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Modulation of Hyperpolarization-Activated Inward Current and Thalamic Activity Modes by Different Cyclic Nucleotides

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The hyperpolarization-activated inward current, Ih, plays a key role in the generation of rhythmic activities in thalamocortical (TC) relay neurons. Cyclic nucleotides, like 3′,5′-cyclic adenosine monophosphate (cAMP), facilitate voltage-dependent activation of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels by shifting the activation curve of Ih to more positive values and thereby terminating the rhythmic burst activity. The role of 3′,5′-cyclic guanosine monophosphate (cGMP) in modulation of Ih is not well understood. To determine the possible role of the nitric oxide (NO)-sensitive cGMP-forming guanylyl cyclase 2 (NO-GC2) in controlling the thalamic Ih, the voltage-dependency and cGMP/cAMP-sensitivity of Ih was analyzed in TC neurons of the dorsal part of the geniculate nucleus (dLGN) in wild type (WT) and NO-GC2-deficit (NO-GC2−/−) mice. Whole cell voltage clamp recordings in brain slices revealed a more hyperpolarized half maximal activation (V1/2) of Ih in NO-GC2−/− TC neurons compared to WT. Different concentrations of 8-Br-cAMP/8-Br-cGMP induced dose-dependent positive shifts of V1/2 in both strains. Treatment of WT slices with lyase enzyme (adenyllyl and guanylyl cyclases) inhibitors (SQ22536 and ODQ) resulted in further hyperpolarized V1/2. Under current clamp conditions NO-GC2−/− neurons exhibited a reduction in the Ih-dependent voltage sag and reduced action potential firing with hyperpolarizing and depolarizing current steps, respectively. Intrathalamic rhythmic bursting activity in brain slices and in a simplified mathematical model of the thalamic network was reduced in the absence of NO-GC2. In freely behaving NO-GC2−/− mice, delta and theta band activity was enhanced during active wakefulness (AW) as well as rapid eye movement (REM) sleep in cortical local field potential (LFP) in comparison to WT. These findings indicate

Abbreviations: AW, active wakefulness; DSWS, deep slow wave sleep; EEG, electroencephalogram; HCN, hyperpolarization-activated cyclic nucleotide-gated channels; IC, internal capsule; Ih, hyperpolarization activated current; LFP, local field potential; LSWs, light slow wave sleep; LTS, low-threshold Ca2+ spike; NO, nitric oxide; nonREM, non-rapid eye movement sleep; nRT, reticular thalamus; PIR, passive infrared recording system; REM, rapid eye movement sleep; Rin, input resistance; RMP, resting membrane potential; SSC, somatosensory cortex; SWS, slow wave sleep; TC, thalamocortical; V1/2, half maximal activation; VB, ventrobasal thalamus.
Cyclic nucleotides, like cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) bind to the hyperpolarization-activated cyclic nucleotide-gated (HCN) channels and stabilize their open state (Zagotta et al., 2003). HCN channels represent the molecular basis of the hyperpolarization-activated current, termed I_h (Pape, 1996). HCN isoforms (HCN1-4) reveal different characteristics with respect to voltage dependency, activation kinetics and cyclic nucleotide sensitivity (He et al., 2014). HCN2 and the HCN4 isoforms (Ludwig et al., 2003; Notomi and Shigemoto, 2004) are strongly modulated by cGMP in thalamocortical (TC) relay neurons (Kanyshkova et al., 2009, 2012). In thalamic neurons of the rodent brain, I_h contributes to the resting membrane potential (RMP) and determines cell type-specific firing patterns and postnatal changes in HCN isoform expression profiles are accompanied by the maturation of sleep-related slow oscillations (Meuth et al., 2006; Kanyshkova et al., 2009).

In the dorsal lateral geniculate nucleus (dLGN), neuronal nitric oxide synthase (nNOS) was found in interneurons and cholinergic afferents arising from the ascending brainstem system (Gabbott and Bacon, 1994). NO exerts an important role in behavioral state-dependent gating of visual information and regulating TC oscillations (Pape and Mager, 1992; Yang and Cox, 2008). Thalamic NO concentrations increase during wakefulness and rapid eye movement (REM) sleep and decrease during slow wave sleep (SWS; Burlet and Cespuglio, 1997), pointing to a possible role of NO in regulation of arousal and REM sleep.

NO has been identified as an important modulator of HCN channel activity mediated by the NO-sensitive soluble NO-GC1 and NO-GC2 (Russwurm et al., 2013). Although cyclic nucleotide-dependent modulation of I_h in the thalamus under physiological and pathophysiological conditions has been assessed before (Pape, 1996; He et al., 2014), it is not known whether I_h in TC neurons is under the simultaneous control of both cAMP and cGMP. To address this issue, we studied I_h properties in wild type (WT) and NO-GC2-deficient mice, in the presence of adenylyl and guanylyl cyclase inhibitors and determined cell type-specific firing patterns and postnatal changes in HCNO isoform expression profiles are accompanied by the maturation of sleep-related slow oscillations (Meuth et al., 2006; Kanyshkova et al., 2009).
Series resistance compensation of temperature of 30–32°C. Access resistances were between 8 MΩ and 20 MΩ. Series resistance compensation of >50% was routinely applied. Recordings started 2–3 min after obtaining the whole-cell configuration. Voltage clamp experiments were controlled by the software Pulse or PatchMaster (HEKA Elektronik) operating on an IBM-compatible personal computer. All recordings were corrected offline for a liquid junction potential of 10 mV (\(V_M = V_P - 10 \text{ mV}\); with \(V_M\) = membrane potential and \(V_P\) = pipette voltage). Care was exercised to monitor series resistance and recordings were terminated whenever a significant increase (>-20%) occurred.

The voltage protocol used to examine \(I_h\) (Kanyshkova et al., 2012) was designed in order to increase the stability of whole cell recordings and account for increasingly fast activation kinetics of the current. Therefore the pulse length was shortened by 500 ms with increasing hyperpolarization (3.5 s pulse length at −130 mV). Steady-state activation of \(I_h\), \(p(V)\), was estimated by normalizing the mean tail current amplitudes (\(I\)) 50–100 ms after stepping to a constant potential from a variable amplitude step using the following function (equation 1):

\[
P(V) = \frac{(I-I_{\text{min}})}{(I_{\text{max}}-I_{\text{min}})} (1)
\]

with \(I_{\text{max}}\) being the tail current amplitude for the voltage step from −130 mV to −100 mV and \(I_{\text{min}}\) for the voltage step from −40 mV to −100 mV, respectively. \(I_h\) activation was well accounted for by a Boltzmann function of the following form (equation 2):

\[
p(V) = \frac{1}{1+\exp\left((V-V_{1/2})/k\right)} (2)
\]

where \(V_{1/2}\) is the voltage of half-maximal activation and \(k\) the slope factor.

The current density was calculated by dividing the \(I_h\) amplitude at −130 mV (i.e., subtracting the instantaneous current amplitude from the steady-state current) by the membrane capacitance obtained during whole cell recordings.

The time course of \(I_h\) activation in TC neurons at a temperature of 30–32°C was best approximated by the following double exponential equation:

\[
I_h(t) = A_1(1-e^{-t/\tau_{\text{fast}}}) + A_2(1-e^{-t/\tau_{\text{slow}}}) (3)
\]

Where \(I_h(t)\) is the time (ms), \(A_1\) and \(A_2\) are current amplitudes (pA), and \(\tau_{\text{fast}}\) and \(\tau_{\text{slow}}\) are time constants (ms), respectively. Currents evoked by voltage steps to −130 mV were analyzed.

A series of hyperpolarizing (500 ms) voltage steps in −10 mV increments were injected from the holding potential of −60 to −130 mV in order to evoke inwardly rectifying potassium (\(I_{KIR}\)) current. \(I_{KIR}\) currents were isolated from \(I_h\) by applying 20 μM ZD7288. \(I_{KIR}\) amplitudes were measured manually as the difference of the peak and the steady state current at the beginning and at the end of voltage pulses, respectively.

**Current Clamp Recordings and Determination of the Intrinsic Electrophysiological Properties**

The active and passive membrane properties of TC neurons were determined in current clamp mode. Recordings were performed at RMP in Ba²⁺-free extracellular solution containing (in mM): NaCl, 125; KCl, 2.5; NaH₂PO₄, 1.25; NaHCO₃, 24; MgSO₄, 2; CaCl₂, 2; dextrose, 10; pH adjusted to 7.35 by bubbling with carbogen. In order to compare the effects of different buffers and Ba²⁺ ions on intrinsic membrane properties of TC neurons, 0.5 mM BaCl₂ was added in HEPES and NaHCO₃ buffered extracellular solutions. Analysis was performed according to established procedures (Leist et al., 2016). Only cells with overshooting APs were included for analysis. The stimulation protocol contained hyperpolarizing and depolarizing current steps (1 s duration, from −230 pA to +370 pA with 40 pA increments; for 8-Br-cGMP experiments, a protocol with steps of 1 s duration, from −120 pA to +260 pA with 20 pA increments, was used). Membrane input resistance (\(R_{\text{in}}\)) was deduced from the slope of the current-voltage (I-V) relationship obtained from current injections of −30 and 50 pA. Membrane time constants (\(\tau_m\)) were obtained by fitting single or double exponentials (FitMaster, HEKA Elektronik) to negative voltage deflections induced by hyperpolarizing current injections of −30 pA. The \(I_h\)-dependent anomalous rectification (or voltage sag) of current injection of −230 pA was calculated as the change between the maximal and steady state voltage deflection (at the end of hyperpolarizing current injection). APs were detected manually by setting an amplitude threshold (\(V_{\text{thres}}\). FitMaster (HEKA Elektronik) and Clampfit 10.7 (Axon Molecular Devices, Sunnyvale, CA, USA) software was used for the analyses.

**Immunofluorescence**

WT mice were transcardially perfused with 4% (w/v) phosphate-buffered paraformaldehyde (PFA). Brains were removed and post fixed overnight in 4% PFA and later in 30% (w/v) sucrose for 48–72 h. Free-floating coronal sections (40 μm) were cut and slices were collected in phosphate-buffered saline (PBS). Sections were rinsed three times for 10 min in PBS. Slices were then incubated for 2 h in PBS supplemented with 10% (v/v) normal goat serum, Triton-X100 (0.3% (w/v)), and 3% (w/v) bovine serum albumin (BSA). Finally sections were incubated with primary antibodies overnight at 4°C. Polyclonal rabbit (rb) anti-NO-GCβ2 (1:1,1000; ab42108, Abcam, USA) antibody was utilized to detect a localization of dLGN. Several studies using this antibody revealed detecting of a single protein band of the correct size in quantitative Western blot experiments, thereby pointing to specificity of the reaction (Backer et al., 2008; Thoonen et al., 2015; product data sheet Abcam). In a similar way the monoclonal mouse anti-postsynaptic density protein 95 (PSD95; 1:1,000; 10011435, Cayman, USA) antibody which marks the postsynaptic membrane revealed a single protein band in Wetsers blot (Yao et al., 2004). After incubation with primary antibodies, sections were washed three times for 10 min in PBS and then transferred to the secondary antibody solution.
oxygenated salt solution (in mM: NaCl, 125; KCl, 2.5; NaHCO\textsubscript{3}, 1.25; MgCl\textsubscript{2}, 2; glucose, 10; pH 7.35 adjusted with NaOH and further incubated in an oxygenated salt solution consisting of (in mM): NaCl, 125; KCl, 2.5; NaHCO\textsubscript{3}, 1.25; MgCl\textsubscript{2}, 2; glucose, 10; CaCl\textsubscript{2}, 1; IBMX, 0.05; pH 7.3) for 2 h. Samples were mechanically homogenized at room temperature. To eliminate any non-solubilized material, samples were centrifuged at 1,000 g for 10 min at 4°C. The supernatant was transferred to a new Eppendorf cup and kept on ice, snap frozen in liquid nitrogen and stored at −80°C before further use.

Prior to cGMP quantification, samples were thawed on ice and 50 µl was used for Bradford protein assay. A cyclic GMP ELISA Kit (Prod. No. 581021, Cayman Chemicals, Ann Arbor, MI, USA) was used to quantify cGMP. Briefly, all samples were treated with trichloroacetic acid (TCA, final concentration 5% (w/v)) to precipitate proteins. After centrifugation, the supernatant was extracted with ether to remove any TCA residuals. An acetylation step for tissue samples was performed according to the manufacturer’s protocol. The final assay set up, luminescent measurement and analysis was performed as suggested by the manufacturer. We used a Fluostar Omega fluorescence reader (BMG Labtech, Ortenberg, Germany) for data acquisition and Microsoft Excel 2011 version 14.0 to analyze the data.

Rhythmic Burst Activity Recordings in Thalamic Slices

Horizontal brain slices were transferred to an interface chamber and recordings were performed at 32 ± 1°C. The superfusion solution consisted of (in mM): NaCl, 125; KCl, 2.5; NaHCO\textsubscript{3}, 26; NaH\textsubscript{2}PO\textsubscript{4}, 1.25; MgCl\textsubscript{2}, 2; CaCl\textsubscript{2}, 2; glucose, 10; pH 7.35 adjusted with carbogen. Rhythmic burst activity was induced through stimulation (1 ms, 1.45 mA) of the internal capsule (IC) using a pair of tungsten electrodes (with 50–100 MΩ resistance). Stimulation electrodes were connected to custom-made amplifier and stimulus isolator, and duration of stimulus was controlled by WinLTPd101 software (WinLTP Ltd, University of Bristol, UK). Network activity was measured in VB using a glass electrode (GC150T-10; Clark Electromedical Instruments, Harvard, UK) with a resistance of 0.5–2 MΩ. Burst firing was characterized by at least three high-frequency spikes with an intra-burst frequency interval of >100 Hz and inter-burst interval not more than 500 ms. Activity was analyzed in a time interval ranging from 50 ms to 100 ms up to 2–3 s after stimulation of the IC. Analysis was performed offline using Clampfit 10.7 and Peak v1.0 software.

Electrode Implantation and LFP Recordings for in vivo Electrophysiology

Mature properties of I\textsubscript{h} were reached in young mice postnatal between P20 and P30. Similar mature sleep pattern were found in young (P18) and adult (P90) rodents based on the analyses of cortical electroencephalogram (EEG) recordings, in which well-developed high amplitude delta waves distinguished NREM sleep from wakefulness. 3 to 5 months old adult male NO-GC2\textsuperscript{-/-} and WT mice were used for the in vivo experiments. Before surgery each animal was kept individually for 1 week in 12 h light/dark conditions (6 a.m.–6 p.m. light on period) and had unrestricted access to water and food. Implantation of the local field potential (LFP) recording electrodes was performed in a stereotactic frame (David Kopf Instruments, Tujunga, CA, USA) in animals under pentobarbital anesthesia (50 mg kg\textsuperscript{-1} i.p.) supplemented by a subcutaneous injection of carprofen (rimadyl; 5 mg kg\textsuperscript{-1}). Holes were drilled into the skull of the right hemisphere for inserting the silver recording electrode within the somatosensory cortex (SSC) (A/P = 0, M/L = 3, depth = −1.2; referenced to Bregma according to the mouse brain atlas), as well as for the reference and ground electrodes, which were placed over the cerebellum. The electrode assembly was fixed to the skull using dental acrylic cement (Pulpdent Glasslute, Watertown, MA, USA). After 1 week of recovery period the mice were habituated to the recording chamber. The LFP signal of each mouse was recorded for 2 h between 6 and 8 a.m. corresponding to the first 2 h of the light period. The LFP signal was amplified with a physiological amplifier (DPA-2F, Science Products, Hofheim, Germany), filtered by a band pass filter with cut-off points at 1 (HP) and 30 (LP) Hz and digitalized with a constant sampling rate of 2 kHz by multichannel continuous recording system (CED1401, Cambridge Electronic Design, Cambridge, England). In parallel, behavioral activity of mice was registered using a Passive Infrared Recording System (PIR, RK2000DPC LuNAR PR Ceiling Mount, Rokonet RISCO Group S.A., Drogenbos, Belgium). Following LFP recordings, animals were deeply anesthetized with an overdose of pentobarbital (i.p. injection) and the brains were removed for histological verification of the correct electrode positions.

Analysis of LFP Activity

LFPs were inspected offline by trained electrophysiologist (blinded for genotype) using Spike2 analysis software (Version 7.08, Cambridge Electronic Design, Germany). Recordings were subjected to Fast Fourier Transformation (FFT) in the 1–30 Hz range. Twenty epochs of 10 s duration were chosen from four behavioral states for power spectral density (PSD) analyses: active wakefulness (AW), REM sleep, light SWS (LSWS) and deep SWS (DSWS). For all epochs the EEG power in the delta (δ = 1–4 Hz), theta (θ = 5–8 Hz), alpha (α = 9–12 Hz)
and beta (β = 13–30 Hz) frequency ranges were calculated. Epochs were scored visually according to the following criteria: AW was characterized by high activity on the PIR and LFP which was dominated by a mixture of theta activity with higher frequencies, the amplitude was generally low. Low frequencies with intermediate amplitude and spindle activity and a low PIR were prevalent during LSWS. During DSWS, the LFP was dominated by low frequency, high amplitude delta activity and a low PIR as well. REM sleep was characterized by high frequency and low amplitude EEG activity, with predominantly theta activity. During REM sleep no movements were detected by PIR, indicating that the animal was immobile except for facial and bodily twitches. For each behavioral state the spectral power of the LFP epochs were assessed via a time frequency analysis (TFA) using Hanning tapering. Assessment of spectral power was performed in 1 s timeframes shifting along the 10 s epochs in steps of 50 ms. TFA was performed for the frequency range of 1–30 Hz using Fieldtrip software, an open-source Matlab-based toolbox for advanced analysis of electrophysiological data (Oostenveld et al., 2011). From each animal 20 epochs of 10 s duration spectral power was averaged over time for different frequency bands. Both raw and normalized EEG power spectra were used for statistical analysis. Normalized power spectra were used to control individual differences in EEG amplitude and PSD. To do so, the total power of 1–30 Hz was set at 100% and percentage for δ, θ, α and β frequencies were calculated.

Computer Modeling
Simulations were conducted within the NEURON simulation tool (Hines and Carnevale, 2001; Meuth et al., 2005) based on a modified version of an intrathalamic network model consisting of four cells (Destexhe et al., 1998). The four-cell model comprised of two spontaneously pacemaking TC neurons and two reticular thalamic (nRT) neurons interacting via GABA_A, GABA_A+B and AMPA synapses. While nRT neuron parameters were not changed, the I_h module of both TC neurons was modified by introducing activation kinetics, V_{1/2}, k and conductance values as obtained from our voltage-clamp recordings in 11 control and 13 knockout cells (Kanyshkova et al., 2009, 2012).

Materials
8-bromo-adenosine 3′,5′-cyclic monophosphate (8-Br-cAMP) sodium salt, 8-bromo-guanosine 3′,5′-cyclic monophosphate (8-Br-cGMP) sodium salt (Tocris, R+D Systems, Wiesbaden, Germany), Cytidine-3′-[1,2,4]Oxadiazolo[4,3-a]quinazolin-1-one (ODQ; Sigma, Munich, Germany) was prepared as a stock solution in DMSO (10 mM) and diluted in ACSF to obtain the final concentration (10 µM). The concentration of DMSO was below 0.1%. Slices were kept for 10 min in ODQ before starting the recordings. In order to block adenyl cyclase, the slices were preincubated with 200 µM SQ 22536 (Tocris) for 2 h. HCN channels were blocked by washing the slices for 10 min with 20 µM ZD7288 (Abcam, Cambridge, UK). Inward-rectifier K^+ channels were blocked by Tertiapin-Q (Tocris).

Data and Statistical Analysis
All results are presented as mean ± SEM if not mentioned otherwise. By default statistical significance was tested using the nonparametric Mann-Whitney test. For normally distributed data Student’s t-test was used (Graph Pad Prism software; Graph Pad, San Diego, CA, USA; OriginLab software, Additive GmbH, Friedrichsdorf, Germany). For multiple comparisons ANOVA testing (Graph Pad Prism) was used. For statistical comparison of NO-GC2−/− and WT mice recorded in vivo, data were subjected to a Repeated-Measures-ANOVA with spectral power as dependent variable, mouse strain (NO-GC2−/−, WT) between subjects factor and frequency band (delta, theta, alpha, beta) as within subjects factor. This analysis was performed for each of the four behavioral states (AW, REM, LSWS, DSWS) using IBM-SPSS Version 22. Differences were considered statistically significant if P < 0.05. *,**,**,** indicate P < 0.05, P < 0.01, P < 0.001, respectively.

RESULTS
Modulation of I_h in TC Neurons by Cyclic Nucleotides
Cyclic nucleotides, like cAMP and cGMP can influence neuronal signaling via modulation of ion channel activity either by direct binding interaction or indirectly by cyclic nucleotide-dependent phosphorylation of ion channels. Modulation by cAMP is a well characterized property of HCN channels in rodent TC neurons, but the role of cGMP is not fully understood. In the present study, thalamic I_h properties were analyzed in WT and NO-GC2−/− mice (Figure 1A). Analyses of activating and deactivating currents revealed that the loss of NO-GC2 was associated with a significant negative shift in voltage-dependency of activation (WT: V_{1/2} = −86.4 ± 0.9 mV, n = 11; NO-GC2−/−: V_{1/2} = −91.8 ± 1.1 mV, n = 13; P < 0.05; Figures 1B,C), whereas current density remained unaltered (WT: I_h = 6.1 ± 0.6 pA/pF, n = 11; NO-GC2−/−: I_h = 6.4 ± 0.8 pA/pF, n = 13; P > 0.05; Figure 1D). Activation kinetics were best approximated by a double-exponential function (Figure 1E), and revealed no changes (WT: τ_{fast}/τ_{slow} = 135 ± 13 ms/806 ± 60 ms, n = 11; NO-GC2−/−: τ_{fast}/τ_{slow} = 146 ± 15 ms/981 ± 117 ms, n = 13; P’s > 0.05; Figure 1F).

We next assessed the effects of cGMP on I_h current properties by supplying defined 8-Br-cGMP concentrations (1, 10, 100 µM) via the recording pipette. The experiments revealed dose-dependent alterations of I_h in both, WT and NO-GC2−/− animals (Figure 2A). While V_{1/2} was positively shifted (Figure 2B), activation kinetics were enhanced (Figure 2C). For better comparison of different sensitivity between the strains, ΔV_{1/2} was calculated (Figure 2D). TC neurons from...
NO-GC2^{−/−} revealed increased sensitivity to all applied concentrations of 8-Br-cGMP (ΔV_{1/2}: 1 μM = 14.2 ± 1.7 mV, n = 7; 10 μM = 16.5 ± 1.2 mV, n = 8; 100 μM = 20.7 ± 1.5 mV, n = 9), compared to WT (ΔV_{1/2}: 1 μM = 3.4 mV, n = 8; 10 μM = 9.6 ± 1.3 mV, n = 11; 100 μM = 10.3 ± 1.1 mV, n = 9; P’s < 0.05; Figure 2D). Preincubation of slices in 200 μM ODQ induced a negative shift of V_{1/2} in WT (V_{1/2} = −93.6 ± 1.3 mV; ΔV_{1/2} = −7.2 ± 1.1 mV, n = 8), while it did not cause significant changes in GC2 knockout mice (V_{1/2} = −94.1 ± 0.9 mV; ΔV_{1/2} = −2.2 ± 0.5 mV, n = 5; Figures 2B,D). No effects of 8-Br-cGMP and ODQ were found on I_{h} current density in both strains (data not shown).

Next, we manipulated intracellular cAMP levels by applying different concentrations of 8-Br-cAMP via the patch pipette. This resulted in a dose-dependent depolarizing shift in voltage-dependent activation (Figure 2E) accompanied by reduced activation time constants (Figure 2F) of I_{h} in both strains. ΔV_{1/2} revealed significant changes during application of 1 μM (ΔV_{1/2}: WT = 8.5 ± 0.9 mV, n = 10; NO-GC2^{−/−} = 17.1 ± 1.6 mV, n = 8; P < 0.05) and 10 μM 8-Br-cAMP (ΔV_{1/2}: WT = 17.4 ± 1.6 mV, n = 11; NO-GC2^{−/−} = 21.9 ± 1.7 mV, n = 8; P < 0.05; Figure 2D).

Preincubation of slices with 200 μM SQ 22536 shifted activation potentials of I_{h} to more negative values in both strains. The effect was stronger in WT (V_{1/2} = −95.5 ± 0.6 ΔV_{1/2} = −9.1 ± 0.6 mV, n = 11), compared to NO-GC2^{−/−} mice (V_{1/2} = −95.2 ± 0.8 ΔV_{1/2} = −3.5 ± 0.7 mV, n = 10; P’s < 0.05; Figures 2D,E). 8-Br-cAMP and SQ 22536 did not change I_{h} current density in TC neurons (data is not shown).

More recently, the modulation of HCN2 and HCN4 channels by cCMP and cUMP has been reported (Zong et al., 2012). Both channel isoforms are major constituents of I_{h} in murine TC neurons (Ludwig et al., 2003; Leist et al., 2016). Thus, we examined the effect of cCMP and cUMP on I_{h} currents in WT neurons. cCMP is a partial agonist of HCN channels with an EC_{50} value of ~30 μM compared to 1 μM for cAMP (Zong et al., 2012). Therefore, we applied 30 μM cCMP and cUMP. Both compounds induced depolarizing shifts of V_{1/2} in TC compared to control cells (cCMP: V_{1/2} = −77.44 ± 2.3 mV; ΔV_{1/2} = 8.9 ± 2.3 mV; n = 6; P’s < 0.05); cUMP: V_{1/2} = −78.9 ± 3.6 mV, ΔV_{1/2} = 7.6 ± 3.5 mV; n = 6; P’s < 0.05; Figures 2D,G).

In order to allow better comparison between voltage and current clamp recordings and to assess potential effects of pH...
buffering conditions, we compared $I_h$ current from WT TC neurons in NaHCO$_3$ and HEPES buffered extracellular solutions in the presence of 0.5 mM BaCl$_2$. Half maximal activation potential of $I_h$ (NaHCO$_3$: $V_{1/2} = -89.7 \pm 0.8$ mV, $n = 6$; HEPES: $V_{1/2} = -88.2 \pm 1.2$ mV, $n = 5$; $P > 0.05$) and current density (NaHCO$_3$: $I_h = 5.2 \pm 0.6$ pA/pF; HEPES: $I_h = 6.9 \pm 1.2$ pA/pF, $n = 5$; $P > 0.05$) did not differ between the two recording conditions (data not shown).

Since Kir channels have been found to be modulated by cyclic nucleotides and are important in setting the RMP and determining the firing pattern of neurons, the effect of 8-Br-cGMP was studied on WT inwardly rectifying potassium
\((I_{\text{KIR}})\) current. \(I_{\text{KIR}}\) was evoked by hyperpolarizing voltage steps and was isolated from \(I_h\) by applying 20 \(\mu\)M ZD7288. Extracellular application of 1 mM 8-Br-cGMP did not change the amplitude of \(I_{\text{KIR}}\) (ZD7288: 231.9 ± 23.9 pA at −130 mV, \(n = 6\); 8-Br-cGMP: 235.8 ± 22.8 pA, \(n = 6\); \(P > 0.05\); Figures 3A,B) in dLGN TC neurons.

**Influence of cGMP on TC Neurons Firing Patterns**

To determine the functional impact of NO-GC2 activity on passive membrane properties and neuronal firing, TC neurons were recorded under current clamp conditions from the RMP (Figure 4A). While the RMP (WT: −67.1 ± 0.9 mV, \(n = 16\); NO-GC2\(^{-/-}\): −69.3 ± 0.9 mV, \(n = 14\); \(P > 0.05\)) did not differ between the strains, \(R_n\) was significantly reduced in NO-GC2\(^{-/-}\) (WT: 224.9 ± 13.1 MΩ, \(n = 16\); NO-GC2\(^{-/-}\): 175.9 ± 8.7 MΩ, \(n = 14\); \(P < 0.05\); Figure 4B). Negative current injections (−230 to −30 pA, 1 s duration) induced significantly smaller voltage sags in the absence of NO-GC2 (WT: 13.5 ± 1.6 mV at −230 pA, \(n = 16\); NO-GC2\(^{-/-}\): 6.2 ± 0.9 mV at −230 pA, \(n = 14\); \(P < 0.05\); Figures 4A,B). Upon release from the hyperpolarizing pulse NO-GC2\(^{-/-}\) mice generated less rebound low-threshold Ca\(^{2+}\) spikes (LTS) with action potentials riddling on top of it. Of 16 WT cells 12 cells showed an LTS which triggered action potentials (number of action potentials per LTS: 1.25 ± 0.3, \(n = 16\)), whereas only 4 out of 14 NO-GC2-deficient cells displayed an LTS triggering action potentials (number of action potentials per LTS: 0.38 ± 0.2, \(n = 14\); \(P < 0.05\); Figure 4B). When TC neurons from NO-GC2\(^{-/-}\) mice were challenged with depolarizing currents (10–370 pA, 1 s duration), tonic activity was characterized by less APs compared to WT mice (WT: 65.1 ± 3.6 at 370 pA, \(n = 16\); NO-GC2\(^{-/-}\): 40.9 ± 4.3 at 370 pA, \(n = 14\); \(P < 0.05\); Figures 4A,B). Preincubation of slices from WT in 10 \(\mu\)M ODQ was performed to mimic the effects of NO-GC2 deficiency under current clamp conditions. No changes in the RMP (control: −67.1 ± 0.9 mV, \(n = 16\); 10 \(\mu\)M ODQ: −69.1 ± 0.9 mV, \(n = 7\); \(P > 0.05\)) and \(R_n\) (control: 224.9 ± 13.1 MΩ, \(n = 16\); 10 \(\mu\)M ODQ: 193.6 ± 19.6 MΩ, \(n = 7\); \(P > 0.05\)) were found (Figure 4C). Voltage sag amplitudes were marginally decreased (control: 13.5 ± 1.6 mV at −230 pA, \(n = 16\); 10 \(\mu\)M ODQ: 7.7 ± 2.8 mV, \(n = 7\); \(P > 0.05\)), generation of LTS was less pronounced (number of action potentials per LTS: control: 1.25 ± 0.3, \(n = 16\); 10 \(\mu\)M ODQ: 0.29 ± 0.2, \(n = 7\); \(P < 0.05\); Figure 4C) and number of APs was reduced in TC cells treated with ODQ (control: 65.1 ± 3.6 at 370 pA, \(n = 16\); ODQ: 48.5 ± 6.4 at 370 pA, \(n = 7\); \(P < 0.05\); Figure 4C).

Next, effects of exogenously increased cGMP concentrations were assessed. Application of 10 \(\mu\)M 8-Br-cGMP via the patch pipette in WT TC neurons resulted in a more positive RMP (control: −70.43 ± 0.69 mV, \(n = 7\); cGMP: −65.57 ± 1.7 mV, \(n = 7\); \(P < 0.05\); Figure 5A) and increased occurrence of LTS. All cGMP treated cells showed LTS (number of action potentials per LTS: control: 0.7 ± 0.3, \(n = 7\); 10 \(\mu\)M 8-Br-cGMP: 1.7 ± 1.8, \(n = 7\); \(P < 0.05\); Figure 5A). Under these conditions, TC neurons fired significantly more tonic APs (Figure 5A). To assess to what extend these changes may be related to modulation of \(I_h\), neurons from WT and NO-GC2\(^{-/-}\) mice were washed with the HCN channel blocker ZD7288 (20 \(\mu\)M) and then subjected to an extracellular application of 8-Br-cGMP (1 mM; Figure 5B). In all cells tested, ZD7288 completely abolished the voltage sag. In WT, ZD7288 strongly hyperpolarized the RMP (control: −64 ± 1.3 mV, \(n = 7\); ZD7288: −70.3 ± 1.5 mV, \(n = 7\); \(P < 0.05\)), while 8-Br-cGMP caused depolarization of the RMP (8-Br-cGMP: −60.7 ± 2.8 mV, \(n = 7\); \(P < 0.05\); Figure 5C). Although ZD7288 did not change the \(R_{in}\) of WT cells (control: 191.9 ± 25.4 MΩ, \(n = 7\); ZD7288: 182.2 ± 16.9 MΩ, \(n = 7\); \(P > 0.05\)), administration of 8-Br-cGMP strongly increased \(R_{in}\) to 272.3 ± 33.4 MΩ (\(n = 7\); \(P < 0.05\); Figure 5C). Tonic firing was only nominally increased after the application of ZD7288 (Figure 5C). With injected currents up to 90 pA, WT TC neurons fired slightly more APs after additional application of 8-Br-cGMP. Stronger current injections however were associated with partial depolarization block (Figure 5B, inset), thereby pointing to the loss of hyperpolarizing influences (Figures 5B, C). When hyperpolarizing K\(^+\) currents are reduced, neurons may enter the state of depolarization block where under sustained input current of increasing strength neurons eventually reduce or stop firing, while membrane potential fluctuations above threshold are present (Bianchi et al., 2012). In NO-GC2\(^{-/-}\) the direction of changes in RMP induced by ZD7288 and 8-Br-cGMP was similar to WT (control: −67.2 ± 1.1 mV, \(n = 6\); ZD7288: −67.3 ± 0.9 mV, \(n = 6\); 8-Br-cGMP: −67.4 ± 1.0 mV, \(n = 6\); \(P > 0.05\); Figure 5C).
FIGURE 4 | Passive and active membrane properties of TC neurons in WT and NO-GC2−/−. (A) Voltage responses of TC neurons recorded under current clamp conditions at rest. Both WT (black) and NO-GC2−/− (red) neurons were injected with a series of hyperpolarizing and depolarizing currents. (B) Box and whisker plot showing the resting membrane potential (RMP) and \( R_{in} \) of NO-GC2−/− (red box) and WT (black box) TC neurons. The amplitude of anomalous rectification induced by hyperpolarizing current injection was less pronounced in NO-GC2−/− (red symbols) compared to WT (black symbols) mice. Upon release from membrane hyperpolarization the occurrence of low-threshold Ca\(^{2+}\) spikes (LTS) and the number of action potentials per LTS was higher in WT neurons (black box) in comparison to NO-GC2−/− (red box). Depolarizing currents evoked less number of action potentials in NO-GC2−/− (red symbols) compared to WT (black symbols) neurons. (C) Preincubation of WT slices in 10 \( \mu \)M ODQ (green box) did not change either RMP nor \( R_{in} \), compared to control conditions (black box). Voltage sag amplitudes were nominally reduced, occurrence of LTS and the number of action potentials per LTS was less pronounced and the number of APs in response to depolarizing current injections was nominally decreased after the blockage of GC (green symbols) in comparison to control (black symbols). ** indicate \( P < 0.05 \), \( P < 0.01 \), respectively.

\[ -71.7 \pm 2.7 \text{ mV, } n = 6; \text{ 8-Br-cGMP: } -63.6 \pm 3.7 \text{ mV, } n = 6; \] 
\[ \text{ P’s } > 0.05; \text{ Figure 5D} \]. Both compounds increased the \( R_{in} \) of NO-GC2-deficient cells (control: 165.9 ± 17.7 MΩ, \( n = 6; \) ZD7288: 227.7 ± 28.4 MΩ, \( n = 6, \) \( P > 0.05; \) 8-Br-cGMP: 278 ± 16.9 MΩ, \( n = 6, \) \( P < 0.05; \) Figure 5D). Both ZD7288 and 8-Br-cGMP increased the excitability of NO-GC2−/− TC neurons, compared to control recordings. Again, AP firing in the presence of both compounds was characterized by a partial depolarization block (Figure 5D). RMP, \( R_{in} \) and firing did not differ between the strains after ZD7288 and cGMP application (Figures 5C,5D). When WT slices were preincubated with 10 \( \mu \)M ODQ, application of ZD7288 (20 \( \mu \)M) marginally hyperpolarized the RMP (ODQ: −65.5 ± 1.6 mV, \( n = 6; \) ZD7288: −70.5 ± 2.4 mV, \( n = 6, \) \( P > 0.05 \)) and increased the \( R_{in} \) (ODQ: 192.1 ± 23.2 MΩ, \( n = 6; \) ZD7288: 221.9 ± 27.3 MΩ, \( n = 6; \) \( P > 0.05 \)) of TC. The number of spikes with depolarizing current injections was slightly increased after the application of ZD7288 (Figure 5E).

**Effect of Ba\(^{2+}\) on Membrane Properties of TC Neurons**

In order to allow better comparison between current and voltage clamp recordings and to assess the contribution of Ba\(^{2+}\)-sensitive inward rectifier and \( K_{2P} \) channels, like TASK and TREK channels, BaCl\(_2\) (0.5 mM) was added to NaHCO\(_3\)- and HEPES-buffered extracellular solutions. Results were compared to the data described above (i.e., Ba\(^{2+}\)-free NaHCO\(_3\)-buffered solution; Figure 6). In both genotypes Ba\(^{2+}\) significantly changed passive and active membrane properties of TC neurons. In the presence of Ba\(^{2+}\), the RMP was strongly depolarized, \( R_{in} \) was increased, voltage sags were unmasked, and the number of APs was significantly increased (Figures 6A–C). In the presence of Ba\(^{2+}\)
FIGURE 5 | Effects of increased intracellular levels of cGMP on the electrical properties of WT and NO-GC2−/− mice TC neurons. (A) Perfusion of WT TC neurons with an intracellular solution containing 10 µM 8-Br-cGMP. Left panel: the RMP of control TC cells (black box) and the cells treated by 10 µM 8-Br-cGMP (blue box). Middle panel: the number of APs per LTS induced upon release from the hyperpolarizing pulse in control (black box) and 10 µM 8-Br-cGMP (blue box) infused cells. Right panel: the number of APs induced by positive current injection in control (black symbols) and 10 µM 8-Br-cGMP (blue symbols) treated cells.

(B) Example traces of WT neurons at -230 pA and 210 pA current injection recorded under control (black heading) condition and in the presence of 20 µM ZD-7288 (gray heading) and 1 mM 8-Br-cGMP (blue heading). Inset: please notice an example of depolarization block with 250 pA depolarizing current injection.

(C) The passive and active membrane properties of WT neurons: the RMP (left panel), $R_{in}$ (middle panel) and the number of APs (right panel) under control conditions (black box, symbols) and in the presence of 20 µM ZD-7288 (gray box, symbols) and 1 mM 8-Br-cGMP (blue box, symbols).

(D) The passive and active membrane properties of NO-GC2 deficient neurons: the RMP (left panel), $R_{in}$ (middle panel) and the number of APs (right panel) under control conditions (red box, symbols) and in the presence of 20 µM ZD-7288 (gray box, symbols) and 1 mM 8-Br-cGMP (blue box, symbols).

(E) The RMP (left panel), $R_{in}$ (middle panel) and the number of APs (right panel) of WT neurons incubated in 10 µM ODQ (green box, symbols) and in the presence of 20 µM ZD-7288 (gray box, symbols). ∗, ∗∗, ∗∗∗ indicate $P < 0.05$, $P < 0.01$, $P < 0.001$, respectively.
voltage sag amplitudes were significantly larger in NO-GC2−/− in comparison to WT (NaHCO3 + Ba2+: WT: 52.74 ± 2.25 mV at −230 pA, n = 12; NO-GC2−/−: 63.38 ± 5 mV at −230 pA, n = 6; HEPES: WT: 53.95 ± 4.84 mV at −230 pA, n = 7; NO-GC2−/−: 65.45 ± 2.8 mV at −230 pA, n = 10, P’s < 0.05; Figures 6B,C).

To assess the possible contribution of IKir to hyperpolarization-induced voltage deflections and firing pattern of TC neurons, WT slices were washed for 15 min with the Kir channel inhibitor Tertiapin-Q, thereafter effects of extracellular application of 8-Br-cGMP on membrane properties were studied. Tertiapin-Q (200 nM) did not change either the RMP (control: −69 ± 0.7 mV, n = 6; Tertiapin-Q: −68.8 ± 1.4 mV, n = 6; P > 0.05), nor Rin (control: 215.5 ± 7.3 MΩ, n = 6; Tertiapin-Q: 239.4 ± 9.9 MΩ; P > 0.05) and voltage sag amplitudes (control: 21.3 ± 2.9 mV at −230 pA, n = 6; Tertiapin-Q: 21.8 ± 3.6 mV at −230 pA, n = 6; P > 0.05; Figure 7A) of TC neurons. Application of 8-Br-cGMP significantly depolarized the RMP (8-Br-cGMP: −62.8 ± 1.4 mV; P < 0.05), increased Rin (8-Br-cGMP: 297.4 ± 19.4 MΩ, n = 6; P < 0.05) and voltage sag amplitudes (8-Br-cGMP: 31.9 ± 4.7 mV, n = 6; P < 0.05; Figure 7A). Firing was slightly increased by Tertiapin-Q and 8-Br-cGMP caused further excitation of TC neurons, eventually resulting in a depolarization block with higher depolarizing current injections (Figure 7A).
Next, the effects of elevated levels of 8-Br-cGMP on TC neurons membrane properties were studied in the presence of Kir and HCN channels inhibitors Tertiapin-Q and ZD7288, respectively (Figure 7B). Co-administration of the two inhibitors significantly hyperpolarized the RMP (control: $-66.8 \pm 0.7$ mV, $n = 6$; Tertiapin-Q + ZD7288: $-77.5 \pm 1.6$ mV, $n = 6$; $P < 0.05$) and nominally increased $R_{\infty}$ (control: $228.3 \pm 0.7$ M$\Omega$, $n = 6$; $P > 0.05$; Figure 7B) of TC neurons. The RMP was significantly depolarized (8-Br-cGMP: $-64.5 \pm 1.7$ mV, $n = 6$; $P < 0.05$) and $R_{\infty}$ was increased (8-Br-cGMP: $438.4 \pm 57.7$ M$\Omega$, $n = 6$; $P > 0.05$) following extracellular application of 1 mM 8-Br-cGMP. Inhibition of Kir and HCN channels resulted in an increased number of APs with small depolarizing current injections and the occurrence of a depolarization block was noticed in the presence of 8-Br-cGMP in TC neurons (Figure 7B).

**GC Activity in dLGN**

The presence and localization of NO-GC2 in the thalamus was analyzed immunohistochemically. A strong fluorescent signal was detected in dLGN (Figure 8A). Higher magnification images revealed expression in the cellular boundaries. Co-staining for NO-GC$\alpha_2$ and the PSD95 revealed a strong overlap of the fluorescent signals, pointing to a postsynaptic localization.

In order to address a potential contribution of cGMP in TC neurons signaling, we quantified the amount of cGMP in tissue from different brain areas at different developmental stages. In all samples (dLGN, VB, SSC) the highest cGMP levels were consistently detected in young animals (postnatal day 7, P7) with the SSC containing the largest amount of cGMP. During postnatal development (P21 and P107) the tissue contained lower cGMP levels (Figure 8B).

**Reduction of Burst Activity in the Thalamic Network in the Absence of NO-GC2**

The intrathalamic network activity is involved in the generation of different thalamic oscillations such as sleep spindle and delta oscillations which critically depend on activation of $I_h$ (Kanyshkova et al., 2009, 2012). Rhythmic bursting is a property of thalamic cells and has frequently been used as a measure of intrathalamic rhythmicity in horizontal thalamic slices conserving axonal connections between nRT and VB TC neurons (Huguenard and Prince, 1994; Yue and Huguenard, 2001). In this model system intrathalamic oscillations are generated by reciprocal interactions between inhibitory nRT neurons and excitatory TC neurons. Stimulation of nRT neurons evokes IPSPs in VB neurons and triggers rebound burst activity in TC. Rebound bursts of TC neurons re-excite the nRT and the cycle starts again. Here dampened oscillatory activity (i.e., up to 6–8 cycles) was induced by stimulation of the IC (containing TC and corticothalamic fibers) and recorded in VB (Figure 9A). Compared to control animals, NO-GC2$^{-/-}$ mice revealed a significantly lower number of bursts in response to a single stimulus (NO-GC2$^{-/-}$: 5.8 $\pm$ 0.3 bursts, $n = 12$; WT: 7.1 $\pm$ 0.5 bursts, $n = 8$; $P < 0.05$; Figures 9B,C).

To further assess the influence of NO-GC2 activity on rhythmic activity in the thalamic network, a mathematical modeling approach was used (Kanyshkova et al., 2009, 2012; Figure 9D). Spontaneous rhythmic bursting was analyzed in an interconnected four-cell model of two TC and two nRT neurons which was used for simulation of the intrathalamic network activity (Kanyshkova et al., 2009, 2012). Compared to WT, the number of bursts generated within the stimulation period of 2 s based on NO-GC2$^{-/-}$-derived parameters was significantly lower (NO-GC2$^{-/-}$: 4.7 $\pm$ 0.5 bursts,
n = 11; WT: 8.0 ± 0.8 bursts, n = 11; P < 0.05; Figure 9E).

**Spectral Power Characteristics of NO-GC2−/− Mice During Different Behavioral States**

Thalamic bursting is involved in the generation of TC rhythms such as delta oscillations which are found on the LFP and EEG recordings during SWS sleep and anesthesia (Steriade et al., 1993; Timofeev, 2011). Therefore, we assessed the effects of NO-GC2 deficiency on cortical oscillations by performing LFP recordings from the SSC of freely moving mice (Figures 10, 11). Recordings made during the first 2 h of the light period were taken for analysis. Absolute spectral power of LFP signals recorded from NO-GC2−/− (n = 4) and WT (n = 5) mice was assessed for the delta, theta, alpha and beta bands and compared between strains during the four different behavioral states (AW, REM, LSWS and DSWS).

During AW both strains show predominant theta activity in the LFP, characteristic for a moving rodent (Figure 10A). NO-GC2−/− mice displayed more power than WT mice within the δ (WT: 51 ± 9.5 μV²/Hz; NO-GC2−/−: 267 ± 41 μV²/Hz; P < 0.05), θ (WT: 132 ± 80 μV²/Hz; NO-GC2−/−: 450 ± 89 μV²/Hz; P < 0.05) as well as within the α frequency band (WT: 19 ± 4 μV²/Hz; NO-GC2−/−: 354 ± 72 μV²/Hz; P < 0.05), whereas the β power did not significantly differ between the strains (WT: 32 ± 4 μV²/Hz; NO-GC2−/−: 32 ± 4 μV²/Hz; P > 0.05; Figures 10B, C). The percentage of EEG frequencies (%) were nominally enhanced in δ (WT: 16.6 ± 2.2%; NO-GC2−/−: 28.6 ± 8.2%; P > 0.05) and significantly decreased in β frequency range in NO-GC2−/− (3.3 ± 0.69%), compared to WT (5.9 ± 0.44%; P < 0.05, Figure 10D) mice.
Fig. 9 | Dysfunction of the NO-GC2 signaling pathway changes thalamic oscillations. (A) Dampened rhythmic activity was induced by exciting neurons in the reticular thalamus (nRT) via stimulation of the internal capsule (IC) in horizontal thalamic slices. Neuronal responses were measured in the ventrobasal thalamus (VB). (B) Example traces of rhythmic burst activities from WT (black) and NO-GC2−/− (red) mice. (C) The number of bursts (red bar) was significantly decreased in NO-GC2−/− compared to WT (black bar) mice. (D) Computer simulation of rhythmic activity in a mathematical model. In this simulation two nRT neurons reciprocally communicate via GABA_A-mediated connections and project to two TC neurons via GABA_A and GABA_B signaling. The feedback from TC neurons is carried by AMPA receptors to both nRT neurons. Rhythmic bursting profiles with Ih properties taken from TC neurons of WT (black) and NO-GC2−/− (red) mice are shown. (E) With Ih parameters set to the values obtained from TC cells of WT (black bar) and NO-GC2−/− (red bar) mice, the number of bursts was significantly lower in NO-GC2−/− mice. ∗, ∗∗∗ indicate P < 0.05, P < 0.001, respectively.

Both strains show a typical REM sleep spectrum, characterized by the presence of theta activity in the LFP (Fig. 11A). NO-GC2−/− mice had a significantly higher spectral power than WT mice in the δ band during REM sleep (WT: 76 ± 28 µV^2/Hz; NO-GC2−/−: 199 ± 31 µV^2/Hz; P < 0.05). However, strains did not differ in power of θ, α and β frequency bands (Figures 11B,C). The normalized values of different frequencies showed increased δ (WT: 14.3 ± 1.58%; NO-GC2−/−: 24.7 ± 3.62%; P < 0.05) and decreased α (WT: 38.92 ± 1.2%; NO-GC2−/−: 28.64 ± 2.4%; P < 0.05) and β (WT: 5 ± 0.42%; P < 0.05; NO-GC2−/−: 3.58 ± 0.33%; Figure 11D) activities in NO-GC2−/− mice.

During LSWS and DSWS there were no strain differences in any of the bands of the raw LFPs, as well as in the normalized data (data not shown).

**DISCUSSION**

The modulation of Ih in TC neurons of different mammalian species by cAMP is well established (McCormick and Pape, 1990; Budde et al., 2005; Leist et al., 2016). Fewer studies have addressed effects mediated by cGMP. While the increase in current and the shift to depolarized potentials in the activation curve by NO donors and cGMP as well as the involvement of GC activity in Ih modulation have been described before (Pape and Mager, 1992; Yang and Cox, 2008), the GC subtype involved in these phenomena has not been yet identified. Experimental data suggested low to moderate mRNA expression of GC α_1, α_2 and β_1 subunits which form the heterodimeric NO-GC1 (α_1β_1) and NO-GC2 (α_2β_1) isoforms in different thalamic nuclei (Giuli et al., 1994; Gibb and Garthwaite, 2001). Notably, V_{1/2} of Ih in TC neurons of NO-GC2−/− mice was found to be more hyperpolarized compared to WT, indicating that the current is controlled by the basal activity of postsynaptic NO-GC2. The control of Ih by basal cGMP production is in good agreement with electrophysiological analyses of CA1 pyramidal neurons (Neitz et al., 2014).

It has recently been suggested that cCMP and cUMP should also be considered in HCN channel-regulated processes (Zong et al., 2018).
FIGURE 10 | Effect of NO-GC2-deficiency on active waking (AW) state. (A) Raw local field potential (LFP) example traces during AW from SSC and movement detector (Passive Infrared Recording System, PIR) in WT (left) and NO-GC2−/− (right) mice. (B) The mean power spectrum of the LFPs between 1 Hz and 30 Hz obtained from 20 epochs of 10 s duration analyzed during the first 2 h of the light on period. (C) The mean values of raw PSD during AW showed enhanced δ (1–4 Hz), θ (5–8 Hz) and α (9–12 Hz) activity in NO-GC2−/− (red bar) compared to WT (black bar) mice. (D) The normalized PSD values are presented as percentage for WT (black bar) and NO-GC2−/− (red bar) mice. *P < 0.05.

et al., 2012) and soluble GC might also synthesize cCMP and cUMP in the presence of NO and Mn2+ (Beste et al., 2012). In agreement with this previous notion, we found that Ih in native neurons is moderately modulated by cCMP and cUMP.

In other neuronal cell types an increase in AP firing induced by NO donors which was sensitive to ODQ has been previously observed, suggesting participation of cGMP-dependent mechanisms (Kim et al., 2004). Intracellular application of 8-Br-cGMP strongly increased tonic firing in WT TC neurons and a number of distinct changes in electrophysiological properties were found in TC neurons following knock out or inhibition of NO-GC2. While NO-GC2−/− mice revealed reduced voltage sag amplitudes and tonic firing, RMP was unchanged. With respect to the number of triggered action potentials, LTS firing was less pronounced in NO-GC2-deficient mice. It has been shown before that the voltage-dependent properties of Ih crucially influence LTS generation and burst firing in TC neurons (Hughes et al., 1998). Positive shifts in the voltage-dependency of Ih increase the amplitude and duration of the LTS while negative shifts have opposite effects. Thus in line with our findings the reduced availability of Ih is associated with a decreased number of action potentials in the LTS.

Although we registered a decreased availability of Ih in TC neurons of NO-GC2−/− mice which is generally in line with some of our functional findings, the intrinsic membrane properties of TC neurons are expected to depend on several membrane currents. Setting of the RMP (Meuth et al., 2006), rhythmic bursting (Amarillo et al., 2014) and tonic firing (Kasten et al., 2007) of rodent TC neurons is based on the dynamic interaction of multiple membrane currents, with some of them being modulated by cGMP in a differential manner. While HCN channels are activated by direct binding of cGMP to the cyclic nucleotide binding domain (CNBD), the situation is more complex when it comes to K2P channels. PKG-dependent activation of TASK-1 and TREK-1 (Toyoda et al., 2010) as well as inhibition of heteromeric TASK-1/TASK-3 (Gonzalez-Forero et al., 2007) channels have been described. In addition, inward rectifier K+ currents have been shown to be inhibited in a cGMP-dependent manner (Dixon and Copenhagen, 1997) and a number of Kir channel subtypes (including members of the Kir2, Kir3 and Kir6 families) are expressed in thalamic cells.
FIGURE 11 | Effect of NO-GC2-deficiency on rapid eye movement (REM) sleep. (A) Raw LFP example traces during REM sleep in WT (left) and NO-GC2−/− (right) mice. Note, during REM sleep no movements are detected on PIR, indicating the immobility of the animal. (B) LFP power spectrum of different frequencies (1–30 Hz) over time. (C) During REM sleep, electroencephalogram (EEG) spectral power density is increased in the δ (1–4 Hz) and θ (5–8 Hz) band in NO-GC2−/− (red bar) compared to WT (black bar) mice. (D) The normalized PSD values are presented as percentage in WT (black bar) and NO-GC2−/− (red bar) mice. ** indicate \( P < 0.05 \), *** indicate \( P < 0.01 \), respectively.

(Thomzig et al., 2005). In TC neurons of different species several K\(_{2P}\) (TASK-1, TASK-3, TREK-1, TREK-2) and Kir channels (Kir2, Kir3) are expressed and have important contributions to the RMP, anomalous rectification and firing pattern (Meuth et al., 2003, 2006; Bista et al., 2015), thereby pointing to a complex scenario when cGMP-dependent effector functions are considered. The knockout of NO-GC2 and application of ODQ which both decrease the basal cGMP levels may have inhibited depolarizing HCN channels and (positively and negatively) modulated diverse hyperpolarizing K\(^{+}\) channels in a way that the net effects on RMP and R\(_{in}\) in the present study were inconspicuous. When taking the effects of exogenous cGMP application also into account, the strong effect of 8-Br-cGMP on RMP, R\(_{in}\) and tonic firing in the presence of ZD7288 (i.e., with HCN channels blocked) clearly points to the involvement of further ion channels. Here the combination of membrane depolarization and increased R\(_{in}\) is in line with the inhibition of TASK1/TASK3 heteromers which are present in TC neurons (Meuth et al., 2006). Indeed, application of Ba\(^{2+}\) which blocks TASK, TREK and Kir channels was associated with strong membrane depolarization, increased R\(_{in}\) and strongly enhanced tonic firing. Specific inhibition of some Kir channel types by Tertiapin-Q however, did not change the RMP and R\(_{in}\) in WT TC neurons. Moreover, extracellular application of 8-Br-cGMP had no influence on I\(_{KIR}\) amplitudes in Voltage clamp recordings. Taken together, the pharmacological manipulations indicate that altered modulation of cGMP-sensitive K\(_{2P}\) channels (Ma et al., 2011) may contribute to the phenotype of NO-GC2-deficient mice. But it does not exclude other ion channels as well.

Sleep is a complex process and controlled by several mechanisms (Borbély and Tobler, 2011) such as homeostatic, allostatic and circadian components. All regulators must ultimately target the thalamus to affect the cellular mechanisms that induce stable and global sleep-related oscillatory activity and allow the state-dependent gating of sensory information (Goulon et al., 2012). NO-dependent signaling is critically involved in the regulation of sleep homeostasis (Kalinchuk et al., 2006) and variations of NO and cGMP levels in the brain have been observed during the sleep-wake cycle (Kostin et al., 2013). We observed a strong increase in delta activity of the EEG as a consequence of NO-GC2 deficiency during AW and REM sleep. The nerve terminals of the mesopontine tegmentum cholinergic neurons which are part of the ascending reticular activating system have the ability to synthesize NO (Vincent, 2000). The release of NO from these thalamic afferents during arousal or REM sleep is followed by depolarization of TC neurons. It is possible that lack of postsynaptically located NO-GC2 receptors and decreased excitability of TC neurons in NO-GC2 mice disrupt the transition to AW and REM sleep and increases periods of slow oscillations in the EEG. The decreased availability to HCN channels may additionally hamper their pacemaker function and slow down oscillatory activity. We suggest that in the thalamus the cellular mechanisms of cGMP action may involve activation of HCN channels in addition to...
cGMP-dependent protein kinases. In addition, complete loss of the HCN2 channel gene and decreased responsiveness of I$_h$ were associated with the appearance of pathological slow high amplitude oscillations (5–7 Hz) in the EEG in form of spike-and-wave discharges (Ludwig et al., 2003; Kuise et al., 2006). These pathological activities were not found in NO-GC2$^{-/-}$. Furthermore, loss of HCN1 expression in the forebrain is associated with increased theta oscillations during AW and REM sleep (Nolan et al., 2004), and deletion of HCN channels auxiliary subunit TRIP8b increases delta oscillations during AW (Zobeiri et al., 2018). Therefore the effects of the NO-GC2 gene knockout are in agreement with reduced activation of I$_h$ in forebrain neurons which result in increased slow frequency bands in EEG recordings.

The involvement of the NO/GC/cGMP pathway in the regulation of sleep and wakefulness are not fully conclusive and in part even contradictory (Cavas and Navarro, 2006). While some studies indicate that NOS inhibition increases SWS and NO is required for arousal (Burlet and Cespuglio, 1997), opposite responses and differential effects on REM and non-REM sleep have been described (Hars, 1999). Nevertheless, the use of NOS inhibitors, NO donors and 8-Br-cGMP in cats in vivo supported the role of cGMP in controlling rhythmic neuronal activity in thalamic and cortical neurons, which may play a role in sleep/wake transition (Cudeiro et al., 2000). Divergent findings might be due to variances in timing and dosage of drug administration, acute pharmacological treatment vs. genetic background (transgenic animals), and NO-independent cGMP signaling (Hess et al., 2005). The fact that cGMP-dependent signaling has multiple effectors throughout the brain which are difficult to control experimentally may have additionally contributed to the ongoing discussion (Russwurm et al., 2013).

Neurons of the nRT reveal intrinsic pacemaking properties and participate in intrathalamic network operations which allow them to generate and synchronize spindle waves (7–15 Hz), a hallmark of early sleep stages (Fuentealba and Steriade, 2005). Since nRT and TC neurons are interconnected in a loop, and we reported decreased LTS characteristics and damped burst activities of nRT neurons and combined electrophysiological recordings in nRT and VB in vivo may be well suited to investigate this aspect in future studies.

Based on our results, we conclude that thalamic HCN channels are modulated by different cyclic nucleotides and that cGMP is a good candidate to regulate intrathalamic and cortical activities. However, the action of cGMP is broad, involving complex signaling pathways and is thus not limited to the modulation of HCN channels. Since reduced basal levels of cGMP decrease the excitability of TC cells, our data are in agreement with previous studies and supports the idea that the role of cGMP in thalamus is excitatory. The increased occurrence of delta and theta band activity during AW characterizes the loss of NO-GC2 as a TC dysrhythmia syndrome (Llinás et al., 1999) and supports the view that slow oscillations are an intrinsic property of the TC system (Timofeev, 2011).

**AUTHOR CONTRIBUTIONS**

MD, RC, EM, AL, MZ, BB, SB and AB performed experiments and analyzed/interpreted data. TB designed and supervised the project and reviewed all experiments. H-CP, DK and GvL provided important scientific input. MD and TB wrote the manuscript. All authors edited and agreed on the final version of the manuscript.

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Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.