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Neuronal synchronization without calcium-dependent synaptic transmission in the hypothalamus

(suprachiasmatic nucleus/intercellular communication/nonsynaptic mechanism/circadian rhythm/patch clamp)

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ABSTRACT **A critical question in understanding the mammalian brain is how populations of neurons become synchronized. This is particularly important for the neurons and neuroendocrine cells of the hypothalamus, which are activated synchronously to control endocrine glands and the autonomic nervous system. It is widely accepted that communication between neurons of the adult mammalian brain is mediated primarily by Ca^{2+}-dependent synaptic transmission. Here we report that synchronous neuronal activity can occur in the hypothalamic suprachiasmatic nucleus without active Ca^{2+}-dependent synaptic transmission. Simultaneous extracellular recordings of neuronal activity in the suprachiasmatic nucleus, which contains the mammalian biological clock, confirmed a circadian rhythm of *synchronized* activity in hypothalamic slices. Ca^{2+}-free medium, which blocks chemical synaptic transmission and increases membrane excitability, produced periodic and *synchronized* bursts of action potentials in a large population of suprachiasmatic nucleus neurons with diverse firing patterns. *N*-Methyl-D-aspartic acid, non-*N*-methyl-D-aspartic acid, and γ-aminobutyric acid type A receptor antagonists had no effect on burst synchrony. Whole-cell patch-clamp recordings confirmed that the Ca^{2+}-free solution blocked evoked postsynaptic potentials and that the mixture of antagonists blocked the remaining spontaneous postsynaptic potentials. Therefore, mechanisms other than Ca^{2+}-dependent synaptic transmission can synchronize neurons in the mammalian hypothalamus and may be important wherever neuronal networks are synchronized.**

There is virtually no physiological information on local neuronal interactions in the hypothalamus, even though synchronization of the neuronal and neuroendocrine elements of the hypothalamus is a critical and fundamental process in the neurobiology of hormone secretion and homeostasis. In freely moving animals, the neuronal activity in the suprachiasmatic nucleus (SCN) of the hypothalamus, which contains the mammalian biological clock (1, 2), exhibits a circadian rhythm where peak activity occurs at the middle of the light phase, even in a surgically isolated hypothalamus (3) or *in vitro* brain slice (4–7). Because these data are derived from neuronal populations, they also imply that the electrical activity generating the pacemaker is synchronized. Determining the mechanism(s) underlying this synchronization is critical to an understanding of circadian rhythm generation and neuronal interactions in the hypothalamus. The Ca^{2+}-independent nonsynaptic mechanism(s) of synchronization we describe here in the SCN provides a crucial clue for understanding the cellular processes underlying circadian rhythm generation, since it is quite likely that the circadian rhythm incorporates the same mechanism.

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Most of the studies on neuronal networks in the mammalian brain have been directed toward chemical synaptic transmission, while other nonconventional forms of communication between neurons have been relatively neglected and probably underestimated. Previous studies have suggested that certain areas of the brain (e.g., hippocampus, inferior olive, and locus coeruleus) exhibit synchronization independent of chemical synaptic transmission, but the activity appears to be tightly synchronized (i.e., neuronal activity occurs in a one-for-one manner throughout the population; see *Discussion*). Here we show that neuronal activity in the hypothalamus can be loosely synchronized (i.e., action potentials generally occur together, but with different firing patterns) after chemical synaptic transmission has been blocked. This significantly extends the brain areas in which a nonchemical synaptic mechanism has been shown to synchronize activity of large neuronal populations in the adult mammalian brain.

MATERIALS AND METHODS

Slice Preparation. Coronal hypothalamic slices (350–500 μm) were prepared 2 h before the dark phase from Sprague–Dawley male rats (100–400 g, except 40–85 g for patch-clamp experiments). Rats were obtained from a single colony for circadian rhythm experiments and maintained on a 12-hr light/12-hr dark schedule for ≥14 days. The slices were studied in an interface chamber (33–35°C, in humidified 95% O_2/5% CO_2).

Solutions. For the circadian rhythm experiments (see Fig. 1), slices were bathed in a physiological solution containing 124 mM NaCl, 3 mM KCl, 1.4 mM NaH_2PO_4, 2.4 mM $CaCl_2$, 1.3 mM $MgSO_4$, 11 mM glucose, and 26 mM NaH_2CO_3. Control solutions in the other experiments (see Figs. 2 and 3) were similar to those for Fig. 1 except that the NaCl concentration was 134 mM, 10 mM Hepes was used as a buffer, and 8 mM NaOH was added to adjust to pH 7.4. In the Ca^{2+}-free medium, $MgCl_2$ was substituted for $CaCl_2$, and 0.1 mM 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, a specific Ca^{2+} chelator, was added. Blockade of *N*-methyl-D-aspartic acid (NMDA), non-NMDA, and γ-aminobutyric acid type A ($GABA_A$) receptors was achieved by bath application of 100 μM DL-2-amino-5-phosphonopentanoic acid, 50 μM 6,7-dinitroquinoxaline-2,3-dione, and 50 μM bicuculline.

Extracellular Recordings. Multiple-unit activity (MUA) was recorded simultaneously with two metal electrodes (90% platinum/10% iridium wire coated with teflon, diameter = 76 μm). The signals were amplified with a differential amplifier

Abbreviations: SCN, suprachiasmatic nucleus; NMDA, *N*-methyl-D-aspartic acid; $GABA_A$, γ-aminobutyric acid type A; MUA, multiple-unit activity; PSP, postsynaptic potential.

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(bandwidth filter = 0.3–3 kHz). Discharges above a threshold (signal-to-noise ratio ≥ 1.5–2.0:1) were counted in 5-min bins and then averaged in 1-h bins. Single-unit activity was recorded by using glass micropipettes filled with 1 M NaCl (resistance = 30–40 MΩ).

Whole-Cell Patch-Clamp Recordings. Glass pipettes were filled with solution containing 120 mM potassium gluconate, 1 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, 4 mM MgATP, 5 mM 1,2-bis(2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid and adjusted to pH 7.2 with KOH. Electrode resistance before seal formation was 4–7 MΩ. Extracellular stimulation applied through a platinum/iridium bipolar electrode to a site 2–3 mm dorsal to the SCN (0.2–1.6 mA and 0.5 ms) evoked an excitatory postsynaptic potential (PSP) starting after a 2- to 5-ms delay with 5–16 mV peak amplitude and duration of 60–120 ms. Input resistance was 657 ± 80 MΩ (mean ± SE; n = 12; range of 350–1150 MΩ) at membrane potentials of −70 to −90 mV. During PSP blockade, no reduction was observed in input resistance (n = 5), indicating that PSPs were not shunted by a leak in the membrane.

RESULTS

Simultaneous recordings of MUA with two electrodes positioned on the slice surface were made for 25 h. One electrode was located in the midventral SCN and the other in the dorsal SCN (100- to 250-μm separation). These recordings confirmed that a circadian rhythm can be obtained in an isolated slice (Fig. 1*A*); peak activity occurred at 06:29 ± 0:26 h circadian time (mean ± SE; n = 6; lights on from 00:00 to 12:00 h circadian time), in agreement with other *in vivo* and *in vitro* studies (3–7). The correlation between the two hourly MUA counts across the circadian rhythm in the same SCN was high (r = 0.99 for Fig. 1*B*; r = 0.73, 0.61, 0.87, and 0.93 in other cases; df = 23; $P < 0.0015$), thus confirming synchrony in circadian neuronal activity (Fig. 1*B*).

To test whether chemical synaptic transmission is required for synchronization of SCN neurons, we bathed hypothalamic slices in Ca^{2+}-free solution, which blocks evoked chemical PSPs (8–10) and also increases membrane excitability (11). After 2.5–5.5 h, periodic bursts of neuronal activity appeared in the SCN. Simultaneous MUA recordings from two locations within one SCN revealed that the bursts always occurred simultaneously (n = 11; Fig. 2*A*). Bursts were never synchronized in opposite SCNs (n = 9; Fig. 2*B*) and differed in phase as well as in interval. Burst synchrony was maintained for 18.5 h (n = 2), and normal activity could be restored within 12 min in normal Ca^{2+} solution. Simultaneous single-unit activity and MUA recordings revealed that 61% of all neurons (n = 23) generated bursts and that most neurons (79% of the bursting neurons or 57% of all neurons) fired action potential(s) only during the MUA bursts. Intraburst interval and burst duration

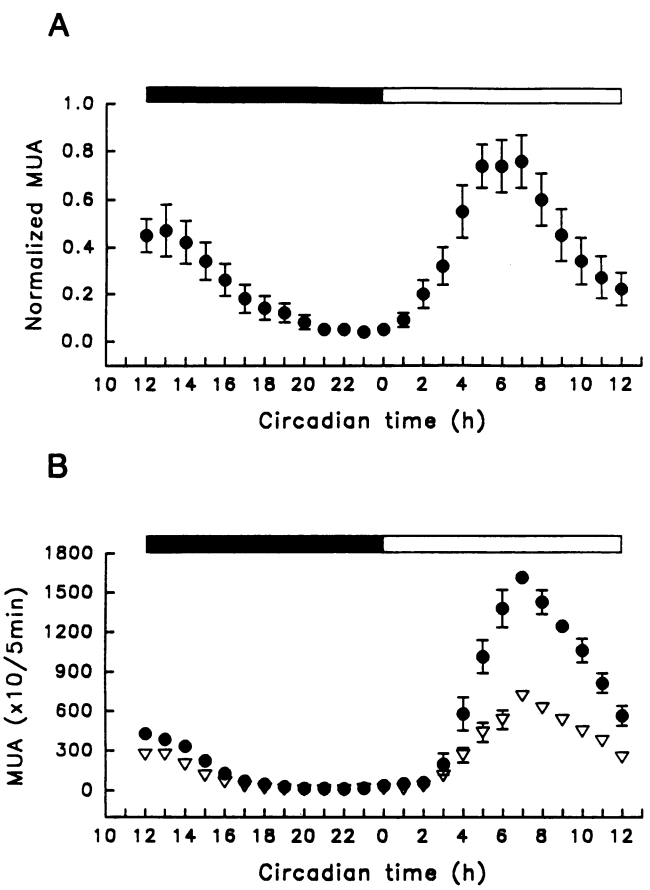

FIG. 1. Circadian rhythm of neuronal activity and comparison of activity recorded simultaneously from two locations in the SCN. (*A*) Average of normalized MUA (●) at each circadian hour from six rats. Normalized MUA was computed by dividing each hourly MUA count by the maximum hourly MUA count of each animal. (*B*) Averaged MUA count per 5 min at each circadian hour from one animal. MUA from the ventral SCN (●) and from 200 μm dorsal in the same SCN (▽) were counted simultaneously. Vertical lines are standard errors. Solid bars represent dark hours and open bars represent light hours.

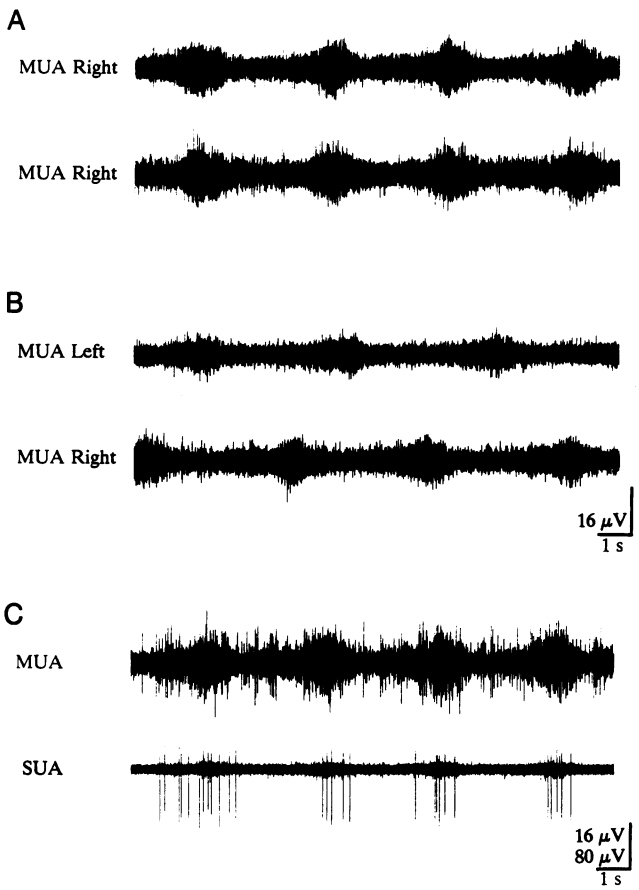

FIG. 2. Simultaneous extracellular recordings during bursting activity in Ca^{2+}-free solution. (*A*) Simultaneous recordings of MUA from two locations (150-μm separation) in the same SCN (right side) indicate synchronous bursts. The interburst interval gradually increased from 1 s to a maximum of up to 10 s after several hours, and the burst duration ranged from 0.5 to 2.0 s. (*B*) Simultaneous recordings of MUA from opposite SCNs (left and right) indicate that the bursts were not synchronous and had different interburst intervals (recorded in the same slice as in *A*). (*C*) Single-unit activity (SUA) recording during a MUA burst in the same SCN; the single-unit activity bursts coincided with the MUA bursts.

varied considerably between neurons and between consecutive bursts of the same neuron. Bursts of action potentials in individual cells occurred simultaneously with periodic increases of MUA amplitude (Fig. 2C), which demonstrates directly that MUA bursts represent action potentials from a population of bursting neurons.

Excitatory amino acid receptor antagonists and bicuculline block all evoked and spontaneous postsynaptic events in SCN neurons (12–15). Although unlikely, spontaneous Ca^{2+}-independent release of neurotransmitters (i.e., not mediated by action potentials) could conceivably contribute to burst synchronization. To test this hypothesis, we bath-applied glutamate (NMDA and non-NMDA) and $GABA_A$ receptor antagonists (100 μM 2-amino-5-phosphonopentanoic acid, 50 μM 6,7-dinitroquinoxaline-2,3-dione, and 50 μM bicuculline) in the Ca^{2+}-free solution. Although these antagonists led to a slight increase in interburst interval, they never altered MUA burst synchrony (n = 6; tested for 60–100 min), indicating that spontaneous release of excitatory and inhibitory amino acids was not required for synchronization. Intracellular recordings have revealed only *fast* PSPs mediated by amino acid receptors (i.e., NMDA, non-NMDA, and $GABA_A$ receptors) in the nucleus (refs. 12–14, 16, and 17; also see Fig. 3). Since all detectable PSPs were blocked, this result also rules out synchronization of SCN neurons through Ca^{2+}-independent transmitter release, which has previously been demonstrated in fish retinal neurons (18).

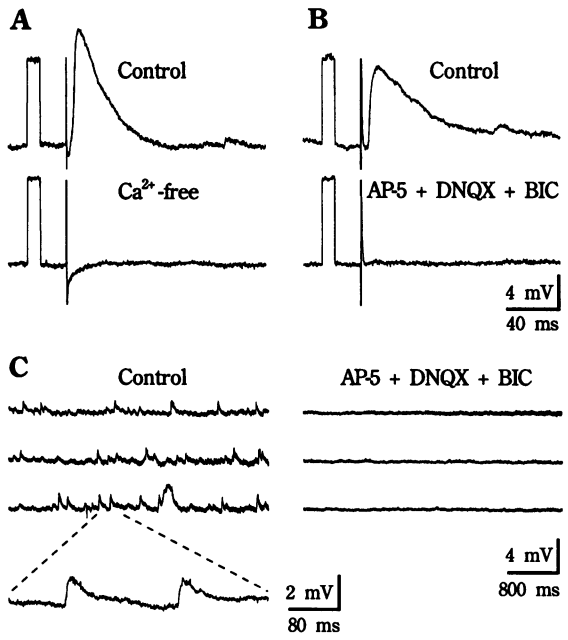

FIG. 3. Effect of Ca^{2+}-free solution or glutamate and $GABA_A$ antagonists on PSPs recorded with the whole-cell patch-clamp technique. (*A*) The upper trace shows an excitatory PSP evoked by extracellular stimulation dorsal to the SCN. The excitatory PSP was completely blocked (lower trace) after 17 min (range of 9–26 min; n = 4) in Ca^{2+}-free solution. Full recovery was obtained after 10 min in normal Ca^{2+} solution (not shown). The membrane potential in both traces was −66 mV. (*B*) The upper trace shows an evoked excitatory PSP from another neuron, which was blocked in a 100 μM 2-amino-5-phosphonopentanoic acid (AP-5), 50 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX), and 50 μM bicuculline (BIC) mixture in normal Ca^{2+} (lower trace) after 13 min (range of 9–13 min; n = 3). The membrane potential in both traces was −78 mV. (*C*) The three upper left traces show continuous recordings of spontaneous PSPs in the same neuron as in *B*. The lower left trace shows two spontaneous PSPs at faster sweep speed and higher gain. The three right traces show a continuous recording during complete blockade of spontaneous PSPs after 12 min (range of 9–14 min; n = 6) in the antagonist mixture and normal Ca^{2+}. All traces in *C* were recorded at a membrane potential of −78 mV. Traces in *A* and *B* contain a calibration pulse (10 mV, 10 ms) before the truncated stimulus artifact.

To assess directly the effect of the Ca^{2+}-free solution and the receptor antagonists on chemical synaptic transmission in SCN neurons, we recorded spontaneous and evoked PSPs by using the whole-cell patch-clamp technique, which allows high resolution of synaptic events. Evoked excitatory PSPs were blocked in Ca^{2+}-free solution or during bath application of the amino acid antagonists (Fig. 3 *A* and *B*). Spontaneous PSPs were not blocked in the Ca^{2+}-free solution (n = 5; data not shown), but they were always abolished after application of the antagonists (Fig. 3*C*). Therefore, high-resolution whole-cell recordings directly demonstrated that all detectable chemical synaptic transmission was blocked in conditions that induced synchronized bursting when antagonists were present.

DISCUSSION

The synchronized bursts in Ca^{2+}-free solution represent a loose type of neuronal synchronization (i.e., many neurons firing within the same time window, but with variable discharge patterns and burst durations). To our knowledge, this type of Ca^{2+}-independent synchronization has not been described previously in the mammalian brain. Normal synchronization of SCN activity during the circadian rhythm (see Fig. 1*B*) has similarities to that seen in Ca^{2+}-free conditions, since at any given time a sample of neurons shows a wide range of firing frequencies (4–7) while still being loosely synchronized. The persistence of the synchronous bursts induced by Ca^{2+}-free solution, despite complete block of all detectable chemical synaptic transmission, demonstrates that a communication mechanism(s) other than Ca^{2+}-dependent synaptic transmission must operate in the SCN. This mechanism can synchronize neuronal activity within a nucleus but not between opposite nuclei (Fig. 2*B*). Communication between opposite nuclei is probably mediated by chemical synaptic transmission (16, 19) and therefore was blocked in the absence of Ca^{2+}.

Neurons in the inferior olive (20) and locus coeruleus (21) generate Ca^{2+}-dependent subthreshold oscillations that are rhythmic and synchronous. Although these oscillations are synchronized by gap junctions, and thus also seem to be independent of chemical synaptic mechanisms, they are Ca^{2+}-dependent and therefore different from the bursts described here. In hippocampal slices, low Ca^{2+} solutions produce synchronized bursts in the absence of active chemical synapses (22–24), but the action potentials are tightly synchronized and generate population spikes during these bursts. The SCN bursts differ from the hippocampal activity (i.e., weaker synchronization whereby action potentials do not occur one-for-one) and therefore represent a different type of synchronization of neuronal activity in the mammalian brain.

The neuronal synchronization in the hypothalamus that we are reporting here opens several additional lines of investigation. This Ca^{2+}-independent mechanism(s) of synchronization in the SCN may depend on electrotonic coupling via gap junctions, electrical field effects (ephaptic interactions), and/or changes in the concentration of extracellular ions (25). Among these three possible mechanisms, electrical field effects seem the most unlikely to operate in the SCN due to the lack of parallel arrangement of neuronal processes in the nucleus. Electrotonic coupling and ionic interactions are more likely to facilitate synchronization in the nucleus, since many SCN cell bodies are tightly packed together with extensive regions of membrane apposition between them (19). Although there is evidence for electrotonic coupling in some areas of the hypothalamus (26), nearly all of the data are derived from anatomical rather than electrophysiological

studies, and virtually no data are available for most hypothalamic areas including the SCN. It should now be possible to determine which hypothetical mechanisms of neuronal interaction are present in the SCN and the role they play in synchronization.

Previous indirect evidence has suggested that neurons in the SCN can be synchronized via nonsynaptic mechanisms. The circadian rhythm of glucose utilization that occurs across cells in the SCN is expressed before chemical synapses are functional (27). Tetrodotoxin application, which blocks synaptic transmission mediated by action potentials in axons, also blocks the circadian rhythm of motor activity while leaving the coordinated timing mechanism of the cellular clock in the SCN unimpaired (28). The nonsynaptic mechanism(s) demonstrated here could explain both observations, since neuronal activity could be synchronized by electrical and/or ionic interactions. A Ca^{2+}-independent mechanism(s) of neuronal synchronization may coordinate the cellular elements in the SCN responsible for circadian rhythms in mammals. Synchronization of neuronal activity in the SCN and other areas of the hypothalamus must be critical to execute the widespread behavioral, physiological, and endocrine effects. Nonsynaptic communication between mammalian neurons has not received the attention it deserves, but accumulating evidence (20–24, 29), as well as the present study, suggests that nonsynaptic mechanisms may have a major role in synchronizing neuronal activity in the mammalian brain.

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