

GABA_B receptor-mediated frequency-dependent and circadian changes in synaptic plasticity modulate retinal input to the suprachiasmatic nucleus

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Key points

- Light entrains the circadian clock by activating intrinsically photosensitive retinal ganglion cells projecting axons through the retinohypothalamic tract (RHT) to the hypothalamic suprachiasmatic nucleus (SCN).
- Initial release probability and synaptic plasticity changes in RHT-SCN synapses depended on the strength of GABA_B receptor (GABA_BR)-mediated presynaptic inhibition.
- The RHT axon terminals are under the tonic inhibitory control of GABA_B receptors. CGP55845 (3 μM) application increased the evoked excitatory postsynaptic current amplitude 30% throughout the light–dark cycle.
- During the light and dark phases the RHT inputs to 55% and 33% of recorded neurons, respectively, were under GABA_B inhibitory control indicating that the tonic GABA inhibition contributes to the circadian variation of transmitter release.
- GABA_BR-mediated presynaptic inhibition depended on the sensitivity of RHT terminals to GABA_BR agonists and diurnal changes of the extracellular GABA concentration around RHT axon terminals in the SCN, and decreased with increasing frequency of RHT stimulation.

Abstract Light is the most important environmental signal that entrains the circadian clock located in the hypothalamic suprachiasmatic nucleus (SCN). The retinohypothalamic tract (RHT) was stimulated to simulate the light intensity-dependent discharges of intrinsically photosensitive retinal ganglion cells projecting axons to the hypothalamus. EPSCs were evoked by paired-pulse stimulation or by application of stimulus trains, and recorded from SCN neurons in rat brain slices. Initial release probability (P_r) and synaptic plasticity changes depended on the strength of GABA_B receptor (GABA_BR)-mediated presynaptic inhibition and could be different at the same GABA_BR agonist concentration. Facilitation caused by frequency-dependent relief of GABA_BR-mediated inhibition was observed when the initial P_r was decreased to less than 15% of control during strong activation of presynaptic GABA_B receptors by (±)baclofen (10 μM), GABA (≥2 mM) or by GABA uptake inhibitor nipecotic acid (≥5 mM). In contrast, short-term synaptic depression appeared during baclofen (10 μM) application when initial P_r was greater than 30% of control. Block of 4-aminopyridine-sensitive K⁺ currents increased the amplitude and time constant of decay of evoked EPSCs (eEPSCs), and decreased the GABA_BR-mediated presynaptic inhibition. The GABA_B receptor antagonist CGP55845 (3 μM) increased the eEPSCs amplitude 30% throughout the light–dark cycle. During light and dark phases the RHT inputs to 55% and 33% of recorded neurons, respectively, were under GABA_B inhibitory control indicating that the tonic inhibition induced by local changes of endogenous GABA concentration contributes to the

circadian variation of RHT transmitter release. We conclude that GABA_BR-mediated presynaptic inhibition decreased with increasing frequency and broadening of presynaptic action potentials, and depended on the sensitivity of RHT terminals to GABA_BR agonists, and diurnal changes of the extracellular GABA concentration around RHT axon terminals in the SCN.

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Abbreviations 4-AP, 4-aminopyridine; eEPSC(s), evoked EPSC(s); PPS, paired-pulse stimulation; eEPSC₁, first eEPSC during PPS or stimulus train application; eEPSC₂, second eEPSC during PPS or stimulus train application; eEPSC_n, successive eEPSC in the stimulus train; GABA_ARs, GABA_A receptors; GABA_BRs, GABA_B receptors; ipRGCs, intrinsically photosensitive retinal ganglion cells; LD cycle, light/dark cycle; *P*, release probability; PPR, paired-pulse ratio; STD, short-term synaptic depression; SCN, suprachiasmatic nucleus; RHT, retinohypothalamic tract; VDCCs, voltage-dependent Ca²⁺ channels

Introduction

The molecular circadian clock located in the hypothalamic suprachiasmatic nucleus (SCN) receives environmental light intensity information directly from the retina via the retinohypothalamic tract (RHT) and indirectly through a projection from the intergeniculate leaflet (Pickard *et al.* 1987). Light activates intrinsically photosensitive retinal ganglion cells (ipRGCs) whose axons project to the SCN through the RHT. ipRGCs fire action potentials, which induce glutamate release from RHT terminals for the duration of a light exposure with the frequency determined by the light intensity (Wong, 2012; Hu *et al.* 2013).

Light pulses applied in the early night or the late night, respectively, phase delay or phase advance the locomotor activity rhythms (Daan & Pittendrigh, 1976). This mechanism adjusts the circadian clock to seasonal changes of light duration. Light-induced phase shifts are strongly regulated by G protein-coupled presynaptic receptors (Ralph & Menaker, 1989; Gillespie *et al.* 1997, 1999; Pickard *et al.* 1999; Sollars *et al.* 2006). GABA_B receptors (GABA_BRs) were unambiguously identified in retinal terminals (Belenky *et al.* 2008). Microinjection of the GABA_B receptor (GABA_BR) agonist baclofen into the SCN of freely-moving animals reduced light-induced phase shifts and *c-Fos* immunoreactivity during the early and late subjective night (Ralph & Menaker, 1989; Gillespie *et al.* 1997, 1999; Novak *et al.* 2004). The phase shift reduction was thought to be due to the presynaptic inhibitory effect of baclofen on RHT synaptic transmission, though GABA_BRs located on GABAergic axonal terminals, and on the cell membrane of SCN neurons could also play a role (Jiang *et al.* 1995; Chen & Van den Pol, 1998). Baclofen activates presynaptic GABA_BRs and attenuates glutamate release by inhibiting voltage-dependent Ca²⁺ channels (VDCCs) located on RHT terminals (Jiang *et al.* 1995; Moldavan *et al.* 2006). During activation of G protein-coupled receptors G_{βγ} subunits directly inhibit VDCCs, shifting them from an easily activated 'willing'

state to a more difficult to activate 'reluctant' state (Herlitze *et al.* 2001). G protein-mediated inhibition was relieved when the binding of the βγ subunits to VDCCs was reduced by strong prepulse cell membrane depolarization (Isaacson, 1998; Herlitze *et al.* 2001; Kajikawa *et al.* 2001), by broadening of action potential waveforms (Brody *et al.* 1997), or by high-frequency stimulation of presynaptic axons (Brenowitz *et al.* 1998; Brenowitz & Trussell, 2001). During high-frequency stimulation the relief of G protein-mediated inhibition was significantly stronger for P/Q-type compared to N-type VDCCs (Brody & Yue, 2000). These observations predict that G protein-mediated inhibition will predominate at low firing frequencies playing the role of a high-pass filter, in contrast to vesicle depletion, which is significant at high frequencies acting as a low-pass filter (Bertram, 2001; Fortune & Rose, 2001). The interplay between these two mechanisms controlling glutamate release from RHT terminals could be critical for the transmission of light entraining signals. The frequency-dependent relief of GABA_BR-mediated inhibition significantly enhances synaptic strength allowing transmission to persist at higher rates of synaptic activity, as occurred at the calyceal synapses (Brenowitz *et al.* 1998; Brenowitz & Trussell, 2001), in hippocampal neurons (Brody & Yue, 2000), and in the rat spinal cord (Lev-Tov & Pinco, 1992). As GABA is the most abundant neurotransmitter in the SCN it was important to study how endogenous GABA modulates RHT synaptic transmission and affects synaptic plasticity (Moore & Speh, 1993). Our goal was to examine the concentration and frequency dependence of synaptic plasticity at RHT synapses induced by activation of presynaptic GABA_BRs.

Our data indicate that short-term synaptic depression (STD) and GABA_BR-mediated presynaptic inhibition are distinct phenomena that cooperatively regulate RHT synaptic transmission and demonstrate an opposite frequency dependence. The magnitude of initial transmitter release as well as the strength of GABA_BR-mediated presynaptic inhibition defined the frequency-dependent

changes in synaptic plasticity. Endogenous GABA inhibited more RHT terminals projecting on to SCN neurons during subjective day than at night. However, due to GABA uptake mechanisms the average value of GABA_BR-mediated tonic inhibition does not change during a diurnal cycle.

Methods

Animal entrainment and preparation of brain slices

The Institutional Animal Care and Use Committee of OHSU approved all experimental procedures involving animals and all efforts were made to minimize pain and the number of animals used. Male Sprague–Dawley rats ($n = 61$, 4–6 weeks old; Charles River Labs, Wilmington, MA, USA) were housed in an environmental chamber (Percival Scientific, Perry, IA, USA) maintained at 20–21°C on a 12:12 h light/dark (LD) cycle, with free access to food and water. Recordings were made during subjective day. Only the effect of endogenous GABA was studied during both the light and dark phases. For subjective day recordings lights-on was at 08.00 h; for night recordings lights-on was at 23.00 h. Zeitgeber time (ZT) was used to define light and dark phases during the LD cycle. By convention, ZT12 was defined as lights-off. During the light phase, rats were deeply anaesthetized with isoflurane (Hospira, Inc., Lake Forest, IL, USA), their brains removed and submerged in an ice-cold Krebs solution consisting of (in mM): NaCl 126, KCl 2.5, NaH₂PO₄ 1.2, MgCl₂ 4.0, CaCl₂ 0.5, glucose 11, NaHCO₃ 26, saturated with 95% O₂ and 5% CO₂ (pH 7.3–7.4, 301–303 mosmol l⁻¹). Coronal (250 μm thick) or horizontal (~500 μm thick) brain slices containing the SCN were cut with a vibrating-blade microtome (Leica VT 1000 S, Leica Biosystems GmbH, Nussloch, Germany). The slices were maintained in a chamber at 30°C for 1.5–2 h before beginning the recordings.

Whole cell patch clamp recording

Recordings were made at 28°C using the whole cell patch clamp technique (Moldavan & Allen, 2010). The artificial cerebrospinal fluid (ACSF) used for the recordings consisted of (in mM): NaCl 132.5, KCl 2.5, NaH₂PO₄ 1.2, CaCl₂ 2.4, MgCl₂ 1.2, glucose 11, NaHCO₃ 22, saturated with 95% O₂ and 5% CO₂ (pH 7.3–7.4, 300–305 mosmol l⁻¹). Microelectrodes with resistances of 7–9 MΩ were pulled from borosilicate glass capillaries (World Precision Instruments, Inc., Sarasota, FL, USA) and filled with a solution containing (in mM): CH₃O₃SCs 102, CsCl 20, CaCl₂ 1, Hepes 10, EGTA 11, CsOH 28, MgATP 3, Tris-GTP 0.3, QX-314 5. Lidocaine *N*-ethyl chloride (QX-314) was included in the patch pipette

solution to block voltage-dependent Na⁺ channels. Cs⁺ was used to block postsynaptic K⁺ channels, including GABA_B-activated K⁺ channels (Jiang *et al.* 1995). To prevent activation of GABA_A receptors (GABA_ARs), the GABA_A receptor (GABA_AR) antagonist picrotoxin (50 μM) was added to the external solution in all experiments. The inclusion of ion channel blockers in the internal solution as well as voltage clamping at -60 mV prevented the activation of voltage-dependent ionic currents in SCN neurons. Individual SCN neurons were visualized with infrared illumination and differential interference contrast optics using a Leica DMLFS (Leica Biosystems GmbH, Nussloch, Germany) microscope with video camera and display (Sony, Tokyo, Japan). On-line data collection and analysis were performed using an EPC-7 patch clamp amplifier (HEKA Elektronik, Lambrecht/Pfalz, Germany), a Macintosh G3 or Mac mini computers, Pulse and Patchmaster programs (HEKA Elektronik, Lambrecht/Pfalz, Germany). The records were filtered at 3 kHz and digitized at 10 kHz. To allow equilibration between the pipette solution and the cell cytoplasm, whole cell patch clamp recording started ~10 min after rupturing the membrane. A small voltage step (2 mV, 5 ms) was applied before optic chiasm or optic nerve stimulation to monitor the series resistance (Moldavan *et al.* 2006). Recordings were made from the ventrolateral part of SCN.

Optic nerve and optic chiasm stimulation

EPSCs were evoked by electrical stimulation of the optic chiasm or optic nerve with a Grass S88 stimulator connected to a stimulus isolation unit (model SIU5B, Grass Medical Instruments, Quincy, MA, USA). The optic nerve was stimulated with a suction electrode in horizontal brain slices with both optic nerves attached (Moldavan & Allen, 2010) and the evoked EPSC (eEPSC) was recorded from neurons located in the ipsilateral SCN (Jiang *et al.* 1995). The optic chiasm was stimulated in the coronal brain slice with a concentric bipolar tungsten microelectrode (outer pole diameter 125 μm; catalogue no. CBASC75, FHC, Bowdoinham, ME, USA). The stimulating electrode was placed in the middle part of the optic chiasm as far as possible from the SCN. The stimulus pulse duration was 0.17 ms and the stimulation intensity 19.1 ± 0.7 V (range 9–28 V, $n = 43$) was set 1.5 times higher than that needed to evoke a threshold response. Paired-pulse stimulation (PPS) and stimulus trains were applied to simulate the discharge frequencies characteristic of ipRGCs. Trains of 25 stimuli (square pulses, 0.08–25 Hz, separated by 40 s intervals between trains) or PPS (2–100 Hz, 3 s interval between paired pulses) were used to study STD and frequency-dependent facilitation induced by relief of baclofen-mediated inhibition. If an

alternate PPS protocol was used it was indicated in the text.

Test agent application

All test agents were bath applied by perfusion with ACSF containing the final concentration of the compound. A complete change of the external solution in the recording chamber (volume $\sim 600 \mu\text{l}$) took less than 30 s at a flow rate of $1.5\text{--}2 \text{ ml min}^{-1}$. (\pm)Baclofen, picrotoxin, GABA, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and QX-314, (\pm)Nipecotinic acid (3-Piperidinecarboxylic acid) were purchased from Sigma (St Louis, MO, USA). (2S)-3-[[[(1S)-1-(3,4-Dichlorophenyl)amino-2-hydroxypropyl](phenylmethyl)phosphinic acid (CGP55845), (-)-Bicuculline methochloride, and 4-aminopyridine (4-AP) were purchased from Tocris Cookson Inc. (Ellisville, MO, USA). Appropriate stocks were made and diluted with ACSF just before application. CGP55845 and 4-AP were dissolved in DMSO. The final concentrations of DMSO in the recording solution during CGP55845 and 4-AP application were 0.01% and 0.5%, respectively. Picrotoxin was dissolved in ethanol. The final concentration of ethanol was 0.1%.

Statistical analysis

eEPSC amplitude was measured as the difference between the peak eEPSC current and the baseline before or after the stimulus artefact, when the optic chiasm or the optic nerve was stimulated, respectively. During PPS the paired-pulse ratio (PPR) was calculated as the ratio (mean eEPSC₂/mean eEPSC₁) of the mean peak amplitude of the second eEPSC (eEPSC₂) to the mean peak amplitude of the first eEPSC (eEPSC₁) (Kim & Alger, 2001). To estimate the magnitude of the drug effect the amplitude of each successive eEPSC (eEPSC_n) in the stimulus train was normalized (in %) to the control eEPSC₁ amplitude (ratio eEPSC_n/eEPSC₁). The ratio described changes of synaptic transmission with reference to the initial transmitter release. The steady-state eEPSC amplitude was calculated as the mean of the amplitude of last 10 eEPSCs in the stimulus train at each stimulus frequency, averaged across all recorded neurons (n) and plotted against stimulus frequency (Hz) (Moldavan & Allen, 2010). To compare the magnitude of STD or facilitation between different conditions (control, drug application) the eEPSC_n amplitude was normalized (in %) to the eEPSC₁ amplitude for each condition and the steady-state amplitude was calculated. Given the variability of the initial transmitter release the mean eEPSC₁ amplitude was calculated from two to three stimulus trains at each stimulus frequency. Short-term synaptic plasticity was defined as facilitation when the ratio eEPSC_n/eEPSC₁

was greater than 1, and as synaptic depression when the ratio eEPSC_n/eEPSC₁ progressively decreased to less than 1 during stimulus trains or PPS. The time constant τ that characterized the decay phase of eEPSC was calculated using the single exponential equation: $I(t) = I_0 + A \exp[-(t-t_0)/\tau]$, where $I(t)$ is the current at any given time t ; I_0 is the residual current, A is the amplitude at time 0, t is the given time, t_0 the initial time ($t = 0$) and τ the time constant. The concentration–response curve for GABA_BR-mediated inhibition of eEPSC induced by the GABA application was fit with the Hill equation; $I = I_{\text{max}} - (I_{\text{max}} - I_{\text{base}}) / [1 + (EC_{50}/[\text{agonist}])^{n_H}]$, where $[\text{agonist}]$ is the GABA concentration, I_{max} is the maximal eEPSC response when $[\text{agonist}]$ is 0, I_{base} is the eEPSC amplitude at maximal $[\text{agonist}]$, EC_{50} is the effective $[\text{agonist}]$ that induced a 50% reduction of eEPSC amplitude and n_H is the Hill coefficient. Student's paired or unpaired t tests (two-tailed) or extra sum of squares F test were used for statistical analysis as appropriate to compare the data sets recorded under different conditions. A confidence level of 95% was used to determine statistical significance. P values are reported throughout the text. Data were averaged across recorded neurons (n) and presented as means \pm S.E.M. Igor Pro (Version 5.03; Wave Metrics, Inc., Lake Oswego, OR, USA), KaleidaGraphTM (version 3.6; Synergy Software, Reading, PA, USA) and Excel 11.6.5 (or 14.1.3) (Microsoft Co., Redmond, WA, USA) were used for curve fitting, data analysis and graphic presentation.

Results

The activation of presynaptic GABA_BRs defined the magnitude of initial transmitter release and frequency-dependent changes of synaptic plasticity

STD occurs when the initial release probability (P_r) is high and facilitation when the initial P_r is low (Del Castillo & Katz, 1954; Millar *et al.* 2002). Reduction of presynaptic Ca^{2+} influx decreases initial P_r and may induce facilitation during repetitive stimulation (Lev-Tov & Pinco, 1992; Brenowitz & Trussell, 2001; Zucker & Regehr, 2002; Moldavan & Allen, 2010). We predicted that inhibiting presynaptic VDCCs with baclofen would decrease the initial P_r in RHT terminals and induce facilitation in contrast to the STD observed in control (Moldavan & Allen, 2010). We designed experiments to study the frequency-dependent GABA_BR-mediated synaptic plasticity. The optic chiasm or optic nerve was stimulated with stimulus trains or paired-pulses to simulate discharges of ipRGCs. RHT stimulation induced AMPA receptor-mediated eEPSCs in SCN neurons (Moldavan & Allen, 2010). At 0.08 Hz PPS (40 s between paired pulses) the PPR was approximately 1.0 and changes in synaptic plasticity were not observed in control or

during baclofen application. Increasing the PPS frequency to 2–100 Hz induced STD in control experiments ($n = 8$, Fig. 1A and C). The PPR progressively decreased from ~ 1.0 to 0.7 at the highest stimuli frequencies (50, 100 Hz) due to reduction of the eEPSC₂ amplitude. In contrast, baclofen ($10 \mu\text{M}$) significantly increased the PPR in a frequency-dependent manner (F test: $F_{3,8} = 72.0$; $P < 0.001$, $n = 8$; Fig. 1A and B, paired t test, P values are shown on Fig. 1A). The facilitation was observed when baclofen ($10 \mu\text{M}$) decreased eEPSC₁ amplitude to 14.8% of control (157.6 ± 20.8 pA in control and 23.3 ± 4.6 pA during baclofen application, $n = 8$) that corresponded to nearly maximal baclofen-mediated presynaptic inhibition (Moldavan *et al.* 2006). The facilitation was maximal at 100 Hz (~ 2 -fold increase of PPR).

Baclofen ($10 \mu\text{M}$) application decreased the initial P_T and enhanced synaptic strength (Fig. 2A–F). Baclofen decreased the eEPSC₁ amplitude during 50 Hz optic nerve PPS (12 s between paired pulses (Fig. 2A and B). The PPR exceeded the control level three times during the entire period of baclofen application and about 20 min of washout (Fig. 2C and D).

Stimulus train application induced STD in the majority of recorded SCN neurons (Moldavan & Allen 2010). During repetitive stimulation the ratio of the amplitude of each successive eEPSC to the amplitude of the first eEPSC (eEPSC_n/eEPSC₁) progressively decreased until it reached steady state (Fig. 3A). STD was not observed during the 0.08 Hz stimulation, thus 12 s inter-

vals between stimuli were sufficient for eEPSC₁ recovery (Fig. 3B). The magnitude of STD was higher during stimulus train application than during PPS due to activity-dependent vesicle depletion (Moldavan & Allen, 2010). Baclofen ($10 \mu\text{M}$)-mediated inhibition was relieved by fast (50 Hz) repetitive stimulation (Fig. 3A and C). In contrast, during low-frequency stimulation (0.08 Hz) the eEPSC_n/eEPSC₁ ratio was not changed indicating a frequency dependence of relief of baclofen-mediated inhibition (Fig. 3B). The baseline current remained stable even at high stimulus frequencies and, therefore, did not significantly contribute to the measured change of the eEPSC amplitude. For example, during 25 Hz stimulation the negative baseline shift was 8.95 ± 0.90 pA and 4.27 ± 1.10 pA (or $4.42 \pm 0.78\%$ and $2.29 \pm 0.75\%$ of the eEPSC₁ amplitude, $n = 10$) in control and during baclofen ($10 \mu\text{M}$) application, respectively.

Next, we studied how the frequency-dependent relief from the baclofen-mediated inhibition depends on the magnitude of the initial transmitter release. To reduce the initial P_T , baclofen concentrations that induced near maximal ($10 \mu\text{M}$) or half-maximal ($1 \mu\text{M}$) inhibition of the eEPSC amplitude were applied (Moldavan *et al.* 2006).

Following baclofen ($10 \mu\text{M}$) application functional criteria were used to separate recorded neurons into two groups. The first group (Group I) included neurons demonstrating facilitation of eEPSCs caused by frequency-dependent relief of baclofen-mediated inhibition during stimulus train application. Records were obtained during optic nerve ($n = 4$) or optic chiasm ($n = 8$) stimulation (Fig. 4A–D). The second group (Group II) of neurons showed activity-dependent STD during baclofen application ($n = 6$, optic chiasm stimulation, Fig. 4E and F). The eEPSC₁ amplitude (% of control) then was measured for each group to evaluate the magnitude of baclofen-mediated presynaptic inhibition (i.e. the reduction of initial P_T). In addition, the steady-state eEPSC amplitude was measured over a range of stimulus frequencies during optic nerve or optic chiasm stimulation with stimulus trains (Fig. 4).

In Group I, repetitive stimulation significantly relieved the presynaptic baclofen-mediated inhibition during optic nerve (Fig. 4A and B) or optic chiasm (Fig. 4C and D) stimulation. When the optic nerve was stimulated, baclofen inhibited the eEPSC₁ to $11.02 \pm 0.02\%$ (range 6.4–16.9%) of control (eEPSC₁ was 14.0 ± 2.9 pA during baclofen application *versus* 127.0 ± 16.7 pA in control, $n = 4$, Fig. 4A). During optic chiasm stimulation baclofen inhibited the eEPSC₁ to $7.96 \pm 0.02\%$ (range 2.8–25.0%) of control (eEPSC₁ was 18.3 ± 4.1 pA during baclofen application *versus* 230.0 ± 47.2 pA in control, $n = 8$, Fig. 4C). The steady-state eEPSC amplitude was attenuated by baclofen over the entire range of applied stimulus frequencies during optic nerve (F test: $F_{3,2} = 309.3$, $P < 0.01$; paired t test, $P < 0.001$) or

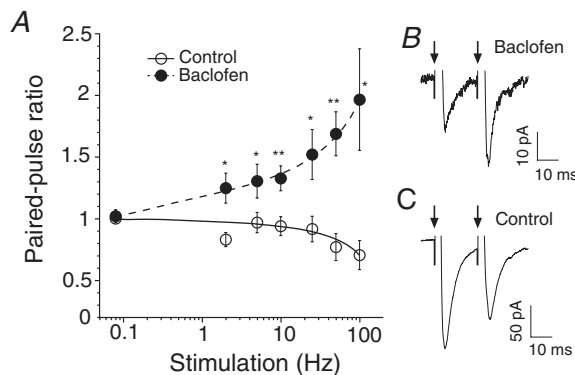


Figure 1. Relief of baclofen-mediated inhibition during paired-pulse stimulation causes frequency-dependent facilitation in retinohypothalamic tract synapses

A–C, paired-pulse stimulation of the optic chiasm. A, paired-pulse ratio plotted against stimulus frequency (control, baclofen $10 \mu\text{M}$, $n = 8$). The paired-pulse ratio equals the mean eEPSC₂/mean eEPSC₁, where the eEPSC₁ and eEPSC₂ are the first and the second eEPSC in the pair of stimuli, respectively. Short-term synaptic depression was observed in control, while frequency-dependent facilitation was observed during baclofen application. B and C, eEPSC recordings made from the same neuron in control (C) and during baclofen application (B); 50 Hz paired-pulse stimulation, arrows – stimuli application. * $P \leq 0.05$, ** $P \leq 0.01$ (paired t test, two-way). eEPSC, evoked excitatory postsynaptic current.

optic chiasm (F test: $F_{3,8} = 190.6$, $P < 0.001$; paired t test, $P < 0.05$) stimulation. Under these conditions, the eEPSC_n amplitude increased in a frequency-dependent manner and reached a maximum at 25 Hz. The increased eEPSC_n amplitude indicated a relief of baclofen-mediated inhibition. To compare changes in synaptic plasticity, the same data were normalized to the eEPSC₁ for each condition (control, baclofen, Fig. 4B and D). During baclofen (10 μM) application the eEPSC_n amplitude progressively increased to 1.5–2.5 times the eEPSC₁ amplitude demonstrating maximal facilitation during 5–25 Hz stimulation ($n = 4$, F test: $F_{3,2} = 37.2$, $P < 0.05$; paired t test, $P < 0.001$ at 5–25 Hz, Fig. 4B) or the optic chiasm ($n = 8$, F test: $F_{3,8} = 4.0$, $P < 0.05$; paired t test, $P < 0.001$, Fig. 4D).

In Group II, where stimulus trains produced only modest relief of baclofen (10 μM)-mediated inhibition, STD was observed. Baclofen inhibited the eEPSC₁ to $30.19 \pm 0.10\%$ of control (eEPSC₁ was 53.2 ± 18.1 pA during baclofen application versus 176.2 ± 30.6 pA in control, $n = 6$, Fig. 4E). Under these conditions no facilitation was observed (Fig. 4F). These data indicate that RHT inputs on to recorded neurons in Group II were less sensitive to baclofen than in Group I.

Similarly, a moderate decrease of STD was observed when baclofen (1 μM) reduced the eEPSC₁ amplitude to $46.5 \pm 0.2\%$ of control (66.5 ± 27.5 pA during baclofen

application versus 143.0 ± 26.8 pA in control ($n = 4$, paired t test, $P < 0.01$, Fig. 4G and H).

Our data clearly show that synaptic plasticity depends on the initial P_r and could be different even at the same baclofen concentration. The magnitude of the reduction of initial P_r by baclofen determined the frequency-dependent changes of synaptic plasticity.

GABA activating presynaptic GABA_BRs modulated retinohypothamic tract synaptic transmission in a concentration- and frequency-dependent manner

We wondered what concentration of GABA is needed to activate GABA_BRs to decrease the eEPSC₁ amplitude to about 10% of control to produce the largest frequency-dependent relief of GABA_BR-mediated inhibition of synaptic transmission. To block GABA_ARs, picrotoxin (50 μM) or bicuculline (20 μM) were applied together with GABA. The concentration–response curve for GABA was fitted with a Hill equation ($\text{EC}_{50} = 0.49 \pm 0.04$ mM, Hill coefficient = 1.86, $n = 15$, see Methods, Fig. 5A). Only GABA concentrations greater than or equal to 2 mM decreased the eEPSC₁ amplitude to less than 10% of control. GABA (5 mM) reduced the eEPSC₁ amplitude to $5.9 \pm 0.7\%$ of control and strongly attenuated the steady-state eEPSC amplitude over

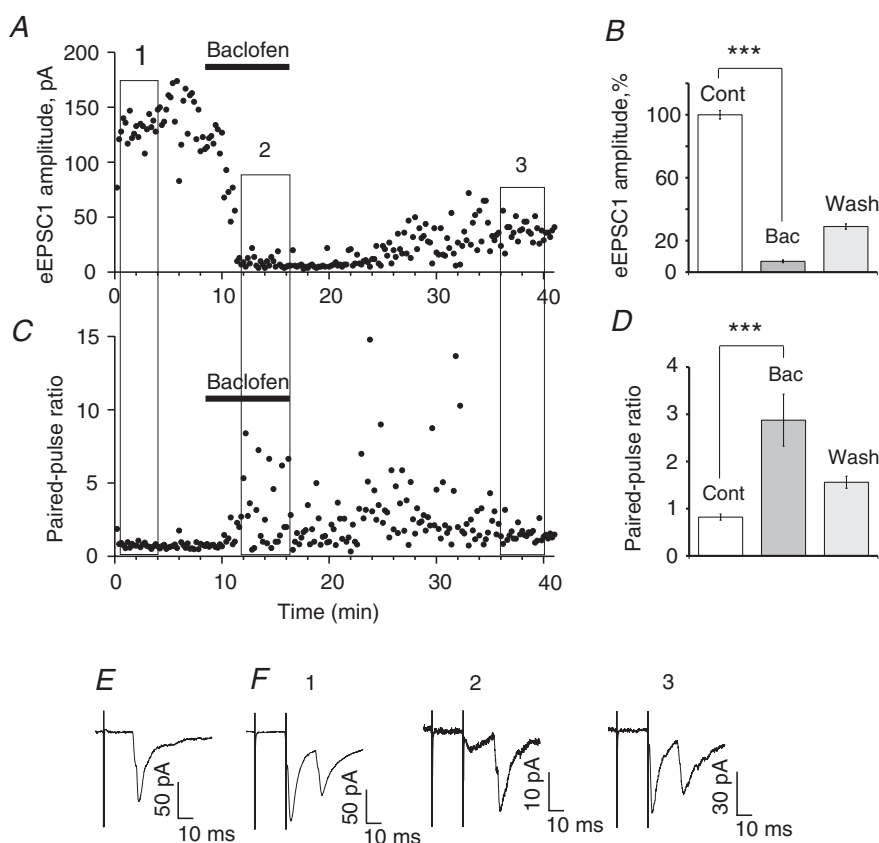


Figure 2. Baclofen-mediated presynaptic inhibition in retinohypothamic tract synapses increased synaptic strength

A, the amplitude of the first eEPSC (eEPSC₁) evoked by 50 Hz PPS of the optic nerve was plotted. At 12 s intervals between pairs of stimuli the eEPSC₁ amplitude remained relatively stable [control (1)]. eEPSC₁ amplitude decreased during baclofen (10 μM) application (2) and partially recovered during washout (3). B, mean eEPSC₁ amplitude is shown for three specified intervals: control, baclofen (10 μM) and washout (average of 20 sweeps, % of control). C and D, 50 Hz PPS revealed an increase of synaptic strength (facilitation) during baclofen application. C, baclofen application increased paired-pulse ratio. D, paired-pulse ratio (mean eEPSC₂/mean eEPSC₁): control, baclofen, washout (average of 20 sweeps). Paired t test, two tail, *** $P < 0.001$. E and F, recordings of eEPSC made from the same neuron. E, 0.08 Hz stimulation (average of $n = 4$ sweeps). F, PPS: control (1), baclofen (2) and washout (3); average of 15 sweeps. Numbers correspond to specified periods (1, 2, 3) on (A) and (C). Bac, baclofen; Cont, control; eEPSC, evoked excitatory postsynaptic current; PPS, paired-pulse stimulation.

the range of stimulus frequencies (F test: $F_{3,2} = 28.0$, $P < 0.05$; paired t test, $P < 0.001$, $n = 4$, Fig. 5B). The same data normalized for each condition (control, GABA) demonstrated frequency-dependent relief of GABA_BR-mediated presynaptic inhibition during GABA (5 mM) application (paired t test, $P < 0.001$ at 1–25 Hz, $n = 4$, Fig. 5C).

Blockade of GABA uptake increased GABA_BR-mediated presynaptic inhibition

We applied nipecotic acid, a transportable blocker of all types of GABA transporters, to inhibit the GABA uptake. As the rate of GABA uptake increases directly proportional to the increase of temperature (Binda *et al.* 2002), experiments were performed at physiological temperature (35°C). Picrotoxin (50 μM) was applied to block the GABA_A current. Nipecotic acid (40 mM) fully blocked the eEPSC (Fig. 6A). The inhibition induced by nipecotic

acid (20 mM) was completely reversed by the GABA_BR antagonist CGP55845 (3 μM) (Fig. 6B). Thus, inhibition of GABA uptake by nipecotic acid caused an accumulation of extracellular GABA that activated presynaptic GABA_BRs inhibiting the eEPSC.

Nipecotic acid (5 mM) decreased the eEPSC amplitude to $10.0 \pm 2.6\%$ of control (two-tailed t test $P < 0.001$, $n = 9$, Fig. 6C). At 0.08 Hz PPS, no difference was found in synaptic plasticity in control and during nipecotic acid application (PPR: 0.99 ± 0.03 and 0.94 ± 0.14 for control and nipecotic acid, respectively; two-tailed t test $P = 0.74$, $n = 8$, Fig. 6D). In contrast, at 50 Hz PPS synaptic depression was observed in control (PPR 0.82 ± 0.07) and facilitation (PPR 1.71 ± 0.23) during nipecotic acid application (two-tailed t test $P < 0.02$, $n = 8$). Thus, when GABA uptake was inhibited, accumulated GABA strongly activated presynaptic GABA_BRs, and decreased initial transmitter release from RHT terminals. Under these conditions high-frequency stimulation relieved GABA_BR-mediated inhibition.

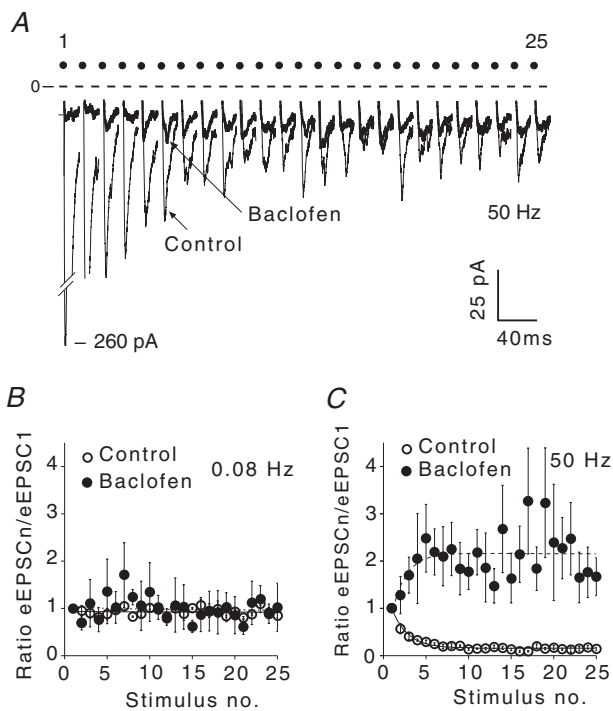


Figure 3. Relief of baclofen-mediated inhibition during high-frequency repetitive stimulation

A, eEPSCs recordings during stimulus train application [50 Hz, optic chiasm stimulation, 25 stimuli in the train; control, baclofen (10 μM), average of 10 sweeps]. Note: these records are not shown on a time scale (each dot shows the stimulus number). B and C, ratio of eEPSC_n/eEPSC₁ amplitude: (B) 0.08 Hz, average of three sweeps (control), five sweeps (baclofen); (C) 50 Hz stimulation, average of five sweeps (control), 10 sweeps (baclofen). eEPSC₁ is the amplitude of the first eEPSC; eEPSC_n is the amplitude of the each successive eEPSC in the train. eEPSC, evoked excitatory postsynaptic current.

Inhibition of the 4-aminopyridine-sensitive K⁺ currents increased initial P_r and restored synaptic transmission altered by baclofen

Increasing the duration of action potential-like depolarizing waveforms in HEK 293 cells relieved G protein-mediated inhibition (Brody *et al.* 1997). Therefore, we predicted that broadening the presynaptic action potential would relieve the baclofen-mediated inhibition of VDCCs in RHT terminals. To broaden the presynaptic action potential, 4-AP (5 mM), a blocker of transient A-type potassium currents, was applied. 4-AP broadened the action potential and induced a slow Ca²⁺ transient followed by the fast transient in the optic nerve (Sun & Chiu, 1999). The elongation of the Ca²⁺ influx induced by 4-AP increased the amplitude ($189.2 \pm 30.5\%$ of control, t test: $P < 0.01$, $n = 8$) and slowed the decay of the eEPSC during 0.08 Hz stimulation of the optic chiasm (Fig. 7A). The eEPSC demonstrated an exponential decay and the time constant (τ) was increased $46.1 \pm 20.6\%$ of control (7.0 ± 1.2 ms during 4-AP application vs. 4.7 ± 0.8 ms in control, two-tailed t test: $P < 0.001$, $n = 7$, Fig. 7C). Baclofen (10 μM) decreased the eEPSC amplitude to $6.5 \pm 1.4\%$ of control ($n = 10$, Fig. 7B). Baclofen did not alter the presynaptic action potential properties (Isaacson, 1998) and did not significantly affect the decay of eEPSC, τ : 4.5 ± 0.6 ms vs. 5.2 ± 0.6 ms in control (τ 86.6 \pm 6.5% of control; two-tailed t test: $P = 0.75$, $n = 5$; baclofen 10 μM). Application of 4-AP relieved the baclofen-mediated inhibition and increased the eEPSC amplitude to $108.3 \pm 21.5\%$ of control (t test: $P = 0.70$, $n = 10$, Fig. 7D). Being applied together with baclofen (10 μM), 4-AP broadened the eEPSC and

increased the tau $142.4 \pm 12.8\%$ of control (6.8 ± 1.5 ms vs. 4.7 ± 0.8 ms in control, two-tailed t test $P < 0.01$, $n = 7$).

The broadening of presynaptic action potential combined with high-frequency stimulation should maximally relieve the baclofen-mediated inhibition. In

addition, this will allow us to answer the question: Does the maximal relief of G protein-mediated inhibition overwhelm vesicle depletion? To test this idea we used 25 Hz stimulus trains to induce frequency-dependent relief of baclofen-mediated inhibition and applied 4-AP to broaden the action potentials. eEPSC amplitude and

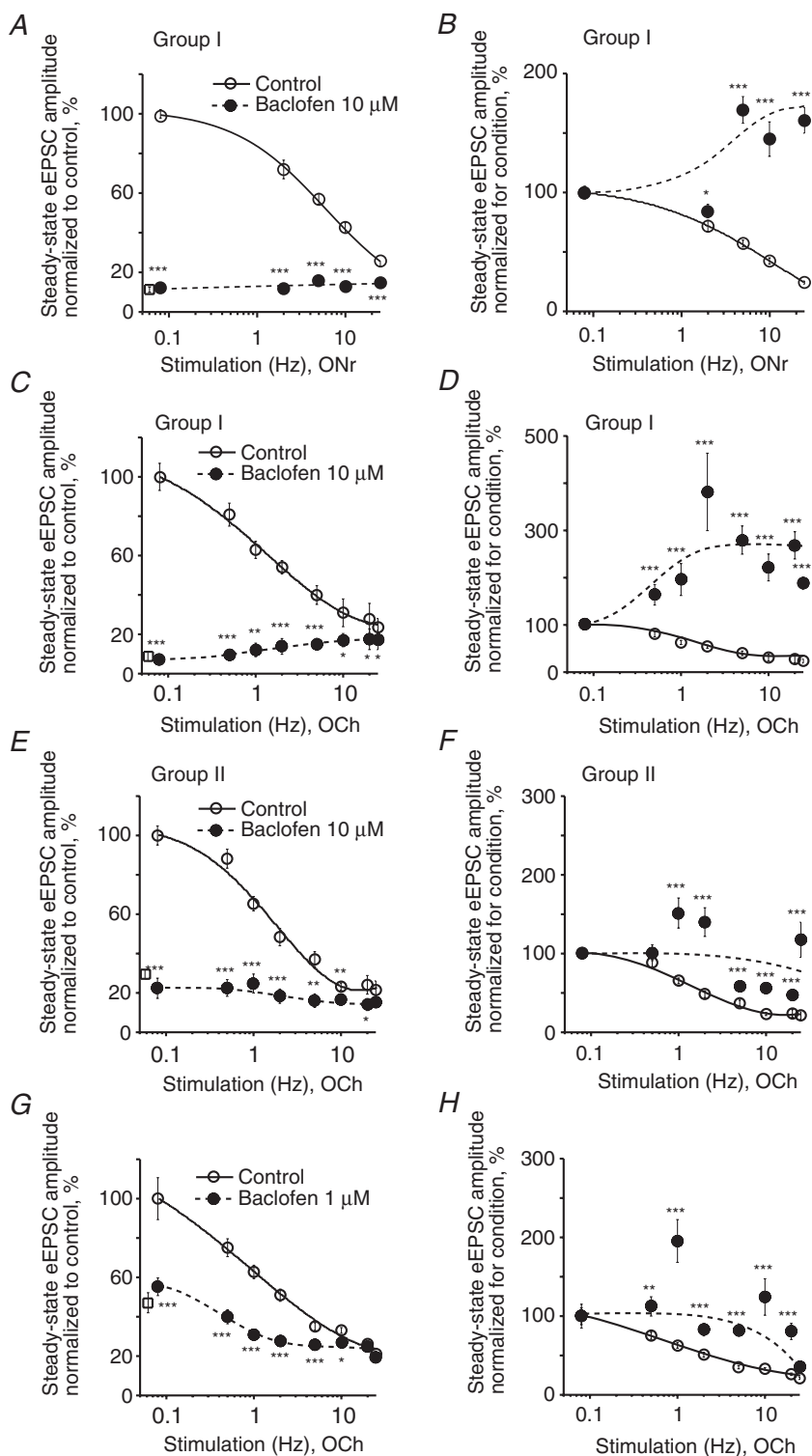


Figure 4. Synaptic plasticity depends on the sensitivity of retinohypothalamic tract terminals to GABA_BR agonist and its concentration

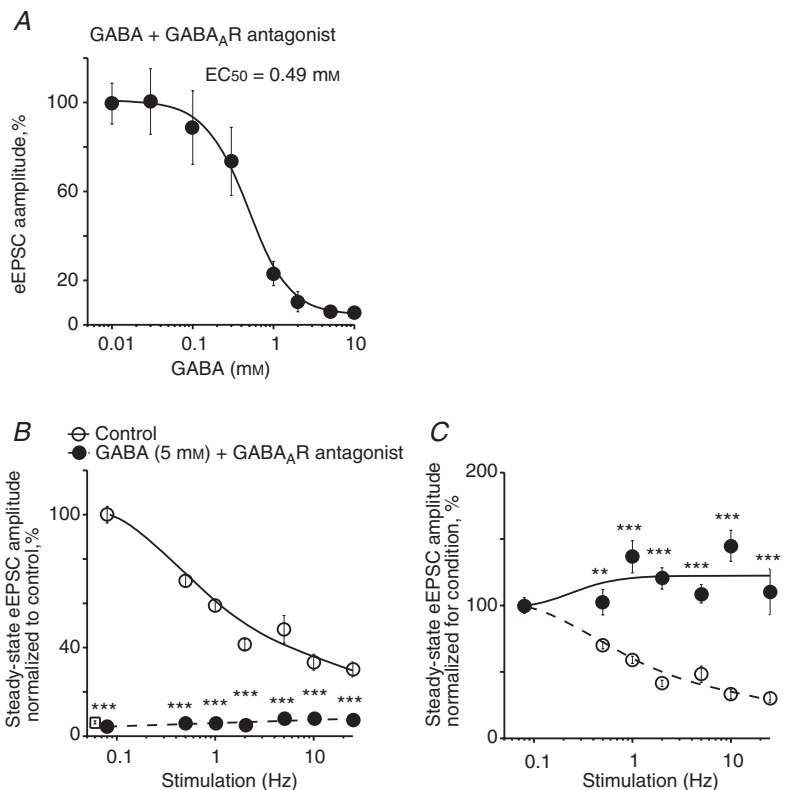
A–H, frequency dependence of the steady-state eEPSC amplitude during 0.5–25 Hz stimulus train application (control, baclofen). A–D, Group I neurons. E and F, Group II neurons. A and B, baclofen 10 μM, ONr stimulation, $n = 4$ neurons; C and D, baclofen 10 μM, OCh stimulation, $n = 8$; E and F, baclofen 10 μM, OCh stimulation, $n = 6$; G and H, baclofen 1 μM, OCh stimulation, $n = 4$. A, C, E and G, to compare the magnitude of the baclofen-mediated inhibition at each stimulus frequency, the amplitude of each successive eEPSC in the stimulus train was normalized (%) to the eEPSC₁ amplitude in control, then the steady-state eEPSC amplitude was plotted (open circles, control; closed circles, baclofen). The mean eEPSC₁ amplitude (% of control) recorded over the range of stimulus frequencies during baclofen application is shown as an open square near the Y-axis. Short-term synaptic depression was observed in control. The eEPSC amplitude during baclofen application did not exceed the steady-state amplitude in control. B, D, F and H, to compare changes in synaptic plasticity between conditions (control, baclofen) the same data were normalized (%) to the eEPSC₁ amplitude for each condition. Paired t test, two tail, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. eEPSC, evoked excitatory postsynaptic current; OCh, optic chiasm; ONr, optic nerve.

the charge transfer were analysed during the last 10 sweeps in the train when the eEPSCn amplitude reached the steady state, and vesicle depletion was maximal for each stimulus frequency. To compare the steady-state amplitude between different conditions [control, baclofen (10 μ M), joint baclofen and 4-AP application], it was normalized (%) to the eEPSC₁ in control (Fig. 7E). Twenty-five Hz stimulation relieved baclofen-mediated inhibition and induced maximal facilitation (eEPSCn amplitude was normalized of eEPSC₁ at each condition, Fig. 7H). 4-AP applied together with baclofen increased initial P_r (eEPSC₁ amplitude) and produced STD during high-frequency stimulation (Fig. 7E and H). During joint 4-AP and baclofen application the steady-state eEPSC amplitude and charge transfer were similar to or higher than control, and significantly exceeded those parameters during baclofen application alone (Fig. 7F and G). Despite the fact that the broadening of action potential by 4-AP in combination with high-frequency stimulation would maximally relieve the baclofen-mediated inhibition, the steady-state eEPSC amplitude demonstrated a frequency-dependent decrease (Fig. 7I).

Thus, even during maximal relief of baclofen-mediated inhibition induced by high-frequency stimulation and by broadening of the presynaptic action potential the steady-state eEPSC amplitude and charge transfer were attenuated by vesicle depletion.

Figure 5. GABA concentration- and frequency-dependent modulated synaptic transmission at retinohypothalamic tract synapses by activating presynaptic GABA_BRs

A, GABA activated presynaptic GABA_BRs and reduced the eEPSC amplitude in a concentration-dependent manner. GABA was applied together with the GABA_AR antagonists: picrotoxin (50 μ M) or bicuculline (20 μ M). eEPSC amplitude (% of control) was measured during 0.08 Hz stimulation. The concentration–response curve was fitted with a Hill equation ($EC_{50} = 0.49 \pm 0.04$ mM, Hill coefficient = 1.86, $n = 15$). B, frequency dependence of the steady-state eEPSC amplitude during optic chiasm stimulation with stimulus trains (control, GABA (5 mM) together with GABA_AR antagonists). Amplitude of each successive eEPSC in the stimulus train was normalized (%) to the eEPSC₁ amplitude in control. The mean eEPSC₁ amplitude recorded over the range of stimulus frequencies during GABA application is shown as an open square near Y-axis ($5.9 \pm 0.7\%$ of control, $n = 4$). C, the same data normalized to the eEPSC₁ amplitude for each condition (control, GABA) demonstrated short-term synaptic depression in control and, frequency-dependent relief of GABA_BR-mediated inhibition during GABA application. Paired t test, two tail, $**P < 0.01$, $***P < 0.001$. eEPSC, evoked excitatory postsynaptic current.



Endogenous GABA modulates the EPSC evoked by retinohypothalamic tract stimulation

The data describing the GABA content and GAD activity in the rat SCN during the LD cycle are controversial. It was reported that they are minimal during the light phase and maximal during the dark phase at ZT 16.00 h (Aguilar-Roblero *et al.* 1993). Though in earlier studies the peak of GABA levels in the rat hypothalamus was detected during the lights-on period (Cattabeni *et al.* 1978). In these studies the diurnal changes of GABA content in the SCN were reported but the extracellular GABA level was not determined. Therefore, to investigate how extracellular GABA modulates RHT synaptic transmission during the LD cycle, CGP55845 (3 μ M) was applied to block presynaptic GABA_BRs and the eEPSC was recorded (Fig. 8C). To confirm that GABA_BRs were completely blocked, baclofen (10 μ M) was applied after CGP55845. Under these conditions, baclofen did not inhibit eEPSCs because GABA_BRs remained blocked at least 30 min after ending CGP55845 application. To confirm that eEPSCs were induced by activation of AMPA receptors, 6-cyano-7-nitroquinoxaline-2,3-dione (20 μ M) was applied after each recording. During light and dark phases the eEPSC was recorded, respectively, from 22 and 21 neurons located in the ventrolateral SCN (Fig. 8B). eEPSC amplitude was measured at 0.08 Hz stimulation that does not induce STD (Moldavan & Allen, 2010). During the light phase (ZT 5.00–9.00 h)

CGP55845 increased the eEPSC amplitude $28.2 \pm 9.1\%$ of control (168.3 ± 9.6 pA) in 55% (12 of 22) neurons and decreased the amplitude $28.4 \pm 5.8\%$ of control (176.4 ± 18.0 pA) in 45% (10 of 22) cells (Fig. 8A and B, left column). During the dark phase (ZT 15.00–18.00 h) CGP55845 increased the eEPSC amplitude $30.8 \pm 16.2\%$ of control (153.1 ± 12.9 pA) in 33% (seven of 21) neurons

and decreased the amplitude $21.6 \pm 3.9\%$ of control (145.0 ± 12.6 pA) in 67% (14 of 21) cells (Fig. 8A and B, right column). Thus, CGP55845 similarly increased eEPSC amplitudes during both light and dark phases (two-tailed *t* test, $P = 0.89$, $n = 19$). At the same time, a significant diversity in the tonic activation of GABA_BRs located on RHT terminals was observed during the LD cycle: CGP55845 increased the eEPSC amplitude in the range 2.5–116.8% of control (the average $29.1 \pm 8.0\%$, two-tailed *t* test, $P < 0.016$, $n = 19$) (Fig. 8E).

We also checked the possibility that stronger electrical stimuli may induce a greater GABA release resulting in more extracellular GABA due to spillover. Therefore, we compared the strength of electric stimuli used to evoke EPSC in two groups of neurons that demonstrated the increase or decrease of eEPSC amplitude, respectively, during CGP55845 application. The magnitude of stimulation was not significantly different between those groups: 20.3 ± 1.1 V (ratio stimulus/threshold = 1.6, $n = 19$) versus 18.3 ± 0.9 V (ratio stimulus/threshold = 1.5, $n = 24$) for neurons that increased and decreased the eEPSC amplitudes, respectively (two-tailed *t* test, $P = 0.17$, $n = 43$). Thus, the increase of eEPSC amplitude during CGP55845 application was not caused by an increase of the stimulus intensity. Both groups of neurons had similar locations in the SCN. Many of the neurons that showed increased eEPSC amplitude were located even farther from the stimulating electrode than neurons in which the eEPSC amplitude was decreased. Thus, the RHT inputs to the neurons that demonstrate an increase of eEPSC amplitude during CGP55845 application were under the tonic inhibitory control of endogenous GABA that activated presynaptic GABA_BRs on RHT terminals.

To study how endogenous GABA modulates synaptic plasticity, the 50 Hz optic chiasm PPS was applied. In neurons under tonic inhibitory control of endogenous GABA (i.e. demonstrated an increase of the eEPSC₁ amplitude during CGP55845 application) the PPR significantly decreased to 0.65 ± 0.04 after CGP55845 application versus 0.85 ± 0.05 in control (two-tailed *t* test, $P < 0.002$, $n = 19$, Fig. 8F). The PPR decreased mainly due to a change of the eEPSC₁ but not eEPSC₂ amplitude (Fig. 8D). During CGP55845 application the eEPSC₁ amplitude increased $29.1 \pm 8.0\%$ while the eEPSC₂ amplitude decreased only $2.8 \pm 4.9\%$. Thus, blocking GABA_BRs increased the initial P_r and the magnitude of STD. Or, in other words, the activation of presynaptic GABA_BRs by endogenous GABA attenuates initial P_r and decreases STD. In the rest of the neurons, in which CGP55845 decreased the eEPSC amplitude, the PPR did not change significantly: 0.8 ± 0.07 and 0.78 ± 0.07 in control and during CGP55845 application, respectively (two-tailed *t* test, $P = 0.83$, $n = 24$).

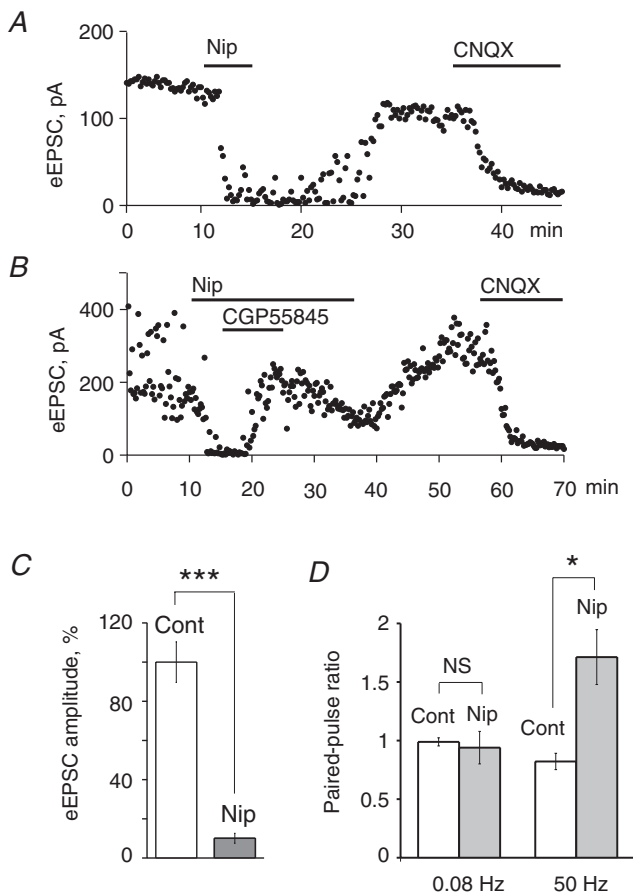


Figure 6. Inhibition of GABA uptake increased extracellular GABA concentration that activated presynaptic GABA_BRs and inhibited retinohypothalamic tract synaptic transmission
 A, inhibition of eEPSC by nipecotic acid (40 mM), a non-selective transportable GABA uptake inhibitor. Nipecotic acid increased the extracellular GABA concentration. In turn, GABA in the presence of the GABA_AR antagonist picrotoxin (50 μM) activated presynaptic GABA_BRs and inhibited RHT synaptic transmission. B, GABA_BRs antagonist CGP55845 (3 μM) recovered eEPSC inhibited by nipecotic acid (20 mM). A and B, CNQX (20 μM) blocked eEPSC induced by optic chiasm stimulation (0.08 Hz). C, nipecotic acid (5 mM) decreased the eEPSC amplitude to about 10% of control ($n = 9$). D, nipecotic acid (5 mM) induced a frequency-dependent facilitation during 50 Hz paired-pulse stimulation: paired-pulse ratio increased 1.7 times versus paired-pulse ratio in control or during low frequency (0.08 Hz) stimulation ($n = 8$). A–D, nipecotic acid was applied together with picrotoxin (50 μM). Paired *t* test, two tail, * $P < 0.05$, *** $P < 0.001$; NS, non-significant changes. CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; eEPSC, evoked excitatory postsynaptic current; Nip, nipecotic acid.

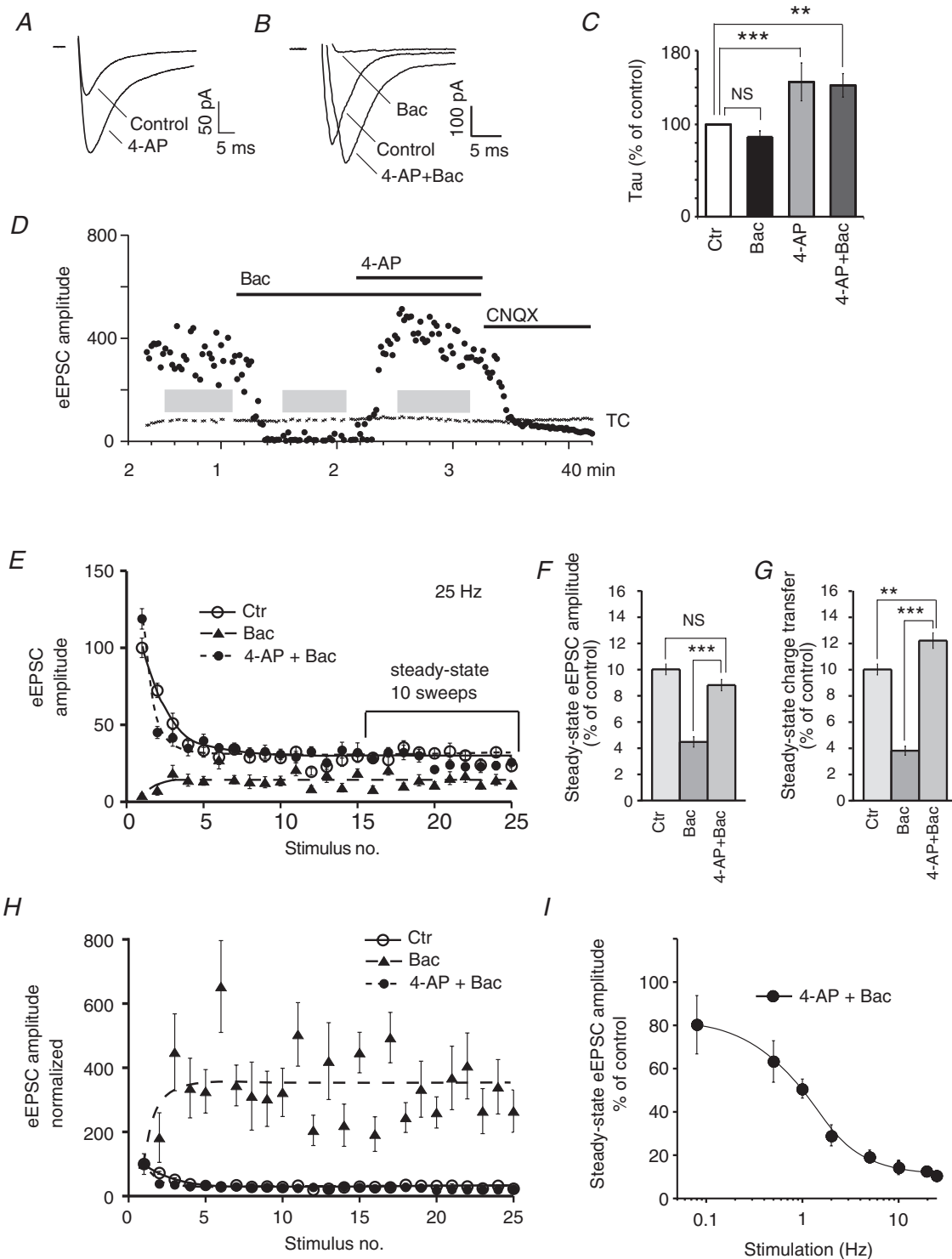


Figure 7. 4-AP restored synaptic transmission altered by baclofen
 A and B, EPSCs evoked by 0.08 Hz optic chiasm stimulation. A, 4-AP (5 mM) increased the amplitude and the decay time of the eEPSC (average of 25 trials). B, eEPSCs were inhibited by baclofen (10 μM). 4-AP applied together with baclofen increased the eEPSC amplitude and lengthened the decay time of eEPSC (average of nine trials). C, time constant of the eEPSC decay calculated for conditions: baclofen, 4-AP, and 4-AP+baclofen applications. The eEPSC decay was fit with single exponential curve and the resulting time constants were compared (% of control, mean of seven eEPSCs for each neuron, n = 7 neurons). D, eEPSC was blocked by baclofen and recovered after 4-AP application (0.08 Hz stimulation). The eEPSC was induced by AMPA receptor activation and was blocked by CNQX (20 μM). E, activity-dependent changes of eEPSC amplitude (control, baclofen, 4-AP and baclofen together). eEPSC

Discussion

Frequency and concentration dependence of GABA_BR-mediated synaptic plasticity

The maximal action potential firing rate of ipRGCs varied between the different types of cells and can exceed 100 Hz (Wong, 2012; Hu *et al.* 2013). Repetitive stimulation of the optic nerve or optic chiasm that simulate the firing of ipRGCs induced a frequency-dependent STD in most RHT synapses on to SCN neurons that reflects activity-dependent reduction of transmitter release when the initial P_r is high (Del Castillo & Katz, 1954; Millar *et al.* 2002; Moldavan & Allen, 2010). We predicted that activation of GABA_BRs on RHT terminals would decrease P_r and increase synaptic strength during high-frequency stimulation. The expression of VDCCs, GABA_BRs, G proteins and extent of modulation of different Ca²⁺ channel subtypes will define the sensitivity of RHT terminals to GABA_BR agonists (Brenowitz & Trussell, 2001; Moldavan *et al.* 2006). The synaptic strength was enhanced by baclofen decreasing the initial P_r . Strong activation of GABA_BRs by baclofen (10 μM) reduced the eEPSC₁ amplitude to less than 15% of control and induced frequency-dependent facilitation in RHT synapses during PPS or stimulus train application. The maximal facilitation was observed during 25–100 Hz stimulation. Some terminals were under the weaker GABA_B inhibitory control, therefore baclofen (10 μM) reduced the eEPSC₁ amplitude only to 30% of control and STD was observed. The initial P_r was higher at the lower baclofen concentration (1 μM), when the eEPSC₁ amplitude decreased to only 43% of control, and stimulation induced STD. Thus, the value of the P_r and synaptic plasticity depended on the sensitivity of RHT terminals to baclofen. If the eEPSC₁ amplitude exceeded 30% of control, STD instead of facilitation was observed. GABA (>2 mM) applied by bath or accumulated due to GABA uptake inhibition decreased the eEPSC₁ amplitude to 10% of control producing a frequency-dependent relief of GABA_BR-mediated inhibition.

Our data agree with previous studies on the calyceal synapse and the spinal cord showing the relief of G protein-mediated inhibition at high stimulus frequencies during strong activation of presynaptic GABA_BRs

(Lev-Tov & Pinco, 1992; Brenowitz & Trussell, 2001). In the presence of baclofen, the maximal eEPSCn amplitude during the facilitation did not exceed the steady-state amplitude observed during STD in control. This indicates that in RHT synapses frequency-dependent facilitation caused by relief of G protein-mediated inhibition does not overcome vesicle depletion.

The functional and behavioural relevance of the relief from G protein-mediated inhibition induced at faster stimulation frequencies is not clear. In contrast to vesicle depletion, which acts as a low-pass filter, the activation of G protein-coupled presynaptic GABA_BRs act as a high-pass filter by increasing the P_r at faster stimulus frequencies (Forsythe *et al.* 1998; Brody & Yue, 2000; Bertram, 2001; Fortune & Rose, 2001). We agree with Brenowitz *et al.* (1998) that: 'The ability to modulate release probability by presynaptic receptors may represent an adaptation to control the rate of vesicle depletion and receptor desensitization in order to maintain maximal synaptic strength at different stimulus rates'.

Blockade of transient A-type potassium currents reversed inhibitory effect of baclofen on eEPSCs

Baclofen-mediated inhibition of presynaptic VDCCs is voltage-dependent. Therefore, strong prepulse depolarization of the presynaptic terminal as well as widening of action potential-shaped waveforms during repetitive stimulation relieved GABA_BR-mediated inhibition (Brody *et al.* 1997; Isaacson, 1998). 4-AP, a blocker of the transient A-type potassium currents, broadens the action potential, induces the delayed depolarization and slows repolarization in the rat optic nerve (Gordon *et al.* 1988; Sun & Chiu, 1999). We predicted, that broadening of the presynaptic action potential by 4-AP in combination with high-frequency stimulation would strongly relieve the baclofen-mediated inhibition in RHT terminals. 4-AP increased the P_r and substantially diminished the baclofen-mediated inhibition in RHT synapses, even during strong activation of presynaptic GABA_BRs (Brody & Yue, 2000; Moldavan *et al.* 2006). Baclofen-mediated inhibition may be altered by several mechanisms. Baclofen does not activate voltage-gated K⁺ channels in the rat optic nerve, in the RHT, or other glutamatergic presynaptic terminals

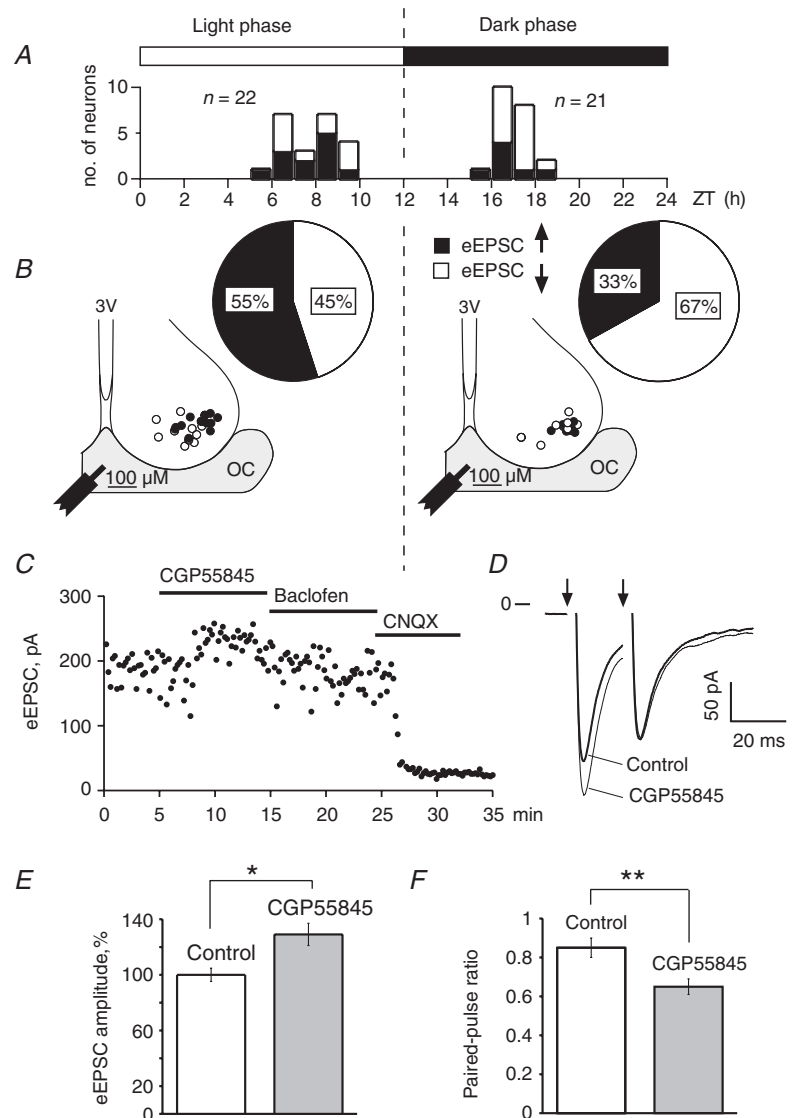
amplitude normalized (%) to the control eEPSC₁ amplitude (eEPSC₁, the first eEPSC in the train). *F* and *G*, eEPSC amplitude (*F*) and charge transfer (*G*) were analysed during steady state (last 10 sweeps in the train). *H*, eEPSC amplitude normalized (%) to the eEPSC₁ amplitude for each condition (control, baclofen, 4-AP and baclofen together). *I*, frequency-dependent decrease of the steady-state eEPSC amplitude during joint 4-AP and baclofen application (0.08–25 Hz stimulation). Unpaired (*C*) or paired (*F* and *G*) *t* test, two tail: ***P* < 0.01, ****P* < 0.001. 4-AP, 4-aminopyridine; Bac, baclofen; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; Ctr, control; eEPSC, evoked excitatory postsynaptic current; TC, test current; grey rectangles (D), stimulation with stimulus trains (optic chiasm stimulation, 25 Hz, 25 stimulus in the train).

(Takahashi *et al.* 1998; Sun & Chiu, 1999; Moldavan *et al.* 2006) and does not change presynaptic action potential properties (Isaacson, 1998; Sun & Chiu, 1999). Therefore, blocking of 4-AP-sensitive K⁺ currents would not directly alter the baclofen-mediated inhibition. Because the baclofen effect is voltage-dependent, the broadening of presynaptic action potential will relieve baclofen-mediated inhibition. 4-AP delays repolarization of presynaptic action potentials, which increased Ca²⁺ entry and may activate VDCCs that were not inhibited by baclofen (Sun & Chiu, 1999; Brody & Yue, 2000; Moldavan *et al.* 2006; Wu *et al.* 2009). Baclofen strongly decreased initial P_r ; therefore, during high-frequency repetitive stimulation the ratio eEPSC_n/eEPSC₁ was higher than 1, consistent with frequency-mediated facilitation. 4-AP enhanced the eEPSC₁ amplitude and the time constant of eEPSC₁ decay. When 4-AP increased the initial P_r , the ratio eEPSC_n/eEPSC₁ was less than

1 and STD was observed. We compared the eEPSC_n induced by prolonged high-frequency stimulation, when STD or frequency-dependent relief of baclofen-mediated inhibition were maximal. During STD (in control or joint 4-AP and baclofen application), eEPSC_n was even higher than during facilitation when baclofen was applied alone. Our data are consistent with previous studies where baclofen in high Ca²⁺/4-AP extracellular solutions caused only small changes in short-term plasticity that casts doubt on changes in the maximal P_r as the mechanism of facilitation (Brody & Yue, 2000). Even under conditions, when baclofen-mediated inhibition was relieved by high-frequency stimulation and by broadening of presynaptic action potential, the eEPSC amplitude and the charge transfer during steady state were in the range observed in control. Thus, the relief of baclofen-mediated inhibition could not overcome the gain control introduced by vesicle depletion in RHT

Figure 8. Presynaptic GABA_BR-mediated tonic inhibition of retinohypothalamic tract synaptic transmission induced by endogenous GABA

A, ZT scale was used to show the time when eEPSC recordings were performed. Distribution of neurons recorded during light and dark phase is shown. n is the quantity of neurons. B, sector diagrams: the ratio (%) of neurons in which the eEPSC amplitude increased (black) or decreased (white) during GABA_BR antagonist CGP55845 (3 μ M) application. Recorded neurons were located in the ventrolateral region of the suprachiasmatic nucleus (coronal slice); OC, optic chiasm; 3V, third ventricle; filled circles, neurons whose retinohypothalamic tract inputs were under tonic inhibitory control of endogenous GABA; open circles, neurons whose retinohypothalamic tract inputs were not inhibited by endogenous GABA; the tip of the stimulus electrode was located in the middle part of the OC. C, eEPSC recording during successive application of CGP55845, baclofen (10 μ M) and CNQX (20 μ M). D, eEPSC recordings during paired-pulse stimulation (50 Hz): control, CGP55845 application; average of 25 sweeps; arrows are stimuli applications. E, eEPSC amplitude was increased by CGP55845 ($n = 19$). F, CGP55845 decreased the paired-pulse ratio due to an increase of the eEPSC₁ amplitude (paired-pulse stimulation, 50 Hz, $n = 19$). Paired t test, two tail, * $P < 0.05$, ** $P < 0.01$. CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; eEPSC, evoked excitatory postsynaptic current; ZT, Zeitgeber.



synapses, in contrast to synapses designed for conduction of high-frequency action potentials (Brenowitz *et al.* 1998; Bertram, 2001). Our data are consistent with the idea that baclofen blocking presynaptic VDCCs regulates transmitter release upstream of the vesicle release mechanism.

Endogenous GABA induces tonic GABA_BR-mediated inhibition of retinohypothalamic tract synaptic transmission

Direct synaptic connections between GABAergic and retinal terminals have not been described in the SCN, although those terminals are located in close proximity to each other (Belenky *et al.* 2008). Therefore, presynaptic GABA_BRs on RHT terminals could be activated by GABA that diffuses from local GABAergic synapses (Moore & Speh, 1993; Sollars *et al.* 2006; Belenky *et al.* 2008) including afferents from the intergeniculate leaflet (Pickard *et al.* 1987; Morin *et al.* 1992) and surrounding hypothalamic tissue (Castel & Morris, 2000). The extracellular GABA concentration in the hypothalamus was detected by microdialysis in the range 0.05–0.07 μM (Yananli *et al.* 2008). The low endogenous GABA concentration and, therefore relatively weak activation of GABA_BRs might explain why microinjection of GABA_B antagonists neither alters phase shifts during the subjective night or increases *c-Fos* expression in the SCN when administered before light exposure, nor induce phase shifts when given alone (Gillespie *et al.* 1997, 1999).

Considering our data, we propose the hypothesis that two mechanisms co-operate regulating extrasynaptic GABA in the SCN. The first is the activity of GABAergic neurons in SCN and, possibly, some external GABAergic inputs and, the second, the GABA uptake mechanism. Recordings in freely moving rats showed that SCN neurons increased their discharge twofold in the day and decreased activity at night (Meijer *et al.* 1998). The firing frequency of SCN neurons recorded from rat brain slices also peaked near midday and gradually decreased during subjective night (Green & Gillette, 1982; Jobst & Allen, 2002; Gribkoff *et al.* 2003). In addition, a larger proportion of SCN neurons are silent during subjective night than during the day. For example, the percentage of silent cells in SCN increased up to ~40% during subjective night in comparison to 20% of the population during the day (Schaap *et al.* 1999; Jobst & Allen, 2002). Thus, more SCN neurons are active and fire faster during the subjective day. Because most SCN neurons are GABAergic the release and synaptic spillover of GABA would be predicted to be stronger during the day than at night. This conclusion is confirmed by measurement of the glutamic acid decarboxylase (GAD65) mRNA level in the rat SCN that was significantly higher in the light than in the dark

(Huhman *et al.* 1996). Our data are in a good agreement with these findings. In our experiments, CGP55845 application increased the eEPSC amplitude in 55% of studied SCN neurons during the subjective day and in 33% during the night. This indicates that more RHT terminals projecting on to SCN neurons are under the inhibitory control of endogenous GABA during the light than during the dark phase. In contrast, baclofen activating presynaptic GABA_BRs inhibited eEPSCs in a concentration-dependent way in almost all studied SCN neurons, and the rate of inhibition was not significantly different during subjective day and night (Moldavan *et al.* 2006). Baclofen is not a substrate for GABA uptake carriers (Ong & Kerr, 1998). Therefore, baclofen could not be removed from the extracellular space by GABA transporters and activates GABA_BRs on all RHT terminals. We suggest that GABA spillover from GABAergic synapses is limited by GABA uptake mechanisms. Therefore, synaptically released GABA activated only GABA_BRs on RHT terminals located in close proximity to GABAergic synapses and did not activate ones on remote RHT terminals. Thus, RHT inputs to 45–67% of SCN neurons were not inhibited by endogenous GABA during the LD cycle but could be inhibited by baclofen. The important role of GABA uptake was confirmed in our experiments. Nipecotic acid blocked GABA uptake, significantly increasing GABA diffusion into the extrasynaptic space and the extracellular GABA concentration. As a result, the eEPSC amplitude was strongly decreased during nipecotic acid application in all studied SCN neurons. Recently we observed expression of GAT-3 and GAT-1 in the SCN that would limit the diffusion of GABA from synaptic cleft (Moldavan *et al.* 2012). GAT-1, -2 and -3 mRNA transcripts found within the neonatal and mature rat optic nerve also indicate the important role of GABA transporters (Howd *et al.* 1997). Because of a strong GABA uptake in the SCN, a high GABA concentration ($\text{EC}_{50} = 0.49 \text{ mM}$ in the presence of picrotoxin) was required to block the eEPSC (Fig. 5). The half-maximal effective concentration of GABA in our experiments was much higher than that required to block Ca^{2+} transient in the trunk of the optic nerve (Sun & Chiu, 1999). This indicates an important role of synaptic and extrasynaptic GABA uptake in the vicinity of RHT terminals.

Despite the variability among SCN neurons, the average increase of eEPSC amplitude during CGP55845 application was similar during subjective day and night. This indicates a relatively stable endogenous GABA concentration around RHT terminals during the LD cycle that is maintained by the GABA uptake mechanism. CGP55845 blocking presynaptic GABA_BRs increased the initial P_r (eEPSC₁ amplitude was on average increased to approximately 30% of control) and enhanced STD. In other words, the endogenous GABA activating presynaptic GABA_BRs decreased the initial P_r and relieved STD.

Conclusion

The GABA content in the SCN is higher during the day and lower at night (Cattabeni *et al.* 1978; Huhman *et al.* 1996). We demonstrated that despite this variation the average magnitude of the presynaptic tonic GABA_BR-mediated inhibition in RHT synapses did not change during the LD cycle. Considering our data, we propose a hypothesis that explains this paradox. The number of active GABAergic SCN neurons is higher during the day and their discharges are stronger than during the night. The possible role of GABAergic inputs to the SCN also could not be rejected. More GABA is released and accumulates in the extracellular space in certain locations in the SCN during the day than at night. As a result, the total GABA concentration in the SCN would increase. Owing to an increase of locations where GABA is released and spills over, the number of RHT inputs inhibited by endogenous GABA also increases. Despite the increased GABA release during the day, strong GABA uptake keeps the concentration of endogenous GABA in the vicinity of RHT terminals in these locations at a relatively consistent level during the LD cycle. Owing to this mechanism, GABA_BR-mediated tonic inhibition was observed in much smaller number (in ~30–60%) of RHT inputs than those that express GABA_BRs, and could be activated by baclofen. Activity-dependent changes of RHT synaptic plasticity were generally dependent on the initial P_r that was defined by sensitivity of RHT terminals to GABA_BR agonists.

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Author contributions

M.G.M. and C.N.A. conceived and designed the experiments, M.G.M. performed the electrophysiological experiments, collected, and analysed the data, M.G.M. and C.N.A. interpreted the data and wrote and revised the manuscript. The experiments were performed in the laboratory of C.N.A. at the Center for Research on Occupational and Environmental Toxicology, Oregon Health & Science University, Portland, OR, USA. All authors read and approved the final version of the manuscript.

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