Membrane Properties and Monosynaptic Retinal Excitation of Neurons in the Turtle Accessory Optic System

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Kogo, Naoki and Michael Ariel. Membrane properties and monosynaptic retinal excitation of neurons in the turtle accessory optic system. J. Neurophysiol. 78: 614–627, 1997. Using an eye-attached isolated brain stem preparation of a turtle, Pseudemys scripta elegans, in conjunction with whole cell patch techniques, we recorded intracellular activity of accessory optic system neurons in the basal optic nucleus (BON). This technique offered long-lasting stable recordings of individual synaptic events. In the reduced preparation (most of the dorsal structures were removed), large spontaneous excitatory synaptic inputs [excitatory postsynaptic potentials (EPSPs)] were frequently recorded. Spontaneous inhibitory postsynaptic potentials were rarely observed except in few cases. Most EPSPs disappeared after injection of lidocaine into the retina. A few EPSPs of small size remained, suggesting that these EPSPs either were from intracranial sources or may have been miniature spontaneous synaptic potentials from retinal ganglion cell axon terminals. Population EPSPs were synchronously evoked by electrical stimulation of the contralateral optic nerve. Their constant onset latency and their ability to follow short-interval paired stimulation indicated that much of the population EPSP’s response was monosynaptic. Visually evoked BON spikes and EPSP inputs to BON showed direction sensitivity when a moving pattern was projected onto the entire contralateral retina. With the use of smaller moving patterns, the receptive field of an individual BON cell was identified. A small spot of light, projected within the receptive field, guided the placement of a bipolar stimulation electrode to activate retinal ganglion cells that provided input to that BON cell. EPSPs evoked by this retinal microstimulation showed features of unitary EPSPs. Those EPSPs had distinct low current thresholds. Recruitment of other inputs was only evident when the stimulation level was increased substantially above threshold. The average size of evoked unitary EPSPs was 7.8 mV, confirming the large size of synaptic inputs of this system relative to nonsynaptic noise. EPSP shape was plotted (rise time vs. amplitude), with the use of either evoked unitary EPSPs or spontaneous EPSPs. Unlike samples of spontaneous EPSPs, data from many unitary EPSPs formed distinct clusters in these scatterplots, indicating that these EPSPs had a unique shape among the whole population of EPSPs. In most BON cells studied, hyperpolarization-activated channels caused a slow depolarization sag that reached a plateau within 0.5–1 s. This property suggests that BON cells may be more complicated than a simple site for convergence of direction-sensitive retinal ganglion cells to form a central retinal slip signal for control of oculomotor reflexes.

INTRODUCTION

In many species, direction sensitivity is initially processed in the retina and sent by axons of direction-sensitive (DS) ganglion cells to different areas of the brain for different purposes. For optokinetic reflexes, this signal is sent to the pretectum and the accessory optic system (AOS). The properties of these nuclei have been studied in different animals (Grasse and Cynader 1982; Gruberg and Grasse 1984; Man- teuffel 1982; Morgan and Frost 1981; Rosenberg and Ariel 1990; Soodak and Simpson 1988). In turtles, the accessory optic nucleus is called the basal optic nucleus (BON). BON cells were suggested to receive monosynaptic inputs from DS retinal ganglion cells and, as a result, show DS spike activity (Rosenberg and Ariel 1991).

To date, however, subthreshold activity and membrane properties of AOS cells have never been studied in any species. To perform intracellular recording in AOS, we utilized an eye-attached isolated brain stem preparation. This preparation had the advantages of other in vitro preparations: mechanical stability and an ability to change ionic concentrations of the superfusate. However, with the eyes attached, there were additional advantages. First, the physiologically known synaptic input can be evoked in AOS by visual stimulation of the retina. When the direction of the movement of the visual pattern is controlled, for instance, a synaptic input in a particular direction can be studied. In addition, a small area of the retina can be stimulated to reduce the number of synaptic inputs by reducing the size of visual stimulation. Second, the type of retinal ganglion cell input to AOS may be quite homogeneous: DS cells with similar preferred directions. Consequently, if electrical microstimulation, as opposed to natural stimulation, is applied to the retina the evoked excitatory postsynaptic potential (EPSP) in an AOS cell is still very likely from the DS retinal ganglion cell(s) nearest to the electrode tip. Third, because the retina is a separate structure from the brain, projection cells (retinal ganglion cells) and target cells (AOS cells) can be bathed separately with different solutions to localize pharmacological effects.

We chose to study turtle AOS for the following three additional reasons. First, because the BON is located quite close to the ventral surface, whole cell recordings are possible, allowing stable recordings of small synaptic potentials. Second, both DS retinal ganglion cells and BON cells show low frequency of spontaneous action potentials, so that individual synaptic events occur that are separated in time from other events. Third, the high resistance of turtle to anoxia keeps the condition of its brain healthy and stable for the long-duration recordings required for quantitative studies.

This in vitro preparation also offers an excellent experimental model in which to study properties of single unitary EPSP as well as the integration of many identified unitary EPSPs by a cell’s membrane. Because the retina is far from
the BON, positioning of recording and stimulating electrodes can be performed easily. Also, retinal ganglion cells that project to BON are arranged in a diffuse topography. Therefore individual ganglion cells can be stimulated separately and multiple retinal sites can even be stimulated simultaneously (unpublished data).

This paper is the first in a series of investigations of BON cells’ intracellular activities in which whole cell recording techniques are used. In this paper, we report basic features of spontaneous synaptic events as well as evoked input (by electrical or visual stimulation) and membrane properties of BON cells.

METHODS

The basic techniques for BON recordings from an eye-attached isolated brain stem preparation have been described elsewhere (Rosenberg and Ariel 1990). Turtles (Pseudemys scripta elegans) were maintained in a room-temperature aquarium before >1 h of cryanesthesia in ice water. The entire brain was removed with the eyes attached. The eyes were hemisection so that visual stimuli could be focused onto each retina.

In these experiments, however, several features have been modified to suit the whole cell recording technique. First, many dorsal brain stem structures were removed, i.e., telencephalon, dorsal thalamus, pretectum, tectum, and cerebellum, to achieve a sheet-like structure so that the tissue would lie flat between two meshes in an interface chamber. In the chamber, the brain was placed ventral side up on the bottom mesh above the source of the superfusate. To hold the tissue stable, a top mesh gently covered the brain stem (although occasionally the thin tissue between the two BONs then tore). Each eye rested in a separate adjacent chamber with its own superfusate source separate from the brain. The chambers for the brain and eyes had independent drainage through small wicks of Kim-Wipe tissue that led to a lower reservoir by gravity.

The brain chamber was covered by a plastic plate with a small hole for the recording electrode to access the tissue. This cover had a gas inlet by which a moisturized 95% O₂-5% CO₂ gas mixture flowed from the caudal brain toward the eyes to form an oxygen-rich interface on the surface of the tissue.

The ionic composition of the superfusate was (in mM) 130 Na, 2.0 K, 3.0 Ca, 2.0 Mg, and 97 Cl (modified from Mori et al. 1981). After bubbling with 95% O₂-5% CO₂ gas, this solution’s pH was ~7.6 ± 0.05 (SD) (Hitzig 1982; Malan et al. 1976; Rahm and Baumgardner 1972; Reeves 1977) and its osmolality was ~274 ± 2 mosmol (Cserr et al. 1988). Pipettes used for recordings had resistances of 5–9 MΩ and were fabricated from Corning No. 7052 glass capillary (A-M Systems) with the use of a horizontal pipette puller (Model P-80, Sutter Instruments). The pipette solution contained (in mM) 124 KMeSO₄, 2.3 CaCl₂, 1.2 MgCl₂, 10.0 N₂-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, 5.0 ethylene glycol-bis-(β-aminoethyl ether)-N,N’,N’’,N’’’-tetraacetic acid, and 2.0 ATP, pH adjusted to 7.3–7.4 and osmolarity occasionally measured at 264 ± 3 mosmol. The equilibrium potentials of ions in this combination of superfusate and pipette solutions, estimated by the Nernst equation, are 90, −107, 6.9, and −68 mV for sodium, potassium, calcium, and chloride, respectively.

In several cases, the intracellular membrane potential was accessed with the use of the membrane perforation agent Amphotericin-B (Sigma, St. Louis, MO) in the patch pipette. In that case, 0.5–0.6 mg/ml of solubilized Amphotericin-B was added to the pipette solution. There were no differences in the EPSP results described here for either ruptured or perforated whole cell recordings.

In most cases, the basal optic tract and BON were visible under a ×25 dissecting microscope, thus facilitating placement of the recording pipette. The pipette’s location was also verified by recording a field potential evoked by optic nerve stimulation. In successful whole cell recordings, seals were >2 GΩ and series resistances were <50 MΩ. Recordings were terminated for recordings with membrane potentials higher than −50 mV or with action potentials that did not exceed 0 mV.

Criteria for BON cells

Once a stable whole cell recording was achieved, four separate criteria were used to identify a BON cell. Recorded neurons were quite homogeneous, so that nearly every cell fulfilled all four criteria.

1) Recording position was close to the ventral surface (<500 μm).

2) BON cells received EPSPs from optic nerve stimulation.

3) EPSPs were verified as monosynaptic: if EPSPs had a short and constant latency (<5 ms); if changing the stimulus intensity did not change this latency; and if paired pulse stimulation of optic nerve showed that responses followed both pulses when the interval was <5 ms.

4) Visual responses were DS. The contralateral retina was stimulated with a full-field pattern moving in 12 different directions to study direction sensitivity of BON cells.

Visual and electrical stimulation of retina

The system for visual stimulation of the retina in this in vitro preparation has been already described (Amamoto and Ariel 1993). That full-field stimulus was a checkerboard pattern generated on a computer monitor (stimulus display), focused through a lens to cover the whole retinal eyecup contralateral to the recording site. This visual pattern moved to either 12 or 18 different directions interrupted by a 1-s pause. A smaller stimulus pattern was used to stimulate only a focal area of the retina. The size of the window of this visual pattern was between 25 and 64 pixels, which equaled 275–704 μm (3.4–8.8°). During visual stimulation, the room was darkened.

Electrical stimulation of the optic nerve or a focal retinal area was performed by passing constant current pulses (duration 0.1 ms) through bipolar tungsten electrodes. To stimulate the optic nerve, a concentric bipolar electrode (Frederick Haer, Brunswick, ME) was placed into the optic disk inside the retinal eyecup. To position a stimulating electrode for focal retinal stimulations, the visual field boundaries of the BON cell were first determined. Then, a small spot was placed on the edge of the visual field on the stimulus display so that its image could guide the stimulating electrode within the retinal eyecup. The location and depth of the electrode were adjusted further to evoke a unitary-like EPSP/excitatory postsynaptic current (EPSC) with minimal stimulus current. For this retinal microstimulation, a small bipolar stimulating electrode (distance between the 2 poles was ~50 μm) was used. To fabricate the stimulating electrode, two finely polished tungsten wires were coated with Epoxylite. Then a theta glass capillary was pulled by the electrode puller and its tip was broken. The coated tungsten wires were inserted in the theta glass so that their tips protruded from the pipette tip. The wires were glued into the pipette so that the tungsten tips were aligned and separated by ~50 μm. The whole electrode was wrapped by aluminum foil, which was connected to ground to reduce the stimulus artifacts.

Conduction lengths for evoked synaptic events were measured after each experiment by first placing fine wire from the optic disk to the BON along the optic nerve and the basal optic tract and...
then by measuring the wire’s length. Distances from the stimulus site(s) to the optic disk were also measured (see Table 1).

**Data analysis**

The membrane current and membrane voltage recordings from BON cells were stored on video tape after digitization (Neurocorder DR390, Neurodata) at a 44-kHz sampling rate, or in a computer with the use of a data acquisition system (P-Clamp, version 6.0, Axon Instruments) with a 20-kHz sampling rate. Further off-line electrophysiological analysis was performed by P-Clamp data analysis programs, except for spontaneous synaptic events, which were analyzed with the use of a program called MINI, graciously provided by Dr. J. H. Steinbach. With the use of MINI, synaptic events were detected by setting a threshold of a differentiated trace so that events were distinguished from the noise and displayed. All waveforms with a notch indicating summation of two coincident synaptic events were disregarded, so that only unitary synaptic potentials were analyzed. The rise time and amplitude of each event were then measured. Amplitude and rise time of unitary EPSPs evoked by retinal microstimulation were measured with the use of P-Clamp.

**R E S U L T S**

**Basic membrane properties**

Whole cell recordings from 83 BON cells were analyzed in this study. In most cases, stable recordings lasted from several hours up to 12 h. Membrane potential, firing properties, current-voltage relationships, and responses to visual and electrical stimulations did not change significantly during the recording period. Changes in electrode series resistance were observed occasionally, and in those cases data collection was not continued. The mean access resistance of these recordings was 28.3 ± 16.8 (SD) MΩ. The mean values of input impedance and membrane time constant were 469 ± 140 MΩ and 26.5 ± 8.7 ms, respectively. The mean resting membrane potential was −59.6 ± 3.7 mV. The mean spike threshold was −45.0 ± 5.5 mV when measured from action potentials evoked by spontaneous EPSPs.

Figure 1 shows averaged traces of 50 action potentials evoked from a BON cell by injection of small current pulses. The afterhyperpolarization was typically biphasic (Fig. 1B). Intracellular injection of hyperpolarizing step current caused a slow depolarizing sag during the hyperpolarization, instead of a monotonic exponential response (Fig. 1C). This depolarization sag was nearly complete within 0.5–1 s. Therefore, under voltage clamp, an early peak value of the membrane current (filled arrow) and a later steady-state value (open arrow) during a voltage step were measured for a current-voltage plot (Fig. 1D). This hyperpolarization-activated current had an average threshold of −61.3 ± 4.8 mV (n = 15).

The relationship between current injection through the electrode and spike responses was further investigated during brief 1-s depolarizing pulses (Fig. 2). Depolarizing the membrane potential above the spike threshold caused a regular frequency of spikes, without any onset transients or spike frequency adaptation. At higher levels of current injection, prominent hyperpolarizations occurred at the offset of high-frequency spike bursts. At even higher levels of current injection, spike height gradually decreased during the burst, ultimately causing spike failure at the highest current injections. Spike frequency changes are quantified in Fig. 2 bottom. Spike frequency is related linearly to current injection (spike gain = 290 spikes·s⁻¹·nA⁻¹) over a large range from spike threshold (<0.02 nA for this cell) through saturation (0.3 nA, resulting in 81.3 ± 2.1 spikes/s).

**Spontaneous synaptic events**

In all cells, various sizes of spontaneous EPSP inputs were observed. Because of the relatively large size of these EPSPs and quiet electronic noise, the signal-to-noise ratio of the recordings was quite high, which facilitated accurate analyses of synaptic events. Most spontaneous EPSPs did not reach spike threshold (Fig. 3A). Therefore, even though BON cells fire spontaneous spikes very infrequently (Rosenberg and Ariel 1990), there were frequent excitatory events occurring in BON cells at subthreshold levels. This was true whether the recording room was illuminated or not and whether the visual stimulus on the retina was a checkerboard, totally white, or totally black. The average frequency of spontaneous EPSPs was 8.8 ± 5.9 Hz and the average amplitude was 2.3 ± 1.3 mV (range: 0.2–12.2 mV) at a membrane potential of −80 mV (n = 18). Data collected when the membrane potential was kept to −60 mV (n = 15) had similar values (8.5 ± 6.3 Hz and 2.3 ± 1.3 mV, respectively).

When lidocaine (a few drops of 0.5% solution) was applied simultaneously to both retinas, the majority of spontaneous EPSPs in BON cells disappeared (Fig. 3B), as did all responses to visual and optic nerve stimulation. Only a few smaller EPSPs remained after lidocaine application, indicating that most EPSPs are of retinal origin. This result was expected because most of the brain structures that are known to project to the BON were removed in this reduced preparation. Figure 3C shows the distribution of EPSP shapes (amplitude vs. rise time) during 1-min periods (□, before lidocaine application; △, after lidocaine application). The average amplitude of the remaining EPSPs was 1.06 ± 0.11 mV, with an average frequency of 1.61 ± 1.49 Hz (n = 3).

In only a few BON cells (n = 5) were spontaneous inhibi-
tory postsynaptic potentials (IPSPs) recorded (Fig. 4). However, these specific brains were not prepared differently than other brains studied with the use of this reduced preparation. At the cell’s resting potential, these events were typically slow hyperpolarizations (~2 mV). At a membrane potential of −70 mV, however, hyperpolarizing events were no longer observed, suggesting that the reversal potential of these ion channels was close to this membrane potential. The absence of IPSPs in most preparations may be due to absence of an essential neural structure(s) that provides inhibitory input to the BON.

Response to visual stimulation

Figure 5 shows an example of responses of a BON cell when a visual pattern that projected onto the contralateral retina was moved in 12 different directions. The membrane potential was initially set to −60 mV during recording in the current-clamp mode (top trace of each set). The polar plot in the middle was made by counting action potentials evoked during 15 s of visual motion (3 5-s presentations) for each of direction (200-s total recording time with 1-s pauses between stimuli). Then the membrane potential was brought to −90 mV and the response to pattern movement was again recorded. EPSPs were clearly DS in the absence of action potentials (bottom trace of each set).

Note that responses to pattern movement had a burst of spikes just after stimulus onset. In fact, when the membrane potential was set to −90 mV, DS EPSPs had a higher frequency at stimulus onset, resulting in a brief membrane depolarization. In all cells tested that had a transient excitation at stimulus onset in the preferred direction, a transient excitation was observed at stimulus onset for all directions of pattern movement.

Response to optic nerve stimulation

The response to optic nerve stimulation (up to 500 μA, 100-μs pulses) was evaluated as a possible measure of the average monosynaptic retinal input to a BON cell. Figure 6 shows an example of EPSPs evoked by stimulation of the contralateral optic disk (with stimulus intensity of 50 and 500 μA for Fig. 6, A and B, respectively). In all cases, these EPSPs had a short rise time (mean 2.9 ± 1.9 ms, range: 0.8–9.8 ms, n = 70) and a long falling phase. As a result, the mean value of the half-width of this response was 17.0±13.0 ms. The average amplitude and onset latency measured at a stimulus intensity of 1.5 times threshold were 10.0 ± 7.9 mV and 3.9 ± 1.3 ms (n = 70), respectively. Ipsilateral stimulation did not evoke any response, even following a train of 500-μA stimulus pulses (n = 6). Average values of various parameters of EPSPs are given in Table 1.

EPSP responses to optic nerve stimulation were determined to be monosynaptic. The first demonstration is that the latency of each EPSP to the same stimulus did not vary (Fig. 6E, expanded from Fig. 6A). However, Fig. 6, A and B, indicates that EPSP responses to different stimulus intensities may have slight differences in latency. The increase of intensity also caused recruitment of EPSPs, and this recruitment sometimes caused a shortening of onset latency (see DISCUSSION).

The monosynaptic nature of EPSPs was also shown with the use of short-interval paired stimuli (Fig. 6, C and D). With a minimum interval of 3.9 ms between two pulses, this cell had an EPSP response to each pulse (Fig. 6D). When the interval was shorter (2.9 ms), the cell did not respond to the second stimulation (Fig. 6C). The average value of this minimum interval for all BON cells was 2.4 ± 0.8 ms.

In some cells, polysynaptic responses could be evoked by optic nerve stimulation, but, in all those cases, monosynaptic
responses were also observed. To demonstrate polysynaptic responses, stimulus intensity was reduced to a subthreshold level (Fig. 7A). Then, a train of those pulses could evoke a slow EPSP (Fig. 7B). In this case, such a slow EPSP was evoked with as few as five pulses, each separated by 2.0 ms. In some cases, long-latency EPSPs with variable onset, independent from the monosynaptic EPSPs, were clearly observed \((n = 8)\). This indicated the involvement of polysynaptic component in BON cell responses to optic nerve stimulation. Figure 7C shows an example of this response. In this case, when the stimulus intensity was low \((50 \mu A)\), only long-latency EPSPs \((\text{mean } 12.9 \pm 0.3 \text{ ms})\) were evoked. When stimulus intensity was increased, this cell started to show earlier EPSPs. Finally, short-latency monosynaptic EPSPs were evoked by 200-\(\mu A\) stimuli. Note the constant onset latency of these early EPSPs, compared with the variability of the long-latency EPSPs’ onset. In some cases, polysynaptic EPSPs were evoked at low stimulus intensity and observed independently as in Fig. 7C, but in other cases the threshold was higher than for the monosynaptic EPSPs, appearing as the humplike shape on the falling phase of the monosynaptic EPSPs. The average onset latency of this response in all eight cases was \(10.5 \pm 1.7 \text{ ms}\) when measured with the stimulus intensity of 1.5 times threshold of this response.

**Response to current microstimulation of the retinal surface**

To stimulate retinal ganglion cells directly, the receptive field needed to be localized. The window size of the visual pattern was reduced so that a smaller number of EPSPs was evoked from the smaller area of the retina. Responses to these smaller visual stimuli were used to define the BON cell’s receptive field and to position a bipolar electrode on the retinal surface for microstimulation. Visual responses could be recorded with the use of a window reduced to as small as 25 pixels on the stimulus display, which equals 275 \(\mu\text{m} (3.4^\circ)\) on the retina. A small white square \((440 \mu\text{m})\) was then centered on the same location on the stimulus display screen. A bipolar stimulation electrode was then placed approximately at the same place as the image of this white square on the retina. With the use of 100-\(\mu\text{s}\) constant current pulses, the position of the electrode was adjusted until an EPSP was evoked at a minimum stimulus intensity.

Figure 8 shows the response of a BON cell to such a
AOS PROPERTIES AND ITS RETINAL INPUTS 619

Microstimulation of the retina. When the stimulus intensity was 10 μA, small homogeneous EPSPs were evoked (Fig. 8A). Similar responses were recorded when the stimulus intensity was increased to 40 and 60 μA (Fig. 8, B and C, respectively). When the stimulus was increased to 70 μA, additional EPSPs were recruited and superimposed on the original small responses (Fig. 8D). The finding that an increase in intensity from 10 μA up to 60 μA still only evoked small EPSPs suggests that these small EPSPs were unitary (input from a single retinal ganglion cell). The larger EPSPs evoked by higher-intensity stimulation appear to result from multiple EPSPs recruited by the spread of current to adjacent ganglion cell inputs along the retinal surface.

As with responses to optic nerve stimulation, unitary EPSPs were shown to be monosynaptic. The consistency of onset latency was examined as shown in Fig. 9A, with the use of the same data as shown in Fig. 8. These latencies were the same for stimulus intensities of 10–70 μA, i.e., for stimuli that only evoked a unitary EPSP and did not spread to recruit other EPSPs. From a different cell, Fig. 9, B and C, shows the result of short-interval paired stimulation of a focal retinal site. The unitary EPSP followed each pulse during paired stimulation having a 3.8 ms interpulse interval, but the second response failed when the interval was shortened to 2.8 ms. The average minimum interval for all unitary EPSPs recorded in this experiment was 3.5 ± 2.0 ms (n = 16).

Finally, repetitive stimulation in the voltage-clamp mode was employed to examine whether the EPSC waveform shape would change, indicating a nonmonosynaptic or nonunitary contribution to the evoked response. EPSCs are shown in Fig. 9, D and E, that were averaged from 20 trials of a high-frequency train of 100 stimuli (each trial was separated by 3.4 s). In Fig. 9D, the shape of the EPSC did not change when the pulse interval within the stimulus train was 80 ms, even up to the 100th EPSC response. In Fig.

**FIG. 3.** Spontaneously occurring excitatory postsynaptic potentials (EPSPs). Voltage traces were recorded at membrane potential of ~80 mV before and after lidocaine was dripped into retinal eyecups (A and B, respectively). Note that only small deflections of voltage recording remained during lidocaine’s effect. C: amplitude/rise time scatterplot showing synaptic events before and after lidocaine application (□ and △, respectively).

**FIG. 4.** Spontaneous depolarizing and hyperpolarizing synaptic events. Voltage traces were recorded at membrane potential of ~60 mV. In addition to EPSPs, this cell showed frequent hyperpolarizing events, considered to be spontaneously occurring inhibitory postsynaptic potentials (IPSPs). Only a few BON cells in these preparations showed these IPSP hyperpolarizations.
Fig. 5. Responses to pattern motion. Full-field patterns were projected on contralateral retina and moved in 12 different directions, each for 5 s separated by 1-s pauses. Membrane potential was first kept at −60 mV (top trace of each set) to measure cell’s spike response as firing frequency as graphed on polar plot (middle). Cell’s membrane potential was then lowered to −90 mV (bottom trace of each set) to show synaptic inputs responding to same pattern. Responses were plotted in visual world coordinates (N, nasal; T, temporal; S, superior; I, inferior). Inset: voltage trace, expanded from spike response during preferred motion shown at top left. Inset shows that spontaneously coincident EPSPs caused membrane potential to reach spike threshold during visual stimulation.

9E, responses are shown for a pulse interval of 40 ms. In that case, the amplitude gradually decayed during the stimulus train, yet the time course of EPSC remained the same.

The average amplitude of these unitary EPSPs was 7.8 ± 5.2 mV and the average onset latency was 5.3 ± 1.1 ms (n = 59). Various measurement parameters of unitary EPSPs are summarized in Table 1 for comparison with EPSPs evoked by optic nerve stimulation. Unitary EPSPs showed a large amplitude variability (Fig. 8), perhaps due to the mechanism of quantal transmission at a synapse. Variance methods (Kuno 1964) were used to estimate the quantal size of unitary EPSPs from these data; this quantal size is supposed to correspond to the size of the miniature potential. The equation used was $M^2/(\sigma^2 - \sigma_0^2)$, where $M$ is an average peak amplitude of EPSPs, $\sigma$ is a standard deviation of the noise-contaminated EPSP amplitude, and $\sigma_0$ is a standard deviation of the baseline noise. The average quantum size estimated this way was 0.96 ± 0.70 mV (n = 9), which corresponded well with the average EPSP amplitude (1.06 mV) in cells after retinal lidocaine application.

Comparison of spontaneous unitary EPSPs with EPSPs elicited by retinal microstimulation

Spontaneous EPSPs were compared with evoked EPSPs by retinal microstimulation (n = 42). An example is shown in Fig. 10, for which two different stimulation sites ($S_1$ and $S_2$) were tested on the retina (Fig. 10A) and unitary EPSP
AOS PROPERTIES AND ITS RETINAL INPUTS

formed by drawing a minimal rectangle around the downward triangles (R2 EPSPs) in Fig. 10D, identifying the square data points of spontaneous EPSPs within that rectangle, clipping those EPSPs from the spontaneous voltage recording, and overlapping them for display (Fig. 10E). The

responses were recorded (R1 and R2, Fig. 10, B and C, respectively). Then, the shapes of spontaneous events were plotted in terms of their amplitude and rise time (Fig. 10D, squares). The shapes of the EPSPs evoked by the retinal microstimulation are also plotted in Fig. 10D (upward and downward triangles). The amplitude/rise time scatterplot indicates that these two evoked EPSPs overlap with different groups of spontaneous EPSPs. Further comparison was per-

FIG. 6. Responses to electrical stimulation of contralateral optic nerve. Membrane potential was held at ~63 mV (this cell’s resting membrane potential). A and B: responses to single 0.1-ms pulses of 50 and 500 µA. In B, action potentials are truncated for clarity. C and D: paired pulse stimulation. In D, 2 stimulations were separated by 3.9 ms and responses were able to follow both stimulations. When interval was shortened to 2.9 ms as in C, however, 2nd response disappeared. E: expansion of same recording as in A, showing constancy of latency of EPSP onset.

FIG. 7. Polysynaptic responses to optic nerve stimulation. A: single sub-threshold stimulus pulses (30 µA, 0.1 ms) were presented to contralateral optic nerve without apparent effect. B: trains of 10 pulses of 30 µA each evoked responses of variable amplitude, indicating temporal summation in polysynaptic pathway. C: 3 sets of voltage traces from another BON cell that showed long-latency EPSPs. When stimulus intensity was 50 µA (top), only EPSPs with onset latency ~ 15 ms were evoked. Increasing stimulus intensity to 100 µA (middle) caused faster response, and 200-µA stimulation (bottom) finally evoked monosynaptic short-latency EPSP component. Note fluctuation of onset timing of long-latency response of top traces compared with fixed short-latency EPSPs in bottom traces.
The resting membrane potential was $-59.6 \text{ mV}$, which is close to that of cortical neurons in juvenile turtles ($-61.1 \pm 11.2 \text{ mV}$) (Blanton et al. 1989). In the case of spinal motoneurons of turtles, it has been reported to range from $-60$ to $-80 \text{ mV}$ (Hounsgaard et al. 1988). The membrane time constant and input impedance measurements were roughly in the same range as those for other neural systems studied with whole cell recording techniques (Blanton et al. 1989). The action potentials of BON cells had biphasic afterhyperpolarizations (Fig. 1B), indicating the contribution of at least two different components during this period (Gustafsson and Wigstrom 1981; Hounsgaard et al. 1988; Lancaster and Nicoll 1987; Storm 1989).

When the BON cell was hyperpolarized, the existence of a slow hyperpolarization-activated current was revealed (Fig. 1C). A depolarizing sag occurred after the onset of hyperpolarizing current, and a postinhibitory rebound depolarization occurred right after the release from the current. Although neuropharmacological features of this current are not described in this report (unpublished data), the behavior of this current is quite similar to that of the hyperpolarization-activated currents first found in cardiac sinoatrial node ($I_h$, Brown and Difrancesco 1980; $I_o$, Yanagihara and Irisawa 1980) and in neurons ($I_o$, Halliwell and Adams 1982; $I_o$, Mayer and Westbrook 1983). The average threshold of this hyperpolarization-activated current in BON cells was $-61 \text{ mV}$, right below the resting membrane potential. Therefore it is possible that this inward current produces negative feedback and thus contributes to keeping the membrane potential at its resting level. It is also possible that this current has other functions that affect the shape of synaptic inputs or firing properties when the cell is hyperpolarized. More detailed properties and possible roles of this current in conjunction with IPSP inputs will be discussed elsewhere (unpublished data).

**Spontaneous activity in BON cells**

In contrast to the infrequent spontaneous action potentials in BON cells, we found that many spontaneous synaptic events occur below spike threshold. However, despite the “extracellular” quietness, BON cells are quite ready to respond to visual stimulation, because their resting membrane potentials ($-59.6 \text{ mV}$) were relatively close to their spike threshold ($-45.0 \text{ mV}$), so that a few coincident inputs would fire spikes (see Fig. 5, inset). Dripping lidocaine onto both retinas eliminated, most, but not all, of the spontaneous synaptic potentials. Therefore these inputs were caused by the spontaneous activity of retinal ganglion cells. The size of these retinal inputs was relatively large, which offered a very high signal-to-noise ratio. The large size of EPSP from retina would help the membrane potential to reach spike threshold when only a few afferent inputs coincided temporally.

It is interesting to consider these features of BON cells with reference to the spike activity of AOS nuclei of other species. In birds and mammals, the firing rate of these cells is quite high, which has been related to the fact that the AOS is very sensitive to visual field motions that either increase or decrease the spontaneous spike rate. The pond turtle, however, resists the detrimental effects of anoxia by lowering
its metabolic rate. As a result, the spontaneous spike rate of neurons may be lowered to zero but BON cells still retain sensitivity to visual field motion by keeping their resting membrane potential close to their spike threshold level.

The remaining synaptic activity after lidocaine (Fig. 3B) could be from spontaneously active neurons elsewhere in the remaining brain tissue or miniature potentials of either the same retinal synapses or synapses from axons whose cell bodies were removed with the dorsal brain stem. The fact that the remaining synaptic potentials were all small suggests that they are miniature potentials. If this is true, the average size of miniature potentials is 1.06 mV, which is still fairly large compared with miniature potentials measured in other systems (for instance, see Lin 1988; Lupica 1992; Walmsley 1987).

Monosynaptic and polysynaptic components of evoked EPSPs

Previous studies have demonstrated the direct connection from retinal ganglion cells to the BON by an antidromic stimulation technique (Rosenberg and Ariel 1991) or by a retrograde labeling technique (Reiner 1981; Zhang and Eldred 1994). Therefore, when EPSPs were evoked electrically by stimulation of the optic nerve or a focal retinal site, monosynaptic features of the EPSPs were expected. In this regard, the following response properties were considered: 1) the constancy of onset latency, 2) the ability to follow short-interval paired stimuli, and 3) the ability to follow high-frequency repetitive stimuli.

All short-latency EPSPs had a constant latency at a given stimulus intensity (Figs. 6E and 9A). In the case of retinal microstimulation, increasing the intensity from threshold neither changed the onset latency nor the average response amplitude. This was true until another EPSP was recruited (Fig. 8). In such a case, because of the different conduction velocity of the recruited EPSP, the onset latency of the whole response was sometimes shortened. Because recruitment of EPSPs required a large increase of the stimulus intensity, a shortening of the latency (if it happened) occurred rather suddenly, instead of gradually while the intensity was increased gradually. It is therefore not likely that these responses are polysynaptic responses.

For optic nerve stimulation, the concentric bipolar electrode had a tip diameter (150 µm) that was smaller than the size of the optic disk (~600–800 µm). Consequently, the
whole optic nerve may not have been stimulated equally and a constant onset latency during increase of the stimulus intensity could not be a criterion of monosynapticity. During increases in optic nerve stimulation, current may spread and activate fibers with faster conduction velocities. In fact, the shortening of the onset latency was often observed as the stimulus intensity increased.

Synaptic response followed the short-interval paired stimulation (Figs. 6, A and B, and 10, B and C, minimum interval of 2.4 ms for optic nerve stimulation and 3.5 ms for retinal microstimulation). The second response appeared with a constant latency in each case. Therefore the second response was perhaps due not to an activation of a polysynaptic response by paired subthreshold stimulation but rather to the activation of a second monosynaptic response. Also, responses followed high-frequency stimulation without changing their time course, indicating that they were all monosynaptic EPSPs. The minimum interval to evoke paired EPSPs was longer for retinal microstimulation than for optic nerve stimulation. This is presumably due to the longer refractory period of the cell soma than of the axon.

Subthreshold train pulse stimulation, on the other hand, evoked EPSPs, indicating the existence of polysynaptic responses. The existence of long-latency EPSPs with variable onset also supports the involvement of a polysynaptic pathway following optic nerve stimulation. This polysynaptic pathway is usually considered to be within the brain. Another possibility is that optic nerve stimulation activated centrifugal fibers that may project to DS retinal ganglion cells (Hayes and Holden 1983; Miles 1971; Uchiyama and Barlow 1994). In either case, features such as a constant EPSP latency, paired responses to short-interval stimuli, and responses to high-frequency stimulation clearly indicate that the short-latency EPSPs are monosynaptic, at least at their early phase. The locus of polysynaptic pathway(s) remains to be determined.

**Unitary input to BON cells from retinal ganglion cells**

By reducing the size of the stimulus pattern window and placing it on the edge of a BON cell’s visual field, we succeeded in evoking just a few DS synaptic inputs. Microstimulation of the same area indeed evoked a response with properties of unitary EPSPs. These responses are more likely to be due to stimulation of ganglion cell somata, because ganglion cell axons in the overlying fiber layer should have a much higher stimulus threshold than the underlying somata. The observation that those evoked EPSPs had the same response shape to a wide range of stimulus intensities indicates that the spread of the stimulus current was quite limited relative to the distances between neighboring DS ganglion cells. In fact, a diffuse distribution of BON projecting ganglion cells in the retina has been reported anatomically (Zhang and Eldred 1994). The total number of those ganglion cells in the turtle retina was 1,500, with the nearest neighbor being \( \sim 100 \) \( \mu \)m away. However, only ganglion cells with a similar preferred direction in a portion of the retina (the receptive field of a BON cell) would project to the same BON cell. It is estimated that the closest neighboring DS ganglion cells projecting to the same BON cell are separated by 424–734 \( \mu \)m (5.3–9.18°) (unpublished data).

The evoked unitary EPSPs had an average amplitude of 7.8 mV. This is relatively large compared with that of unitary
EPSPs reported in different system and different animals. In mammalian hippocampus, for instance, the sizes of unitary EPSPs reported previously were in a range of 89–563 μV (in CA1 cell, evoked by a CA3 cell stimulation) (Friedlander et al. 1990) or 2.26 mV on average (in CA3 pyramidal cell evoked by dentate hilar mossy cell stimulation) (Scharfman 1994). The spinal motor neurons in mammals received unitary EPSPs of a mean size of 270 μV (Kuno and Miyahara 1969) or 100 μV (Finkel and Redman 1983). The only available data of the turtle CNS were recorded in the medulla and indicate a unitary EPSP size of 0.6–0.8 mV (Selenov and Shik 1982). Our observation of large unitary EPSPs is confirmed, however, by the large size of spontaneous synaptic events in BON cells. These large unitary EPSPs will offer a great advantage in the analysis of the detailed properties of the individual inputs because they are integrated at the BON cell membrane.

Besides the amplitude variability for any individual afferent input, we found that the amplitude and rise time of evoked EPSPs differed for different unitary inputs (Figs. 10 and 11). Variability was also observed in spontaneously occurring EPSPs (squares in Figs. 3C and 10D). One factor may be that the location of synapses on the dendrites varies (Rall 1967). Another possibility is that each synapse uses a different ratio of the mixed transmitter receptors, such as two glutamate receptors, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid and N-methyl-d-aspartate (for review, see Nicoll et al. 1990). Pharmacological studies can determine whether different receptors contribute to EPSP amplitude variability.

Whatever the mechanism, the shape differences for different synapses resulted in clustering of data points on amplitude/rise time scatterplots, as shown in Fig. 11. Unless the data points of these clusters overlap each other by chance, these EPSP shapes within these clusters can be useful “signatures” of individual synapses to identify individual afferent inputs from a sample of EPSPs. This feature was utilized to select a particular EPSP group from a continuous recording of spontaneous EPSPs, as shown in Fig. 10. The shapes of these collected spontaneous EPSPs were indeed quite similar to the shape of the evoked unitary EPSPs, suggesting that inputs originating from a single retinal ganglion cell can be extracted from the population of spontaneous or visually evoked EPSPs. Unfortunately, there are far too many EPSPs during full-field stimulation to identify with good reliability the responses of a single afferent input from among dozens of other visually responsive inputs (see Fig. 5). Use of a visual pattern restricted to a small region of

FIG. 11. Amplitude/rise time scatterplots of 25 evoked unitary EPSPs. These plots were generated from shape measurements of sets of unitary EPSPs from 25 retinal microstimulation sites (like the 2 sets of triangles shown in Fig. 10). Data were derived from 16 cells in 13 preparations, but are plotted here with identical axis scaling for comparison. Note that many unitary EPSPs had characteristic shapes that resulted in compact clusters of data points in amplitude/rise time scatterplot.
the receptive field may limit the number of stimulated ganglion cell afferents, so that fewer unitary EPSP shapes may be recorded in the BON cell.

BON cell contribution to visual information processing

The spike response of BON cells to moving patterns that were recorded at the resting membrane potential with the whole cell patch configuration in this reduced preparation showed essentially the same visual response as the response recorded extracellularly in an intact turtle brain stem in vitro. It therefore appears that any nonretinal inputs to the BON that were removed in this reduced preparation have little obvious effect on the visual response to these full-field visual stimuli. Moreover, whole cell recording of ruptured BON cells appears not to disturb the response properties of these cells.

Hyperpolarization during full-field stimulation prevented spike activity, allowing comparison of the retinal input and the spike output (Fig. 5). Both show a transient excitation at stimulus onset. It is not known how BON cells act to shape the spatiotemporal properties of retinal input and further enhance motion detection of optokinetic stimuli. Stimulation detection in another retinal target, the lateral geniculate nucleus, appears to be related to two different modes of activity, called continuous and bursting modes, which change the visual sensitivity and input/output function of these neurons (Guido et al. 1992). The BON cells, on the other hand, had linear responses in firing frequency to depolarizing current injections when near their resting potentials.

Hyperpolarizing current injection through the patch pipette may reveal other insights into the visual information processing performed by these cells. An inward current was activated when the membrane potential was slightly hyperpolarized from its resting membrane potential. As discussed above, this characteristic slowly activating current may have an important role in fine tuning of BON cell activity, especially when the cells are hyperpolarized. Indeed, BON cells may be hyperpolarized under certain conditions, because IPSPs were recorded in some brain preparations (see Kogo and Ariel 1996). The hyperpolarization-activated current may help create more phasic responses in BON cells to excitatory inputs after prolonged hyperpolarization.

In conclusion, the BON is a second-order nucleus that integrates and relays DS signals from the retina to higher-order brain structures. Because of some complex features of cells in this nucleus, it is possible that the BON performs more than simple signal-conserving relay function. These experiments, with their stable whole cell recordings of large unitary EPSPs, offer a means to a detailed investigation of signal processing in the AOS. Further studies of spatial summation of unitary EPSPs, physiological functions of IPSP inputs, and hyperpolarization-activated currents should clarify the complex functions of the BON.

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