Disrupted Thalamic T-Type Ca\(^{2+}\) Channel Expression and Function During Ethanol Exposure and Withdrawal

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INTRODUCTION

Chronic alcoholism produces profound disruptions in both resting and event-related electroencephalogram (EEG) activity patterns (Porjesz and Begleiter 2003), as well as in associated diurnal behaviors (Kubota et al. 2002). In addition, when alcoholics suddenly decrease their alcohol consumption or abstain completely, they may undergo alcohol withdrawal with symptoms ranging from cognitive deficits to seizures (Saitz 1998). Symptom severity may progress with subsequent withdrawals, resembling a process of kindling (Becker 1998). Current treatments address the acute effects of ethanol as well as undergo compensatory transcriptional and functional changes in response to chronic intermittent ethanol (CIE) exposure (Kumar et al. 2009; Walter and Messing 1999). Because acute alcohol has been shown to inhibit T-type currents in both thalamic neurons and cell expression systems (Jokovic et al. 2005; Mu et al. 2003; Shan et al. 2008), we tested the hypothesis that chronic, intermittent ethanol exposure disrupts the normal expression patterns of thalamic T-type channels in a way that promotes network hyperexcitability during periods of alcohol withdrawal. We found that normal variations in T-type channel mRNA were disrupted by CIE in tandem with changes in T-type channel physiology that serve to promote bursting within subcortical networks. These changes occurred in tandem with alterations of EEG theta rhythms and disrupted theta rhythms could be restored through treatment with the T-type channel blocker, ethosuximide. In concert with known ethanol effects on other neuronal properties, disruption of T-type channel function by

In rodent models, repeated ethanol exposures increase the severity of subsequent withdrawal-related hyperexcitability, leading to altered sleep architecture (Kubota et al. 2002; Veatch 2006), heightened anxiety (Overstreet et al. 2002), and abnormal EEG activity (Veatch and Becker 2002; Veatch and Gonzalez 1996). The neural structures involved in this progressive disruption form a network composed of medial thalamic, limbic and prefrontal regions, all of which are especially vulnerable to the adverse effects of chronic ethanol (Fadda and Rossetti 1998).

Within this network, both the hippocampus and thalamus act as rhythm generators, giving rise to normal neural activity such as theta oscillations and sleep spindles as well as pathological activity such as seizures (Buzsaki 2002; Jeannmonod et al. 1996; McCormick and Contreras 2001; Steriade 2005). In the thalamus, rhythmic activity is mediated and sustained by transient (T-type) Ca\(^{2+}\) channels participating in this activity. We therefore examined T-type channel gene expression and physiology in the thalami of C57Bl/6 mice during a 4-wk schedule of chronic intermittent ethanol exposures in a vapor chamber. We found that chronic ethanol disrupts the normal daily variations of both thalamic T-type channel mRNA levels and alters thalamic T-type channel gating properties. The changes measured in channel expression and function were associated with an increase in low-threshold bursts of action potentials during acute withdrawal periods. Additionally, the observed molecular and physiological alterations in the channel properties in wild-type mice occurred in parallel with a progressive disruption in the normal daily variations in theta (4–9 Hz) power recorded in the cortical electroencephalogram. Theta rhythms remained disrupted during a subsequent week of withdrawal but were restored with the T-type channel blocker ethosuximide. Our results demonstrate that a key ion channel underlying the generation of thalamic rhythms is altered during chronic ethanol exposure and withdrawal and may be a novel target in the management of abnormal network activity due to chronic alcoholism.
chronic ethanol is a novel mechanism by which the brain may become hyperexcitable in response to chronic ethanol.

**Methods**

**Animals and experimental designs**

All experiments were performed in accordance with procedures approved by the Institutional Animal Care and Use Committee of Wake Forest University and in agreement with National Institutes of Health and United States Department of Agriculture guidelines, including measures to eliminate suffering and to minimize the use of animals. Ethanol was chronically administered by inhalation (Becker and Hale 1993), a well-characterized rodent exposure model that has been shown to induce both alcohol dependence and withdrawal symptoms (Becker and Lopez 2004; Veatch 2006). Briefly, individually housed 8-wk-old male C57Bl/6 mice (Harlan, Indianapolis, IN) were placed in a sealed Plexiglas vapor chamber modified from Goldstein (1972) in a room with a 12 h light/dark schedule with lights off at 5 pm and lights on at 5 am. For each bout of exposure, ethanol (95%) was volatilized and delivered to the chamber at a rate of 2.0 l/min by a vacuum pump for 16 h (5 pm to 9 am). This, in combination with air being delivered at a rate of 20 l/min, maintained an ethanol concentration in the chamber of 14–16 mg/l air [15.6 ± 0.5 (SE) mg/l]. An 8 h period of abstinence allowed complete clearance of ethanol from circulation prior to the next cycle of intoxication (Becker 1994; Becker and Hale 1993). At the beginning of each lights off period (5 pm), both air and ethanol exposed mice were treated with a subcutaneous injection of saline containing the alcohol dehydrogenase inhibitor pyrazole (100 mg/kg, Sigma Aldrich, St. Louis, MO). Pyrazole was prepared daily by dissolving in saline. Blood ethanol concentrations (BECs) achieved under these conditions remained relatively stable from one bout of intoxication to the next (mean BEC = 186.0 ± 9.4 mg/dl during the 4 wk exposure); however, average BECs during the third and fourth week of exposure were significantly lower than the first week of exposure (Supplementary Fig. S1). BECs for all mice were measured by taking 5 μl blood samples from the tail (stored in vials containing 45 μl of 6.25% trichloroacetic acid) and analyzed using a NAD-ADH enzyme assay (Diagnostic Chemicals, Oxford, CT).

Data were collected throughout the intermittent exposure paradigm at time points corresponding to either immediately following the end of ethanol exposure (9 am, EXP) or during the subsequent acute withdrawal period (5 pm, WD), for a number of intermittent exposures and withdrawals. For example, the time point for tissue harvested from animals immediately following the end of a second intermittent exposure is indicated as 2 EXP, whereas the time point for tissue taken 8 h later is described as 2 WD. For multiple week experiments, mice were exposed to intermittent ethanol exposures Monday through Friday; 10 WD, 15 WD, and 20 WD time points indicate 8 h following the end of the last ethanol exposure for the second, third, and fourth weeks, respectively.

**RNA isolation and real-time RT-PCR**

RT-PCR methods used in this study were similar to those described previously (Graef et al. 2010; Nordskog et al. 2006). Briefly, isolated midline thalamic tissue was stored in RNAlater (Ambion, Austin, TX) according to manufacturer’s instructions. After removal from storage, tissue was immediately homogenized and total RNA was isolated using Qiagen RNeasy spin columns (Qiagen, Valencia, CA). cDNA was produced with the SuperScript III First Strand Synthesis System (Invitrogen, Carlsbad, CA). Real-time PCR was performed on an ABI 7300 thermal cycler (Applied Biosystems, Foster City, CA) using Taqman Universal PCR Mix (Applied Biosystems), Taqman-specific gene assays (Applied Biosystems), and 2 ng sample cDNA. Changes in relative gene expression were quantified using the ΔΔCt method (Livak and Schmittgen 2001). Each measurement was run in triplicate and normalized to 18 s ribosomal RNA gene expression. Data are represented as the group means ± SE.

**Electrophysiological recordings**

Midline thalamic T-type currents were recorded from cells corresponding to the same time points as described for gene expression analysis. Brain slices were prepared and whole cell patch clamp recordings of T-type currents were performed according to established protocols (Alexander et al. 2006; Carden et al. 2006; Graef et al. 2009). Thalamic slices were continuously perfused with oxygenated artificial cerebrospinal fluid (ACSF) at a flow rate of 1.5–2 ml/min. Patch pipettes (5–10 MΩ) were filled with an internal solution containing (in mM) 100 gluconic acid, 100 CsOH, 10 NaCl, 10 HEPES, 20 TEA-Cl, 1 EGTA, and 4 ATP (pH 7.3 with 2 N CsOH, osmolality 270–290 mosM) for voltage-clamp experiments and (in mM) 117 K-gluconate, 13 KCl, 1 MgCl2, 0.07 CaCl2, 10 HEPES, 0.1EGTA, and 2 Na-ATP (pH 7.3 with 2 N KOH, osmolality 270–290 mosM) for current-clamp experiments. Experimentally determined junction potentials, +14 mV for voltage-clamp experiments and +13 mV for current-clamp experiments, were corrected for post hoc. Cellular activity was acquired with an AxoClamp 2B amplifier (Axon Instruments), digitized with a Digidata 1322 (Axon Instruments) and analyzed using pCLAMP 9.0 software (Axon Instruments). For all voltage-clamp experiments, tetrodotoxin (TTX, 1 μM; Alomone Laboratories, Jerusalem, Israel) was included in the ACSF to block sodium action potentials.

**Whole cell patch clamp analysis**

Whole cell electrophysiological data were analyzed as previously described (Graef et al. 2009). Briefly, average peak activation and inactivation currents were normalized to the peak of the maximally available current (\(I_{\text{max}}\)). This relative current was plotted as a function of prepulse potential and fitted with a Boltzmann equation. The decay of maximally elicited T-type currents for all voltage-clamp recorded cells was fit in Clampfit 9.0 (Axon Instruments) with a standard exponential decay function. Peak window currents were estimated by finding the intersection of the averaged, fitted activation and inactivation curves with a custom MATLAB function. The area under the two curves (AUC) was determined using the trapezoidal rule in Prism (GraphPad), with the units corresponding to relative units multiplied by millivolts (RU·mV).

**Integrate-and-fire or burst model**

Methods for the development of the modified integrate-and-fire or burst (IFB) model have been described previously (Graef et al. 2009). Parameters used in the model to generate data were collected empirically (Table 1), with additional parameters given in Supplementary Table S1. Because there were no significant differences between the \(V_{\text{so}}\) activation values for all conditions and time points, single \(V_{\text{so}}\) activations for ethanol-exposed animals (\(-79.3 ± 1.3, n = 20\)) and ethanol-withdrawal animals (\(-74.4 ± 1.1, n = 22\)) were used for the model, whereas the \(V_{\text{so}}\) activation values for both control groups (9 am and 5 pm) given in Table 1 were used. The equation used for this model was

\[
\frac{dV}{dt} = I_{\text{app}} - I_c - I_r
\]

An action potential was generated when the membrane potential reached a defined threshold (\(V_{\text{th}}\)), such that

\[
V(t) = V_a \Rightarrow V(t^+) = V_{\text{reset}}
\]

Additional parameters were derived for the model based on experimental data collected during voltage-clamp experiments. A constant

1 The online version of this article contains supplemental data.
Inactivation V50, mV potentials per burst. values for the model parameters are given in Table 1. We then number of action potentials per burst at each of the 441 points. The model at 21 different holding potentials following 21 different We then generated contour plots of elicited bursts by running the Wireless EEG conductance leakage current (I), was incorporated in the form \( I = g_L (V - E_L) \) with \( E_L \) being the reversal potential of the leak current and \( g_L \) being calculated from the input resistance \( (R_p) \) and capacitance-derived cell surface area \( (S_c) \), using the following equation, \( g_L = 1/(R_m \times S_c) \). The low-threshold Ca\(^{2+}\) current, \( (I_{T}) \), is given by \( I_T = I_{ratio}(V_{m}) - E_{Ca} \), with \( E_{Ca} \) being the Ca\(^{2+}\) reversal potential, \( I_{ratio} \) being the fraction of T-type current available based on empirical steady-state inactivation and activation measurements fitted with a Boltzmann equation, and \( g_L \) being the maximal T-type conductance derived from the experimentally determined maximum T-type current \( (I_{max}) \) and holding potential \( (V_{hold}) \) with the following equation, \( g_L = I_{max}(V_{hold}) - E_{Ca} \). The equations characterizing the activation \( (m) \) and inactivation \( (h) \) of \( I_T \) were adopted from Smith et al. (2000) with

\[
m - 1 \text{ if } V > V_h; m = 0 \text{ if } V < V_h
\]

\[
\frac{dh}{dt} = -h/\tau_h \text{ if } V > V_h; \frac{dh}{dt} = -h/\tau_h \text{ if } V < V_h
\]

We then generated contour plots of elicited bursts by running the model at 21 different holding potentials following 21 different hyperpolarizing current injections of 500 ms and plotted the number of action potentials per burst at each of the 441 points. The values for the model parameters are given in Table 1. We then derived a burst index from each plot that was calculated by multiplying the total percentage of elicited bursts (number of elicited bursts divided by 441) by the average number of action potentials per burst.

**Wireless EEG**

Mice were implanted subcutaneously with telemetric physiologic monitors [Model F20-EET; Data Sciences International (DSI), Arden Hills, MN] that simultaneously recorded EEG, electromyogram (EMG), temperature, and activity. Briefly, animals were anesthetized with ketamine and xylazine followed by the implantation of electrodes for recording EEG signals and EMG signals. For placement of EEG wire leads, holes slightly larger than the coil diameter of the transmitter lead wire were drilled in the skull 2 mm on either side of the midline suture half way between bregma and lambda. The exposed portions of the leads were placed between the skull and underlying dura. Wires were secured to the skull with dental acrylic. EMG leads were placed in the neck muscles and secured with sutures. The signal transmitter body was placed subcutaneously over the dorsal thorax. Mice were allowed to recover from surgery for 1 wk prior to recording. The exposure paradigm during the 5-wk recording period was as follows: 1 wk baseline followed by 4 wk intermittent ethanol exposure (5 PM to 9 AM on days 1–5, no exposure days 6 and 7). One EEG and one EMG channel were continuously recorded over the entire exposure paradigm at a sampling rate of 500 Hz along with average activity counts and body temperature measurements every 10 s.

**EEG analysis**

EEG data were divided into 10-s epochs and analyzed with a conventional FFT power spectra function in 0.5-Hz increments using Neuroscore software (DSI). A custom program written in MATLAB was used to filter the raw power spectra into a 4–9 Hz theta power band. Each 10-s epoch was then normalized to the maximum epoch within its respective 24-h period (5 PM to 5 PM) for each band. Normalized 10-s EEG and activity epochs were then further averaged into 1-h bins. All data in the manuscript are presented as means ± SE with tests for significance provided within RESULTS.
RESULTS

Ethanol exposure and withdrawal alters T-type channel expression patterns

All three T-type channel isoforms (CaV3.1, CaV3.2, and CaV3.3) have been shown to display different functional characteristics and distribution patterns throughout the brain (for review, see Perez-Reyes 2003). For example, CaV3.3 has slower kinetics than CaV3.1 and CaV3.2 (Chemin et al. 2002), and CaV3.2 demonstrates a more depolarized steady-state voltage dependence (McRory et al. 2001). In addition, CaV3.1 is the more predominant isoform in thalamic relay neurons with CaV3.2 and CaV3.3 showing greater expression in the GABAergic thalamic reticular neurons (Talley et al. 1999). We examined the time course of thalamic T-type Ca\(^{2+}\) channel gene expression for C57Bl/6 mice that were exposed to either air or chronic intermittent ethanol. Changes in gene expression for all cohorts were determined at time points corresponding to immediately following the end of the ethanol exposure period (EXP, 9 AM) or 8 h later during acute withdrawal (WD, 5 PM). We have previously shown that all three T-type channel isoforms displayed diurnal changes in gene expression in the thalamus and that this pattern was modulated for CaV3.2 and CaV3.3 by chronic ethanol consumption through a liquid diet (Nordskog et al. 2006). Here we found significant variation between time points in all three T-type isoform expression levels in the thalamic reuniens nucleus (RE) from air-exposed controls, with increased expression in the morning (9 AM), corresponding to the early inactive period (n = 4), and lower expression during the afternoon (5 PM), corresponding to the beginning of the active period (n = 4, P < 0.05 unpaired t-test, Fig. 1).

During the multiple, intermittent ethanol exposure and withdrawal periods, mRNA levels for both CaV3.2 and CaV3.3 were altered with a significant increase in CaV3.2 expression immediately following two ethanol exposures (2 EXP), and a significant upregulation of both CaV3.2 and CaV3.3 beginning during the withdrawal periods following the third (3 WD) and fourth (4 WD) exposures, respectively (n = 4 for all points, *P < 0.05, ANOVA with a Bonferroni’s post test, compared with time-matched air controls). Gene expression results for all mice were relative to air-exposed controls (9 AM). CaV3.1 mRNA levels did not change at any of the time points tested during the ethanol exposure and withdrawal paradigm.

Ethanol exposure and withdrawal changes T-type channel gating properties

Multiple, intermittent ethanol exposures altered both CaV3.2 and CaV3.3 expression levels in the thalamus. We therefore sought to determine whether there were corresponding functional changes by measuring whole cell T-type currents from RE neurons during the same exposure and withdrawal time points.

T-type currents can be evoked by first hyperpolarizing the cells to deinactivate the T-type Ca\(^{2+}\) channels then depolarizing to a level close to the resting membrane potential to activate them. Voltage clamp protocols employing this paradigm have previously been used in our lab to determine the voltage-dependent inactivation and activation properties of the whole cell, T-type current (Graef et al. 2009).
mV, n = 7; Air 5 pm: $V_{50} = -89.3 \pm 1.1$ mV, n = 10; $P < 0.01$ 1-way ANOVA with a Bonferroni’s post test).

Even though the voltage-dependent gating properties exhibit a hyperpolarizing shift during the early active period (5 PM), there was no apparent change in the estimated peak window current between the two control conditions (Air 9 AM: 20% of maximum current at $-79.3$ mV, AUC = 1.3 RU · mV; Air 5 PM: 19% of maximum current at $-82.9$ mV, AUC = 1.57 RU · mV; peak represent by the intersection of the activation and inactivation curves). The window current, illustrated by the shaded region under the two curves, is the fraction of channels that are not fully inactivated and remain available to conduct T-type current around a set of membrane potentials (Crunelli et al. 2005; Hughes et al. 1999). Estimates of the window current can therefore be used to approximate the amount of spontaneous T-type channel activity around physiologically relevant membrane potentials.

Following a fourth intermittent ethanol exposure, the inactivation curve was shifted to the left (Fig. 2B), resulting in a significantly more hyperpolarized $V_{50}$ value as compared with air-exposed animals (EtOH EXP: $V_{50} = -88.7 \pm 1.0$ mV, n = 9, Air 9 AM: $V_{50} = -83.6 \pm 0.6$ mV, n = 7; $P < 0.05$ 1-way, ANOVA with a Bonferroni’s post test). However, during WD 1 day following four intermittent ethanol exposures, the inactivation curve was shifted in the opposite direction, producing a significantly more depolarized $V_{50}$ value as compared with controls (Fig. 2D, EtOH WD: $V_{50} = -82.5 \pm 1.4$ mV, n = 6, Air 5 PM: $V_{50} = -89.3 \pm 1.1$ mV, n = 10; $P < 0.001$, 1-way ANOVA with a Bonferroni’s post test). In addition to the shift in the $V_{50}$ values, a larger peak window current was estimated from the steady-state activation and inactivation curves during the ethanol withdrawal condition (Air 5 PM: 19% of the maximum current at $-82.9$ mV, AUC = 1.57 RU · mV; EtOH WD: 25% of the maximally-elicited T-type current at $-79.4$ mV; AUC = 2.04 RU · mV). No apparent difference was seen in the estimated peak window current between the control and EtOH EXP conditions (Air 9 AM: 20% of maximum current at $-79.3$ mV, AUC = 1.3 RU · mV; EtOH EXP: 20% of the maximum current at $-82.9$ mV, AUC = 1.69 RU · mV).

Although the voltage-dependent gating properties during withdrawal are significantly shifted to more depolarized potentials, they are similar to those recorded from air-exposed controls during the time point corresponding to the inactive period (9 AM). Additionally, the significant hyperpolarizing shift seen immediately following a fourth ethanol exposure resembles control voltage-dependent properties measured during the early active period (5 PM). These data indicate that ethanol exposure and withdrawal induce complex changes in T-type channel properties that disrupt the normally occurring daily variations observed in air-exposed animals. Furthermore, withdrawal from intermittent ethanol exposures appears to increase the available T-type current at physiologically relevant membrane potentials as suggested by the increases in estimated peak window current.

Figure 3 shows the average $V_{50}$ values for the steady-state inactivation curves generated for several time points over the course of a 4-wk multiple intermittent ethanol exposure and withdrawal paradigm. During this period, the average voltage-dependent inactivation $V_{50}$ values were seen to become significantly more hyperpolarized immediately following the second and fourth exposures (Fig. 3). During the withdrawal periods, the average inactivation $V_{50}$ values became progressively more depolarized and stayed significantly depolarized for up to 2 days following the fourth withdrawal period. The average $V_{50}$ eventually returns to values indistinguishable from controls at 4 wk after the initial week of intermittent ethanol exposure, indicating that these changes are transient. Recordings from additional cohorts of mice during the withdrawal period following 2, 3, and 4 wk of chronic intermittent ethanol exposure demonstrated that the steady-state inactivation $V_{50}$ values reach a plateau around $-80$ mV, and remain depolarized one day.
control animals (Fig. 4B), suggesting that Ca\textsubscript{V}3.2 channels make little, if any contribution to the native, whole cell T-type current in RE thalamic neurons during the early active period.

Withdrawal from chronic ethanol alters low-threshold Ca\textsuperscript{2+} bursts

As described earlier, low-threshold Ca\textsuperscript{2+} bursts can be elicited in thalamic neurons through hyperpolarizing current pulses followed by a return to holding potentials near resting membrane voltages. Using this protocol, we tested several different hyperpolarizing prepulse currents ranging from 25 to 375 pA followed by a rebound step to various holding potentials that ranged from −50 to −79 mV to determine whether or not a low-threshold Ca\textsuperscript{2+} burst could be elicited under those conditions. We then plotted the results along with the number of spikes per elicited burst to generate a burst map. The burst maps for RE neurons from air-exposed (5 pm) and 1 day post 20 WD are shown in Fig. 5, B and C, with a representative trace from the indicated points given on the graphs. In Fig. 5A, these traces are overlapped to show a longer T-type Ca\textsuperscript{2+} mediated voltage deflection during the withdrawal period, which allows for the generation of more action potentials. This longer deflection could be explained by the slower current decay kinetics measured during the course of the multiple, intermittent ethanol exposure and withdrawal paradigm (Table 1). The average number of action potentials per burst is quantified in Fig. 5D, demonstrating that RE neurons recorded during withdrawal from repeated ethanol exposures exhibit significantly more action potentials per burst (4 WD: 4.45 ± 0.08, n = 5; 1 day post 4 WD: 5.54 ± 0.06, n = 3; 1 day post 20 WD: 6.48 ± 0.06, n = 4) as compared with control RE neurons (3.94 ± 0.03, n = 4; *P < 0.01 ANOVA with Bonferroni’s multiple comparison post test).

We then employed a modified IFB computational model that we have previously used (Graef et al. 2009) to model the ethanol-induced alterations in whole cell T-type currents, thereby reproducing the changes in low-threshold Ca\textsuperscript{2+} bursts seen during our chronic ethanol exposure paradigm. Figure 6, A and B, shows the burst map generated by the IFB model for the air WD and 1 day post 20 WD time periods seen in Fig. 5, B and C, along with representative traces generated by the model for the indicated times given on the graphs. We then calculated a burst index from the generated burst maps for each time point that T-type currents were measured as a means to gauge neuronal excitability (Fig. 6C). Alterations in the burst index indicate increased excitability over the week of multiple intermittent exposures, with maximum excitability, as measured by the burst index, coming 1 day after 4 wk of intermittent ethanol exposure. These changes in both measured and modeled RE burst responses indicate a progressive increase in excitability during multiple, intermittent bouts of ethanol exposure that may lead to the generation of a hyperexcitable population of neurons during subsequent periods of withdrawal.

Chronic ethanol produces progressive disruptions in EEG theta power

We next sought to determine the effect of 4 wk of intermittent ethanol exposure in vivo on global brain activity as
measured by surface EEG. It has been demonstrated that multiple intermittent withdrawals produce a progressive disruption of EEG activity that takes the form of brief high-amplitude activity in the hippocampus (Veatch and Becker 2002). These events, referred to as brief spindle episodes (BSEs), have a frequency of 7–9 Hz and bear a strong resemblance to the ictal activity seen in absence epilepsy, a nonconvulsive seizure disorder. In addition, increases in the power and

FIG. 4. Contribution of CaV3.2 to changes in thalamic T-type Ca\(^{2+}\) channel currents during repeated ethanol exposures. A: the application of the CaV3.2-specific inhibitor, ascorbate, rapidly reduces the whole cell T-type current of a RE neuron during acute withdrawal from 4 intermittent ethanol exposures. B: ascorbate-inhibition of T-type current progressively increases with the amount of intermittent ethanol exposures; however, it has no effect on RE thalamic T-type currents from air-exposed animals. *\(P < 0.05\), **\(P < 0.01\), paired t-test.

FIG. 5. Midline thalamic neurons exhibit more spikes per low-threshold Ca\(^{2+}\) burst during withdrawal from chronic ethanol exposure. Burst profiles of RE neurons following different hyperpolarizing prepulses (y axis) while holding at various membrane potentials (x axis) with the number of spikes per burst indicated on the z axis. A: representative traces from a control RE neuron (black) and ethanol withdrawal RE neuron (gray) are overlapped to show a longer Ca\(^{2+}\) deflection and more spikes in the ethanol withdrawal condition. B: burst profile for a control cohort of mice during the withdrawal period after 4 wk of air exposures with a representative trace shown on the graph. C: the burst profile for RE neurons from mice 1 day following withdrawal from 4 wk of multiple, intermittent ethanol exposures (20WD) with a representative trace shown on the graph. D: significant increase in the average number of action potentials generated per low-threshold Ca\(^{2+}\) burst for WD mice 1 day following 4 wk of intermittent ethanol exposures (*\(P < 0.01\), compared with air exposed controls, 1-way ANOVA with a Bonferroni’s post test).
coherence of thalamocortical activity in this frequency range (4–9 Hz) are associated with several pathological conditions (Jeanmonod et al. 1996; Llinas et al. 1999; Sarnthein and Jeanmonod 2007, 2008). Therefore because of the strong recurrent connections between the RE, hippocampus and mPFC (DiPrisco and Vertes 2006; Dollerman-Van der Weel and Witter 1996, 2000; Dollerman-Van der Weel et al. 1997; Herkenham 1978; McKenna and Vertes 2004; Vertes et al. 2006, 2007), we examined ethanol-induced alterations in surface EEG theta rhythms (4–9 Hz).

Figure 7Ai shows the average, normalized theta (4–9 Hz) signal power for eight mice during the 7-day baseline period. Similar to our experiments on T-type channel gene expression and functional gating-properties, a normal, daily variation in theta power was seen with lower overall raw theta power during the dark (5 to 5 AM) period as compared with the light (5 AM to 5 PM) period (dark: 225.0 ± 8.0 μV², Light: 270.2 ± 6.1 μV², n = 8, P < 0.001, paired t-test). A higher normalized theta power during the baseline light period is shown in Fig. 7Aii ([arrow]) and is consistent with our molecular and physiological data demonstrating increased baseline gene expression and increased channel function at 9 AM as compared with 5 PM.

Figure 7, Bi and Ci, plots the normalized theta power for the first and fourth week of intermittent ethanol exposures, respectively. A peak in normalized power during the acute withdrawal period (1–5 PM) following each ethanol exposure can be seen for both the first and fourth weeks of ethanol exposure.

![Fig. 6. Modeled burst responses during 4 wk of repeated ethanol exposures and withdrawals. Contour plots generated by the integrate-and-fire or burst (IFB) model using the measured T-type current properties from air-exposed, control mice (A) and mice exposed to 4 wk of intermittent bouts of ethanol (B) during the withdrawal time period (1 day post 20 WD). Representative traces for the indicated points are also shown (scale bar, 100 ms, 20 mV). C: a burst index was calculated for all time points by multiplying the percent of total bursts (number of elicited bursts divided by 441 test points) by the number of spikes per burst and plotted.](http://jn.physiology.org/)

![Fig. 7. Chronic intermittent ethanol exposures disrupt baseline theta patterns. Ai: normalized theta electroencephalographic (EEG) power during 1 wk of continuously recorded baseline shows lower power during the dark period as compared with the light period (n = 8). Aii: an average 24 h plot of the normalized theta power during the baseline week shows higher power at 9 AM as compared with 5 PM. Signal power for the 4–9 Hz theta band during the 1st and 4th weeks of 5 intermittent ethanol exposures (M-F, 5 PM to 9 AM) are also shown (Bi and Ci) along with a corresponding average 24 h period (Bii and Cii). □, 12 hr dark (5 PM to 5 AM) period; ▪, 12-hr light (5 AM to 5 PM) period.](http://jn.physiology.org/)
(Fig. 7, Bi and Cii; n = 8). The higher normalized theta power at 5 PM and lower normalized power at 9 AM (Fig. 7, Bii and Cii) is consistent with the increase in channel function observed during each acute ethanol withdrawal period and decrease in channel function immediately following ethanol exposure. This indicates that the measured increases and decreases in T-type channel function occurred in parallel with the increases and decreases in theta power we observed over the intermittent ethanol exposure paradigm.

We then wanted to determine if these alterations in theta power continued into a subsequent week of withdrawal and if blocking the observed ethanol-mediated increases in T-type channel function with ethosuximide would have an effect on any persistent alterations. Ethosuximide has been shown to inhibit low-threshold T-type Ca2+ currents in both the thalamic ventrobasal nucleus (Coulter et al. 1989) and thalamic reticular nucleus of the rat (Huguenard and Prince 1994). Figure 8, Ai and Bi, plots the average, normalized theta (4–9 Hz) EEG power during the subsequent week of withdrawal for both saline (n = 3) and ethosuximide-treated (n = 5) mice, respectively. Figure 8Aii shows that at the beginning of the dark period, the normalized theta power remained elevated in saline-treated mice. This is in contrast to ethosuximide-treated animals, where the normalized theta power was lower during the beginning of the dark period following daily intraperitoneal injection of ethosuximide (Fig. 8Bii; injections given at 5 PM—the onset of the dark period). This indicates that blocking T-type channel function during withdrawal with ethosuximide corresponds to significant reductions in theta power during ethanol withdrawal.

To statistically compare each condition, we divided the normalized theta power during the acute withdrawal period (1–5 PM) by the normalized theta power during the dark period (5 PM to 5 AM). This ratio was significantly greater during the first exposure of ethanol week one as compared with a baseline ratio between the same time periods (Ci; M, n = 8, P < 0.01, ANOVA with Bonferroni’s post test). The normalized theta ratio then progressively decreases toward baseline values during subsequent ethanol exposures and becomes significantly lower during the fifth exposure of ethanol week 1 (Ci; F, n = 8, P < 0.05, ANOVA with Bonferroni’s post test). This normalized theta ratio remained significantly lower during the fourth week of ethanol exposure (Cii; n = 8, P < 0.05, ANOVA with Bonferroni’s post test). For saline-treated mice, the normalized theta ratio continued to remain significantly reduced as compared with baseline values and was significantly lower than ethanol week 4 values as well (Cii; n = 3, P < 0.01 as compared with respective baseline treatment group, P < 0.05 as compared with respective ethanol week four treatment group, ANOVA with Bonferroni’s post test).

For ethosuximide-treated mice, the normalized theta ratio during the week of withdrawal was not statistically different from either baseline or ethanol week 4 as compared with its respective treatment group for both conditions (Cii; n = 5). These data indicate that inhibiting T-type channel function with ethosuximide treatment had a similar effect as ethanol exposure on theta power. Furthermore, while the diurnal variation in theta power appears to become independent of the light/dark cycle and entrained to the ethanol exposures, this variation is no longer apparent during a subsequent untreated week of withdrawal but is restored with ethosuximide. This indicates that the increase in T-type channel function continues into the withdrawal week and that inhibiting this increase restores the normal entrainment of the diurnal variation in theta power to the light/dark cycle.

These data show that chronic intermittent ethanol exposure produces a profound disruption of the normal variations in theta power that persists during a subsequent week of withdrawal and can be ameliorated with the T-type channel blocker ethosuximide. Taken together with our data showing progres-
sive disruptions in both the expression and function of thalamic T-type channels that also began during the first week of intermittent ethanol exposures, these results suggest a potential association between ethanol-mediated changes in T-type channels and theta rhythms that warrants further investigation.

DISCUSSION

In this study, we have demonstrated the effects of multiple intermittent ethanol exposures on the expression and function of T-type calcium channels in midline thalamic neurons. Our results show normal variations in relative T-type channel mRNA levels and steady-state inactivation properties between daily time points corresponding to active and inactive periods that were significantly altered during chronic ethanol exposure. Additionally, shifts in T-type channel voltage-dependent gating properties corresponded to an increased ability to elicit low-threshold Ca^{2+} bursts during acute withdrawal periods. The changes in thalamic T-type channel properties occurred in parallel with progressive alterations in theta (4–9 Hz) EEG power in vivo, such that measured increases and decreases in T-type channel function corresponded to observed increases and decreases in normalized theta power, respectively. Furthermore, disrupted diurnal variations in theta power during a subsequent week of withdrawal could be restored by the T-type Ca^{2+} channel blocker ethosuximide. Our results suggest that along with well-established synaptic changes, alterations in intrinsic membrane properties mediated by augmented thalamic T-type channel function may also play a role in the disrupted balance between excitation and inhibition that results from chronic ethanol exposure.

T-type channel diurnal variation and effects of multiple exposures and withdrawals

Our findings showing that chronic ethanol exposure alters thalamic CaV3.2 and CaV3.3 mRNA levels support previous work from our lab (Nordskog et al. 2006) demonstrating that all three T-type channel isoforms displayed a diurnal gene expression pattern in mouse thalamus with peak expression exhibited during the inactive phase and lower expression during their active period. Diurnal variations have also been shown in rat suprachiasmatic neurons for both CaV3.1 gene expression (Nahm et al. 2005) and T-type current (Kim et al. 2005). Our molecular and physiological data demonstrate a similar variation with higher expression and increased channel function at a time point corresponding to the early inactive period as opposed to lower expression and reduced function at the beginning of the active phase. It is well established that cholinergic brain stem efferents depolarize thalamic relay neurons during waking hours, thereby supporting tonic firing and reducing T-type channel-mediated bursts (Francesconi et al. 1988; Hu et al. 1989; McCormick 1989). Our results add to this framework by suggesting that normal diurnal variations in thalamic T-type channel gating properties can help stabilize these firing modes by further inhibiting or enhancing low-threshold thalamic bursts.

Our data also suggest a relationship between daily variations in thalamic T-type channel function and diurnal fluctuations in theta power. Increased thalamic T-type channel function occurred in parallel with higher normalized theta power during the inactive period, and decreased thalamic T-type channel function occurred in parallel with lower normalized theta power during the active period. Additionally, the ethanol-mediated increase in T-type channel function observed during acute withdrawal periods corresponded to increases in normalized theta power. Furthermore, we measured decreases in theta power in response to both ethanol exposure and ethosuximide treatment that are consistent with the acute inhibitory effects of both ethanol and ethosuximide on T-type channel function. These data reveal a relationship between ethanol-mediated alterations in thalamic T-type channel function and theta power fluctuations that warrants further investigation.

T-type channel alterations in different models of hyperexcitability

Our results bear some similarities to our previous study of T-type channel function in a model of chemically induced epilepsy (Graef et al. 2009). In the current study, we also observed increased thalamic excitability, but we did not record overt seizure activity during withdrawal periods. The absence of ethanol withdrawal seizures could be partially explained by the less dramatic and transient changes in thalamic T-type channels as compared with the persistent alterations seen in an acquired model of epilepsy (Graef et al. 2009). Another possible reason is that additional voltage-gated channels undergo significant changes in acquired models of epilepsy (Graef and Godwin 2010) which may not occur to the same degree during ethanol exposure and withdrawal. Additionally, the strain of mice used in this study, C57Bl/6, has been shown to exhibit less severe ethanol withdrawal symptoms as compared with more sensitive strains such as DBA/2Js (Crabbe 1998; Crabbe and Phillips 1993; Roberts et al. 1992). It will be important to...
determine if ethanol-induced T-type alterations are exaggerated in vulnerable strains and in what capacity other structures and channels contribute to this sensitivity.

Potential role of the midline thalamus in abnormal theta rhythms during ethanol exposure

Abnormal theta EEG activity has been reported in alcoholics and animals models of chronic ethanol exposure and withdrawal, indicating a potential role for altered theta frequencies in the development of alcoholism and withdrawal symptoms (Ehlers and Sławecki 2000; Kubota et al. 2002; Rangaswamy et al. 2003). Specifically, increases in resting EEG theta coherence have been reported in alcoholics that were suggested to arise from altered thalamocortical function (Porjesz and Rangaswamy 2007). The interplay between thalamic relay neurons and cells of the thalamic reticular nucleus (TRN) produces sleep-related oscillations (Steriade 2003, 2005). Alterations within this thalamic circuitry can lead to disruptions in normal brain rhythms that have been associated with several pathological conditions such as neurogenic pain, epilepsy, and movement disorders. These alterations have been termed thalamocortical dysrhythmias and display a significant increase in medial thalamic T-type channel-mediated bursts as well as augmented power and thalamocortical coherence in the theta (4–9 Hz) band range (Jeanmonod et al. 1996; Llinás et al. 1999; Sarnthein and Jeanmonod 2007, 2008). Recent work by Jia et al. (2008) demonstrated the potentiating effects of acute ethanol on the tonic inhibition of thalamocortical neurons via extrasynaptic GABA_A receptors containing the α4 subunit. Therefore potential ethanol-induced alterations in thalamocortical circuitry mediated by disruptions in the interaction between thalamic T-type channels and GABA_A receptors could play a role in the theta alterations we observed during the 4 wk of multiple intermittent ethanol exposures.

In addition to the thalamus, EEG theta band disruption could also be partly due to the reported effects of ethanol on hippocampal activity (Becker and Veatch 2002; Veatch and Gonzalez 1996). Hippocampal-dependent theta oscillations have been shown to arise from inputs coming from both the brain stem and medial septum (Buzaki 2002; Colom et al. 1987; Oddie et al. 1994). The CA1 region also has dense excitatory projections to the mPFC; however, there are no direct cortical feedback connections. Instead mPFC axons form synaptic contacts with the dendrites of RE neurons projecting to the CA1, suggesting that the RE is a critical node involved in modulating activity between the CA1 and mPFC (Vertes et al. 2007). The RE also sends glutamatergic projections to the medial septum (Bokor et al. 2002; Herkenham 1978; Vertes et al. 2006), placing these midline thalamic neurons in an advantageous position to modulate both hippocampal-cortical and septohippocampal activity (Fig. 9). The changes in theta rhythms we observed during chronic ethanol exposure and withdrawal may be consistent with augmented T-type channel bursts from thalamic RE neurons that are distributed onto neuronal populations in the hippocampus, medial septum, and mPFC. Indeed our results support this notion by demonstrating that the ethanol-induced disruptions in baseline theta frequencies we observed persisted into a subsequent week of withdrawal and were restored with the T-type channel blocker ethosuximide. This is in agreement with an earlier study that demonstrated ethosuximide was able to reduced withdrawal symptoms in ethanol-dependent mice (Kaneto et al. 1986).

Finally it is also important to note that in addition to midline thalamus, neurons in the CA1, medial septum, and cortex all express T-type channels (Talley et al. 1999). Potential changes in T-type channel mediated bursts within these regions might also have contributed to the observed ethanol-induced theta disruptions as well as played a role in the ethosuximide effects we saw during the withdrawal week. Future experiments investigating the effects of chronic ethanol on the intrinsic excitability of these neuronal populations will help to further clarify the role of these channels in ethanol-mediated alterations in theta rhythms.

In conclusion, our study illustrates unique functional properties of native T-type channels in midline thalamus, including daily variations in functional channel properties. We demonstrated a disruption of this baseline variation during a schedule of chronic intermittent ethanol exposures. Furthermore, the increase in T-type channel function during acute withdrawal periods translated into a greater tendency for these neurons to burst, indicating a strong T-type channel contribution to altered excitability during withdrawal. Finally, these changes occurred in parallel with disruptions in EEG theta power that could be ameliorated with the administration of the T-type channel blocker ethosuximide during withdrawal, suggesting a functional role for these channels in the appearance of altered theta activity during chronic intermittent ethanol exposure. Our results reveal T-type Ca^{2+} channels as a promising new target in the understanding of disrupted network activity and hyperexcitability occurring with chronic alcoholism and withdrawal.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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