Research report

Physiological and anatomical properties of the suprachiasmatic nucleus of an anophthalmic mouse

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Abstract

Congenitally anophthalmic mice (ZRDCT-AN) have circadian rhythms which ‘free-run’ and are not light modulated. Their rhythms differ from those of controls in: duration of circadian period, length of active phase, and pattern/intensity of activity. Three different populations have been described based upon wheel-running: rhythmic with stable period, rhythmic with unstable period and arrhythmic. Circadian rhythms are generated by neurons in the suprachiasmatic nuclei (SCN) of the hypothalamus. To better understand whether intrinsic properties of SCN neurons differ in anophthalmic and sighted mice, we examined the electrical activity of these neurons in slices, using single unit recordings, ionophoresis and bath perfusion of agonists and antagonists of known SCN neurotransmitters. Lucifer yellow was injected to characterize morphology. In controls, in daytime, units fired at a higher rate (44% at \(5\) Hz) than at night (21% at \(5\) Hz) and with regular interspike intervals versus irregular intervals nocturnally. In anophthalmics four firing patterns were observed as follows: (1) irregular at \(<5\) spikes/s (70% of the total); (2) regular at \(\geq 5\) spikes/s; (<10%); (3) irregular bursts (20%); (4) regular bursts (<1%). Most neurons were inhibited by GABA, but a few were excited in controls. Blocking synaptic transmission with low Ca/ high Mg increased the frequency and regularized the pattern of previously irregular discharges both in anophthalmics and controls. Bicuculline (10^{-5} M), a GABA antagonist, had a similar effect. These data suggest that the characteristic irregular firing pattern of anophthalmics, and of controls at night, results from extrinsic, at least in part, GABAergic input.

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1. Introduction

The paired suprachiasmatic nuclei (SCN) of the hypothalamus are located immediately dorsal to the optic chiasm. They receive direct input from the retina [5,25] and play an important role in regulation of circadian oscillations related to a number of physiological functions including sleep–wake cycles, release of hormones and core body temperature [10,14,16,17,24].

Neurons in ventral parts of the nuclei generate circadian rhythms, which are synchronized (entrained) to environmental light–dark cycles by retinal input [15]. Glutamate is the principal neurotransmitter from the retina [5,8,13]. Almost all SCN neurons contain GABA in addition to a number of other neuropeptides, some of which show diurnal variations [1,6,19,26,27,32,35]. In sighted animals, spontaneous electrical activity in individual SCN neurons exhibits diurnal variations in firing rate [9,11,12]. During daytime the SCN is characterized by a preponderance of rapidly firing units (>5 Hz), whereas at night, units firing greater than 5 Hz are rare.

Mutant anophthalmic mice, whose optic primordia are resorbed during embryonic development, are mostly born without eyes or optic nerves. Ocular development, morphology and neurochemistry of the SCN in these mice have been studied by Chase and Chase [7], Silver [30], Laemle...
and Rusa [20] and Laemle et al. [19]. The SCN are present in anophthalmic mice, but exhibit conformational differences from those of sighted, control mice. Notably, while the nuclei in sighted mice are bilaterally symmetrical, in mutants they may be asymmetrical in size and location (Fig. 1). One or both nuclei may be entirely absent. Through recording of wheel running activity, three populations of anophthalmic mice have been described: rhythmic with stable circadian period; rhythmic with unstable circadian period; and arrhythmic [18]. The basis for the differences in circadian rhythms is unknown, since physiological properties of individual SCN neurons have never been examined in anophthalmic mice. It should be noted that blind humans are likewise known to have circadian rhythm disturbances [28].

The present study was designed to test the hypothesis that neurons in the SCN of anophthalmic mice have different electrical properties than those of control mice. Brain slices were used to characterize neuronal activity in the SCN of anophthalmic mice and compare it with that of controls. The effects of naturally occurring neurotransmitters, their agonists and antagonists also were tested.

2. Methods

Twenty-five mice, 19 anophthalmic (ZRDCT-AN) and six sighted congenic controls (ZRDCT-N) were used in these experiments. The mice were kept in constant temperature on a light–dark cycle of LD 12:12. They were euthanized by cervical dislocation under ether anesthesia, and the brain was rapidly removed to cold (approximately 0°C) normal Krebs–Ringer solution containing 124 mM NaCl, 5 mM KCl, 2.4 mM CaCl, 1.3 mM MgSO, 1.26 mM KH PO, 26 mM NaHCO, and 10 mM D-glucose. The brain was transected at the intercollicular level and, using cyanoacrylate glue, the cut caudal surface of the block containing the forebrain was fixed on a stage in the chamber of a simple vibratome. Coronal sections were cut at 450 μm and transferred to an incubation chamber filled with Krebs–Ringer solution bubbled with 95% O and 5% CO. Slices were incubated at 34°C for at least 2 h before starting experiments. For recording, SCN slices were mounted on a plexiglass mesh submerged in the recording chamber, and covered with a nylon mesh on which a silver wire coil was placed to prevent movement of the tissue. The chamber was perfused with oxygenated Krebs–Ringer solution (34°C) at 3 ml/min. Single unit discharges were recorded with a glass micropipette filled with Krebs–Ringer solution (3–5 MΩ). The electrode was advanced into the SCN until a spontaneous action potential was encountered (Fig. 1). Spikes, displayed on an oscilloscope, were recorded extracellularly and amplified with a Dagan AC-amplifier. After a unit was encountered and stabilized, GABA, AMPA, and NMDA were ionophoretically applied for 1 s each in an automated sequence at 30 s intervals, through a separate three-barrel micropipette advanced to near the recording site. For positioning, the tips of both ionophoretic and recording micropipettes were painted with black waterproof ink to enhance visualization under the dissecting microscope. GABA was prepared at 0.5 M in distilled water; pH 3.5; amino acids were dissolved in 0.15 M NaCl at pH 7.5. The ionophoretic current, passed using a Neurophore Model BH-2 Control Unit, was −10 to −30 nA for AMPA and NMDA, and +2 to +20 nA for GABA. Spikes were simultaneously fed into a Gateway 2000 computer containing a data analyzer (Computer Disk Recorder, Model Lab-PC). Two hundred seventy-three neurons were recorded; 173 from anophthalmic and 100 from control mice.

The Chi-square statistical test was applied to the data using SAS software. Comparisons were made between: (1) control neurons during daytime and night time; (2) anophthalmic and control neurons during night time; (3) anophthalmics and controls during daytime; and (4) total populations of anophthalmic versus control neurons.

![Fig. 1. Electrode placement and morphology of the murine SCN.](image-url)
Modified Krebs–Ringer with low Ca\(^{2+}\) (0.1 mM) and high Mg\(^{2+}\) (4.3 mM) was bath perfused to block synaptic transmission. Bicuculline (10\(^{-5}\) M) was perfused to block GABA\(_A\) receptors. Serotonin (5-HT) was perfused at 5\(\times\)10\(^{-5}\) M, as well as the 5-HT agonist (+)8-OH-DPAT at 5\(\times\)10\(^{-5}\) M, and the 5-HT antagonist cinanserin HCl, at 5\(\times\)10\(^{-5}\) M. Spontaneous activity was recorded from some cells which were impaled with a special recording electrode containing Lucifer yellow CH (Sigma, 10% in distilled water). These cells were then injected with Lucifer yellow through the same electrode by application of 2 nA negative current pulses of 250 ms duration at 2 Hz for 1.5–2 min. The slices were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer and visualized under a fluorescence microscope. Selected neurons were optically sectioned at 2–3 µm with an Olympus Fluorview confocal microscope.

3. Results

In general, fewer spontaneously firing neurons were encountered in the SCN of anophthalmic mice than of control mice. SCN neurons of both controls and mutants showed a range of firing patterns and frequencies. In control mice, neurons fired predominantly at regular intervals during daytime (Fig. 3A) and at irregular intervals at night (Fig. 3B). In addition, firing frequencies tended to be higher during the day. In daytime 44% fired at \(\geq 5\) Hz, while only 21% fired at \(\geq 5\) Hz at night (Fig. 2).

There was no statistically significant difference in the distribution of firing frequencies (\(P=0.28\)).

In contrast, SCN firing rates in anophthalmic mice were primarily 1–4 spikes/s, with only 16% firing at \(\geq 5\) Hz, regardless of the time of sacrifice as shown in Fig. 2. The differences in the distribution of firing frequencies between anophthalmics and controls were statistically significant (\(P \leq 0.0001\)) when compared during nighttime, daytime or both combined. Four different firing patterns were observed. Those units (\(\leq 10\%\)) with a rate of \(\geq 5\) spikes/s fired at regular intervals, and were similar to daytime controls (Fig. 3A). Units firing at lower rates exhibited three different spike patterns (Fig. 3B,C,E). Most (ca. 70% of the total number of units), fired at irregular intervals, similar to control units at night (Fig. 3B). Other neurons fired in bursts, approximately 20% at irregular intervals (Fig. 3E,F), and less than 1% of the total, at regular

![Fig. 3. Extracellular unit recordings of spontaneous action potentials. (A) Daytime unit from control mouse firing at regular intervals at a rate of 6 Hz. Approximately 10% of anophthalmic units displayed similar spontaneous action potentials. (B) Unit from anophthalmic mouse firing at irregular intervals. Similar units were recorded from control mice at night. (C) Unit from anophthalmic mouse firing in bursts at regular intervals of 1 burst/s. This pattern was typical of 20% of units from anophthalmic mice. Burst activity was not seen in control mice. Horizontal scale bar=10 s. (D) Magnification of last two bursts in Fig. 3C, shows that regular interval bursts typically consisted of a single action potential followed by a train of 6 to 8 spikes of diminishing amplitude. Horizontal scale bar=2 s. (E) Unit from anophthalmic mouse firing in irregular bursts. This pattern was recorded from less than 1% of anophthalmic units. Horizontal scale bar=20 s. (F) Magnification of the last burst in Fig. 3E shows that irregular bursts were characterized by a train of 6 to 10 action potentials of relatively constant amplitude. Horizontal scale bar=8 s.](image-url)
intervals (Fig. 3C,D). Irregular bursts were characterized by a train of six to 10 action potentials of constant amplitude (Fig. 3E,F). Regular interval bursts typically consisted of a single action potential followed by a train of six to eight spikes of diminishing amplitude. Burst activity did not occur in control mice.

In order to determine whether the diverse firing patterns were intrinsic properties of individual SCN neurons, or whether they were extrinsically imposed, synaptic transmission was blocked by bath perfusion with low Ca$^{2+}$ (0.1 mM) and high Mg$^{2+}$ (4.3 mM) Krebs–Ringer. Regular interval neurons of both control and anophthalmic mice responded with a marked increase in firing rate (Fig. 4). In addition, irregularly firing neurons in both control and anophthalmic mice became regularized as shown in Fig. 5. This was reversed by wash out with normal Krebs–Ringer. Because these data suggested that there is an inhibitory input to SCN neurons, attempts were made to identify the transmitter(s) involved. Thus, 5-HT ($5 \times 10^{-5}$ M), the 5-HT agonist (+)-8-OH-DPAT, the 5-HT antagonist, cinanserin HCl ($10^{-4}$ M), GABA and the GABA$_A$ antagonist, bicuculline ($10^{-3}$ M), were bath perfused. Neither 5-HT agonists nor antagonists altered the rate or pattern of action potentials of irregular units. In contrast, bicuculline increased the firing rate of both regular and irregular units. Further, the discharge of irregular units became regularized (Fig. 6). This effect was reversed by wash out.

Individual anophthalmic SCN neurons exhibited rapid time-dependent changes in firing pattern related to phase of circadian period (Fig. 7). The mouse illustrated in this experiment was euthanized at 04:30 h, approximately 4 h prior to the normal cessation of the active phase and the beginning of the resting phase of its circadian period. Following a 2-h preparation and incubation time, SCN recordings were made for 4 h. Between 06:30 and 08:00 h many neurons were characterized. At 08:15 a single neuron was selected for detailed analysis. This neuron fired irregularly, with some burst activity. Between 08:31 and 08:55 h the interspike interval increased, and by 09:50 the cell was quiescent. At 09:55 its viability was confirmed by perfusion with low Ca$^{2+}$ and high Mg$^{2+}$. This elicited a robust discharge at relatively constant intervals. When the low Ca$^{2+}$/high Mg$^{2+}$ was washed out with normal Krebs–Ringer solution, the cell returned to its quiescent state.

Twenty-eight anophthalmic and 23 control SCN neurons

![Fig. 4. High frequency regularly firing units in both control (A) and anophthalmic (B) mice show increased firing rates to blocking of synaptic transmission induced by bath perfusion with low Ca$^{2+}$ (0.1 mM) and high Mg$^{2+}$ (4.3 mM). Three traces are shown for each mouse. The upper trace shows the spontaneous action potential. The middle trace shows an increased firing rate elicited by low Ca$^{2+}$ and high Mg$^{2+}$. The bottom trace shows return to baseline following wash out with normal Krebs–Ringer solution.](image)
Ringer on this activity. Our data showed that the spontaneous activity of SCN neurons in anophthalmic mice differs in frequency and discharge pattern from control mice. Responses of SCN neurons in anophthalmic mice to ionophoreses of AMPA, NMDA and GABA was similar to that of controls, although anophthalmic neurons appeared to be more sensitive to NMDA. Pacemakers are defined as neurons which fire spontaneously in the absence of all synaptic input [3]. Continued firing of SCN neurons of control and anophthalmic mice during blockade of synaptic transmission with low Ca\textsuperscript{2+} and low Mg\textsuperscript{2+} confirmed their identity as pacemaker cells. Pacemakers have been described elsewhere in the mammalian nervous system, particularly in the brainstem [4,21,29,31].

Anophthalmic mice have a genetic mutation, which causes regression of the optic primordia in utero, prior to formation of retinal ganglion cells. Thus, these mice never develop a retina or optic nerve and never have retinal input to the SCN [7,30,33]. We have previously demonstrated that anophthalmic mice do not perceive light [18]. The current experiments showed that glutamate receptors are retained on SCN neurons in the absence of retinal or other photic input by virtue of the fact that firing rates increased in response to bath perfusion of NMDA and AMPA. Furthermore, NMDA elicited a greater degree of excitation in SCN neurons of anophthalmic mice than in controls. The increased response to NMDA could be due to an increase in the number, or sensitivity, of NMDA receptors but elicited a more vigorous response in the mutant. All responses were dose-dependent. Bath perfusion of GABA inhibited all 28 units studied in anophthalmic mice and most but not all units in controls (Fig. 9). Four of 23 units were excited in the latter. It should be noted that control slices were exposed to GABA only during the daytime portion of the circadian period.

Intracellular injection of Lucifer yellow showed three morphologically distinct classes of neuron that were excited by AMPA and NMDA: simple bipolar, curly bipolar and monopolar (Fig. 10A). There were no discernible differences between anophthalmics and controls. The monopolar cell in Fig. 10A is from the SCN of an anophthalmic mouse. It has a long dendritic processes which courses close to the base of the hypothalamus and was followed as far as the midline. Its dendrites are beaded and sparsely spinous. Fig. 10C shows two bipolar cells filled with Lucifer yellow.

4. Discussion

The current study has used brain slices to characterize and compare spontaneous activity in SCN neurons of congenitally anophthalmic and sighted, congenic control mice, and to investigate the effect of various agents. These included NMDA, AMPA, GABA, bicuculline, 5-HT, its agonists and antagonists, and low Ca\textsuperscript{2+} high Mg\textsuperscript{2+} Krebs–Ringer on this activity. Our data showed that the spontaneous activity of SCN neurons in anophthalmic mice differs in frequency and discharge pattern from control mice. Responses of SCN neurons in anophthalmic mice to ionophoreses of AMPA, NMDA and GABA was similar to that of controls, although anophthalmic neurons appeared to be more sensitive to NMDA. Pacemakers are defined as neurons which fire spontaneously in the absence of all synaptic input [3]. Continued firing of SCN neurons of control and anophthalmic mice during blockade of synaptic transmission with low Ca\textsuperscript{2+} and low Mg\textsuperscript{2+} confirmed their identity as pacemaker cells. Pacemakers have been described elsewhere in the mammalian nervous system, particularly in the brainstem [4,21,29,31].

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GABA was primarily inhibitory (control mice, 19 of 23 or 83% of neurons; anophthalmic mice, 28 of 28 neurons). While there is general agreement that GABA is inhibitory to SCN neurons at night, reports of daytime effects are inconsistent [2,22,23]. Therefore, in sighted mice, the current study focused on the effect of daytime exposure to GABA. In 17% of neurons (4 of 23) tested during the daytime, GABA induced excitation. Both responses, inhibitory and excitatory, were dose-dependent. Our data support the observations of Wagner et al. [37], who described excitation of SCN neurons by GABA in sighted rats during the daytime. These authors suggested that diurnal changes in the effect of GABA are related to changes in intracellular Cl\textsuperscript{−} concentration, with a high daytime concentration being responsible for the excitatory effects of GABA. One hundred percent of anophthalmic neurons were inhibited by GABA. In most of the current experiments it was not known whether brain slices from anophthalmic mice were obtained during subjective day or subjective night. Circa-
Fig. 7. Time dependent changes in spontaneous firing of a single unit in an anophthalmic mouse. Recording is shown for a 2-h period beginning approximately 15 min prior to the onset of the resting phase of the mouse’s circadian period at 08:30 h. Top trace: 08:15 h, approximately 15 min prior to the end of wheel-running; 08:31 h, time of transition from active to resting phase; 08:55 h, firing has become irregular; 09:23 h, spike interval has increased and amplitude has decreased; 09:50 h, the neuron has become completely quiescent; 09:55 h, bath perfusion with low Ca\(^{2+}\) and high Mg\(^{2+}\) for 5 min results in resumption of firing, confirming the continued viability of the neuron; 10:15 h, wash out with normal Krebs–Ringer causes the unit to return to its quiescent state.

dian rhythms of anophthalmic mice free-run with a period of 24.2 h. The 24.2 h period comprises approximately 10.3 h of subjective night and 13.8 h of subjective day [18]. It is highly unlikely that all 19 mice were sacrificed during the same phase of their circadian period. If, as is more likely, some mice were sacrificed during subjective day and others during subjective night, our data would suggest that the excitatory effect of GABA is related to photic input rather than mechanisms intrinsic to SCN neurons. Future studies of anophthalmic mice whose free running rhythms have been pre-determined, as discussed by Laemle and Ottenweller [18] will clarify this issue.
Fig. 8. Excitation by AMPA and NMDA and inhibition by GABA of control and anophthalmic neurons. Agonists were ionophoresed during the daytime for 1 s each.

Studies of the anophthalmic SCN using cresyl violet and Golgi preparations have shown a high degree of variability among individual animals [20,30,34]. SCN are frequently asymmetrical in size, location and morphology. In addition, the number of neurons in a pair of nuclei varies from fewer than 3000 to more than 25,000 compared to approximately 23,000 in control mice [30]. Thus, it is not surprising that we recorded fewer spontaneously firing units from anophthalmic SCN than controls. Although the size of individual SCN neurons is comparable in control and anophthalmic mice, basal dendrites appear to be abnormally atrophied in Golgi preparations of the anophthalmic SCN [30]. These are the dendrites that normally extend into the optic chiasm.

Five cell classes have been described in the SCN of the rat, based on dendritic morphology [34]. Combining intracellular injection of Lucifer yellow, confocal microscopy and physiological recording, our experiments have identified three distinct morphological classes of SCN neuron, monopolar, simple bipolar and curly bipolar,
circadian period and of the active phase, as well as patterns of locomotor activity, anophthalmic mice have been categorized into three groups [18]. Elucidating physiological differences in properties of SCN neurons in these groups is critical for a thorough understanding of the mechanisms by which the SCN regulates circadian rhythms. Fundamental differences were found in the physiological activity of the SCN in sighted vs. anophthalmic mice. In the latter, units were more irregular and tended to fire at a lower frequency.

Two basic types of units were recorded in control animals: high frequency regularly spaced (80%); and low frequency irregularly spaced (20%). In addition to these types, which accounted for approximately 10% and 70%, respectively in anophthalmics, two other types of unit were seen in the latter. These were burst units, which fired at either irregular (20%) or regular intervals (<1%). We never recorded a bursting unit in a normal animal and there are no descriptions of such SCN units in the mammalian literature. However, burst units have been recorded in the medial septal nucleus-diagonal band of Broca [36]. It should be noted that bursting pacemaker activity commonly occurs in the invertebrate nervous system [38]. Although the four types of unit in the mutants may represent four different neuronal populations, it also is possible that they reflect different stages of the circadian cycle in a single neuron. However, during the limited time in which an individual unit was held, we never saw a transition. Alternatively, there may be two basic types of neurons in both anophthalmic and control SCN—regularly firing (more than 80% of control neurons) and irregularly firing (more than 90% of anophthalmic neurons)—with a mechanism unique to anophthalmic mice superimposed to induce bursting discharges in some neurons. One possibility is that the synaptic sites vacated due to a lack of retinal input are replaced by afferents from other loci including recurrent collaterals from the recorded cells.

The current study shows that there is no intrinsic difference in electrical properties of SCN neurons in anophthalmic and control mice. When isolated from all afferent input their firing patterns are identical. The change in firing from irregular to regular by blocking synaptic transmission with low Ca$^{2+}$ and high Mg$^{2+}$, suggests that the firing pattern in most anophthalmic units and in control neurons at night, is due to the presence of inhibitory synaptic input. Regularization of the discharge following bath perfusion with the GABA$_A$ antagonist bicuculline, provides strong evidence that these synapses must be GABAergic.

References


