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Synchronous hippocampal bursting reveals network excitability defects in an epilepsy gene mutation

(tottering mouse/paroxysmal depolarizing shift/afterhyperpolarization/norepinephrine/cornu ammonis region 3 pyramidal neurons)

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ABSTRACT **A mutation at the tottering locus (*tg*, recessive, on chromosome 8) stimulates noradrenergic locus coeruleus axon terminal outgrowth and predisposes the brain to generalized spike-wave epilepsy in the young mouse. In an isolated synaptic circuit studied *in vitro*, the hyperinnervated mutant hippocampal pyramidal neurons respond normally when individually activated; however, latent neuronal signaling defects emerge during synchronous network bursting, revealing two conditional excitability phenotypes: a voltage-dependent prolongation of a complex synaptic response, the paroxysmal depolarizing shift, and a β-adrenoreceptor-linked attenuation of the afterhyperpolarization. In this target brain region, the *tg* locus transforms neuronal excitability without altering measured intrinsic membrane properties, indicating that gene control of inherited epileptic traits may be mediated in part by activity-dependent modulation of network behavior favoring synchronous neuronal firing.**

Inherited molecular defects in central nervous system control mechanisms can express epileptic phenotypes in the mouse with distinct patterns of paroxysmal neuronal synchronization. One fundamental question regarding the emergence of neuronal synchrony in epilepsy is whether the intermittent electrical phenotype reflects an underlying conditional excitability defect contingent upon certain patterns of cerebral network behavior, or a static membrane defect whose influence is felt continuously throughout all modes of neuronal signaling. For example, modulation of voltage- and ligand-gated ion channel behavior by alterations in neurotransmitter-mediated intracellular second messenger levels might produce transient excitability changes within a synaptic pathway that appear principally during conditions favoring bursting, while intrinsic defects in passive membrane properties might produce more persistent shifts in excitability.

We explored these alternatives by *in vitro* intracellular analysis of a recessive gene error at the tottering locus (*tg*, on chromosome 8 between *Os* and *myd*) expressing a generalized spike-wave seizure phenotype in neocortex and hippocampus of the young mouse (1, 2). The hippocampal cornu ammonis region 3 (CA3) subfield was selected as the target brain region for our study, since this region possesses a low threshold for synchronous neuronal burst generation (3, 4). We find that mutant hippocampal CA3 pyramidal neurons show no alterations in several measured intrinsic membrane properties but display abnormally hyperexcitable responses during synchronous network bursting. The two excitability defects include a voltage-dependent prolongation of an epileptiform neuronal burst response, the paroxysmal depolarizing shift (PDS), and an attenuation of the post-PDS afterhyperpolarization (post-PDS AHP). Post-PDS AHP reduction is a feature of models of focal epileptogenesis (5) but has not been linked previously to generalized epilepsies. The prolonged PDS bursting is a new phenotypic defect.

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MATERIALS AND METHODS

***In Vitro* Hippocampal Slices.** Experiments were performed on 18 *tg* and 17 +/+ C57BL/6J adult mice maintained in breeding colonies at the Baylor College of Medicine in accordance with National Institutes of Health guidelines. Transverse hippocampal slices (450 μm thick) were cut by using standard techniques and were incubated in an air/fluid interface chamber at a constant temperature of 32 ± 1°C for a period of at least 1 hr before commencing intracellular recordings. Slices were superfused with a continuously oxygenated (95% O_2/5% CO_2) artificial cerebrospinal fluid (aCSF) with the following composition: 124 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 2 mM $CaCl_2$, 2 mM $MgSO_4$, 26 mM $NaHCO_3$, and 10 mM glucose at pH 7.4. Bursting in the CA3 region was induced by increasing the K^+ concentration in the aCSF to 10 mM.

Intracellular Electrophysiological Recordings. Intracellular recordings were carried out by using glass microelectrodes filled with 3 M potassium acetate and having a resistance of 25–70 MΩ. In some experiments, 10–20 mM BAPTA {[1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*″-tetraacetic acid]} was added to the electrolyte in the microelectrode. All recordings were carried out with a single electrode-clamp amplifier in the discontinuous current clamp mode (switching frequency, 3 KHz). Data from cells that were determined to be healthy based on the following objective criteria were used for analysis: for cells in 3 mM extracellular potassium ($[K^+]_o$)-containing saline, (*i*) a resting membrane potential more negative than −50 mV, (*ii*) input resistance greater than 25 MΩ, and (*iii*) the presence of action potentials that overshoot the baseline; for cells bursting in 10 mM $[K^+]_o$, (*i*) a resting membrane potential more negative than −46 mV and (*ii*) the presence of overshooting action potentials. Input resistance was not used as a strict acceptance criterion in this group, since it could not be accurately measured in every cell because of large baseline fluctuations produced by increased spontaneous synaptic activity.

Analysis of Data. Measurements of durations and amplitudes of recorded waveforms were carried out by a semiautomated procedure involving initial visual determination of the average membrane potential (baseline) 100 ms prior to the PDS response in the digitized trace. All subsequent measurements were made by computer detection of baseline crosses and peak excursions of the waveforms. Peak PDS amplitude

Abbreviations: PDS, paroxysmal depolarizing shift; AHP, afterhyperpolarization; CA3, cornu ammonis region 3; NE, norepinephrine; *tg*, tottering; aCSF, artificial cerebrospinal fluid.

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was measured by using a digital filtering algorithm to disregard action potentials and sharp fluctuations at the crest of the depolarizing envelope without smoothing by interpolation. This method selects local minima from actual values of membrane potentials during this period, whose first- and second-order derivatives with respect to time are equal to and greater than zero, respectively. The largest membrane potential value was taken to be the peak amplitude of the PDS. The peak post-PDS AHP amplitude was determined as the maximum point of downward deflection in the hyperpolarization following the PDS. Mean PDS amplitudes from 11 +/+ and 18 *tg/tg* neurons were plotted as a function of holding potential and compared with curves drawn by linear regression by using the least-squares-fit routine. Resting membrane potential and input resistance were measured in all cells. Input resistance and membrane time constant were measured from the voltage responses of cells held at a membrane potential between −70 to −80 mV to 100-ms hyperpolarizing current pulses. The membrane time constant was determined from the best fit of the charging phase of the hyperpolarizing voltage response, generated by a multiexponential curve-fitting program. Peak spike-induced AHP amplitudes in cells exposed to 3 mM $[K^+]_o$-containing saline were measured by determining the peak amplitude of the late AHP evoked by 100-ms intracellular depolarizing pulses. Pulses of sufficient amplitude to evoke four to five action potentials during depolarization were selected for spike-induced AHP comparisons. The mean amplitude of the depolarization elicited in a given cell during the above procedure was used as a measure of spike-frequency accommodation for comparison between genotypes. All statistical comparisons were done by using the Student's unpaired two-tailed *t* test.

RESULTS

Intracellular current-clamp recordings were carried out in CA3 pyramidal neurons held at various membrane potentials in hippocampal slices from adult *tg/tg* and +/+ mice. Spontaneous epileptiform bursts (PDSs) induced by 10 mM $[K^+]_o$ were significantly (60%) prolonged in the mutant (190 ± 12 ms, $n = 18$) compared with +/+ (119 ± 9 ms, $n = 11$; $P \leq 0.001$) at membrane potentials between −47 and −54 mV (mean *tg/tg*, −50.8 ± 0.4 mV; +/+, −50.9 ± 0.5 mV; Fig. 1*A*). Time to peak (*tg/tg*, 70 ± 5 ms, $n = 18$; +/+, 46 ± 4 ms, $n = 11$; $P \leq 0.006$) and decay time from peak to baseline

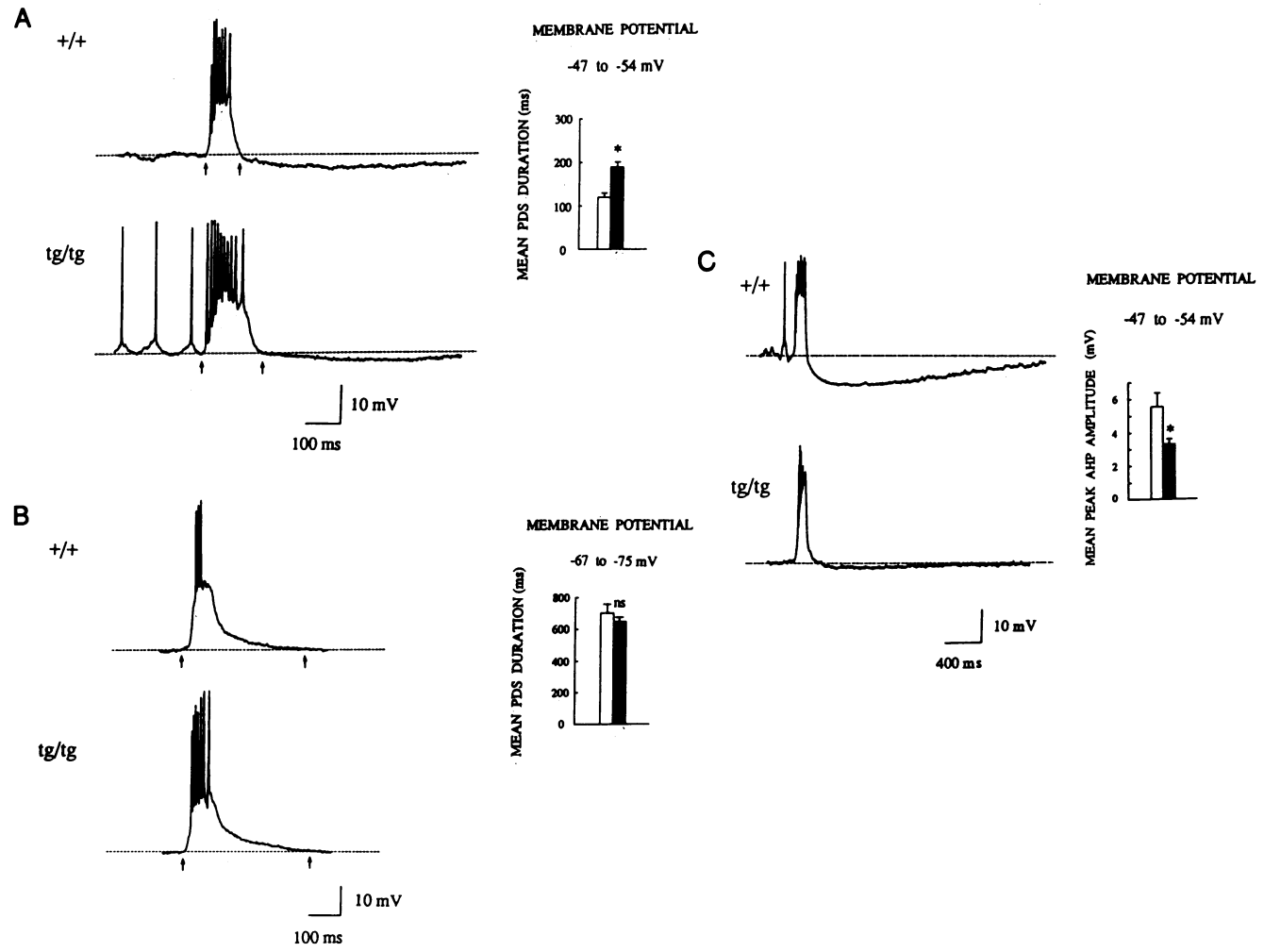

FIG. 1. Gene-linked differences in neuronal excitability revealed during network bursting. (*A*) A pair of representative traces showing PDSs obtained by intracellular recordings from +/+ and *tg/tg* CA3 pyramidal neurons in hippocampal slices bursting during superfusion with aCSF containing 10 mM K^+. The membrane potential (V_m) of these cells was held at −50 mV. At this V_m, the mean PDS duration in mutant neurons is prolonged by 60% compared with that in +/+ neurons. The group data (*Right*) are obtained from 11 +/+ (open bar) and 18 *tg/tg* (solid bar) neurons ($P \leq 0.001$; Student's unpaired *t* test). (*B*) Hyperpolarization of the same cells to a V_m of −70 mV prolongs both PDS durations, but there is no difference between the two genotypes [data from 7 +/+ (open bar) and 8 mutant (solid bar) neurons shown in the histogram]. (*C*) Representative traces obtained from +/+ and mutant CA3 pyramidal neurons displayed on a longer time scale reveal gene-linked differences in amplitude of the post-PDS AHP. There is a 40% reduction in the mean peak post-PDS AHP amplitude in *tg/tg* neurons compared with +/+ neurons. The histogram shows data from 11 +/+ (open bar) and 18 *tg/tg* (solid bar) cells ($P \leq 0.002$).

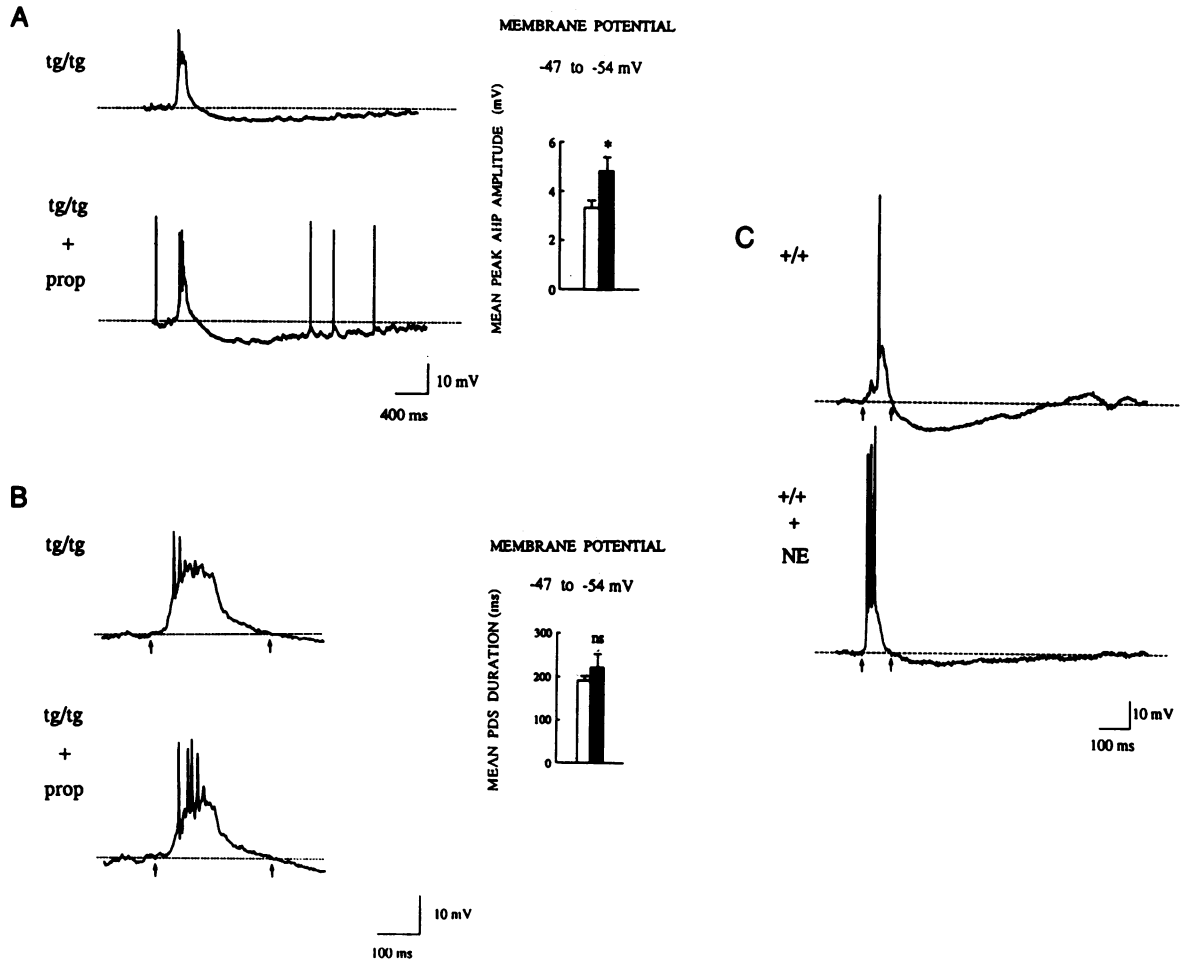

FIG. 2. Role of NE in the *tg* mutant excitability phenotype. (*A*) Traces showing a PDS–AHP complex from a single *tg/tg* CA3 pyramidal neuron before (*tg/tg*) and during bath exposure to 1 μM propranolol (*tg/tg* + prop). There is a 46% enhancement of peak post-PDS AHP amplitude in the presence of the β-blocker. The histogram shows results from neurons in the absence ($n = 11$; open bar) and presence ($n = 6$; solid bar) of propranolol ($P \leq 0.05$). (*B*) Traces taken from the same cell as in *A* displayed on a shorter time scale and group data showing no significant difference in PDS duration between neurons in the absence ($n = 11$; open bar) and presence ($n = 6$; solid bar) of propranolol ($P > 0.05$). The reduced duration at half-maximal amplitude seen in the bottom trace in the presence of propranolol was not consistently observed. There was no significant difference in the latter parameter between *tg* cells in the presence (100 ± 9.9 ms; $n = 7$) and absence of propranolol (91 ± 3.5 ms; $n = 18$). (*C*) Bath application of 50 μM NE to a +/+ neuron bursting in 10 mM $[K^+]_o$ aCSF reduces the post-PDS AHP but does not prolong PDS duration.

(*tg/tg*, 119 ± 10 ms, $n = 18$; +/+, 73 ± 9 ms, $n = 11$; $P \leq 0.005$) of the PDS were also prolonged in the mutant. No difference in mean PDS duration was found between mutant (647 ± 27 ms, $n = 8$) and +/+ (701 ± 57 ms, $n = 7$; $P > 0.05$) CA3 neurons at hyperpolarized potentials (−67 to −75 mV; mean *tg/tg*, −69.6 ± 0.5 mV; +/+, −71.7 ± 0.9 mV; Fig. 1*B*). The mean PDS duration in both genotypes at these hyperpolarized potentials was considerably longer than that at the depolarized membrane potentials, probably reflecting the reversal of chloride-dependent γ-aminobutyric acid $GABA_A$-receptor-mediated synaptic inhibition at the soma (6, 7). Mean PDS durations measured at half-maximal PDS amplitude did not differ significantly between the genotypes at both depolarized holding potentials (*tg/tg*, 91 ± 3.5 ms, $n = 18$; +/+, 78 ± 5.5 ms, $n = 11$; $P > 0.05$) and hyperpolarized holding potentials (*tg/tg*, 98 ± 4.1 ms, $n = 5$; +/+, 90 ± 11.3 ms, $n = 5$; $P > 0.05$). Mean peak amplitudes of the PDS at all membrane potentials examined did not significantly differ in the two genotypes, and there was no apparent difference in the extrapolated reversal potentials of the PDS obtained by linear regression. Mutant neurons showed a tendency toward more pronounced spike firing during the PDS than did +/+ cells.

A second abnormality in *tg/tg* neurons was a 40% reduction in the mean peak amplitude of post-PDS AHP (3.3 ± 0.3 mV, $n = 18$) compared with +/+ neurons (5.5 ± 0.8 mV, $n = 11$; $P < 0.002$) (Fig. 1*C*). Intracellular injections of a calcium chelator, BAPTA, reduced the peak post-PDS AHP amplitude by 20–50% in both genotypes (*tg/tg*, $n = 3$; +/+, $n = 3$) (data not shown), confirming that one component of the slow post-PDS AHP in murine CA3 neurons is a calcium-mediated hyperpolarizing conductance, similar to the Ca^{2+}-dependent K^+ current in rat neurons that is sensitive to β-adrenoreceptor agonists (8). No consistent difference in the total duration of post-PDS AHP could be detected, since it was highly variable in both genotypes. No difference in the spontaneous burst discharge frequency exists between the two genotypes (9).

We tested the possibility that these burst-dependent alterations in network excitability might arise from excessive β-adrenoreceptor activation by 10 mM $[K^+]_o$-induced norepinephrine (NE) release, since locus coeruleus axon terminals hyperinnervate *tg* forebrain regions and contribute to the development of spontaneous seizure expression in this mutant (10, 11). Bath application of a competitive β-adrenoreceptor antagonist propranolol to bursting slices at a concentration (1 μM) known to block the NE-mediated reduction of

Table 1. Intrinsic membrane properties measured in +/+ and *tg/tg* CA3 pyramidal neurons

Intrinsic membrane properties*	CA3 pyramidal neurons +/+	CA3 pyramidal neurons *tg/tg*	*P*
	3 mM $[K^+]_o$		
Resting membrane potential, mV	60.6 ± 2.1 (n = 9)	63.3 ± 1.9 (n = 11)	>0.05
Input resistance, MΩ	39.7 ± 2.9 (n = 7)	43.6 ± 3.6 (n = 10)	>0.05
Membrane time constant, ms	45.2 ± 5.9 (n = 5)	49.8 ± 4.51 (n = 6)	>0.05
Peak AHP† amplitude, mV	4.9 ± 1.2 (n = 7)	4.0 ± 0.3 (n = 9)	>0.05
	10 mM $[K^+]_o$		
Resting membrane potential, mV	55.63 ± 1.81 (n = 11)	54.21 ± 1.17 (n = 18)	>0.05
Input resistance, MΩ	18.12 ± 2.62 (n = 8)	21.65 ± 2.19 (n = 12)	>0.05

*Mean ± SEM.
†Spike-induced.

spike-induced AHP (8) significantly enhanced the peak amplitude of the *tg* post-PDS AHP, correcting the mutant phenotype (Fig. 2*A*). The mean peak amplitude of the *tg* post-PDS AHP in the presence of propranolol (4.8 ± 0.6 mV, n = 6) was equivalent to that obtained in control CA3 cells in aCSF containing 10 mM $[K^+]_o$, and +/+ cells showed no significant alteration by β-adrenoreceptor blockade (data not shown).

In contrast, PDS duration was unaffected by acute exposure to propranolol in either genotype (Fig. 2*B*). Furthermore, bath exposure of +/+ neurons (n = 5) to NE in concentrations (10–50 μM) sufficient to reduce the post-PDS AHP did not prolong the PDS (Fig. 2*C*). These two lines of evidence suggest that excess α- or β-adrenoreceptor activation in the adult mutant cannot fully account for the inherited alteration in hippocampal network behavior, although the possibility of indirect consequences of persistently increased NE during development on adult network excitability cannot be excluded.

Recordings carried out in standard aCSF with a physiological concentration of $[K^+]_o$ (3 mM) to evaluate intrinsic membrane properties of mutant CA3 pyramidal neurons revealed no abnormalities in the resting membrane potential, input resistance, membrane time constant, and spike-induced AHP (Table 1), as reported previously in *tg* CA1 neurons (12). The mean amplitude of depolarization needed to induce four to five spikes did not significantly differ in the two genotypes (+/+, 16.9 ± 2.3 mV, n = 7; *tg/tg*, 16.8 ± 2.5 mV, n = 9; $P > 0.05$), suggesting that spike frequency accommodation is unlikely to be altered in *tg* cells. Robust excitatory and inhibitory postsynaptic potentials could be induced in these cells by stimulation of mossy fibers at the hilus, however these responses could not be adequately normalized for meaningful comparison between genotypes.

DISCUSSION

These results demonstrate that an as yet unidentified molecular defect at the *tg* locus produces latent functional alterations in CA3 pyramidal cell excitability that emerge under conditions of synchronous neuronal discharge. The differential sensitivity of the abnormal PDS prolongation and the diminished post-PDS AHP amplitude to NE and propranolol exposure indicates that the *tg* mutation alters these complex but separable membrane responses by two distinct cellular mechanisms. Attenuation of the post-PDS AHP is predominantly mediated via excess β-adrenoreceptor activation by mutant locus coeruleus axon terminals hyperinnervating CA3 neurons. The post-PDS AHP is composed of multiple hyperpolarizing conductances (13–16) of which a NE-sensitive calcium-mediated K^+ current is an important component. While this current is similar, if not identical, to the spike-induced AHP (16), we found in mutant cells a small but statistically insignificant reduction in the latter. This may be due to the fact that in the isolated *tg* hippocampus, NE is presumably released in excessive amounts only during high $[K^+]_o$-induced bursting (conditions of measurement of the post-PDS AHP) and not during normal firing in 3 mM $[K^+]_o$ saline (conditions of measurement of the spike-induced AHP). Additionally, NE may be reducing other conductances contributing to the post-PDS AHP but not the spike-induced AHP, such as the γ-aminobutyric acid receptor $GABA_A$ (17) conductance.

The earlier component of the mutant phenotype, the voltage-dependent prolongation of the mutant PDS, cannot be rapidly reversed by β-adrenergic receptor blockade or mimicked in +/+ cells by brief NE exposure, and the exact mechanism remains to be defined. Several activity-dependent processes could account for the prolonged depolarizing membrane response during this complex giant excitatory postsynaptic potential (EPSP) (18), including modulation of a voltage-dependent ion channel (19), unmasking (20) or potentiation (21, 22) of excitatory synapses, or attenuation of concurrent synaptic inhibition (23, 24). Although any of these mechanisms might be altered independently of the aberrant noradrenergic input, it is worth noting that increases in neuronal cAMP levels (25) and potentially in other second messengers (26) altering protein phosphorylation modulated by excessive NE might contribute to longer lasting plasticity (27–29) in hyperinnervated regions of the *tg* nervous system.

The cellular origins of generalized, nonconvulsive spike-wave epilepsies are unknown but may involve diffuse excitability increases throughout cortical and thalamoreticular pathways (30, 31). If the burst-related signaling abnormalities in the *tg* mutant hippocampal cortex are also expressed in NE-sensitive (32, 33) and other cells of the neocortex, thalamus, and other NE-hyperinnervated forebrain areas (10) involved in spike-wave seizure generation, they could favor widespread recruitment of network bursting. In particular, the tendency to generate a prolonged PDS could intensify synchronization of the neuronal population, and the diminished post-PDS AHP could compromise post-PDS neuronal refractoriness, promoting the repeated occurrence of the synchronous discharge.

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