Essential Thalamic Contribution to Slow Waves of Natural Sleep

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Slow waves represent one of the prominent EEG signatures of non-rapid eye movement (non-REM) sleep and are thought to play an important role in the cellular and network plasticity that occurs during this behavioral state. These slow waves of natural sleep are currently considered to be exclusively generated by intrinsic and synaptic mechanisms within neocortical territories, although a role for the thalamus in this key physiological rhythm has been suggested but never demonstrated. Combining neuronal ensemble recordings, microdialysis, and optogenetics, here we show that the block of the thalamic output to the neocortex markedly (up to 50%) decreases the frequency of slow waves recorded during non-REM sleep in freely moving, naturally sleeping-waking rats. A smaller volume of thalamic inactivation than during sleep is required for observing similar effects on EEG slow waves recorded during anesthesia, a condition in which both bursts and single action potentials of thalamocortical neurons are almost exclusively dependent on T-type calcium channels. Thalamic inactivation more strongly reduces spindles than slow waves during both anesthesia and natural sleep. Moreover, selective excitation of thalamocortical neurons strongly entrains EEG slow waves in a narrow frequency band (0.75–1.5 Hz) only when thalamic T-type calcium channels are functionally active. These results demonstrate that the thalamus finely tunes the frequency of slow waves during non-REM sleep and anesthesia, and thus provide the first conclusive evidence that a dynamic interplay of the neocortical and thalamic oscillators of slow waves is required for the full expression of this key physiological EEG rhythm.

Introduction

Slow waves and their neuronal counterpart, the cortical and thalamic oscillations between depolarized UP states and hyperpolarized DOWN states (Steriade et al., 1993a; Contreras and Steriade, 1995; Petersen et al., 2003; Siroti and Buzsáki, 2005; Crunelli et al., 2012), are the main EEG hallmark of non-rapid eye movement (non-REM) sleep (Crunelli and Hughes, 2010; Brown et al., 2012) and are also observed during anesthesia (Chauvette et al., 2011). The physiological importance of these waves of natural sleep is emphasized by their ability to group together other EEG rhythms of non-REM sleep (Steriade, 1997) and by their putative role in the consolidation of recently acquired memories (Tononi and Cirelli, 2001; Marshall et al., 2006; Ji and Wilson, 2007).

The mechanisms underlying the generation of EEG slow waves, however, remain controversial. Because (1) lesions of thalamic nuclei do not suppress slow waves in anesthetized cats (Steriade et al., 1993b) and (2) UP and DOWN states are recorded in neocortical slices (Sanchez-Vives and McCormick, 2000; Cossart et al., 2003) and in an isolated cortical gyrus in vivo during anesthesia (Timofeev et al., 2000), these EEG slow waves are exclusively and consistently viewed as a cortically generated rhythm (Sanchez-Vives and McCormick, 2000; Timofeev et al., 2000; Chauvette et al., 2011; Brown et al., 2012). However, (1) increasing thalamic inhibition alters EEG slow waves in anesthetized rats (Doi et al., 2007) and suppresses whisking-induced cortical UP states in head-restrained mice (Poulet et al., 2012); (2) UP and DOWN states, and associated slow waves, can be recorded in thalamic slices (Hughes et al., 2002, 2004; Blethyn et al., 2006); and (3) selective thalamic degeneration modifies slow waves of non-REM sleep in humans (Gemignani et al., 2012). These findings, together with other mechanistic in vitro studies and investigations in anesthetized animals (for review, see Crunelli and Hughes, 2010), question the current cortico-centric view of slow wave generation and led us to suggest that the full expression of these EEG waves of natural sleep requires a dynamic interplay of cortical and thalamic oscillators (Crunelli and Hughes, 2010). Unfortunately, the resolution of this controversy is still hampered by the lack of any study that has directly and systematically addressed this issue in unrestrained, naturally waking-sleeping...
animals. Moreover, our current mechanistic knowledge of slow waves of natural sleep continues to be clouded by the speculative extrapolations of findings obtained in anesthetized conditions.

Using a combination of neuronal ensemble recordings, thalamus-selective pharmacological inactivation, and optogenetic activation of thalamocortical (TC) neurons in naturally sleeping or anesthetized rats, here we show, for the first time, that the thalamus is required for finely tuning the frequency of slow waves during non-REM sleep and anesthesia. Moreover, we demonstrate that the entrainment of EEG slow waves by selective thalamic activation is dependent on T-type calcium channels. Together, these results provide the first conclusive evidence that cortical and thalamic oscillators are necessary for the full expression of slow waves of non-REM sleep.

Materials and Methods

All experimental procedures were performed in accordance with the United Kingdom Animals (Scientific Procedure) Act 1986 and local ethics committee guidelines. All efforts were made to minimize animal suffering and the number of animals used. Experiments were performed on male adult Wistar rats (260–400 g, Harlan Laboratories), maintained on a normal diet and under an 8:00 A.M. to 8:00 P.M. light-on regimen.

Experiments in anesthetized rats

Surgery. After anesthesia induction with 5% isoflurane, rats received an intraperitoneal injection of ketamine (120 mg/kg) and xylazine (20 mg/kg). Anesthesia was then maintained with a constant flow of ketamine (120 mg/kg) and xylazine (20 mg/kg). Anesthesia was then maintained with a constant flow of ketamine (120 mg/kg) and xylazine (20 mg/kg). Anesthesia was then maintained with a constant flow of ketamine (120 mg/kg) and xylazine (20 mg/kg). Anesthesia was then maintained with a constant flow of ketamine (120 mg/kg) and xylazine (20 mg/kg). Anesthesia was then maintained with a constant flow of ketamine (120 mg/kg) and xylazine (20 mg/kg). Anesthesia was then maintained with a constant flow of ketamine (120 mg/kg) and xylazine (20 mg/kg). Anesthesia was then maintained with a constant flow of ketamine (120 mg/kg) and xylazine (20 mg/kg). Anesthesia was then maintained with a constant flow of ketamine (120 mg/kg) and xylazine (20 mg/kg). Anesthesia was then maintained with a constant flow of ketamine (120 mg/kg) and xylazine (20 mg/kg). Anesthesia was then maintained with a constant flow of ketamine (120 mg/kg) and xylazine (20 mg/kg).

For measuring the spatial extent of the action of 3,5-dichloro-N-[1-(2,2-dimethyl-tetrahydro-pyran-4-ylmethyl)-4-fluoro-piperidin-4-ylmethyl]-benzamide (TTA-P2), a potent and selective T-type channel antagonist (Uebel et al., 2009; Dreyfus et al., 2010), or of the sodium channel blocker TTX in the thalamus (i.e., see experiments illustrated in Fig. 3), a 1 mm hole was drilled unilaterally above the ventrobasal thalamic nucleus (VB), the thalamus was carefully removed, and a 32-channel silicone probe (10 mm length, 0.6 mm width, 1–4 μM) with four shanks (200 μm recording point distance, 15 μm thickness) (NeuroNexus Technologies) was slowly lowered in the VB (dorsoventral [DV] = -4.5 to -5.5 mm). A second hole in the same hemisphere was used to slowly lower (200 μm every 5 min) a microdialysis probe (CMA 12 Elite, 2 mm dialysis membrane length, 20 kDa cutoff, with a 16° angle with respect to the vertical axis; see Fig. 3A) to a final position that was between 0.05 and 1 mm away from the silicone probe. The 16° angle positioning of the dialysis probe was dictated by the space constraints of the silicone and dialysis probes connecting devices on the animal skull.

For measuring the effect of TTX and TTA-P2 (applied by microdialysis in the VB) on slow and spindle waves during anesthesia (i.e., see experiments illustrated in Fig. 4), EEG electrodes were implanted as above, and two microdialysis probes (one in each VB) were slowly lowered fully vertically until their tips rested 6.5 mm below the skull (i.e., in the most ventral part of the VB). In some rats, a silicone probe was also inserted unilaterally in the VB with a 16° angle with respect to the vertical axis.

For measuring the effect of systemic TTA-P2 injection (i.e., see experiments illustrated in Fig. 5), rats were implanted with EEG electrodes and a unilateral silicone probe in the VB (as described above).

Systemic and microdialysis solutions. For intraperitoneal injection, TTA-P2 was dissolved in saline containing 4% DMSO and the pH adjusted with potassium hydroxide (1 m). Control intraperitoneal injections contained 4% DMSO in saline. For reverse microdialysis injection, TTA-P2 was dissolved in aCSF with 4% DMSO. Tetrodotoxin citrate (TTX) was dissolved in aCSF. Flow rate of the microdialysis injection was set at 1 μl/min.

Injections. For the systemic injections, once stable EEG slow waves were recorded for at least 30 min and high amplitude well isolated units were present in some of the silicone probe channels, a control period of at least 40 min was recorded before injecting intraperitoneally either saline/ DMSO or TTA-P2 while continuing recording for at least another 2 h. For intrathalamic drug application, reverse microdialysis injection of aCSF was initiated as soon as the microdialysis probes were in position, and continued for at least 1 h before electrical recordings commenced. Once stable, high-amplitude, well-isolated units could be recorded from...
with 16-bit resolution. EEG recordings were digitized using the Plexon made, signals were digitized with a Plexon recorder/64 system at 20 kHz width DC to 500 Hz). When combined unit and EEG recordings were AMP (Pecs) pre- (bandwidth 0.1–500 Hz) and main-amplifiers (bandwidth 0.8 Hz to 54 kHz) and a Plexon recorder/64 transcardially perfused.

continued for at least 1 h. At the end of the recording session, rats were ing either TTX or TTA-P2. Recordings and dialysis application then continued for at least 40 min was initiated. This was followed either by continuation of aCSF application or the inlet tubing was switched to a solution contain- ing either TTX or TTA-P2. Recordings and dialysis application then continued for at least 1 h. At the end of the recording session, rats were transcardially perfused.

Recordings. Thalamic signals were amplified with an Omnetics pream- plifier (gain 20, bandwidth 0.8 Hz to 54 kHz) and a Plexon recorder/64 channel amplifier (gain 7500–12,500, bandwidth 1–6000, Plexon).

The EEG signal was amplified with a combination of SuperTech Bio- AMP (Pecs) pre- (bandwidth 0.1–500 Hz) and main-amplifiers (bandwidth DC to 500 Hz). When combined unit and EEG recordings were made, signals were digitized with a Plexon recorder/64 system at 20 kHz with 16-bit resolution. EEG recordings were digitized using the Plexon recorder input via the IP16 event input break- out panel. The digitized data were converted to Spike2 format (version 5.13, CED). For all further analyses, data were converted to a raw bi- nary format using tools of the freely available Kusters, Neuroscope, and NDManager software suite (Hazan et al., 2006). EEG data were low-pass filtered with a windowed sinc filter at 100 Hz and downsampled to 200 Hz.

Experiments in freely moving rats
Surgery. Rats were anesthetized and implanted with EEG electrodes, as described above, and an EMG electrode was positioned in the neck mus- cle. Two or four microdialysis guide cannulae (one or two in each VB, respectively) (i.e., see experiments illustrated in Fig. 6 for two probes in each VB) were slowly lowered fully vertically so their tip was just above the VB (DV = −4.4 mm) (Paxinos and Watson, 2007). When two guide cannulae were inserted in each VB, their AP co- ordinates were as follows: −2.6 and −3.8 mm (Paxinos and Watson, 2007). Both EEG elec- trodes and guide cannulae were fixed to the skull with dental acrylic cement. Rats were allowed at least 7 d to recover from surgery (single housed) and to habituate to the recording cage (4 h/d). At the end of the recording sessions, rats were tran- scardially perfused.

Injections. For the systemic injections, on the day of the experiment rats were transferred to the recording cage, connected to the electrical recording and microdialysis apparatus (as ap- propriate), and allowed to move freely in their cage for at least 1 h before any recording com- menced. A control period of 1.5 h was then recorded before an intraperitoneal injection of either saline or TTA-P2 was made while continuing recording for at least another 2 h. Four days were allowed between two consecutive recording days in each rat. For intrathalamic drug application, 24 h before recording, mi- crodialysis probes were slowly inserted into the brain to replace the dummy probe in the guide cannulae. On the day of the experi- ments, rats were transferred to the recording cage and connected to the dialysis probes and electrical recording apparatus. aCSF dialysis was initiated immediately while the rats were allowed to habituate for 1 h. A control period of 2.5 h was then recorded while administer- ing aCSF (same conditions as described for the anesthetized condition). Animals were then recorded for an additional 2 h while receiving either aCSF or drug-containing aCSF (in a random order on consecutive re- cording days, each separated by at least 4 d). Video recording was performed simultaneously with electrical recordings in all experi- ments in freely moving rats.

TTA-P2 levels in the VB. The concentration of TTA-P2 in samples taken from the inlet and outlet dialysis tubes was measured, following protein precipitation with acetonitrile, by liquid chromatography-mass spectrometry under a validated analytical protocol (Shipe et al., 2008; Uebele et al., 2009). TTA-P2 concentration in the brain tissue outside the dialysis membrane was estimated according to the equilibrium equation described previously (Chan and Chan, 1999).

Optogenetics
Viral injection. pAAV-CaMKIIα-bChR2(H134R)-mCherry plasmids (K. Deisseroth laboratory, Addgene plasmid 26975) were packaged into recom-

Figure 2. Detection of slow and spindle waves. A, Representative EEG wavelet power spectrum of slow wave frequency band (0.2–4.5 Hz) during ketamine-xylazine anesthesia. B, Representative EEG broadband power spectrum. Dashed lines indicate the frequency band shown in A. C, Expanded EEG trace (black) (from time period marked in A with arrow) illustrating the identification of slow waves (green circles) by means of negative to positive zero-crossing detection on the 0.2–4.5 Hz bandpass-filtered signal (green trace). Waves with peak-to-peak amplitude (red triangles to blue triangles) of <60% of the mean peak-to-peak amplitude were discarded. D, Autocorrelogram of EEG slow waves detected as shown in C from the first 20 min of data shown in A. E, Representative raw (middle), 5–12.5 Hz bandpass-filtered (bottom) EEG traces showing spindle waves (black arrows) and wavelet power spectrum (top) with detected spindle wave episodes (green lines) during anesthesia. F, Average frequency distribution of spindles during anesthesia (n = 7 rats). G, H, Data for spindles during natural sleep are illustrated in the same format as E and F, respectively (n = 6 rats).

some of the silicone probe channels, recording of a control period of at least 40 min was initiated. This was followed either by continuation of aCSF application or the inlet tubing was switched to a solution contain- ing either TTX or TTA-P2. Recordings and dialysis application then continued for at least 1 h. At the end of the recording session, rats were transcardially perfused.

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binant AAV2 vectors and serotyped with AAV1 coat proteins. Viral suspensions were titrated to 1.14 × 10^11 genome copies/ml (GC) (University of Pennsylvania Vector Core). Concentrated stock virus was diluted with 0.1 M PBS tinted with Fast Green FCF (Sigma), giving a final viral concentration of 5.70 × 10^6 to 2.28 × 10^7 GC/µl for injection. The dorsal surface of the skull was exposed, a small craniotomy made over one VB (AP = −3.14 mm, ML = 2.80 mm from bregma) (Paxinos and Watson, 2007), and the dura reflected. A 10 µl Gastight Hamilton syringe and 34 G6 needle were front filled with mineral oil and diluted virus. Needles were then lowered slowly into the thalamus (DV = −5.75 mm from the pia) and left in place for 10 min. A 1 µl virus injection site (AP, ML, DV) was posed, a small craniotomy made over one VB, and the needle was slowly retracted. Rats were then individually housed and allowed to recover with ad libitum access to food and water for a minimum of 3 weeks to allow viral gene expression.

**Optical stimulation and recording.** Rats previously injected with RAVV-CaMKIIe-hChR2 (H134R)-mCherry were anesthetized and EEG screws and two microdialysis probes (one in each VB) were implanted (as above) with a 16° angle with respect to the vertical axis. A 200 µm multimode (0.39 NA) optic fiber (CFM121.20; Thorlabs) was custom-glued to a 32-channel silicone probe, with the fiber tip ~400 µm above the top recording sites. The fiber ferrule was connected to a compatible patch-cord and 473 nm laser diode (70 mW Stradus; Vortran Laser Technology), and the silicon probe and EEG wires connected to a Digital Lynx 10SX recording system (with Hybrid Input Boards; Neuralynx) via HS-36 unity gain preamplifiers. Data preprocessing and spike sorting were performed with the Klusters software and a 1322A Digidata (Molecular Devices), synchronized with a 1401a (CED) via USB, and were visually examined by an expert neurophysiologist. Using the Klusters software, all spikes were computed with their respective ChR2 firing rate and exit point before being manually edited by an expert neurophysiologist.

**Burst analysis.** Bursts were defined as two or more spikes that were preceded by at least 100 ms of silence and had interspike intervals (ISIs) <10 ms, and were visually examined post hoc. For each burst, the following parameters were calculated (Fig. 1): (1) ISI, (2) interburst interval, (3) number of spikes per second in 5 min windows (spike rate), (4) number of bursts per second in 5 min windows (burst rate), (5) ratio of all spikes taking part in a burst, (6) number of spikes in each burst, and (7) ISI as a function of the ISI number within a burst (i.e., burst signature). Units with a decelerando burst signature (Fig. 1C,D) were classified as TC neurons, whereas those with a burst signature and spike autocorrelation typical of thalamic reticular neurons (Huguenard and Prince, 1992) were discarded.

**Detection of slow waves and spindles under anesthesia.** To quantify EEG slow waves beyond power spectral analysis, a slow wave detection algorithm similar to those described previously (Mölle et al., 2009; Nir et al., 2011) was implemented (Fig. 2A–D). On the 0.2 to 4.5 Hz band-pass filtered signal, all negative to positive zero-crossings were detected as slow waves. To discard spurious slow waves, the local minimum and maximum around a crossing were determined. If the difference between these was <60% of the mean maximum-to-minimum distance, the slow clustered by an expectation maximization algorithm (Klustakwik; Harris et al., 2000) on the basis of their first three principal components. All results of the automatic clustering were verified post hoc by visual inspection. Units were excluded from further analyses if more than one high amplitude cell was present on a single channel, if their autocorrelation did not show a refractory period of at least 2 ms, and if their spike amplitude markedly changed during the experimental session.

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Spindles and 1.0 cycle for slow waves at a frequency resolution of 0.1 Hz for spindles and 0.01 Hz for slow waves (Kronland-Martinet et al., 1987). Using a wavelet ridge extraction method, each oscillatory epoch of the EEG was extracted with an energy threshold to detect its beginning and end (see Figs. 2G, 6A, and 7A) (Roux et al., 2007; Garcia and Fourcaud-Trocmé, 2009). The boundary frequencies of wave detection were chosen as from 0.5 to 4 Hz for slow waves (see Fig. 6B) and from 6 to 14 Hz for spindle oscillations (see Fig. 2G,H). The threshold was defined as 3 times the average energy during a non-REM sleep period during the control session. Slow and spindle waves with <2 and 3 cycles, respectively, were discarded. When overlapping oscillations were detected, the wave with the highest energy was selected.

Statistical analysis. Group comparisons were performed using the Mann–Whitney U test. Paired data were tested with Wilcoxon’s signed rank test. All quantitative data in figures and text are given as mean ± SEM.

Histology
Electrode and microdialysis probe tracking. Before insertion, silicone and microdialysis probes were immersed for 1/2 h and 5 min, respectively, in a 1% Vybrant DiI (Invitrogen) dye solution. At the end of the experiment, rats were injected with a lethal dose of urethane (40%), and the brains were removed and placed in a 0.1 M PFA solution for 48 h. The brains were then transferred and stored in a 0.1 M PBS. Sections (100 µm thick) containing the VB were cut with a vibratome (Leica VT1000S) and mounted on coverslips to measure the relative position of microdialysis and silicone probes, which were visualized using a fluorescent microscope (Leica).

Immunofluorescence. Rats were given an overdose of ketamine-xylazine and transcardially perfused with 4% PFA. Brains were fixed in 4% PFA and then cryoprotected in 20% (w/v) sucrose in 0.1 M PBS. Each brain was blocked to give coronal sections, mounted onto a freezing microtome (Leica), and cut into 50 µm sections. Free-floating sections were processed for NeuN and RFP immunofluorescence. Briefly, sections were washed 3 × 10 min with fresh 0.1 M PBS and blocked for 1.5 h in 1 × PGT (0.1 M PBS, 3% NGS, and 0.2% Triton X-100 (Sigma-Aldrich)). Sections were gently shaken at room temperature for 2 h, then overnight at 4°C with primary antibodies against both NeuN (1:300 mouse monoclonal, Millipore), and RFP (1:1000 Living Colors DsRed rabbit polyclonal, Clontech) in 1 × PGT. Sections were rinsed 3 × 10 min with fresh 0.1 M PBS and incubated for 2 to 3 h at room temperature with 1:200 goat anti-mouse AlexaFluor-488 (Invitrogen) and 1:200 goat anti-rabbit AlexaFluor-594 (Invitrogen) secondary antibodies in 1 × PGT. Sections were washed 3 × 10 min in fresh 0.1 M PBS and mounted onto gelatin-subbed Superfrost Plus microscope slides (Thermo Scientific). Slides were coverslipped with Vectashield fluorescent mounting medium (Vector Laboratories) and visualized using a fluorescence microscope (Leica).

Results
Effect of thalamic inactivation on slow waves during anesthesia
We first abolished the somatosensory thalamic output to the cortex by bilateral reverse microdialysis of TTX in the ventrobasal thalamus (VB) of ketamine-xylazine anesthetized rats, while

![Block of thalamic firing decelerates EEG slow waves during anesthesia](image-url)

Figure 4. Block of thalamic firing decelerates EEG slow waves during anesthesia. A. Spike raster plots (top three traces; *bursts* from 3 VB TC neurons and EEG from S1 (bottom trace) show the effects of 50 µM TTX and 300 µM TTA-P2 dialysis in the VB. The predominant burst firing during aCSF is virtually abolished by TTX and TTA-P2, an effect accompanied by slowing of the EEG rhythm. B. Event-triggered averages of raw EEG traces centered on the middle point of DOWN to UP state transitions were calculated after 1 h of aCSF, TTX, and TTA-P2 dialysis (n = 438, 243, and 222 transitions, respectively). C. Normalized (to predrug period), time-dependent decrease of slow waves by TTX (n = 5) and TTA-P2 (n = 5) (drug dialysis starts at 0). D, E. EEG power spectra 60 min after start of drug dialysis. E. Normalized (to predrug period), time-dependent reduction of spindle waves by TTX and TTA-P2. C–E. Solid lines indicate the mean; color shadings indicate SEM. In this and the following figures, illustrated drug concentrations during microdialysis are those of the inlet dialysis tube (for brain concentration delivered by dialysis probes, see Fig. 3E). In this experiment and those depicted in Figures 5, 6, and 7, the dialysis probes were inserted in a fully vertical position.
simultaneously recording slow waves from primary somatosensory cortex (S1) and the firing of different single TC neurons in the VB. TTX abolished action potentials in TC neurons around the VB injection site (predrug: 2.51 ± 0.11 spikes/s, TTX: 0.001 ± 0.002, n = 11 rats, n = 33 neurons, p < 10^(-7), Wilcoxon signed-rank test) (Fig. 3B1,B2, blue lines, C, bottom plot; see also Fig. 4A, unit recordings). This effect led to a concomitant marked reduction (45 ± 1.4%) in the number of EEG slow waves after 1 h of drug dialysis (Fig. 4A,B) and thus to a frequency decrease from 1.19 ± 0.02 to 0.63 ± 0.03 slow waves/s (n = 5, p = 0.0017, Mann–Whitney U test compared with aCSF, n = 6) (Fig. 4A–D). The duration of both UP and DOWN states was increased by TTX from 0.40 ± 0.01 (UP) and 0.42 ± 0.03 s (DOWN) to 0.68 ± 0.07 and 0.58 ± 0.06 s, respectively (p < 0.006, Mann–Whitney U test compared with aCSF) (Fig. 4B). In the same rats, the simultaneously recorded spindle waves, which are a thalamically generated rhythm (Morison and Bassett, 1945; Steriade et al., 1985; De Gennaro and Ferrara, 2003; Astori et al., 2011), were abolished by TTX (predrug: 0.13 ± 0.02 spindles/s, TTX: 0.009 ± 0.008, p = 0.0016) (Fig. 4E), indicating the effectiveness of our thalamic inactivation by microdialysis administration of TTX.

Because T-type calcium channels play a key role in the thalamic output to cortex (Llinás and Jahaens, 1982; Crunelli et al., 1989; Deleuze et al., 2012) and underlie TC neuron UP states (Hughes et al., 2002), we next investigated the effect on slow waves of the potent and selective T-type calcium channel antagonist, TTA-P2 (Uebele et al., 2009; Dreyfus et al., 2010), directly applied in the VB by reverse microdialysis. This drug produced a block of high-frequency bursts of TC neurons, which was dependent on its concentration in the dialysis inlet tube and on the distance between the recorded neuron and the dialysis probe (Fig. 3B1; see also unit recordings in Fig. 4A). Interestingly, single action potential firing (which accounted for ~18.4 ± 1.2% of the total firing) was also markedly decreased by TTA-P2 (Fig. 3B2) (see Fig. 4A, unit recordings) so that, at a distance of 500 µm from the dialysis probe, only 3.3 ± 2.1% of total spikes (i.e., in bursts and as single action potentials) remained after 1 h of TTA-P2 application. We chose a microdialysis inlet tube concentration of 300 μM TTA-P2 because: (1) it virtually abolished the total TC neuron firing in a region that almost fully covered the mediolateral extent of the VB (Fig. 3C,D), and (2) it resulted in a tissue concentration around the probe of ~42 μM (based on a 14% recovery rate) (for explanation, see Fig. 3E), which is well in the range of concentrations that we previously showed to be required to abolish intrinsic and synchronically driven TC neuron firing in the VB in vitro (Dreyfus et al., 2010, their Fig. 1C). Thus, TTA-P2 applied in the VB at 300 μM elicited a clear reduction (25 ± 3%, n = 5, p = 0.02 compared with aCSF, n = 6) of slow waves (Fig. 4A,B), leading to a frequency decrease from 1.00 ± 0.02 to 0.69 ± 0.03 slow waves/s (p = 0.020) (Fig. 4A,C,D). The durations of UP and DOWN states were both prolonged from 0.59 ± 0.04 (UP) and 0.58 ± 0.02 (DOWN) to 0.77 ± 0.06 and 0.70 ± 0.03 s, respectively (p < 0.05, Mann–Whitney U test compared with aCSF) (Fig. 4B). Moreover, in the same experiments, spindle waves were markedly suppressed (88 ± 5%) by microdialysis injection of TTA-P2 in the VB (aCSF: 0.15 ± 0.01 spindles/s; TTA-P2: 0.019 ± 0.008; p = 0.02) (Fig. 4E), indicating the effectiveness of our thalamic inactivation by microdialysis administration of TTA-P2.

TTA-P2 also elicited a dose-dependent decrease in slow waves when applied systemically (Fig. 5A–D), with a similar ED_{50} on burst (0.18 ± 0.05 mg/kg) (Fig. 5E) and total firing (0.26 ± 0.06 mg/kg) (Fig. 5F). In particular, at a dose (3 mg/kg) that abolished
burst firing in the VB (Fig. 5E), TTA-P2 produced a stronger decrease (63 ± 1%, n = 3, p = 0.018) of slow waves (Fig. 5B) than an equipotent (on burst firing) intra-VB application (compare with Fig. 3B1), indicating that T-type calcium channels in other thalamic nuclei and brain regions effectively contribute to the full expression of EEG slow waves in S1 during anesthesia.

Effect of thalamic inactivation on slow waves during natural sleep

Because anesthesia does not fully reproduce the spatiotemporal dynamics of slow waves during non-REM sleep (Chauvette et al., 2011; Nir et al., 2011; Vyazovskiy et al., 2011), we then applied TTX and TTA-P2 by reverse microdialysis in the thalamus of naturally sleeping-waking rats. The same inlet dialysis concentration of TTA-P2 (300 μM) and one-probe-per-VB configuration that was effective during anesthesia had no effect on slow waves of natural sleep, nor did 1 and 3 mM TTA-P2, most likely because the volume of tissue where a firing block was achieved with these higher TTA-P2 concentrations was not much bigger than the region affected by 300 μM (Fig. 3B1, B2, D). We therefore enlarged the thalamic area affected by microdialysis application by implanting a group of rats with four dialysis probes, so that in each VB one probe was close to its rostral and one to its caudal end (see Materials and Methods). In these animals, TTA-P2 (3 mM) induced a significant decrease (23 ± 7.8%, n = 7, p = 0.0095, Mann–Whitney U test compared with aCSF, n = 12) in the peak of the instantaneous frequency distribution of slow waves during non-REM sleep (predrug: 2.19 ± 0.09 Hz; TTA-P2: 1.72 ± 0.13 Hz), as did TTX (50 μM) (TTX: 0.92 ± 0.13 Hz, 50 ± 13% reduction, n = 5, p = 0.0061) (Fig. 6A–D). As expected, in the same animals, slow spindles were more potently decreased by TTX and TTA-P2 (94 ± 7% and 48 ± 6%, respectively) than slow waves (predrug: 0.36 ± 0.02 spindles/s; TTX: 0.05 ± 0.03, TTA-P2: 0.19 ± 0.02; p = 0.001 for both compared with aCSF) (Fig. 6D, E).
As it was observed during anesthesia, also in naturally sleeping rats systemic injections of TTA-P2 dose-dependently blocked burst and total firing in VB TC neurons during non-REM sleep (with an ED_{50} of 0.55 ± 0.03 and 1.71 ± 0.11 mg/kg, respectively) (Fig. 7F,G), as well as slow waves and sleep spindles (Fig. 7A–E). In particular, a dose of 10 mg/kg of TTA-P2 markedly reduced slow wave (saline: 1.94 ± 0.13 Hz, n = 6; TTA-P2: 0.8 ± 0.01 Hz, n = 4 rats) and sleep spindle frequencies (saline: 0.32 ± 0.13 spindles/s, TTA-P2: 0.05 ± 0.01) by 73 ± 5% (p = 0.0095) and 86 ± 4% (p = 0.00031), respectively (Fig. 7E).

Entrainment of EEG slow waves by optogenetic stimulation of TC neurons during anesthesia

Because thalamic inactivation by either TTX or by selective block of thalamic T-type calcium channels with TTA-P2 decreased the frequency of slow waves during anesthesia and natural sleep, the thalamic input to the neocortex should be able to entrain these EEG waves. Because electrical stimulation of the thalamus leads to antidromic excitation of the somatotopic cortical region, which may in turn affect the cortical slow wave oscillator, we addressed this question using selective optogenetic activation of channelrhodopsin2-expressing TC neurons (Fig. 8) with short (5, 20, or 100 ms) 473 nm light pulses at stimulation frequencies that ranged from 0.75 to 4 Hz (n = 7 rats) (Fig. 9). At the cellular level, each light pulse elicited a single high-frequency burst of action potentials, which was invariably followed by a 100–250 ms period of electrical silence before firing resumed (Fig. 9C). For stimulation frequencies from 0.75 to 1.5 Hz, this pattern of TC neuron activation elicited a clear peak in the EEG wavelet (Fig. 9A) and FFT power spectrum (Fig. 9D) at the respective stimulation frequency, which had higher amplitude than the peak of the control EEG (i.e., with no light stimulation) (Fig. 9D, black line). As seen from the light-pulse triggered averages (Fig. 9B), light stimulation for frequencies >1.5 Hz not only failed to entrain EEG slow waves but also markedly flattened the power spectra eliminating the peak present at ~1 Hz during the control condition (i.e., without light stimulation; Fig. 9D, black line). When T-type calcium channels in VB were blocked by microdialysis of 300 μM TTA-P2 (n = 3 rats) (Fig. 10A), the cellular burst response during stimulation was virtually abolished (Fig. 10B), and no entrainment of slow waves occurred for stimulation frequencies between 0.75 and 1.5 Hz (Fig. 10C, D). Moreover, a similar block of EEG slow wave entrainment was observed after systemic injection of TTA-P2 (3 mg/kg) at these stimulation frequencies (data not shown). Finally, no significant effect of TTA-P2 injected locally (Fig. 10C2, D) or systemically (data not shown) was observed at stimulation frequencies ≥ 1.75 Hz.

**Discussion**

Our results conclusively demonstrate that the full manifestation of EEG slow waves during non-REM sleep in freely moving, naturally waking-sleeping rats requires a dynamic interplay of cortical and thalamic neuronal ensembles because inactivation of the thalamic output to the neocortex brings about a marked deceleration of slow waves and selective excitation of TC neurons entrains EEG slow waves.

**Contribution of thalamic oscillators to sleep rhythms**

There is a general consensus that an isolated neocortex can generate and maintain the UP and DOWN state dynamics that underlie EEG slow waves of natural sleep via an intricate balance of excitation and inhibition that is mostly generated by synaptically driven cortical slow wave oscillators (Sanchez-Vives and McCormick, 2000; Timofeev et al., 2000; Bazhenov et al., 2002; Cossart et al., 2003; Shu et al., 2003; Le Bon-Jego and Yuste, 2007; Beltramo et al., 2013). In line with this interpretation, the cortical firing (during UP states) and the electrical silence (during DOWN states) impose similar UP/DOWN state transitions on thalamic neurons, making the thalamus fully subservient to corticofugal activity. Over the last 10 years, however, this view has been challenged by solid experimental evidence demonstrating that an isolated thalamus as well as single TC and nucleus reticularis thalami (NRT) neurons *in vitro* can sustain slow waves and an intrinsic UP/DOWN states dynamics, respectively, which are similar to those observed during natural sleep (Crunelli et al., 2003; Le Bon-Jego and Yuste, 2007).
A competing oscillators (Contreras and Steriade, 1995, their Fig. 9; Slězia et al., 2011; Ushimaru et al., 2012). The effect of this thalamic modulation will also be important in determining the slight acceleration of slow waves observed from sleep Stage 2 to 4 (Crunelli and Hughes, 2010; Brown et al., 2012) because the intrinsic slow wave thalamic oscillator increases its frequency with the progressive hyperpolarization that accompanies non-REM sleep deepening (Hughes et al., 2002; Crunelli and Hughes, 2010). Support to the thalamic modulation of EEG slow waves also comes from data showing that an acceleration of these waves is accompanied by a phase shift of the TC neuron firing toward the start of an UP state (Slězia et al., 2011).

In the same animals where slow waves of non-REM sleep were decreased in frequency, the simultaneously recorded sleep spindles, which are a thalamically generated rhythm (Morrison and Bassett, 1945; Steriade et al., 1983; De Gennaro and Ferrara, 2003; Astori et al., 2011), were abolished by inactivation of the same thalamic region. This demonstrates that, whereas both cortex and thalamus are required for the full expression of slow waves and spindles of natural sleep, the relative contribution of these brain areas to these oscillations is markedly different, reflecting the diverse cellular/network generators that underlie these two EEG rhythms.

By comparing, for the first time, slow waves during natural sleep and anesthesia under the same laboratory conditions, we could identify that a larger area of thalamic inactivation was necessary to obtain a significant effect on slow waves during non-REM sleep compared with anesthesia. This indicates that the thalamic modulation of slow waves during anesthesia is very different from that during natural sleep, stressing the diverse nature of the cortical and thalamic neuronal dynamics underlying these behavioral states, the limitations associated with extrapolating results from one experimental condition to the other, and a potentially different involvement of the “core” and “matrix” thalamic projection systems (Jones, 2001).

Thalamic T-type calcium channels and the slow rhythm
Our investigation is the first to provide direct evidence that the T-type calcium channels of thalamic (i.e., TC and NRT) neurons are required for the full expression of slow waves during natural sleep, although we could not distinguish between TC and NRT neuron contribution because of TTA-P2 spread to the latter neuronal population at the highest concentrations. A previous study reported an increased number of awakenings in mice carrying a supposedly thalamic-selective deletion of CaV3.1 T-type channels expression (Anderson et al., 2005). However, the presence of recombination in piriform cortex, some hypothalamic nuclei, and other brain areas questions the selectivity of this genetic approach and weakens its conclusions. Another study in anesthe-
tized CaV3.1 T-type calcium channel KO mice (Lee et al., 2004) suggested a contribution of these channels to δ waves (defined as power in the 1–4 Hz frequency band) but not to slow waves (defined as power in the <1 Hz frequency band). Our results in anesthetized rats instead indicate that slow waves peaking at ~1 Hz are decreased in number by TTA-P2, leading to a slowing down in frequency to ~0.6 Hz. Although these contradictory data may be the result of the use of different species or anesthetics, they may also result from the different wave classifications. Indeed, it is debatable (Luczak and Barthó, 2012) whether EEG waves occurring at the upper end of the δ frequency range (2–4 Hz) in naturally sleeping rats do represent a separate entity from slow (<2 Hz) waves because the underlying UP and DOWN state firing dynamics of slow waves in the 2–4 Hz band is similar to that in 0.5–2 Hz band (Ji and Wilson, 2007, their Fig. 2).

It is not surprising that the vast majority of the total TC neuron firing (i.e., high-frequency bursts plus single action potentials) depends on T-type calcium channels (i.e., it is blocked by TTA-P2). Indeed, although these channels are classically viewed as underlying only high-frequency bursts of TC neurons, recent evidence has indicated that the continuous opening of a very small number of T-type channels that occurs at ~−60 mV generates a depolarizing window current (Dreyfus et al., 2010, their Fig. 2) that is crucial for the maintenance of the UP state (Hughes et al., 2002; Crunelli and Hughes, 2010). This strong reliance of the thalamic output on T-type channels also indicates that during slow waves synaptic activity, and in particular cortical inputs to the thalamus, are not sufficient to drive a TC neuron output in the absence of these channels.

Resetting and entrainment of EEG slow waves can be achieved by whisker stimulation (Civitello and Contreras, 2012) or by optogenetic activation of cortical neurons in anesthetized rats (Kuki et al., 2013), whereas long (>4 s) optogenetic stimulation of TC neurons in head-restrained mice can induce UP states in neocortical neurons that do not outlast the light stimulus (Poulet et al., 2012). Our results show that short (5 ms) optogenetic stimuli, which evoke high-frequency bursts in TC neurons, strongly entrain EEG slow waves, and that this response is only present when thalamic T-type calcium channels are functionally active. This strengthens the suggestion that thalamic T-type channel-mediated bursts may critically control UP states in related cortical territories during non-REM sleep by finely tuning the frequency of the EEG slow waves that occur during this behavioral state (Crunelli and Hughes, 2010). In contrast, a recent imaging study has reported that thalamic slow waves always follow cortical slow waves during anesthesia (Stroh et al., 2013). However, these results shed little light on the relative contribution of thalamic and cortical neuronal ensembles to slow waves of natural sleep, because, as indicated by Wester and Contreras (2013), they may be confounded by the experimental conditions, in particular the use of an anesthetic agent that is known to markedly block T-type calcium channels (Jokovic and Todorovic, 2010; Eckle et al., 2012).

In conclusion, these findings provide conclusive demonstration of our hypothesis (Crunelli and Hughes, 2010) that both cortical and thalamic population activities are required for the full manifestation of EEG slow waves of natural sleep, and suggest that a decreased thalamic output to the neocortex resulting from thalamic pathologies (Schmahmann, 2003; Kopollem et al., 2009; Parnaudeau et al., 2013) will bring about a slowing down of slow waves during non-REM sleep with deleterious consequences for memory processes.

References


Figure 10. Thalamic entrainment of EEG slow waves requires thalamic T-type calcium channels during anesthesia. A, EEG traces showing the effect of 20 ms, 473 nm light pulses (vertical blue lines) at 1 and 2 Hz during aCSF and TTA-P2 dialysis in VB. B, Raster plots of firing of 4 VB TC neurons in response to 20 ms pulses (at 1 Hz) during aCSF (B1) and TTA-P2 dialysis (B2). *Bursts. The corresponding EEG trace is superimposed. B3. Light pulse-triggered spike rates (red trace is right-shifted for clarity) from 41 stimulation epochs. C, EEG power spectra in response to 20 ms light stimulation at 1 (C1) and 2 (C2) Hz (arrows) during thalamic aCSF (blue, n = 7 rats) and TTA-P2 (red, n = 3 rats) dialysis compared with power spectra without light stimulation. Black represents aCSF; green represents TTA-P2. D, The power values at a given stimulation frequency (obtained from spectra as in C) for different light stimulation frequencies are plotted during aCSF and TTA-P2 dialysis. Error bars indicate SEM. *p < 0.05 for 5 and 20 ms pulses (Mann–Whitney U test).


