Presynaptic GABA$_B$ Receptors Regulate Retinohypothalamic Tract Synaptic Transmission by Inhibiting Voltage-Gated Ca$^{2+}$ Channels

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Moldavan, Mykhaylo G., Robert P. Irwin, and Charles N. Allen. Presynaptic GABA$_B$ receptors regulate retinohypothalamic tract synaptic transmission by inhibiting voltage-gated Ca$^{2+}$ channels. J Neurophysiol 95: 3727–3741, 2006; doi:10.1152/jn.00909.2005. Presynaptic GABA$_B$ receptor activation inhibits glutamate release from retinohypothalamic tract (RHT) terminals in the suprachiasmatic nucleus (SCN). Voltage-clamp whole cell recordings from rat SCN neurons and optical recordings of Ca$^{2+}$-sensitive fluorescent probes within RHT terminals were used to examine GABA$_B$-receptor modulation of RHT transmission. Baclofen inhibited evoked postsynaptic currents (EPSCs) in a concentration-dependent manner equally during the day and night. Blockers of N-, P/Q-, T-, and R-type voltage-dependent Ca$^{2+}$ channels, but not L-type, reduced the EPSC amplitude by 66, 36, 32, and 18% of control, respectively. Joint application and resting Ca$^{2+}$ influx and Ca$^{2+}$ concentration in RHT terminals. Tertiapin did not alter the evoked EPSC and baclofen-induced inhibition, indicating that baclofen does not inhibit glutamate release by activation of Kir3 channels. Neither Ba$^{2+}$ nor high extracellular K$^+$ modified the baclofen-induced inhibition. 4-Aminopyridine (4-AP) significantly increased the EPSC amplitude and the charge transfer, and dramatically reduced the baclofen effect. These data indicate that baclofen inhibits glutamate release from RHT terminals by blocking N-, T-, and P/Q-type Ca$^{2+}$ channels, and possibly by activation of 4-AP-sensitive K$^+$ channels, but not by inhibition of R- and L-type Ca$^{2+}$ channels or by Kir3 channel activation.

INTRODUCTION

Suprachiasmatic nucleus (SCN) neurons of mammals contain a biological clock that synchronizes physiological processes with changing environmental conditions. To maintain proper temporal coupling between the circadian clock and the environment, neural systems have evolved to sense changing conditions and communicate that information to the clock. The daily change in light intensity (day/night) is the most important of these environmental signals. The SCN receives light intensity information by the retinohypothalamic tract (RHT), a direct glutamatergic projection from retinal ganglion cells (RGCs) to the SCN (Berson et al. 2002; Card and Moore 1991; Castel et al. 1993; Liou et al. 1986).

Light’s effect on circadian clock timing can be modified by neurotransmitters activating presynaptic receptors, thereby reducing glutamate release from RHT terminals. For example, activation of 5-HT$_{1B}$ receptors reduces c-fos expression and the magnitude of light-induced phase shifts (Pickard et al. 1996, 1999). Similarly, activation of $\gamma$-aminobutyric acid type B (GABA$_B$) receptors inhibits light-induced phase advances and phase delays (Colwell et al. 1993; Gillespie et al. 1997; Ralph and Menaker 1989). Baclofen, a GABA$_B$ agonist, reduces the light-induced c-fos expression in the SCN that is often used as a physiologic measure of the activation of SCN neurons by light stimulation (Colwell et al. 1993; Gillespie et al. 1997; Pickard et al. 1996). Thus GABA$_B$ receptors as well as 5-HT$_{1B}$ receptors “act to prevent photic information from reaching the SCN” (Colwell et al. 1993; Pickard et al. 1996). In hypothalamic slices baclofen reduces the amplitude of excitatory postsynaptic currents (EPSCs) evoked by optic nerve stimulation without altering the intrinsic activity of postsynaptic glutamate receptors (Jiang et al. 1995). GABA$_B$-receptor activation also reduces the frequency but does not alter the distribution of miniature EPSC amplitudes in SCN (Jiang et al. 1995). Further, the GABA$_B$ antagonist phaclofen potentiates the field potential induced by optic nerve stimulation, consistent with a tonic GABA$_B$-receptor-mediated inhibition (Gannon et al. 1995). Some of the RGCs that possess G-protein–coupled GABA$_B$ receptors may contribute axons to the RHT (Chen et al. 2004; Slaughter 1995). These data are consistent with the hypothesis that activation of presynaptic GABA$_B$ receptors inhibits glutamate release from RHT terminals (Gannon et al. 1995; Jiang et al. 1995).

Currently, the specific mechanism(s) by which GABA$_B$ receptors regulate neurotransmitter release at RHT terminals is not known. In the hippocampus, GABA$_B$-receptor activation inhibits voltage-dependent Ca$^{2+}$ channels and activates K$^+$ channels through a G(i/o)-type G-protein–mediated pathways (Andrade et al. 1986; Sodickson and Bean 1996). The GABA$_B$-receptor–mediated effect is attenuated by pertussis toxin uncoupling the G(i/o)-protein from the GABAB receptor. Loading G-protein beta-gamma subunits (G$\beta$G) into the calyceal nerve terminal partially occluded the inhibitory effect of baclofen on presynaptic Ca$^{2+}$ currents (Kajikawa et al. 2001). G$\beta$G directly inhibits Ca$^{2+}$ channels, putting them into a “reluctant” state (Bertram et al. 2003; Zamponi and Snutch 1998). Activation of GABA$_B$ receptors suppresses GABA release from the terminals of cultured SCN neurons through a G-protein–mediated inhibition of N- and P/Q-type Ca$^{2+}$ channels (Chen and Van den Pol 1998). Also baclofen-induced...
inhibition of N-type Ca\(^{2+}\) channels was found in axons of neonatal rat optic nerve (Sun and Chiu 1999). In addition, baclofen activates fast inactivating A-type K\(^+\) channels and Ca\(^{2+}\)-activated K\(^+\) channels (SK channels) (Bettler et al. 2004; Saint et al. 1990). We performed experiments to test the hypothesis that activation of presynaptic GABA\(_B\) receptors acts to decrease evoked glutamate release from RHT terminals by inhibiting voltage-dependent Ca\(^{2+}\) channels or by activating K\(^+\) channels.

**METHODS**

**Preparation of SCN brain slices**

Male Sprague–Dawley rats (4–6 wk old) were housed in an environmental chamber (Percival Scientific, Perry, IA) maintained at 20–21°C on a 12 h light/12 h dark schedule. During the lights-on phase, rats were deeply anesthetized with halothane, their brains removed and submerged in an ice-cold Krebs solution consisting of (in mM): NaCl 126, KCl 2.5, NaH\(_2\)PO\(_4\) 1.2, MgCl\(_2\) 4.0, CaCl\(_2\) 0.5, glucose 11, and NaHCO\(_3\) 26, saturated with 95% O\(_2\)-5% CO\(_2\) (pH 7.3–7.4, 301–303 mOsm). Coronal 250- to 300-m thick slices of the hypothalamus containing the SCN were cut with a vibrating-blade microtome (Leica VT 1000 S, Nussloch, Germany). 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.

**Whole cell patch-clamp recording**

Recordings were made at 28–30°C using the whole cell patch-clamp technique from 1 to 8 h after slicing (Fig. 1A). The recording solution was identical to the slicing solution, but contained (in mM): NaCl 130, NaHCO\(_3\) 22, CaCl\(_2\) 2.4, and MgCl\(_2\) 1.2 (pH 7.3–7.4, 300–305 mOsm). Microelectrodes with resistances of 7–9 MΩ were pulled from borosilicate glass (World Precision Instruments, Sarasota, FL) and filled with a solution containing (in mM): CsCH\(_3\)O\(_2\)S 105, CsCl 20, CaCl\(_2\) 1, HEPES 10, EGTA 11, CsOH 25, ATP 3, GTP 0.3, and QX-314 5. QX-314 was included in the patch pipette solution to block voltage-activated Na\(^+\) channels and K\(^+\) channels. Cs\(^+\) was used to block K\(^+\) channels including GABA\(_B\)-activated K\(^+\) channels (Jiang et al. 1995). To prevent activation of GABA\(_A\) receptors, by GABA released from SCN GABAergic neurons after RHT stimulation, picrotoxin 50 μM was added to the external solution. Individual SCN neurons were visualized with infrared illumination and differential interference contrast optics using a Leica DMLF microscope with video camera and display (Sony). On-line data collection and analysis were performed using an EPC-7 patch-clamp amplifier (HEKA Electronic, Lambrecht, Germany), a Macintosh G3 computer, and Pulse and PulseFit (HEKA). The records were filtered at 3 kHz and digitized at 10 kHz.

To allow equilibration between the pipette solution and the cell cytoplasm contents, whole cell patch-clamp recording usually started 10 to 25 min after rupturing the cell membrane. During this time there was a rundown of the EPSC amplitude, which reached a plateau then remained stable for the next 1–1.5 h. Series resistance was typically 17–63 MΩ (mean 33 ± 0.7 MΩ, n = 148) and was monitored by applying a small voltage step (1 mV, 5 ms) before optic chiasm stimulation. SCN neurons were voltage clamped at −60 mV. During whole cell recording the series resistance remained stable and only recordings with series resistance changes of <10% were included in the analysis. Only cells that showed significant recovery (to 60–100%) from test agent application (except o-agonotoxin-TK, o-conotoxin GVIA, mibefradil, and SNX-482 experiments) were included in the analysis.

**Optic chiasm stimulation**

EPSCs were evoked by electrical stimulation of the optic chiasm. A bipolar concentric tungsten electrode (FHC, Bowdoinham, ME) connected to a stimulus isolation unit (model SIU5B, Grass Medical Instruments) was placed in the optic chiasm and stimulated using a Grass S88 stimulator (Grass Medical Instruments). The pulse duration was 0.13–0.17 ms and the stimulation intensity was set 1.5–2 times higher than that needed to evoke a threshold response and usually varied between 8 and 40 V. EPSCs were elicited by electrical stimuli (square pulses) at 0.08 Hz.

**Test agent application**

All test agents were applied by perfusion of the recording chamber with artificial cerebrospinal fluid (ACSF) containing the final concentration of the compound. Chamber volume was about 400 µl; a complete change of the external solution took <30 s at a flow rate of 1.5–2 ml/min. (±)Baclofen (GABA\(_B\) receptor agonist, 0.03–30 µM), mibefradil (T-type Ca\(^{2+}\) channel blocker, 20 μM), nimodipine (L-type Ca\(^{2+}\) channel blocker, 10 μM), lidocaine N-ethylchloride (QX-314), d-(-)-2-amino-5-phosphonovaleric acid (d-APV, 50 μM), CNQX (10 μM), picrotoxin (50 µM), and DMSO were purchased from Sigma (St. Louis, MO). o-Agatoxin TK (P/Q-type Ca\(^{2+}\) channel blocker, 100 or 500 nM), o-conotoxin GVIA (N-type Ca\(^{2+}\) channel blocker 1 μM), SNX-482 (R-type Ca\(^{2+}\) channel blocker, 150 nM), tetrodotoxin (TTX, 750 nM), and tertiapin (a potent inhibitor of Kir3.1, Kir3.4, Kir1.1, and K\(_{Ca}\) inwardly rectifying K\(^+\) channels, 10 or 100 nM) were purchased from Alomone Labs (Jerusalem, Israel). 4-Aminopyridine (4-AP, 1–5 mM) was purchased from Sigma (St. Louis, MO) and Tocris Cookson (Ellisville, MO). Appropriate stocks were made and diluted with ACSF just before application. o-Agatoxin TK, o-conotoxin GVIA, SNX-482, tertiapin, mibefradil, and baclofen, in experiments with the Ca\(^{2+}\) channel blockers, were applied by perfusion through a micropipette with an internal diameter of 100 µm. ACSF applied through micropipette contained the final concentration of test agent. The micropipette was placed close to the slice surface upstream of the SCN and the ACSF containing the toxins flowed out of the perfusion micropipette in the same direction to the 

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**FIG. 1.** Schema of recording and test agent application onto suprachiasmatic nucleus (SCN) neurons in the rat brain coronal slices. A: whole cell patch-clamp recording. B: Ca\(^{2+}\) probe injection into the optic chiasm for Ca\(^{2+}\) imaging.
flow of ACSF in the recording chamber (Fig. 1A). The flow from micropipette completely covered the SCN. These Ca\(^{2+}\) channel blockers were applied for 20–40 min.

**Optical measurements**

Stock solutions (2.5 mM) of Fluo-4 acetoxymethyl ester (AM) (K\(_f\) = 345 nM) or Fura-Red AM (K\(_f\) = 140 nM; Molecular Probes, Eugene, OR) dissolved in DMSO (Mallinkrodt, Hazelwood, MO) were diluted with double-strength ACSF (containing in mM NaCl 240, KCl 5, HEPES 20, pH 7.4 without Mg\(^{2+}\), Ca\(^{2+}\), or PO\(_4\)^{3-}\) to a final concentration of 250 \(\mu\)M, and briefly triturated. Glass pipettes with tips of approximately 5 \(\mu\)m were filled with probe solution and pressure injected (Picospritzer II; General Valve, Fairfield, NJ) over 20–40 min into the optic chiasm approximately 250–300 \(\mu\)M away from the SCN. The pipette tip was positioned at an angle pointing away from the SCN. A suction pipette was placed adjacent to the injection site to remove any extraneous probe (Fig. 1B). In addition, the laminar flow (2 ml/min) of ACSF in the chamber was aimed away from the SCN to further reduce the possibility of depositing probe outside of the optic chiasm. Further, the likelihood of the probe getting into SCN neurons is low because rats of this age (>4 wk) have minimal uptake of AM probes into neurons while presynaptic terminals load well (Colwell 2000; Yuste 2000). After injection, 2–3 h were allowed for probe transport into the RHT terminals. For experiments with Fluo-4 AM, a small amount of Texas Red-dextran (Molecular Probes) was added to the solution to help visualize the time course of probe loading. Fluorescent images were obtained with an upright microscope (DM LFS; Leica) with a water immersion objective (HCX Pro L63X/0.9W U-V-I; Leica). To capture fast Ca\(^{2+}\) transients, probes) was added to the solution to help visualize the time course of flow of ACSF in the recording chamber (Fig. 1A). The flow from micropipette completely covered the SCN. These Ca\(^{2+}\) channel blockers were applied for 20–40 min.

**Math and statistical analysis**

To calculate the inhibitory effect of baclofen on each Ca\(^{2+}\) channel type we used the equation: \[ Ic = \left(1 - \frac{[Ib - It]}{Ib}\right) \times 100\% , \]
where Ic is the percentage occlusion of baclofen effect by a Ca\(^{2+}\) channel blocker, It is the percentage inhibition of the EPSC by the Ca\(^{2+}\) channel blocker, Ib is the percentage inhibition of the EPSC induced by baclofen alone, and Itb is the percentage inhibition of EPSC produced by baclofen and the Ca\(^{2+}\) channel blocker together.

The peak amplitude, charge transfer, and 10–90% rise time for EPSCs were averaged across neurons (five current traces were averaged for each neuron). EPSC amplitudes were measured as the difference between the peak EPSC current and the baseline current before the stimulus artifact. Data for each neuron represent an average of EPSC amplitudes from five sweeps over 1 min. Between neurons, these EPSC amplitudes were normalized and presented as the means ± SE. Charge transfer represented the area enclosed by the EPSC and was analyzed during a 0.15-s period. To compare experimental protocols, amplitude, charge transfer, and rise time of EPSC were expressed as a percentage of control. Igor Pro (Version 5.0, Wave Metrics, Lake Oswego, OR) was used for curve fitting and data analysis. ANOVA, two-tail paired t-test, and unpaired t-test were performed using StatView 5.0.1 (SAS Institute, Cary, NC) or Excel 11.1.1 (Microsoft, Redmond, WA). A confidence level of 95% was used to determine statistical significance.

**RESULTS**

**GABA\(_B\) activation inhibits evoked EPSCs**

Joint application of CNQX (10 \(\mu\)M) and APV (50 \(\mu\)M) with picrotoxin (50 \(\mu\)M) was used to confirm that the currents activated in SCN neurons by stimulation of the optic chiasm were EPSCs mediated by glutamate release. CNQX (10 \(\mu\)M) and APV (50 \(\mu\)M) applied together significantly decreased the EPSC amplitude to 11.0 ± 1.9 pA or 7.3 ± 5.5% of control (control amplitude 156 ± 16.8 pA, \(n = 5\), \(P < 0.0001\); Fig. 2). This reduces the EPSC amplitude and confirms that stimulation of the optic chiasm evokes EPSCs mediated by glutamate-receptor activation.

Baclofen in the presence of picrotoxin (50 \(\mu\)M) reversibly reduced the amplitude of EPSCs evoked by low-frequency (0.08-Hz) optic chiasm stimulation (Fig. 3, A and B). The magnitude of the EPSC amplitude suppression was determined during the third through sixth minutes of baclofen application.

![FIG. 2. Inhibition of evoked excitatory postsynaptic currents (EPSCs) by \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and \(N\)-methyl-D-aspartate (NMDA) receptor antagonists. CNQX (10 \(\mu\)M) and \((-\))-2-amino-5-phosphonovaleric acid (2-APV, 50 \(\mu\)M) were applied together in the presence of picrotoxin (50 \(\mu\)M). Each point represents the mean amplitude of 5 evoked EPSCs. Inset: examples of individual EPSCs.](http://jn.physiology.org/doi/10.1152/jn.02324.2006)
Baclofen inhibited evoked EPSCs during both the subjective day and the subjective night in a concentration-dependent manner (0.03–30 μM, Fig. 3C). During the subjective day, baclofen (30 μM) produced an 83.3 ± 3.4% inhibition (n = 4), which was not different from the inhibition observed during the subjective night (82.0 ± 3.1%, n = 6, P = 0.80). The recordings were performed during the light phase from Zeitgeber Time (ZT) 4 to ZT11 (n = 42 neurons) and during the dark phase from ZT12 to ZT21 (n = 75 neurons), respectively. The IC₅₀ for baclofen was estimated to be 0.71 ± 0.12 μM during the subjective day and was not different from the IC₅₀ of 0.87 ± 0.20 μM (P = 0.52) estimated during the subjective night. There was also no significant difference between the concentration–response curves determined during either the subjective day or night (F-test, P = 0.80). These data indicate that GABA₉ receptors located on RHT terminals in the SCN inhibited glutamate release in a concentration-dependent manner and there was no diurnal variation in the presynaptic effect of GABA₉ receptors. The next studies were designed to determine whether GABA₉ receptors regulate the function of Ca²⁺ or K⁺ channels in RHT terminals to modulate transmitter release.

Dependency of RHT synaptic transmission on extracellular Ca²⁺

Because the relationship between the presynaptic Ca²⁺ concentration and glutamate release is unknown for RHT terminals, we examined the effects of changing the extracellular Ca²⁺ concentration and glutamate release is unknown for RHT terminals. Decreasing the [Ca²⁺]ₑ from 2.4 to 1.2 mM significantly reduced the EPSC amplitude to 52.9 ± 3.8% of control (98.1 ± 5.0 pA, P < 0.0001, n = 8), whereas a reduction to 0.6 mM further significantly reduced the EPSC amplitude to 20.6 ± 3.5% of control (P < 0.0001, n = 7, Fig. 4A). The EPSC amplitude increased as [Ca²⁺]ₑ was raised from 0.6 to 2.4 mM, saturated when the extracellular [Ca²⁺]ₑ reached 2.4 mM, and remained stable through 3.5 mM [Ca²⁺]ₑ (Fig. 4B).

Optical measurements of Ca²⁺ in RHT terminals

GABA₉-receptor activation could inhibit EPSCs by reducing the action potential–evoked increase in the presynaptic terminal Ca²⁺ concentration required to trigger neurotransmitter release. Because of the nonlinearity of the relationship between Ca²⁺ entry and transmitter release a relatively small decrease in the evoked presynaptic Ca²⁺ transient might significantly reduce glutamate release. To test this hypothesis we measured the changes in RHT terminal Ca²⁺ probe fluorescence after optic chiasm stimulation. We first confirmed that the optical measurements were from RHT terminal Ca²⁺ and not from postsynaptic SCN neurons. Stimulation of the optic nerve produced a rapid increase in Ca²⁺ probe fluorescence (transients) that was fully blocked by TTX (750 nM). Simultaneous application of CNQX (10 μM), APV (50 μM), and picrotoxin (50 μM) did not change the RHT Ca²⁺ transients, demonstrating that activation of postsynaptic AMPA, NMDA, or GABA₆ receptors did not confound the RHT-presynaptic Ca²⁺ signal (Gompf et al. 2005). Visual inspection of RHT terminals demonstrated probe fluorescence in terminal processes, but not in neuronal cell bodies (Gompf et al. 2005). These data strongly support the conclusion that the Ca²⁺ signal measured was presynaptic. Because presynaptic Ca²⁺ transients might reach the micromolar range, it is possible that high-affinity Ca²⁺ probes such as Fluo-4 or Fura-Red may become saturated. However, presynaptic Ca²⁺ transients could be enhanced by 4-AP (1 mM) or by stimulation of the optic chiasm with pulses trains (Gompf et al. 2005). Therefore changes in Ca²⁺ probe fluorescence after a single stimulating pulse were within the linear range for the Ca²⁺ concentration response of the probe.
Relationship between presynaptic Ca\(^{2+}\) entry and neurotransmitter release

To examine the relationship between the evoked EPSC amplitude and evoked presynaptic Ca\(^{2+}\) transients, the extracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(e\)) was varied from 0.6 to 3.5 mM while electrically stimulating the optic chiasm. Concentration–response curves for the [Ca\(^{2+}\)]\(e\) and either EPSC amplitude or Ca\(^{2+}\) transients demonstrated a saturating nonlinear relationship, with near saturation at about 2.4 mM [Ca\(^{2+}\)]\(e\) (Fig. 4B). At 0.6 mM [Ca\(^{2+}\)]\(e\), presynaptic Ca\(^{2+}\) transient amplitudes in RHT terminals were reduced nearly 45% compared with a roughly 80% reduction of the EPSC amplitude (Fig. 4C). This relationship implies that small changes in the amplitude of RHT terminal Ca\(^{2+}\) transients equate to a much larger reduction in the EPSC amplitude.

Effect of baclofen on electrically induced RHT presynaptic Ca\(^{2+}\) transients

Recordings of evoked Ca\(^{2+}\) transients and resting Ca\(^{2+}\) concentration were made using Fluo-4 AM; loaded RHT terminals with either photometry or Ca\(^{2+}\) imaging techniques (six and eight experiments, respectively) showed similar results (Fig. 4D). Experiments were performed in the presence of CNQX, APV, and picrotoxin. Baclofen (30 μM) significantly attenuated (87.9 ± 1.3% of control, \(P < 0.0001\)) evoked Ca\(^{2+}\) transients and reduced the baseline Ca\(^{2+}\) probe fluorescence 1.38 ± 0.25% (\(P < 0.01\), \(n = 14\)). Because of the need for fast recordings, we used a single-wavelength Ca\(^{2+}\) probe in the linear range to calculate ΔF/Fo [i.e., (peak – baseline)/baseline]. This assumes that the baseline Ca\(^{2+}\) concentration does not change with drug exposure. However, we have observed that baclofen lowers resting RHT terminal Ca\(^{2+}\) (Fig. 4E), and thus the evoked Ca\(^{2+}\) transient could underestimate the ability of baclofen to lower presynaptic Ca\(^{2+}\). Because measured Ca\(^{2+}\) transients reflect only activated terminals, whereas resting Ca\(^{2+}\) reflects all the terminals, we were unable to combine the baclofen-induced reduction of resting Ca\(^{2+}\) with the reduction in the Ca\(^{2+}\) transient. Nonetheless, the reductions observed in EPSCs (roughly 80%) versus presynaptic calcium transients (nearly 12%) during baclofen application are consistent with the nonlinear relationship between Ca\(^{2+}\) entry into presynaptic terminals and neurotransmitter release (Fig. 4C).

Dependency of baclofen effect on extracellular Ca\(^{2+}\)

Reducing [Ca\(^{2+}\)]\(e\) to 0.6 mM decreased the EPSC amplitude during baclofen (10 μM) application to 2.1 ± 0.2% of control,
a value that was significantly lower ($P < 0.0001$) than that when baclofen was applied at 2.4 mM [Ca$^{2+}]_e$ (26.3 $\pm$ 3.0% reduction). Decreasing [Ca$^{2+}]_e$ to 1.2 mM during baclofen application reduced the evoked EPSC amplitude to 5.0 $\pm$ 1.1% ($P < 0.0001$) of control. The magnitude of baclofen’s inhibition was stable between 2.4 and 3.5 mM [Ca$^{2+}]_e$, which produced maximal EPSCs and Ca$^{2+}$ transients (Fig. 4B). These data indicate a strong correlation between the magnitude of baclofen’s inhibition, Ca$^{2+}$ transient amplitude, EPSC amplitude, and [Ca$^{2+}]_e$.

Role of presynaptic voltage-dependent Ca$^{2+}$ channels in glutamate release from RHT terminals

Selective Ca$^{2+}$ channel blockers were used to identify the voltage-dependent Ca$^{2+}$ channels that regulate neurotransmitter release from RHT terminals. $\omega$-Conotoxin GVIA (1 $\mu$M) significantly reduced the evoked EPSC amplitude (66.1 $\pm$ 3.3%), consistent with an important role for N-type Ca$^{2+}$ channels in mediating glutamate release from RHT terminals ($P < 0.0001$, $n = 40$; Fig. 5, A and B). Application of $\omega$-agatoxin TK (500 nM) produced a 36.1 $\pm$ 2.5% reduction ($P < 0.0005$, $n = 49$; Fig. 5, C and D), whereas a lower $\omega$-agatoxin concentration (100 nM) produced a nonsignificant 20.3 $\pm$ 4.8% reduction ($P = 0.5$, $n = 4$). SNX-482 (150 nM) induced a significant 17.5 $\pm$ 3.0% reduction of the EPSC amplitude ($P < 0.03$, $n = 15$; Fig. 5, E and F), and nimodipine (10 $\mu$M) a nonsignificant reduction of 5.6 $\pm$ 2.7% ($P = 0.53$, $n = 5$; Fig. 5, G and H). These data show that R-type but not L-type Ca$^{2+}$ channels participate in glutamate release from RHT terminals. Mibefradil (20 $\mu$M) produced a significant 31.5 $\pm$ 3.1% reduction of the EPSC amplitude ($P < 0.0002$, $n = 5$; Fig. 5, I and J), indicating that T-type Ca$^{2+}$ channels contribute to the release of transmitter from RHT terminals.

The lack of a complete inhibition of the EPSCs by a single Ca$^{2+}$ channel blocker is consistent with a model in which multiple Ca$^{2+}$ channel types contribute to glutamate release from RHT terminals (Mintz et al. 1995; Regehr and Mintz 1994). To test this possibility selective blockers were applied in combination (Fig. 6). The order of $\omega$-conotoxin and $\omega$-agatoxin application was varied so that in half of the 14 experiments $\omega$-agatoxin was applied first (Fig. 6, B and C). Joint application of $\omega$-conotoxin, $\omega$-agatoxin, SNX-482, nimodipine, and mibefradil produced the largest reduction (87.1%) in the mean EPSC amplitude and the residual of 13% was completely blocked by Cd$^{2+}$ (100 $\mu$M) (Fig. 8B). In each case the observed inhibition produced by application of toxins in different combinations was less than that predicted by summing their individual inhibitory effects (Fig. 6A). These data indicate that activation of high-voltage–activated N-, P/Q-, R-type and low-voltage–activated T-type Ca$^{2+}$ channels demonstrate cooperatively in regulation of glutamate release from RHT terminals. The small difference between the observed and predicted inhibition produced by SNX-482 with either $\omega$-conotoxin or $\omega$-agatoxin suggests a minimal overlap in effect of these blockers on glutamate release. In contrast, overlap of $\omega$-conotoxin and $\omega$-agatoxin actions was greater ($\approx 33\%$).

![Graph](http://jn.physiology.org/)

**FIG. 5.** Inhibition of evoked EPSCs by voltage-dependent Ca$^{2+}$ channel blockers. A and B: $\omega$-conotoxin GVIA (1 $\mu$M), C and D: $\omega$-agatoxin TK (500 nM). E and F: SNX-482 (150 nM). G and H: nimodipine (10 $\mu$M). I and J: mibebradil (20 $\mu$M). A, C, E, G, and I: examples of EPSCs recorded before, during, and after blocker application. B, D, F, H, and J: control EPSC amplitude plotted vs. the EPSC amplitude (test EPSC) in the presence of Ca$^{2+}$ channel blocker. Each point represents an individual experiment. Dashed line, 0% inhibition; solid line, mean % inhibition of EPSC amplitude by the Ca$^{2+}$ channel blocker.

Identification of the presynaptic Ca$^{2+}$ channels inhibited by GABA$_B$-receptor activation

Experiments were performed to determine whether GABA$_B$-receptor activation decreased RHT transmission by inhibiting voltage-dependent Ca$^{2+}$ channels. The EPSC amplitudes recorded when baclofen was applied on a background of prior
toxin exposure were compared with the EPSC amplitudes in the presence of only baclofen. Occlusion of the baclofen effect by one of the toxins would suggest that baclofen is acting by inhibiting the activity of that channel.

Baclofen (0.3 and 10 µM) evoked a significant reduction of the EPSC amplitude (26.6 or 73.7% respectively; Fig. 7, A and B). The maximal inhibitory effect occurred 3.3 ± 0.3 min (n = 13) after beginning the baclofen application. After washout, the EPSC amplitude recovered to 70–100% of control for 25–40 min. To determine whether there was an interaction between GABA_A receptors and N-, P/Q-, T-, R-, or L-type Ca^2+ channels, various Ca^2+ channel blockers were applied alone or together with baclofen (0.3 or 10 µM) (Fig. 7). The ω-conotoxin (1 µM) plus baclofen (0.3 or 10 µM) effect (73.7 ± 6.0%, n = 12 or 92.1 ± 1.7%, n = 7 reduction, respectively) was significantly greater than the effect of ω-conotoxin alone (Fig. 7, C and D). From experiments combining ω-conotoxin with baclofen at low concentration (0.3 µM) and a nearly maximal concentration (10 µM), we calculate 72.2 and 48.6% (respectively) of baclofen’s effect was mediated by inhibition of N-type Ca^2+ channels (Fig. 8A) (see Math and statistical analysis). ω-Agatoxin (500 nM) and baclofen (0.3 or 10 µM) were applied together to study a possible interaction between GABA_A receptors and P/Q-type channels (Fig. 7, E and F), and reduced the EPSC amplitude 62.2 ± 5.5% (n = 12) or 96.1 ± 0.5% (n = 4), respectively. These data indicate that P/Q-type Ca^2+ channels contribute 15.4% at low and 6.8% at high baclofen concentrations to baclofen’s inhibition of evoked EPSCs (Fig. 8A).

GABA_A-receptor inhibition of R-type Ca^2+ channels was studied by applying SNX-482 (150 nM) together with baclofen (0.3 or 10 µM). This resulted in a 45.6 ± 6.1% (n = 6) or 92.9 ± 1.7% (n = 5) reduction, respectively (Fig. 7, G and H). Because SNX-482 did not significantly reduce baclofen’s inhibition from baclofen alone, we conclude that baclofen does not modulate R-type Ca^2+ channels at either low or high concentrations (1.1 and 1.0%, respectively; Fig. 8A).

GABA_A-receptor–mediated inhibition of T-type Ca^2+ channels was studied by applying mibefradil (20 µM) together with baclofen (0.3 or 10 µM). Mibefradil plus baclofen produced a 50.8 ± 5.3% (n = 8) or 82.4 ± 6.2% (n = 6) reduction, respectively, for 0.3 and 10 µM baclofen (Fig. 7, I and J). The component of baclofen’s effect occluded by mibefradil was estimated to be 51.9 and 23.9% in low and high concentrations of baclofen, respectively. Thus mibefradil has a greater effect at lower than higher baclofen concentrations, and indicates a strong effect of baclofen on T-type Ca^2+ channels. In contrast, evoked EPSCs inhibited by application of baclofen (10 µM) together with nimodipine (10 µM) were not significantly different from baclofen alone (Fig. 7K).

Joint application of ω-agatoxin, ω-conotoxin, SNX-482, nimodipine, mibefradil, and baclofen (10 µM) inhibited EPSCs 97.5 ± 1.9% compared with 81.8 ± 1.1% inhibition evoked by these toxins alone (n = 3). Thus the blocker cocktail does not completely inhibit the evoked EPSCs. Because baclofen (10 µM) applied with this cocktail almost completely blocked the remaining EPSCs, we calculated that the residual inhibitory baclofen effect was 15.7% (Fig. 8B).

Finally, because the application of Ca^2+ channel blockers alone or jointly cannot completely block glutamate release, it suggests the existence of Ca^2+ currents resistant to the applied blockers. These remaining Ca^2+ currents were almost completely inhibited by the addition of baclofen (10 µM), Ca^2+ (100 µM) in the same experiments also completely blocked the EPSC (Fig. 8B).

**Baclofen does not activate presynaptic K^+ channels**

GABA_A receptors couple to Gi/0-type G-proteins and activate G-protein–activated inward rectifier K^+ channels
We designed experiments to determine whether the GABAB-receptor–mediated inhibition of glutamate release at RHT terminals could be attributable, in part, to an activation of presynaptic Kir3 channels. To block Kir3 channels we used a peptide toxin, tertiapin, a potent blocker of Kir3.1, Kir3.4, Kir1.1, and K Ach inwardly rectifying K+ channels and Ba2+ (BaCl2).

Tertiapin (10 nM) application produced a small nonsignificant decrease in the EPSC amplitude to 95.9 ± 7.9% of control (control 70.4 ± 10.0 pA) and did not change the charge.

**FIG. 7.** Occlusion of baclofen effects on EPSC amplitude by Ca2+ channel blockers. A–K: histograms indicate the inhibition produced by baclofen alone (0.3 or 10 μM) and by application of Ca2+ channel blocker alone and together with baclofen. Left column: baclofen 0.3 μM. Right column: baclofen 10 μM. A and B: baclofen alone. C and D: baclofen and conotoxin (1 μM) (C, n = 12; D, n = 7). E and F: baclofen and agatoxin (500 nM) (E, n = 12; F, n = 4). G and H: baclofen and SNX-482 (150 nM) (G, n = 5; H, n = 5). I and J: baclofen and mibefradil (20 μM) (I, n = 8; J, n = 6). K: baclofen and nimodipine (10 μM) (n = 5). Current traces are individual EPSCs recorded in control, channel blocker, and channel blocker plus baclofen conditions. NS, nonsignificant; *P < 0.05, **P < 0.01, ***P < 0.001.
The effect of baclofen alone (Fig. 9, and the charge transfer (tertiapin (10 nM) reduced the EPSC amplitude to 23.7% on evoked EPSC in the same neuron. EPSC recording: control, cocktail, baclofen, and cocktail; effect of Cd2+

Inset: EPSC recording: control, cocktail, baclofen, and cocktail; effect of Cd2+

Application of 4-AP (5 mM) significantly increased the EPSC amplitude to 153 ± 14.6% of control (control 109.0 ± 9.4 pA), the charge transfer (n = 9; Fig. 9, C–F), and the amplitude of the evoked Ca2+ transients (Gompf et al. 2005). Baclofen added together with 4-AP reduced the EPSC amplitude to 75.8 ± 9.0%, a value that was significantly different from the effect of baclofen alone (n = 9; Fig. 9, C–E). After baclofen application the charge transfer remained 31% larger than in control (131.2 ± 24.2% of control) and more than fourfold larger than baclofen alone (30.0 ± 3.3% of control; Fig. 9F). These data show that application of 4-AP significantly decreases the magnitude of the baclofen-induced inhibition. After joint application of 4-AP and baclofen (10 μM), the EPSC amplitude remained larger than the control EPSC amplitude in two neurons (Fig. 9D).

4-AP and Ba2+ increased the charge transfer (225.0 ± 24.1 and 144.1 ± 18.0% of control, respectively). Charge transfer (131.2 ± 24.2% of control, P < 0.006) remained significantly higher than in control when baclofen was applied together with 4-AP. Ba2+ or tertiapin together with baclofen induced a charge transfer (29.9 ± 9.1 and 32.5 ± 6.1% of control, respectively) that was not significantly different (P = 0.99 and P = 0.72) from baclofen alone (30.0 ± 3.2%). Unlike Ba2+, 4-AP increased the 10–90% rise time of the EPSC [108.8 ± 13.3% (P = 0.96, n = 5) and 174.4 ± 26.3% of control, respectively]. Application of baclofen (10 μM) with BaCl2 or with 4-AP increased the EPSC rise time in both cases (129.6 ± 35.8 and 252.3 ± 34.8%, respectively). These data demonstrate that the baclofen effect on the rise time in the presence of Ba2+ was not significantly different from that of baclofen alone (135.9 ± 20.7%, P = 0.12). Thus Ba2+, unlike 4-AP, does not alter the EPSC rise time when applied alone or together with baclofen. Thus 4-AP and Ba2+ significantly increased the EPSC amplitude and charge transfer. Ba2+, unlike 4-AP, did not change the EPSC rise time in either the presence or the absence of baclofen. The charge transfer remained significantly larger than control when baclofen was applied together with 4-AP, in contrast to Ba2+ or tertiapin, which did not alter baclofen’s effect.

\[ \text{Effect of increasing the extracellular K}^+ \text{ on EPSC inhibition evoked by baclofen} \]

To evaluate whether changes in the presynaptic membrane potential could alter the effect of baclofen on glutamate release, we varied the extracellular KCl concentration from 2.5 to 25 mM, and correspondingly decreased the NaCl concentration, to balance osmolality. Increasing the extracellular K+ ([K+]o) depolarizes presynaptic terminals (Hoss and Labkovsky 1986). As expected, raising the [K+]o resulted in an exponential increase in resting presynaptic Ca2+ concentration, and a biphasic concentration–response relationship for both evoked EPSCs and presynaptic Ca2+ transient amplitudes (Fig. 10D). The EPSC was completely blocked by [K+]o > 20 mM (Fig. 10A). These data suggest that strong depolarization of optic nerve terminals can completely block EPSCs by blocking action potential propagation. In contrast, raising [K+]o to 7–12 mM increased the EPSC amplitude (Fig. 10, B, C, and D) to

\[ \text{FIG. 8. Occlusion of baclofen’s inhibition of EPSCs by Ca}^2+ \text{ channel blockers. A: occlusion baclofen’s effect by } \omega \text{-conotoxin GVIA (1 μM), mibebradil (20 μM), } \omega \text{-agatoxin TK (500 nM), and SNX-482 (150 nM). Data in histogram: normalized % of full effect of baclofen (0.3 and 10 μM, respectively). White squares: quantity of neurons. B: occlusion of baclofen’s effect by a cocktail of Ca}^2+ \text{ channel blockers: } \omega \text{-conotoxin GVIA, } \omega \text{-agatoxin TK, SNX-482, mibebradil, and effect of Cd}^2+. \text{ Baclofen 10 μM. Inset: EPSC recording: control, cocktail, baclofen, and cocktail; effect of Cd}^2+ \text{ on evoked EPSC in the same neuron.} \]
139.5 ± 9.7% of control (control 78.6 ± 10.9 pA, n = 16). The EPSC amplitude inhibition induced by baclofen (10 μM) did not change significantly after increasing of [K+]e (Fig. 10D). Thus the depolarization of RHT terminals evoked by increasing [K+]e does not significantly alter baclofen’s inhibitory effect on EPSCs.

**DISCUSSION**

Environmental light information is conveyed to the SCN from RGCs that project glutamatergic fibers by the RHT terminating on SCN neurons (Berson et al. 2002; Card and Moore 1991; Castel et al. 1993; Liou et al. 1986; Moore et al. 1995). The majority of RGCs that project to the SCN express the photopigment melanopsin (Gooley et al. 2003; Morin et al. 2003; Sollars et al. 2003). Synaptic transmission at RHT–SCN neuron synapses and light’s effect on circadian clock timing can be modulated by activation of presynaptic GABA_B receptors reducing glutamate release from RHT terminals (Colwell et al. 1993; Gannon et al. 1995; Gillespie et al. 1997; Jiang et al. 1995; Ralph and Menaker 1989). Disruption of presynaptic
modulation of RHT transmission has significant effects on light entrainment of the circadian clock. Activation of either GABAB or 5-HT1B receptors reduces the magnitude of light-induced phase shifts (Gillespie et al. 1997, 1999; Pickard and Rea 1997; Pickard et al. 1999). The GABAB and 5-HT systems may overlap in their regulation of photic input to the circadian system. Presynaptic 5-HT1B receptors inhibit GABA release in the SCN that may contribute to the tonic GABAB-receptor–mediated inhibition (Bramley et al. 2005; Gannon et al. 1995; Sollars et al. 2006). 5-HT1B-knockout mice have smaller light-induced phase shifts than would be predicted, possibly arising from disinhibition of GABAB signaling by removal of 5-HT inhibition of GABA release (Sollars et al. 2006). Mice with the 5-HT1B receptors knocked out have a delayed phase angle of entrainment (Sollars et al. 2006). Interestingly a similar delayed phase angle of entrainment is present in humans with seasonal affective mood disorders (Lewy et al. 1987).

We have shown that GABA<sub>B</sub> receptor activation reduces transmission at RHT synapses by inhibiting primarily N-, T-, and P/Q-type voltage-gated Ca<sup>2+</sup> channels that are required for glutamate release. R-type voltage-gated Ca<sup>2+</sup> channels also contribute to glutamate release but are not affected by activation of presynaptic GABA<sub>B</sub> receptors. L-type Ca<sup>2+</sup> channels and K<sub>i</sub>3 channels contribute neither to glutamate release from RHT terminals nor to GABA<sub>B</sub> presynaptic inhibition. The magnitude of the effect on RHT synaptic transmission was dependent on the GABA<sub>B</sub> agonist concentration but was not dependent on the circadian time.

Role of voltage-dependent Ca<sup>2+</sup> channels in glutamate release from RHT terminals

Selective Ca<sup>2+</sup> channel blockers were used to determine the contribution of specific types of Ca<sup>2+</sup> channels to RHT transmission. These toxins do not have postsynaptic effects on glutamatergic responses nor do they alter the number of stimulated afferents (Luebke et al. 1993; Mintz et al. 1995). We have shown that N-, P/Q-, T-, and R-type, but not L-type, Ca<sup>2+</sup> channels contribute the Ca<sup>2+</sup> required for glutamate release from RHT terminals. During low-frequency stimulation (0.08 Hz) the EPSC amplitudes were reduced nearly 70% by N-type channel inhibition, nearly 30% by either P/Q-type or T-type channel inhibition, and nearly 20% by R-type Ca<sup>2+</sup> channel inhibition. Blocking L-type channels with nimodipine had no significant effects on RHT synaptic transmission. ω-Agatoxin produced almost twice the reduction of the evoked EPSC at 500 than at 100 nM, suggesting that Q-type (IC<sub>50</sub> = 90 nM) channels have the same importance in regulating release as P-type channels (IC<sub>50</sub> = 1–3 nM).

To study which types of Ca<sup>2+</sup> channels are involved we used specific blockers (see above). However, mibebradil is not completely selective for T-type Ca<sup>2+</sup> channels. Our observations on mibebradil effects on EPSCs can be explained in two different ways. First, mibebradil specifically blocks low-voltage–activated T-type Ca<sup>2+</sup> channels (Lacinova 2004). In this case, we would conclude T-type Ca<sup>2+</sup> channels contribute to mediating transmitter release from RHT terminals. Alternatively,
mitubradil blocks several Ca\(^{2+}\) channel types to reduce the evoked EPSC amplitude (Bezprozvanny and Tsien 1995; Liu et al. 1999; Viana et al. 1997). Our data were consistent with the first possibility because in the presence of N-, P/Q-, R-, and L-type Ca\(^{2+}\) channel blockers mibefradil blocked an additional roughly 10% of the EPSC amplitude. This interpretation must be made with caution because we cannot exclude the blocking effect of mibefradil on Na\(^{+}\) and K\(^{+}\) channels (Liu et al. 1999).

Multiple types of Ca\(^{2+}\) channels with overlapping functions mediate glutamate release at RHT terminals. Application of any two toxins reduced the EPSC amplitude less than would be expected from the sum of their individual ability to reduce EPSC amplitude (Fig. 6). The sum of the inhibition produced individually by N-, P/Q-, and T-type Ca\(^{2+}\) blockers was greater than inhibition produced by their joint application. For example, the overlap between the effect of N- and P/Q-type Ca\(^{2+}\) channels was estimated to be 33%, but there was no overlap with R-type Ca\(^{2+}\) channels. These data are consistent with the activity of multiple Ca\(^{2+}\) channels overlapping to initiate transmitter release. Previous studies have similarly demonstrated that blocking synaptic currents by selective antagonists of N- and P/Q currents sum to >100% (Mintz et al. 1995; Reid et al. 1998; Wheeler et al. 1996). A model was proposed in which “multiple types of Ca\(^{2+}\) channels exert synergistic control over individual release sites, and that the domains of several Ca\(^{2+}\) channels must overlap at each release site” (Mintz et al. 1995). The regulation of glutamate release from RHT terminals by multiple types of voltage-dependent Ca\(^{2+}\) channels is consistent with such a model.

Joint application of \(\omega\)-conotoxin GVIA, \(\omega\)-agatoxin TK, mibefradil, SNX-482, and nimodipine in saturating concentrations evoked an 87% decrease of the control EPSC amplitude and should completely block N-, P/Q-, T-, R-, and L-type Ca\(^{2+}\) channels. The component of the EPSC (13%) that was not inhibited by these blockers was inhibited by Cd\(^{2+}\) and might reflect the contribution of SNX-482–resistant R-type Ca\(^{2+}\) channels (Tottene et al. 2000). In axons of the rat optic nerve and RGCs nearly 40–60% of the evoked Ca\(^{2+}\) influx accounted for N-type Ca\(^{2+}\) channels, whereas P/Q-type Ca\(^{2+}\) channels make little, if any, contribution (Guenther et al. 1994; Sun and Chiu 1999). In RGCs, L-type Ca\(^{2+}\) channels mediate 25% of the Ca\(^{2+}\) current (Guenther et al. 1994). Immunocytochemical studies confirmed the presence of L-type Ca\(^{2+}\) channels in axons of adult rat optic nerve, although they contribute little to evoked Ca\(^{2+}\) influx (Brown et al. 2001; Sun and Chiu 1999). The insignificant contribution of L-type channels in transmitter release can be explained by localization of L-type channels far from release sites at these axonal terminals.

During electrophysiological recordings, we noted that there was a large variation of the magnitude of toxin effects (Fig. 5). For example, \(\omega\)-conotoxin produced as much as 100% inhibition and as little as 20% inhibition. One explanation is that delivery of the toxin varied across the experiments but this is unlikely because baclofen application from the same micropipette showed a consistent effect. An alternate explanation is that different RHT fibers have different complements of Ca\(^{2+}\) channels (Reid et al. 2003). Those that rely on N-type channels for glutamate release would be more sensitive to block by \(\omega\)-conotoxin than those that rely more on P/Q- or R-type channels. Our data suggest that the different types of Ca\(^{2+}\) channels can vary considerably in different RHT terminals (Fig. 5).

\textbf{Presynaptic effect of baclofen on voltage-dependent Ca\(^{2+}\) channels}

GABA\(_{B}\)-receptor activation reduces the release of glutamate from axon terminals of the RHT (Jiang et al. 1995). The magnitude of the effect is dependent on the GABA\(_{A}\)-agonist concentration but is not dependent on the circadian time (Fig. 3). The IC\(_{50}\) for baclofen-evoked inhibition during the subjective day was not significantly different from that during the subjective night (IC\(_{50}\) \(\approx 1\) \(\mu\)M) and was similar to those measured in the rat optic nerve and in the calyx of Held (Sun and Chiu 1999; Takahashi et al. 1998). Further the concentration–response curves recorded during the day or night were not significantly different (Fig. 3).

Activation of GABA\(_{B}\)-receptor inhibitors reduced the release of glutamate from RHT terminals as measured by a reduction in the EPSC amplitude. Blocking specific types of voltage-dependent Ca\(^{2+}\) channels attenuated the magnitude of the GABA\(_{B}\)-agonist–induced reduction. The GABA\(_{B}\)-receptor regulation is not uniform across all types of Ca\(^{2+}\) channels. We estimate that depending on the baclofen concentration (0.3–10 \(\mu\)M) that was applied, nearly 49–72% of the presynaptic inhibition produced by GABA\(_{B}\)-receptor activation was mediated by block of N-type Ca\(^{2+}\) channels, nearly 24–52% mediated by block T-type Ca\(^{2+}\) channels, and nearly 7–15% mediated by block of P/Q-type Ca\(^{2+}\) channels. The effect of GABA\(_{B}\)-receptor activation was not mediated by R-type (nearly 1% inhibition) or L-type Ca\(^{2+}\) channels. Similarly, baclofen-induced inhibition was occluded by block of N-type Ca\(^{2+}\) channels in axons of neonatal rat optic nerve (Sun and Chiu 1999). Recently G-proteins were shown to bind more strongly to Cav2.2 (\(\alpha\) 1B, N-type) than Cav2.1 (\(\alpha\) 1A, P/Q-type) Ca\(^{2+}\) channels (Agler et al. 2003). This may explain why baclofen inhibits N-type more than P/Q-type Ca\(^{2+}\) channels. These effects of baclofen are mediated by G\(\beta\)\(\gamma\) that directly inhibits Ca\(^{2+}\) channels, putting them into a reluctant state (Kajikawa et al. 2001; Zamponi and Snutch 1998).

\textbf{Effect of baclofen on resting and evoked presynaptic Ca\(^{2+}\) transients}

The relationship between evoked EPSC amplitude and [Ca\(^{2+}\)] in, was sigmoidal, whereas the relationship between presynaptic Ca\(^{2+}\) transients and [Ca\(^{2+}\)] in, approximated a hyperbolic response (Mintz et al. 1995). Reduction of Ca\(^{2+}\) influx in presynaptic terminals by baclofen has previously been reported in different brain structures (Barnes-Davies and Forsythe 1995; Dittman and Regehr 1996; Isaacson 1998; Wu and Saggau 1997). A similar effect of baclofen on presynaptic Ca\(^{2+}\) influx and EPSC amplitude (correspondingly about 50 and 90% reduction of initial value) was shown in presynaptic terminals of the calyx of Held (Sakaba and Neher 2003). The Ca\(^{2+}\) concentration in RHT terminals after optic chiasm stimulation was reduced by GABA\(_{B}\)-receptor activation, and glutamate release from RHT terminals was nonlinearly dependent on changes of the Ca\(^{2+}\) concentration in those terminals. We observed baclofen (30 \(\mu\)M) inducing roughly 12% reduction in presynaptic Ca\(^{2+}\) transients compared with nearly 80% reduc-
tion in EPSC amplitudes. Although this is consistent with the nonlinear relationship discussed above, the reduction of Ca\(^{2+}\) transients by baclofen was less than expected.

There are several possible reasons for this difference. First, the Ca\(^{2+}\) channels, which mediate transmitter release, are believed to be located near the vesicles to be released. However, we are recording the bulk Ca\(^{2+}\) in the terminals, which may underestimate the change in Ca\(^{2+}\) required for release. Second, nonlinearity of a single-wavelength fluorescent Ca\(^{2+}\) probe may underestimate Ca\(^{2+}\) responses. Directly quantifying the magnitude of the Ca\(^{2+}\) level with a dual excitation probe conceivably would have eliminated this problem. However, single-wavelength excitation recordings provide the fast data acquisition needed for measurement of Ca\(^{2+}\) transients in presynaptic terminals. Finally, it is important to note that in our studies baclofen reduced resting Ca\(^{2+}\). This reduction is not included in determining the roughly 12% reduction of Ca\(^{2+}\) transients. Therefore this may result in an underestimation of the inhibition by baclofen. Previous studies examining the effect of baclofen on presynaptic Ca\(^{2+}\) influx in the hippocampus and cerebellum have not shown this reduction in resting Ca\(^{2+}\) (Dittman and Regehr 1996; Wu and Saggu 1997). These studies differed by examining different regions of the brain and by using different Ca\(^{2+}\) indicators. Overall, our data are consistent with a nonlinear relationship between presynaptic RHT Ca\(^{2+}\) concentration and glutamate release, such that GABA\(B\) receptor activation producing a small reduction in the evoked presynaptic Ca\(^{2+}\) transient can have a large effect on inhibiting glutamate release.

Role of K\(^{+}\) channels in RHT transmission and in modulation of baclofen effect

Tertiapin and BaCl\(_2\) were used to study whether the GABA\(B\) agonist baclofen activates Kir3 channels on RHT presynaptic terminals. Tertiapin did not change the EPSC amplitude or charge transfer. Neither tertiapin nor Ba\(^{2+}\)-blocked baclofen-induced presynaptic inhibition in RHT synapses. These data are in agreement with studies showing that Ba\(^{2+}\) does not block presynaptic inhibition induced by baclofen in excitatory (glutamatergic) synapses (Cui et al. 2000; Takahashi et al. 1998; Thompson and Gahwiler 1992). Similarly, baclofen does not directly increase a K\(^{+}\) conductance in the optic nerve (Sun and Chiu 1999). Moreover, G-protein–activated Kir current can be detected in the presynaptic terminal but it cannot be activated by baclofen (Takahashi et al. 1998). Baclofen-induced inhibition does not require activation of Kir3 channels on excitatory terminals because the presynaptic effect of GABA\(B\) receptors was unaltered in mutant mice lacking the GIRQ2 gene (Lüscher et al. 1997). Thus our data show that baclofen does not activate Kir3 channels in RHT axon terminals, unlike its effect on RGCs (Chen et al. 2004).

More than 60% of outward K\(^{+}\) currents in presynaptic terminals are sensitive to 4-AP and are very important for regulation of synaptic transmission (Forsythe 1994; Reiff and Guenther 1999). 4-AP potentiates EPSCs by broadening presynaptic action potentials and slowing action potential repolarization (Ishikawa et al. 2003; Sun and Chiu 1999). 4-AP application onto the optic nerve induced a delayed inward Ca\(^{2+}\) current that forms a second “hump” on the action potential (Sun and Chiu 1999). The “hump” was blocked partially by baclofen, although the Ca\(^{2+}\) transient remained unaffected (Sun and Chiu 1999). These data are intriguing because 4-AP application increased Ca\(^{2+}\) influx and the EPSC amplitude and dramatically decreased the baclofen-induced inhibition in RHT terminals (Fig. 9D) (Gompf et al. 2005; Liang et al. 2002). The 4-AP effect was so strong that after baclofen application charge transfer remained 31% larger than in control.

The mechanism of disinhibition of baclofen’s presynaptic effect by 4-AP was not clear. One explanation was that baclofen’s effect could be altered by depolarization of presynaptic terminal. It is known that depolarization of the nerve terminal relieves baclofen- and G\(\beta\gamma\)-induced inhibition of presynaptic Ca\(^{2+}\) channels (Isaacson 1998; Kajikawa et al. 2001). Another explanation is that 4-AP blocks voltage activated K\(^{+}\) channels (Kv) Kv3.1/Kv2.1 and enhances Na\(^{+}\) and Ca\(^{2+}\) influx in presynaptic terminals (Ishikawa et al. 2003; Kirsch and Drew 1993). Elevation of axonal [Na\(^{+}\)] also induces Ca\(^{2+}\) influx into the axon (Verbny et al. 2002). Increasing the Ca\(^{2+}\) influx could partially relieve baclofen-induced inhibition (Fig. 4B). At the same time it is unclear why depolarization of RHT terminals evoked by Ba\(^{2+}\) application or by high [K\(^{+}\)] did not reduce the baclofen effect. Thus we can state only that 4-AP-sensitive K\(^{+}\) currents were important for baclofen-induced inhibition because baclofen significantly increased the transient outward K\(^{+}\) current (\(I_{A}\)) and shifts the activation of \(I_{A}\) to more positive potentials (Saint et al. 1990; Takeda et al. 2004). The effects of GABA\(B\) receptor activation on \(I_{A}\) could be similar to other G-protein–coupled receptors (cannabinoid, opioidergic, and serotonergic) whose presynaptic inhibition used an opening 4-AP–sensitive K\(^{+}\) channel by G-proteins (CHILDERS et al. 1993; KISHIMOTO et al. 2001). We conclude that activation of GABA\(B\) receptors inhibits voltage-dependent Ca\(^{2+}\) channels but does not activate Kir3 channels in RHT presynaptic terminals and GABA\(B\) inhibitory effect could be reduced by block of 4-AP–sensitive K\(^{+}\) currents.

Comparing GABA\(B\) presynaptic inhibition of evoked EPSCs and IPSCs in the SCN

It is important to note similarities and differences in GABA\(B\) presynaptic inhibition of EPSCs and inhibitory postsynaptic currents (IPSCs) in the SCN. Presynaptic GABA\(B\)-receptor activation inhibits IPSCs recorded from single autaptic SCN cells (Chen and Van den Pol 1998). Comparison with our data shows that baclofen dose-dependently inhibits both EPSCs and IPSCs. Activation of presynaptic GABA\(B\) receptors suppresses transmitter release through G-protein–coupled inhibition of N- and P/Q-type Ca\(^{2+}\) channels and have no effect on L-type Ca\(^{2+}\) channels when EPSCs and IPSCs were studied (Chen and Van den Pol 1998). There is no evidence for a contribution of Kir3 channels in the presynaptic baclofen effect on IPSC (Chen and Van den Pol 1998). However, it has been shown that there are differences in the effect of baclofen on EPSCs and IPSCs. Baclofen presynaptically inhibits IPSCs by activation of GABA\(B\) autoreceptors, with a larger effect on P/Q- and lesser effect on N-type Ca\(^{2+}\) channels (Chen and Van den Pol 1998). This observation is different from the baclofen effect on EPSCs in brain slices of adult rats, where inhibition of N- and T-type Ca\(^{2+}\) channels was larger than that of P/Q-type Ca\(^{2+}\) channels. Our data demonstrate that baclofen has a strong inhibitory
effect on presynaptic T-type \( Ca^2+ \) channels and does not regulate glutamate release by inhibiting R-type \( Ca^2+ \) channels. It is important to be cautious when comparing the ratio of N-type and P/Q-type \( Ca^2+ \) channels in GABAergic axonal terminals of SCN cells and in glutamatergic RHT terminals because the experimental models were different (cell culture from neonatal pups and brain slices from adult rats). In short- and long-term culture models, different types of \( Ca^2+ \) channels begin to participate in transmitter release at different times during synapse development and maturation (Scholz and Miller 1995). Also, \( Ca^2+ \) channel expression might be different in adult and young animals (Forti et al. 2000). Additional studies of the effect of \( Ca^2+ \) channel blockers on IPSCs in adult animals are necessary to answer this question.

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PRESYNAPTIC GABA_B RECEPTORS ON RHT TERMINALS


