zitman et al.

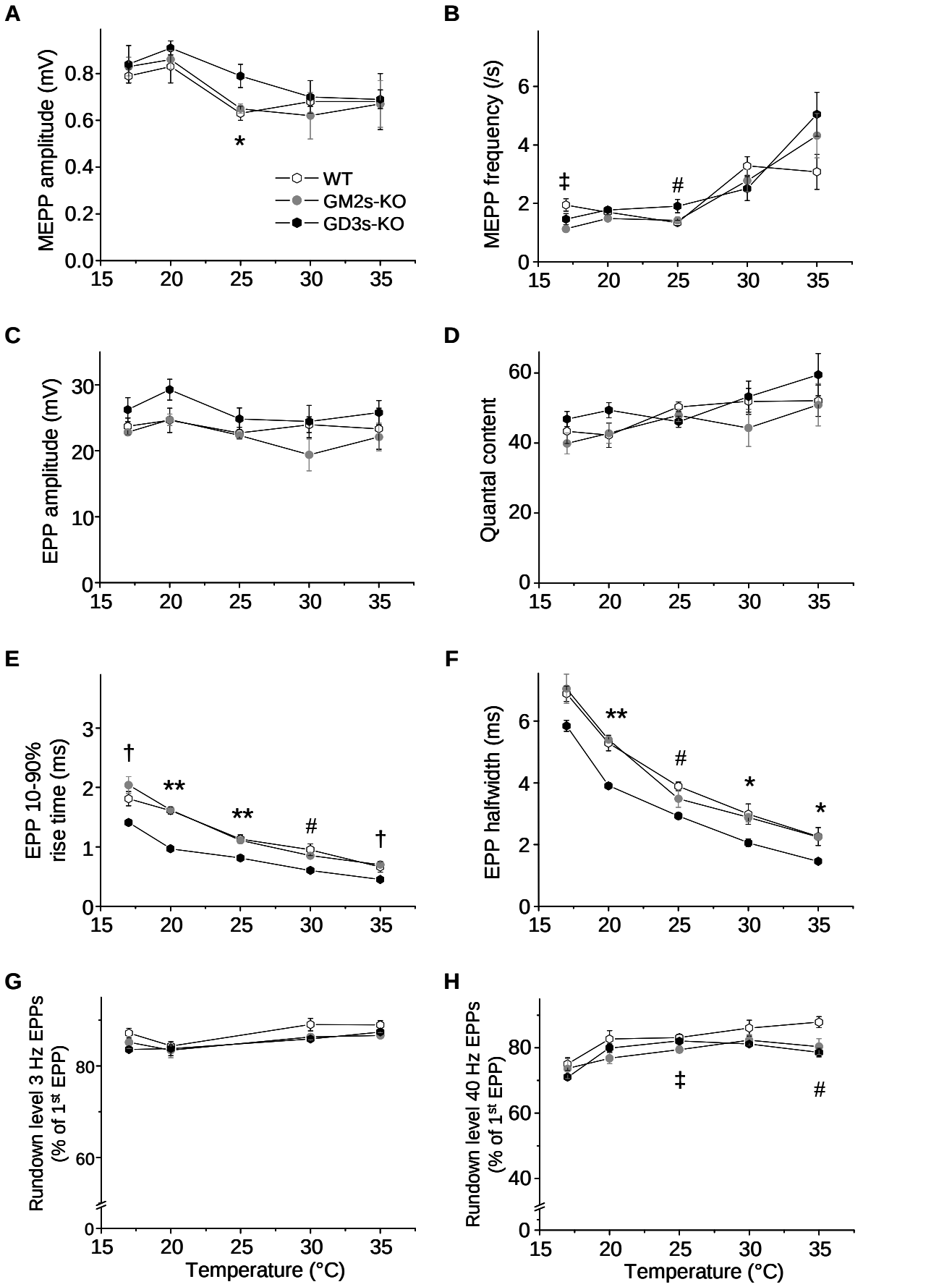

Figure 7, Zitman et al.