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GABAergic signaling induces divergent neuronal Ca^{2+} responses in the suprachiasmatic nucleus network

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Abstract

Intercellular communication between γ-aminobutyric acid (GABA)ergic suprachiasmatic nucleus (SCN) neurons facilitates light-induced phase changes and synchronization of individual neural oscillators within the SCN network. We used ratiometric Ca^{2+} imaging techniques to record changes in the intracellular calcium concentration ($[Ca^{2+}]_i$) to study the role of GABA in interneuronal communication and the response of the SCN neuronal network to optic nerve stimulations that mimic entraining light signals. Stimulation of the retinohypothalamic tract (RHT) evoked divergent Ca^{2+} responses in neurons that varied regionally within the SCN with a pattern that correlated with those evoked by pharmacological GABA applications. $GABA_A$ and $GABA_B$ receptor agonists and antagonists were used to evaluate components of the GABA-induced changes in $[Ca^{2+}]_i$. Application of the $GABA_A$ receptor antagonist gabazine induced changes in baseline $[Ca^{2+}]_i$ in a direction opposite to that evoked by GABA, and similarly altered the RHT stimulation-induced Ca^{2+} response. GABA application induced Ca^{2+} responses varied in time and region within the SCN network. The NKCC1 cotransporter blocker, bumetanide, and L-type calcium channel blocker, nimodipine, attenuated the GABA-induced rise of $[Ca^{2+}]_i$. These results suggest that physiological GABA induces opposing effects on $[Ca^{2+}]_i$ based on the chloride equilibrium potential, and may play an important role in neuronal Ca^{2+} balance, synchronization and modulation of light input signaling in the SCN network.

Keywords

chloride cotransporter; circadian rhythm; retinohypothalamic tract; synaptic transmission

Introduction

Suprachiasmatic nucleus (SCN) neurons possess a molecular clock consisting of interconnected positive and negative gene transcription feedback loops that oscillate with periods ranging from 19 to 28 h (Welsh *et al.*, 1995; Herzog *et al.*, 1998). To function as a regulator of behavioral and physiological circadian rhythms, these neurons require cell-to-cell communication to synchronize individual neuronal oscillators to a common period close to 24 h (Quintero *et al.*, 2003; Schaap *et al.*, 2003; Yamaguchi *et al.*, 2003; Aton *et al.*, 2005; Aton & Herzog, 2005) and the ability to be dynamically reset by environmental signals, of which light is the most important (Ding *et al.*, 1997; Gillette & Mitchell, 2002; Challet, 2007). Input signals from light-sensitive retinal ganglion cells (RGC) mediated via glutamatergic terminals of the retinohypothalamic tract (RHT) can reset the oscillators to a new phase (Hattar *et al.*, 2002; Yan & Silver, 2002; Warren *et al.*, 2003; Hirota & Fukada,

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2004; Naito *et al.*, 2008). Light evokes phase-advances during the late night and phase-delays during the early night, but has no effect on circadian timing during the day (Shirakawa & Moore, 1994; Ebling, 1996; Gillette & Mitchell, 2002; Challet, 2007). The magnitudes of these phase shifts depend on the timing and the intensity of light stimuli (Takahashi *et al.*, 1984; Cui & Dyball, 1996; Gooley *et al.*, 2001; Berson *et al.*, 2002; Morin *et al.*, 2003; Sollars *et al.*, 2003; Warren *et al.*, 2003). In addition, a light pulse or optic nerve stimulation can increase, decrease or not affect the action potential firing frequency of SCN neurons (Meijer *et al.*, 1986, 1998; Cui & Dyball, 1996; Jiao & Rusak, 2003).

γ-Aminobutyric acid (GABA) can both excite and inhibit SCN neurons (Wagner *et al.*, 1997). GABA can induce phase-advances (Smith *et al.*, 1990) and $GABA_A$ antagonists can block light-induced phase-delays (Ralph & Menaker, 1989). Thus, within the SCN, neurons must integrate incoming glutamatergic and peptidergic (e.g. PACAP; Castel *et al.*, 1993; Hannibal *et al.*, 2000; Kopp *et al.*, 2001) signaling from the RHT, and synchronize and stabilize to the new phase via GABAergic and peptidergic (e.g. VIP; Moore *et al.*, 2002; Belenky *et al.*, 2008) signaling from communicating neurons within the SCN network.

The activity of neurons in a network even in small brain areas receiving multiple changing inhibitory and excitatory synaptic inputs is very complex. Single-cell electrophysiological experiments generally do not take into account the complexity of neural circuits even in reduced preparations such as brain slices, and application of pharmacological agents alters the activity of all the neurons in the preparation not just in the neuron being recorded. Recently, several research groups have used imaging techniques together with calcium indicators to study the function of neuronal networks (Gobel & Helmchen, 2007; Gobel *et al.*, 2007; Zhang *et al.*, 2007). We used a similar experimental strategy to study the role of GABAergic neurotransmission in the response of the SCN network to optic nerve stimulations that mimic entraining light input signals.

Materials and methods

Tissue preparation

Male Spraque-Dawley rats (Charles River, Wilmington, MA, USA) were housed for at least 1 week on a 12 : 12 h light : dark schedule. During the light phase, 4-6-week-old rats were anesthetized with isofluorane (Novaplus, UK), their brains removed and coronal hypothalamic slices (250 μm) containing the SCN were cut with a vibrating blade microtome (Leica VT1000S, Nussloch, Germany) while the tissue was surrounded by ice-cold artificial cerebrospinal fluid (ACSF) containing (in mM): NaCl, 120; KCl, 2.5; NaH_2PO_4, 1.2; $MgCl_2$, 5; $CaCl_2$, 0.5; glucose, 10; $NaHCO_3$, 26; adjusted to 300 mOsm with sucrose and saturated with 5% CO_2 and 95% O_2. The slices were maintained in a recording chamber (35°C) with a continuous laminar flow (1-2 mL/min) of an ACSF solution consisting of (in mM): NaCl, 120; KCl, 2.5; NaH_2PO_4, 1.2; $MgCl_2$, 1.2; $CaCl_2$, 2.4; glucose, 10; HEPES, 10; $NaHCO_3$, 26; adjusted to 300 mOsm and bubbled with 5% CO_2 and 95% O_2. The Institutional Animal Care and Use Committee of OHSU approved, in advance, all procedures involving animals.

Calcium imaging

Multiple SCN neurons maintained in hypothalamic slices were loaded with Ca^{2+}-sensitive probes for the simultaneous recording of Ca^{2+} responses (Fig. 1A-C). We modified the method of Yuste (Yuste, 2000; Irwin & Allen, 2007), as CNS neurons in brain slices (older than 4 weeks) are more difficult to load with acetoxymethyl (AM) esters. Freshly prepared ice-cold SCN slices were treated with warm (30°C) ACSF containing fura-2 AM (50 μM) for 1-2 min (Invitrogen, CA, USA; from stock 3.3 mM in dimethylsulfoxide), followed by

incubation with fura-2 AM (10-25 μM) in ACSF for 1-2 h. The slice was washed for at least 30 min prior to recording to allow for fura-2 AM deesterification.

Additional experiments were performed using microelectrodes that were filled with an internal solution to which bis-fura-2 hexapotassium salt (250 μM) was added (Invitrogen, Carlsbad, CA, USA; Irwin & Allen, 2007). Upon entering whole-cell mode, the SCN neuron including the dendrites rapidly filled with fluorescent probe.

Ca^{2+} measurements were obtained by recording a pair of images at excitation wavelengths of 340 and 380 nm, a 400-nm DCLP dichroic and an emission filter of 510 ± 40 nm (Chroma Technology, Rockingham, VT, USA). Excitation light was supplied via a monochronometer (Polychrome IV; Till Photonics, Martinsried, Germany) with a 10-nm bandwidth and passed through a UG11 optical filter to restrict harmonic wavelengths above 400 nm. Optical recordings were made with a cooled charge-coupled device camera (CCD, ORCA-ER 12 bit level, Hamamatsu, Japan) with acquisition time and binning adjusted to minimize photobleaching and maximize recording speed. Experiments were controlled via the digital imaging software Metafluor (Molecular Devices, Sunnyvale, CA, USA). Optical data were converted to relative fluorescence intensity units for each cell. The data were generally obtained from neurons located near the surface of the slice and intensely loaded with fura-2 AM. Data were presented as the emission ratio (R) at 510 ± 40 nm following excitation at 340 and 380 nm and background subtraction at each wavelength. Background fluorescence values were obtained from regions adjacent to each cell (including autofluorescence, residual probe in the interstitial space, and dendrites and cells out of the focal plane). Because of the background fluorescence associated with this slice preparation, the data are presented as measured ratios and not converted to $[Ca^{2+}]_i$ units. However, an approximation for $[Ca^{2+}]_i$ can be made utilizing the formula $\sim[Ca^{2+}]_i = (R - R_{min})/(R_{max} - R) \times K_d$, where K_d is the disassociation constant for fura-2 AM. The observed Ca^{2+} ratios ranged from a minimum of 0.34 (R_{min}) to a maximum of 3.7 (R_{max}), and assuming a K_d of 224 nM, a ratio of 1 corresponds to ~55 nM and a ratio of 2 to ~219 nM. Neurons were identified by an increase in Ca^{2+} ratio in response to *N*-methyl-D-aspartate (NMDA; 200 μM for 5 s; Fig. 1C). Cells that did not respond to NMDA (200 μM, 5 s) or were dim were excluded. Ca^{2+} signals were initially sorted into three groups (increase, decrease or no response) by visual inspection. In a subset of the data where the Ca^{2+} signals were quantified, the response was defined as a change from baseline of more than 2 standard deviations. Neurons were considered to have a Ca^{2+} response to the $GABA_A$ antagonist gabazine by a sustained increase or decrease of the Ca^{2+} (340 nm/380 nm) ratio = 0.1 units. The localization of neurons to the dorsomedial (DM) and ventrolateral (VL) regions of the SCN were determined using the SCN coordinates for each neuron based on the calibrated 4 × brightfield image for each slice. The *X, Y* coordinate (0, 0) was the intersection of two perpendicular lines, one along the third ventricle and the other drawn along the ventral border of the SCN (Fig. 1A). A designation of VL was made when the coordinate $X = Y$ for the neuron, and DM when the coordinate $X < Y$.

Test agents were applied using a perfusion device consisting of an eight-barrel array of tubes emptying into a common tip positioned approximately 500-1000 μm from the SCN and controlled electronically. The RHT was stimulated by 100 pulses (200 μs each) at 20 Hz via a bipolar electrode placed in the optic chiasm about 200 μm from the SCN (Fig. 1A).

Electrophysiology

Cell-attached and whole-cell patch-clamp recordings of SCN neurons were performed 0.5-8 h after slice preparation. Microelectrodes had an outside tip diameter of approximately 1 μm and resistances of 3-8 MΩ. For whole-cell recording experiments the microelectrode filling solution contained (in mM): K-gluconate, 140; KCl, 5; HEPES, 10; ATP, 4; GTP, 0.4;

adjusted to pH 7.3 with KOH at (280–300 mOsm). Following microelectrode contact with a SCN neuron, negative pressure was applied to form seals with resistances of 1-5 GΩ. For whole-cell experiments additional negative pressure was used to rupture the cell membrane. For loose-seal recordings, the GABA-induced Ca^{2+} response was first determined followed by contacting the neuron with a microelectrode containing (in m_M): NaCl, 140; KCl, 2.5; HEPES, 10; MgCl, 1.2; $CaCl_2$, 2.4; adjusted to pH 7.3 with NaOH at (280–300 mOsm) and applying gentle negative pressure to form a loose seal (< 1 GΩ). Membrane voltage was measured in current-clamp mode with an Axoclamp 2A amplifier (Axon Instruments, Union City, CA, USA) or a HEKA EPC9 amplifier (HEKA, Lambrecht, Germany), and recorded using the data acquisition program Pulse (HEKA, Lambrecht, Germany), corrected for the liquid junction potential (-13 mV) and analysed using Igor (Wavemetrics, Lake Owsego, OR, USA).

Chemicals

GABA, NMDA, gabazine (SR 95531 hydrobromide), muscimol and CGP55845 were obtained from Tocris Bioscience (Ellisville, MO, USA). Fura-2 AM and bis-fura-2 were purchased from Invitrogen (Carlsbad, CA, USA). Other chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA).

Data and statistical analysis

Igor Pro (Version 6.0.4, Wavemetrics, Lake Oswego, OR, USA) was used for curve-fitting and data analysis. Unless otherwise stated, data are presented as the mean ± SEM, and *t*-tests are two-tailed. *t*-Tests and Chi-square analysis were performed using Excel 11.5.1 (Microsoft, Redmond, WA, USA). ANOVA, Kolmogorov-Smirnov and equality of variances *F*-test were performed using StatView 5.0.1 (SAS Institute, Cary, NC, USA). A *P*-level of = 0.05 was used to determine statistical significance.

Results

Time-dependence of the baseline Ca^{2+} concentration in SCN neurons

Simultaneous imaging of multiple neurons at different times of the circadian cycle and specific locations within the SCN (Fig 1A-C) was performed to examine the role of GABA in interneuronal communication and modulation of light-induced signaling to the SCN. We observed that stimulation of the RHT induced a range of postsynaptic Ca^{2+} responses that paralleled divergent responses seen with GABA applications (Fig. 1D).

To determine if endogenous GABA activity is modulating RHT signaling to the SCN, its importance in interneuronal communication, and if there are regional differences within the GABAergic SCN network, we first determined the variations of baseline Ca^{2+} at different times in the circadian phase, and time and regional variation in Ca^{2+} responses to exogenously applied and endogenous GABA, followed by the roles of $GABA_A$ and $GABA_B$ receptor subtypes, L-type Ca^{2+} channels, and the chloride cotransporter NKCC1 in the divergent postsynaptic responses to GABA.

During the day, SCN neurons had higher and more variable baseline Ca^{2+} ratios than during the night (Figs 1C and 2). The mean baseline Ca^{2+} ratio for each neuron was calculated based on the Zeitgeber time (total 1172 neurons) and demonstrated a diurnal change in the Ca^{2+} ratio (Fig. 2A; Colwell, 2000; Ikeda *et al.*, 2003a). The variance of Ca^{2+} ratios during the day was significantly ($P < 0.0001$) different from that during the night (Fig. 2B and C). The distribution was narrower during the night-time and broader in the daytime. $[Ca^{2+}]_i$ in SCN neurons varies with spontaneous action potential firing frequency (Irwin & Allen, 2007). The larger variance of the daytime baseline Ca^{2+} is consistent with the observations

(Gillette, 1986; Jobst & Allen, 2002; Ikeda *et al.*, 2003a) that during the day a greater number of SCN neurons are firing action potentials at higher frequencies. The change in variance of the Ca^{2+} ratio with time implies that there are quiescent and firing populations of SCN neurons.

Whole-cell patch-clamp recordings with bis-fura-2 in the patch microelectrodes were used to further examine the relationship between membrane potential and $[Ca^{2+}]_i$ (Fig. 3). Membrane potential hyperpolarization interrupted action potential firing and lowered the $[Ca^{2+}]_i$, while a further hyperpolarization of membrane potential did not further reduce $[Ca^{2+}]_i$. This indicates that there is a 'floor' to the voltage- $[Ca^{2+}]_i$ relationship when voltage-dependent Ca^{2+} channels are closed and hyperpolarization below approximately -60 mV results in no further reduction in $[Ca^{2+}]_i$ (Fig. 3B). Depolarizing the membrane potential resulted in a rapid increase in the spontaneous action potential firing frequency with a concomitant elevation of $[Ca^{2+}]_i$. We have previously shown that firing frequencies beyond 10-20 Hz did not yield a further increase in $[Ca^{2+}]_i$ (Irwin & Allen, 2007). Taken together, these data suggest that there was a sigmoid relationship between membrane potential and the change in $[Ca^{2+}]_i$ with a 'ceiling' associated with rapid firing of SCN action potentials (Fig 3B).

We used tetrodotoxin (TTX) to evaluate the contribution of action potential firing to the $[Ca^{2+}]_i$ of SCN neurons within the network. In fura-2 AM-loaded slices, application of TTX (0.5 μ_M), to inhibit Na^+-dependent action potentials, reduced the Ca^{2+} ratio (= 0.1 ratio units) in significantly more neurons during the day (47% of 188 neurons) than during the night (26% of 62 neurons; $\chi^2_1 = 8.88$, $P = 0.0029$). The baseline Ca^{2+} ratio of 1.59 ± 0.03 was reduced by -0.27 ± 0.02 ratio units during the day ($t_{88} = 13.69$, $P < 0.0001$), and a baseline of 1.41 ± 0.06 was reduced by -0.23 ± 0.04 ratio units during the night ($t_{15} = 6.47$, $P < 0.0001$). Note that while during the day more neurons had a reduction of baseline Ca^{2+}, the magnitude of the change was similar. These data were consistent with an interpretation that the higher amplitude and greater variance of the Ca^{2+} ratio observed during the day was due to a greater number of SCN neurons firing spontaneous action potentials during the day than at night (Colwell, 2000).

GABA has divergent effects on intracellular Ca^{2+} in SCN neurons that varies with time and region

GABA is generally viewed as an inhibitory neurotransmitter in the CNS (Roberts, 1984; Paul, 1995). However, several studies have observed excitatory GABA responses in neurons from several regions of the CNS including the SCN (Ben-Ari *et al.*, 1989; Obrietan & van den Pol, 1996; Wagner *et al.*, 1997, 2001; Staley & Smith, 2001; De Jeu & Pennartz, 2002; Ikeda *et al.*, 2003b; Stein & Nicoll, 2003; Choi *et al.*, 2008; Shulga *et al.*, 2008; Zemkova *et al.*, 2008). We tested the hypothesis that GABA has dual effects in SCN neurons that vary in time and location, where GABA can elevate $[Ca^{2+}]_i$ in some SCN neurons while reducing the $[Ca^{2+}]_i$ in others. We found that GABA (200 μ_M, 10 s) induced three types of Ca^{2+} responses in SCN neurons (Figs 1D and 4A): Ca^{2+} elevation [GABA(Ca+)]; Ca^{2+} reduction [GABA(Ca-)]; and those that did not respond [GABA(Ca0)]. We confirmed that the GABA-induced rise of Ca^{2+} was associated with membrane depolarization by performing loose-seal recordings (Jobst & Allen, 2002) from GABA(Ca+) neurons ($n = 5$; Fig. 4B). The Ca^{2+} response of SCN neurons to GABA application varied with the baseline Ca^{2+} ratio (Fig. 4C). In neurons with low baseline Ca^{2+} ratios there were fewer GABA(Ca-) neurons and more GABA(Ca0) neurons, while at higher Ca^{2+} baselines there were more GABA(Ca-) than GABA(Ca0) neurons, suggesting that when the baseline Ca^{2+} was low a GABA(Ca-) neuron was not able to further lower Ca^{2+} and may appear as a GABA(Ca0) neuron. The percentage of GABA(Ca+) neurons was reduced as the baseline Ca^{2+} increased. Testing 760 day and 292 night neurons, we found that a significantly lower percentage of GABA(Ca+)

neurons was observed during the day (46.6%), than during the night (65.8%). Conversely, the percentage of GABA(Ca-) neurons decreased from 28.3% during the day to 8.2% at night (χ^2_2= 52.41, $P < 0.0001$). The percentage of GABA(Ca0) neurons did not differ between the day (25.1%) and the night (26.0%; $\chi^2_1 = 0.09$, combined GABA(Ca+ and Ca-) vs. GABA(Ca0), $P = 0.76$; Fig. 4D). The percentage of GABA(Ca+) neurons was proportionally greater in the DM than the VL SCN (Figs 4E and 5). Conversely, a higher percentage of GABA(Ca-) and GABA(Ca0) neurons were observed in the VL than the DM SCN. Therefore we conclude that activation of GABA receptors induced divergent effects on $[Ca^{2+}]_i$ that varied between the day and night, and location within the SCN.

Blocking the chloride cotransporter NKCC1 attenuated GABA-induced elevation of Ca^{2+}

Inhibiting the sodium-potassium-chloride cotransporter NKCC1 activity reduced GABA-induced membrane depolarization (Khirug *et al.*, 2008) in SCN neurons (Choi *et al.*, 2008). SCN neurons were exposed to GABA before and after application of the NKCC1 blocker bumetanide (10 μM; Fig. 6A). The bumetanide effect on GABA-induced transients increased over time, reaching a maximal response after ~300 s (Fig. 6B). Bumetanide significantly attenuated daytime GABA-induced Ca^{2+} transients (65%) in GABA(Ca+) neurons and increased the magnitude of GABA-induced reductions of the Ca^{2+} ratio in GABA(Ca-) neurons (37%; Fig. 6C). These data show that the activity of the NKCC1 cotransporter is required for GABA-induced elevations of $[Ca^{2+}]_i$ in SCN neurons.

Blocking L-type voltage-dependent Ca^{2+} channels attenuated GABA(Ca+)-induced Ca^{2+} transients

L-type voltage-dependent Ca^{2+} channels mediate a significant proportion of the depolarization-induced Ca^{2+} transients in SCN neurons (Irwin & Allen, 2007). Therefore, we examined whether the L-type Ca^{2+} channels contributed to GABA(Ca+) responses in SCN neurons. SCN neurons were treated during the day, with GABA (200 μM, 10 s) or the selective $GABA_A$ agonist muscimol (50 μM, 10 s) before and after the application of the L-type Ca^{2+} blocker nimodipine (20 μM for = 60 s; Fig. 7A). Nimodipine attenuated GABA- and muscimol-induced increases of Ca^{2+} in GABA(Ca+) neurons ($n = 30$, $P < 0.00001$; Fig. 7B). Further, nimodipine application was associated with a -0.16 ± 0.02 ratio unit reduction of the baseline Ca^{2+} ratio (1.40 ± 0.05, $n = 40$, $t_{39} = 7.49$, $P < 0.0001$ daytime neurons), suggesting that L-type Ca^{2+} channels play a role in setting the baseline Ca^{2+} in SCN neurons during the day. These data suggest that GABA increases the $[Ca^{2+}]_i$ of SCN neurons in part by depolarizing the membrane potential and activating L-type voltage-dependent Ca^{2+} channels.

The contributions of postsynaptic $GABA_A$ and $GABA_B$ receptors as mediators of the GABA-induced Ca^{2+} response

To evaluate the contributions of $GABA_A$ and $GABA_B$ receptors in the GABA-induced Ca^{2+} response, SCN slices were treated with GABA in the absence and presence of the $GABA_A$ antagonist gabazine with or without the $GABA_B$ antagonist CGP55485 (Fig. 8A). Simultaneous inhibition of $GABA_A$ and $GABA_B$ receptors eliminated the GABA-induced Ca^{2+} changes. Inhibiting $GABA_A$ receptors eliminated the GABA-induced Ca^{2+} elevations, but not all reductions (Fig. 8A and B). However, inhibiting the $GABA_B$ receptors first did not alter the GABA-induced Ca^{2+} elevations but attenuated a delayed Ca^{2+} reduction that occurred in some neurons (Fig. 8C). We evaluated the selective $GABA_B$ agonists, baclofen and muscimol, on both GABA(Ca+) and GABA(Ca-) neurons. Baclofen lowered the $[Ca^{2+}]_i$ in both GABA(Ca+) (Fig. 8D) and GABA(Ca-) (Fig. 8E) neurons; while muscimol-induced both increases and decreases of $[Ca^{2+}]_i$ corresponding to the GABA responses. Baclofen induced a similar reduction of $[Ca^{2+}]_i$ in both GABA(Ca+) and GABA(Ca-) neurons that persisted for some time after the application was discontinued.

The effect of endogenous GABA in the SCN network on Ca^{2+} homeostasis

As the effect of pharmacological application of GABA on $[Ca^{2+}]_i$ in SCN neurons appeared to vary with regional location and activity phenotype, we evaluated whether endogenous GABA has a role in regulating Ca^{2+} homeostasis. SCN neurons were characterized as to the Ca^{2+} response induced by blocking $GABA_A$ receptors and contrasted with the pharmacological Ca^{2+} response to GABA. If endogenous GABA has a role in setting the level of intracellular Ca^{2+} in SCN neurons then an application of a $GABA_A$ blocker would be expected to reduce the baseline $[Ca^{2+}]_i$ in GABA(Ca+) neurons and elevate the baseline $[Ca^{2+}]_i$ in GABA(Ca-) neurons (Fig. 8F). SCN neurons were separated into three groups based on the baseline Ca^{2+} ratio response to gabazine (10 μ_M; Fig. 9). Similarly, bicuculline, a $GABA_A$ antagonist, has been shown to increase or decrease spontaneous firing of SCN neurons (Wagner *et al.*, 1997; Shirakawa *et al.*, 2000; De Jeu & Pennartz, 2002; Choi *et al.*, 2008). In neurons responding to both GABA and gabazine, 75% of GABA(Ca+) neurons (n = 125) were gabazine(Ca-), and 68% of GABA(Ca-) neurons (n = 77) were gabazine(Ca+) ($\chi^2_1 = 38.03$, $P < 0.0001$). Differences between the pharmacological GABA response and the expected gabazine response may be attributed in part to a residual $GABA_B$ effect on $[Ca^{2+}]_i$. The Ca^{2+} responses to gabazine application demonstrated a distribution within the SCN similar to that predicted using GABA agonists, such that the mean change in Ca^{2+} induced by gabazine was in the opposite direction to that elicited by GABA. GABA(Ca-) and GABA(Ca0) neurons responding to gabazine had a similar change in the Ca^{2+} ratio, suggesting that pharmacological GABA(Ca0) neurons have physiologically inhibitory GABA responses (Fig. 9, bottom right). Collectively, these data suggest that physiological levels of GABA in the SCN network have a role in modulating the $[Ca^{2+}]_i$.

RHT signal modulation by GABA in the SCN network

Because GABA may have a role modulating light signaling to the SCN network, we determined if endogenous GABA alters the SCN neuronal response to RHT stimulation-induced transient changes in Ca^{2+}. We have previously demonstrated that electrical stimulation of the RHT evokes an elevation of $[Ca^{2+}]_i$ in SCN neurons by inducing action potentials (Irwin & Allen, 2007). When the spontaneous firing frequency was low, as occurs at night, RHT stimulation evoked large Ca^{2+} transients, while small Ca^{2+} changes occurred during high-frequency firing. These studies were performed using a $GABA_A$ blocker to eliminate interneuronal GABAergic neurotransmission. In the absence of $GABA_A$ receptor antagonists, stimulation of the RHT induced a range of postsynaptic Ca^{2+} responses comprising basic three types: a transient elevation in Ca^{2+} [RHT(Ca+)]; a transient reduction in Ca^{2+} [RHT(Ca-)]; or no alteration in the Ca^{2+} ratio [RHT(Ca0)] (Figs 1D and 10). GABA was applied to assess the pharmacological Ca^{2+} response 'phenotype' for each neuron. Most of the RHT(Ca+)-responding neurons also were GABA(Ca+) (~90%), and RHT(Ca-)-responding neurons also were GABA(Ca-) (~70%), with little day and night differences (Fig. 11, bottom right).

Regional variation of RHT stimulation induced Ca^{2+} transients in the SCN network

The RHT stimulation-induced postsynaptic Ca^{2+} transients in the SCN network demonstrated a regional variation similar to the Ca^{2+} response pattern observed from application of pharmacological GABA (Figs 5 and 11). GABA application was used to assess the pharmacological phenotype of each neuron (Fig. 10), while gabazine was used to assess the effect of physiological GABA following RHT signaling within the SCN network. The use of $GABA_B$ antagonists was limited, as presynaptic $GABA_B$ receptors reduce the glutamate release from RHT axon terminals (Jiang *et al.*, 1995; Moldavan *et al.*, 2006). Gabazine application altered the RHT stimulation-induced Ca^{2+} responses in 77% of 247 neurons. An increase by gabazine [RHT-Gz(Ca+)] was defined as an increase in the RHT(Ca+) Ca^{2+} transient or a reduction in RHT(Ca-) response, and a decrease [RHT-

Gz(Ca-)] in Ca^{2+} was defined as a reduction of the RHT(Ca+) Ca^{2+} transient or an increase in RHT(Ca-) response, and little or no change [RHT-Gz(Ca0)]. The effect of gabazine on the RHT stimulation-induced Ca^{2+} transients resulted in a decrease of the Ca^{2+} response in 53%, an increase in 21% and no change in 25% of 135 GABA(Ca+) neurons, and an increased Ca^{2+} response in 57%, decreased in 23% and did not change in 20% of 74 GABA(Ca-) neurons (χ^2_2= 28.35, $P < 0.0001$). The gabazine response on Ca^{2+} transients was opposite in 59%, paralleled in 16% and did not change in 25% of 171 SCN neurons with corresponding combined pharmacological GABA and RHT Ca^{2+} response phenotypes (χ^2_2 = 40.89, $P < 0.0001$). In some neurons without an initial Ca^{2+} response to RHT stimulation, application of gabazine unmasked a Ca^{2+} elevation in 15% and a decrease in 3% of 412 neurons.

These data suggest that GABA plays a role in modulating postsynaptic responses to RHT signaling. Because most SCN neurons are GABAergic, the ability of GABA release within the SCN network to modulate intracellular Ca^{2+} in neighboring neurons may emerge as a major level of control within the SCN.

Discussion

Activation of GABA receptors by endogenous GABA or application of pharmacological GABA agonists produced changes of the $[Ca^{2+}]_i$ in SCN neurons that was dependent on the time of day, type of GABA receptors activated and the regional location of the neuron. RHT stimulation, mimicking light entraining input, induced elevations and reductions in $[Ca^{2+}]_i$ with a localization pattern that frequently paralleled the GABA 'pharmacological phenotype'. GABA-induced Ca^{2+} increases in the SCN were associated with membrane depolarization and activation of L-type Ca^{2+} channels, and had a higher proportion during the night than the day. These data are consistent with previous studies demonstrating that activation of $GABA_A$ receptors excites SCN neurons, based on the values of the chloride equilibrium potential and the membrane potential (Wagner *et al.*, 2001), with a subsequent elevation of $[Ca^{2+}]_i$ (Obrietan & van den Pol, 1996; Ikeda *et al.*, 2003b; Choi *et al.*, 2008; Shulga *et al.*, 2008). Activation of $GABA_A$ and $GABA_B$ receptors produced a reduction in $[Ca^{2+}]_i$. $GABA_A$ receptors can induce hyperpolarization due to an inward Cl^- current, that may reduce $[Ca^{2+}]_i$ via a reduction or elimination of action potential firing (Irwin & Allen, 2007) and by closing voltage-dependent calcium channels. Additionally, it is conceivable that the Ca^{2+} reduction may also be augmented by other mechanisms, such as intracellular sequestration, extrusion or closing of other Ca^{2+} channels. The $GABA_B$ receptor-mediated effect is likely secondary to activation of a hyperpolarizing K^+ current (Jiang *et al.*, 1995). In a third group of SCN neurons the Ca^{2+} was not altered by GABA application [GABA(Ca0)] or RHT stimulation [RHT(Ca0)]. Because the physiological firing state of an individual neuron influences the Ca^{2+} response from RHT stimulation (Irwin & Allen, 2007), it is conceivable that a GABA(Ca-) quiescent neuron with a low $[Ca^{2+}]_i$ during the night or a GABA(Ca+) high $[Ca^{2+}]_i$ neuron firing action potentials during the day may not be able to further lower or raise $[Ca^{2+}]_i$, respectively. In most neurons with similar responses to RHT stimulation and GABA, application of gabazine to block $GABA_A$ receptor activity altered the RHT stimulation Ca^{2+} response in an opposite manner to the original response. Similar experiments could not be performed with $GABA_B$ receptor antagonists, as presynaptic $GABA_B$ receptors are present on presynaptic RHT terminals (Jiang *et al.*, 1995; Moldavan *et al.*, 2006). An increase of $[Ca^{2+}]_i$ within SCN neurons during the night phase can shift the circadian clock (Gillette & Mitchell, 2002; Challet, 2007) and is thought to be required to initiate the transduction events leading to photic entrainment (Ding *et al.*, 1994, 1998). Therefore, these data suggest that endogenous GABA activity within the SCN network can modulate the neuronal response to RGC light input signaling and may also play an important

role in integrating the incoming light information to synchronize SCN neurons to produce a unified circadian rhythm.

$GABA_A$ receptors are located postsynaptically on SCN neurons and presynaptically as autoreceptors (Gao *et al.*, 1995; Belenky *et al.*, 2003), while $GABA_B$ receptors are present postsynaptically (Jiang *et al.*, 1995; Belenky *et al.*, 2008) with high expression in dorsal SCN neurons (Belenky *et al.*, 2008) and presynaptically on RHT terminals (Moldavan *et al.*, 2006). To evaluate the contributions of postsynaptic $GABA_A$ and $GABA_B$ receptors as mediators of the GABA-induced Ca^{2+} response, we applied gabazine to block $GABA_A$ receptors and eliminated the GABA-induced rise in $[Ca^{2+}]_i$, whereas blocking both $GABA_A$ and $GABA_B$ receptors eliminated both GABA-induced reductions and elevations of $[Ca^{2+}]_i$. The $GABA_A$ agonist muscimol produced divergent effects on $[Ca^{2+}]_i$ that paralleled the GABA responses; whereas the $GABA_B$ agonist baclofen reduced $[Ca^{2+}]_i$ often with a longer duration, consistent with activation of a G-protein-coupled receptor and K^+ current.

Results from exogenous application of neurotransmitters can be difficult to interpret, as these agents are often applied at possible supraphysiological levels, the concentration may vary secondary to uptake mechanisms, and the levels may not accurately reflect the duration and location associated with either synaptic or tonic concentrations (Walker & Semyanov, 2008). Therefore, we applied gabazine to block endogenous $GABA_A$ receptor activity within the SCN network. Gabazine generally altered the baseline $[Ca^{2+}]_i$ in a direction opposite to that of the pharmacological GABA phenotype and localization pattern within the SCN network. This suggests that the heterogeneous pharmacological GABA phenotype reflects the 'physiological GABA phenotype'. Correspondingly, the $GABA_A$ blocker bicuculline inhibits multiunit electrical activity in the dorsal SCN but increases firing in the ventral SCN (Albus *et al.*, 2005).

Recent evidence suggested that the functional organization of the SCN has broad divisions, such as core and shell (Moore *et al.*, 2002), DM and VL (Masumoto *et al.*, 2006; Belenky *et al.*, 2008), or more complex divisions (Morin & Allen, 2006; Morin, 2007) based on physiological characterizations and localizations of peptides, receptors and gene products. Similar to the GABA-induced excitation and inhibition SCN localization observed by Choi *et al.* (2008), we found that the percentage of GABA(Ca+) neurons was proportionally greater in the DM than the VL half, while the percentage of GABA(Ca-) and GABA(Ca0) neurons was greater in the VL than the DM SCN. The excitatory and inhibitory pattern of physiological GABA modulation of $[Ca^{2+}]_i$ within the SCN suggests a network of neighboring SCN neurons with divergent responses to GABA but grouped by regional localization with a preponderance of phenotypes.

We confirmed that the $[Ca^{2+}]_i$ of SCN neurons demonstrates rhythmicity, with intracellular Ca^{2+} levels having a larger mean and variance during the day than the night (Colwell, 2000; Ikeda et al., 2003a). Application of TTX to inhibit firing of Na^+-dependent action potentials reduced the baseline Ca^{2+} ratio in more neurons during the day than at night, consistent with a model in which $[Ca^{2+}]_i$ varied with the frequency of spontaneous action potential firing. These data were in agreement with our previous whole-cell current-clamp recordings of SCN neurons, establishing that the $[Ca^{2+}]_i$ is higher in SCN neurons with depolarized membrane potentials and faster action potential firing frequencies (Irwin & Allen, 2007). The greater amplitude and variance of the Ca^{2+} ratio observed during the day is, therefore, likely due to the greater number of SCN neurons firing spontaneous action potentials during the day than at night. Similarly, Colwell demonstrated a TTX-sensitive day-night difference in $[Ca^{2+}]_i$ (Colwell, 2000). However, our previous observations using the Ca^{2+} indicator cameleon demonstrated a TTX-insensitive rhythm of Ca^{2+} released from intracellular stores

(Ikeda *et al.*, 2003a). Collectively, these data suggest that cameleon and fura-2 AM are measuring two different pools of Ca^{2+} regulated by different signaling mechanisms.

For a neuronal network to function there needs to be integration of both excitatory and inhibitory inputs. Within the SCN neuronal network, GABA signaling may serve both functions, as the equilibrium potential for the $GABA_A$ chloride channel is near the resting membrane potential in SCN neurons (Wagner *et al.*, 1997, 2001; De Jeu & Pennartz, 2002; Choi *et al.*, 2008). The transmembrane chloride gradient is regulated by two chloride transporters, the sodium-potassium-chloride cotransporter (NKCC1) that increases and the potassium-chloride cotransporter (KCC2) that decreases the intracellular chloride concentration ($[Cl^-]_i$; Payne *et al.*, 2003; Stein & Nicoll, 2003). Modification of the expression or activity of these transporters will alter the chloride equilibrium potential and the magnitude and direction of $GABA_A$-activated currents. We used the NKCC1 cotransporter blocker bumetanide (Kahle *et al.*, 2008) to lower the $[Cl^-]_i$ and significantly attenuated the GABA-induced rise of Ca^{2+} in GABA(Ca+) neurons and further reduced Ca^{2+} in GABA(Ca-) neurons. NKCC1 expression is higher in the dorsal SCN during the night than the day (Choi *et al.*, 2008), and KCC2, responsible for chloride efflux, is predominately expressed in the ventral SCN (Belenky *et al.*, 2008), and is consistent with our observation of a higher percentage of GABA(Ca+) neurons in the DM SCN at night (Fig. 4D). These observations demonstrate that modulation of $[Cl^-]_i$ leads to GABA producing both excitatory and inhibitory effects on $[Ca^{2+}]_i$ in SCN neurons.

Data from several studies indicate that GABA mediates both excitatory and inhibitory neurotransmission in the SCN (Wagner 1997, 2001; De Jeu & Pennartz, 2002; Ikeda *et al*, 2003; Choi *et al.* 2008). However, the results of these studies differ in critical ways, for reasons that remain unknown. Wagner *et al.* (1997, 2001) reported that excitatory responses predominated during the day, while De Jeu & Pennartz (2002) found that GABA primarily inhibits SCN neurons during the day but excites approximately half of the neurons at night. Recently, Choi *et al.* (2008) found GABA inhibitory and excitatory effects occurring during both the day and night, but found excitatory responses were more common at night especially in the dorsal SCN. We demonstrated a greater number of SCN neurons with GABA(Ca+) responses during the night than the day, similar to the data reported by De Jeu & Pennartz (2002) and Choi *et al.* (2008). At night, more GABA(Ca+) neurons were present in the DM SCN, and GABA(Ca-) responses predominated in the VL SCN, not dissimilar to the dorsal localization of excitatory responses observed by Choi *et al.* (2008). These studies indicate that the response of a SCN neuron to GABA depends on many factors, including the time of day, regional localization and its activity state.

Our observations suggest a model where physiological GABA release within the SCN network divergently modulates the response to RHT input signaling. In neurons with high NKCC1 activity (high $[Cl^-]_i$), opening $GABA_A$ receptors removes Cl^- and facilitates depolarization, increasing firing frequency and increasing $[Ca^{2+}]_i$ (Fig. 12A). In neurons with high KCC2 activity (low $[Cl^-]_i$), $GABA_A$ activity moves Cl^- into the neuron hyperpolarizing the cell beyond the depolarizing effect of RHT signaling, slowing the firing rate and decreasing $[Ca^{2+}]_i$ (Fig. 12B). Lastly, it is likely that many if not most neurons are in a balance that varies over time where the chloride level, set by cotransporters, may serve a gating role in the SCN network, and may neutralize the effects of RHT input without altering the membrane potential or firing frequency resulting in no change in $[Ca^{2+}]_i$. Further work is needed to further test this model and explore the role of internal calcium stores, co-released peptides and the reduction of $[Ca^{2+}]_i$ by the $GABA_B$ receptor. In conclusion, these data suggest that GABA has both excitatory and inhibitory communication based on the chloride equilibrium potential and may play an important role in neuronal

$[Ca^{2+}]_i$ balance, synchronization and modulation of light input signaling within the SCN neuronal network.

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Abbreviations

ACSF	artificial cerebrospinal fluid
AM	acetoxymethyl
DM	dorsomedial
GABA	γ-aminobutyric acid
NMDA	*N*-methyl-D-aspartate
RGC	retinal ganglion cells
RHT	retinohypothalamic tract
SCN	suprachiasmatic nucleus
TTX	tetrodotoxin
VL	ventrolateral

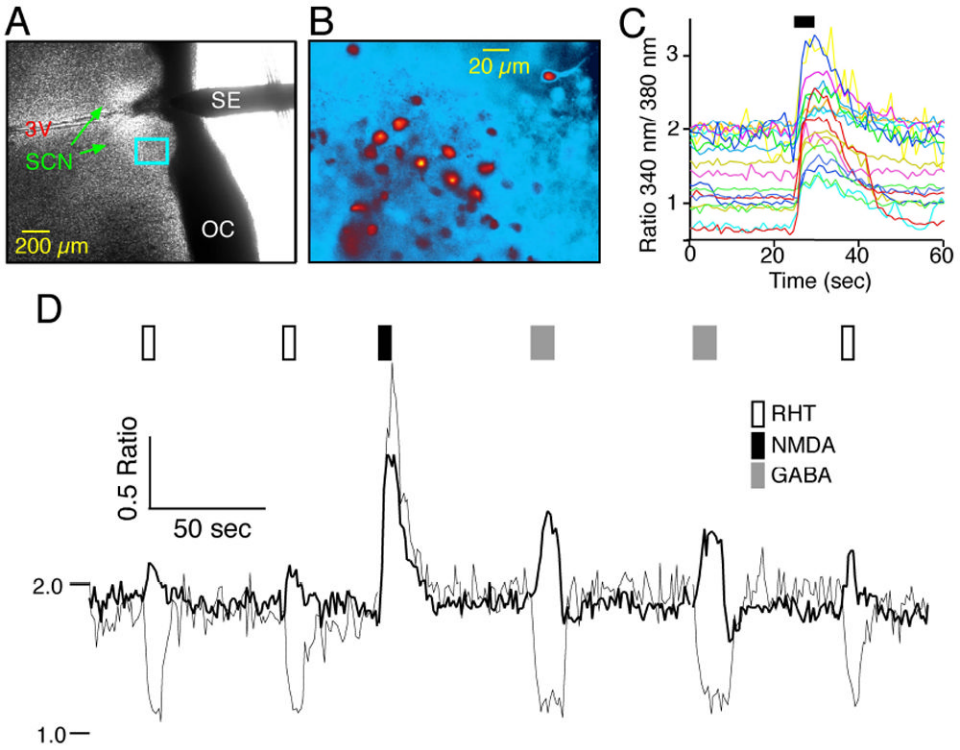

Fig. 1. Measurement of intracellular Ca^{2+} in the suprachiasmatic nucleus (SCN)

(A) Example of the recording setup showing a coronal hypothalamic slice containing the SCN and 3rd ventricle (3V) with a bipolar stimulating electrode (SE) used to stimulate the retinohypothalamic tract (RHT) positioned in the optic chiasm (OC). (B) Higher power fluorescent pseudo-color image of fura-2 AM-loaded SCN neurons located within the rectangle in (A). (C) *N*-methyl-D-aspartate (NMDA; 200 μM, 5 s) induced a transient increase in the Ca^{2+} ratio. Note the range of baseline Ca^{2+} ratios in these SCN neurons during the day (Zeitgeber time = 5 h). (D) Example showing postsynaptic Ca^{2+} in two SCN neurons with similar responses to NMDA (200 μM, 5 s), but divergent responses to stimulation of the RHT (100 pulses, 200 μs at 20 Hz) via a bipolar electrode placed in the OC (A) that paralleled the response to application of g-aminobutyric acid (GABA; 200 μM, 10 s).

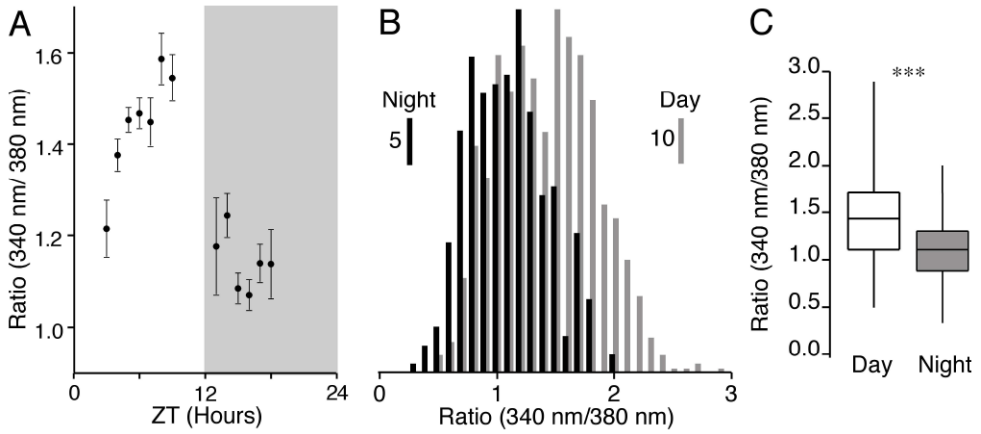

Fig. 2. SCN neuronal baseline Ca^{2+} is dependent on the time of day

(A) The mean baseline Ca^{2+} ratio varied with the time of the day. Each point represents the mean ± SEM of the Ca^{2+} ratio (total 1172 neurons, mean $n = 90$ and range 8–236 neurons) at the indicated times. The darkened box indicates the night phase. (B) Distribution of the baseline Ca^{2+} ratios in SCN neurons recorded during the day ($n = 861$) and night ($n = 311$), respectively. The scale bars indicate the number of cells in each bin. (C) A box plot of the data in (B) showing the range (vertical line), 25^{th} and 75^{th} percentiles (box) and median (horizontal line) for day and night SCN neurons. The variance in Ca^{2+} ratios significantly differed between the day and night (equality of variances $F_{860,310} = 1.65$, $^{***}P < 0.0001$), and were shifted higher during the day compared with night (Kolmogorov-Smirnov test $\chi^2_2 = 104.8$, $^{***}P < 0.0001$). ZT, Zeitgeber time.

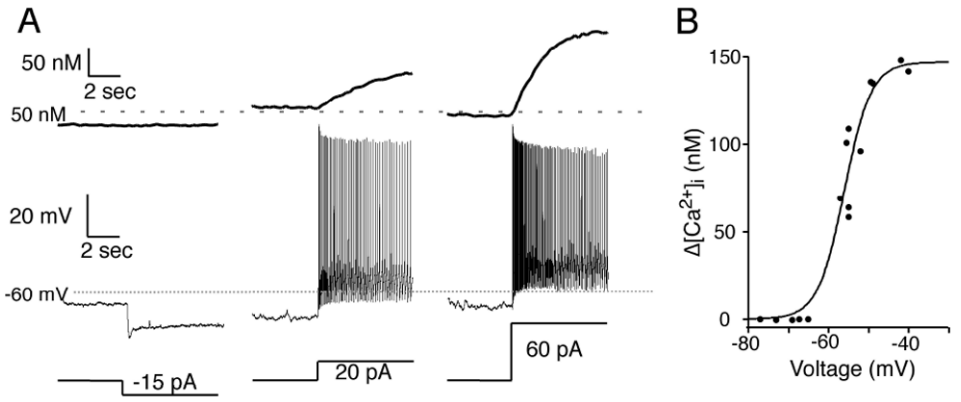

Fig. 3. The $[Ca^{2+}]_i$ in SCN neurons varied with membrane potential and the spontaneous action potential firing frequency

(A) A SCN neuron recorded during the day with a microelectrode filled with an internal solution containing the Ca^{2+}-sensitive probe bis-fura-2 and external ACSF containing picrotoxin (50 μ_M) was hyperpolarized to inhibit spontaneous action potentials (not shown) followed by small current steps (bottom trace) to further hyperpolarize or depolarize the membrane potential (middle trace). A reduction of membrane potential did not lower the estimated $[Ca^{2+}]_i$, while depolarizing steps produced a rapid increase in spontaneous action potential firing with a concomitant elevation of $[Ca^{2+}]_i$ (top trace). (B) Summary of the neuron in (A) showing a sigmoid relationship between the voltage reached from each hyperpolarizing and depolarizing current step and the corresponding change in somatic $[Ca^{2+}]_i$. During action potential firing membrane voltage was estimated by a line halfway between the action potential threshold and peak of the afterhyperpolarization. Estimated $[Ca^{2+}]_i$ was calculated as previously described (Irwin & Allen, 2007).

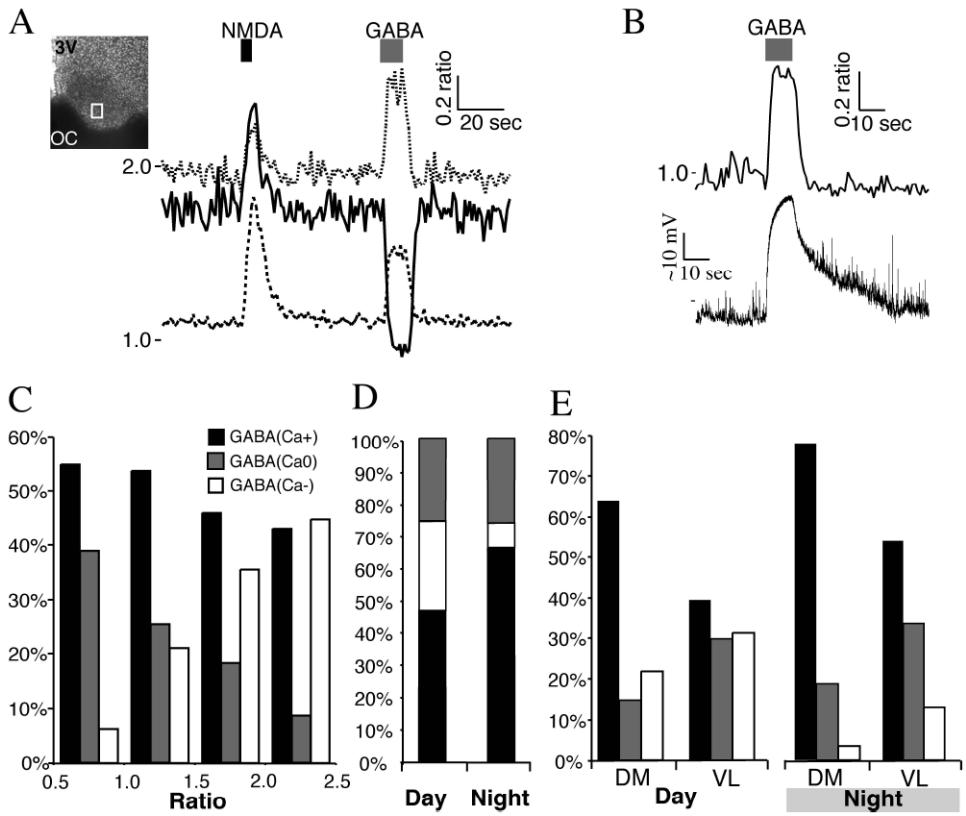

Fig. 4. γ-Aminobutyric acid (GABA) induced divergent Ca^{2+} responses in SCN neurons

(A) SCN neurons imaged during the day, and treated with *N*-methyl-D-aspartate (NMDA; 200 μM, 5 s), which induced an increase in the Ca^{2+} ratio in all neurons. GABA (200 μM, 10 s) application induced divergent Ca^{2+} responses. The box within the SCN indicates the region recorded. Note the range of baseline Ca^{2+} concentrations. (B) Example of a GABA-induced rise in Ca^{2+} ratio and membrane depolarization in a SCN neuron recorded during the night using a loose-seal recording electrode. (C) The Ca^{2+} response to GABA (200 μM, 10 s) application varied with the baseline Ca^{2+} ratio. Ca^{2+} responses were separated into three groups: a transient elevation in the Ca^{2+} ratio [GABA(Ca+)]; a decrease in the Ca^{2+} ratio [GABA(Ca-)]; or no change in the Ca^{2+} ratio [GABA(Ca0)]. Data are the percentage of neurons in each response group based on the baseline Ca^{2+} ratio (range 0.5-1.0, $n = 257$ neurons; 1.0-1.5, $n = 395$; 1.5-2.0, $n = 306$; 2.0-2.5, $n = 56$). Note that the percentages of GABA(Ca-) and GABA(Ca0) neurons appeared reversed between low and high baseline Ca^{2+} ratios. (D) The percentage in the SCN neuronal response to GABA (200 μM, 10 s) varied between the day ($n = 760$) and night ($n = 292$). Note the change in the GABA(Ca+) and GABA(Ca-) groups, but not in the percentage of GABA(Ca0) neurons responding. (E) The percentage of SCN neurons with GABA-induced changes in the Ca^{2+} ratio varied between the dorsomedial (DM; $n = 226$ day and 145 night neurons) and ventrolateral (VL; $n = 534$ day and 147 night) regions of the SCN. The percentage of GABA(Ca+) neurons was higher in the DM during both the day and night. Conversely, the percentage of GABA(Ca-) and GABA(Ca0) neurons was higher in the VL in both day and night. 3V, 3rd ventricle; OC, optic chiasm.

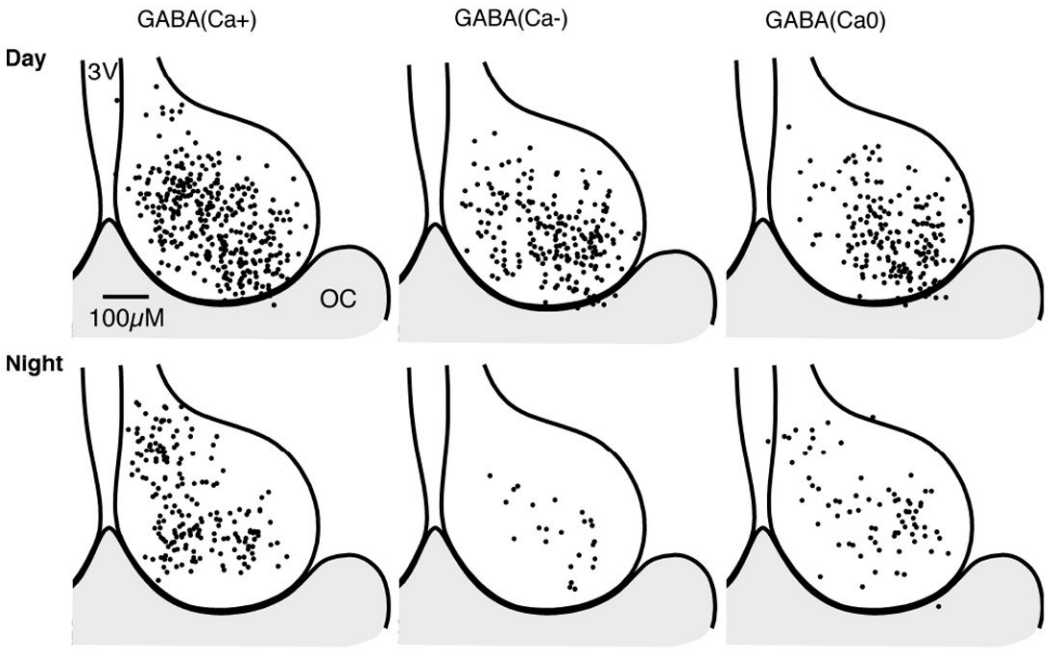

Fig. 5. γ-Aminobutyric acid (GABA)-induced Ca^{2+} responses of SCN neurons varied regionally

Regional and time of day variation of GABA-induced Ca^{2+} responses in SCN neurons (see Fig. 4D and E). The position for each neuron is superimposed on a representative drawing of the SCN, with the 3rd ventricle (3V) on the left and optic chiasm (OC) at the bottom. Note that while the number of cells in the day and night were not equal, the relative proportions varied between the day and the night.

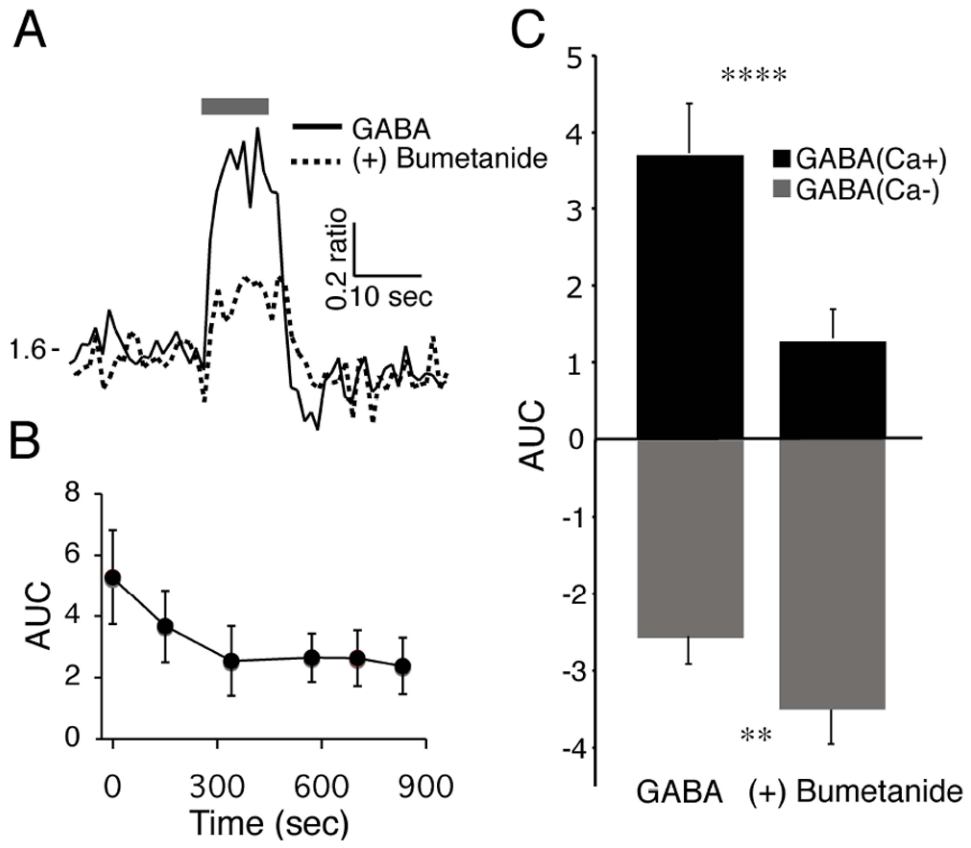

Fig. 6. Blocking the chloride cotransporter NKCC1 attenuated the γ-aminobutyric acid (GABA)-induced elevation of Ca^{2+}

(A) Example of the Ca^{2+} response in a SCN neuron loaded with fura-2 AM and treated with GABA (200 μM, 10 s) during the day before and after bumetanide (10 μM, > 300 s) application. (B) The maximal bumetanide attenuation of the GABA-induced Ca^{2+} transient required > 300 s of treatment. The *y*-axis represents the area under the Ca^{2+} ratio curve [AUC (Ca^{2+} ratio/s), mean ± SEM] during the first 10 s of GABA treatment in 13 GABA(Ca+) neurons. (C) Bumetanide attenuated GABA-induced Ca^{2+} transients in GABA(Ca+) neurons and further reduced the Ca^{2+} ratio in GABA(Ca-) neurons. Data are the mean ± SEM of the AUC (0-10 s) of three different SCN slices for GABA(Ca+) ($n = 39$, $t_{38} = 5.80$, $^{****}P < 0.00001$) and GABA(Ca-) ($n = 29$, $t_{28} = 2.89$, $^{**}P = 0.007$) SCN neurons before and after treatment with bumetanide (> 300 s).

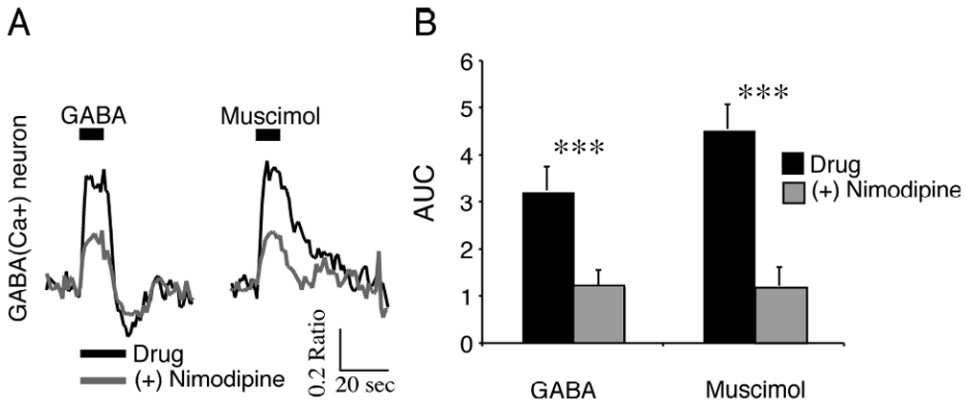

Fig. 7. Blocking L-type voltage-dependent Ca^{2+} channels attenuated γ-aminobutyric acid (GABA)-induced increases of $[Ca^{2+}]_i$

(A) Ca^{2+} responses induced by GABA (200 μM, 10 s) or muscimol (50 μM, 10 s) in a GABA(Ca+) neuron during the day before and after nimodipine (20 μM) application. Both GABA- and muscimol-induced Ca^{2+} transients were attenuated. (B) Nimodipine attenuated GABA- and muscimol-induced Ca^{2+} transients in GABA(Ca+) neurons. Data are the mean ± SEM of the area under the curve (AUC; Ca^{2+} ratio/s over 0-10 s of GABA treatment) for GABA(Ca+) ($n = 30$, $t_{29} = 6.03$ GABA, $t_{29} = 10.6$ muscimol, ***$P < 0.00001$) SCN neurons.

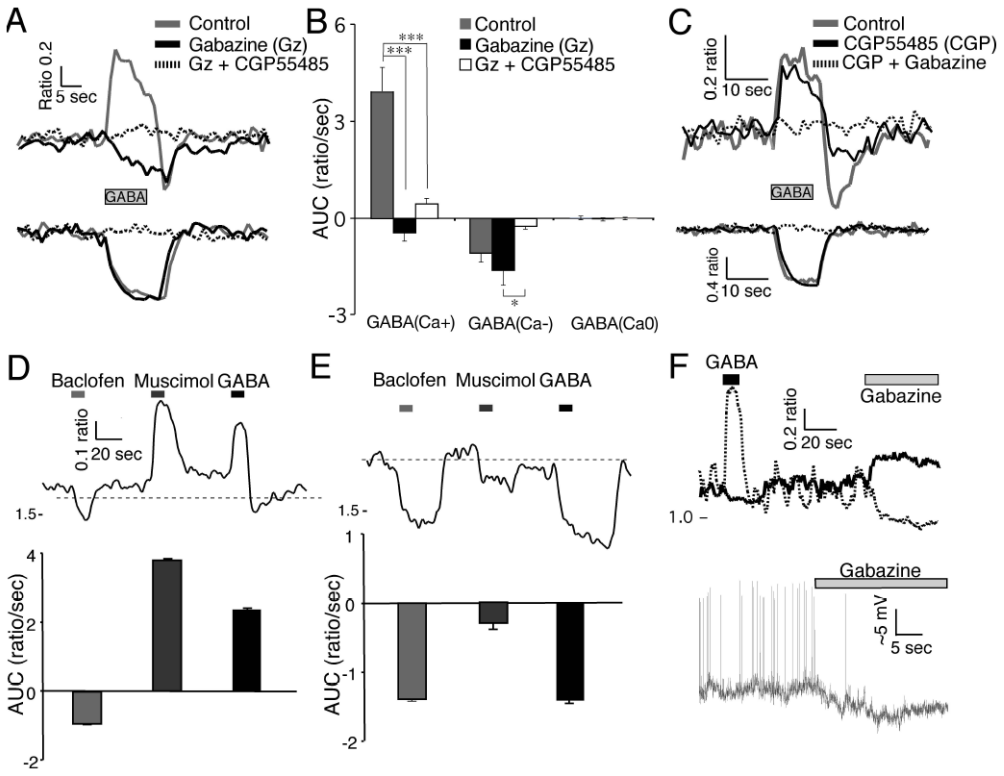

Fig. 8. γ-Aminobutyric acid $(GABA)_A$ and $GABA_B$ receptor-mediated components of the GABA-induced Ca^{2+} response

(A) GABA (200 μM, 10 s) induced Ca^{2+} responses before and during treatment with gabazine (10 μM) followed by gabazine together with CGP55485 (3 μM) in a GABA(Ca+) (top) and GABA(Ca-) (bottom) neuron. Note that the addition of gabazine in this GABA(Ca-) neuron did not attenuate the GABA-induced reduction in Ca^{2+}. (B) Gabazine inhibited the GABA-induced rise of Ca^{2+} but did not alter the GABA-induced reduction of Ca^{2+}. Simultaneous inhibition of $GABA_A$ and $GABA_B$ receptors eliminated the GABA-induced Ca^{2+} changes. Data are the mean ± SEM of the area under the curve (AUC; Ca^{2+} ratio/s over 0-10 s of GABA treatment) of neurons (GABA(Ca+) ($n = 19$, $F_{2,36} = 28.91$, $P < 0.0001$, power = 1.00); GABA(Ca-) ($n = 12$, $F_{2,22} = 6.56$, $P = 0.0058$, power 0.875); GABA(Ca0) ($n = 4$, $F_{2,6} = 0.198$, $P = 0.826$, power = 0.069) from three different SCN slices with repeated-measures ANOVA analysis and *post hoc* Bonferroni *t*-test adjusted for three comparisons [(GABA(Ca+) ***$P < 0.0001$, Gz vs. Gz + CGP55485 $P = 0.46$; GABA(Ca-) control vs. Gz $P = 0.49$, control vs. Gz + CGP55485 $P = 0.13$, *$P = 0.0048$). (C) Example showing GABA-induced Ca^{2+} response during CGP55485 application followed by CGP55485 together with gabazine in GABA(Ca+) (top) and GABA(Ca-) (bottom) neurons. Note that the addition of gabazine in this GABA(Ca-) neuron eliminated the GABA-induced reduction in Ca^{2+}. (D) Effects of baclofen (10 μM), muscimol (50 μM) and GABA (200 μM) on GABA(Ca+) neurons. The top trace is an example of Ca^{2+} responses in a GABA(Ca+) neuron. Below is the mean ± SEM of the AUC in 69 GABA(Ca+) neurons. (E) Effects of baclofen, muscimol and GABA as in (D), but with GABA(Ca-) neurons. Below is the mean ± SEM of the Ca^{2+} change in 21 GABA(Ca-) neurons. (F) The top traces show GABA- and gabazine-induced changes in the baseline Ca^{2+} ratio in two SCN neurons during the night. Gabazine induced a reduction in the baseline Ca^{2+} ratio in the GABA(Ca+) neuron (dashes) and an elevation of the baseline Ca^{2+} ratio in the GABA(Ca-) neuron (solid). The bottom

trace shows an example of gabazine-induced hyperpolarization in a GABA(Ca+) SCN neuron during the night recorded using a loose-seal recording electrode.

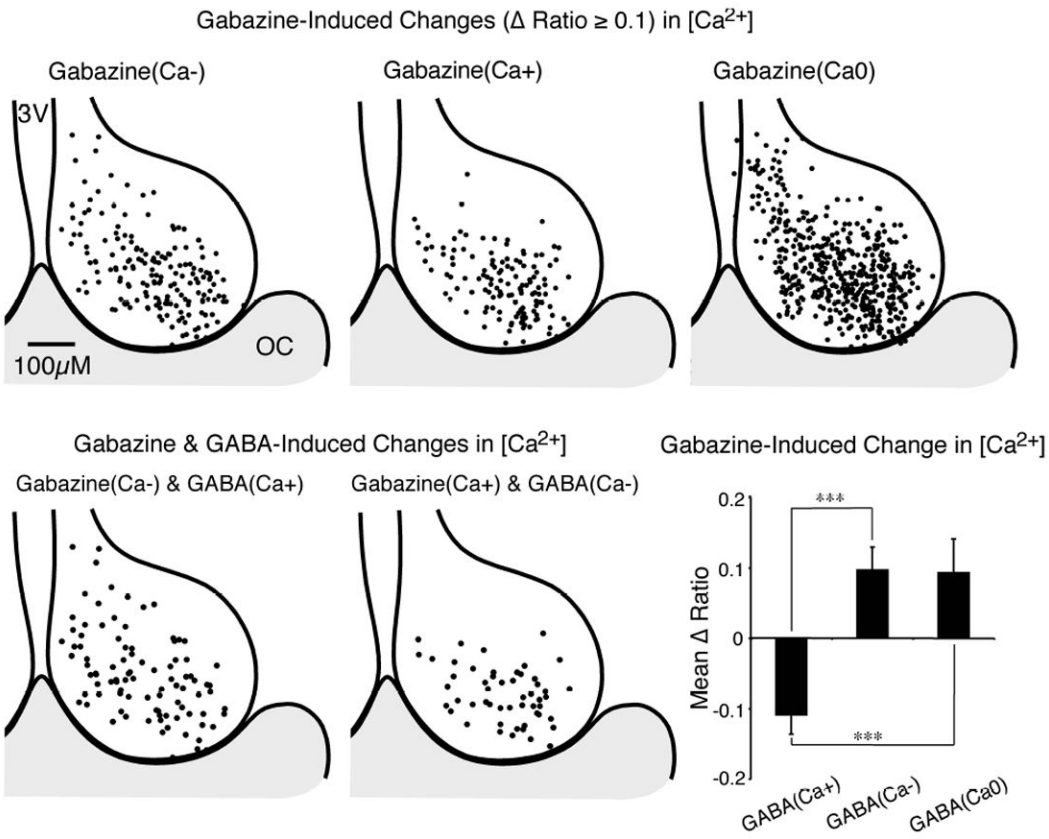

Fig. 9. Inhibiting endogenous γ-aminobutyric acid $(GABA)_A$ alters $[Ca^{2+}]_i$ in SCN neurons

(Top) Regional variation of gabazine-induced (10 μM) changes in baseline Ca^{2+} ratio in SCN neurons. Responses were separated into three groups: a decrease of the Ca^{2+} ratio [gabazine(Ca-), top-left]; an increase of the Ca^{2+} ratio [gabazine(Ca+), top-middle]; and a non-responsive group Ca^{2+} [gabazine(Ca0), top-right]. The position for each neuron was superimposed on a representative drawing of the SCN, with the 3rd ventricle (3V) on the left and optic chiasm (OC) at the bottom. (Bottom left) Regional localization of individual SCN neurons with a GABA (200 μM)-induced increase in Ca^{2+} and a gabazine-induced decrease in Ca^{2+} (see Figs 5 and 8F). (Bottom middle) Regional variation of individual SCN neurons demonstrating both a GABA-induced decease and a gabazine-induced increase of $[Ca^{2+}]_i$. (Bottom right) The mean change in the baseline Ca^{2+} ratio produced by gabazine was in an opposite direction to the Ca^{2+} response evoked by GABA. Data are the mean ± SEM of the Ca^{2+} ratio change in gabazine-responsive neurons for GABA(Ca+) ($n = 125$), GABA(Ca-) ($n = 77$) and GABA(Ca0) ($n = 65$) neurons ($F_{2,264} = 14.74$, $P < 0.0001$, power = 1.00) by ANOVA with Bonferroni t-tests adjusted for three comparisons, ***$P < 0.0001$. Note that GABA(Ca-) and GABA(Ca0) respond similarly ($P = 1$).

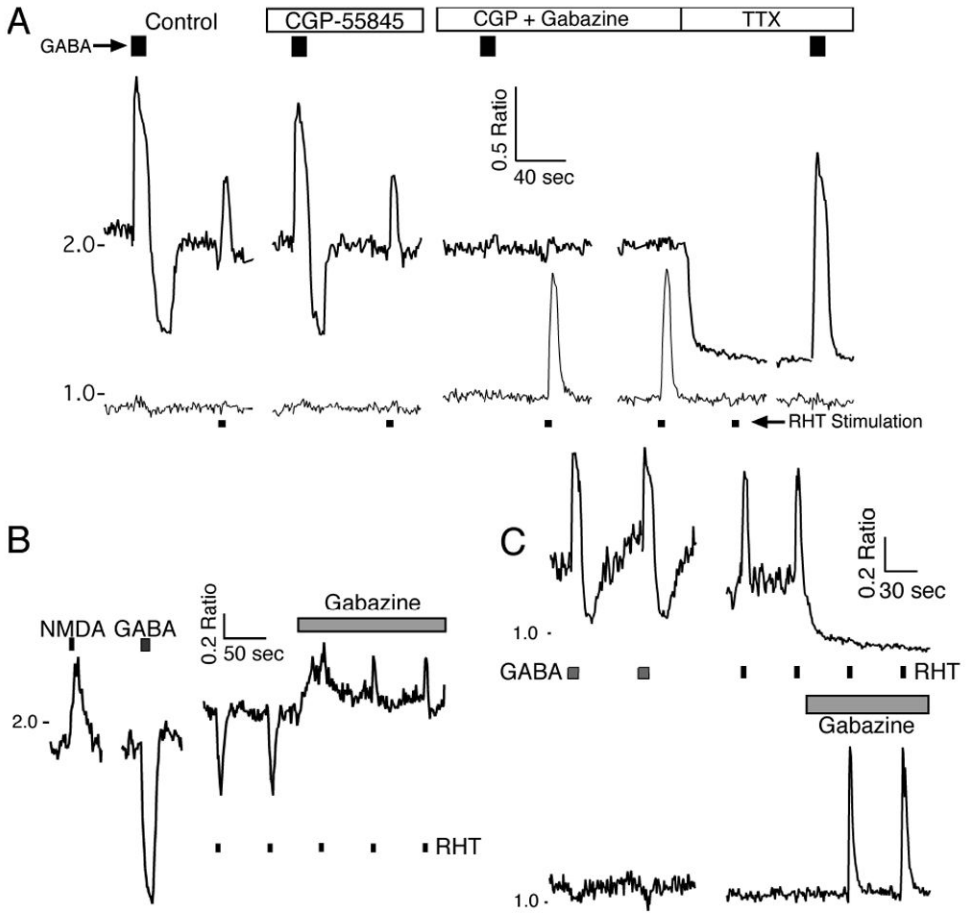

Fig. 10. Retinohypothalamic tract (RHT) stimulation induces a range of postsynaptic Ca^{2+} responses

Stimulation of the RHT (100 pulses, 200 μs at 20 Hz) via a bipolar electrode placed in the optic chiasm (Fig. 1A) induced a transient elevation in Ca^{2+} [RHT(Ca+)], a transient reduction in Ca^{2+} [RHT(Ca-)] or no alteration in the Ca^{2+} ratio [RHT(Ca0)]. (A) The Ca^{2+} response to RHT stimulation and g-aminobutyric acid (GABA; 200 μM, 10 s) in two SCN neurons recorded during the day. CGP55845 (3 μM) application did not alter the RHT-induced Ca^{2+} signal in either cell, but the addition of gabazine (10 μM) eliminated the RHT-induced Ca^{2+} transient in the GABA(Ca+) neuron and induced a RHT-evoked Ca^{2+} response and a small elevation in the baseline Ca^{2+} ratio in the GABA(Ca0) neuron. Tetrodotoxin (TTX; 0.5 μM) eliminated the RHT-induced Ca^{2+} transients in both neurons. Note that GABA induced a biphasic Ca^{2+} response in the neuron with the higher baseline Ca^{2+} ratio (top trace), and the $GABA_B$ blocker reduced the duration of the later phase. Blocking both $GABA_A$ and $GABA_B$ eliminated both phases, and TTX reduced the baseline Ca^{2+} ratio but did not eliminate the rise in Ca^{2+} induced by GABA. (B) Example of a RHT(Ca-) response in a GABA(Ca-) SCN neuron during the day. Gabazine elevated the baseline Ca^{2+} and changed the direction of the RHT-induced Ca^{2+} response [RHT-Gz(Ca+)]. (C) Example showing the Ca^{2+} response following RHT stimulation in GABA(Ca+) and GABA(Ca-) neurons recorded during the night. Gabazine lowered the baseline Ca^{2+} in the GABA(Ca+) neuron and eliminated the RHT-induced transient elevation of Ca^{2+} [RHT-Gz(Ca-)]. In contrast, gabazine increased the RHT-induced Ca^{2+} elevation in a GABA(Ca-) neuron. NMDA, *N*-methyl-D-aspartate.

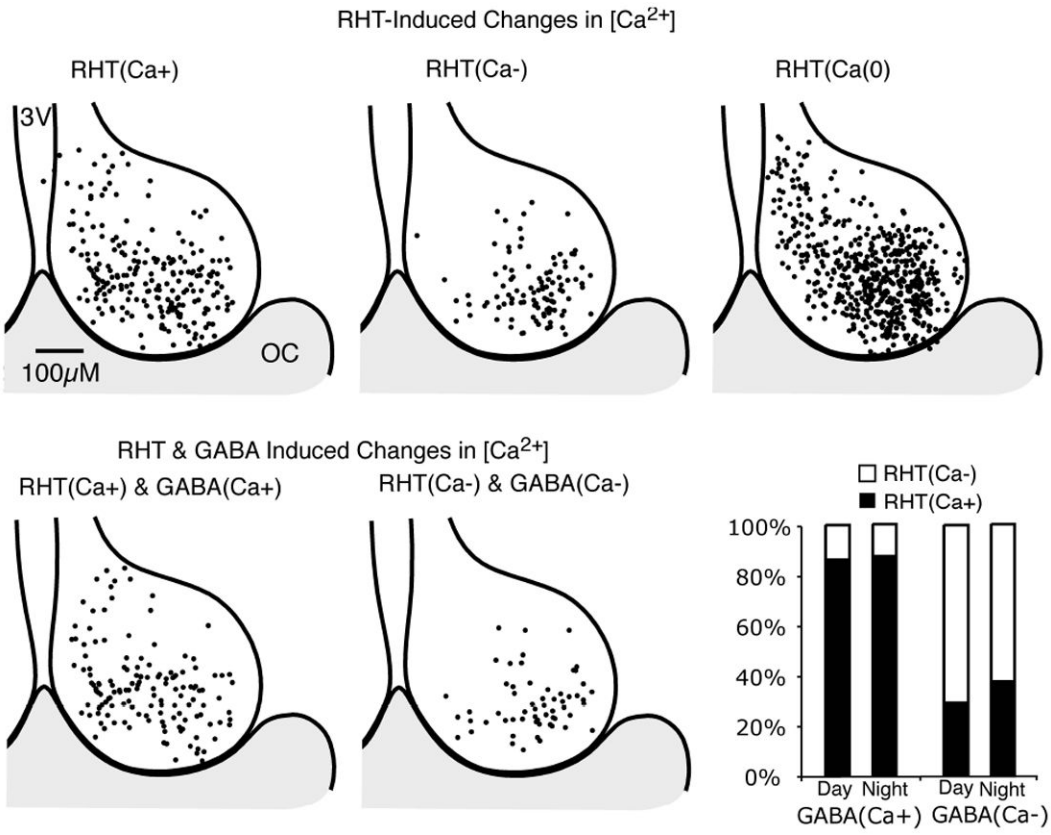

Fig. 11. Regional variation of retinohypothalamic tract (RHT) stimulation induced Ca^{2+} transients in the SCN

(Top) Regional variation in Ca^{2+} responses following optic chiasm (OC) stimulation (100 pulses at 20 Hz). Responses were separated into three groups: a transient elevation in Ca^{2+} [RHT(Ca+)]; a reduction [RHT(Ca-)]; and no alteration in the Ca^{2+} ratio [RHT(Ca0)]. The position for each neuron was superimposed on a representative drawing of the SCN, with the 3rd ventricle (3V) on the left and OC at the bottom. The distribution appears similar to that of the Ca^{2+} response to GABA (Fig. 5). (Bottom left) Regional variation of individual SCN neurons that showed both a RHT stimulation-induced and GABA-induced (200 μ_M) increase in Ca^{2+}. (Bottom middle) Regional variation of individual SCN neurons with a RHT- and GABA-induced decease of Ca^{2+}. (Bottom right) The percentage of GABA(Ca+) (day n = 94, night n = 71) and GABA(Ca-) (day n = 76, night n = 8) SCN neurons also responding to RHT stimulation.

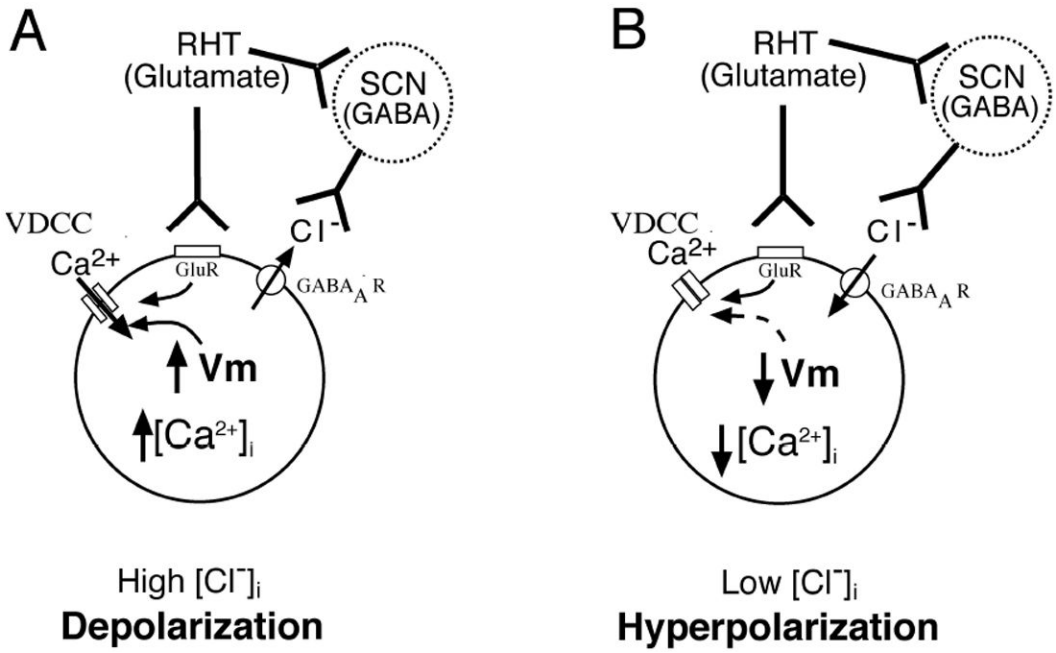

Fig. 12. Theoretical model of glutamatergic light input signaling to the g-aminobutyric acid (GABA)ergic suprachiasmatic nucleus (SCN) network

The effect of GABA on membrane potential and $[Ca^{2+}]_i$ is a balance, with excitatory and inhibitory effects of GABA neurotransmission within the network driven by the chloride gradient that is under control by the cotransporters, NKCC1 and KCC2. (A) Depolarization induced by external light input signaling to glutamate receptors (GluR) via the retinohypothalamic tract (RHT) is facilitated by GABA release from other SCN neurons (dashed circle) within the network to a SCN neuron (solid circle) with a high intercellular chloride concentration ($[Cl^-]_i$), where opening of $GABA_A$ receptors ($GABA_AR$) moves Cl^- out of the neuron, further depolarizing the membrane potential (V_m), opening voltage-dependent calcium channels (VDCC) and increasing $[Ca^{2+}]_i$. (B) In neurons with low baseline $[Cl^-]_i$, GABA released from the SCN network opens $GABA_AR$ and moves Cl^- into the neuron hyperpolarizing the cell beyond the depolarizing effect of RHT signaling and decreasing $[Ca^{2+}]_i$. A balance may also occur between the depolarizing action of RHT light input signaling with a hyperpolarizing effect from GABA release within the network, and no change in membrane potential, firing frequency or $[Ca^{2+}]_i$ occurs.