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Citation

Kaja, S. (2007, February 6). *Synaptic effects of mutations in neuronal Cav2.1 calcium channels*. Retrieved from <https://hdl.handle.net/1887/9750>

Version: Corrected Publisher's Version

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Note: To cite this publication please use the final published version (if applicable).

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No Direct Effect of Topiramate on Wild-Type and Mutant Ca_v2.1 Channels Mediating Acetylcholine Release at Mouse Neuromuscular Synapses

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To be submitted

Abstract

Topiramate (TPM) is an anti-convulsant drug that has recently been used successfully in migraine prophylaxis. However, the mode of action of TPM has not yet been elucidated. Several reports have suggested that neuronal voltage-gated Ca^{2+} (Ca_v) channels can be modulated by TPM. Neuronal $\text{Ca}_v2.1$ (P/Q-type) channels are involved in presynaptic neurotransmitter release and were found to be mutated in familial hemiplegic migraine type 1 (FHM1). We could previously show that mutations in *Cacana1a*-encoded $\text{Ca}_v2.1$ channels in FHM1 R192Q knock-in mice and in *Tottering* mice (*Tg*, P601L mutation) cause abnormalities in acetylcholine (ACh) release at the neuromuscular junction (NMJ). Here, we tested whether TPM has a direct modulating effect on the function of (mutant) $\text{Ca}_v2.1$ channels. However, application of a high concentration (50 μM) TPM did not affect $\text{Ca}_v2.1$ mediated ACh release at *ex vivo* NMJs of wild-type, R192Q KI and *Tg* mice. Our studies indicate that TPM lacks a direct acute effect on (mutant) presynaptic $\text{Ca}_v2.1$ channels.

Acknowledgments

Topiramate was a gift of Dr. Richard Shank (Johnson & Johnson). This work was supported by grants from the Prinses Beatrix Fonds (#MAR01-0105, to JJP), the Hersenstichting Nederland (#9F01(2).24, to JJP), the KNAW van Leersumfonds (to JJP), the European Community (EUROHEAD, LSHM-CT-2004-504837, to MDF), Johnson & Johnson Pharmaceutical Inc., and the Netherlands Organisation for Scientific Research, NWO (VICI 918.56.602, to MDF).

Introduction

Topiramate (TPM) was introduced about a decade ago as novel anti-epilepsy drug (Shank et al., 1994), and many research efforts have focused on the elucidation of its pharmacotherapeutic mechanism. Shown to exert effects on voltage-gated Na^+ channels, to enhance GABA-evoked and to inhibit kainite-evoked currents, TPM also possesses weak carbonic anhydrase activity that lowers systemic pH and may contribute indirectly to its beneficial action by influencing pH-dependent aspects of ion channel behaviour (reviewed in Shank et al., 2000; White, 2005). There have been conflicting reports on whether one of the mechanisms of action is a direct effect on the function of voltage-gated Ca^{2+} (Ca_v) channels. In dentate granule cells, TPM has been shown to reduce Ca_v1 (L-type) currents, and to transiently increase non- Ca_v1 currents (Zhang et al., 2000). Qian and Noebels (2003) reported a lack of effect of TPM on presynaptic Ca^{2+} influx at hippocampal synapse. Two reports have demonstrated a direct inhibiting action of 10–50 μM TPM on $\text{Ca}_v2.3$ (R-type) channels, in both heterologous expression systems and hippocampal slice preparations (McNaughton et al., 2004; Kuzmiski et al., 2005).

TPM has recently become employed in migraine prophylaxis (for reviews, see D'Amico et al., 2005; White, 2005), following the results of three double-blind placebo-controlled multi-center studies that reported that TPM is effective in reducing migraine frequency (Diener et al., 2004; Silberstein et al., 2004; Brandes et al., 2004). Furthermore, long-term TPM treatment has been shown to successfully suppress cortical spreading depression, the mechanism considered to underlie migraine aura, in a rat *in vivo* experimental model (Ayata et al., 2006).

CACNA1A, the gene encoding the $\text{Ca}_v2.1$ (P/Q-type) pore forming subunit has been found mutated in familial hemiplegic migraine type 1 (FHM1) (Ophoff et al., 1996; Plomp et al., 2001). $\text{Ca}_v2.1$ channel dysfunction may also play a role in typical migraine, but this is thus far unclear (Terwindt et al., 2001; Kirchmann et al., 2006). We have recently described the generation of a knock-in (KI) mouse model for FHM1, carrying the R192Q mutation in *Cacna1a*-encoded $\text{Ca}_v2.1$ channels (Van den Maagdenberg et al., 2004). We used the peripheral neuromuscular junction (NMJ), where acetylcholine (ACh) release is exclusively mediated by $\text{Ca}_v2.1$ channels, to study the synaptic consequences and identified increases in ACh release, indicating increased Ca^{2+} influx through R192Q-mutated channels (Van den Maagdenberg et al., 2004; Kaja et al., 2005). Similar findings were obtained at NMJs of natural mutant Tottering (*Tg*) mice, which carry a P601L single amino-acid change in their $\text{Ca}_v2.1$ channels (Fletcher et al., 1996; Plomp et al., 2000; Kaja et al., 2006). However, homozygous *Tg* mice show some compensatory presynaptic $\text{Ca}_v2.3$ channel expression at the NMJ, not present in R192Q KI mice (Kaja et al., 2005; Kaja et al., 2006).

In order to investigate whether TPM acts directly on wild-type or mutated $\text{Ca}_v2.1$ Ca^{2+} channels we measured ACh release before and following the acute application of a therapeutically relevant concentration of 50 μM TPM at NMJs of wild-type, R192Q KI and *Tg*-mutated mice.

Materials and Methods

Mice

All animal experiments were in accordance with national legislation, Leiden University guidelines and the Guiding Principles in the Care and Use of Animals approved by The Japanese Pharmacological Society.

R192Q KI mice were raised from original breeder pairs generated in our laboratory and genotyped as described previously (Van den Maagdenberg et al., 2004). *Tg* mice were raised from original breeder pairs obtained from Jackson Laboratories (Bar Harbor, ME, USA).

Mice were genotyped as described previously (Plomp et al., 2000). Male and female homozygous R192Q KI and *Tg* mice together with their respective wild-type controls (littermates wherever possible) were used at ~3 months of age, with the investigator blinded for genotype. As both *Tg* and R192Q KI mice were maintained on the same BL6/C57J background, data from wild-type mice of either background has been pooled.

Ex vivo NMJ electrophysiology

Mice were euthanized by carbon dioxide inhalation. Hemi-diaphragms with phrenic nerve were dissected and kept in Ringer's medium (in mM: NaCl 116, KCl 4.5, CaCl₂ 2, MgSO₄ 1, NaH₂PO₄ 1, NaHCO₃ 23, glucose 11, pH 7.4) at room temperature and continuously bubbled with 95% O₂ / 5% CO₂. Intracellular recordings of miniature endplate potentials (MEPPs) and nerve stimulation-evoked endplate potentials (EPPs) were made at NMJs at 28 °C using standard micro-electrode equipment. At least 30 MEPPs and EPPs were recorded at each NMJ, and 5-15 NMJs were sampled per experimental condition per muscle. Muscle action potentials were blocked by 3 μ M μ -conotoxin-GIIB (Scientific Marketing Associates, Barnet, UK). For EPP recording, the phrenic nerve was electrically stimulated at 0.3 or 40 Hz. Procedures for analysis of MEPPs and EPPs and calculation of quantal contents, i.e. the number of ACh quanta released per nerve impulse, have been described before (Van den Maagdenberg et al., 2004).

To test for possible effects of TPM on ACh release, EPPs and MEPPs were recorded in the presence of 50 μ M TPM, following a one-hour pre-incubation with the drug. In some experiments, we measured synaptic signals under slightly depolarizing conditions (10 mM K⁺ in the Ringer's medium).

Statistical analysis

Paired or unpaired Student's t-tests were used where appropriate, on grand mean values with *n* as the number of mice tested, and 5-15 NMJs tested per muscle, per condition. *P* < 0.05 was considered to be statistically significant. Data is presented as mean $\pm$ S.E.M.

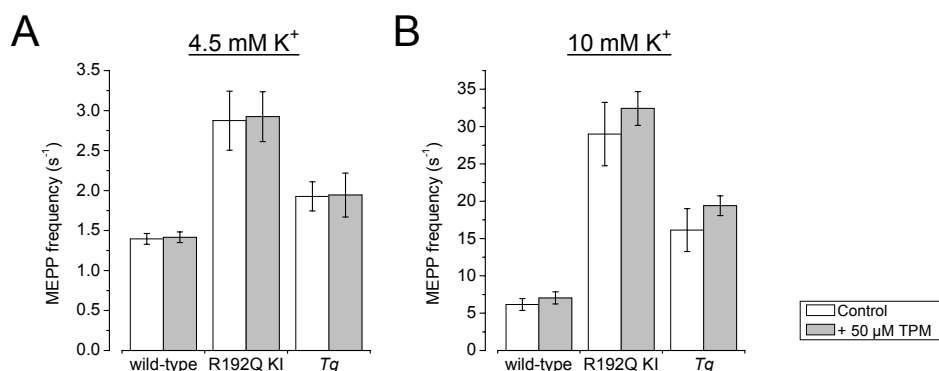


Figure 1. Lack of effect of TPM on spontaneous, unquantal ACh release at wild-type and R192Q- and *Tg*-mutant NMJs. (A) MEPP frequency was measured using standard microelectrode equipment before and in the presence of 50 μ M TPM. The drug did not affect MEPP frequency at NMJs of wild-type (*n*=13, *p*=0.80), FHM1 R192Q KI (*n*=8, *p*=0.76) or *Tg* mice (*n*=9, *p*=0.89). (B) Spontaneous release increases over-proportionally at both R192Q KI and *Tg* NMJs under mildly depolarizing conditions (10 mM K⁺) (Plomp et al., 2000; Van den Maagdenberg et al., 2004). Subsequent application of 50 μ M TPM did not affect MEPP frequency at wild-type (*n*=14, *p*=0.37), FHM1 R192Q KI (*n*=7, *p*=0.49) or *Tg* NMJs (*n*=8, *p*=0.34).

Results

TPM does not affect spontaneous ACh release

We first measured spontaneous unquantal release (MEPP frequency) from motor-nerve terminals of wild-type, R192Q KI and *Tg* mice (Figure 1A). MEPP frequencies were $1.40 \pm 0.07 \text{ s}^{-1}$ ($n=13$), $2.87 \pm 0.31 \text{ s}^{-1}$ ($n=8$) and $1.93 \pm 0.18 \text{ s}^{-1}$ ($n=9$), respectively, in line with previous observations of increased MEPP frequency at R192Q- and *Tg*-mutated NMJs (Plomp et al., 2000; Van den Maagdenberg et al., 2004). MEPP frequencies remained unaffected by the application of 50 μM TPM to the preparation (Figure 1A). Following a one-hour incubation with the drug, frequencies were $1.42 \pm 0.07 \text{ s}^{-1}$ at wild-type ($n=13$, $p=0.80$), $2.92 \pm 0.32 \text{ s}^{-1}$ at R192Q KI ($n=8$, $p=0.76$) and $1.94 \pm 0.27 \text{ s}^{-1}$ at *Tg* NMJs ($n=9$, $p=0.89$). All MEPP amplitudes were unaffected by TPM (Table 1).

Under mildly depolarizing conditions (10 mM K^+) spontaneous ACh release increases over-proportionally at both R192Q KI and *Tg* NMJs (Plomp et al., 2000; Van den Maagdenberg et al., 2004). Application of 50 μM TPM under this condition did not affect MEPP frequencies (Figure 1B). The values for wild-type were 6.2 ± 0.8 and $7.1 \pm 0.8 \text{ s}^{-1}$ without and in the presence of TPM, respectively ($n=14$, $p=0.37$). At R192Q synapses, MEPP frequency was $29.0 \pm 4.3 \text{ s}^{-1}$ before, and $32.4 \pm 2.3 \text{ s}^{-1}$ in the presence of TPM ($n=7$, $p=0.49$). Similarly, *Tg* MEPP frequency was 16.1 ± 2.9 and $19.4 \pm 1.3 \text{ s}^{-1}$ before and after addition of TPM, respectively ($n=8$, $p=0.34$).

Parameter	Genotype	Control	+ 50 μM TPM	p
MEPP amplitude (mV)	wild-type	0.95 ± 0.02	0.98 ± 0.03	0.28
	R192Q KI	0.96 ± 0.08	1.00 ± 0.05	0.29
	<i>Tg</i>	1.18 ± 0.08	1.17 ± 0.05	0.65
0.3 Hz EPP amplitude (mV)	wild-type	24.5 ± 0.7	25.7 ± 0.8	0.12
	R192Q KI	25.0 ± 0.9	26.1 ± 1.3	0.35
	<i>Tg</i>	25.9 ± 1.2	25.7 ± 1.1	0.89
40 Hz EPP amplitude rundown level (% first EPP)	wild-type	82.3 ± 0.9	82.2 ± 0.9	0.93
	R192Q KI	81.8 ± 0.7	80.5 ± 1.5	0.26
	<i>Tg</i>	77.9 ± 0.6	79.2 ± 0.6	0.34

Table 1. No effect of TPM on MEPP and EPP amplitudes.

Quantal size, measured as MEPP amplitude was not affected by 50 μM TPM. Similarly, rise and decay kinetics of MEPPs remained unaltered (data not shown). TPM did not change nerve-stimulation (0.3, 40 Hz) evoked EPPs at either genotype. The 40 Hz EPP rundown level was determined as the mean amplitude of the 21st to the 35th EPP expressed as percentage of the amplitude of the first EPP of the train.

Nerve stimulation-evoked ACh release is unaffected by TPM

We then measured 0.3 Hz nerve stimulation-evoked ACh release at NMJs (Figure 2A, Table 1). EPP amplitudes of all genotypes were unchanged by 50 μM TPM (Figure 2B, Table 1). Consequently, the quantal content at wild-type NMJs was 37.8 ± 1.2 and 39.7 ± 1.3 before and during 50 μM TPM, respectively ($n=13$, $p=0.14$). At R192Q KI NMJs, quantal content was 40.1 ± 3.2 and 39.6 ± 2.5 before and during TPM, respectively ($n=8$, $p=0.54$). The 0.3 Hz-evoked ACh release in *Tg* mice was similarly unaffected, with quantal contents 34.9 ± 2.4 before and 33.1 ± 1.2 in the presence of TPM ($n=5$, $p=0.13$).

Some effects of TPM on $\text{Ca}_v2.1$ channel function may only be revealed at high-frequency use of the channel. We therefore measured ACh release upon 40 Hz nerve stimulation, before and in the presence of TPM. EPP rundown profiles before addition of TPM were comparable to those reported by us previously for the three genotypes (Figures 2C, D; Table 1; Plomp et al., 2000; Kaja et al., 2005; Kaja et al., 2006). Application of TPM to the preparations, however, did not alter any 40 Hz EPP amplitude profiles (Figures 2C, D; Table 1).

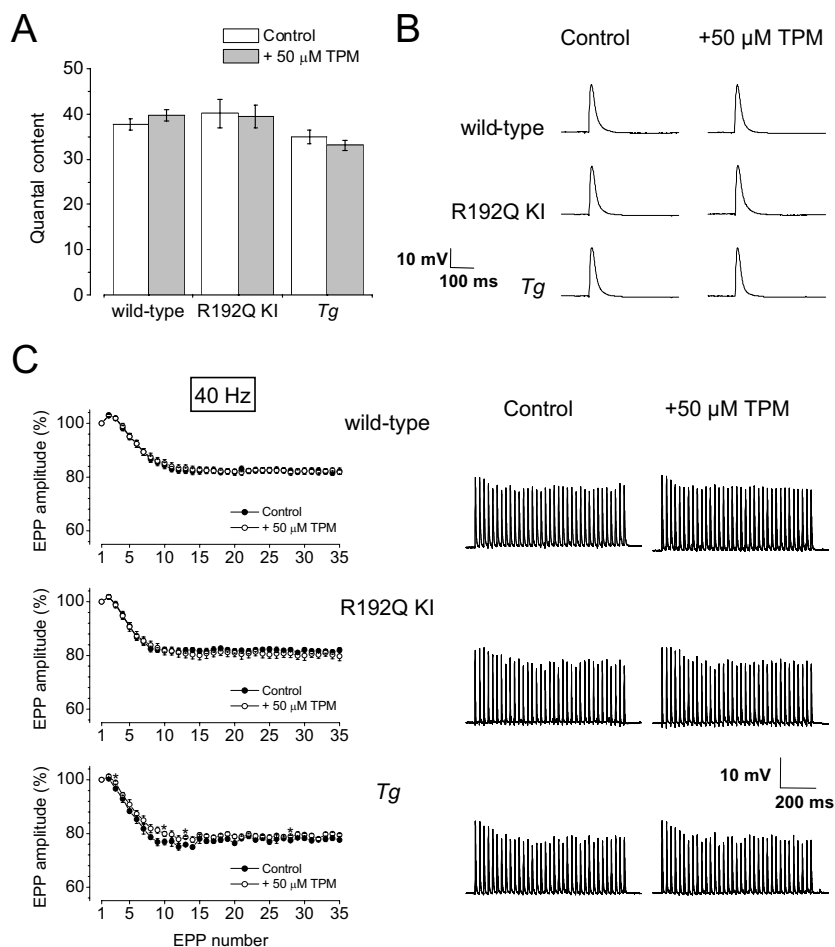


Figure 2. No effect of TPM on nerve stimulation-evoked ACh release at wild-type and R192Q- and *Tg*-mutant NMJs. (A) Quantal content upon 0.3 Hz nerve stimulation was not affected by the subsequent addition of 50 μ M TPM (wild-type: $n=13$, $p=0.14$; R192Q KI: $n=8$, $p=0.79$; *Tg*: $n=5$, $p=0.13$). (B) EPP amplitudes and kinetics did not differ between genotypes and were unaffected by application of 50 μ M TPM. Representative sample traces are shown. (C) Rundown profiles of EPP amplitudes at 40 Hz stimulation were not different between control and 50 μ M TPM conditions at wild-type, FHM1 R192Q KI and *Tg* NMJs. See also Table 1. Representative sample traces of 40 Hz EPP trains are shown for control (left column) and 50 μ M TPM condition (right column).

Discussion

In order to test the hypothesis of a direct, acute action of TPM on (mutant) $\text{Ca}_v2.1$ channel function, we investigated the effects of acute application of TPM on $\text{Ca}_v2.1$ -mediated ACh release from motor-nerve terminals of wild-type and *Cacna1a* mutant R192Q KI and *Tg* mice. We used TPM at a concentration of 50 μ M, which is well above the high end of the range of therapeutic serum concentrations (White, 2005). All electrophysiological parameters of ACh release in the control condition, before the addition of TPM, were similar to those reported by us previously for wild-type and R192Q- and *Tg*- $\text{Ca}_v2.1$ -mutant NMJs (Plomp et al., 2000; Van den Maagdenberg et al., 2004; Kaja et al., 2005; Kaja et al., 2006). However, none of these investigated parameters at NMJs of wild-type mice were affected by 50 μ M TPM. This indicates that TPM does not act directly on native $\text{Ca}_v2.1$ channels, as pre-

synaptic Ca^{2+} influx required to trigger ACh release at the mouse NMJ is exclusively mediated by $\text{Ca}_v2.1$ channels (Uchitel et al., 1992; Van den Maagdenberg et al., 2004; Kaja et al., 2005; Kaja et al., 2006). Furthermore, the R192Q (FHM1) and P601L (*Tg*) mutations in the encoding *Cacna1a* gene seem not to confer TPM-sensitivity onto $\text{Ca}_v2.1$ channels, as TPM did not affect ACh release at NMJs of mice with these mutations.

The monogenic sub-type of migraine with aura, FHM1, serves as a model to study the pathophysiology of typical migraine, which is thought to be a complex polygenic neuro-vascular disorder (Van den Maagdenberg et al., 2004). The present results suggest that the beneficial effects of TPM treatment in reducing migraine attack frequency cannot be attributed to direct acute modulation of $\text{Ca}_v2.1$ channels. However, long-term effects, e.g. through indirect modulation of the phosphorylation state of $\text{Ca}_v2.1$ channel (Yokoyama et al., 2005) or alteration of G-protein mediated inhibition of presynaptic Ca_v channels (Stevens, 2004) cannot be excluded on the basis of our present experiments.

$\text{Ca}_v2.3$ channels have been shown to be inhibited by TPM (McNaughton et al., 2004; Kuzmiski et al., 2005). At the *Tg* NMJ there is a small compensatory contribution (~15%) of $\text{Ca}_v2.3$ channels to evoked ACh release (Kaja et al., 2006). A possible effect of TPM on ACh release at *Tg* NMJs, may have escaped detection, due to relatively small contribution of $\text{Ca}_v2.3$ channels. It may be of interest to study the effect of TPM on ACh release at NMJs of $\text{Ca}_v2.1$ knock-out mice, which is largely (~50%) $\text{Ca}_v2.3$ -dependent (Urbano et al., 2003; S. Kaja and J.J. Plomp, chapter 7).

In conclusion, our experiments provide strong evidence that TPM does not acutely alter the biophysical properties of (mutant) presynaptic $\text{Ca}_v2.1$ channels. However, chronic treatment experiments are required to identify possible long-term effects of TPM on (mutant) $\text{Ca}_v2.1$ channels.

