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Original Research

Cortical Tonic Inhibition Gates the Expression of Spike-and-Wave Discharges Associated with Absence Epilepsy

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Abstract

Objective: Absence seizures result from aberrant thalamocortical processing that confers synchronous, bilateral spike-and-wave discharges (SWDs) and behavioral arrest. Previous work has demonstrated that SWDs can result from enhanced thalamic tonic inhibition, consistent with the mechanism of first-line antiabsence drugs that target thalamic low-voltage-activated calcium channels. However, nearly half of patients with absence epilepsy are unresponsive to first-line medications. In this study we evaluated the role of cortical tonic inhibition and its manipulation on absence seizure expression. **Methods**: We used video-electroencephalogram (EEG) monitoring to show that mice with a γ-aminobutyric acid type A ($GABA_A$) receptor mutation (γ2R43Q) display absence seizures. Voltage-clamp recordings in brain slices from wild type and γ2R43Q mice were used to evaluate the amount of tonic inhibition and its selective pharmacological modulation. Finally, we determined whether modulating tonic inhibition controls seizure expression. **Results**: γ2R43Q mice completely lack tonic inhibition in principal neurons of both layer 2/3 cortex and ventrobasal thalamus. Blocking cortical tonic inhibition in wild type mice is sufficient to elicit SWDs. Tonic inhibition in slices from γ2R43Q mice could be rescued in a dose-dependent fashion by the synthetic neurosteroid ganaxolone. Low-dose ganaxolone suppressed seizures in γ2R43Q mice. **Conclusions**: Our data suggest that reduced cortical tonic inhibition promotes absence seizures and that normal function can be restored via selective pharmacological rescue. These results, together with previous findings, suggest that deviations of tonic inhibition either above or below an optimal set point can contribute to absence epilepsy. Returning the thalamocortical system to this set point may provide a novel treatment for refractory absence epilepsy.

Keywords: epilepsy; absence epilepsy; tonic inhibition; neurosteroids; ganaxolone

1. Introduction

Absence seizures are characterized by periods of behavioral arrest and amnesia without overt convulsions [1]. In patients, these generalized seizures typically last 2–15 seconds with corresponding bilateral, synchronous ~3 Hz electrographic spike-and-wave discharges (SWDs) [2]. The generalized nature of absences, however, should not be interpreted to mean that there is no specific focus or generating region.

Current first-line medications to control absence seizures associate this disorder with pathological thalamic relay neuron activity [3]. Ethosuximide and valproic acid are two commonly prescribed medications to treat absence seizures, with approximately equal efficacy (~55%) [4,5]. Both agents act as T-type Ca^{2+} channel blockers, thus directly inhibiting Ca^{2+}-dependent plateau potentials and bursting in thalamic relay neurons [6]. Cope *et al.* [7] demonstrated that the propensity of thalamic relay neurons to express T-type Ca^{2+} channel-mediated bursts is positively correlated with the level of γ-aminobutyric acid type A-associated (GABAergic) tonic inhibition. Several rodent models of absence epilepsy display aberrantly enhanced tonic inhibition in thalamic relay neurons [8–10], leading to the conclusion that enhanced thalamic tonic inhibition is "necessary and sufficient for the generation of typical absence seizures" [9]. However, it remains to be understood why current first-line medications have limited efficacy in controlling human absence epilepsy.

Absence epilepsy is a thalamocortical disorder, thus a "cortical focus theory" for absence seizures has also been proposed in many absence animal models [11–15]. The cortical focus theory is rooted in findings that SWD initiation was localized to the perioral region of the somatosensory cortex in multiple animal models of absence epilepsy [11,14,16–19]. Local injection of certain drugs into this area was enough to suppress absence seizure expression [20–23]. Direct administration of the endogenous neurosteroid allopregnanolone (ALLO), or its synthetic derivative ganaxolone (GANX), into the primary somatosensory

cortex of SWD-expressing Wistar Albino Glaxo Rijswijk (WAG/Rij) rats reduced both the number and duration of SWDs [20].

ALLO and GANX are positive allosteric modulators of γ-aminobutyric acid type-A ($GABA_A$) receptors, having their largest effects at δ subunit-containing $GABA_A$ receptors and being relatively selective for this subtype at low concentrations [24]. GANX has been evaluated as an antiepileptic drug in humans [25–27] for the treatment of infantile spasms [28] and has shown efficacy with minimal side effects as a treatment for catamenial epilepsy [24] and partial seizures [29]. In animal studies of partial seizures, subcutaneous administration of GANX displayed antiseizure potential even at relatively low doses (3 mg/kg) [30]. However, in two animal models of absence epilepsy (pentylenetetrazole, PTZ; gamma-hydroxybutyric acid, GHB) GANX exacerbated absence seizures and even produced SWDs in wild-type rats when either systemically administered at $\geq$20 mg/kg [31] or administered directly into the ventrobasal thalamus [20]. These findings might be explained if high GANX concentrations stimulated thalamic tonic inhibition via δ subunit-containing $GABA_A$ receptors, as observed in previous studies [8–10]. These findings suggest a dichotomy of effects for neurosteroids regarding absence epilepsy: direct administration into the somatosensory cortex decreases SWD generation and duration, whereas systemic administration of high concentrations results in sedation and SWD exacerbation or generation.

This heritable epilepsy in humans has been traced to an arginine-to-glutamine substitution at position 43 of the $GABA_A$ receptor γ2 subunit (γ2R43Q) that confers a variety of phenotypes, the most common being Childhood Absence Epilepsy (CAE) and febrile seizures [32]. Patients expressing this mutation show evidence of a hyperexcitable cortex and diminished intracortical inhibition [33] that is believed to contribute to SWDs [34]. Patients affected with the γ2R43Q mutation are all heterozygous. Heterozygous knock-in (RQ) mice display absence-like seizures, generalized SWDs and an early developmental onset of seizure susceptibility, all consistent with observations from affected human families [35]. RQ mouse cortical neurons also display a higher spontaneous-firing rate [36].

In this study we used continuous video- electroencephalogram (EEG) monitoring and voltage-clamp electrophysiology to show that RQ mice that display SWDs also lack tonic inhibition in both cortical layer 2/3 pyramidal and thalamic relay neurons. We then used selective pharmacology to modulate cortical tonic inhibition in wild-type mice, introducing a novel model of absence epilepsy employing a previously unexplored mechanism in which decreased cortical tonic inhibition is sufficient to trigger SWDs. Finally, we suppressed the expression and duration of SWDs in absence (RQ) mice by rescuing the lost cortical tonic inhibition via low-dose systemic GANX administration. Together with previous findings, our data suggest that an optimal level of tonic inhibition must be maintained throughout the thalamocortical circuit to ensure normal function and offer a new therapeutic option (low-dose GANX) for patients where SWDs are caused by alternative mechanisms.

2. Methods

2.1 EEG Implantation and Monitoring of SWDs

Our study used male and female wild-type Harlan C57BL/6J-OlaHsd and γ2R43Q knock-in mice bred into a background of Harlan C57BL/6J-OlaHsd mice. Behavioral and electrographic markers of absence epilepsy in these animals were confirmed by video-EEG monitoring. Surgery and electrode implantation were performed as described by Nelson *et al*. [37]. Briefly, P24 mice were implanted under isoflurane (#sc-363629Rx; Santa Crus Biotechnology, Dallas, TX, USA) anesthesia (1%–2% in 100% O_2) for chronic EEG recordings with gold plated miniature screw electrodes over the right and left frontal and parietal cortices, and one over the cerebellum as a reference. Two vinyl-coated braided stainless steel wire electrodes were placed in the nuchal muscle for electromyogram (EMG) recording of muscle activity. All electrodes were gathered into a flexible cable and connected to the Multichannel Neurophysiology Recording system (Tucker-Davis Technologies, TDT, Alachua, FL, USA). EEG and EMG signals were collected continuously at a sampling rate of 256 Hz (digitally filtered between 0.1 and 100 Hz). Continuous EEG recordings with occasional video monitoring were made and SWDs were manually scored off-line. Animals were given a 3-day recovery period after surgery before initiating SWD-scoring. A SWD event was defined as a brief (~2 seconds long) ~6 Hz signal synchronized across all EEG leads, with a corresponding lack of signal in the EMG lead. Only SWD events that occurred >2 min from slow-wave-sleep periods were used for quantification. SWD event durations were measured from the first synchronized positive peak signal to the last synchronized positive peak within an event. "Seizures" were defined as groups of SWD events separated from other events by <30 seconds. Ictal intervals were defined as the time between the beginnings of consecutive seizures. All animal procedures followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Wisconsin-Madison (No. A3368-01). All facilities were inspected and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

2.2 Drugs and Injection Schedule

L655,708 (L9787), GANX (G7795) and THIP (T101; 4,5,6,7-tetrahydroisoxazolo[5,4-c]-pyridine-3-ol HCl) were all obtained from Sigma (St. Louis, MO, USA). L655 and GANX were dissolved in a 30% dimethyl

sulfoxide (DMSO)-saline solution (v/v), whereas THIP was dissolved in 100% saline. Mice were intraperitoneally (i.p.) injected with 2 mg/kg doses of L655, 2 and 5 mg/kg doses of GANX, or 0.5 and 1.5 mg/kg doses of THIP. 160 µL of solution was injected for each drug. L655 was administered to wild type (RR) mice 2 and 4 hours after lights out for 2 consecutive days beginning 5 days after surgery. These mice were not injected for the subsequent 2 days but were given vehicle injections on day 9. GANX or THIP injections were administered to RQ mice 1 and 4 hours after lights out. Drug injections for RQ mice began on day 5 post surgery and consisted of 2 injections of one drug and dose, with a different drug and dose for days 6, 10, and 11. No injections were given to RQ mice on days 7–9.

2.3 Whole-Cell Patch Clamp Experiments

Horizontal slices (400 µm) were prepared from the brains of RR and RQ mice of either sex (16–26 days old). All procedures were approved by the University of Wisconsin IACUC. Mice were anesthetized with isoflurane, decapitated, and the brain was removed and placed in an ice-cold cutting solution containing (in mM): 125 NaCl, 25 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 0.5 $CaCl_2$, 3.35 $MgCl_2$, 25 D-Glucose, 13.87 sucrose, and bubbled with 95% O_2 and 5% CO_2. Slices were cut using a vibratome (Leica VT 1000S, Global Medical Imaging; Ramsey, MN, USA) and placed in a bubbled incubation chamber containing standard artificial cerebrospinal fluid (aCSF) (in mM): 125 NaCl, 25 $NaHCO_3$, 2.5 KCl, 1.25 NaH_2PO_4, 2 $CaCl_2$, 1 $MgCl_2$, 25 D-Glucose, at room temperature for 1 hour before being used for recordings. Whole cell patch-clamp recordings were made from somatosensory cortical layer 2/3 pyramidal cells or ventrobasal thalamic relay cells, visualized using an upright differential interference contrast microscope (Axioskop FS2, Zeiss; Oberkochen, Germany). Patch pipettes were pulled from thin-walled borosilicate glass (World Precision Instruments; Sarasota, FL, USA) with a resistance of 3–5 MΩ when filled with an intracellular solution containing (in mM): 140 KCl, 10 EGTA, 10 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 20 phosphocreatine, 2 Mg2ATP, 0.3 NaGTP (pH 7.3, 310 mOsm). Voltage clamp (–60 mV) recordings were made in a submerged chamber, perfused with bubbled aCSF (4 mL/min) containing (500 nM) tetrodotoxin, (25 µM) DNQX, and (50 µM) AP5 at room temperature using a MultiClamp 700B amplifier (Axon Instruments; Foster City, CA, USA), filtered at 4 kHz and digitized at 10 kHz using a Digidata 1322A analog-digital interface (Axon Instruments). Data were acquired to a Macintosh G4 (Apple Computer; Cupertino, CA, USA) using Axograph X v1.1.4 (Molecular Devices; Sunnyvale, CA, USA).

Data segments (30 seconds) just prior to and 90 seconds after drug administration were analyzed to quantify inhibitory tonic currents. All-point amplitude histograms were computed for each segment and fit with a Gaussian function only to the outward current portions relative to the peak in order to omit components arising from inward miniature inhibitory post-synaptic currents (mIPSCs) [38]. Tonic current was calculated as the difference between the fitted Gaussian means before and after (100 nM or 1 µM) THIP, (30 nM) ALLO or (10 nM) GANX administration. Current density (pA/pF) was calculated by dividing the current by cell capacitance. Bicuculline (100 µM; bicuculline methiodide #2503, Tocris Bioscience, Minneapolis, MN, USA)) was added at the conclusion of some experiments for each drug tested to verify full current block and, therefore, only GABAergic contribution.

2.4 Statistics

The Kruskal-Wallis test of medians was used to compare multiple groups with a Dunn's post-hoc evaluation. Tonic current amplitude and density data were normally distributed; therefore, an analysis of variance (ANOVA) was used to compare multiple groups with a Bonferroni post-hoc evaluation. A p-value of <0.05 is considered significant. MATLAB (version 2013b, Mathworks Inc, Natick, MA, USA) and Prism (macOS v10.0.0, GraphPad Software, Boston, MA, USA) software were used.

3. Results

3.1 RQ Mice Express SWDs and Absence Epilepsy

The γ2R43Q mutation confers absence seizures and generalized SWDs in humans [32] and knock-in (RQ) mice [35], consistent with results presented in this study. Fig. 1 illustrates bilateral, synchronous (~6 Hz) SWDs in a RQ mouse using continuous EEG and EMG recordings. Quantification was done off-line after recordings were completed. A "seizure" was classified as two or more individual SWD events occurring <30 seconds apart. SWDs were assessed for individual event duration, events per seizure, seizure duration and ictal intervals. EEG and EMG recordings from one RQ mouse during a seizure are presented (Fig. 1a,b), along with quantified SWD assessment for three different RQ mice (Fig. 1c). All RQ mice assessed with EEG and EMG monitoring presented with synchronized SWDs across all EEG leads with coincident cessation of EMG activity. Some spindle activity but no SWDs were observed in drug-naïve wild type (RR) mice.

3.2 GABAergic Tonic Inhibition Is Abolished in RQ Cortical and Thalamic Principal Neurons

Although RQ mice express slightly decreased IPSC amplitudes [35], this mutation has also been shown to hinder $GABA_A$ receptor assembly, trafficking and surface expression [39–43]. Based on studies in transfected cultured neurons, Eugène *et al.* [42] reported that this mutation may contribute to absence epilepsy by reducing tonic inhibition. To directly test this hypothesis in RQ animals, we examined tonic inhibition levels in brain slices from RR and RQ

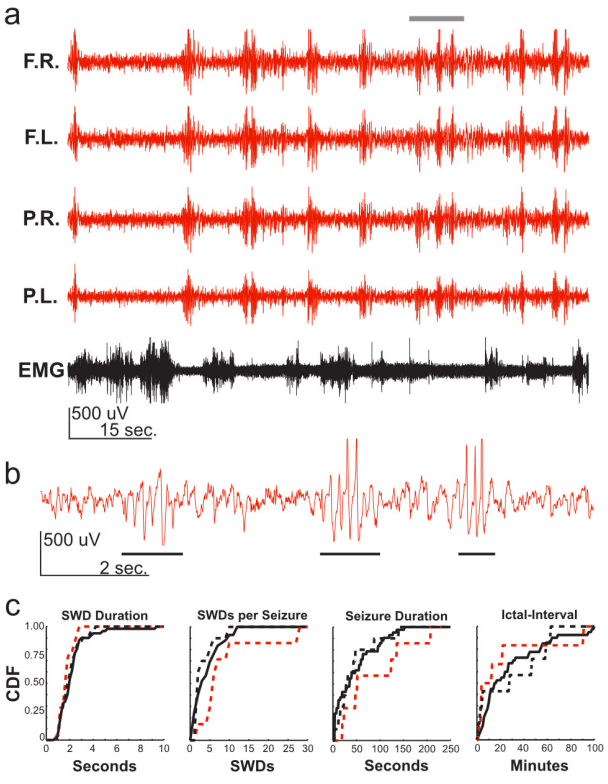

Fig. 1. RQ mice express spike-and-wave discharges (SWDs) associated with absence epilepsy. (a) Electroencephalogram (EEG) recording of an RQ mouse (red). Top trace to bottom trace: frontal right cortex (F.R.); frontal left cortex (F.L.); parietal right cortex (P.R.); parietal left cortex (P.L.); electromyogram (EMG). Note the brief yet frequent (~11 times during the 1.5-minute segment) synchronized events that occur across all EEG leads during the absence of signal in the EMG. (b) Expanded F.R. EEG recording from grey bar in (a) (10 seconds). Note the brief ~6 Hz SWD events (black bars) that occur 3 times during the 10 second trace. (c) Cumulative probability distributions from three different RQ mice (solid & dashed black lines and red dashed line represent individual mice) all display similar characteristics for SWD event durations, SWDs per seizure, seizure durations and ictal-intervals. SWDs were not detected in litter-mate control mice (not shown). CDF, cumulative density function; RQ, γR43Q mutant.

knock-in mice. Using whole cell voltage clamp recordings, we found that whereas RR neurons exhibit a substantial inhibitory tonic current, this current was entirely abolished in RQ mice somatosensory cortical layer 2/3 neurons (mean $\pm$ standard error of the mean (SEM) in pA, N) (RR: 6.0 $\pm$ 0.8, 4; RQ: –1.2 $\pm$ 1.6, 4, $p < 0.05$; Fig. 2a,b), as well as in thalamic relay neurons (RR: 6.9 $\pm$ 2.2, 9; RQ: 0.6 $\pm$ 0.7, 6, $p < 0.05$; Fig. 2c,d). One-sample *t*-test indicated that tonic currents in RQ were indistinguishable from zero ($p > 0.45$).

3.3 $GABA_A$ Receptor Function or Expression Is Altered in a Region-Specific Manner

The tonic current in RR somatosensory cortical layer 2/3 cells (Fig. 2a) was completely blocked by the α5 subunit-selective inverse agonist L655,708 (L655: 30 µM; Fig. 3a), consistent with previous studies showing this subunit is responsible for most or all of the native tonic inhibition in these neurons [44].

In contrast, application of the agonist THIP (1 µM, a concentration previously shown to be selective for δ subunit-containing receptors [45]) evoked currents of similar magnitude in RR and RQ cortical neurons (mean $\pm$ SEM in pA, N) (RR: 21.4 $\pm$ 5.7, 4; RQ: 23.8 $\pm$ 2.2, 5; p = 0.67; Fig. 3b). A similar profile of effect was observed with allopregnanolone (ALLO: 30 nM; Fig. 3c), a neurosteroid that also selectively activates δ subunit-containing $GABA_A$ receptors [46,47]. Together, these results suggest that receptors containing the δ subunit are present in cortical neurons and can be recruited by both exogenous drugs and endogenous modulators, providing potential pharmacological pathways to rescue cortical tonic inhibition in cases where it has been genetically compromised.

Distinct from cortical layer 2/3 neurons, thalamic relay neurons rely solely on δ subunit-containing $GABA_A$ receptors to produce inhibitory tonic currents [7,45,48]. We found that RQ relay neurons in the ventrobasal thalamus responded to THIP (1 µM) with 47% of the current produced in RR thalamic neurons (mean $\pm$ SEM in pA, N) (RR: 131.7 $\pm$ 31.2, 5; RQ: 69.3 $\pm$ 22.4, 4, $p < 0.05$; Fig. 3d). Similarly, in RQ thalamic neurons, ALLO (30 nM) produced 39% of the current observed in RR (RR: 34.7 $\pm$ 6.5, 5; RQ: 13.7 $\pm$ 3.8, 3, $p < 0.05$; Fig. 3e). These results suggest that RQ mouse thalamic relay neurons express reduced levels and/or activation of δ subunit-containing $GABA_A$ receptors.

3.4 Blocking Cortical Tonic Inhibition in Wild-Type Mice Produces SWDs

Previous work has demonstrated that a positive correlation between SWDs and thalamic inhibitory tonic current amplitude exists [7], leading to the conclusion that enhanced GABAergic tonic inhibition is "necessary and sufficient" to cause typical absence epilepsy [9,10]. In this study, we found (by using the α5 subunit-selective blocker L655) that altering thalamic tonic inhibition is not "necessary" for SWD generation and, furthermore, that the loss of α5 subunit-mediated tonic inhibition (present mainly in cortex and hippocampus) is "sufficient" to produce SWDs (Fig. 4). Inhibitory tonic currents in somatosensory cortical layer 2/3 principal neurons are generated by α5 subunit-containing $GABA_A$ receptors, evident by the total block of this current by the α5 subunit-selective inverse agonist L655 (Fig. 3a). This is in contrast to thalamic relay neurons that do not express a5 subunit-containing $GABA_A$ receptors [49,50]. Intraperitoneal (i.p.) administration of L655, at a concentration (2 mg/kg) known to bind the majority of α5

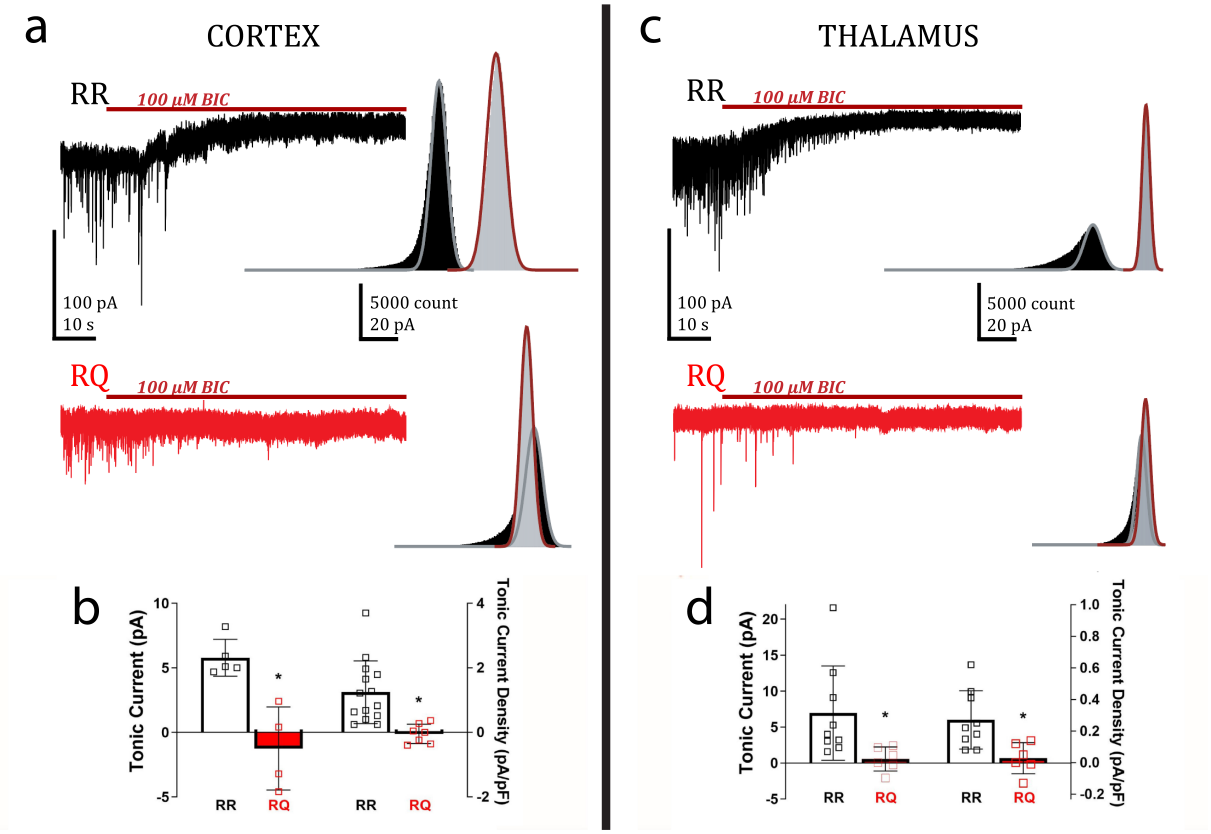

Fig. 2. Tonic currents are abolished in RQ cortex and thalamus. (a) Example voltage-clamp traces for RR (black) and RQ (red) cortical layer 2/3 cell recordings during 100 µM Bicuculline administration (maroon bars). Insets; Corresponding all-points amplitude histograms for data before (black) and after (grey) bicuculline administration. Histograms were fit with a Gaussian function (dark grey and maroon traces) only on the right side of the distribution, thus omitting components due to phasic miniature inhibitory post-synaptic currents (mIPSCs). (b) Tonic current amplitude (pA) (left axis) and tonic current density (pA/pF) (right axis) are abolished in RQ cortical cells (*$p < 0.05$) compared to control. (c,d) Same as a-b, but for ventrobasal thalamic relay neurons. RR, wild type.

subunit-containing receptors [51], produced SWDs (~6 Hz) in RR mice (RRL6) that are electrographically similar, yet distinct, to SWDs observed in RQ mice (Fig. 4a,b). L655 administration induced hallmark synchronous and bilateral SWDs accompanied by behavioral arrest, although these L655-induced SWDs (L6-SWDs) display longer event durations ($p < 0.05$), fewer events per seizure ($p < 0.05$) and shortened seizure durations ($p < 0.01$) compared to RQ (Fig. 4c). The appearance of SWDs 3-days post final L655-injection (Fig. 4d, hour 1 vehicle) highlight lingering plasticity and epileptogenesis induced by the earlier acute insults that provoked SWDs.

3.5 Rescuing Cortical Tonic Inhibition Attenuates SWDs in RQ Mice

Although RQ principal cortical neurons lack inhibitory tonic currents (Fig. 2a), these neurons also display an inhibitory conductance in response to selective δ subunit-selective $GABA_A$ receptor agonists THIP (1 µM) and ALLO (30 nm) (Fig. 3b,c). This finding is consistent with the presence of latent δ subunit-containing $GABA_A$ receptors in RQ cortical neurons and suggests that the lost tonic inhibition in these neurons can be rescued. We used whole-cell patch-clamp recordings to titrate a concentration of selective-δ subunit-containing $GABA_A$ receptor modulators that rescued wild-type tonic inhibition levels in RQ cortical neurons. We found (Fig. 5a) that a low concentration of THIP (100 nM) or the synthetic neurosteroid GANX (10 nM) can activate a latent inhibitory conductance in RQ cortical neurons equal to the inhibitory tonic current observed in RR cortical neurons.

Using video-EEG monitoring, we investigated if treatment with the δ subunit-selective $GABA_A$ receptor agonist (GANX) could ameliorate the SWDs observed in RQ mice. RQ mice were i.p. injected twice a day with GANX for 4 out of 7 days (Fig. 5b). Two concentrations of GANX (2 and 5 mg/kg) were tested for amelioration of SWD expression. We found that only the lower concentration (2 mg/kg) of GANX significantly ($p < 0.05$) decreased RQ-SWD expression (Fig. 5c). This low dose GANX treatment (2 mg/kg) also decreased seizure duration ($p < 0.05$) and event duration ($p < 0.001$) (Fig. 5d). The efficacy of the

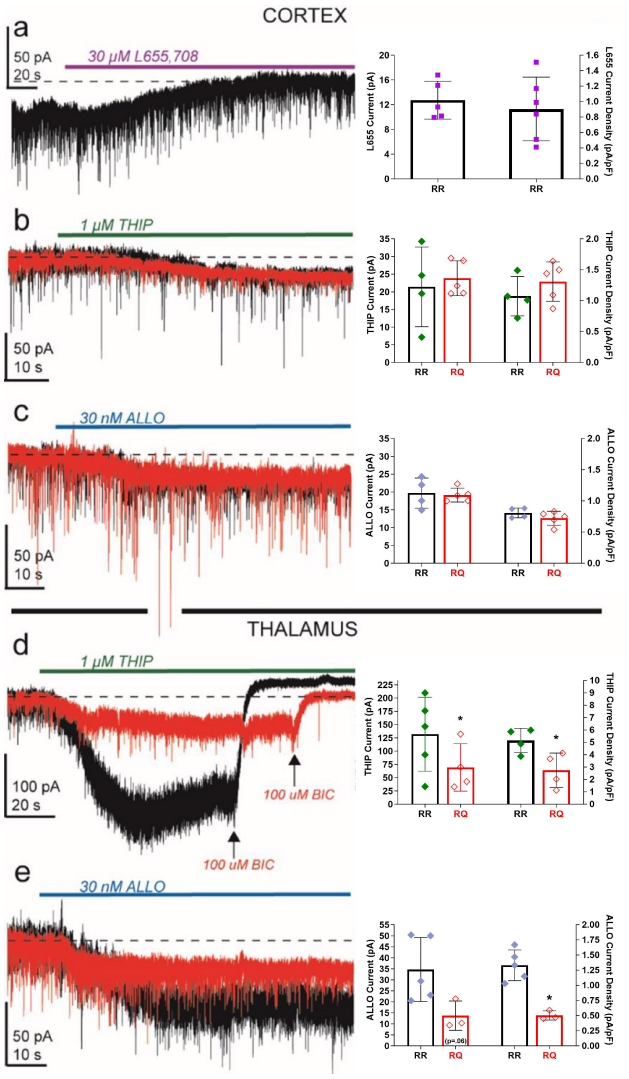

Fig. 3. RQ mice display region- and subunit-specific changes in tonic inhibition. (a) Example voltage-clamp traces for RR cortical layer 2/3 cell recordings during 30 μM L655,708 administration (purple bar). The current density blocked by L655,708 was not significantly different than that blocked by bicuculline (see Fig. 2). In cortical neurons, both THIP. (b) (1 μM; green bar) and allopregnanolone. (c) (ALLO; 30 nM; blue bar) induce indistinguishable current amplitude and density in RQ (red traces) compared to RR (black traces). In thalamic relay neurons, however, THIP (d) and ALLO-induced (e) current densities are significantly reduced in RQ compared to RR (~50%; $*p < 0.05$). ALLO, allopregnanolone; THIP, 4,5,6,7-tetrahydroisoxazolo[5,4-c]-pyridine-3-ol HCl.

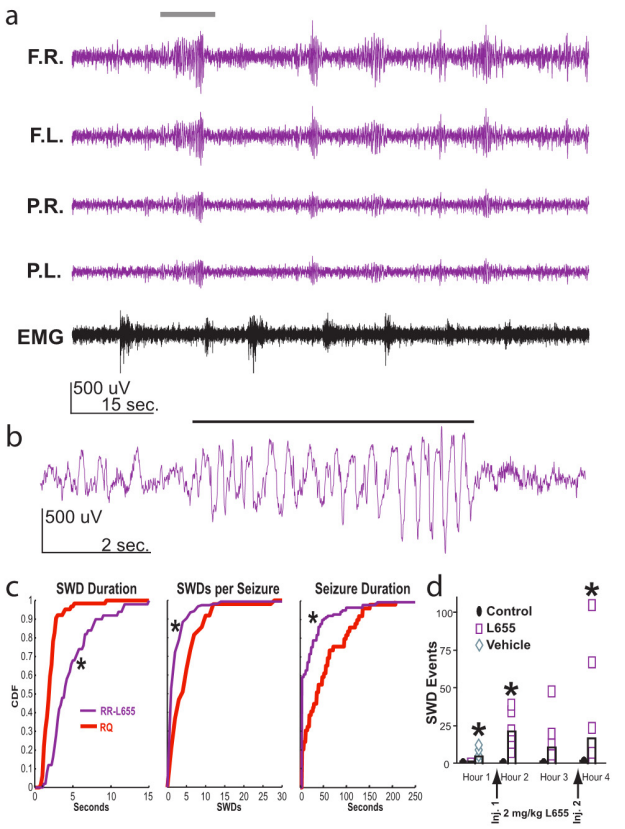

Fig. 4. Blocking cortical tonic inhibition produces SWDs in wild-type mice. (a) Electroencephalogram (EEG) recording of a wild-type (RR) mouse i.p. injected with 2 mg/kg of the $GABA_A$ receptor $\alpha 5$ subunit-selective inverse agonist L655,708 (RRL6; purple). Similarly to RQ mice, note the brief yet frequent (~6 times during the 1.5 min. trace) synchronized events that occur across all EEG leads during the absence of signal in the EMG. (b) Expanded F.R. EEG recording from grey bar in (a) (10 seconds) displays prolonged ~6 Hz SWD event (black bar). (c) Cumulative distributions show RRL6 mice (purple line) display significantly longer SWD event durations ($*p < 0.05$), fewer SWDs per seizure ($*p < 0.05$) and shorter seizure durations ($*p < 0.05$) than RQ mice (red line). (d) Quantification of SWDs show that RRL6 mice did not display SWDs (purple boxes) prior to L655 injection (Hour 1: L655, middle purple bars) but do display SWDs for hours following injection (Hour 2: $*p < 0.05$; Hour 4: $*p < 0.05$). Black outlined bars represent median values. SWDs were still present in RRL6 mice 3 days after the final L655 dosing (vehicle: Hour 1, right turquois diamonds; $*p < 0.05$).

low GANX dose (2 mg/kg), being half the ED_{50} dose that protects against partial seizures [30,51,52], suggests that the mechanism diminishing SWDs in RQ mice involves activation of latent δ subunit-containing $GABA_A$ receptors in cortical neurons.

4. Discussion

The major findings from this study are that the loss (RQ: Fig. 2) or decrease (RRL6: Fig. 4) of cortical tonic inhibition accompanies a SWD-expressing phenotype and that pharmacological replacement of cortical tonic inhibition (RQ-GANX: Fig. 5c) decreases SWD expression. These findings are consistent with the conclusion that cor-

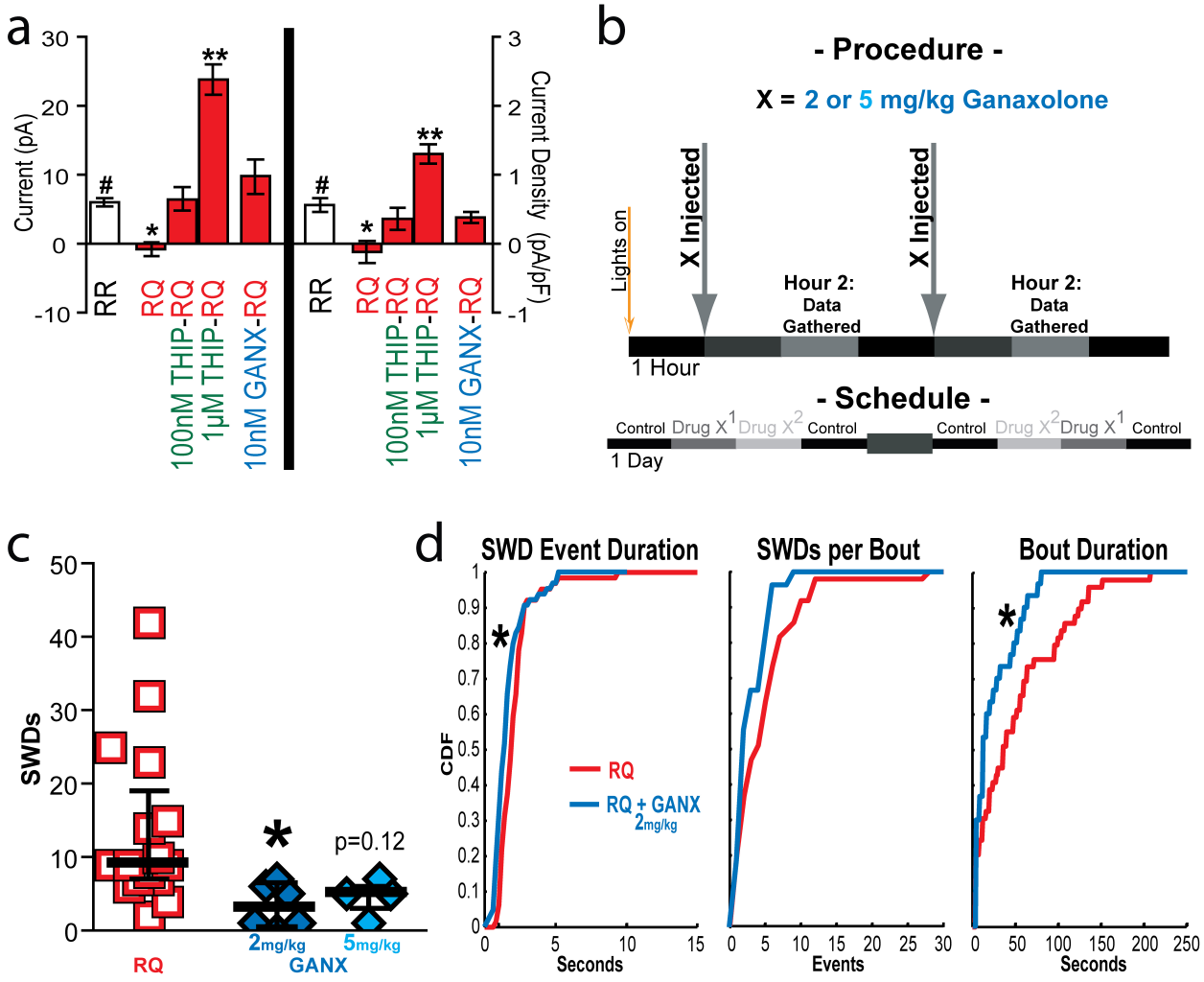

Fig. 5. Rescuing cortical tonic inhibition alleviates SWDs in RQ mice. (a) Voltage-clamp experiments reveal that tonic current amplitude (left y-axis) and density (right y-axis) levels can be rescued in RQ (red bars) cortical layer 2/3 neurons with 100 nM THIP or 10 nM ganaxolone (GANX) treatment, whereas a 1 µM THIP produces 2–4 times more holding current amplitude (**$p < 0.01$) and density (**$p < 0.01$) than that observed in untreated RR neurons (#). (b) Schematic depicts administration and data collection (Hour 2) times and drug-day schedules investigating treatment conditions for RQ mice. GANX (2 and 5 mg/kg) solutions were i.p. injected into RQ mice twice a day for 4 out of 7 days. (c) RQ-SWD event quantification shows that the 2 mg/kg GANX (*$p < 0.05$) (Dark Blue) treatment decreased SWD expression compared to control hours, while 5 mg/kg GANX ($p = 0.12$) (Light Blue) treatment trends towards ameliorating SWD expression. (d) Cumulative distributions of RQ-SWD activity shows that SWD event duration (*$p < 0.05$) and seizure duration (*$p < 0.05$) were decreased with the 2 mg/kg GANX treatment.

tical tonic inhibition levels inversely regulate SWD expression. Therefore, the causal link between absence epilepsy and inhibitory tonic currents is at the least bidirectional: *increased* thalamic tonic inhibition [7] or *decreased* cortical tonic inhibition can both lead to epileptiform activity.

The link between absence seizures and increased δ subunit-associated $GABA_A$ receptor activation in thalamic relay neurons is well established [8–10]. The current leading hypothesis from this evidence is that persistent hyperpolarization of thalamic relay neurons favors T-type Ca^{2+} channel availability [7,45], making these neurons more susceptible to rhythmic bursting and insensitive to sensory input, considered to be a necessary condition for SWD generation [9,10]. Consistent with this hypothesis, ethosuximide and valproic acid, two different T-type Ca^{2+} channel blockers, decrease thalamic relay bursting and are currently the main treatment options to treat absence epilepsy. However, the efficacy of either drug for this condition is at only ~55% [4]. The evidence presented in this study suggests a second classification of absence seizure etiology, separate from altered thalamic activity, that likely accounts for at least a portion of the remaining ~45% of patients that are currently non-responsive to the main treatment options.

Our findings also suggest that SWDs are not linked to a specific $GABA_A$ receptor subtype ($\alpha5$ or δ), but rather linked to cortical tonic inhibitory tone. Rescuing cortical tonic inhibition in RQ mice via activation of δ-subunit-associated $GABA_A$ receptors with GANX, and the subsequent decrease in SWD expression (Fig. 5) indicates that SWD expression can be regulated by δ-subunit-associated tonic inhibition. Additionally, the selective decrease/block of $\alpha5$ subunit-associated inhibition (RRL6), which results in SWD expression (Fig. 4) indicates that SWDs can also be regulated by $\alpha5$ subunit-associated tonic inhibition levels. Collectively, these results provide good evidence that the gating control of SWD expression is not necessarily linked to any specific $GABA_A$ receptor subtype but rather to the general level of cortical tonic inhibitory tone.

Optimal Tonic Inhibition

Studies suggest a dichotomy of effects for neurosteroids in absence epilepsy: lower levels can ameliorate SWDs in RQ mice, whereas higher concentrations can result in SWD exacerbation or generation [20]. Based on the results from this study, we suggest a concentration-dependent relationship of thalamocortical tonic inhibition in regard to SWDs and absence seizure modulation. Evaluation of genetic (Genetic Absence Epilepsy Rat from Strasbourg (GAERS), stargazer, lethargic) and pharmaco-induced (GHB, PTZ) rodent models of absence epilepsy provide ample evidence that excessive thalamic tonic inhibition triggers SWDs [7,31]. However, the novel RRL6-absence animal model introduced here, and the beneficial effects of low-dose GANX treatment in RQ mice, combine to indicate that a reduction in cortical tonic inhibition also results in SWDs. These findings suggest that an 'optimal level' of tonic inhibition in the thalamocortical circuit is a requirement for normal function and that deviation either above or below this optimal range results in aberrant thalamocortical function, SWDs and absence seizures.

5. Conclusion

Transcranial magnetic stimulation (TMS) studies of human patients harboring the γ2R43Q mutation display evidence of a hyperexcitable cortex, increased intracortical excitability and facilitation, and a decreased intracortical inhibition [33]. This evidence is in-line with the conclusion that these patients display a reduced expression of cortical tonic inhibition and that low-dose ganaxolone treatments might be beneficial in helping control seizure outbreaks. Although the number of individuals harboring the γ2R43Q mutation makes up only a small percentage of all those that suffer absence seizures, there are recent findings that provide optimism that a larger portion of the general absence population would respond positively to this same low-dose ganaxolone treatment. One recent study employed a thalamocortical computational model that was optimized via neuronal dynamics captured and measured during SWD events observed in polygenic (GAERS) or pharmaco-induced (GHB) absence seizure animals. This investigation concluded that the synchronous, seizure-perpetuating output of thalamic relay neurons during SWDs is not governed by intrinsic T-type Ca^{2+} channel bursting behaviors but rather by the excitability and top-down driving power of cortical pyramidal neurons [15]. This discovery is significant because it shifts the regional control of SWD generation and expression into the cortex for two well-studied absence rodent models (GAERS, GHB) previously regarded as primary evidence that enhanced thalamic tonic inhibition is the "necessary and sufficient" hallmark of typical absence epilepsy [9]. Although it has yet to be investigated, it is possible that a low-dose ganaxolone treatment would ameliorate the SWDs observed within these two absence rodent models, as well as within human patients with absence epilepsy that are refractory to the current first-line medications.

Availability of Data and Materials

All data points generated or analyzed during this study are included in this article and there are no further underlying data necessary to reproduce the results.

Author Contributions

KPM and MVJ jointly conceived and designed all experiments for this study with guidance from ABN, SP and CC. SP developed and provided the RQ mouse model. KPM and ABN performed experiments. KPM, MVJ, ABN and CC analyzed data. KPM and MVJ wrote the manuscript. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

All animal procedures followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved (Animal Welfare Assurance No. A3368-01) by the IACUC of the University of Wisconsin-Madison. Facilities were inspected and accredited by AAALAC. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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Conflict of Interest

The authors disclose that KPM, MVJ, ABN, and CC are included on the United States patent US9629853B2 titled "Uses of Ganaxolone". The authors declare no conflict of interest.

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