RESEARCH REPORT

Diversity in the structure of action potential-mediated neural connectivity within rat supra chiasmatic nucleus

Cheolhong Min1* | Hyun Kim1* | Wonshik Choi1,2 | Kyoung J. Lee1

1Department of Physics, Korea University, Seoul, Korea
2Center for Molecular Spectroscopy and Dynamics, Institute for Basic Science, Seoul, Korea

Correspondence
Kyoung J. Lee, Department of Physics, Korea University, Seoul 02841, Korea.
Email: kyoung@korea.ac.kr

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Abstract
Action potential (AP)-mediated cell-to-cell communication is essential for the frequency-locking and phase-synchronization of the clock cells within the biological master clock, suprachiasmatic nucleus (SCN). Nevertheless, the morphology of its network connectivity is largely unexplored. Here, with an optimized optogenetic light-stimulation and scanning protocol, we report some key characteristics of the inhibitory receptive field (IRF), the area which brings inhibitory synaptic currents to a given target cell, and basic statistics of the inhibitory network connections of rat SCN clock cells. ChR2 transfected, slice cultures of rat SCN were stimulated by a blue power LED light in a repetitive box-scanning modes, while a target cell was whole-cell patched. The registered inhibitory postsynaptic currents, which were brought by light-induced APs of presynaptic neurons, were mostly GABAergic. The sizes and shapes of IRFs of SCN cells were very diverse, and the number of presynaptic cells making up the IRF of a given target cell followed an exponential distribution with an average value of 8.9 approximately, according to our clustering analysis which is based on a hybrid measure $D$, combining the physical distance $r$ and the difference in the current amplitudes of two different sites. Although this estimate inevitably depends on the construct of the measure $D$, it is found not so sensitive on the parameter $w$, which weighs the relative significance of the current amplitude different with respect to the physical distance $r$: The average number of presynaptic neurons varies $<26\%$ over a significant range of $0<w<30$. On average, the presynaptic connection number density around a target cell falls off as an exponentially decreasing function of $r$. But, its space constant ($\sim 210.7 \, \mu m$) is quite large that long-range ($>210.7 \, \mu m$) neural connections are abundant ($>66.9\%$) within the SCN.

KEYWORDS
neural connectome of SCN, node-degree distribution, receptive field of SCN clock cells

Abbreviations: AP, action potential; ARF, action-potential receptive field; AVP, arginine vasopressin; DMD, digital mirror device; GRP, gastrin-releasing peptide; IRF, inhibitory receptive field; LED, light emitting diode; SCN, suprachiasmatic nucleus; SD, standard deviation; VIP, vasoactive intestinal polypeptide

*Equally contributing two first authors.

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INTRODUCTION

The suprachiasmatic nucleus (SCN) is the master clock in mammals, which orchestrates all the circadian rhythms of behavior, metabolism, and physiology in mammals. It is formed by a pair of two nuclei, which are located in the hypothalamus, where the two optical nerve tracks emanating from the two eyes cross (Mohawk & Takahashi, 2011; Welsh, Takahashi, & Kay, 2010). Each SCN nucleus is composed of approximately 10,000 neuronal cells that can be viewed as a nonlinear oscillator generating its own, almost sinusoidal, circadian rhythm (Welsh, Logothetis, Meister, & Reppert, 1995). The rhythmic activity can be seen as modulations in the spontaneous firing rate of action potentials (APs) (Colwell, 2011; Jones, Tackenberg, & McMahon, 2015) as well as in the expression levels of various clock genes (Abrahamson & Moore, 2001) and in the concentration of cytosolic-free calcium ions (Hong, Jeong, Min, & Lee, 2012; Ikeda, 2004; Ikeda et al., 2003).

A heterogeneous clock-cell population makes up the SCN (Schaap et al., 2003). Most notably, clock cells’ intrinsic circadian periods cover a broad range (20–28 hr), as clearly demonstrated by Welsh et al. (1995) with dissociated cell cultures. Thus, the coherent (nearly) 24-hr rhythmicity of an intact SCN is possible only when several inter-cellular coupling mechanisms are at work. First of all, neuro-peptides like vasoactive intestinal polypeptide (VIP) and arginine-vasopressin (AVP), which are released by the SCN cells, diffuse to their neighboring cells and influence their circadian phases (Antle & Silver, 2005; Aton, Colwell, Harmar, Waschek, & Herzog, 2005). Physically, such a diffusion-mediated, neighboring, cell-to-cell interaction (i.e., non-synaptic neural communication) is short-range in space, because of its slowness. Another important cell-to-cell coupling mechanism for the SCN is mediated by APs and chemical synapses. Several earlier studies recognized the significance of this mechanism as for the proper function of the SCN. For example, an application of tetrodotoxin (TTX) to the organotypic slice culture of the SCN reduced the amplitude of circadian oscillations in SCN neurons (Yamaguchi et al., 2003), and the overall sinusoidal circadian waveform became disrupted (Maywood, O’Neill, Chesham, & Hastings, 2007). Buhr, Yoo, and Takahashi (2010) reported increasing phase-decoherence following a TTX application. The AP-triggered synaptic transmissions through GABA_\(_A\) receptors, which are known as the predominant type of ionotropic receptor mediating intra-communication in the SCN (Albus, Vansteensel, Michel, Block, & Meijer, 2005; Aton, Huettner, Straume, & Herzog, 2006; Evans, Leise, Castanon-Cervantes, & Davidson, 2013; Liu & Reppert, 2000; Liu et al., 2007; Moore & Speh, 1993), can be conceptually long-range covering the entire range of the SCN through the neural network. As a matter of fact, several studies (Albus et al., 2005; Evans et al., 2013; Liu et al., 2007) have suggested that GABAergic synaptic transmission is essential in synchronizing and resetting circadian rhythms in the SCN. Nevertheless, the structure of the SCN neural network is largely unexplored.

Jacomy, Burlet, and Bosler (1999) reported that there are on average 5.4 VIP contacts per AVP clock cell and 1.7 AVP contacts per VIP clock cell (of adult male Sprague-Dawley rats), estimations based on immunocytochemical and electron-microscopic images. A more recent study, which was based on opto-genetic mapping experiments conducted with ChR2-transgenic, male and female, mice (P25–P35), facilitated by a statistical inference, suggested that SCN neurons have approximately two to three presynaptic connections on average and VIP– neurons have more VIP+ presynaptic connections than the VIP+ neurons do (Fan et al., 2015). None of these studies, however, has addressed the morphology of the neural network or the receptive field of individual SCN neuronal cells, where “receptive field” means the topographic area covered by the presynaptic neurons of a given target cell. Characterizing the receptive field is indispensable for understanding the working principles of the SCN and its functions as a master clock, as the dynamics of a coupled network of nonlinear oscillators such as the SCN depends very much on the nature of cell-to-cell connections (Kuramoto, 2011; Nkomo, Tinsley, & Showalter, 2013; Strogatz, 2000).

Accordingly, in this paper we developed and used a custom-built digital optogenetic mapping system alongside an optimally designed spatiotemporal light stimulation/scanning protocol for measuring the areal sizes and shapes of GABA_\(_A\) receptor-mediated inhibitory receptive fields (IRFs) of different target SCN cells that were chosen randomly over many different locations throughout the entire (organotypic cultures of 3–5 days old, male and female, Sprague-Dawley rat) SCNs. The analyses were essentially based on light-evoked GABA_\(_A\)-mediated, inhibitory postsynaptic currents (IPSC). For that matter, whole-cell (instead of gramicidin-perforated) patch was used in order to clamp the concentration of intracellular Cl\(^-\) ([Cl\(^-\)]\(_i\)) always low enough relative to that of outside ([Cl\(^-\)]\(_o\)) so that the connectivity artifacts possibly caused by the circadian modulation in [Cl\(^-\)] (Klett & Allen, 2017) and the subsequent (inhibitory-excitatory) switching action of GABA_\(_A\) receptor channels over a day/night circadian cycle (Cho et al., 2008; De Jeu & Pennartz, 2002) could be avoided. Finally, based on a clustering algorithm we analyzed the number and locations of presynaptic sources that made up the receptive fields.

We find that GABA_\(_A\) receptor-mediated cell-to-cell connections within the SCN are sparse (in comparison to average thousands of synaptic connections per neuron in the rat or mice brain); yet, our estimate of the number of presynaptic connections (on average, 8.9 per one responsive cell) is significantly larger than what were claimed
earlier. The afferent connections converge from all different areas within the nucleus and often they are very far-reaching. On average, the presynaptic connection number density around a target cell is best described by an exponentially decreasing function of \( r \), yet its space constant is quite large (210.0 μm) that long-range synaptic connections are abundant inside the SCN.

2 | MATERIALS AND METHODS

2.1 | SCN slice culture and ChR2 viral transfection

All the animal experiments in this study were approved by our institutional review board for animal research, the Korea University Institutional Animal Care and Use Committee (KUIACUC-20170405-2). The following methods were carried out in accordance with the approved guidelines.

Organotypic SCN slice cultures were prepared as described in our previous paper (Hong et al., 2012). Briefly, postnatal 3–5 days old (P3–5) Sprague-Dawley rats (Crl: CD [SD], Orient Bio., South Korea) bred under a 12:12 hr light-dark cycle at room temperature (22–24°C) were used: total number of animals used = 56). The rats were decapitated using surgical instruments and their brains were removed and immersed in a dish containing ice-cold slush of oxygenated 95% O2, 5% CO2 dissecting solution (in mM: 138.6 NaCl, 3.35 KCl, 0.6 NaH2PO4, 21.0 NaHCO3, 9.9 d-glucose, 0.5 CaCl2, 5.0 MgSO4, Sigma-Aldrich). A 400-μm thick coronal hypothalamic slice containing both SCNs was cut using a vibroslicer (NVSL; World Precision Instrument, FL). Generally, three coronal brain slices were obtained per brain, of which only the middle one was used for this study. Then, the slices were transferred to a cell insert cup (PICM03050, Millicell-CM; Millipore, MA) and placed in a standard six-well plate (140675; Nunc). Within each brain slice, the diameter of each SCN was about 400 μm. A culture medium containing 1 ml of 57% heat-inactivated horse serum (Gibco), 25 g/ml d-glucose (Sigma-Aldrich), 14% Eagle’s balanced salt solution (Gibco), 1x glutamax (glutamax-I; Gibco), kanamycin (Gibco), 28% Eagle’s basal medium (Gibco) was used. The organotypic cultures were maintained in a commercial incubator at 36°C with 5% CO2, and the culture medium was replaced twice per week.

The ChR2 AVV virus (rAAV2/hsyn-hsyn-hchR2 (H134R) - EYFP-WPRE-PA, 5.7 × 10^{12} virus per ml dialysed with 350 mM NaCl and 5% d-Sorbitol in PBS, UNC, GTC, Vector Core) solution (0.5–1 μl) was injected in the vicinity of the 3rd ventricle and rostrocaudal center of the brain slices by using a micropipette after the brain slices were cultured for 7–8 days in vitro. The degree of ChR2 expression was assessed by eYFP fluorescence imaging on an inverted epifluorescence microscope (with 10× or 20× object lens, IX70; Olympus) by using the light passed through a 465–495 nm bandpass filter from a mercury arc lamp. Typically, two to three weeks after the injection of the ChR2 AVV virus, the level of ChR2 expression reached its peak. Only the SCN slices fully expressing ChR2 were used for this study.

2.2 | Patch-recording

A ChR2-expressing brain slice culture was carefully cut out from the cell inserts cup, trimmed, and transferred to a recording chamber, which was superfused with ACSF (124 mM NaCl, 10 mM d-glucose, 26.2 mM NaHCO3, 3.5 mM KCl, 1 mM NaH2PO4, 1.3 mM MgSO4, 2 mM CaCl2, 25–30°C, pH 7.4, 50 mg/l gentamicin) with a flow rate of about 0.3 ml/min at room temperature. The ACSF solution was continuously bubbled with 95% O2/5% CO2. An internal solution (containing 10 mM HEPES, 2 mM MgCl2, 1 mM CaCl2, 11 mM EGTA, 2 mM K2-ATP, pH 7.3 with KOH; osmolality 285–290 mosmol/kg) was used for the whole-cell patch microelectrode pipette (2 μm tip diameter; 5 MΩ resistance). A whole-cell patch recording was used, over glicemicin-perforated patch recording, primarily to suppress the possible modulation of [Cl\(^-\)] inside the cytosol, which could be caused by the circadian modulation of potassium-chloride cotransporters (Klett & Allen, 2017), as our optogenetic mapping is based on the inhibitory (primarily Cl\(^-\)) current mediated by GABA\(_A\) receptor channels. In addition, perforated patch method usually takes a much longer time (>30 min) than the whole-cell patch method (<5 min) for stabilization, and the dye (Alexa 594) loading for the target cell visualization is also much more effective with a whole-cell patch. Membrane potential and current were measured by using a pCLAMP 9.0 data acquisition/analysis software program (Axon Instruments, Union City, CA) with an Axon 700A amplifier and a Digidata 1332 in gap-free mode. Sampling rate was 20 kHz. The holding potentials of 0 and −70 mV were used to measure IPSCs and excitatory postsynaptic currents, respectively. Other details on the whole-cell patch recordings can be found in our previous paper (Hong et al., 2010).

2.3 | Light stimulation and mapping

ChR2-transfected, organotypic slice cultures of rat SCN were stimulated by a blue power LED (M470L2-C1, Thorlabs; 477 nm excitation wavelength, intensity 3.8 mW/cm² at the sample plane), while the whole-cell patch recording was performed for a randomly chosen SCN neuron. For creating different spatiotemporal light-scanning modes, a custom-controlled digital mirror device (DMD; Texas Instrument) was used in combination with a microscope (TCS SL & DM IRB, Leica, Germany) on an optical table. Once a target SCN neuron was selected and successfully patched under the inverted microscope, a small blue
LED light box (50 × 50 μm²) was (point-)scanned across the sample SCN with a pattern controlled by a laboratory-built C# program. Selection of a suitable size of light box for point-scan is a delicate matter as it not only relates to the spatial resolution of the resulting IRF map but also directly influences the amount of ChR2 channel-mediated cations current being generated on ChR2 channel-expressing neurons (see Figure S1). We determined the scan box size of 50 × 50 μm² is, generally large enough to cause a presynaptic neuron to fire and at the same time small enough not to host multiple presynaptic neurons simultaneously (see Figures S2 and S3 for a detailed assessment). Thus, throughout the paper we used the light box size of 50 × 50 μm² for constructing IRFs.

A rectangular area of 4.8 × 10⁵ μm², which spans a large portion of an SCN, is covered by 192 grid boxes, each of which has an area of 50 × 50 μm². Normally, the light-box stimulation was sequentially irradiated from top to bottom and from the left to the right. For all cases, each stimulation delivered a light pulse (exposure time) of 10 ms per each grid box, which was normally repeated either two or five times at an interval of 100 or 200 ms. Then, the full scan (i.e., complete scan of the whole area of 4.8 × 10⁵ μm²) was repeated five times for all experiments. Because of the technical difficulty associated with the whole-cell patch-clamping technique and the large amount of time needed for scanning the sample in order to construct the IRF map, in practice, only one (or two at best) IRF map could be mapped out for a given SCN culture sample. The patch-clamp recording device was synchronized with the DMD light-control unit by an external trigger signal.

2.4 | Target cell visualization

The morphology of the patched neuron was visualized by adding Alexa 594 dye (25 μM, Sigma) in the patch pipette solution. After the connectome-mapping experiment was over, the sample was transferred to a laser scanning confocal microscope (with 10× or 20× water-immersion objective lens, FV500 & BX51WI, Olympus, Japan) and the shapes of the target neurons were imaged.

2.5 | Pharmacological tests

Several different pharmacological agents were used to examine the nature of neural connections. TTX (2.5 μM, Sigma) is a well-known sodium-channel blocker, which silences all AP activity; 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μM; Sigma) is an AMPA/KA GluR antagonist; and bicuculline (BIC, 20 μM; Tocris) is a γ-aminobutyric acid type A (GABA_A) antagonist. Stock solutions were made with phosphate-buffered saline, and a working solution was prepared by diluting the stock solution with regular ASCF. For the Ca²⁺-free medium, MgCl₂ was substituted for CaCl₂, and 0.1 mM 1,2-bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), a specific Ca²⁺ chelator, was added in the ACSF.

2.6 | Data and image analysis

All off-line signal analysis and image processing was made by our custom-made programs based on Matlab subroutines.

2.7 | Statistical analysis

Averages are expressed as mean ± SD. For Student's t-test, significance was accepted when p < 0.05. For all histograms, maximum likelihood estimation method was used for a good fit with chi-square test p < 0.05.

2.8 | Clustering analysis for distinguishing different presynaptic sources

Independent presynaptic neurons that comprise a given IRF are identified following a DBSCAN clustering algorithm (Schubert, Sander, Ester, Kriegel, & Xu, 2017), which classifies elements (i.e., grid boxes) into different clusters (i.e., presynaptic neurons) based on the distance function \( D = \sqrt{r^2 + (w \Delta I_{\text{max}})^2} \) that we defined, where \( r \) is the physical distance between two grid boxes, \( \Delta I_{\text{max}} \) is the difference in the \( <\Delta I_{\text{max}} >/s \) obtained at the two grid boxes, and \( w \) is a weighting factor controlling the relative significance between \( r \) and \( \Delta I_{\text{max}} \). There are several reasons behind the selection of DBSCAN over other well-known clustering algorithms. First, DBSCAN does not require one to specify the number of clusters in the data a priori, as opposed to, for example, k-means clustering or hierarchical clustering: In fact, one of the main purposes of the clustering analysis for us is to find the number of clusters (independent presynaptic sources) itself. Second, it is able to find arbitrarily shaped clusters quite well: We find the shapes (or ARFs) of SCN neurons are often elongated and vary a lot from one to the others. Third, it can also recognize arbitrarily sized (including very small domains of few grid boxes) as separate clusters. Fourth, DBSCAN requires just two parameters: minPts and \( \varepsilon \), where minPts stands for the minimum number of points and \( \varepsilon \) is the maximum radius of the neighborhood. We chose \( \text{minPts} = 1 \), so that well-separated single grid boxes can be recognized as an independent presynaptic source. We decreased the parameter value of \( \varepsilon \) (from 145 μm) iteratively until the resulting maximum cluster size becomes smaller than the area of 15 grid boxes (15 × 50x50 μm²), which was the maximum size of ARF that we measured in our experiment. In our analysis, we have kept the value of \( w = 10 \) for all cases, and \( r \) and \( \Delta I_{\text{max}} \) were measured in the unit of number of
grids and pA, respectively. Our choice of \( w = 10 \) confers approximately a 1:1 balance between the physical distance and the difference in the amplitudes of the average IPSCs of two grid points.

3 | RESULTS

3.1 | Mapping IRF with light-evoked IPSCs

Figure 1A shows a complete neural connectome mapping system, including a ChR2-transfected organotypic slice culture of a rat SCN (shown with pseudo-color green), a laboratory-built light-box point-scanning system, and a patch-clamp recording device that monitors the postsynaptic currents of the chosen target cell (marked with pseudo-color red in Figure 1A) in a whole-cell voltage clamping mode. Once a target cell was chosen and successfully patched, its spontaneous IPSCs were monitored continuously at a holding potential of 0 mV, to be compared later with light-evoked IPSCs. The peak amplitudes of spontaneously generated IPSCs fluctuated significantly in time; and for the marked target cell, they were measured to be 22.7 ± 8.4 pA (mean ± SD of \( n = 2,529 \) successive IPSC peaks; see Figure 1B). Then, a small light box (mesh size of 50 × 50 μm²) was point-scanned from left to right and from bottom to top of the rectangular area (16 × 12 grid covering a total area of 800 × 600 μm²), and the whole sequence was repeated five times periodically. For each visit of a grid box, a light ON-pulse of 10 ms duration was delivered twice in succession at a time interval of 200 ms; otherwise, the light was off during the remaining time of each 1 s window before the light box moved on to the next grid box (see Figure 1C). The two time-cued, repetitive stimulations were employed in order to distinguish light-evoked postsynaptic currents from spontaneous random synaptic currents that were autonomously generated. Therefore, during a complete mapping session each grid box was stimulated 10 times in total.

Figure 1C shows four representative average (detrended) current traces (with SD values shown as the background shades) of the target cell, recorded while a pair of two successive light box-stimulations was given at four different grid boxes labeled by a, b, c and d (as marked in Figure 1A). An AP-evoked IPSC having a significant peak amplitude was registered at the target cell whenever a light-box stimulation was delivered either at the grid box of a or b (\( P_{\text{max}}^{\text{a}} = 26.6 ± 5.8 \) pA, \( P_{\text{max}}^{\text{b}} = 27.7 ± 12.3 \) pA, [mean ± SD of the first peaks of a and b]): As an example, the five raw traces of the evoked IPSCs corresponding to the average profile of Figure 1Ca are given in Figure 1D. The average form of evoked IPSCs (over five repetitions) fits well to a function \( I(t) = I_0 e^{-\alpha t}(1-e^{-\beta t}) \) with \( I_0 \), \( \alpha \) and \( \beta \) being the peak current amplitude and time constants for the rising and decaying phases, respectively; see the green dotted lines in Figure 1C as an example. Only randomly scattered spontaneous events were recorded for the case of c and d, and their emergences are not correlated with the times of stimulations (see the corresponding raster plots in Figure 1E); thus, the average current traces over five repetitive runs smoothed them out as shown in Figure 1Cc and Cd. In fact, the grid box d actually lay within the third ventricle (3V) area, which was devoid of any neurons; so, there should be no light-evoked IPSCs originating from the box d. In general, the amplitude of the induced IPSC varied significantly depending on the location where the light stimulation was given as well as on the cell being monitored. Another notable feature of the evoked IPSCs was that their amplitudes got reduced with a premature stimulation. In general, the IPSC peaks following the second light box-stimulation were smaller than the matching first peaks, as is clearly reflected in the time trace a and b of Figure 1C. This amplitude reduction seemed to result from the long recovery time of the ChR2 cation channels of the presynaptic neurons, as will be discussed later.

Several features are noteworthy in the raster plots of Figure 1E, where each dot marks the peak positions of (both evoked and spontaneous) IPSCs and the color of each dot represents the local peak value (\( P_{\text{max}}^{\text{d}} \)) of detrended IPSC. First of all, as expected for SCN neurons, many of which autonomously fire at a high frequency (5–10 Hz), there were many spontaneous random events of IPSCs across the entire grid that...
were uncorrelated with the times of light-box stimulations: The highlighted grid boxes c and d, matching the third and fourth current time traces in Figure 1C, are a typical such example. Second, there is an issue of fidelity. That is, stimulating one of the presynaptic neurons (grid boxes) does not always guarantee an IPSC for the postsynaptic target cell. In order to achieve “100% fidelity,” there should be at least one almost immediate IPSC registered at the target cell every time a light
pulse is delivered, both at \( t = 0 \) and at \( t = 200 \) ms. None of the grid boxes in Figure 1E actually meets this 100% fidelity requirement. By far, the grid box a shows the highest fidelity, of 90%, by missing only one peak that should have come following the second stimulation of the fifth full-scan round (the missing peak position is marked by an empty square in the blown-up image of a in Figure 1E or its corresponding raw IPSC trace shown in Figure 1D); likewise, the grid box b is also missing two peaks matching the second stimulation of the second and third full-scan rounds (see the blown-up image of b in Figure 1E). In general, several peaks can be skipped and missed, and typically skipping episodes are more pronounced with the second stimulation than with the first of each pair.

Clearly, there are quite a few spontaneous IPSC events, which cannot be easily distinguished from evoked IPSCs, registered at the target cell. For a given cell, they look very much alike; for example, the maximum IPSC amplitude statistics is barely distinguishable from one another (see Figure 1B), except that the evoked IPSCs may have a slightly larger mean maximum amplitude 26.7 pA than that (22.7 pA) of spontaneous (stimulation-independent) IPSCs. Therefore, spontaneously emerging IPSCs seriously hinder our SCN neural connectome analysis and warrant the multiple rounds of the time-correlated stimulation protocol that we have developed and used for this study. The large fluctuation as well as the imperfect fidelity of evoked IPSCs also makes it difficult to determine the IRF. Hence, we need to set up reasonable criteria to decide which grid box contains one (or perhaps more) presynaptic neuron(s) to be a part of the IRF of a given target neuron.

Subsequently, we require the following conditions for a grid box to be a part of the IRF. First, all evoked IPSCs that are meaningful must have an average peak amplitude >10 pA. Second, at least three significant IPSC responses must be generated, for a given set of five stimulations (either for the first or the second set) and their times of emergence must be repeatable within a time window of 20 ms. Third, the average IPSC (over five trials) should fit well to a profile of \( I(t) = e^{-\alpha t} (1 - e^{-\beta t}) \) with a fitting score better than 0.8 (out of 1.0). According to this working definition, the IRF of the target neuron marked in Figure 1A is fragmented and dispersed over the entire nucleus, as it is indicated by the red shade in Figure 1E. The same rule was applied for mapping the IRFs of 94 cells (from 56 slice cultures) that were whole-cell patched successfully. Among them, 40 cells had no IRF whatsoever and they were considered non-responsive.

3.2 Diversity in the IRFs of SCN neurons

The IRF morphologies of SCN neurons were found to be quite diverse, as illustrated by the three examples given in Figure 2. Figure 2a–c are a heat map of IRF showing the values of \(<F_{\text{max}}^\text{}} \rangle\) in color for the grid boxes within the relevant IRF, where \(<F_{\text{max}}^\text{}} \rangle\) represents the mean of five IPSC peak values registered at the target cell when the corresponding grid box was light-stimulated. The first example of Figure 2a, includes only several scattered grid boxes, which are “sprinkled” over nearly the entire nucleus. Meanwhile, the example of Figure 2b shows a rather huge IRF, covering almost the whole nucleus. And its \(<F_{\text{max}}^\text{}} \rangle\)s are generally much larger than those of Figure 2a. Lastly, the case shown in Figure 2c is quite unusual: The target cell has very short extensions; nevertheless, it has a quite sizable IRF, which is localized in the ventrolateral area, far away from the target cell body which lies in the core area.

In order to see if there are any classifiable patterns of IRFs, we have measured the IRFs of 54 (randomly chosen) responsive target cells. The IRF areas fit well to an exponential distribution with an average value of \(6.6 \times 10^4 \, \mu m^2\), as shown in Figure 2d. Very large IRFs (of about \(2.0 \times 10^5 \, \mu m^2\)) covering almost the entire SCN do exist, but they are not so common. About 46% of the 54 responsive cells had an IRF smaller than \(3.0 \times 10^4 \, \mu m^2\), an area covered by 12 small grid boxes: The example given in Figure 2a belongs to this subpopulation. Even within this ‘small IRF’ subgroup, the proximity of the IRF to the target cell and the spatial dispersiveness of the IRF are observed to vary widely from one cell to another (see Figure 3). As we will discuss shortly, the small clusters of (1–3) grid boxes, which are isolated from each other, most likely represent a single presynaptic source.

3.3 Responses to TTX, bicuculline, and calcium depletion in the bath

The light-stimulation-evoked IPSCs that we have measured are essentially AP-mediated, since all of them completely disappear in the presence of TTX, as shown in Figure 4a: Current traces following a pair of two successive light box stimulation are shown, before (blue) and after (red) the TTX application, only for two representative grid boxes (one very near [left frame] and the other [right frame] quite a distance away from the soma of the target neuron) are shown (see Figure S4a for all other grid boxes). The result was the same for all different SCN slice cultures (\(n = 14\)) that were tested. This result is somewhat unexpected, since the earlier optogenetic mapping study carried out by Fan et al. (2015) with acute brain slices containing mouse SCN suggested otherwise. The light-box stimulation could evoke IPSCs again after TTX was washed off (green lines of Figure 4a). Also, as shown in Figure 4b (and Figure S4b) there are no IPSCs in the absence of calcium ions in the bath medium (\(n = 3\) out of 3 slices), which are essential for the generation of synaptic current. These two results together confirm
that the measured currents are indeed through synaptic connections.

In addition, the measured IPSCs are surely made through a GABAergic inhibitory connection, as the application of bicuculline, a strong antagonist of GABA\textsubscript{A} receptors, completely silences the currents, as shown in Figure 4c (also see Figure S4c) \((n = 7\) out of 7 slices). Here, it is also noteworthy that the inward cation current generated by the direct light stimulation given to the target cell (its soma or neurites) itself surfaced, as the application of bicuculline blocks out all IPSCs (see the red line in the left frame of Figure 4c). Essentially, the same effect can be noticed in the TTX study: There are sizable inward cation currents in the presence of TTX (see the red line in the left frame of Figure 4a). In other words, in both cases, before the intervention of the pharmacological agents, the light-induced cation currents were masked by large inhibitory currents. What is also clear from the left frame of Figure 4c is that the size of cation current diminishes significantly as the light-box stimulation repeats (five times for this experiment). We believe this reduction is due to the slow recovery time of the ChR2 cation channel (Berndt et al., 2011): According to the red-line profile shown in the blown-up inset, the full recovery of a light-stimulated ChR2-transfected cell seems to take longer than 100 ms. We note that the sharp and deep first inward current peak (marked by a black arrow in the left frame of Figure 4c) reflects the inward sodium current associated with an AP on top of the light-induced cation current. After all, only the first among five successive light stimulations given directly to the target cell produced an AP. Most likely, this lack of recovery is responsible for the lower fidelity associated with the second light pulse stimulation discussed earlier.

3.4 Identifying independent presynaptic neurons within a given IRF

Given a heat map of \(\langle I^{\text{max}} \rangle\) like the ones shown in Figure 2, it is not obvious how many independent presynaptic neurons actually form the IRF, especially when the IRF includes, simply connected, large clusters of grid boxes as in the example of Figure 2b. Nevertheless, few things are quite certain. First of all, it is uncommon that any, isolated, individual grid boxes of the size 50 \(\times\) 50 μm\textsuperscript{2} hold more than one presynaptic neuron, as we have examined carefully with an example (see Figures S2 and S3 for a detailed analysis) and as several earlier works have suggested very sparse neural connectivity within the SCN (Fan et al., 2015). Conversely, we have quite the opposite issue: One presynaptic SCN neuron generally occupies more than a single grid box of the size 50 \(\times\) 50 μm\textsuperscript{2} hold more than one presynaptic neuron, as we have examined carefully with an example (see Figures S2 and S3 for a detailed analysis) and as several earlier works have suggested very sparse neural connectivity within the SCN (Fan et al., 2015). Conversely, we have quite the opposite issue: One presynaptic SCN neuron generally occupies more than a single grid box of the size 50 \(\times\) 50 μm\textsuperscript{2} within a large IRF domain, since SCN neurons have long extensions and can be excited by the openings of ChR2 cation channels in neurites near the soma as well as the soma itself. Also, the stimulating light shone into a specific grid box can spillover
to the adjacent grid boxes (on the 2D focal plane) due to un-
avoidable light scattering by the SCN sample as well as due to
the 3D nature of our LED box illumination, but we believe
that the extent of the spillover is at most smaller than 50 μm
(see more details in Supporting Information). Accordingly,
we first need to check how much ChR2 cation currents are
generated as the light box is scanned around a cell body
and what is the typical area (around the soma) in which the
amount of induced current is significant enough to evoke an
AP.

Figure 5A illustrates a typical heat map of ChR2 cation
currents around a target SCN cell expressing ChR2 channels.
The cell was voltage-clamped at −70 mV and the light-evoked
cation current was measured as the light box was scanned
in its vicinity; for each visit of a grid box, five light pulses
were delivered at a time interval of 100 ms. The measured
inward cation currents are mostly ChR2 channel-mediated
currents; in other words, the presence of excitatory synaptic
currents is generally not significant: For example, the total
(AMPAkainate receptor-mediated) excitatory receptive field
was measured to be 3.7(±3.0) × 50 × 50 μm² (mean [±SD],
n = 7), which is only 54% of the average effective size of an
SCN neuron (i.e., ARF = 1.7 × 10⁵ μm²) (see Supporting
Information and Figure S5). Generally, the shape of hot

FIGURE 3 The diversity in the shape and range of small inhibitory receptive fields of SCN neurons. The target-cell morphologies are
fluorescence-labeled (pseudo-color red); and the red dots mark only the positions of target cell bodies. The small clusters of (connected 1–3 grid
boxes) most likely represent a single presynaptic source. [Colour figure can be viewed at wileyonlinelibrary.com]
zone in the evoked cation current heat map correlates with the shape of target neuron but much more rounded; and the reason why the hot zone being quite large around the cell body seems to originate from the light scattering by the tissue sample. Although the amplitude of the cation current diminishes dramatically as the distance from the soma increases (see Figure 5A), for a significant area around the cell body the cation current is large enough to generate an AP (see Figure 5B-a). On the other hand, note that although the light stimulation given to the grid box “b” generated some inward cation current, it was not sufficient enough to generate an AP (see Figure 5B-b). Subsequently, we define the ARF to be the collection of grid boxes around the cell body that, upon receiving a light stimulation, generate an AP that swings over 30 mV in a whole-cell patch recording mode (see Figure 5C). With this working definition, our measurement of ARF is 1.7 $(\pm 1.5) \times 10^4 \mu m^2$ (mean $\pm SD$, based on $n = 14$ randomly chosen cases out of 94 cells used for the IRF mapping). Thus, as for estimating the independent number of presynaptic cells within a receptive field, the intrinsic spatial resolution of our optogenetic light-stimulation is no better than $R = \sqrt{ARF/\pi} = 73 \mu m$. That is, if two presynaptic neurons (actually, their somas) in a given IRF are separated by a distance less than $R = 73 \mu m$, we cannot distinguish them, as their ARFs may overlap each other—this issue is analogous to that of the Rayleigh separation distance in optics. We can further validate the above estimate of $R$ directly by the procedure that we coin as independence test (see Supporting Information, Sec. 6 and Figure S6).

The average peak amplitude of evoked IPSC, $<I_{max}>$, can be an important signature characterizing a given presynaptic neuron (see Fan et al., 2015), but we find it, in general, so noisy and often quite so small (see Figure 1B), compared to the level of noisy baseline current fluctuation ($\sigma = 7.0 \mu A$, an average over $n = 36$ different target cells), that it alone is not sufficient enough for distinguishing one presynaptic neuron from the others. Subsequently, based on the combined measure (i.e., distance function $D$) of the difference in $<I_{max}>$ and the physical distance of two grid boxes within a given IRF, we performed a DBSCAN clustering analysis (see Materials and Methods) to identify different presynaptic neurons. Two exemplary cases are illustrated in Figure 6. By far, the case of Figure 6a is the simplest case: A small IRF that includes only a handful number of scattered grid boxes (represented
as dots) that are separated into four clusters, where each of them is representing a different presynaptic neuron. The clusters (mostly single grid dots) marked in different colors are physically well separated from each other with a distance greater than the estimated “Rayleigh separation distance” of $R = 73 \, \mu m$, thus, they may well be considered as an independent presynaptic source; on the other hand, even though the two blue dots are separated from each other with a distance larger than $R = 73 \, \mu m$, (according to our clustering algorithm) they are classified as being belonged to the same cluster because their $\langle I_{\text{max}} \rangle$s are very similar to each other (or, $\Delta I_{\text{max}}$ is too small). Figure 6b illustrates another typical case. Namely, a simply connected domain, whose grid boxes show widely different values of $\langle I_{\text{max}} \rangle$s: Applying the same clustering algorithm, it is separated into four different clusters.

Subsequently, the same clustering algorithm is applied for 36 different IRFs and the histogram of the number of presynaptic sources in Figure 6c summarizes the result. Unfortunately, the other 18 cells (out of total 54 cells used for Figure 2d) were not included in this analysis, as their soma locations with respective to their matching IRFs could not be determined accurately. We find that the resulting number of clusters is not so sensitive on the value of the weighting parameter $w$ inside the distance function $D$: The mean value (of $n = 36$) varies from 8.0 to 11.2 over the range of $0 < w < 30$ (see Figure S7 for further detail). Although it is noisy, the histogram fits
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3.4 | The physical range of presynaptic connections for SCN neurons

On average, the presynaptic neurons are scattered quite uniformly in all directions around their postsynaptic neurons.
(not shown). And what is surprising is that the physical range of GABA_A receptor-mediated synaptic connections within the SCN is quite large as shown in Figure 6d. The number density of the presynaptic connections around a target cell falls off as an exponentially decreasing function of r, yet, its space constant is very large 210.7 μm that long-range (>210.7 μm) neural connections are quite popular (66.9%, n = 216 from 323 cases considered) within the SCN. This property is equally applicable for cells in dorsal region as well as in ventral region.

4 | DISCUSSION

Identifying functional cell-to-cell neural network connectivity at the physical scale of an individual cell in a densely packed SCN is a daunting task. We have undertaken the challenge by using a laboratory-built optogenetic mapping system and by optimizing a stimulation and recording protocol for quantifying GABA_A receptor-mediated synaptic connections. When several factors—sparse neural connectivity, typical sizes and shapes of SCN neurons and their ChR2 cation current-induced ARFs, the desirable spatial resolution of IRF map, and the amount of time necessary for a complete IRF mapping—are considered together, a point-scan light-box of map, and the amount of time necessary for a complete IRF mapping analysis; on the other hand, ours was based on several (2 or 5) repetitions of pairs of two temporally correlated pulses, thus, it excluded any time-uncorrelated spontaneous APs. Third, another significant difference is that Fan et al. (2015) used acute brain slices of P25-35 mutant mice expressing ChR2 channels specifically either for VIP+ neurons or AVP+ neurons, while we used organotypic cultures of P3-5 rats expressing ChR2 essentially in all SCN neurons. The differences in the age and animal type could potentially lead to a rather different neural circuit connectivity (Ansari, Agathagelidis, Lee, Korf, & Gall, 2009).

In summary, we could provide <I>max</I> heat maps (or IRFs) and further identify independent presynaptic sources comprising the IRFs for the responsive SCN cells that were randomly selected from all different locations within many organotypic cultures of P3-5 rat SCNs. We found that (a) the probability distribution of the IRF sizes of responsive SCN cells is best described by an exponential function; (b) the shape of IRF is very diverse and very random, (c) the node-degree (i.e., number of presynaptic connections per individual cell) distribution also fits well into an exponential function with a mean value of 8.9; thus, there exists small subpopulation of cells that have quite a few afferent connections; and (d) the afferent synaptic connections originate from various locations within the entire SCN: On average, presynaptic neurons are scattered randomly in all directions and long-range (>210.7μm), afferent, neural connections are quite abundant within the SCN.

As far as we understand, the work of Fan et al. (2015) is the only pre-existing optogenetic mapping study on the SCN. Their system is similar to ours, yet, has several differences; thus, some comparisons would be useful at this point. First of all, they used a gaussian laser beam of diameter ~20 μm with a spatial sampling interval of 50 μm on a square grid with a typical laser power of 100 μW (or, if the light was assumed uniform, light intensity ~3.2 mW/cm²) with 2 ms exposure time for a pulse stimulation. Since the laser spot size of 20 μm is significantly smaller than the sampling lattice interval of 50 μm, some presynaptic neurons could be potentially dropped out from the actual receptive field of a target neuron during their optogenetic mapping: In comparison, we used a homogeneous LED light box of 50 × 50 μm² with a light intensity of 3.8 mW/cm² and 10 ms pulse exposure time, fully covering a rectangular area of 4.8 × 10⁵ μm². Interestingly, however, their measured (average) "excitability profile," whose definition is very similar to what we have defined as "ARF," suggests that the average ARF of SCN neurons even under their experimental condition and protocol was as large as what we have discussed in Figure 5. Again, the large ARFs could be attributed to the fact that (a) physically, SCN neurons have quite long (bipolar) neurite extensions and (b) the stimulating light can diffuse into the neighboring grids. Second, the analysis of Fan et al. (2015) was based on single-pulse perturbations, so, it is possible that some spontaneously generated APs slipped into their optogenetic mapping analysis; on the other hand, ours was based on several (2 or 5) repetitions of pairs of two temporally correlated pulses, thus, it excluded any time-uncorrelated spontaneous APs. Third, another significant difference is that Fan et al. (2015) used acute brain slices of P25-35 mutant mice expressing ChR2 channels specifically either for VIP+ neurons or AVP+ neurons, while we used organotypic cultures of P3-5 rats expressing ChR2 essentially in all SCN neurons. The differences in the age and animal type could potentially lead to a rather different neural circuit connectivity (Ansari, Agathagelidis, Lee, Korf, & Gall, 2009).

Combining experimental measurements with a statistical inference, Fan et al. (2015) suggested that an adult mouse SCN neuron has a 0.06% probability of receiving input from a single given presynaptic VIP neuron (or about 0.66 VIP+ presynaptic neural connection per each cell, assuming the total number of VIP+ cells is around 1,100 (Welsh et al., 2010). In addition to the sparseness, their analysis on multiple presynaptic VIP partners suggested that VIP input is biased toward a subset of neurons. Basically, our experimental investigation has revealed a similar set of findings but with a quite larger figure. First of all, the average number of afferent neural connections is estimated to be 8.9. This figure is considerably larger than the estimated value of 0.66 given by Fan et al. (2015), but is closer to the estimations (on average, 5.4 VIP contacts per AVP clock cell and 1.7 AVP contacts per VIP clock cell [of adult male Sprague–Dawley rats] given by Jacomy et al. (1999), which were based on immunocytochemical and electron-microscopic images. The large discrepancy between our estimate and that of Fan et al. (2015) could be attributed to the several aforementioned differences. In addition, we should point out that in both studies the estimated values rely on some subjective assumptions: (in our
The action of GABA-receptor channels was suppressed and they are inhibitory/excitatory switching action of GABA-receptor channels through different electrodes, estimated most SCN cells sent and received approximately one to four connections with exponentially fewer connections. Their correlation analysis also suggested that excitatory connections are almost as common as inhibitory connections (with the inhibitory 58% and the excitatory 42%). Knowing that at a given time a wide range of circadian phases is supported by the cells in their SCN slice culture, their assessment seems natural, since the inhibitory/excitatory switching action of GABA_A-receptor channels over a circadian cycle is a salient feature of the SCN. Again, in our current investigation, the potential switching action of GABA_A-receptor channels was suppressed and they were kept inhibitory. When (AMPAkainate receptor-mediated) excitatory currents were looked for at a holding potential of −70 mV under otherwise the same whole-cell patch recording mode, they were insignificant (see Supporting Information, Sec. 5).

Some drawbacks of our current experimental investigation need to be addressed. First of all, we have used organotypic cultures of P3–5 rat SCNs for our current investigations, and all the mapping experiments were carried out at least 3 weeks from the point of brain decapitation and slicing. During that long period of time, the sample had become noticeably thinner and expanded as the astrocytes moved out to the periphery of the SCN. Accordingly, we cannot rule out the possibility of some neural network restructuring during the sample preparation. Unfortunately, we could not estimate how significant that change was. That is, as far as preserving the neural connectivity, it would be better to use acute brain slices over organotypic brain slice cultures, as it was done by Fan et al. (2015). Yet, an acute brain slice also has its drawback to include served axons of presynaptic neurons and dendrites of postsynaptic neurons, and they can make the interpretation of the receptive field ambiguous as it was discussed by Fan et al. (2015). Another deficiency of our optogenetic mapping study is that all SCN neurons including the target neuron itself express ChR2 channels. Thus, delivering a light-box perturbation near the target neuron causes a huge cation current to the target neuron, that any small inhibitory currents brought by adjacent presynaptic cells could be masked by it, being unnoticed. In addition, our current experiments do not distinguish many different types of peptidergic cells (e.g., AVP, VIP, GRP); therefore, we cannot discuss any bias in the directional connectivity of different subpopulations of SCN cells or their reciprocal connectivity that were discussed earlier by (Fan et al., 2015; Jacomy et al., 1999; Romijn, Sluiter, Pool, Wortel, & Buijs, 1997) and more recently by Buijs et al. (2017) and Varadarajan et al. (2018).

Finally, we should point out again that the dynamics of a coupled network of nonlinear oscillators like the SCN clock cells depend critically on the nature of their connections (Kuramoto, 2011; Strogatz, 2000). Therefore, it is imperative to gain insights on the nature of the connectivity of the SCN clock cells as for understanding their collective dynamics (Jeong et al., 2016) and subsequently its related biological function. Currently, optogenetic light-stimulation is the best method for mapping a functional SCN network. In this work, we developed an optimized light-box scanning protocol for the SCN and carefully analyzed the light-induced responses to provide some of the most fundamental statistical information regarding the network of rat SCN (node-degree distribution and presynaptic source density vs. distance). In the future, one can set-up an “adjacency matrix” that faithfully reflects the statistical information and use it for the coupling of spatially distributed (packed) circadian oscillators of the SCN. We expect that the observed long-range connectivity would, in general, confer a tight phase coherence to the system for one thing. On the other hand, the AP-mediated synaptic coupling may not be sufficient enough to account for the (various) progressive circadian waves reported by earlier experimental studies (Antle & Silver, 2005; Jeong et al., 2016; Welsh et al., 2010). In order to realize this wave nature, the SCN cells must have some means of local diffusive couplings, and we speculate that non-synaptic neurotransmission of various peptides (eg. AVP, VIP) can account for the local coupling. As the mapping data accumulate more in the future, we will be able to incorporate position-dependent (e.g., core vs. shell) projection statistics into the adjacent matrix.

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CONFLICT OF INTEREST
The authors declare no competing interests.
DATA ACCESSIBILITY

Data are available from the corresponding author on request.

AUTHOR CONTRIBUTIONS

CHM carried out experiments, analyzed data; HK analyzed data; WC helped building experimental setup; and KJL designed study, analyzed data, and wrote paper; CHM and HK are two first authors who contributed equally to this work. All authors approved the manuscript.

ORCID

Kyoung J. Lee https://orcid.org/0000-0003-4415-5760

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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