GABAergic Tonic Inhibition, Thalamocortical Function, and Absence Epilepsy.

By

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ABSTRACT

One in every 10 persons will suffer a seizure. One in every one hundred people develops epilepsy in their lifetime. Epilepsy can manifest at any age and via a vast range of mechanisms, including genetics, stroke, diabetes, trauma, sleep apnea, barometric pressure, music, lights, and in some cases, just because; in two-thirds of those inflicted with epilepsy, the cause is unknown. Thirty percent of those diagnosed with epilepsy are children, and of this population, ten percent will be diagnosed with a form appropriately titled Childhood Absence Epilepsy (CAE). CAE is genetically determined and characterized by brief periods of behavioral arrest, accompanied by glassy staring and an 'absence' of cognitive function. Absence seizures are petit mal, generalized (i.e. appear bilaterally across cortical EEG leads) seizures that present without overt convulsions and are hallmarked by the occurrence of spike-and-wave discharges (~3 Hz) that reverberate between the thalamus and cortex.

Under normal conditions, the thalamocortical circuit underlies our experience of consciousness. However, absence epileptics transition conscious states rapidly and transiently, and thus, implicate an aberrant thalamocortical circuit in this epilepsy. The mammalian brain works appropriately only when there is a proper balance between excitation and inhibition. An imbalance in the excitatory-inhibitory (E/I) ratio is associated with abnormal sensory processing and unconsciousness. Decreases in the E/I ratio have been linked to abnormalities such as impaired social interaction and autistic behaviors, and mental retardation (Rett Syndrome). Equally, increased E/I ratios lead to prolonged neocortical circuit activity, stimulus hypersensitivity, cognitive impairments and epilepsy.

The investigations presented in this thesis focus on how a loss of or decrease in a tonic (constant) form of inhibition in the CNS, and in particular within the thalamocortical circuit, spontaneously leads to transitory episodes of unconsciousness. Findings of our investigations elucidate a new classification of absence epilepsy, marked by discovery of a novel absence animal model, and the breakthrough of a successful treatment for this newly defined subclass, which has the potential to remedy the substantial (~40%) ineffectiveness of current anti-absence medications.

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CHAPTER 1

Background and Rational.

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INTRODUCTION

The mammalian brain works appropriately only when there is a proper balance between excitation and inhibition. An imbalance in the excitatory-inhibitory (E/I) ratio is associated with abnormal sensory processing and unconsciousness (Zhang and Sun, 2011; Massimini et al., 2012). Increased E/I ratios lead to prolonged neocortical circuit activity, stimulus hypersensitivity, cognitive impairments and epilepsy (Hagerman and Hagerman, 2002; Gibson et al., 2008: reviewed in Zhang and Sun, 2011). Equally, decreases in the E/I ratio have been linked to abnormalities such as impaired social interaction and autistic behaviors, and mental retardation (Rett Syndrome) (Tabuchi et al., 2007; Dani et al., 2005; reviewed in Zhang and Sun, 2011). It has been well studied and determined that the E/I ratio changes during development, with excitation decreasing and inhibition increasing, and that deviations in these changes for either can disrupt the E/I ratio (reviewed in Zhang and Sun, 2011). The investigations and findings presented in this dissertation focus on how altered inhibition in the CNS spontaneously leads to transitory episodes of unconsciousness.

INHIBITION IN THE CNS

The $GABA_A$ receptor is the main inhibitory neurotransmitter receptor in the central nervous system (CNS). Most fast synaptic inhibition in the mammalian CNS is mediated by the neurotransmitter GABA (γ-aminobutyric acid) via activation of the $GABA_A$ receptor. The receptor is a pentameric transmembrane protein, containing an integral chloride ion channel. Synaptic release of GABA, and subsequent binding to an

extracellular binding site, leads to activation, then desensitization of the receptor. The increased chloride conductance produces a resistive shunt and hyperpolarization that inhibit action potential generation. In addition, $GABA_A$ receptor-mediated inhibitory postsynaptic currents (IPSCs) are critically involved in generating rhythmic activity that is highly correlated with cognitive states (Traub et al., 1989; Mizumori et al., 1990; Cobb et al., 1995; Ylinen et al., 1995; Penttonen et al., 1998; Traub et al., 1999; Whittington et al., 2000). Enhancing receptor function produces sedation or unconsciousness, whereas receptor blockade or malfunction results in seizures (Cheng and Brunner, 1985; Olsen et al., 1999; Bernard et al., 2000). Mutations in this receptor are associated with heritable epilepsy (Macdonald et al. 2006).

GABAergic inhibition comes in 'phasic' and 'tonic' forms (Mody and Pearce, 2004). Phasic inhibition is the classical IPSC produced by synaptic transmission, whereas tonic inhibition is a constant receptor activation by low levels (e.g., ~1 µM) of ambient GABA. Phasic inhibition is involved in regulating temporal aspects of neural activity, such as spike timing and synchronous oscillations associated with specific cognitive states (Traub et al., 1989; Cobb et al., 1995; Whittington et al., 2000). Tonic inhibition, in contrast, provides a constant shunt and hyperpolarization, and thus is thought to shunt membrane potentials, decrease membrane resistance, increase the amount of current required for action potential generation (i.e. rheobase) (Bonin et al., 2007), and regulate network excitability (Coulter and Carlson, 2007, Bonin et al., 2007; Semyanov et al., 2003; Marchionni et al., 2007; Nieto-Gonzalez et al., 2010). Changes in tonic inhibition are associated with changes in membrane resistance (Semyanov et al., 2003) and neuronal excitability (Marchionni et al., 2007; Nieto-Gonzalez et al., 2010). Tonic

inhibitory currents are mediated by α5 or δ subunit-containing $GABA_A$ receptors, both of which can be located outside of the synapse (extrasynaptic). Global knockout of the α5 subunit results in hyperexcitable networks and compensatory up-regulation of the δ subunit (Glykys and Mody, 2006).

Tonic inhibition has recently been linked to spike-and-wave discharge (SWD) generation and absence seizures (Cope et al., 2009). Multiple rodent models of absence epilepsy (GAERS, stargazer, lethargic, tottering) display *enhanced* thalamic inhibitory tonic currents, and selective activation of this current produces SWDs and absence seizures in rats (Cope et al., 2009; Errington et al., 2011). These discoveries have lead to the conclusion that enhanced thalamic tonic inhibition is a "necessary and sufficient condition for nonconvulsive typical absence seizure generation" (Crunelli et al., 2011; Errington et al., 2011).

It is noteworthy here to point out that thalamic relay neurons can fire in distinct 'tonic' and 'burst' modes, and that enhanced tonic inhibition may shift thalamic relay neurons into the burst firing mode. Tonic inhibition regulates neuronal membrane potentials and can transition thalamic relay neurons between firing modes (Cope et al., 2005). The tonic firing mode results from a depolarized membrane potential and produces classic sodium channel-based action potentials. Conversely, a hyperpolarized membrane potential deinactivates T-type calcium channels and, upon repolarization, produces a calcium channel-based potential plateau that induces a high frequency 'burst' of sodium channel-based action potentials. The transition between tonic and burst firing modes in thalamic relay neurons, and the preferential expression of one mode over the other, has implications in sensory processing, perception, and cognitive states (i.e.

wake/sleep). The finding that enhanced thalamic tonic currents promotes absence seizures, which include brief moments of unconsciousness (explained in detail below), exemplifies the influence tonic inhibition can have on human behavior.

ABSENCE EPILPESY

Absence epilepsy (AE) is characterized behaviorally by loss of consciousness (absence) without overt convulsions that is typically accompanied by an electrographic ~3 Hz spike-and-wave discharge (SWD) signature that presents bilaterally on an electroencephalogram (EEG) (McCormick and Contreras, 2001; Panayiotopoulous, 2008). The loss of consciousness associated with these seizures is accompanied by gaze fixation and a sudden stoppage of movement, and includes a sudden onset and termination without any noticeable triggers. AE prevalence is ~1 in 50,000, accounts for ~10% of seizures in children with epilepsy and presents in three types: childhood AE, juvenile AE, and epilepsy with myoclonic absence (Jallon et al, 2005; Panayiotopoulous, 2008). Juvenile AE onset ranges from 9 – 21 years of age and mainline anti-absence medication (valproic acid) treatment leads to lasting SWD cessation and normal cognitive ability years later in the majority of patients. Childhood AE (CAE) manifests most frequently between 4-9 years of age, though it can be observed earlier, and affects females predominantly. The loss of consciousness in CAE usually lasts 5-30 seconds and is repeated several times a daily. Prognosis is positive, with cessation of seizures in ~60% of these patients treated with anti-absence mediations. Typical CAE medications include ethosuximide, valproic acid, and also phenytoin and lamotrigine (Lenkov et al., 2012).

ABSENCE EPILPESY AND THE THALAMOCORTICAL CIRCUIT

The thalamocortical circuit underlies our experience of consciousness, with the cortex anchoring the majority of the load. Thalamocortical connection profiles change during the transition to unconsciousness, elucidated via anesthetic studies, with cortical-thalamic connectivity building while cortical-cortical communication becomes disrupted (Alkire et al., 2008; Kim et al., 2011). Absence epileptics transition conscious states rapidly and transiently, implicating an aberrant thalamocortical circuit in these patients.

Absence seizures are petit mal, generalized seizures (i.e. appear bilaterally across cortical EEG leads) involving synchronous SWDs at ~3 Hz. These SWDs include synchronous bursts of neuronal populations in, and between, the cortex and thalamus, with full incorporation of the thalamocortical loop being required for SWDs associated with absence epilepsy (reviewed in McCormick and Contreras, 2001; Huguenard and McCormick, 2001). The current leading explanations for absence seizure and SWD generation are the Cortical Focus Theory (Meeren et al., 2002) and a thalamus-focused theory, originally known as the 'thalamic clock' theory, which argues altered thalamic function underlies seizure generation (Cope et al., 2009; Errington et al., 2011).

Although SWD origin is still controversial, it is likely that the cellular and network mechanisms responsible for sustaining the characteristic ~3 Hz SWD reside in the thalamus, because this region is capable of generating such discharges *in vitro* (Destexhe et al., 1996; McCormick and Contreras, 2001). These rhythms involve a) intrinsic membrane conductances, in particular T-type Ca^{2+} channels, that allow thalamic neurons to fire rebound bursts, b) reciprocal excitation and inhibition between thalamocortical relay neurons and GABAergic neurons in the Thalamic Reticular

Nucleus (TRN), and c) lateral GABAergic inhibition between TRN neurons. The mechanistic framework involves a ping-pong-like alternation of excitation and inhibition, as follows:

1) Thalamocortical relay cells receive large GABAergic inhibitory postsynaptic potentials (IPSPs, mediated by both $GABA_A$ and $GABA_B$ receptors) from GABAergic neurons in TRN. This hyperpolarization removes T-type inactivation, and is followed by rebound bursts (T-burst) in the relay cells. There are also extrasynaptic $GABA_A$ receptors on relay cells that contain $\alpha4$ and δ subunits and mediate a tonic inhibitory current (Cope et al., 2005; Chandra et al., 2006).

2) Activation of T-type Ca^{2+} channels in thalamic relay neurons provides a plateau depolarization, upon which bursts of spikes can be launched. However, because of T-type channel inactivation, these bursts can only occur if preceded by a large hyperpolarization that removes inactivation.

3) Relay cells project to TRN (and also to cortex) where they provide excitatory drive that initiates T-type channel-dependent bursts that in turn drive the IPSPs that hyperpolarize relay cells and allow them to fire another rebound burst.

4) TRN cells laterally inhibit each other via $GABA_A$ receptor-mediated IPSCs. This lateral inhibition ensures that only a small fraction of TRN cells may participate in an individual burst. TRN cells are not thought to express any tonic inhibitory current (Cope et al., 2005).

5) Finally, cortical cells project excitatory synapses to both TRN cells and relay cells, thus the effect of cortical input is to activate the intrinsic rhythmic firing of the thalamic circuit.

These properties allow restricted subregions of thalamic circuitry to engage in rhythmic, partially synchronous bursting activity that is presumably required for thalamocortical information processing under normal conditions and produces pathological reverberations during SWDs. Although the looping connections between the cortex and thalamus are three dimensional *in vivo*, an *in vitro* thalamocortical brain slice has been discovered that preserves some connectivity between these regions (Agmon & Connors, 1991; Krook-Magnuson et al., 2008).

CAE CAUSED BY THE γ2R43Q MUTATION IN THE GABA$_A$ RECEPTOR

Several heritable human epilepsies have recently been traced to mutations in the GABA$_A$ receptor (reviewed in Macdonald et al., 2006). Of these mutations, the one that has received the most study is an arginine-to-glutamine substitution at position 43 of the γ2 subunit (γ2R43Q) (Wallace et al., 2001). Human patients harboring the γ2R43Q mutation present with a variety of epileptic phenotypes, the most common being CAE and febrile seizures, but also including myoclonic seizures, myoclonic astatic epilepsy, atonic seizures, partial seizures, and the very severe Dravet's syndrome (Wallace et al., 2001; Scheffer and Berkovic, 1997; Singh et al., 1999). Studies of these patients show evidence of a hyperexcitable cortex, increased intracortical excitability and facilitation, and decreased intracortical inhibition (Fedi et al., 2008). This evidence matches the hypothesis that a hyperexcitable cortical condition is thought to contribute to the SWDs seen in patients with this mutation (Marini et al., 2003). Studies using heterologous expression systems (oocytes, HEK293 and COS7 cells) investigating the functional consequences of the γ2R43Q mutation uncovered alterations in receptor function,

assembly, trafficking and surface expression (Bowser et al., 2002; Goldschen-Ohm et al., 2010; Kang & Macdonald, 2004; Sancar & Czajkowski, 2004; Hales et al., 2005; Eugene et al., 2007). Interestingly, this γ2 subunit mutation decreases trafficking of multiple GABA$_A$ subunits, including the α5 subunit (Eugene et al., 2007), which as mentioned earlier is an extrasynaptic receptor responsible for tonic inhibition.

Our collaborator Dr. Steven Petrou has provided us with γ2R43Q knock-in mice. The human patients affected with the γ2R43Q mutation are all heterozygous, and heterozygous (RQ) knock-in mice display absence–like seizures and generalized EEG SWDs that are similar to their human counterparts (Tan et al., 2007). The mice also have an early developmental onset of seizure susceptibility, consistent with the CAE observed in humans (Chiu et al., 2008), and seizure severity that depends on their genetic background, also consistent with the multiplicity of seizure phenotypes observed in the affected human families (Tan et al., 2008). Thus, these animals are an excellent model for CAE and the first model, to our knowledge, whose epilepsy is caused by introduction of a human epilepsy gene.

QUESTIONS AND RATIONAL

Surprisingly, it was discovered that synaptic inhibition in brain slices from γ2R43Q knock-in mice is only subtly affected, with the mutation producing only a ~15% reduction in IPSC peak amplitude (Tan et al., 2007). Could this *subtle* reduction in synaptic inhibition really be the root cause of SWD and absence seizure generation? Another notable discovery, on the other hand, is the consensus of many laboratories that this mutation alters receptor trafficking and surface expression of GABA$_A$ receptors, and

most importantly, α5 subunit-containing GABA$_A$ receptors (Eugene et al., 2007). The investigators that found the mutation reduced α5 subunit-containing GABA$_A$ receptor surface expression also found consequential reductions in tonic GABAergic currents. A caveat to their investigation, however, is that γ2R43Q knock-in mouse neurons were not used but instead, hippocampal-cultured γ2R43Q-transfected neurons were employed. Thus, the question still remained as to whether the γ2R43Q mutation was actually reducing surface expression of α5 subunit-containing GABA$_A$ receptors and their associated tonic currents within the mammalian brain. And furthermore, if this were the case, could the reduction of tonic GABAergic currents possibly be underlying the hyperexcitable cortical environment, SWDs and absence seizures displayed by humans harboring this mutation?

One of the earliest discoveries by this investigator into how the γ2R43Q mutation confers absence epilepsy and SWDs was that yes, indeed, mammalian neurons in γ2R43Q knock-in mouse brain slices *lack* tonic inhibition (Chapter 2). Ensuing from this initial find was a bidirectional inquiry into how the mutation abolished tonic inhibition and how this loss of tonic inhibition manifests absence seizures. To investigate, we utilized multiple methods that span molecular, cellular, network and *in vivo* levels.

INVESTIGATIONS

In the first wave of our investigation, presented in Chapter 2, we use whole-cell patch clamp electrophysiology to show that γ2R43Q principal neurons of the thalamocortical circuit display a *subtle* reduction in IPSC amplitude but *lack* tonic inhibition. Using Western blotting, we assess subcellular protein levels to show that the

mutation does indeed alter the trafficking of many GABA$_A$ receptor subtypes, a finding that corroborates previous investigations. We then use multielectrode array recordings in thalamocortical brain slices to show that a reduction in tonic inhibition manifests as hyperexcited cortical, and decreased thalamic T-bursting, neuronal behaviors.

In Chapter 3 we move our investigation into the mouse and use video-EEG recordings to assess *in vivo* SWDs and present a novel absence animal model, which reveals that a reduction in cortical tonic inhibition is enough to generate SWDs. We then show that pharmacological rescue of the lost tonic inhibition in γ2R43Q mice suppresses SWD expression. To achieve this rescue, we use whole-cell patch clamp electrophysiology to titrate the level of a synthetic neurosteroid, Ganaxolone, required to activate wild-type levels of tonic inhibition in γ2R43Q knock-in mice. The results of this investigation leads us to hypothesize that an optimum level of tonic inhibition in the thalamocortical circuit is required for normal functioning and any deviation from this optimum, either less or more, results in aberrant thalamocortical function, SWDs and absence seizures.

In the final investigative chapter (4), we return back to the thalamocortical brain slice and multielectrode array recordings to elucidate the neuronal and population features that are altered upon deviations from the optimal level of tonic inhibition in the thalamocortical circuit. Our results suggest that absence epilepsies characterized by decreased tonic inhibition produce a hypercommunicative cortex that underlies SWD generation; a consequence that can be successfully treated with low doses of Ganaxolone.

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CHAPTER 2

Tonic Inhibition is Abolished in GABA$_A$ Receptor γ2R43Q Knock-in Mice with Absence Epilepsy and Febrile Seizures.

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ABSTRACT

The γ2R43Q GABA$_A$ receptor mutation confers absence epilepsy in humans, and γ2R43Q knock-in mice (RQ) display absence seizures and generalized spike-and-wave discharges reminiscent of their human counterparts. Previous work on several rodent models led to the conclusion that elevated tonic inhibition in thalamic neurons is necessary and sufficient to produce typical absence epilepsy. In contrast, here we used patch-clamp electrophysiology in brain slices to show that RQ mice entirely lack tonic inhibition in principal cells of layer II/III somatosensory cortex and ventrobasal thalamus. Additionally, protein quantification and multielectrode electrophysiology show that the mutation interferes with trafficking of GABA$_A$ receptor subunits involved in generating tonic currents, leading to increased cortical firing and decreased thalamic bursting rates. Together with previous work, our results suggest that an optimum level of tonic inhibition is required for normal thalamocortical function, such that deviations in either direction away from this optimum enhance susceptibility to absence seizures.

INTRODUCTION

Several human epilepsies have been traced to mutations in the GABA$_A$ receptor[1,2], a pentameric transmembrane protein containing an integral chloride ion channel that regulates action potential generation via shunting or hyperpolarization. The mutation that has received the most study is an arginine-to-glutamine substitution at position 43 of the γ2 subunit (γ2R43Q)[3]. Human patients harboring the γ2R43Q mutation present symptoms from a variety of epileptic phenotypes, the most common being Childhood Absence Epilepsy (CAE) and febrile seizures[3]. γ2R43Q knock-in mice (RQ) display absence seizures and generalized EEG spike-and-wave discharges (SWDs) similar to pathology exhibited by humans[4] (see Sup. Fig.). Absence seizures consist of brief losses of consciousness typically lasting 2-15 s, along with bilateral, synchronous 3-Hz spike-and-wave discharges (SWDs)[5]. Human patients with the γ2R43Q mutation show evidence of a hyperexcitable cortex compared to unaffected family members, displaying increased intracortical excitability, decreased intracortical inhibition and increased facilitation in response to paired-pulse stimulation[6]. These findings support the hypothesis that a hyperexcitable cortical condition is thought to contribute to SWDs in these patients[7]. A similar "cortical focus theory" for absence seizures was proposed after SWD generation was localized to the somatosensory cortex in a different mouse model[8,9]. The exact origin of SWDs in γ2R43Q human patients has not been identified.

The functional effects of the γ2R43Q mutation have been studied in heterologous expression systems (oocytes, HEK293 and COS7 cells), but have led to conflicting results. On one hand, the γ2R43Q mutation has been shown to alter receptor function by slowing receptor deactivation, enhancing desensitization, and reducing benzodiazapine

sensitivity[10,11]. However, others have observed little effect on receptor function[12]. In contrast, several studies agree that the mutation alters GABA$_A$ receptor assembly, trafficking or surface expression[12,13,14,15,16,17]. Interestingly, this mutation in the γ2 subunit appears to also affect trafficking of other subunits including α1, α3, β2, β3, and α5[13,16,17]. The α5 subunit participates in extrasynaptic tonic inhibition in cortex, and is decreased by the γ2R43Q mutation[16]. Whether the γ2R43Q mutation also affects trafficking of the δ subunit, which contributes to thalamic tonic inhibition, is not yet known.

Tonic inhibition has recently been linked to SWD generation and absence seizures[18]. Multiple rodent models of absence epilepsy (GAERS, stargazer, lethargic, tottering) display increases in thalamic inhibitory tonic current, and selective activation of this current produces SWDs and absence seizures in rats[18,19]. To understand how altered thalamic inhibitory tonic currents could produce SWDs, we must consider the anatomy and functional connectivity of neurons in the thalamocortical network.

Thalamic relay neurons can fire in distinct 'tonic' and 'burst' modes. The tonic firing mode occurs when the membrane is steadily depolarized, and consists of classical sodium channel-dependent action potentials. The burst firing mode, in comparison, occurs when the membrane is hyperpolarized such that T-type voltage-gated calcium channels are allowed to deinactivate. A subsequent depolarization then results in a high frequency burst of sodium channel-dependent action potentials riding atop a calcium channel-dependent plateau potential. Thus, increased hyperpolarizing tonic inhibition may shift thalamic relay neurons into the burst firing mode[20], which may increase the drive onto GABAergic thalamic reticular nucleus (TRN) neurons. In turn, TRN neurons

transmit hyperpolarizing IPSPs back onto thalamic relay neurons, further promoting relay neuron burst firing. This reverberation between relay and TRN neurons is critical for sustaining SWDs[21,22,23]. Indeed, even in studies supporting a cortical origin of SWDs[9], seizure activity spread to the thalamus within a few hundred milliseconds, consistent with the idea that robust absence seizures are a product of the full thalamocortical network[22,24].

Here we show, using thalamocortical slices, that tonic inhibition is abolished in layer II/III neurons of somatosensory cortex and relay neurons of ventrobasal thalamus of RQ mice. Through Western blotting and voltage-clamp electrophysiology, we show that the loss of tonic inhibition is accompanied by altered expression or trafficking of the GABA$_A$ receptor subunits responsible for mediating tonic currents in these areas. Using multielectrode arrays, we further show that loss of tonic inhibition increases cortical firing rates, but decreases bursting throughout the thalamocortical circuit, consistent with a depolarization of thalamic relay neurons that shifts them away from the burst firing mode. Selective pharmacological blockade of cortical tonic current in wild type (RR) slices also increases cortical firing rates, paralleling the increased cortical firing in RQ slices, and consistent with the increased cortical excitability observed in γ2R43Q human patients. Together these results suggest that the combined loss of cortical and thalamic tonic inhibition in RQ mice enhances susceptibility to absence seizures.

RESULTS

Synaptic inhibition is reduced in RQ thalamus and cortex.

The RQ mutation hinders GABA$_A$ receptor assembly, trafficking and surface expression[12,13,14,15,16,17], and decreases cortical mIPSC amplitude in RQ mice[4]. Our analysis of mIPSCs corroborated the latter finding, showing a decrease (31%) in mIPSC amplitude in somatosensory cortical layer II/III neurons (mean ± SEM in pA, N; RR: 26.2 ± 2.9, 9; RQ: 18.1 ± 1.4, 8, p<0.05; Fig. 1a & 1b) and also a decrease (34%) in thalamic relay neurons (RR: 41.1 ± 4.8, 9; RQ: 27.1 ± 2.1, 7, p<0.05; Fig. 1d & 1e). mIPSC frequency was unaffected in both areas (Fig. 1c & 1f). Weighted decay time-constants for cortical layer II/III neurons are not different for RQ (mean ± SEM in ms, N; 24.0 ± 1.6, 4) compared to RR (19.0 ± 2.2, 5), but are increased for RQ thalamic neurons compared to RR (RR: 8.5 ± 0.8, 7; RQ: 13.8 ± 0.6, 5, p<0.001) (data not shown).

GABAergic tonic inhibition is abolished in RQ cortical and thalamic neurons.

Although reductions in synaptic inhibition are a potential mechanism for hyperexceitability and absence epilepsy in the RQ mice, the γ2R43Q mutation may affect other processes as well. For example, based on studies in transfected cultured neurons, Eugène et al.[16] proposed that this mutation may contribute to absence epilepsy by reducing tonic inhibition. Therefore, to directly test this hypothesis in an animal model, we examined tonic inhibition in slices from RR and RQ 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 mutant somatosensory

cortical layer II/III neurons (mean ± SEM in pA, N) (RR: 5.8 ± 0.6, 5; RQ: -1.2 ± 1.6, 4, p<0.05; Fig. 2a & 2b), as well as in thalamic relay neurons (RR 10.6 ± 3.6, 9; RQ: 0.6 ± 0.7, 6, p<0.05; Fig. 2c & 2d). One-sample t-test indicated that tonic currents in RQ were indistinguishable from zero (p>0.45).

GABA$_A$ receptor function or expression is altered in a region-specific manner.

The tonic current in RR somatosensory cortical layer II/III cells (Fig. 2a) was completely blocked by the α5 subunit-selective inverse agonist L655,708 (30 μM; Fig.3a), matching previous studies showing that the α5 subunit is responsible for most or all of the native tonic inhibition in these neurons[25]. Thus the loss of cortical tonic inhibition in RQ mice may involve reduced expression or function of the α5 subunit (see below).

In contrast, application of the agonist THIP (1 μM, a concentration previously shown to be selective for δ subunit-containing receptors[20,26]) evoked currents of similar magnitude in RR and RQ cortical neurons (mean ± SEM in pA, N) (RR: 21.4 ± 5.7, 4; RQ: 23.8 ± 2.2, 5; p=0.67; Fig. 3b). A similar profile of effects was observed with allopregnanolone (30 nM; Fig. 3c), a neurosteroid that also selectively activates δ subunit-containing receptors[27,28]. 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, potentially providing pharmacological avenues to rescue cortical tonic inhibition in cases where it has been genetically compromised.

In contrast to cortical layer II/III neurons, thalamic relay neurons rely solely on δ subunit-containing GABA$_A$ receptors to produce inhibitory tonic currents[18,20,29,30]. We found that RQ thalamic neurons responded to THIP with 47% of the current produced in RR thalamic neurons (mean ± SEM in pA, N) (RR: 131.7 ± 31.2, 5; RQ: 69.3 ± 22.4, 4, p<0.05; Fig. 3d). Similarly, in RQ thalamic neurons, allopregnanolone produced 39% of the current observed in RR (RR: 34.7 ± 6.5, 5; RQ: 13.7 ± 3.8, 3, p<0.05; Fig. 3e). These results suggest that δ subunit-containing GABA$_A$ receptors are either expressed at lower levels, or have reduced activation, in thalamic relay neurons of RQ mice compared to RR mice.

Expression of GABA$_A$ receptor subunit proteins involved in tonic inhibition is reduced in RQ neurons.

To test whether the loss of tonic inhibition in cortical and thalamic neurons was related to changes in the GABA$_A$ receptor subunit proteins involved in tonic inhibition, we examined the levels of these proteins in whole tissue subcellular fractions (plasma membrane (PM) and intracellular organelles (ER)) using Western blotting. We calculated 'total protein' (PM + ER) and 'surface trafficking' (PM/ER) levels for all proteins assessed (α1, α4, α5, γ2, and δ). Surface trafficking was further normalized to α1-trafficking levels because previous research showed that the R43Q mutation did not alter α1 membrane trafficking[4]. We found that RQ somatosensory cortex showed a marked decrease in total α5 subunit protein expression (mean ± SEM fraction of RR expression, N) (0.66 ± 0.14, 8, p<0.05), as well as a decrease in total γ2 subunit expression (0.76 ±

0.07, 4, p<0.05; Fig. 4b). Membrane surface trafficking was also reduced for the α4 (RQ: .59 ± .10, 8. P<0.05), α5 (RQ: 0.53 ± 0.11, 6, p<0.05), and δ (RQ: 0.60 ± 0.09, 6, p<0.05) (Fig. 4c) subunit proteins. Thus, the loss of cortical tonic inhibition we observed is consistent with the reduced expression of α5 subunits in cortical neuronal surface membranes.

In contrast to the cortex, thalamic tonic inhibition is mediated by receptors containing the obligatory pairing of GABA$_A$ receptor α4 and δ subunits. We found that RQ thalamus showed a reduction in total δ subunit protein levels (0.78 ± 0.07, 6, p<0.05; Fig. 4B). Although we did not find a reduction in the trafficking of thalamic δ subunit protein (1.17 ± 0.35, 4, p=0.72), there was a decrease in surface trafficking of α4 subunits (0.40 ± 0.06, 5, p<0.05; Fig. 4c). Unlike RQ somatosensory cortex, RQ thalamus showed an increase in total γ2 subunit levels (1.27 ± 0.08, 6, p<0.05; Fig. 4b), but a decrease in γ2 subunit trafficking to the membrane surface (0.43 ± 0.15, 4, p<0.05; Fig. 4c).

Firing rates and bursting behaviors are altered in RQ thalamocortical slices.

Although tonic inhibition is known to contribute to neuronal responsiveness, its role in thalamocortical network activity has not been studied in detail. To explore this role, we used multielectrode extracellular recording arrays to examine neuronal spiking and burst firing in somatosensory cortex and ventrobasal thalamus of RR and RQ thalamocortical slices, as well as RR thalamocortical slices treated with L655,708 (30 μM) to selectively block α5 subunit-mediated tonic current in cortical neurons (RR-L655). Cumulative distribution plots of the average firing rates in cortical neurons show

increased firing rates for RQ (median rate in Hz, [25 : 75 percentiles], N; Kruskal-Wallis p-value) (0.06, [0.02 : 0.14], 230, p<0.01) and RR-L655 (0.17, [0.06 : 0.22], 96, p<0.01) compared with RR (0.04, [0.01 : 0.09], 328; Fig. 5c). Conversely, RQ thalamic neurons displayed decreased firing rates (0.02, [0.00 : 0.13], 216, p<0.01) compared to RR (0.11, [0.03 : 0.52], 444), whereas RR-L655 thalamic neurons did not (0.18, [0.02 : 0.73], 122; Fig. 5c).

We assessed bursting activity using two definitions of bursts: i) 'generic' bursts, reflecting any tendency to fire in groups of spikes, and ii) 'T-bursts', reflecting the temporal structure characteristic of thalamic relay neurons firing in burst mode, mediated by T-type calcium channel-dependent plateau potentials (see Methods). The 'burst fraction' quantified the probability that a neuron fired bursts versus lone spikes.

For generic bursts, the cortical burst fraction was lower than the thalamic burst fraction in all conditions (RR: p<0.01; RQ: p<0.05; RR-L655: p<0.05; Fig. 5d). The thalamic burst fraction was reduced in RQ compared to RR, but not in RR-L655 (RR: 0.43, [0.18 : 0.64], 130; RQ: 0.29, [0.09 : 0.49], 108, p<0.05; RR-L655: 0.35[0.20 : 0.59], 84; Fig. 5d). There were no differences observed among cortical burst fractions (RR: 0.21, [0.14 : 0.34], 99; RQ: 0.18, [0.10 : 0.32], 110; RR-L655: 0.23, [0.16 : 0.33], 80; Fig. 5d).

Similar to the generic burst fraction, RR cortex had a lower T-burst fraction (0.03, [0.01 : 0.09], 99, p<0.01) than RR thalamus (0.16, [0.08 : 0.31], 130, Fig. 5d). Selective blockage of cortical tonic inhibition in RR slices with L655,708 did not alter T-burst fraction in either cortex (0.03, [0.02 : 0.04], 80) or thalamus (0.15, [0.04 : 0.27], 84).

However, the T-burst fraction was lower in RQ thalamus compared with RR thalamus (0.05, [0.01 : 0.16], 108, p < 0.01; Fig. 5d), and was not significantly different than in RQ-cortex (0.03, [0.00 : 0.09], 110). Together these data suggest that thalamic, not cortical, tonic inhibition is a primary regulator of T-burst activity in the thalamocortical network.

Closer examination of T-bursts in RR slices revealed that thalamic neurons displayed more spikes per burst than cortex (cortex: 2, [2 : 2], 99; thalamus: 2, [2 : 3], 130; p<0.01); as well as longer burst durations (in ms; cortex: 4.1, [0.6 : 5.7], 99; thalamus: 5.1, [3.6 : 7.9], 130; p<0.01). Neither RQ nor RR-L655 neurons differed from control in the number of spikes per burst (RQ cortex: 2, [2 : 2], 110; RQ thalamus: 2, [2 : 3], 108; RR-L655 cortex: 2, [2 : 2], 80; RR-L655 thalamus: 3, [2 : 4], 84) or in burst durations (RQ cortex: 3.2, [0.8 : 6.0], 110; RQ thalamus: 6.4, [2.6 : 9.1], 108; RR-L655 cortex: 3.6, [1.5 : 5.2], 80; RR-L655 thalamus: 6.1, [3.5 : 8.9], 84). Similar to control, thalamus displayed more spikes per burst (p<0.01) and longer T-bursts (p<0.01) than cortex in both RQ and RR-L655.

Discussion

Our major findings are that mice expressing the γ2R43Q mutation entirely lack GABAergic tonic currents in both somatosensory cortical layer II/III pyramidal (Fig. 2a & b) and ventrobasal thalamic relay neurons (Fig. 2c & d), and that these deficiencies increase cortical firing rates (Fig. 5c) and decrease thalamic firing rates and T-bursting (Fig. 5d). The loss of tonic currents in RQ mice is correlated with decreases in surface

trafficking of different GABA$_A$ receptor subunits responsible for generating these currents in cortex and thalamus (Fig. 4). Selective pharmacological blockade of cortical tonic currents increased cortical firing rates as expected, but did not affect thalamic firing rates or bursting behaviors. These results are consistent with the loss of tonic currents causing neuronal depolarization that renders cortical neurons hyperexcitable and shifts thalamic relay neurons away from a burst-firing mode.

Mutation or over-expression of the γ2 subunit of the GABA$_A$ receptor was previously shown to interfere with receptor assembly or trafficking[13,14,15] of multiple GABA$_A$-subunits, including the α5 subunit that mediates tonic inhibition in mouse somatosensory cortex[16,25] and the δ subunit that mediates tonic inhibition in thalamus[18,20,29,30,31]. Our results match with these findings, showing a decrease in membrane trafficking for multiple subunits in the cortex (α5 & δ) and thalamus (α4 & γ2) (Fig. 4), and also complement previous evidence that the R43Q mutation impairs surface expression of functional GABA$_A$ receptors that could result in reduced synaptic inhibition (IPSCs)[12,13,14,15,16,17] (Fig. 1). In addition to absence epilepsy, however, this mutation also causes febrile seizures in humans and RQ mice[3,32,33]. Thus, the question arises as to whether the observed changes in tonic and phasic inhibition contribute differentially or synergistically to the absence and febrile seizure phenotypes. This issue is complicated somewhat by the variable penetrance of the absence phenotype, even amongst mice that share the C57Bl6 background[4,7,33,(present study)], possibly due to subtle differences in genetic background between colonies or in rearing conditions. Reid and colleagues (2013) recently showed that C57Bl6 RQ mice that do not display absence seizures continue to express febrile seizures, demonstrating that the two phenotypes are

dissociable in the presence of the mutation. The C57Bl6 RQ mice studied here have absence seizures (Sup. Fig. 1), have changes in both tonic and phasic inhibition and have altered thalamocortical signaling, but we have not yet measured their febrile seizure sensitivity. Thus, conclusive determination of whether the dissociation between the two phenotypes is more closely related to changes in tonic or phasic inhibition will require further research.

We showed that, in RR cortical neurons, the $\alpha5$ subunit-selective inverse agonist L655,708 blocked as much tonic current as did the broad-spectrum $GABA_A$ receptor antagonist bicuculline, confirming that most or all of the active tonic current in these neurons is mediated by $\alpha5$ subunit-containing receptors. Thus, the loss of tonic current in RQ cortical neurons is consistent with a reduction in protein expression and trafficking of the $\alpha5$ subunit in RQ, as confirmed by Western blotting (Fig. 4b & c).

We also showed that RQ thalamic neurons lack tonic currents. Furthermore, in these neurons, δ subunit-selective activators (i.e., THIP and allopregnanolone) produced less current in RQ compared to RR, suggesting dysfunction of δ subunit-containing receptors. Although Western blotting did not reveal a reduction in δ subunit surface trafficking, it did show a reduction in total δ subunit expression along with a reduction of $\alpha4$ subunit trafficking, which is the partner for the δ subunit required to form functional receptors that mediate tonic inhibition in thalamic neurons[30]. Therefore, we propose that the loss of tonic inhibition in cortical and thalamic neurons in mice expressing the mutant $\gamma2R43Q$ subunit is caused by a dysregulation of the assembly/trafficking of *non-mutant* subunits, namely $\alpha5$ in cortex and $\alpha4$ and δ in thalamus.

Reduction of inhibitory tonic currents is linked to membrane depolarization[34], increased neuronal firing[25] and enhanced synaptic summation[35]. Our findings that RQ somatosensory cortical layer II/III neurons lack inhibitory tonic current and exhibit increased firing rates are consistent with these previous conclusions and with the hyperexcitable cortex of human patients harboring the γ2R43Q mutation[6,7]. Although cortex and thalamus are both involved in SWDs, cortical hyperexcitability appears to be a prerequisite for SWD generation[8,9,36], and thus the loss of cortical tonic inhibition may be a key cause of the increased intracortical excitability, increased facilitation, and the development of SWDs seen in humans harboring the γ2R43Q mutation[6].

Thalamic relay neurons can function in either tonic or burst firing modes depending on the average membrane potential, which in turn can be influenced by the level of GABAergic tonic current[20]. Thus, depolarization resulting from the loss of tonic inhibition may shift thalamic neurons away from burst firing mode. Consistent with this idea, our multielectrode recordings revealed that RQ thalamic neurons have a reduced probability of burst firing compared with RR. Interestingly, the average thalamic firing rate was lower in RQ than in RR, suggesting that the depolarization caused by loss of tonic inhibition is relatively subtle: enough to reduce burst firing but not enough to itself promote strong tonic firing. Furthermore, selective blockade of cortical tonic inhibition with L655,708 increases the firing rate in cortex only, leaving thalamic firing and the bursting behaviors in both cortex and thalamus unaffected. Taken together, these results suggest that cortical and thalamic tonic inhibition have distinct and separable roles in regulating thalamocortical circuit function.

Previous work demonstrates a correlation between absence seizures and *enhanced* tonic inhibition in thalamic relay neurons of several rodent models[18], leading to the conclusion that enhanced tonic GABAergic inhibition is a "necessary and sufficient condition for nonconvulsive typical absence seizure generation"[37,38]. However, our finding that γ2R43Q knock-in mice entirely lack tonic inhibition in thalamic relay neurons demonstrates that enhanced thalamic tonic inhibition is not necessary to produce absence seizures. Instead, together with the aforementioned work, our data suggest that an optimal level of tonic inhibition throughout the thalamocortical circuit is necessary for normal thalamocortical processing, such that either increases or decreases away from this optimum are sufficient to enhance susceptibility to absence epilepsy. Importantly, we also show that cortical tonic inhibition is absent and that cortical neurons have elevated firing rates in RQ mice. Future genetic or pharmacological models of region-specific deficits in tonic inhibition will be helpful for dissecting the contributions of tonic inhibition in cortex versus thalamus to regulating absence epilepsy.

Despite the absence of endogenous tonic inhibition, we show that the δ subunit-selective activators THIP and allopregnanolone can recruit tonic currents in both thalamus and cortex of RQ mice. We therefore propose that absence epilepsies can be divided into multiple classes, two distinct examples of which are characterized by either an increase[18,19] or a decrease (e.g., γ2R43Q) in tonic inhibition. Therefore, appropriately titrated doses of tonic current activators may have high therapeutic benefit for rescuing normal function in the latter class.

FIGURE LEGENDS:

Figure 1 - mIPSC amplitude is decreased in RQ slices.

A) Example voltage-clamp trace (left) and corresponding miniature inhibitory postsynaptic currents (mIPSCs) (right) for a wild type (RR) (top: black) and a mutant (RQ) (bottom: grey) cortical layer II/III pyramidal cell. Overlaid white traces are the average mIPSCs. B) Cumulative amplitude distributions (top) and median mIPSC amplitudes (bottom) for cortical cells, showing a reduction in mIPSC amplitude for RQ compared to RR ($p < 0.05$, asterisk). Bars represent the mean of medians. C) Cumulative inter-event interval (IEI) distribution (top) and medians (bottom) for cortical neurons, showing no difference in RQ compared to RR. D-F) Same as A-C, but for thalamic relay neurons in RR and RQ slices.

Figure 2 - Tonic currents are abolished in RQ cortex and thalamus.

A) Example voltage-clamp traces for RR (above: black) and RQ (below: grey) cortical layer II/III cell recordings during 100 µM Bicuculline administration (grey 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) only on the right side of the distribution, thus omitting components due to phasic 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.

Figure 3 - RQ mice display region- and subunit-specific changes in tonic inhibition.

A) Example voltage-clamp traces for RR cortical layer II/III cell recordings during 30 µM L655,708 administration (grey bar). The current density blocked by L655,708 is not significantly different than that blocked by bicuculline (see Fig. 2). B) Both THIP (1 µM) and C) allopregnanolone (ALLO; 30 nM) induce indistinguishable current amplitude and density in RQ (grey traces) compared to RR (black traces). D) In thalamic relay neurons, however, THIP- and E) ALLO-induced current densities are significantly reduced in RQ compared to RR (~50%; $p<0.05$).

Figure 4 - GABA$_A$ receptor subunit trafficking is altered in a region-specific manner.

A) Western Blots for 5 GABA$_A$ receptor subunits for RR and RQ plasma membrane (PM) and intracellular organelle (ER) fractions. Calreticulin (a marker for endoplasmic reticulum) and actin were used as controls for fraction specificity and loading quantity, respectively. B) Measures of total protein (PM + ER) display decreases in α5- and γ2-subunit ($p<0.05$) levels in RQ cortex (grey bars) and a decrease in δ-subunit ($p<0.05$) in RQ thalamus compared to RR (white bars). C) Protein trafficking to the cell surface (evaluated as the PM/ER ratio) was also reduced for the α4-, α5- and δ-subunits in the cortex ($p<0.05$). In thalamus, trafficking to the surface was reduced for the γ2-subunit ($p<0.05$). Although δ subunit trafficking in the thalamus was not reduced, there was a reduction in trafficking of the α4 subunit ($p<0.05$), the obligatory partner for δ subunit-mediated tonic currents in thalamus.

Figure 5 - RQ slices display elevated cortical firing and reduced thalamic bursting.

A) A thalamocortical slice with two multielectrode arrays (black ovals) placed in layer II/III cortex (upper) and ventrobasal thalamus (lower). B) Top: A segment of recording from an electrode located in thalamus. Bottom: Expanded segments, corresponding to the black bars in the recording above, and illustrating burst firing of two different neurons (see Methods). C) Cumulative distribution functions (CDF) of mean firing rates for cortex (CTX, upper) and thalamus (Thal, lower), for RR, RQ, and RR in the presence of L655,708 (L655). For cortex, both RQ and L655 display increased firing rates compared with RR ($p<0.01$). In thalamus, RQ displays reduced firing rates compared to RR ($p<0.01$), whereas no change is observed for RR or L655. D) CDF plots for generic burst fractions (upper) and T-burst fractions (lower, see Methods). For generic burst fraction, thalamus displayed a higher burst fraction than cortex in all conditions. In thalamus, RQ burst fraction was reduced compared to RR ($p<0.05$), whereas L655 was not. In cortex, neither RQ nor L655 differed from RR. For T-burst fraction, RR thalamus displayed a higher value than RR cortex ($p<0.01$), RQ cortex ($p<0.01$), and RQ thalamus ($p<0.01$), whereas neither RQ area was different than RR cortex.

Supplemental Figure - RQ mice display SWDs *in vivo*.

A) Electroencephalogram (EEG) recording of an RQ mouse. 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 high number (~11 times during the 1.5 minute trace) of 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 that occurs multiple times during the 10-second trace (bars). C) Cumulative distributions from two different RQ mice (solid and dashed lines) over two days of recording show similar characteristics from both animals, whereas SWDs were not observed in litter-mate control mice (not shown). D) Normalized non-rapid eye movement (NREM) slow-wave activity (SWA) power across mouse age shows that RQ mice (circles and grey line) had a steeper decline in SWA with age compared to wild-type mice (black line from ref. 46) ($R^2 = 0.682$, RQ: -4.399 per day, WT: -0.526 per day; $p < 0.001$ by multiple regression analysis). SWA levels are an indicator of how many neurons are simultaneously entering an up-state, and thus, more cortical activity[48].

Figure 1 - mIPSC amplitude is decreased in RQ slices.
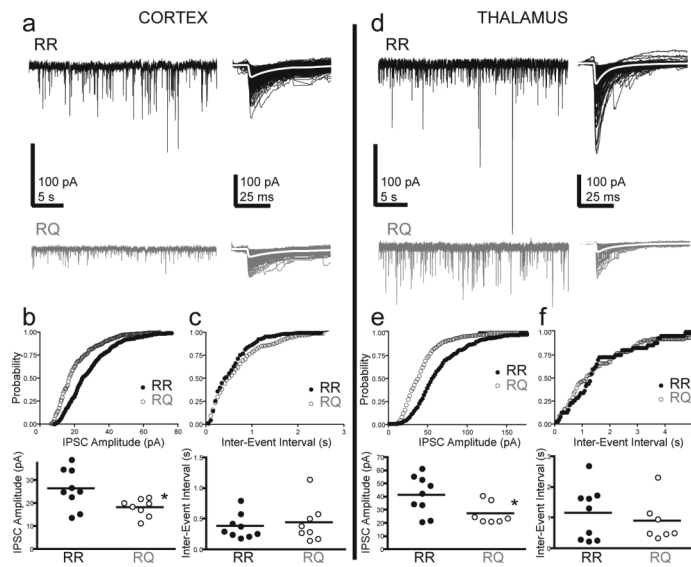
Figure 2 - Tonic currents are abolished in RQ cortex and thalamus.
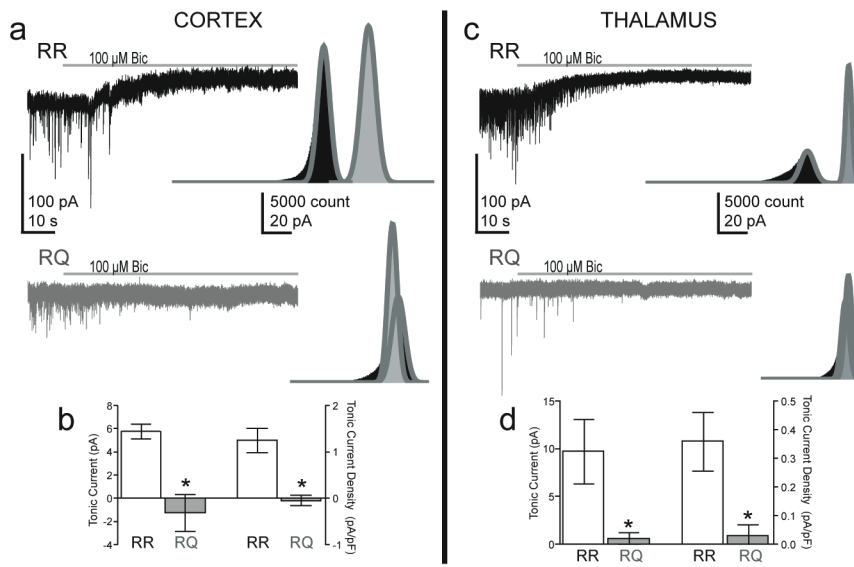
Figure 3: RQ mice display region- and subunit-specific changes in tonic inhibition.
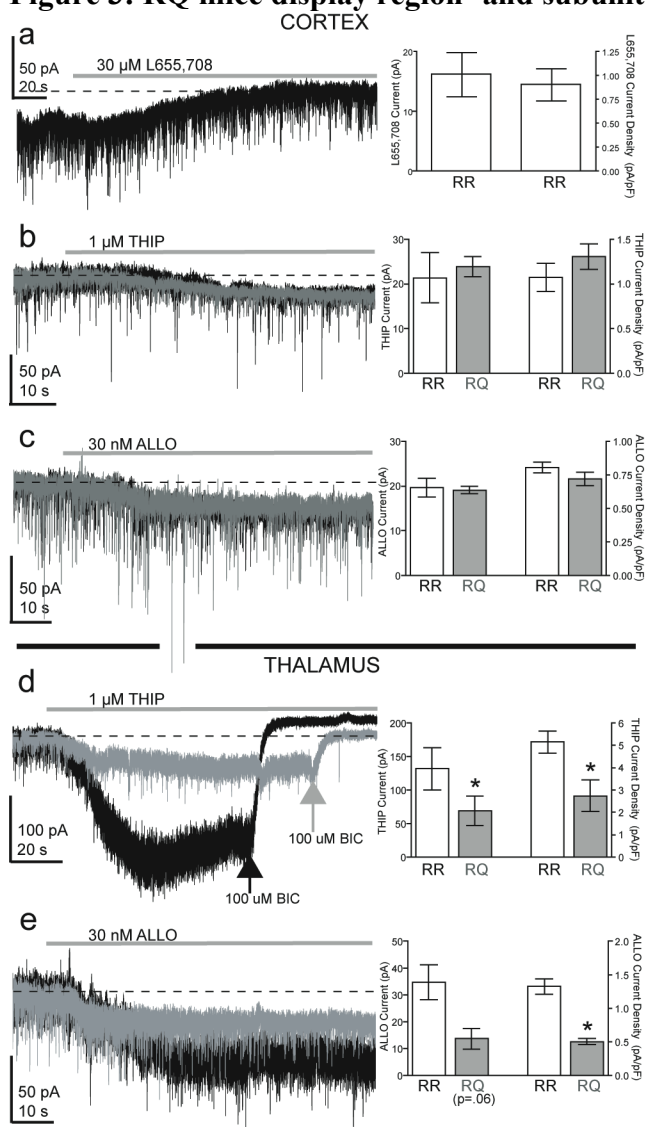
Figure 4: GABA$_A$ receptor subunit trafficking is altered in a region-specific manner.
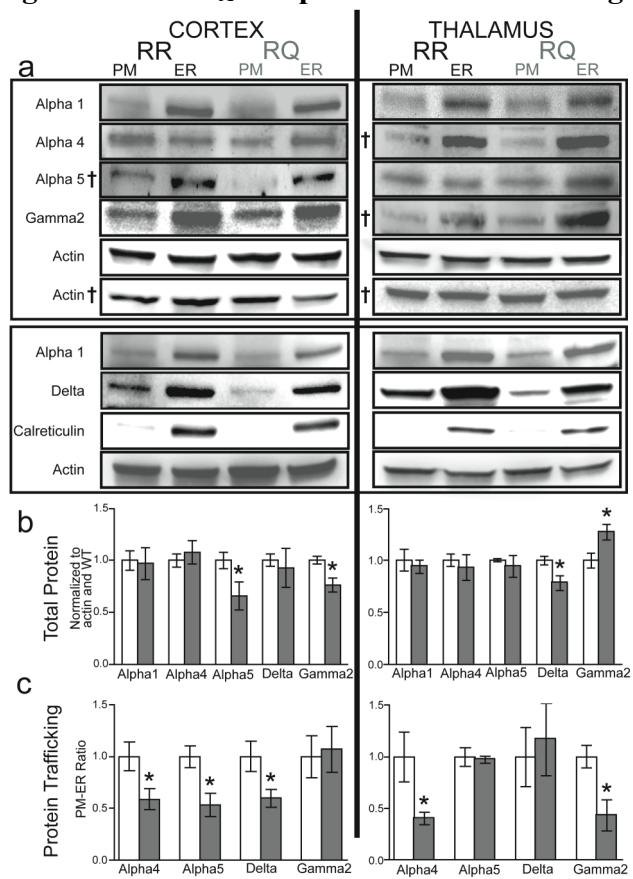
Figure 5: RQ slices display elevated cortical firing and reduced thalamic bursting.

Multichannel Recording
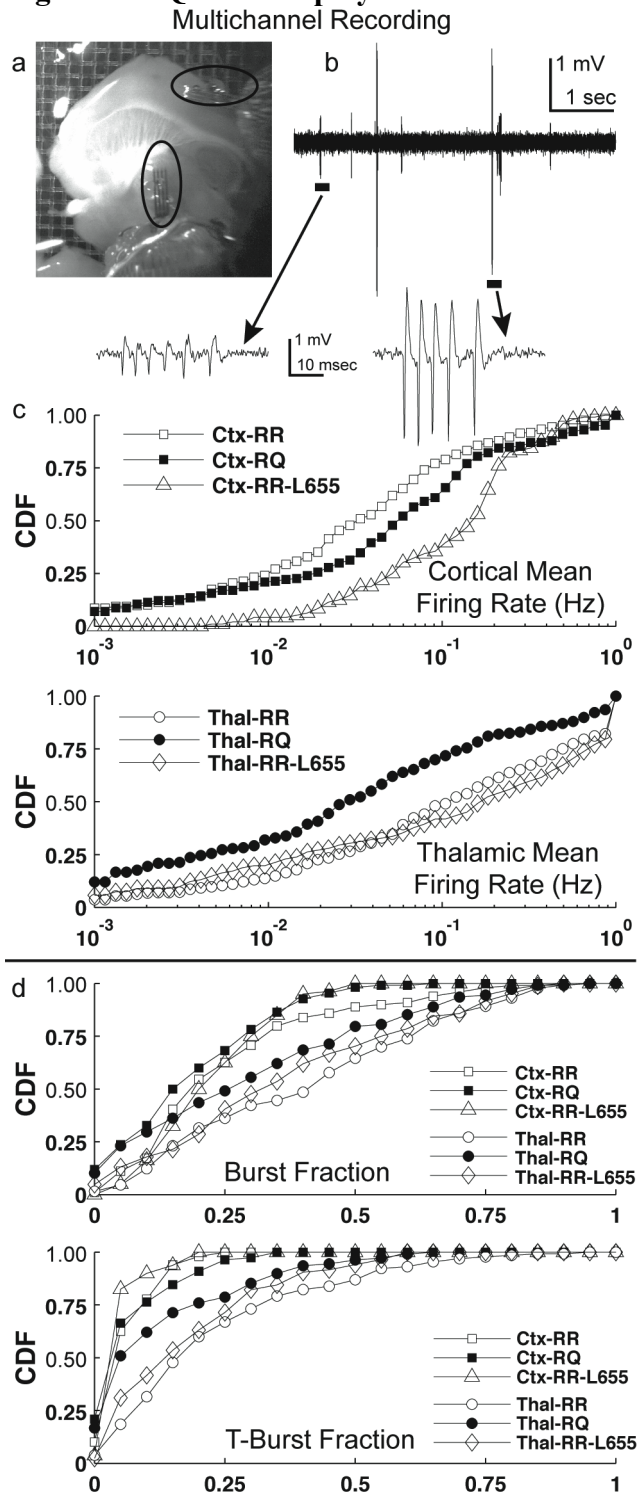
Supplemental Figure: RQ mice display SWDs *in vivo*.
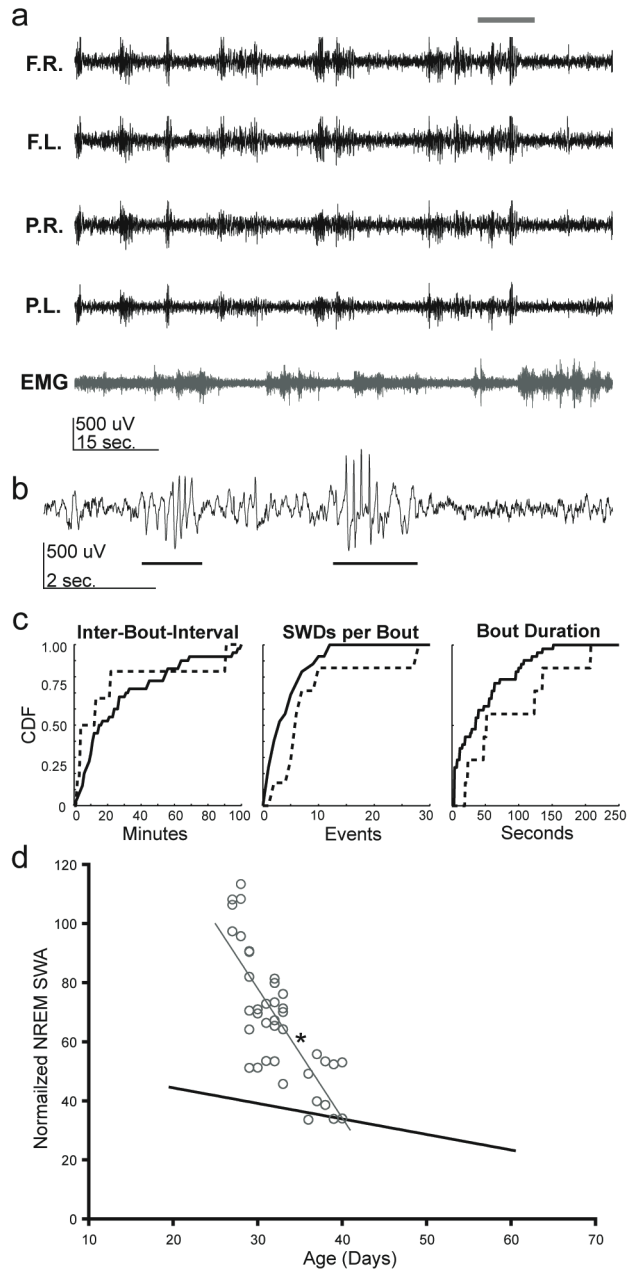
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AUTHOR CONTRIBUTIONS

K.P.M. and M.V.J. jointly conceived and designed all experiments for the study with guidance from S.P., S.M.J., A.R., and C.C.; K.P.M., W.B.P., and A.B.N. performed experiments; K.P.M. and M.V.J. analyzed data; K.P.M and M.V.J. wrote the manuscript.

Methods

Whole-cell Patch Clamp Experiments

Horizontal slices (400 μm) were prepared from the brains of C57BL/6J mice of either sex (16 – 26 days old). All procedures were approved by the University of Wisconsin Institutional Animal Care and Use Committee. Mice were anesthetized with isoflurane, decapitated, and the brain was removed and placed in ice-cold cutting solution containing (in mM): 125 NaCl, 25 NaHCO$_3$, 2.5 KCl, 1.25 NaH$_2$PO$_4$, 0.5 CaCl$_2$, 3.35 MgCl$_2$, 25 D-Glucose,13.87 M 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) and placed in an incubation chamber containing standard 2 mM CaCl$_2$,1 mM MgCl$_2$ artificial cerebrospinal fluid (ACSF) at room temperature for 1 hour before being used for recordings. Whole cell patch-clamp recordings were made from somatosensory cortical layer II/III pyramidal cells or vetrobasal 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) with a resistance of 3-5 MΩ when filled with intracellular solution containing (in mM): 140 K-gluconate, 10 EGTA, 10 HEPES, 20 phosphocreatine, 2 Mg$_2$ATP, 0.3 NaGTP (pH 7.3, 310 mOsm). Recordings were made in a submerged chamber at room temperature using a MultiClamp 700B amplifier (Axon Instruments; Foster City, CA), 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) using Axograph X v1.1.4 (Molecular Devices; Sunnyvale, CA).

Data segments (120 s) prior to bath application of bicuculline (100 μM) were analyzed for miniature inhibitory postsynaptic currents (mIPSCs), using the variable amplitude template-matching algorithm in Axograph (τ_1=0.64 msec, τ_2=14.95 msec).

Additional segments (30 s) just prior to and 90 s after bicuculline 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 phasic mIPSCs[39]. Tonic current was calculated as the difference between the fitted Gaussian means before and after bicuculline administration. Current density (pA/pF) was calculated by dividing the current by cell capacitance. Similar fitting was used to measure the currents produced by THIP (4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol) and allopregnanolone (ALLO).

Multichannel Electrode Array Recordings

Thalamocortical slices (400 μm)[40,41] were prepared as above and placed on an interface chamber perfused with 3-5 ml/min of low-Mg^{2+} (200μM) ACSF. Two to four 16-channel arrays (4X4, NeuroNexus; Ann Arbor, Michigan) were inserted into somatosensory cortex or ventrobasal thalamus. Data were acquired continuously using Tucker-Davis Technologies (TDT) SH16 headstages, Medusa preamplifiers, and RX5 Pentusa Base Station (TDT; Alachua, FL) at a 12.2 kHz sampling frequency. Recordings were box filtered between 100 and 5KHz and digitized at 10 KHz. Spikes were detected as events larger than 2.5 standard deviations above baseline noise, with 5 millisecond segments surrounding each spike captured for analysis. Spikes were sorted by principal

component analysis of spike waveforms, followed by clustering of waveforms projected into the space spanned by the first three principal components using the Klustakwik algorithm[42,43]. Homewritten Matlab (MathWorks, Natick, NJ) code was used to analyze firing and bursting of each neuron, based on the timestamps of the sorted spikes. 'Generic' bursts were defined to reflect any tendency to fire in groups of spikes, and were detected as groups of spikes separated from other groups by gaps of ≥ 50 msec. We also used a measure specifically reflecting the expected statistics of thalamic neuron bursting mediated by T-type calcium channels ('T-bursts'), which were detected as events with an interburst gap of ≥ 100 msec combined with an intraburst gap of ≤ 8 msec[44,45]. The 'burst fraction' in both cases was computed as the number of bursts containing 2 or more spikes divided by the total number of bursts.

Subcellular Fractionation and Western Blotting

To evaluate differences in $GABA_A$ receptor subunit protein expression and trafficking, somatosensory cortex and ventrobasal thalamus were dissected from horizontal slices (1200 μm) prepared as above, and immediately placed on dry ice then stored at -80º C. Samples were thawed and suspended in 50 μL 0.1% Triton buffer with protease inhibitors (Sigma; St. Louis, MO) and further disrupted with 3-5 pumps of a fine-tipped syringe. After 10 minutes at room temperature, samples were centrifuged at 8,000g for 10 minutes at 4° C. The supernatant (organelle) fraction was then transferred to another chilled tube and the pellet (plasma membrane) fraction was resuspended in 50 μL Triton buffer[46,47].

A Bradford protein assay (Bio-Rad; Hercules, CA) was performed on all samples to quantify protein concentration. Loading buffer was added, samples were boiled, and proteins were one-dimensionally separated on Mini-PROTEAN TGX (Bio-Rad) gels (10%), then transferred to polyvinylidene difluoride membranes (Immobilon®-P, Millipore; Billerica, MA). Membranes were probed with antibodies against GABA$_A$ receptor subunits α1 (#OPA1-04100: Thermo Scientific; Waltham, MA), α4 (#AB5459: Millipore), α5 (#AB9678: Millipore), δ (#868-GDN: PhosphoSolutions; Aurora, CO), and γ2 (#OPA1-04111: Thermo Scientific). Actin (#691001: MP Biomedicals; Solon, OH) and the endoplasmic reticulum-enriched protein calreticulin (#06-661: Millipore) were also probed and used as loading and organelle fraction controls, respectively[46,47]. Some gels were stripped with Restore™ PLUS Western Blot Stripping Buffer (Thermo Scientific) and re-blotted for a second protein. Corresponding secondary antibodies (1:20k) (Santa Cruz Biotechnology; Santa Cruz, CA) were applied and immunolabeling of membranes was detected via SuperSignal West Femto (Thermo Scientific) chemiluminescence using a UVP ChemiDoc-IT™ Imaging System controlled by Image Acquisition and Analysis software (VisionWorks LS: UVP; Upland, CA).

Statistics

When comparing normally distributed data, two groups were assessed with a t-test and comparisons of three or more were assessed with ANOVA. When comparing non-normally distributed data, a Kruskal-Wallis examination of medians was used to compare multiple groups.

Supplemental Methods - RQ mice display SWDs *in vivo*.

In human patients expressing the γ2R43Q mutation, penetrance of the absence epilepsy phenotype depends strongly on genetic background[3]. This dependence also applies to knock-in mice, such that penetrance can vary even between colonies of γ2R43Q mice that are nominally of the same background strain[4,7,33,49,(present study)]. Thus it is important to correlate the epilepsy phenotype with putative underlying cellular or network mechanisms. The present study used 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. Details of surgery and electrode implantation are described in Nelson et al.[50]. Briefly, RQ mice, under isoflurane anesthesia, were implanted 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 reference. Two vinyl-coated braided stainless steel wire electrodes were placed in the nuchal muscle for EMG recording. Continuous video-EEG recordings were made and SWDs were scored off-line. A SWDs event was defined as a brief (~2 seconds long) ~7 Hz signal synchronized across all EEG leads, with a corresponding lack of signal in the EMG recording. SWDs "bouts" were defined as groups of SWD events separated from other events by <1 minute.

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CHAPTER 3

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

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ABSTRACT

Synchronous and bilateral spike-and-wave discharges accompany nonconvulsive behavioral and cognitive arrest during seizures associated with absence epilepsy. Previous investigation of multiple absence animal models suggests that the underlying cause of absence seizures is an increase in thalamic inhibitory tonic currents. In contrast, in this study we provide evidence that the level of cortical tonic inhibition also regulates absence seizure expression. Using continuous video-EEG recordings to monitor absence seizures and spike-and-wave discharge expression we show that pharmacological blockade of cortical tonic inhibition provokes absence seizures in wild-type mice. Furthermore, we show that pharmacological rescue of cortical tonic inhibition in an absence mouse (γ2R43Q) model, which lacks tonic inhibition, suppresses absence seizure and spike-and-wave discharge expression. Collectively, these results suggest an optimum level of tonic inhibition in the thalamocortical circuit is required for normal functioning and that a deviation from this optimum results in aberrant thalamocortical function, SWDs and absence seizures.

INTRODUCTION

Absence seizures are characterized by periods of behavioral arrest, accompanied by glassy staring with loss of cognitive function, often without overt convulsions (McCormick and Contreras, 2001). These generalized seizures include a brief loss of consciousness typically lasting 2-15 s with corresponding bilateral, synchronous ~3 Hz electrographic spike-and-wave discharges (SWDs) (Panayiotopoulos et al., 2008). The generalized nature of absences, however, should not be rigidly interpreted to mean that there is no specific focus or generating region. SWD initiation has been localized to the perioral region of the somatosensory cortex in multiple animal models of absence epilepsy (Meeren et al., 2002; Meeren et al., 2005; van Luijtelaar & Sitnikova, 2006; van Luijtelaar et al., 2006; van Luijtelaar et al., 2011; L'uttjohann & van Luijtelaar, 2012; Pinault, 2003; Polack et al., 2007; Bazyan & van Luijtelaar, 2013) and local injection of different pharmaceutical agents into this area suppresses absence seizures in multiple SWD-expressing animal models (Manning et al., 2004; Chen et al., 2011; Bazyan & van Luijtelaar, 2013). A "cortical focus theory" for absence seizures has been proposed and many absence animal models support this conclusion (Meeren et al., 2005; Steriade and Contreras, 1998; Sitnikova and van Luijtelaar, 2004; van Luijtelaar and Sitnikova, 2006; Polack et al., 2007).

The γ2R43Q mutation, an arginine-to-glutamine substitution at position 43 of the $GABA_A$ receptor γ2 subunit, confers absence epilepsy and febrile seizures in humans (Wallace et al., 2001). Compared to unaffected family members, patients harboring the γ2R43Q mutation display increased intracortical excitability, decreased intracortical inhibition, and increased cortical facilitation (Fedi et al., 2008). Similarly, γ2R43Q

knock-in (RQ) mice display absence seizures, generalized SWDs (~ 6 Hz), and increased cortical excitability (Tan et al., 2007; Chapter 2). Results from several labs conclude that the γ2R43Q mutation alters the membrane trafficking of several GABA$_A$ receptor subunits to the cell surface (Kang & Macdonald, 2004; Sancar & Czajkowski, 2004; Hales et al., 2005; Eugene et al., 2007; Chapter 2), including the α5 (Eugene et al., 2007) and δ (Chapter 2) subunits in cortex and thalamus, respectively. The altered trafficking of these specific subunits results in the loss of GABAergic tonic currents in principal neurons of the thalamocortical loop, leading to increased cortical excitability and decreased thalamic bursting behaviors (Chapter 2).

Recently a link has been established between tonic inhibition and absence-associated SWD generation, though this link is currently isolated to, and believed to be 'required' in, thalamic relay neurons (Fariello & Golden, 1987; Crunelli et al., 2011; Errington et al., 2011). Evidence of this link includes findings that the *increase* of GABAergic tonic currents in thalamic relay neurons is sufficient to produce SWDs in wild-type rats, and multiple rodent models of absence epilepsy (GAERS, stargazer, lethargic, tottering) express *increases* in thalamic inhibitory tonic currents (Fariello & Golden, 1987; Crunelli et al., 2011; Errington et al., 2011). The present study, however, demonstrates that this link must be expanded to include tonic inhibition in cortical neurons. Using continuous video-EEG monitoring and selective pharmacological manipulation of cortical tonic inhibition, we show that *decreasing* cortical inhibitory tonic currents is also 'sufficient' to produce SWDs in wild-type (RR) mice, and that *rescuing* the lost cortical tonic currents in RQ mice suppresses SWD expression.

RESULTS

RQ mice express SWDs and absence epilepsy.

The γ2R43Q mutation confers absence seizures and generalized EEG SWDs in humans[3] and knock-in (RQ) mice (Tan et al, 2007). Figure 1 illustrates bilateral, synchronous (~6 Hz) SWDs in a RQ mice using continuous EEG and electromyogram (EMG) recordings. Quantification was done off-line after recordings were completed. A SWD 'bout' was classified as two or more individual SWD events occurring <30 seconds apart. SWDs were assessed for individual event duration, inter-bout-intervals (IBI), events per bout, and bout duration. EEG and EMG recordings from one RQ mouse during a SWD bout are presented (Fig. 1A & 1B), along with quantified SWD assessment for three different RQ mice (Fig. 1C). All RQ mice assessed with EEG and EMG monitoring presented synchronized SWDs across all EEG leads with coincident cessation of EMG activity. No SWDs were observed in naïve wild-type (RR) mice. All values for all groups and conditions and measures are presented in Table 1.

Blocking cortical tonic inhibition produces SWDs in wild-type mice.

It has been demonstrated that a positive correlation between SWDs and thalamic inhibitory tonic currents exists (Cope et al., 2009), leading to the conclusion that enhanced GABAergic tonic inhibition is "necessary and sufficient" condition to cause typical absence epilepsy (Crunelli et al., 2011; Errington et al., 2011). In sharp contrast, here we demonstrate that altering thalamic tonic inhibition is not necessary for SWD generation, and furthermore, blockade of cortical tonic inhibition is sufficient to produce

SWDs (Fig. 2). Previously we reported that inhibitory tonic currents in somatosensory cortical layer II/III principal neurons are generated by $\alpha5$ subunit-containing $GABA_A$ receptors and that this current is blocked by the $\alpha5$ subunit-selective inverse agonist L655,708 (L655) (Chapter 2). Intraperitoneal (i.p.) administration of L655, at a concentration (2 mg/kg) known to bind the majority of $\alpha5$ subunit-containing receptors (Atack et al., 2006), produced SWDs (~6 Hz) in RR mice (RRL6) that are electrographically similar to SWDs seen in RQ mice (Fig. 2A & 2B). However, L655 induced SWDs (L6-SWDs) display fewer events per bout ($p<0.001$) and shortened bout durations ($p<0.001$) compared to RQ, while individual L6-SWD event duration was longer ($p<0.001$). Also noteworthy is the appearance of SWDs 3 days after the last L655 injection (Fig. 2D, hour 1 vehicle), suggesting lingering plasticity following initial insult and SWD induction.

$GABA_A$ receptor δ subunit-selective agonists rescue tonic inhibition in RQ cortical principal neurons.

Although RQ principal cortical neurons lack inhibitory tonic currents (Chapter 2), these neurons also display an inhibitory conductance in response to selective δ subunit-associated $GABA_A$ receptor agonists (1 μM THIP (Cope et al., 2005; Adkins et al., 2001; Chapter 2), 30 nM allopregnanolone (ALLO) (Fodor et al., 2005; Rajasekaran et al., 2010; Chapter 2). 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 agonists that rescued wild-type tonic inhibition levels in RQ cortical neurons. We found that a low concentration (10 nM) of Ganaxolone (GANX) (Fig. 3B), a synthetic neuroactive steroid related to ALLO (Citraro et al., 2006), activates a latent inhibitory conductance in RQ cortical neurons equal to the inhibitory tonic current observed in RR cortical neurons.

Rescuing cortical tonic inhibition attenuates SWDs in RQ mice.

Low doses of selective δ subunit-containing $GABA_A$ receptor agonists rescue tonic inhibition in RQ principal cortical neurons (Fig. 3B). Using video-EEG monitoring, we investigated if treatment with these δ subunit-selective $GABA_A$ receptor agonists (GANX or THIP) could ameliorate the SWDs observed in RQ mice.

RQ mice were i.p. injected twice a day with GANX or THIP for 4 out of 7 days (for drug schedule see Fig. 4A). Multiple concentrations of GANX (2 and 5 mg/kg) and THIP (0.5 and 1.5 mg/kg) were tested for their ability to suppress SWD expression. Only the lowest concentration (2 mg/kg) of GANX was significantly ($p < 0.05$) effective in decreasing RQ-SWD expression (Fig. 4B). This low dose of GANX treatment also decreased bout duration ($p < 0.05$) and event duration ($p < 0.001$), but did not affect the number of SWDs per bout. The selective efficacy of the low GANX dose (2 mg/kg), which is half the ED_{50} dose that protects against partial seizures (Gasior et al., 1999; Kaminski et al., 2004), suggests that the mechanism that diminishes SWDs in RQ mice involves activation of latent δ subunit-containing $GABA_A$ receptors in cortical neurons.

DISCUSSION

The major findings from this study are that the loss (RQ) (Fig. 1) or decrease (RR-L655) (Fig. 2) of cortical tonic inhibition results in a SWD-expressing phenotype, and pharmacological replacement of cortical tonic inhibition (RQ-GANX: Fig. 3C) suppresses SWD expression (Fig. 4). These findings are consistent with the conclusion that the amount of cortical tonic inhibition regulates SWD expression. Thus, the causal link between absence epilepsy and inhibitory tonic currents is at least bidirectional: *increased* thalamic tonic inhibition (Cope et al., 2009) or *decreased* cortical tonic inhibition; both can 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 (Fariello & Golden, 1987; Crunelli et al., 2011; Errington et al., 2011). The current hypothesis from this evidence is that persistent hyperpolarization of thalamic relay neurons (Cope et al., 2005, 2009) makes these neurons more susceptible to rhythmic bursting and insensitive to sensory input, which is considered to be a necessary condition for SWD generation (Crunelli et al., 2011; Errington et al., 2011). 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 for absence epilepsy. However, the efficacy of either drug for this condition is at only ~55% (Glauser et al., 2010). The evidence presented in this study suggests a second classification for absence seizure generation that is separate from altered thalamic activity, and could apply to at least a portion of the remaining ~45% of patients that are currently not treatable by the main treatment options.

In vitro examination of thalamic relay bursting behaviors in thalamocortical mouse brain slices detected a decrease or no change in bursting behaviors compared to control for RQ and L655-treated (RR) brain slices, respectively (Chapter 2). Additionally, RQ thalamic relay neurons display no inhibitory tonic currents (Chapter 2). These results suggest that neither increased thalamic tonic inhibition nor the resulting increased susceptibility to rhythmic bursting is essential for SWD generation. Furthermore, the SWDs expressed endogenously in WAG/Rij rats is quenched by local application of ganaxolone or allopregnanolone into somatosensory cortex, a treatment that would not directly affect thalamic relay neuron tonic inhibitory levels or bursting susceptibility (Citraro et al., 2006). It is likely, however, that this treatment to WAG/Rij rats increased cortical tonic inhibitory levels, further suggesting that cortical inhibitory levels regulate SWD expression.

Our findings suggest that SWDs are linked to general cortical tonic inhibition levels and not to a specific tonic current-associated $GABA_A$ receptor subtype ($\alpha5$ or δ). Rescuing RQ cortical tonic inhibition via activation of δ-subunit-associated $GABA_A$ receptors with GANX, and the subsequent decrease in SWD expression (Fig 4), indicates that SWD expression can be regulated by δ-subunit-associated tonic inhibition. Conversely, the selective decrease/block of $\alpha5$ subunit-associated inhibition (RR-L655), which results in SWD expression (Fig. 2), indicates that SWD expression can also be regulated by $\alpha5$ subunit-associated tonic inhibition.

We found evidence of long-lasting aberrant thalamocortical function after inducing SWDs with L655 in wild-type mice. Mice that were injected twice a day for 2

consecutive days with L655 still displayed SWDs 3 days after the last injection (Fig. 2D: vehicle, Hour 1, p<0.05). Given that the half-life of L655 in multiple animal models (rat, dog, rhesus monkey) is 0.3 – 0.5 hours (Atack et al., 2009), this result suggests lingering pro-epileptic plasticity of the thalamocortical circuit follows initial seizure insult. Similar pro-seizure susceptibility has been noted in other epilepsy-induced animal models (Yu et al., 2013; Peng et al., 2004; Houser & Esclapez, 2003). Getting the earliest possible therapeutic intervention should be a high priority for individuals suffering from absence epilepsy.

Optimal Tonic Inhibition

GANX is the only neurosteroid evaluated as an anti-epileptic drug in humans (Monaghan et al., 1999; Nohria et al., 2010; Reddy and Rogawski 2012). It has been clinically studied for the treatment of infantile spasms (Kerrigan et al., 2000) and shown to be effective, with minimal side effects (sedation), as a treatment for catamenial epilepsy (Reddy and Rogawski, 2009) and partial seizures (Laxer et al., 2000) in adults. However, investigation of GANX in animal models of absence epilepsy (PTZ, GHB) uncovered that it exacerbates absence seizures and can even produce SWDs in wild-type rats when administered at $\geq$ 20 mg/kg (Snead III; 1998). Thus, how can we suggest GANX can be effective as a treatment for absence epilepsy?

Neurosteroids activate $GABA_A$ receptors directly but are known to produce the largest magnitude effects at δ subunit-containing $GABA_A$ receptors and are selective for this receptor subtype only at lower concentrations (Reddy and Rogawski, 2009). The

current results suggest a dichotomy of effects for neurosteroids in the CNS: higher concentrations result in general sedation and SWD generation or exacerbation, and lower levels produce normal functionality and ameliorate SWDs in RQ mice. Similarly, we suggest a concentration dependent consideration of thalamocortical tonic inhibition in regards to SWDs and absence seizure generation.

Evaluation of polygenic (GAERS, stargazer, lethargic) and pharmaco-induced (GHB, PTZ) rodent models of absence epilepsy provide substantial evidence that too much thalamic tonic inhibition triggers SWDs (Cope et al., 2009; Snead III, 1998). Equally, here we present a novel absence animal model (L655) and treatment for a known absence animal model (RQ) that indicate reduced cortical tonic inhibition in SWDs. These findings suggest that an optimum level of tonic inhibition in the thalamocortical circuit is required for normal functioning and that deviation from this optimum in either direction results in aberrant thalamocortical function, SWDs and absence seizures.

FIGURE AND TABLE LEGENDS

Figure 1 - RQ mice express SWDs associated with absence epilepsy.

A) Electroencephalogram (EEG) recording of an RQ mouse. 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 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). Note the brief ~6 Hz SWD events (grey bars) that occur 3 times during the 10-second trace. C) Cumulative distributions from three different RQ mice (solid, dashed, and dash-dotted lines represent each mouse) show similar characteristics from all animals for interbout-interval, SWDs per bout, bout duration and SWD event duration. SWDs were not observed in litter-mate control mice (not shown).

Figure 2 - 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 $\alpha5$ subunit-selective inverse agonist L655,708 (RR-L655). Similar to RQ mice, note the brief yet frequent (~6 times during the 1.5 minute 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 (grey bar). C) Cumulative distributions show RR-L655 mice display significantly less SWDs per bout ($p<0.05$), shorter bout durations ($p<0.05$), yet longer SWD event durations ($p<0.05$) than RQ mice. D) Quantification of SWD events shows that RR-L655 mice did not display SWDs prior to L655,708 injection (Hour 1), but did show SWDs after each hour of injection (Hour 2, $p<0.05$; Hour 4, $p<0.05$). Interestingly, SWDs were still present in RR-L655 mice 3 days after the last L655,708 treatment (vehicle: Hour 1, $p<0.05$).

Figure 3 - GABA$_A$ receptor δ subunit-selective agonists rescue tonic inhibition in RQ cortical neurons.

A) Example voltage-clamp traces for RR (black-behind) and RQ (grey-front) cortical layer II/III cell recordings during 1 µM THIP (top) and 30 nM allopregnanolone (ALLO: bottom) treatments. Both GABA$_A$ receptor δ subunit-selective agonist treatments induce indistinguishable current amplitudes and densities in RQ compared to RR. B) Example voltage-clamp trace for RQ cortical layer II/III cell recording during a 10 nM ganaxolone (GANX) treatment also shows an increase in the holding current, similar to THIP and ALLO. C) Tonic current amplitude (left y-axis) and density (right y-axis) quantifications reveal normal RR tonic inhibition levels can be rescued in RQ with 100 nM THIP and 10 nM GANX treatments, whereas 1 µM THIP treatment in RQ produces 2-4 times more holding current amplitude ($p<0.05$) and density ($p<0.05$) than that observed in untreated RR neurons.

Figure 4 - Rescuing cortical tonic inhibition alleviates SWDs in RQ mice.

A) Schematic depicting administration times and drug schedule investigating 4 drug-treatment conditions in RQ mice. GANX (2 and 5 mg/kg) or THIP (0.5 and 1.5 mg/kg) solutions were i.p. injected in RQ mice twice a day for 4 out of 7 days. B) RQ SWD event quantification during the second hour after drug administration shows the 2 mg/kg GANX treatment decreased SWD expression compared to control hours ($p<0.05$). C) Cumulative distributions of RQ SWD activity after 2 mg/kg GANX treatment shows that bout ($p<0.05$) and SWD event ($p<0.05$) durations are decreased after treatment.

Table 1 – Measures and statistics.

All measures (mean or median), spreads (SEM or 25%:75%), N (number of samples) and statistical significance (ANOVA or Kruskal-Wallis) for all groups and conditions compared are presented according to associated Figure.

Figure 1 – RQ mice express SWDs associated with absence epilepsy.
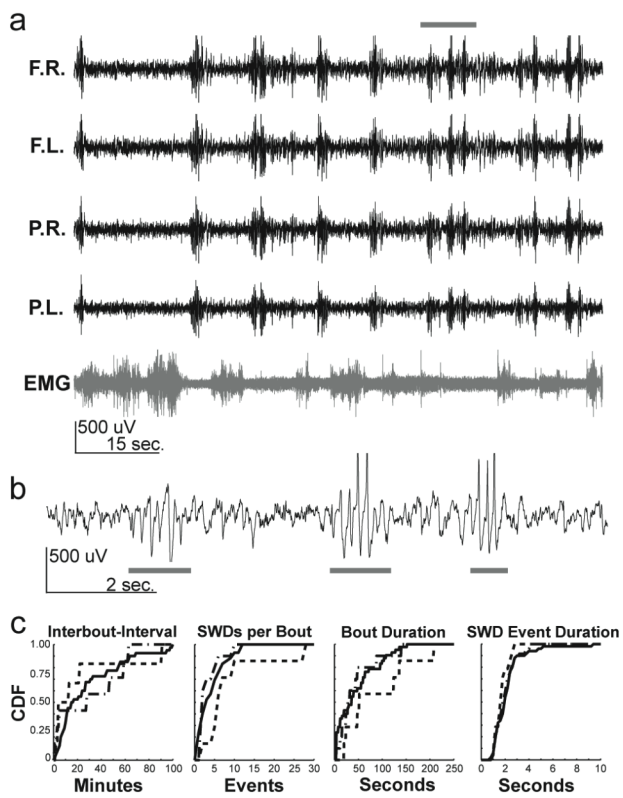
Figure 2 – Blocking cortical tonic inhibition produces SWDs in wild-type mice.
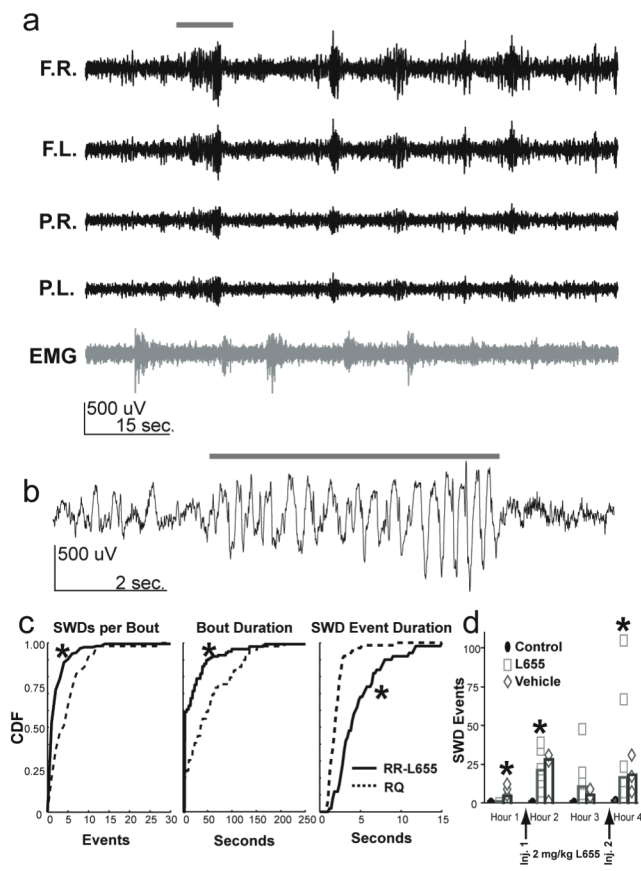
Figure 3 – GABA$_A$ receptor δ-subunit selective agonists rescue tonic inhibition in principal RQ cortical neurons.
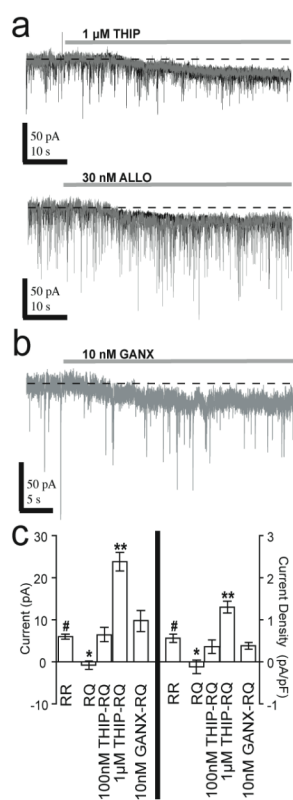
Figure 4 – Rescuing cortical tonic inhibition attenuates SWDs in RQ mice.
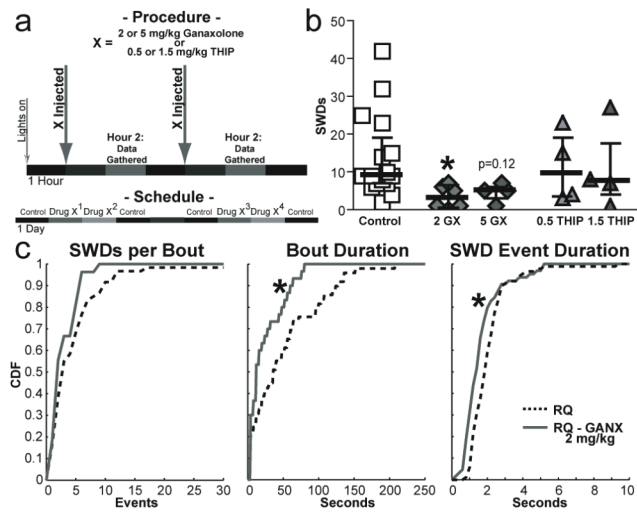
Table 1 – Measures and statistics.

Figure	Measure	Animal	Central Tendency	Spread			N	Significance
	Interbout-Interval (min.)	RQ 1	16.2	8.4	:	44.5	40 Bouts	
	median; 25%:75%	RQ 2	8.7	3.7	:	21.5	6	
	Kruskal-Wallis	RQ 3	27.5	2.9	:	59.1	7	
	SWDs per Bout	RQ 1	3.0	1.0	:	6.5	42 Bouts	
	median; 25%:75%	RQ 2	6.0	3.5	:	10.0	7	
Figure 1	Kruskal-Wallis	RQ 3	2.0	0.0	:	5.5	10	
	Bout Duration (sec.)	RQ 1	36.0	6.0	:	72.0	42 Bouts	
	median; 25%:75%	RQ 2	52.0	24.0	:	136.0	7	
	Kruskal-Wallis	RQ 3	30.0	16.0	:	43.0	10	
	SWD Event Duration (sec.)	RQ 1	2.0	1.4	:	2.5	50 SWDs	
	median; 25%:75%	RQ 2	1.7	1.3	:	1.9	14	
	Kruskal-Wallis	RQ 3	1.9	1.4	:	2.5	24	

	Measure	Group	Central Tendency	Spread			N	Significance
	SWDs per Bout							
	median; 25%:75%	RQ	3.0	2.0	:	6.0	59 Bouts	
	Kruskal-Wallis	RL6	1.0	0.0	:	3.0	155	p<0.001
	Bout Duration (sec.)							
	median; 25%:75%	RQ	36.0	12.0	:	80.0	59 Bouts	
	Kruskal-Wallis	RL6	4.0	0.0	:	36.0	137	p<0.001
	SWD Event Duration (sec.)							
	median; 25%:75%	RQ	1.9	1.4	:	2.4	64 SWDs	
	Kruskal-Wallis	RL6	4.0	2.8	:	6.5	50	p<0.001
	SWD Events							
	median; 25%:75%	Control	0.0	0.0	:	0.0	4 Days	
	Hour 1	L655	0.0	0.0	:	0.0	3	
Figure 2	Kruskal-Wallis	Vehicle	3.5	0.5	:	10.5	6	RR p<0.05
		Control	0.0	0.0	:	0.0	4	
		L655	20.0	11.0	:	37.0	6	RR p<0.05
	Hour 2	Vehicle	27.0	1.0	:	31.0	3	
		Control	0.0	0.0	:	0.0	4	
		L655	9.5	0.5	:	34.0	6	
	Hour 3	Vehicle	4.0	1.0	:	9.0	3	
		Control	0.0	0.0	:	0.0	4	
		L655	15.5	1.5	:	86.0	6	RR p<0.05

Hour 4	Vehicle	17.0	7.0	:	31.0	3	

| | | | Central | | | | |
Measure	Genotype	Drug	Tendency	Spread		N	Significance
	RR	-	6.1	±	0.6	5 Cells	
	RQ	-	-0.8	±	1.0	5	RR p<0.05
Holding Current (pA)	RQ	100 nM THIP	6.5	±	1.7	4	
mean; SEM	RQ	1 μM THIP	23.9	±	2.2	5	RR p<0.05
ANOVA / (T-test)	RQ	10 nM GANX	9.8	±	2.5	4	
	RR	-	0.56	±	0.11	5	
	RQ	-	-0.11	±	0.16	5	RR p<0.05
Current Density (pA/pF)	RQ	100 nM THIP	0.36	±	0.16	4	
mean; SEM	RQ	1 μM THIP	1.30	±	0.14	5	RR p<0.05
ANOVA / (T-test)	RQ	10 nM GANX	0.38	±	0.08	4	

Figure 3

| | | Central | | | | | |
Measure	Drug	Tendency	Spread			N	Significance
RQ	-	9.0	7.0	:	19.0	16 Hours	
	2 mg/kg GANX	3.0	0.5	:	6.5	6	RQ p<0.05
SWDs per Hour	5 mg/kg GANX	5.0	3.0	:	6.0	4	
median; 25%:75%	0.5 mg/kg THIP	9.5	3.5	:	19.0	4	
Kruskal-Wallis	1.5 mg/kg THIP	7.5	4.0	:	17.5	4	

Figure 4

SWDs per Bout							
median; 25%:75%	RQ	3.0	2.0	:	6.0	59 Bouts	
Kruskal-Wallis	RQ-GANX 2 mg/kg	2.0	1.0	:	5.0	27	
Bout Duration (sec.)							
median; 25%:75%	RQ	36.0	12.0	:	80.0	59 Bouts	
Kruskal-Wallis	RQ-GANX 2 mg/kg	12.0	4.0	:	46.0	30	p<0.05
SWD Event Duration (sec.)							
median; 25%:75%	RQ	1.9	1.4	:	2.4	88 SWDs	
Kruskal-Wallis	RQ-GANX 2 mg/kg	1.4	1.0	:	2.0	64	p<0.001

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AUTHOR CONTRIBUTIONS

K.P.M. and M.V.J. jointly conceived and designed all experiments for the study with guidance from S.P. and C.C.; K.P.M. and A.B.N. performed experiments; K.P.M. and M.V.J. analyzed data and wrote the manuscript.

METHODS

EEG implantation and monitoring of SWDs

The present 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 are described in Nelson et al. (2013). Briefly, P24 mice were implanted, under isoflurane 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 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 scored off-line. Animals were given a 3-day recover period after surgery before SWD scoring began. 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. SWD "bouts" were defined as groups of SWD events separated from other events by <30

seconds. Interbout-intervals were defined as the time between the beginnings of consecutive bouts.

All animal procedures followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals and facilities were reviewed and approved by the IACUC of the University of Wisconsin-Madison, and were inspected and accredited by AAALAC.

Drugs and Injection Schedule

L655,708 (L9787), GANX (G7795) and THIP (T101) were all obtained from Sigma (St. Louis, MO). L655 and GANX were dissolved in a 30% DMSO-saline solution (v/v), while 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 uL of solution was injected for each drug. L655 was administered to RR mice 2 and 4 hours after lights out (Fig 2) 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. Ganxolone or THIP injections were administered to RQ mice 1 and 4 hours after lights out (Fig. 4). Drug injections for RQ mice began on day 5 after 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.

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 Institutional Animal Care and Use Committee. Mice were anesthetized with isoflurane, decapitated, and the brain was removed and placed in ice-cold cutting solution containing (in mM): 125 NaCl, 25 NaHCO$_3$, 2.5 KCl, 1.25 NaH$_2$PO$_4$, 0.5 CaCl$_2$, 3.35 MgCl$_2$, 25 D-Glucose,13.87 M 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) and placed in an incubation chamber containing standard artificial cerebrospinal fluid (aCSF) (in mM): 125 NaCl, 25 NaHCO$_3$, 2.5 KCl, 1.25 NaH$_2$PO$_4$, 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 II/III pyramidal 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) with a resistance of 3-5 MΩ when filled with intracellular solution containing (in mM): 140 KCl, 10 EGTA, 10 HEPES, 20 phosphocreatine, 2 Mg$_2$ATP, 0.3 NaGTP (pH 7.3, 310 mOsm). Voltage-clamp (-60 mV) recordings were made in a submerged chamber at room temperature using a MultiClamp 700B amplifier (Axon Instruments; Foster City, CA), 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) using Axograph X v1.1.4 (Molecular Devices; Sunnyvale, CA).

Data segments (30 s) just prior to and 90 s 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 phasic mIPSCs[39]. Tonic current was calculated as the difference between the fitted Gaussian means before and after (100 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) was added at the conclusion of at least one experiment for each drug tested to verify full current block and, thus, only GABAergic contribution.

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, thus an ANOVA was used to compare multiple groups with a Bonferroni post-hoc evaluation. Matlab and Prism software was used.

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CHAPTER 4

Ganaxolone Ameliorates Aberrant Corticothalamic Behaviors in Brain Slices from γ2R43Q Knock-In Mice with Absence Epilepsy.

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ABSTRACT

Recent investigation has uncovered that absence seizures and associated spike-and-wave discharges can be generated via activation or blockade of tonic inhibitory currents in the thalamus or cortex, respectively. In the first classification, which results from increased thalamic tonic inhibition, it is agreed that the resulting increased burst tendencies of thalamic relay neurons drives circuit synchrony into spike-and-wave discharges. The latter classification, however, has yet to be thoroughly investigated and no specific explanation beyond cortical excitability has been suggested. A major underlying characteristic of spike-and-wave discharges is synchronous bursts of cellular populations throughout the thalamocortical circuit. Therefore, here we assess population bursting, cellular behaviors and network communication using multielectrode array recordings in thalamocortical brain slices. We compare results between wild-type mouse brain slices before and after pharmacologically decreasing cortical tonic inhibition with L655,708, and before and after rescuing tonic inhibition with Ganaxolone in brain slices from mice that lack tonic inhibition and suffer absence epilepsy (γ2R43Q mice). We found that decreasing cortical tonic inhibition increased intracortical and thalamocortical communication, and that rescuing lost tonic inhibition ameliorated aberrant thalamic cellular behaviors and normalized cortical and thalamic population bursting behaviors.

INTRODUCTION

Along with loss of cognitive function without overt convulsions, the hallmark characteristic of absence seizures is the occurrence of bilateral, synchronous ~3 Hz spike-and-wave discharges (SWDs) that travel throughout the thalamocortical circuit (McCormick and Contreras, 2001; Panayiotopoulous, 2008). These SWDs include synchronous bursts of neuronal populations in both the cortex and thalamus, with a suggested initiation site localized to somatosensory cortex and immediate propagation following into the underlying thalamus (Errington, 2013; Meeren et al., 2002; Polack et al., 2007; Polack et al., 2009). Full incorporation of the thalamocortical loop is known to be required for SWDs associated with absence epilepsy (reviewed in McCormick and Contreras, 2001; Huguenard and McCormick, 2001).

A strong case has been made that inhibitory tonic currents play a crucial role in the generation and expression of SWDs (Cope et al., 2009, Chapter 2 & 3). Inhibitory tonic currents are persistent hyperpolarizing currents generated by extrasynaptic $GABA_A$ receptors activated by spillover of GABA from the synaptic cleft (Farrant and Nusser, 2005; Belelli et al., 2009). Cope and colleagues (2009) uncovered that thalamic inhibitory tonic currents are *increased* in multiple animal models of absence epilepsy, and that over-activating extrasynaptic $GABA_A$ receptors in thalamic relay neurons is enough to produce SWDs in wild-type rats (Cope et al., 2009). By contrast, we show have shown that the *loss* of cortical tonic inhibitory currents is also enough to produce SWDs, and that rescue of lost cortical tonic inhibition in an absence animal (γ2R43Q) model that lacks tonic inhibition reduces SWD expression in these animals (Chapter 3). Collectively, these

findings suggest that a balance of inhibitory tonic currents throughout the thalamocortical circuit is necessary for normal thalamocortical function.

Falling into the classification of absence epilepsy resulting from decreased tonic inhibition, the $GABA_A$ receptor γ2R43Q mutation confers Childhood Absence Epilepsy (CAE) (Wallace et al., 2001) and a hyperexcitable cortex in humans (Fedi et al., 2008), and results in absence seizures, SWDs (~ 6 Hz), and increased cortical excitability in knock-in mice (Tan et al., 2007; Chapter 2 & 3). We previously discovered that treating γ2R43Q knock-in (RQ) mice with low-doses of Ganaxolone (GANX) reduces the SWDs observed in these mice (Chapter 3). GANX, a synthetic neuroactive steroid derivative of allopregnanolone, was shown to activate tonic inhibition in cortical layer II/III principal neurons, though presumably it also activates GABAergic tonic inhibition in multiple cell types throughout the thalamocortical circuit, including thalamic relay neurons. Thus, although GANX reduced SWDs in these mice, it is still unclear exactly what physiological changes take place in the thalamocortical circuit following GANX administration that make it effective as a treatment.

In the present study we use a thalamocortical brain slice, which preserves synaptic connections between cortex and thalamus (Agmon & Connors, 1991; Krook-Magnuson et al., 2008), and multielectrode array recordings to assess thalamocortical firing rates, bursting probabilities, communication profiles, and population events that are altered in RQ mice and subsequently rescued with the addition of a low-dose (30 nM) of GANX. We also investigated these thalamocortical features in wild-type (RR) following blockade of cortical tonic inhibition with L655,708 (L655) , an inverse agonist to α5 subunit-associated $GABA_A$ receptors that produces SWDs when administered to naive mice

(Chapter 3). Wild-type (RR), γ2R43Q (RQ), GANX-treated RQ, and L655-treated RR thalamocortical brain slices were assessed for "resting state" cellular firing rates, bursting probabilities and correlation levels, and "population behaviors" that include population burst events, burst threshold and peak frequencies, and afterquiets, which we believe to be a measure of tonic inhibition.

RESULTS

Multichannel array recordings reveal thalamocortical slices display repeating neuronal firing patterns and population bursting behaviors.

Multichannel array recordings of thalamocortical brain slices (Fig. 1A & B), along with principal component analysis (PCA) clustering and spike waveform discrimination (Fig. 1C & D) allow for observations and analysis of simultaneous, multicellular activities across the cortex and thalamus (Fig. 1E). Thalamocortical slices exhibit spatiotemporal firing patters that replay at random over several hours of recording (Fig. 1E & F) suggest communication throughout the thalamocortical circuit on a cellular and population level. Thalamocortical brain slices can be kept alive for ~4-6 hours and continue to express communication within and across areas of the thalamocortical circuit during the entirety of this time (Fig. 1G).

Ganaxolone rescues lost RQ thalamic firing and bursting behaviors.

The γ2R43Q mutation interferes with the trafficking of $GABA_A$ receptor subunits to the cell surface (Kang & Macdonald, 2004; Sancar & Czajkowki, 2004; Hales et al.,

2005; Eugene et al., 2007; Chapter 2), resulting in a loss of inhibitory tonic currents in principal neurons of the thalamocortical loop that increases cortical firing rates and decreases thalamic relay neuron bursting (Chapter 2). Attempting to return these altered cellular behaviors to normal levels, we treated RQ thalamocortical brain slices with (30 nM) GANX. Unexpectedly, RQ cortical neurons treated with GANX expressed a significant increase ($p < 0.05$) in over all firing rates (Fig 2A), firing rates that are already elevated ($p < 0.05$) above rates in RR (Chapter 2) (Fig. 2B). GANX also increased ($p < 0.05$) thalamic firing rates (Fig. 2A), but in this case, GANX returned RQ thalamic firing rates back to RR levels (Fig. 2B). In an examination of bursting behaviors, addition of GANX to RQ thalamocortical slices returned the likelihood that RQ thalamic relay neurons would fire a T-burst ($p < 0.05$) back to control (RR) (Fig. 2D). We also observed that GANX-RQ thalamic relay neurons produced more spikes per T-burst ($p < 0.05$) and longer lasting T-bursts ($p < 0.05$) (Fig. 2E) than untreated RQ neurons, suggesting that increasing inhibitory tonic currents with GANX in these neurons not only causes them to fire T-bursts more frequently, but each T-burst is also more robust. All population statistics are located in Table 1.

Ganaxolone returns aberrant RQ thalamocortical communication back to wild-type levels in thalamus, but not in cortex or between cortex and thalamus.

Correlated neuronal activity is often interpreted as a measure of synchrony and functional connectivity, and has been linked to information processing and cognitive states, such as attention (Salinas and Sejnowski, 2001; Engel et al., 2001). We assessed 'resting state' neuronal connectivity in thalamocortical brain slices by measuring

correlated neuronal activity in RR, RQ (Fig. 3A), RQ treated with GANX, and RR treated with L655,708 (L655) thalamocortical brain slices. Selectively reducing cortical tonic inhibitory currents in RR with the addition of L655 increased (p<0.05) correlated firing behaviors within cortex and between cortex and thalamus, but did not alter correlated firing levels within thalamus (Fig. 3B). Interestingly, compared to RR, RQ thalamocortical slices displayed a decrease (p<0.05) in correlated neuronal firing within cortex, within thalamus, and between cortex and thalamus (Fig. 3B). Treating RQ slices with GANX rescued thalamic (p<0.05) correlated firing, and although slightly increasing correlations within cortex and between cortex and thalamus, did not fully return these measures to RR levels (Fig. 3B). A noticeable pattern appears between intracortical and thalamocortical communication profiles regardless of intrathalamic levels (decreased in both for RQ and increased in both for L655), suggesting that the cortical correlation profile dominates resting state thalamocortical communication levels.

Ganaxolone ameliorates aberrant RQ population behaviors throughout the thalamocortical circuit.

SWDs, the underlying electrographic signature of absence seizures, include synchronous, pathological reverberations of cortical and thalamic population bursts. We observed spontaneous, synchronous population bursts occurring in thalamocortical brain slices (Fig. 4A). In RR slices we also noticed a corresponding "afterquiet" immediately following these population bursts, where the over-all firing rate reached or closely approached zero (Fig. 4A). We believe this corresponding afterquiet is a measure of active tonic inhibition where a synchronous population burst increases the ambient

GABA concentration in the extracellular milieu and subsequently quenches neuronal activity. To assess population behaviors, we quantified the number of population bursts and corresponding afterquiets, and measured population burst threshold and peak firing frequencies. RR brain slices displayed population bursts in both the cortex and thalamus, with a corresponding afterquiet occurring about ~75% of the time in the cortex and ~33% of the time in thalamus (Fig. 4B & C). The number of population bursts was significantly increased in RQ cortex ($p<0.05$) and showed an increased ($p=0.056$) tendency in RQ thalamus (Fig. 4B). RQ thalamocortical brain slices also displayed a decreased number of afterquiets in the cortex ($p<0.05$) (Fig. 4B) and a decreased population burst to afterquiet ratio in both the cortex ($p<0.05$) and the thalamus ($p<0.05$) (Fig. 4C). Although the addition of GANX to RQ slices did not produce an appreciable increase in afterquiets (Fig. 4B & C), the number of population bursts became indistinguishable from RR in the cortex (t-test: $p=0.63$) and was significantly decreased in the RQ thalamus ($p<0.05$) (Fig. 4B). GANX also returned RQ cortical ($p<0.05$) population burst threshold ($p<0.05$) and peak frequency amplitudes ($p<0.05$) back to control levels (Fig. 4D). GANX treatment did not produce appreciable afterquiets, but it did return population bursting behaviors throughout the thalamocortical circuit back to wild-type levels, and in particular, recovered cortical population bursting behaviors.

DISCUSSION

The major finding from this study is that GANX returns cortical population bursting characteristics (Fig. 4), along with multiple measures of thalamic cellular behaviors (Fig. 2 & 3), back to wild-type levels in "resting state" thalamocortical brain

slices. We also discovered that decreasing cortical tonic inhibition (L655) in RR slices increased correlated firing in the cortex and between the cortex and thalamus (Fig. 3), a finding that was opposite from that observed in RQ, which does not endogenously express cortical tonic inhibition.

The low dose (30 nM) of GANX that we administered to RQ thalamocortical slices produces wild-type levels of tonic inhibition in RQ cortical layer II/III principal neurons (Chapter 2). Thus, it was expected that rescuing RQ cortical tonic inhibitory currents via GANX administration would reduce activity levels and decrease the elevated firing rates observed in RQ cortex. However, unexpectedly, we observed an *increase* in overall firing rates in RQ cortex after GANX administration. Although at first glace this finding appears contradictory, this contradiction can be resolved considering that the thalamocortical connectivity is intact in these experiments and that GANX increases bursting and functional connectivity, as explained more fully below.

Thalamic relay neurons display two firing modes: singularly occurring, Na-based action potentials and Ca^{2+}-based rebound bursting (T-bursts). The likelihood that a thalamic relay neuron will fire in one or the other of these two firing modes is intertwined with the neuron's resting membrane potential, a potential that is regulated by the active amount of tonic inhibition (Cope et al., 2005). RQ thalamic relay neurons display a decreased probability of T-burst firing; a finding consistent with membrane depolarization resulting from the loss of tonic inhibition in these neurons (Chapter 2). Here we find that adding GANX to thalamocortical slices increases ($p < 0.05$) the likelihood that RQ thalamic relay neurons will fire T-bursts back to wild-type levels (Fig. 2D). Assessment of individual T-burst properties also uncovered that GANX increases

the number of spikes occurring within a T-burst and lengthens the duration of each T-burst in RQ thalamic neurons (Fig. 2E). The fact that RQ thalamic neurons treated with GANX are more likely to fire T-bursts that also include more spikes is consistent with the finding that RQ thalamic firing rates are increased ($p<0.05$) after GANX administration (Fig. 2A). We also discovered that RQ thalamic firing correlations are elevated back to regular levels after GANX treatment (Fig. 3B). Collectively, these findings are consistent with GANX increasing inhibitory tonic currents in RQ thalamic relay neurons, which returns aberrant RQ thalamic activities (firing rates, T-bursting behaviors, firing correlations) back to normal behavior.

Our findings that RQ thalamocortical slices display decreased correlations (Fig. 2B) are consistent with previous findings. Functional magnetic resonance imaging (fMRI) of 'resting state' thalamocortical connectivity in patients with absence epilepsy also uncovered decreased connectivity in the default mode network (DMN) (Lou et al., 2010). Paradoxically, although there is an obvious increase in correlated neuronal activity during SWDs, it appears that neurons that participate in SWDs are less connected at resting states (i.e. periods between SWDs) (Fig. 2B; Lou et al., 2010). The observation that GANX elevates overall cortical firing rates while rescuing RQ thalamic behaviors suggests that elevated thalamic activities could induce elevated cortical activity. In depth evaluation of neuronal connectivity and drive, for example using Granger causality statistics (Cadotte et al., 2010), and the use of stimulation protocols would elucidate how much thalamic T-bursting contributes to cortical firing rates.

SWDs associated with absence epilepsy include synchronous, pathological population discharges in cortex and thalamus (McCormick and Contreras, 2001;

Huguenard and McCormick, 2001; Panayiotopoulous, 2008). We observed spontaneous, synchronous population discharges in the cortex and the thalamus in thalamocortical brain slices (Fig. 1G & Fig. 4A). Along with population discharges in thalamocortical slices, we also noticed a cessation of firing frequency following population discharges in wild-type slices. We have labeled this firing rate cessation as an afterquiet (AQ) and suggest that it is the result of tonic inhibition activated by a substantially elevated ambient GABA concentration following a population discharge. This momentary increase in the ambient GABA concentration could activate available extrasynaptic $GABA_A$ receptors and provide a transient hyperpolarizing shunt that inhibits cellular excitability (Coulter and Carlson, 2007; Bonin et al., 2007; Semyanov et al., 2003; Marchionni et al., 2007; Neito-Gonzalez et al., 2011).

Not surprisingly, afterquiets were reduced ($p < 0.05$) throughout the thalamocortical circuit in RQ brain slices (Fig. 4B & C). Adding GANX to RQ thalamocortical slices did not produce an increase in measurable afterquiets (Fig. 4B & C), a finding that is also not surprising considering that GANX concentration levels do not fluctuate with neuronal activity and therefore would not produce responsive inhibition following population bursts. We did, however, find an increase in the frequency of population burst events in RQ cortex ($p < 0.05$) that was returned back to RR levels after the addition of GANX ($p = 0.63$) (Fig. 4B). This result was also observed in RQ thalamus where an increased trend, compared to RR, in population bursting frequency ($p = 0.06$) was significantly decreased after GANX treatment ($p < 0.05$) (Fig. 4B). Furthermore, GANX also recovered wild-type threshold ($p < 0.05$) and peak ($p < 0.05$) frequencies for population bursts in RQ cortex, and increased population peak ($p < 0.05$)

frequencies in RQ thalamus (Fig. 4D). These results suggest that, although GANX does not increase the likelihood of a responsive afterquiet, it does dampen (frequency) and recover (threshold & peak) normal population behaviors throughout the thalamocortical circuit. These recoveries are consistent with tonic inhibitory shunting, albeit GANX-induced, of neuronal noise and thus allowing for improved cellular filtering of surrounding activity. This finding hints that GANX combats SWD generation by promoting appropriate intracortical communications suggesting that appropriate intracortical communication is more important for SWD generation than afterquiets.

Interestingly, the only appreciable changes from control observed in RR slices treated with L655 were increases in cortical-cortical and cortical-thalamic firing rate correlations (Fig. 3B). As previously argued, thalamocortical correlations are likely dominated by intracortical correlations (Fig. 3B). Considering that L655-treated wild-type mice display SWDs and absence seizures (Chapter 3), our L655 data here connect SWD generation with a hypercommunicative intracortical environment. This result is consistent with the model that GANX suppresses SWD generation by promoting appropriate intracortcial communication. Furthermore, although the thalamocortical "resting state" does not express increased thalamocortical correlations in RQ brain slices (Fig. 2B), an increased facilitation in response to paired-pulse stimulation compared to unaffected family members has been observed in human patients that harbor the γ2R43Q mutation (Fedi et al., 2008), suggestive of a hypercommunicative intracortical environment. Collectively, these considerations suggest that absence epilepsies characterized by decreased tonic inhibition produce a hypercommunicative cortex that

underlies SWD generation, a consequence that can be successfully treated with low doses

of GANX (Chapter 3).

FIGURE LEGENDS:

Figure 1 – Multichannel electrode array recordings of mouse brain slices.

A) A 4X4 electrode array. B) Four arrays placed in the areas shown. C & D) Principal component analysis (PCA) clustering and spike waveforms ("noise" waveforms are gray and were discarded). E) Three examples (30 sec each, RR) of spontaneously repeating activity patterns traveling between thalamus and cortex, suggesting causal connectivity. F) Crosscorrelograms of the same thalamic cell with two different cortical cells. G) Multichannel recording activity of all simultaneously recorded cells from one slice each of RR (left) and RQ (right) mice. Top Panels) Each row shows the firing rate (in 1 min. bins, color-code at top) versus time during the experiment. For RR, cells 1-23 and 71-93 were in thalamus, and 24-70 were in cortex. For RQ, cells 1-33 and 66-91 were in thalamus and 34-65 were in cortex. Bottom Panes) The mean firing rate over all recorded cells, as a function of time during the experiment. The peaks (red circles) were detected as excursion of the first derivative above 1.5X its standard deviation, and line up with the vertical bands in the top panels and represent population events like those shown in E.

Figure 2 – Ganaxolone alters firing behaviors of RQ thalamocortical neurons.

A) Cumulative distribution plots of all RQ cortical (left) and thalamic (right) neuronal firing rates with and without GANX (30 nM) show GANX increases RQ firing rates in both areas ($p < 0.05$). B) Compared to control (RR), RQ cortical firing rates are increased before ($p < 0.05$) and after ($p < 0.05$) the addition of GANX. RQ thalamic firing rates, however, which are decreased ($p < 0.05$) compared to RR, are indistinguishable from RR firing rates after the addition of GANX. C) Top: A four second long segment of recording from an electrode located in thalamus of a thalamocortical brain slice. Bottom: Expanded segments (40 msec), corresponding to the black bars in the recording above, illustrating burst firing of two different neurons. D) Cumulative distribution plots of T-burst fractions for thalamic relay neurons from RR (filled squares), RQ (open circles), and RQ slices treated with GANX (filled circles). The addition of GANX to RQ slices returns RQ T-burst fractions to RR levels. E) Further evaluation of thalamic T-burst behaviors shows that the addition of GANX to RQ slices increases the number of (left) spikes per T-burst ($p < 0.05$) and lengthens (right) T-burst duration ($p < 0.05$) in RQ neurons. The increase of T-burst fraction, spikes per T-burst, and T-burst duration in GANX-treated RQ relay neurons is consistent with an increase in overall firing rate observed in GANX-treated RQ thalamus. All statistical measures are presented in Table 1.

Figure 3 – Neurons in the RQ thalamocortical circuit display less correlated firing.

A) Example firing rate rasters (left) for RR (top) and RQ (bottom) slices including all cells recording during the experiment. Areas are designated with a letter ('T': thalamus; 'C': cortex) and white lines show the cell number cutoff for each area. Adjacent are intercell correlation coefficient matrices (right) for both RR and RQ, with each cell-to-cell interaction generating a correlation coefficient (color code) B) Correlation coefficient cumulative probability distributions for each condition (RR: black lines; RR+L6: light grey lines; RQ: red lines; RQ+GANX: dotted red lines) are presented for cortical-cortical (left), thalamus-thalamus (middle), and thalamus-cortical (right) interactions. Cortical, thalamic, and thalamocortical correlations are decreased in RQ slices compared to RR. Treating RQ slices with GANX shifts distributions towards RR levels, and treating RR with L655 increases cortical and thalamocortical correlations.

Figure 4 – The RQ thalamocortical circuit produces more population bursts and less corresponding afterquiets than control.

A) Colorcoded spike rates of many cells in RR or RQ cortex (top) and mean firing rates across all cells (bottom). Note selected periods of population bursts (red circles) and associated periods of an "after-quiet" (blue circles). Also note that certain cells "rev up" activity prior to the population burst. B) Top) Quantification of population bursts (PB) and after-quiets (AQ) (mean ± SEM) for cortex (left) and thalamus (right) show RQ slices express more bursts ($p<0.05$) and less after-quiets ($p<0.05$) in cortex compared to RR. Treating RQ slices with GANX does not produce after-quiets in RQ ($p<0.05$) but does dampen the number of population bursts in both cortex and thalamus ($p<0.05$). C) Ratios of after-quiets to population bursts show a decrease in the number of corresponding AQs for each cortical or thalamic population burst in RQ slices compared to RR ($p<0.05$). D) Cumulative distribution plots for (top) population burst threshold (Hz) and (bottom) peak (Hz) levels. In cortex, RQ population burst threshold ($p<0.05$) levels are increased, but peak ($p<0.05$) levels are decreased, compared to RR. Treating RQ with GANX returns both to RR levels. In thalamus, RQ slices treated with GANX show an increased population peak level compared to RQ ($p<0.05$).

Table 1 – Measures and statistics.

All measures (mean or median), spreads (SEM or 25%:75%), N (number of samples) and statistical significance (ANOVA or Kruskal-Wallis) for all groups and conditions compared are presented according to associated Figure.

FIGURES AND TABLES:

Figure 1 – Multichannel electrode array recordings of mouse brain slices.

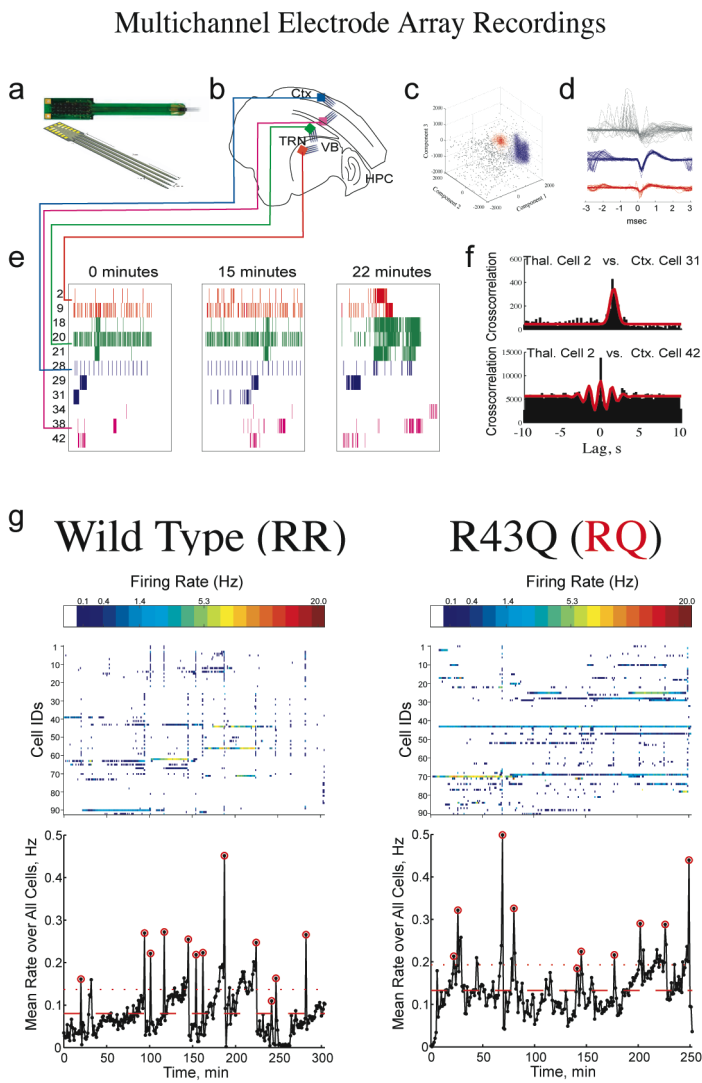Multichannel Electrode Array Recordings

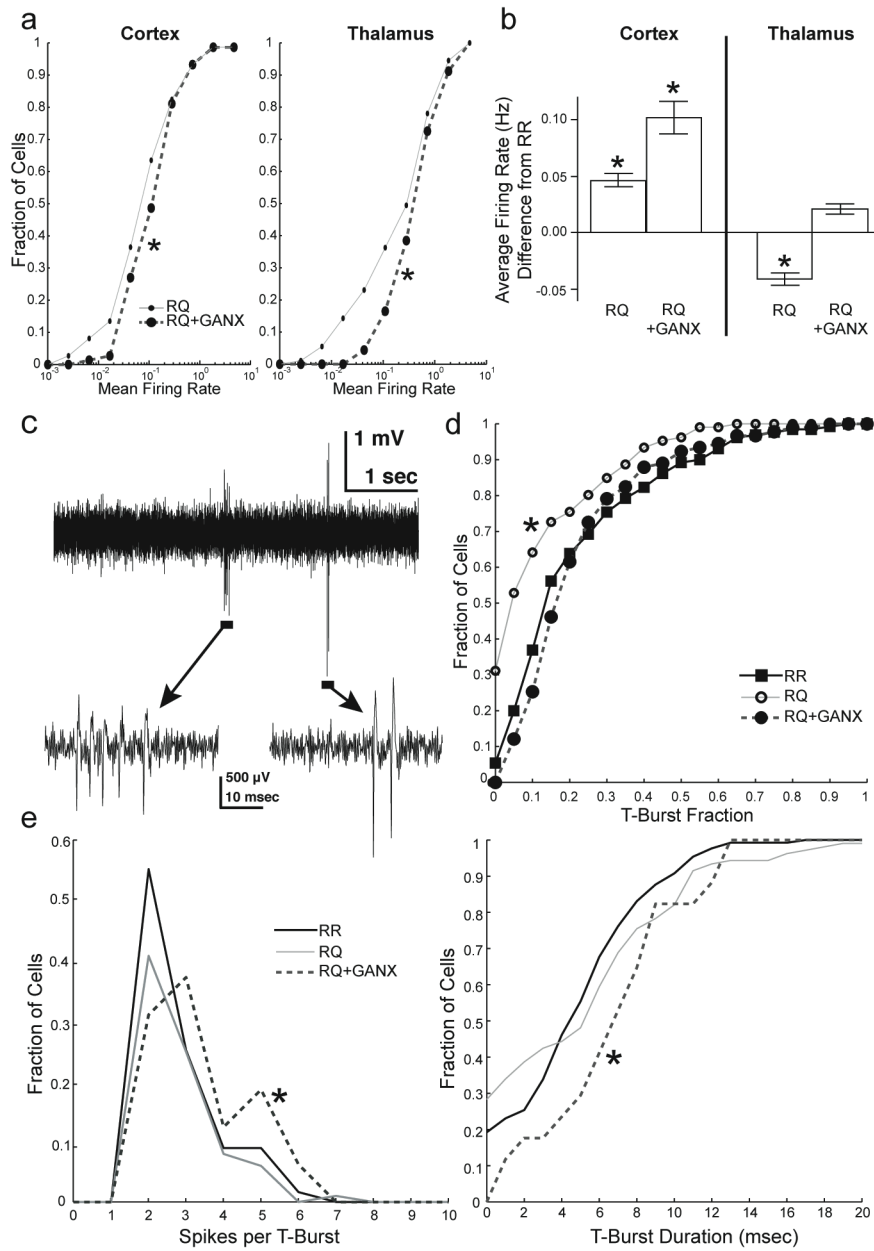**Figure 2 – Ganaxolone alters firing behaviors of RQ thalamocortical neurons.**

Figure 3 – Neurons in the RQ thalamocortical circuit display less correlated firing activity than in wild-type.
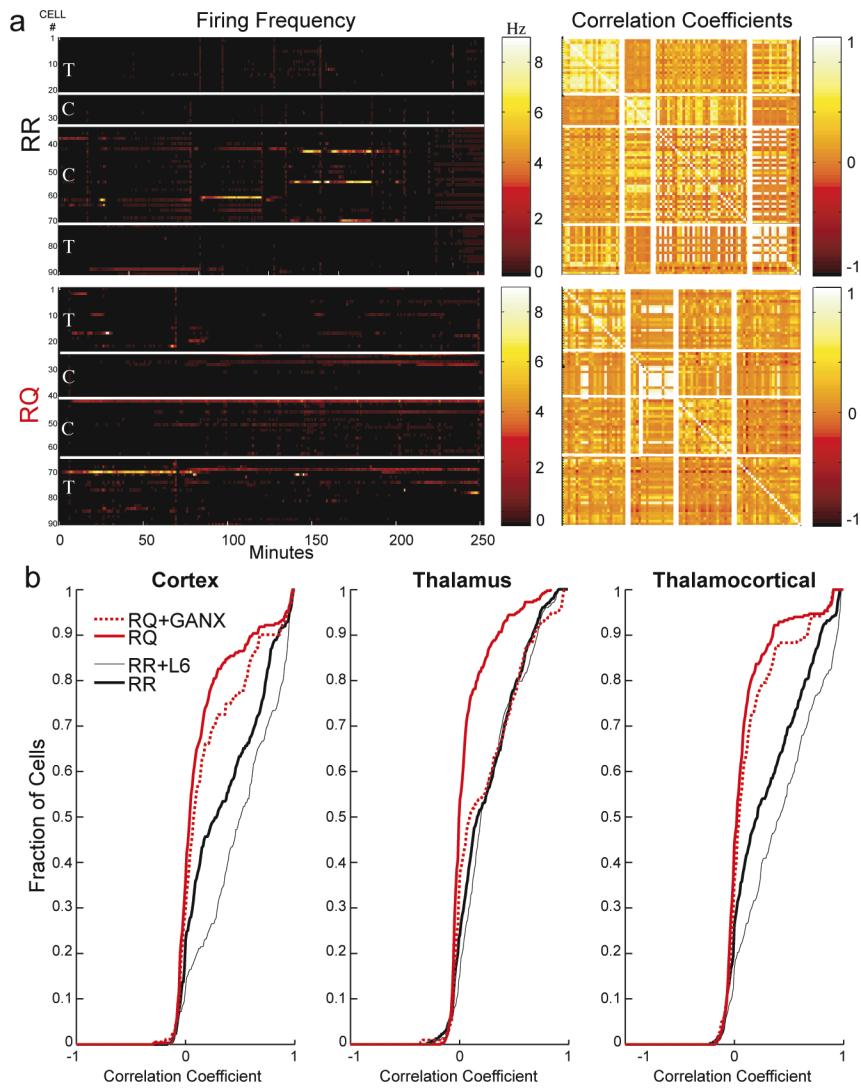
Figure 4 – The RQ thalamocortical circuit produces more population bursts and less corresponding afterquiets than wild-type.

a   Wild Type (RR)   R43Q (RQ)
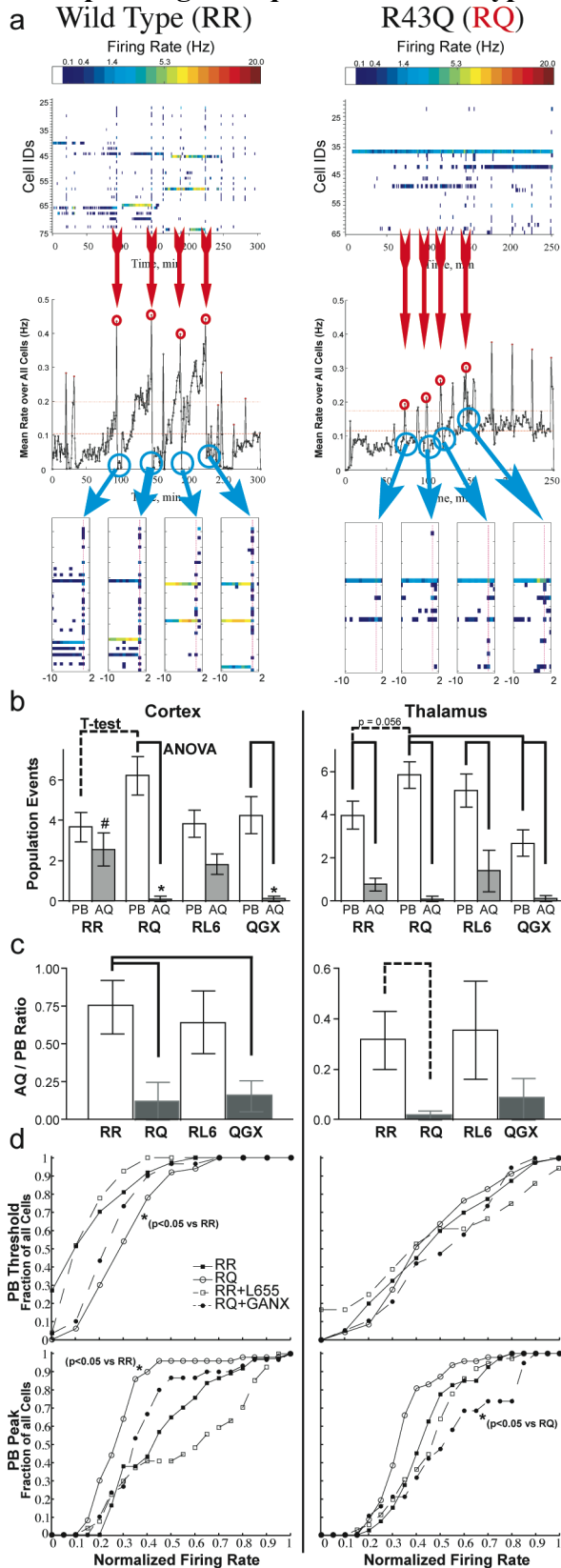
Table 1 – Measures and statistics.

	Activity: Statistical Measure	Area	Genotype	Drug	Central Tendency	Spread			N	Significance
	Firing Rate (Hz) median; 25%:75% Kruskal-Wallis	Cortex	RQ	-	0.07	0.03	:	0.18	74 Cells	
			RQ	GANX	0.13	0.05	:	0.25	74	RQ*
		Thalamus	RQ	-	0.32	0.05	:	0.73	74	
			RQ	GANX	0.45	0.19	:	0.88	74	RQ*
							:			
							:			
Figure 2	T-burst Fraction median; 25%:75% Kruskal-Wallis	Thalamus	RR	-	0.14	0.07	:	0.30	130	
			RQ	-	0.04	0.00	:	0.18	106	RR* RQG*
			RQ	GANX	0.16	0.10	:	0.28	91	
							:			
							:			
	Spikes per Burst (#) median; 25%:75% Kruskal-Wallis	Thalamus	RR	-	2.00	2.00	:	3.00	130	
			RQ	-	2.00	2.00	:	3.00	106	
			RR	L655	3.00	2.00	:	4.00	101	RR*
			RQ	GANX	3.00	2.00	:	4.00	68	RR* RQ*
							:			
	Burst Duration (msec) median; 25%:75% Kruskal-Wallis	Thalamus	RR	-	5.00	2.30	:	7.40	130	
			RQ	-	5.70	0.20	:	7.80	106	
			RR	L655	6.30	3.50	:	8.90	101	
			RQ	GANX	7.10	5.30	:	9.20	68	RR* RQ*
							:			
Figure 3	Correlations (C.C.) median; 25%:75% Kruskal-Wallis	Cortex	RR	-	0.29	0.02	:	0.70	335	
			RQ	-	0.04	-0.03	:	0.21	281	RR* RL6*
			RR	L655	0.51	0.22	:	0.85	237	RR* RQ* RQG*
			RQ	GANX	0.08	-0.01	:	0.41	171	RR* RL6
		Thalamus	RR	-	0.17	0.02	:	0.48	402	
			RQ	-	0.01	-0.04	:	0.10	320	RR* RL6* RG*
			RR	L655	0.21	0.06	:	0.46	272	RQ*
			RQ	GANX	0.11	-0.01	:	0.49	212	RQ*
		Cortex- Thalamus	RR	-	0.22	0.00	:	0.59	335	
			RQ	-	0.03	-0.03	:	0.12	281	RR* RL6*
			RR	L655	0.42	0.10	:	0.73	237	RR* RQ* RQG*
			RQ	GANX	0.05	-0.01	:	0.20	171	RR* RL6*
	Popuation Events (#)	Cortex	RR	-	3.70	0.75			10 Slices	
			RQ	-	6.25	0.96			8	RR(t-test)*
			RR	L655	3.86	0.67			7	

Measure	Region	Agent	Drug	Value	SEM / 25%	:	75%	n	Significance
Peaks		RQ	GANX	4.29	0.92			7	
mean; SEM									
ANOVA / (T-test)	Thalamus	RR	-	4.00	0.65			10	
		RQ	-	5.88	0.61			8	
		RR	L655	5.14	0.77			7	
		RQ	GANX	2.71	0.61			7	RQ-P*
	Cortex	RR	-	2.6	0.82			10	
		RQ	-	0.13	0.13			8	RQ-P* / RR-AQ*
Popuation Events (#)		RR	L655	1.86	0.51			7	
Afterquiets		RQ	GANX	0.14	0.14			7	RQG-P* / RR-AQ*
mean; SEM									
ANOVA	Thalamus	RR	-	0.80	0.29			10	RR-P*
		RQ	-	0.13	0.13			8	RQ-P*
		RR	L655	1.43	0.97			7	RL6-P*
		RQ	GANX	0.14	0.14			7	RQG-P*
	Cortex	RR	-	0.75	0.18			10	
		RQ	-	0.13	0.13			8	RR*
Afterquiet / Peak		RR	L655	0.65	0.21			7	
Ratio		RQ	GANX	0.16	0.10			7	RR*
mean; SEM									
ANOVA / (T-test)	Thalamus	RR	-	0.32	0.12			10	
		RQ	-	0.02	0.02			8	RR(t-test)*
		RR	L655	0.36	0.19			7	
Figure 4		RQ	GANX	0.08	0.08			7	
	Cortex	RR	-	0.04	0.00	:	0.13	37 Events	
Population Event		RQ	-	0.15	0.09	:	0.20	50	RR* RL6*
Threshold levels (Hz)		RR	L655	0.05	0.03	:	0.10	27	RQ*
median; 25%:75%		RQ	GANX	0.12	0.09	:	0.16	30	RQ*
Kruskal-Wallis	Thalamus	RR	-	0.22	0.12	:	0.32	40	
		RQ	-	0.20	0.14	:	0.29	47	
		RR	L655	0.20	0.09	:	0.40	36	
		RQ	GANX	0.29	0.16	:	0.36	19	
	Cortex	RR	-	0.42	0.27	:	0.60	37	
Population Event		RQ	-	0.28	0.20	:	0.33	50	RR* RL6*
Peak levels (Hz)		RR	L655	0.63	0.26	:	0.82	27	RQ*
median; 25%:75%		RQ	GANX	0.35	0.28	:	0.44	30	
Kruskal-Wallis	Thalamus	RR	-	0.43	0.35	:	0.50	40	
		RQ	-	0.32	0.27	:	0.38	47	RL6* RQG*
		RR	L655	0.47	0.33	:	0.55	36	RQ*
		RQ	GANX	0.51	0.37	:	0.78	19	RQ*

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AUTHOR CONTRIBUTIONS

K.P.M. and M.V.J. jointly conceived and designed all experiments for the study with guidance from S.P. and S.J.; K.P.M. performed experiments; K.P.M. and M.V.J. analyzed data and wrote the manuscript.

Methods

Multichannel Electrode Array Recordings

Thalamocortical slices (400 µm) (Agmon & Connors; 1991; Krook-Magnuson et al., 2008) were prepared from the brains of C57BL/6J mice, of either sex, without (RR) and with (RQ) the γ2R43Q mutation (16 – 26 days old). All procedures followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals and facilities were reviewed and approved by the IACUC of the University of Wisconsin-Madison, and were inspected and accredited by AAALAC. Mice were anesthetized with isoflurane, decapitated, and the brain was removed and sectioned in an ice-cold cutting solution containing (in mM): 125 NaCl, 25 NaHCO$_3$, 2.5 KCl, 1.25 NaH$_2$PO$_4$, 0.5 CaCl$_2$, 3.35 MgCl$_2$, 25 D-Glucose,13.87 M 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) and then immediately placed in a interface chamber perfused with 3-5 ml/min of bubbled (95% O$_2$ and 5% CO$_2$), 37°C low-Mg^{2+} (200µM) aCSF (in mM): 125 NaCl, 25 NaHCO$_3$, 2.5 KCl, 1.25 NaH$_2$PO$_4$, 2.8 CaCl$_2$, 0.2 MgCl$_2$, 25 D-Glucose. Two to four 16-channel arrays (4X4, NeuroNexus; Ann Arbor, Michigan) were inserted into somatosensory cortex or ventrobasal thalamus. Data were acquired continuously using Tucker-Davis Technologies (TDT) SH16 headstages, Medusa preamplifiers, and RX5 Pentusa Base Station (TDT; Alachua, FL) at a 12.2 kHz sampling frequency. Recordings were box filtered between 100 and 5KHz and digitized at 10 KHz. Spikes were detected as events larger than 2.5 standard deviations above baseline noise, with 5 millisecond segments surrounding each spike captured for analysis. Spikes were sorted by principal component analysis of spike waveforms, followed by clustering of waveforms projected

into the space spanned by the first three principal components using the Klustakwik algorithm (Harris et al., 2000; Wild et al., 2012).

Firing rates, T-bursting, correlations and population behaviors

Homewritten Matlab (MathWorks, Natick, NJ) code was used to analyze firing characteristics of each neuron, based on the timestamps of the sorted spikes. 40-minute long segments before and after drug addition were used for analysis. Mean neuronal overall firing rates, in 1-minute bins, were determined for each cell for each condition. Cumulative distributions were produced from all cells in each condition. These distributions were used for comparison for overall firing rates.

Using a measure specifically reflecting the expected statistics of thalamic neuron bursting mediated by T-type calcium channels ('T-bursts'), we detected events with an interburst gap of $\geq$ 100 msec combined with an intraburst gap of $\leq$ 8 msec (Lu et al., 1992; Ramcharan et al., 2005). The 'T-burst fraction' was computed as the number of T-bursts containing 2 or more spikes divided by the total number of events (T-burst events + single spike events). T-burst events were further assessed for spikes per burst and burst durations.

Firing rate correlations were computed for all pairs of cells and all areas (cortex-cortex, thalamus-thalamus, and cortex-thalamus) for each recording. Cumulative distributions of firing rate correlation coefficients were produced from all cell-to-cell comparisons in each area and condition and were used for comparison.

Individual cell firing rates were normalized to that cell's maximum firing rate. Mean normalized firing rates across all cells, as a function of time, for each condition and

area were used to compare area-specific population burst and afterquiet (see below) events. Population burst peaks and afterquiets were detected as excursion of the first derivative above 0.5X its standard deviation. The criteria for afterquiets also included cutoff of activity $\leq$ 0.025 Hz. The number of events (bursts and afterquiets) was detected for each area and experiment, and mean values were compared for all conditions. Afterquiet-to-population burst ratios were also calculated and compared for each condition. Cumulative distributions for all population burst threshold (mean population rate corresponding to the peak in the first derivative) and peak (mean population rate corresponding to the max firing rate immediately following the peak in the first derivative) frequencies were calculated and compared across conditions.

Drugs

GANX (G7795) and L655 (L9787) were obtained from Sigma (St. Louis, MO). Each was dissolved in 0.5 mL DMSO and subsequently added to 1L of bubbled (95% O_2 and 5% CO_2), 37°C low-Mg^{2+} aCSF prior to being used for experimentation.

Statistics

All values used for statistical comparison for all measures and groups are presented in Table 1. Most data were not normally distributed, so a Kruskal-Wallis examination of medians was used to compare multiple groups in these conditions. Population burst and afterquiet events, as well as afterquiet-to-population burst ratios, were normally distributed, thus an ANOVA (solid bars) was used for comparison in these cases. However, when strong trends that fell just short of significance with an ANOVA

were observed, we performed a T-test (dashed bars) on selected comparisons (RR vs RQ). This type of analysis has drawbacks that can lead to false-positives, however, we found *many* patterns by taking this approach.

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CHAPTER 5

Conclusions and Future Directions.

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NOTABLE FINDINGS

The major findings from the first phase (Chapter 2) of our investigations are that mice expressing the γ2R43Q mutation entirely lack GABAergic tonic currents in principal neurons of the thalamocortical circuit. We also discovered that the loss of tonic currents in these neurons manifests as increased cortical firing rates and decreased thalamic T-bursting probabilities, consistent with a resulting depolarization that renders cortical neurons hyperexcitable and shifts thalamic relay neurons away from a burst-firing mode. Selective pharmacological blockade (L655) of cortical tonic currents increased cortical firing rates as expected, but did not affect thalamic firing rates or bursting behaviors. Corroborating previous findings, we also found that the loss of tonic inhibition is correlated with decreases in surface trafficking of $GABA_A$ receptor subunits responsible for generating these currents in cortex ($\alpha 5$) and thalamus (δ).

The second phase (Chapter 3) of investigation discovered a novel model (RR-L655) of absence epilepsy, uncovering that the loss (RQ) or decrease (RR-L655) of cortical tonic inhibition generates SWDs. We also discovered that low levels of GANX can activate tonic inhibition in RQ cortical neurons, and that *in vivo* GANX administration suppresses SWD expression in RQ mice. These findings are consistent with the conclusion that the amount of cortical tonic inhibition regulates SWD expression and thus expands the causal link between absence epilepsy and tonic inhibitory currents, suggesting both a thalamic and a cortical contribution. Consequentially, we postulate that

an optimum level of tonic inhibition in the thalamocortical circuit is required for normal functioning and any deviation from this optimum results in aberrant thalamocortical function, SWDs and absence seizures.

In the last phase (Chapter 4) of inquiry, we found that GANX returned cortical population bursting characteristics and thalamic cellular behaviors back to wild-type levels, findings that are consistent with a rescue of tonic inhibition by GANX. We also discovered that decreasing cortical tonic inhibition (L655) increased correlated firing rates in the cortex and between the cortex and thalamus. Considered together, our results suggest that decreased tonic inhibition in cortical neurons produces a hyper-communicative neuronal environment and underlies SWDs generation.

Collectively, our investigations suggest the following mechanism for how the γ2R43Q mutation confers SWDs and absence seizures: this γ2-subunit mutation 1) interrupts appropriate GABA$_A$ receptor trafficking, 2) abolishing tonic inhibitory currents that 3) results in a hypercommunicative cortical environment that 4) manifests as SWDs and absence seizures.

INTERRUPTION OF APPROPRIATE GABA$_A$ RECEPTOR TRAFFICKING

Thalamocortical principal neurons in γ2R43Q mouse brain slices show decreased total protein levels for GABA$_A$ subunits in a region-specific manner that matched observed decreases in tonic inhibitory currents. Somatosensory cortical layer II/III neurons display decreased total α5 subunit protein levels, and ventrobasal thalamic relay neurons exhibit decreased total δ subunit protein levels (Chapter 2). Beyond decreasing total protein levels, the results of subcellular protein level ratio analysis suggest that the

γ2R43Q mutation promiscuously decreases subunit (α4, α5, δ, and γ2) surface membrane trafficking. Based on previous heterologous expression system investigation (Hales et al., 2005), it is believed that mutation at the γ2 R43 location increases γ2 and α subunit interactions, suggesting that the γ2R43Q mutation is causing a preferential coupling of mutated γ2 subunits with α subunits, including the α4 and α5 subunits. Furthermore, mutated γ2 subunit complexes improperly aggregate and become trapped in the endoplasmic reticulum (ER), reducing surface expression of any mutated γ2-coupled subunit (Sancar and Czajkowski, 2004; Hales et al., 2005; Kang and Macdonald, 2004; Eugene et al., 2007). This consideration could explain all the decreases in subunit trafficking that we observe in RQ neurons.

In order for extrasynaptic δ subunit-associated GABA$_A$ receptors to function, these subunits must co-associate with α4 subunits (Maguire et al., 2005). Thus, we would predict that preferential coupling of mutated γ2 subunits with α4 and α5 subunits should decrease surface trafficking of these subunits and α4 subunit-coupled δ subunits. The decrease in γ2 subunit trafficking is likely the result of immunohistochemical antibodies not differentiating mutated and non-mutated γ2 subunits, and thus ER protein levels would consist of a large pool of mutated γ2 subunit proteins, which would then display decreased trafficking levels. Although our investigation does not, nor could it, uncover the exact mechanism that the γ2R43Q mutation utilizes to alter subunit surface expressions, it does corroborate previous findings from expression systems within mammalian neurons. Collective findings suggest that increased coupling and ER trapping of many GABA$_A$ receptor subunits underlies the abolishment of inhibitory tonic currents in RQ neurons.

RQ THALAMOCORTICAL PRINCIPAL NEURONS *LACK* TONIC INHIBITION

Examination of GABAergic tonic currents via whole-cell patch-clamp electrophysiology revealed that principal excitatory cells of the thalamocortical circuit *lack* tonic inhibition (Chapter 2). Reduction of inhibitory tonic currents is linked to membrane depolarization (Hoshino, 2011), increased neuronal firing (Sebe et al., 2005) and enhanced synaptic summation (Chen et al., 2010). Our observations that the loss of tonic inhibition in thalamocortical neurons was associated with an increase in cortical firing rates and a decrease in thalamic bursting probabilities are consistent with these functions of tonic inhibition.

Using a α5 subunit-selective inverse agonist (L655), we showed that the majority of tonic inhibition in somatosensory cortical layer II/III neurons is α5 subunit-associated. We further produced an increase in cortical firing rates in RR brain slices with addition of L655 without decreases in thalamic bursting probability, suggesting that L655 reduces cortical tonic inhibition without altering tonic inhibition levels in the thalamus. The similar increase in cortical firing rates in RQ and L655-treated RR brain slices suggests that cortical activity levels are regulated by tonic inhibition. Collectively, these findings suggest that increased intracortical excitability and increased paired-pulse facilitation observed in humans that harbor the γ2R43Q mutation (Fedi et al., 2008) could be the result of abolished cortical tonic inhibition.

We also showed that RQ thalamic neurons lack tonic inhibition. We also corroborated our finding that the thalamus exhibits decreased δ subunit protein levels by showing that δ subunit-selective activators (i.e., THIP and allopregnanolone) produced

less current in RQ compared to RR. Assessment of thalamic T-bursting behaviors showed that RQ thalamic relay neurons were less likely to fire, consistent with a loss of the hyperpolarizing tonic inhibition and a transition away from a burst firing mode in these neurons.

It is noteworthy here to mention the discrepancy of our findings with those that claim that *enhanced* thalamic tonic inhibition is a "necessary" and "required" condition for SWD generation (Crunelli et al., 2011; Errington et al., 2011). Although this discrepancy cannot be fully reconciled, in the 'Optimal Tonic Inhibition' section of this discussion I present an updated model, which incorporates previous findings with those from these investigations, for how thalamocortical tonic inhibition levels regulate absence seizure genesis.

TONIC CURRENTS AND CORTICAL COMMUNICATION

Correlated neuronal activity is often interpreted as a measure of synchrony and functional connectivity, and is linked to information processing and cognitive states, such as attention (Salinas and Sejnowski, 2001; Engel et al., 2001). In the third phase of our investigations (Chapter 4), we observed an increase in cortical-cortical and cortical-thalamic firing rate correlations after reducing cortical tonic inhibition with L655 in RR thalamocortical brain slices. Interestingly, L655 has been investigated as a cognitive enhancer in rodent models (Sabb et al., 2010; Atack et al., 2006) and a similar negative allosteric modulator of $\alpha 5$ subunit-containing $GABA_A$ receptors (RG-1662) is currently being investigated in human trials to treat Down's Syndrome (McCabe and McCabe, 2013). Considering that heightened correlations and functional connectivity is linked to

cognitive states, it is not surprising that L655, and similar agents, could help increase or recover cognition in deficient situations. However, with consideration of our findings that L655 induces SWDs in wild-type mice, findings that are discussed in detail in the next section, the use of such agents in the general population may not be advisable.

Surprisingly, and importantly, the only appreciable changes observed in RR slices treated with L655 were the increases in firing rate correlations. As previously argued in Chapter 4, thalamocortical correlations appeared to be driven primarily by intracortical correlations. Furthermore, because thalamic relay neurons do not express appreciable levels of α5 subunit-containing GABA$_A$ receptors (Hortnagl et al., 2013) and NRT does not express tonic inhibition, in consideration of thalamocortical processing, it appears safe to assume the majority of the effect produced by L655 is on cortical neurons. Thus, the results suggest that selectively increasing intracortical communication can result in SWD generation and absence seizures.

In contrast to the L655 results, RQ thalamocortical brain slices exhibit multiple aberrant thalamocortical cellular and population behaviors. We found that RQ thalamocortical brain slices displayed *decreased* correlation within each area and also between areas. Although this result is unexpected, previous investigations suggest thalamocortical 'resting state' connectivity is decreased also in patients with absence epilepsy (Lou et al., 2010). Furthermore, there is evidence that individuals with the γ2R43Q mutation display increased intracortical excitability, decreased intracortical inhibition, and increased cortical facilitation (Fedi et al., 2008). Furthermore, the increased number of population burst events observed in RQ cortex of thalamocortical slices, and the similar trend (p = 0.06) observed in RQ thalamus suggests that neuronal

recruitment is heightened in these areas. Consistent with these results, low doses of GANX, titrated to recover wild-type levels of tonic inhibition in RQ cortical neurons, decreased the number of population burst events and returned population event behaviors (event threshold and peak frequencies) back to wild-type levels. Thus, GANX appears to dampen the increased network excitability associated with the loss of tonic inhibition in RQ cortex (Coulter and Carlson, 2007, Bonin et al., 2007; Semyanov et al., 2003; Marchionni et al., 2007; Nieto-Gonzalez et al., 2010). In addition, GANX treatment also increased thalamic T-burst probability, which is also consistent with a membrane hyperpolarization induced by increased tonic inhibition (Cope et al., 2005; Bonin et al., 2007).

Interestingly, we observed what is believed to be a consequence of tonic inhibition manifested in population behaviors in thalamocortical brain slices. We noted and quantified the occurrence of a brief cessation in neuronal activity immediately following population discharges in both the cortex and thalamus. We hypothesize that this brief neuronal activity cessation, or afterquiet, is a result of a transient increase in the ambient GABA concentration resulting from the population discharge. Quantifying this afterquiet in RR brain slices revealed a coincidence with population bursts of ~75%. In RQ cortex, however, this coincidence was reduced to ~10%, consistent with a lack of tonic inhibition. The afterquiet coincident rate in RR cortex after treatment with L655 did not decrease, as might be expected, but rather stayed relatively regular at ~65%. Treating RQ brain slices with GANX also did not alter coincidence levels (~10%), though this was expected because, unlike transient fluctuations in ambient GABA concentrations following population bursts, GANX levels stay relatively constant (30 nM).

INTRACORTICAL COMMUNICATION REGULATES SWD EXPRESSION

Collectively, the evidence discussed thus far supports the model that the critical consequenceof lost or decreased tonic inhibition in the thalamocortical circuit is the manifestation of a hypercommunicative intracortical environment, which promotes SWD generation. Utilizing video-EEG monitoring to assess SWD expression *in vivo*, our investigation revealed that the selective blockade of cortical tonic inhibition in wild-type mice with L655 was adequate to produce absence seizures (Chapter 3). Conversely, we also discovered that *in vivo* treatment with low doses of GANX in RQ mice suppressed endogenous SWD expression. In addition to decreasing the frequency of SWDs, evaluation of SWD expression characteristics revealed that GANX also decreased the durations of SWD bouts and of individual SWD events. Jointly, these findings imply that GANX is ameliorating SWD expression across multiple time scales; decreasing the duration of the ~6 Hz recurrent signals associated with individual SWDs, and a general dampening down of the network excitability responsible for generating SWDs. This multi-temporal amelioration of SWD characteristics is consistent with a tonic inhibition that shunts action potential generation by increasing rheobase (Bonin et al., 2007), and also hyperpolarizes membrane potentials to regulate general network excitability (Coulter and Carlson, 2007, Bonin et al., 2007; Semyanov et al., 2003; Marchionni et al., 2007; Nieto-Gonzalez et al., 2010). GANX also returns aberrant thalamic bursting behaviors in RQ thalamocortical brain slices back to wild-type levels, suggesting that *in vivo* administration of GANX to RQ mice is also returning thalamic activity back towards a wild-type phenotype. However, the SWDs observed in L655-treated mice, along with the

increased correlated firing behaviors in L655-treated thalamocortical brain slices, underscore the importance that intracortical communication levels have on SWD generation.

ENHANCED THALAMIC TONIC CURRENTS AND SWD EXPRESSION

Previously, most studies found that *enhanced* thalamic inhibitory tonic currents were linked to absence epilepsy. Evaluations of polygenic (GAERS, stargazer, lethargic) and pharmaco-induced (GHB, PTZ) rodent models of absence epilepsy provide substantial evidence that increased thalamic tonic inhibition triggers SWDs and absence seizures (Cope et al., 2009; Snead III, 1998). Most convincingly, select activation (THIP) of δ subunit-containing GABA$_A$ receptors in thalamic relay neurons has proven to be sufficient in generating SWDs and absence seizures in rats (Cope et al., 2009; Errington et al., 2011). These discoveries have lead to the conclusion that enhanced thalamic tonic inhibition is a "necessary and sufficient condition for nonconvulsive typical absence seizure generation" (Crunelli et al., 2011; Errington et al., 2011).

Neurosteroids activate GABA$_A$ receptors directly but are known to produce the largest magnitude effects at δ subunit-containing GABA$_A$ receptors and are particularly selective for these receptors at low concentrations (Reddy and Rogawski, 2009). The naturally occurring neurosteroid, allopregnanolone, and the precursor steroid, progesterone, have been investigated for effects on SWD expression. Both drugs exacerbate SWDs in WAG/Rij rats after systemic administration, with the pro-epileptic action of progesterone being depended on its conversion to allopregnanolone (van Luijtelaar at al., 2001; van Luijtelaar et al., 2003). SWD expression profiles in female

WAG/Rij rats include circadian fluctuations coinciding with progesterone levels, showing increased SWD and progesterone levels at the last portion of the light and first portion of the dark cycle of each day (van Luijtelaar at al., 2001).

GANX is a synthetic neurosteroid derivative of allopregnanolone. It is the only neurosteroid evaluated as an antiepileptic drug in humans (Monaghan et al., 1999; Nohria et al., 2010; Reddy and Rogawski 2012) and has been clinically shown to be effective as a treatment for catamenial epilepsy (Reddy and Rogawski, 2009) and for partial seizures (Laxer et al., 2000) in adults. GANX treatment also produces minimal side effects, the most common of which is sedation, which makes it a good candidate for treatment. Investigation of GANX in animal models of absence epilepsy (PTZ, GHB) showed that it too exacerbates absence seizures, similarly to its endogenous neurosteroids counterparts, and is even known to produce SWDs in wild-type rats when administered at ≥ 20 mg/kg (Snead III; 1998). Thus, although the suggestion that GANX can be effective as a treatment for absence epilepsy may be counterintuitive, the results presented here clearly show it has promise in this regard.

OPTIMAL TONIC INHIBITION

Our discovery of a novel animal model (L655) of absence epilepsy supplies evidence that *enhanced* thalamic tonic inhibition is not necessary for generating the SWDs associated with absence seizures. Our findings and previous results suggest a link between absence seizures, SWD generation, and inhibitory tonic currents within the thalamocortical circuit. We propose that the findings from our investigations can be

reconciled with previous discoveries when considering that an *optimal level* of tonic

inhibition throughout the thalamocortical circuit might regulate SWD generation.

Figure 1 illustrates how an evenly distributed level of tonic inhibition in the two

main areas of interest for absence epilepsy, the cortex and the thalamus, can produce a

balance of normal thalamocortical function (Fig. 1A: Wild-Type). However, as shown

throughout the schematic, fluctuations away from this evenly distributed level, or

optimum level, of tonic inhibition result in abnormal thalamocortical function and the

generation of SWDs. Each main area of interest possesses an endogenous level of tonic

inhibition, represented in the Figure as vertical columns. The height of these columns

represents the amount of tonic inhibition for the specific area. As our, and others,

research has shown, the level of cortical tonic inhibition is regulated by the availability

and activation of $\alpha 5$ subunit-containing $GABA_A$ receptors. For our purposes, and

simplicity, the represented level of cortical tonic inhibition ('C') is limited to the level

expressed in somatosensory cortical layer II/III principal neurons. Likewise, the height of

the darker, thalamic column (white 'T') is indicative of the level of inhibitory tonic

currents activated in ventrobasal thalamic relay neurons. This thalamic column height

also indicates the level of available and activated δ subunit-containing $GABA_A$ receptors,

because this receptor subtype is responsible for generating the majority, if not all, of tonic

inhibition in these neurons (Cope et al., 2005). Although the magnitudes of tonic currents

from each area are shown to be even in the schematic, the actual levels of tonic inhibition

are not equal, with baseline levels of conductance in the thalamus being about twice that

measured in the cortex (Chapter 2). For purposes of this explanation, however, it is only

important to consider fluctuations in the general ratio of tonic current contributions

between the two regions rather than comparing the absolute contributions. As discussed, enhanced thalamic tonic currents likely elevate bursting rates, and reduced cortical tonic currents likely produce a hypercommunicative cortical network. Although either of these resulting behaviors are associated with SWD generation, the mechanistic link between altered tonic currents and SWDs is unclear.

The schematic presents outcomes to the general balance of the thalamocortical circuit after fluctuations occur in the contributing levels of tonic inhibition. For instance, following the arrow directly beneath the middle Wild-Type picture (A) leads to a representation of what occurs following L655 blockade of cortical tonic inhibition (B). In this case, the height of the light grey bar, which represents the level of active tonic inhibition in the cortex following L655 administration, shrinks compared to Wild-Type. The result of this loss of cortical tonic inhibition, and representative shrinkage of the cortical light grey column, is that the balanced beam tilts down to the left and normal function falls prey to SWDs.

Following the arrow left of the center Wild-type picture (A), we see a representation of the γ2R43Q knock-in mice (C). Note the shrinkage of *both* column supports of the representative, thalamocortical balance beam. In this case, although the balance of the system is not tilted, the balance beam of normal function is descended into SWDs. From this R43Q picture (C), note how GANX treatment (D) rescues toinc currents in both areas and helps elevate the normal function back above SWD interference. Because neurosteroids are going to preferentially activate tonic inhibition in thalamic relay neurons over that in cortical neurons, most likely due to receptor quantity, although both areas show some rescue of activated tonic inhibition, the thalamus displays

more. Regardless, although GANX treatment may not restore a perfect, elevated balance to the thalamocortical circuit, it does elevate normal function above SWD domination.

Also represented in the figure are pictures for intrathalamic administrations of THIP, which produce SWDs in wild-type mice; systemic administration of THIP or GANX, both of which produce SWDs at high doses; Absence animal models (GAERS, stargazer, lethargic, GHB, PTZ) that display enhanced thalamic tonic inhibition are also pictured.

Interestingly, the majority of absence epilepsy models display an imbalance of normal tonic current expressions with alterations suggesting a decrease in the cortical-to-thalamic ratio of tonic contributions. This may very well *not* be a red herring. For example, the L655 model (B) shows decreased cortical tonic currents compared to thalamus. Likewise, both examples of THIP induced absence epilepsy show decreased cortical tonic contributions compared to thalamus. This result is indicative of a cortical : thalamic ratio for SWD expression always being ≤1, with the one example of SWD expression equally 1 being the R43Q model (C). The discovery of an α5 subunit-selective agonist or a δ subunit-selective antagonist is needed to elucidate the result of an unbalance in normal thalamocortical balance due to a ratio discrepancy of ≥1. Although δ subunit-knockout mice are available and, at first glace, could be a potential model for this inquiry, it is known that cerebellar neurons in δ-KO mice compensate for the lost δ subunit-associated current with an up-regulation of a potassium conductance (Brickley et al., 2001). Furthermore, the discovery of such agents may also be helpful in returning balance to the thalamocortical circuit in situations where thalamic tonic inhibition is

heightened, as in animal models of absence epilepsy such as GAERS, stargazer, lethargic, GHB, PTZ, THIP and GANX. This is illustrated in Figure 1 by the dashed arrows from multiple models converging on the picture at the very top.

FUTURE DIRECTIONS

Causality measures and thalamocortical communication

Because the thalamocortical brain slice that we use preserves the connectivity of the circuit, obvious neuronal communications and patterns can be observed in multielectrode recordings (illustrated in Chapter 4, Fig. 1). Currently we have assessed these communications via correlation rates and population bursting behaviors, but it would create a clearer picture of connectivity to assess these repeating patterns for causality. Using statistical methods, such as Granger Causality, we can elucidate directionality and drive strength underlying connectivity on a neuron-to-neuron, or an area-to-area (i.e. cortex and thalamus), basis (Granger, 1969; Seth, 2005; Seth and Edelman, 2007). Casual statistics gauge the causal relationship between two time series, which in our case are neuronal action potential trains. It is based on a notion of predictability and takes into account not only the history of one neuron's firing profile, but also the profile of another neuron. If the firing profile of the second neuron affects the future firing rate of the first neuron, then these neurons are said to be causal to one another and a statistical p-value is generated corresponding to a degree of significance. The more significant this value is, the more strength is ascribed to that casual connection. Causality may be unidirectional, or bidirection. Fig 2 illustrates an example Granger

Causality analysis on synthetic data, as well as on spiketrain rasters collected with multielectrodes in thalamocortical brain slices. Utilizing this type of investigation we could uncover the important properties underlying how a hypercommunicative intracortical condition might lead to SWDs, and also discover how varying tonic inhibition levels in the thalamocortical circuit alters signaling directionality, revealing if information flow from one area to another is altered in conditions more susceptible to SWDs and absence seizures.

Burst triggered averages (BTAs)

The thalamocortical drive from thalamic relay neurons can display at least two tendencies corresponding to the bimodal firing patterns intrinsic to these neurons. The enhanced thalamic tonic inhibition discovered in multiple animal models of absence epilepsy suggest that an increased tendency for thalamic neurons to fire in the burst firing mode may underlie the generation of SWDs. By contrast, our current data suggest that a hypercommunicative intracortical environment possibly underlies the generation of SWDs in the thalamocortical circuit during absence seizures. It could be possible that a hypercommunicative cortex may result in the same phenomenon that would be produced by an increase in thalamic relay bursting tendencies; an increased communication between the cortex and thalamic relay neuron bursts.

Because the thalamocortical circuit is kept intact in the brain preparation we use, it is possible to assess the responsiveness that the cortex exhibits to thalamic relay neuron bursts. Burst triggered averages (BTAs) of cortical activity can be assessed for each

condition that we have investigated. Figure 3 illustrates this from data gathered by multielectrode array recordings. By going to the time-stamp of each thalamic relay neuron burst that occurred during a multelectrode recording, we can produce a corresponding firing profile of each and every cortical neuron within each experiment. The cortical firing profile, which surrounds the occurrence of each thalamic burst, can be averaged across all cortical neurons and for all thalamic bursts. Subsequent normalization of this average to the number of bursts in each experiment will produce a cortical response firing probability plot corresponding to thalamic bursts. This probability plot can then be compared across all groups and conditions (i.e. RR vs RQ; RR vs RR-L655; RQ vs RQ-GANX), and any alterations in cortical selective responsiveness to a thalamic relay neuron burst can be elucidated. If the hypercommunicative intracortical profile that we observe in SWD-susceptible conditions would be more responsive to thalamic bursts, the production of a BTA plot could uncover this hyper-connection.

IN CLOSING

The work presented herein defines a new classification of absence epilepsy that is characterized by a loss or decrease of inhibitory tonic currents within the thalamocortical circuit, and particularly within the cortex. The consequential tip in the excitatory – inhibitory ratio of the cerebrum to a hyper state of communication underscores the generation of spike-and-wave discharges and onset of absence seizures. Discovery of a successful treatment for this condition with low dose application of a drug possessing minimal side effects clarifies the importance of our investigations. In addition, although

our discovery of this subclass of absence epilepsy manifested from investigations surround a point mutation, the general nature of our findings, marked by discovery of a novel absence model, has the potential to remedy the ineffectiveness ($\sim 40\%$) of current anti-absence medications. An initial, first-line prescription of Ganaxolone, at doses low enough to be harmless in unresponsive cases, would provide a screening approach to treatment that potentially could categorize patient subclass early and increase long-term positive prognosis. Genetic screening of patients could also categorize subclass, with patients harboring GABAergic mutations being those most likely to be responsive to Ganaxolone treatment. Our investigations have provided an avenue for 'personalized medicine' to treat this disease, an avenue that is not common to many pathologies.

FIGURE LEGENDS

Figure 1 – The Balance Model of 'Optimal level of thalamocortical tonic inhibition' for SWD generation.

A schematic representation depicting multiple states of thalamocortical tonic inhibitory conditions that produce 'normal function' and abnormal function, SWDs, in the thalamocortical circuit. A) Wild-type conditions. The lighter-grey bar on the left with a set-in 'C' represents the level of cortical tonic inhibition. The darker column towards the right, with an inset white 'T', depicts the level of thalamic tonic inhibition. The balanced beam resting atop the tonic current columns represent a 'balanced' thalamocortical circuit that supports 'Normal Function'. B) The L655 condition. Notice the shrunken light grey bar on the left suggesting cortical tonic inhibition is reduced in this condition. The level of thalamic tonic inhibition is unaltered. In this condition, the balance beam is titled off-center because of the lost cortical support, dipping 'Normal Function' of the thalamocrortical circuit into SWD. C) R43Q conditions. Note that both tonic current representative columns are shrunken, dropping the level 'Normal Function' down into SWDs. D) R43Q + GANX condition. Note the recovery of tonic inhibition in both cortex and thalamus, elevating thalamocortical 'Normal Function' above the level of SWD expression. Conditions of multiple other known SWD-susceptible animal models are presented. Known animal models that show deviations from Wild-Type tonic current level profiles are pointed to via solid arrows. GAERS, stargazer, lethargic, GHB, and PTZ (up and right from Wild-Type (A)) display the same Balance Model profile that is developed in models produced via i.p. injection of THIP or GANX, or by intrathalamic injection of THIP. Hypothesized profiles presented with dashed arrows.

Figure 2 – Granger Causality detects causal connectivity underlying spontaneous and reproducible patterns of activity in thalamocortical brain slices.

A) Causal connectivity between Poisson neurons was enforced by probabilistically coupling the spikes in one neuron to another. Coupling was from neurons 1->4->5->3 and separately from neuron 2->6. The matrix of significant Granger causal connections (p <0.01) is shown at center. The connectivity graph is shown at the right, and matches the simulated causal coupling. Thus Granger Causality accurately detects causal relationships among spiketrains. B) Three examples of a spontaneously repeating pattern. The six cells showing the strongest firing were selected for display and analysis (18 cells participated in these patterns, out of 93 cells recorded). Each row is a different cell (cell ID#s at left), and each tick mark is a spike. Cells 31- 39 were recorded on a separate array from cells 45-70. C) The matrices of Granger causal connections that reached a significance level of p<0.001. Grey squares are unidirectional connections, black squares are bidirectional. D) Connectivity graphs deduced from the matrices in B. Green are unidirectional and red are reciprocal connections. Note the overal similarity in causal connectivity across different repetitions of the pattern. Also note that #45 appears to be the "source" of the pattern in the lower group, which then jumps to the upper group via a connection from #70 to #31.

**Figure 3 – Burst triggered averages from thalamocortical brain slices.**

A) Example multielectrode array recording traces of 2 cortical neurons (top) and 1 thalamic neuron (bottom) that depict a T-burst in the thalamic cell provoking action potential generation in both cortical neurons, albeit cortical cell #2 is expressing a much higher responsive firing rate than cortical cell #1. B) Burst triggered spiking averages of a cortical spiking probability in response to a thalamic T-type Ca^{2+} burst (T-burst) (occuring at time 0) (from 100 msecs before and 300 msecs after time 0; binned at 10 msec bins). Graph displays 4 conditions, with all conditions producing a noticeable average spiking rate peak centered around time zero, which depicts the onset of a T-burst. C) Cumulative distributions of average firing rates for all cortical neurons (left, top) occurring within the first 10 msecs immediately after the onset of a thalamic T-burst. Cumulative distributions are presented for average firing rates of only cells participating in activity (Active Cortical Population), and the distributions for both of these conditions for activity occurring 120 – 150 msecs after T-burst onset.

FIGURES

Figure 1 – The Balance Model of 'Optimal level of thalamocortical tonic inhibition' for SWD generation.

Figure 2 – Granger Causality detects causal connectivity underlying spontaneous and reproducible patterns of activity in thalamocortical brain slices.
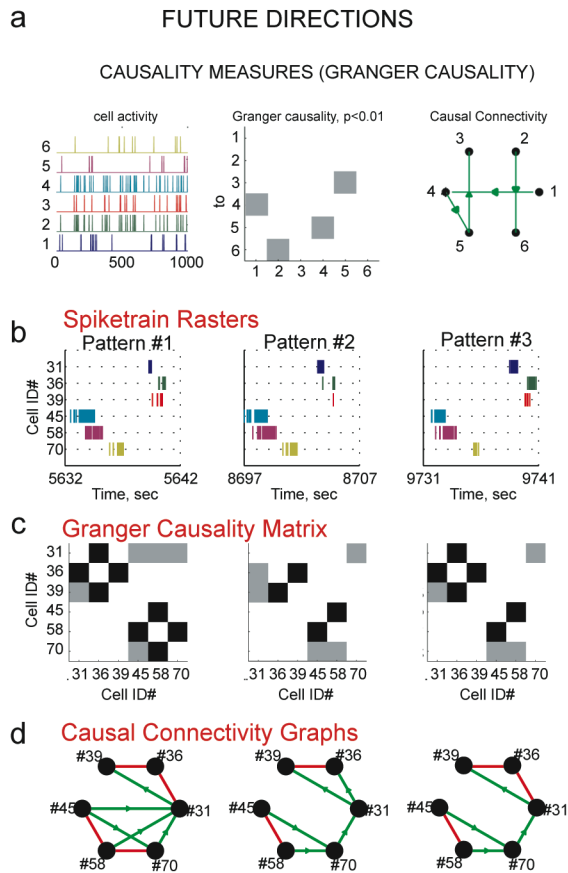
- Granger Causality analysis can be used to determine anatomical origin and causal progression of spikes and bursts.

- Granger Causality is based on the conceptually simple idea that if one can improve prediction of one time series (B) by taking into account not only the history of B but also the history of another time series (A), then series A contains information that affects the future of B. Thus, A is said to be Granger Causal to B.

- Spiketrains can be analyzed pair-wise for all combination of recorded cells, generating a matrix of causal 'weights' and a matrix of p-values. These matrices are not necessarily symmetric, which allows for a designation of "upstream" and "downstream" cells. Thus, we will be able to evaluate what cells (and their regions) preferentially drive network activity.

Figure 3 – Burst triggered averages from thalamocortical brain slices.
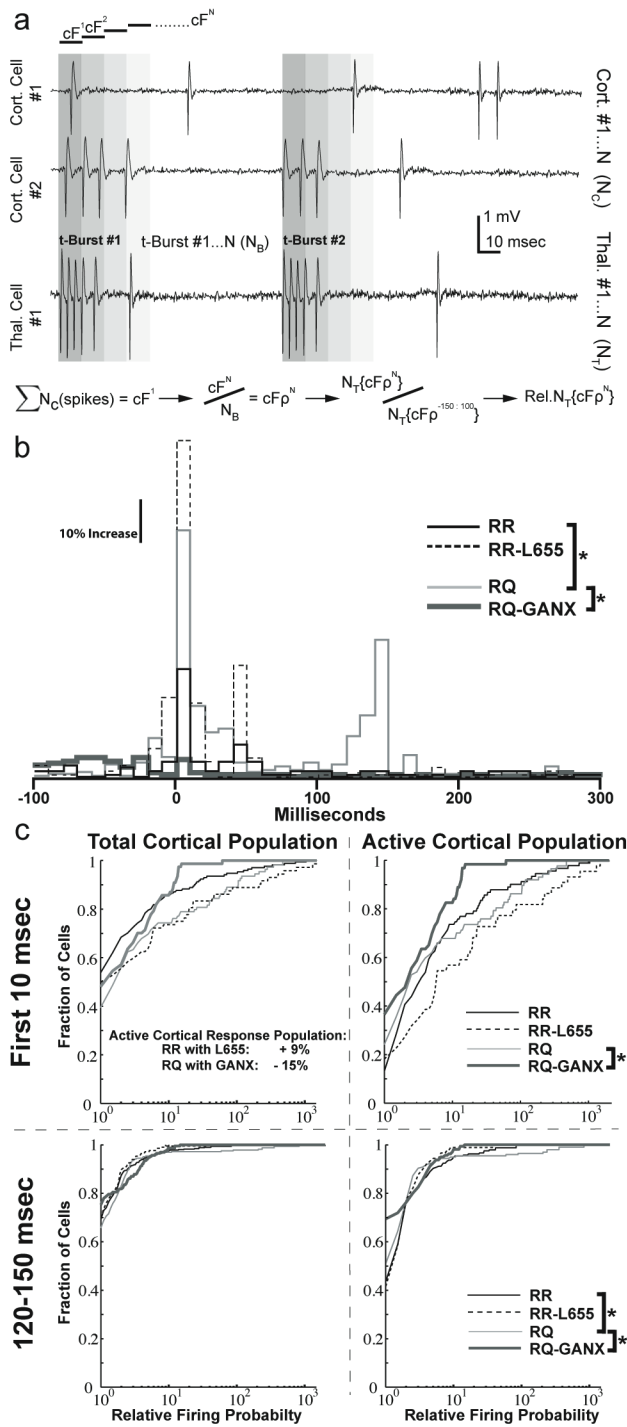
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