Polarization-sensitive and light-sensitive neurons in two parallel pathways passing through the anterior optic tubercle in the locust brain

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**Abbreviated title:** Polarization-sensitive neurons

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ABSTRACT

Many migrating animals use a sun compass for long-range navigation. One of the guiding cues used by insects is the polarization pattern of the blue sky. In the desert locust *Schistocerca gregaria*, neurons of the central complex, a neuropil in the center of the brain, are sensitive to polarized light and might serve a key role in compass navigation. Visual pathways to the central complex include signal processing in the upper and lower units of the anterior optic tubercle. To determine whether these pathways carry polarization-vision signals, we have recorded the responses of interneurons of the optic tubercle of the locust to visual stimuli including polarized light. All neurons of the lower unit but only one out of five recorded neurons of the upper unit of the tubercle were sensitive to linearly polarized light presented in the dorsal visual field. These neurons showed polarization opponency, or a sinusoidal modulation of activity, during stimulation through a rotating polarizer. Two types of bilateral interneurons preferred particular e-vector orientations, reflecting the presence of bilateral pairs of these neurons in the brain. We show here for the first time neurons with projections to the lateral accessory lobe that are suited to provide polarization input to the central complex. All neurons of the tubercle, furthermore, responded to unpolarized light, mostly with tonic activity changes. These responses strongly depended on stimulus position and might reflect navigation-relevant signals such as direct sunlight or visual landmarks that are integrated with polarization responses in neurons of the lower unit.

Keywords

polarization vision; central complex; compass orientation; insect brain; *Schistocerca gregaria*
INTRODUCTION

Animals moving through their environment are guided by a variety of sensory cues they exploit to control their direction of locomotion and to calculate distances. Spatial orientation in mammals is characterized by internal representations of the spatial relationship between the animal’s body and its surrounding space. “Place cells” and “head direction cells” are well studied examples of neurons encoding spatial properties in the brain of rats and other mammals (Best et al. 2001; Taube and Bassett 2003; Poucet et al. 2003). These neurons signal the location of the animal within familiar terrain and encode the azimuthal orientation of the animal’s head irrespective of its location. Activity of these neurons is influenced by distal visual and non-visual cues, but also by motion-related, idiothetic information. Insects rely on a system for spatial orientation based on landmark orientation in familiar terrain and on vector orientation based on a sky-compass in unfamiliar terrain or when landmarks are not available (Giurfa and Capaldi 1999; Wehner 2003).

Many insect species have evolved a specialized region in their compound eye, the dorsal rim area that is highly adapted to detect the polarization pattern of the blue sky (Labhart and Meyer 1999; Homberg and Paech 2002). Common characteristics of dorsal rim areas that favor polarization vision include precise alignment of microvilli in two orthogonal blocks in each ommatidium, enhanced cross-sectional area and reduced length of rhabdoms, and homochromacy of dorsal rim photoreceptors (UV receptors in bees and ants, blue receptors in crickets and locusts; reviewed by Labhart and Meyer 1999). Behavioral experiments in honeybees, desert ants, crickets, flies, and locusts showed that the dorsal rim area is essential for polarotactic orientation and, in bees and ants, for compass orientation (Brunner and Labhart 1987; von Philipsborn and Labhart 1990; Wehner 2003; Mappes and Homberg 2004). Polarization-sensitive interneurons (POL-neurons), found in several areas of the insect brain, are involved in signaling the orientation of the animal relative to the sky polarization pattern (Labhart and Meyer 2002; Wehner 2003; Homberg 2004). POL-neurons were studied in
peripheral visual neuropils of the optic lobe in cockroaches (Kelly and Mote 1990; Loesel and Homberg 2001), crickets (Labhart et al. 2001), and ants (Labhart 2000). More recently in the desert locust and cricket, POL neurons were discovered in the central complex (Vitzthum et al. 2002; Sakura and Labhart 2005), a neuropil in the median protocerebrum involved in spatial orientation, right-left maneuvering, and other aspects of motor control (reviewed by Strauss 2002; Homberg 2004). POL neurons of the central complex have receptive fields oriented toward the zenith and show broad distribution of their preferred e-vector orientations (Labhart and Meyer 2002; Wehner 2003; Homberg 2004). Although direct evidence is still lacking, these features render these neurons particularly well suited to provide the directional component of an internal compass signal.

A recent anatomical study in the locust suggests that a small area in the brain, the anterior optic tubercle (AOTu), is a relay station in the neural pathway from polarization-sensitive photoreceptors to the central complex (Homberg et al. 2003; Fig. 1A). We showed that two parallel pathways originating in the medulla provide, via two subunits of the AOTu, visual input to two subunits of the central complex, the upper and lower divisions of the central body. To provide direct evidence for a role of the AOTu in polarization vision, we characterized the responses of AOTu neurons to visual signals, in particular polarized light. Our present study supports the hypothesis that the AOTu contributes prominently to the sky-compass system. The lower unit of the AOTu plays a role in interhemispheric exchange of polarized light information and acts as an input stage for POL neurons of the central complex. Both units of the AOTu, furthermore, show responses to unpolarized light stimuli that may directly mediate information about solar position and thus may also be relevant to navigational tasks.
METHODS

Preparation

Adult locusts (Schistocerca gregaria) were taken from crowded laboratory cultures at the University of Marburg, 1-3 weeks after imaginal moult. Animals were anesthetized by cooling for 20-30 minutes. After removal of the legs the locusts were waxed to a metal holder. The head capsule was opened frontally by cutting dorsally between frons and vertex, laterally along the inner boundaries of the lateral ocelli and the outer boundaries of the ocular sutures, and ventrally parallel to the epistomal suture. After cutting of the antennal nerves, the cuticle, tracheal air sacs, and fat body were removed to expose the brain. To minimize movements, all muscles in the head capsule were transected. The metal holder with the locust was mounted vertically into the experimental setup (Fig. 1B). A stainless steel platform was inserted between the esophageal connectives. It supported the brain from posterior and served as a ground electrode. In some preparations the brain was additionally stabilized by slightly pushing a stainless steel ring against its frontal surface. If these measures did not suffice to obtain stable recordings, the abdomen and the entire gut were removed, and the thorax was filled with cotton tissue and vaseline to prevent desiccation. To facilitate microelectrode penetration, we used forceps to remove the neural sheath above the target area. During the whole experiment, the brain was submersed in locust saline (Clements and May 1974).

Electrophysiology

Microelectrodes, with a resistance of 70-200 MΩ in the tissue, were drawn from omega-shaped borosilicate capillaries (inner diameter: 0.75 mm, outer diameter: 1.5 mm; Hilgenberg, Malsfeld, Germany) with a Flaming/Brown puller (P-97, Sutter, Novato, CA). Their tips were either filled with a 5% aqueous solution of Lucifer yellow (Sigma, Deisenhofen, Germany) or with 4% Neurobiotin (Vector Laboratories, Burlingame, UK) in 1 M KCl. The shanks were filled with 0.1 M LiCl (Lucifer electrodes) or 1 M KCl (Neurobiotin electrodes). Intracellular
signals were amplified and filtered (10x, 2 kHz low pass) with a custom built amplifier and monitored with an audiomonitor and a digital oscilloscope (Hameg HM 205-3; Hameg, Frankfurt/Main, Germany). After sampling at 25 kHz with a Digidata 1322A (Axon Instruments, Union City, CA) the data were stored on a personal computer, using pClamp 9 (Axon Instruments). To compensate for shifts in baseline, some recordings were digitally filtered (10 Hz high pass). After recording, the tracer was injected iontophoretically into the cell with constant hyperpolarizing current (3-5 nA, 1-7 min, Lucifer yellow), or constant depolarizing current (3 nA, 1-7 min, Neurobiotin).

**Visual stimulation**

Experiments were carried out in the dark with two experimental setups. On the first setup, light stimuli were provided by a xenon arc (XBO 150 W) and filtered through an infrared short pass filter. Since locust polarization-sensitive photoreceptors are blue-sensitive (Eggers and Gewecke 1993), light was passed through a standard glass light guide (Schölly, Denzlingen, Germany; spectral range ~400-800 nm), connected to a perimeter. Either a linear polarizer (HN38S, Polaroid, Cambridge, MA) or a neutral density filter of equal transmission was moved into the light path. Stimuli were applied from the zenith and, for unpolarized light flashes, also from lateral to the right and left eye (0° elevation, in a few experiments 30° elevation, duration of unpolarized light stimuli 1.5-3 s). The angular extent of the stimulus at the locust’s eye was 4.7°, its irradiance was 125 µW/cm². To test for polarization sensitivity, the polarizer was rotated through 180° (angular velocity: 12.9-34°/s) either clockwise (0°-180°) or counterclockwise (180°-0°) as seen by the animal (Fig. 1B). An orientation parallel to the longitudinal axis of the animal was defined as 0°.

At a second experimental setup, polarizer and neutral density filter were illuminated through a standard glass light guide that was connected to a 150 W halogen bulb (3200 K; irradiance at the locust head: 13 µW/cm², visual angle: 2.1°; spectral range ~400-800 nm).
Lateral light stimuli could be applied by additional light guides (standard glass), illuminated by 150 W halogen bulbs. These stimuli appeared at a visual angle of 3° and an irradiance of 2-4 μW/cm². After weak responses to unpolarized light were encountered in some neurons, the halogen light source was replaced by a stronger xenon arc (XBO 75 W) and quartz optics to increase the intensity of the stimuli and to expand the spectral range to UV (irradiance, 67 μW/cm², visual angle: 2.1°, spectral range ~280-800 nm; polarizer HNP’B, Polaroid). Under both illuminations, the polarizer was rotated through 360° in either direction. Angular velocity was 20°/s or 21.8°/s.

Ocular dominance was tested in both experimental setups, by shielding one eye from the light source with a handheld piece of cardboard. Additionally in some experiments, the ipsilateral eye was painted black (Marabu Decorlack matt, water based) during the recording to test for contralateral input.

**Data analysis**

Relative times of action potentials were evaluated by the threshold detection algorithm of pClamp 9. Mean background activities during darkness were obtained from counts of spikes in 1-s time intervals before each visual stimulus divided by the respective time. For single stimuli, tonic responses were scored as excitatory, if the spiking activity during the last second of stimulation (A_{stim}) was higher than the mean background activity plus the standard deviation, and as inhibitory if A_{stim} was lower than the mean background activity minus the standard deviation. For comparison of different recordings from the same cell type (TuTu1 and LoTu1 neurons), relative activity changes in response to unpolarized light stimuli were determined as A_{stim} divided by the number of spikes during one second immediately before stimulus onset (A_{pre}). The responses of these neurons to unpolarized light were statistically evaluated using the Wilcoxon test for paired samples (significance level α = 0.05).
Neuronal responses to polarized light were analyzed using Oriana 2.02a (Kovach Computing Services, Anglesey, Great Britain) for circular statistics and Origin 6.0 (Microcal, Northhampton, CA) for curve fitting. To investigate the presence of periodicity (= polarization sensitivity) in the neuronal responses to the rotating polarizer, we used the Rayleigh test (Batschelet 1981). We specifically tested whether the distribution of e-vector angles assigned to each spike in the recording trace showed directedness. Significant deviation from a random distribution indicating polarization sensitivity, was set at a significance level of $\alpha = 0.01$. To obtain e-vector response plots of the polarization-sensitive neurons, means of spike frequencies ($\pm$ SD) were determined during consecutive 10° bins from two to four revolutions of the polarizer. These were plotted against the bin centers as a function of e-vector angle. e-vector angles eliciting maximal spike activity ($\Phi_{\text{max}}$) were determined by fitting $\sin^2$-functions to the data sets using the Origin 6.0 implementation of the nonlinear least-squares Levenberg-Marquardt algorithm. The match between the dataset and the fit is described by the coefficient of determination ($0 \leq R^2 \leq 1$). To test whether the distribution of $\Phi_{\text{max}}$ angles in multiple recordings from the same cell type (LoTu1- and TuTu1 neurons) was significantly different from randomness, Rao’s spacing test was used (Batschelet 1981).

**Histology**

After recording and Neurobiotin/Lucifer injection, brains were dissected out of the head capsule and fixed for 1 h at room temperature or overnight at 4°C either in 4% paraformaldehyde in 0.1 M phosphate buffer (Lucifer preparations) or in fixative containing 4% paraformaldehyde, 0.25% glutaraldehyde, and 0.25% saturated picric acid in 0.1 M phosphate buffer (Neurobiotin preparations). Brains were then embedded in gelatine/albumin and fixed overnight in 8% formaldehyde in 0.1 M phosphate buffer. After sectioning at 35 μm with a vibrating blade microtome (VT 1000S, Leica, Wetzlar, Germany) the Neurobiotin
preparations were incubated for 18 h with Streptavidin conjugated to horseradish-peroxidase (Amersham Buchler, Braunschweig, Germany) at 1:200 in phosphate buffered saline with 0.5% Triton X-100. The Lucifer yellow preparations were first incubated with rabbit anti-Lucifer yellow antiserum, then with goat-anti-rabbit antiserum and finally with a peroxidase-antiperoxidase conjugate (Homberg and Würden 1997). After incubation, Lucifer and Neurobiotin preparations were stained with 3,3’-diaminobenzidine tetrahydrochloride, hydrogen peroxide, and nickel ammonium sulfate as described by Vitzthum et al. (2002). Neurons were reconstructed from consecutive sections using a microscope with camera lucida attachment. Photomicrographs were made using a Zeiss Axioskop compound microscope equipped with a Polaroid DMC digital camera. All positional information refers to the body axis of the animal. The terms ipsi- and contralateral refer to the position of the cell body.

RESULTS

This study is based on 112 intracellular recordings obtained from neuronal processes in the AOTu. Dye-injections were successfully completed in 92 neurons which were categorized into seven morphological types. Twenty neurons that could not be stained were assigned to morphological types based on their physiological response properties. The unique combination of high background activity, polarization opponency, and reduction with brief total inhibition of spiking activity in the sinusoidal response to the rotating polarizer (see below) allowed to assign 15 recordings from unstained neurons to TuTu1. Five unstained neurons were identified as LoTu1 based on characteristic combinations of low background activity and activation by all e-vector angles. The arborizations of the recorded cell types in the AOTu were restricted either to the upper or the lower subunit but never extended into both units. All neurons recorded from the lower unit, but only one neuron from the upper unit were polarization-sensitive. The neurons were termed after their main projection areas and enumerated if necessary. In some preparations, more than one cell was stained. In these cases
one of the neurons, interpreted as the recorded cell, was usually stained dark blue to black, while others appeared in shades of gray. Colabelling of certain cell types did not occur on a regular basis, and in all cases, the weakly labelled neurons had a major neurite in close proximity to the recording site. Therefore, dye leakage through injuring of neighboring cells rather than electrical coupling through gap junctions appears as the most likely reason for colabelling of neurons in our experiments. No systematic differences in polarization sensitivity (types of responsive neurons, polarization-opponency, ocular dominance, and e-vector tuning) were observed with the three types of polarized light stimulation, probably owing to the fact that locusts are sensitive to polarized light in the blue range (Eggers and Gewecke 1993). However, responses to unpolarized light flashes were clearly stronger and more robust with xenon arc stimulation (both with quartz and standard glass optics).

Neurons of the intertubercle tract

Three types of neuron, termed tubercle-tubercle neuron 1 (TuTu1) and lobula-tubercle neuron 1 and 2 (LoTu1, LoTu2) interconnected the AOTu of the right and left brain hemispheres via the intertubercle tract. Two of these neurons, LoTu1 and LoTu2, had additional bilateral ramifications in the anterior lobe of the lobula.

TuTu1 neurons

Stable recordings from TuTu1 neurons were obtained in 48 experiments. Their cell bodies are in the inferior lateral protocerebrum close to the antennal lobe. TuTu1 neurons have ramifications in the lower unit of the ipsilateral AOTu. An axonal fiber projects through the intertubercle tract and gives rise to a second tree of arborizations in the lower unit of the contralateral AOTu (Fig. 2A). Ipsilateral, presumably dendritic ramifications are smooth and are concentrated either in the outer or the inner hemisphere of the lower unit. These arborizations are less dense than the beaded contralateral terminals, which extend throughout
the lower unit of the AOTu (Fig. 2A). Neurons with cell bodies in the right hemisphere were recorded 23 times, neurons with cell bodies in the left hemisphere 10 times, and 15 cells were not stained. Graded changes in membrane potential were not observed in any of the recordings and, correspondingly, the soma and presumed dendritic input arborizations were always in the brain hemisphere contralateral to the recording site. Since all of the 15 unstained neurons were recorded in the right AOTu, their cell bodies had to be in the left brain hemisphere. In 13 recordings two sibling TuTu1 neurons were stained. In 8 cases the somata were located on the same side. In two recordings three TuTu1 neurons were stained, but in both cases only two cell bodies were in the same brain hemisphere, suggesting that TuTu1 neurons exist as two bilateral pairs of neurons.

TuTu1 neurons had a mean background activity of 31.6 (SD 10.2) impulses/s and a maximum activity of 63.0 (SD 19.2) impulses/s. The neurons responded to stimulation with unpolarized light depending on spatial position and type of light source. Lateral stimulation with halogen light (0° elevation) did not elicit a response in 12 out of 13 experiments. In contrast, stimulation with xenon light led to tonic inhibitions when applied to both eyes from the zenith but had no significant effect when applied from lateral to the ipsi- or contralateral eye (Fig. 2B).

TuTu1 neurons showed polarization opponency, i.e. they were maximally excited by a particular $e$-vector ($\Phi_{\text{max}}$) and were maximally inhibited by an $e$-vector perpendicular to $\Phi_{\text{max}}$ (Fig. 2C). Ocular dominance was tested in 8 experiments. When the ipsilateral eye was shielded and/or painted black, the amplitude of the POL-response (maximum activity – minimum activity) was strongly reduced in 7 experiments, but some residual response remained (Fig. 2D). Shielding the contralateral eye had little or no effect on the polarization response (Fig. 2D).

The distribution of $\Phi_{\text{max}}$ angles was significantly different from randomness (Rao’s spacing test, $p < 0.01$) and differed between TuTu1 neurons with somata in the left brain
hemisphere (Fig. 2E) and those with somata in the right brain hemisphere (Fig. 2F).

Recordings from TuTu1 with somata in the left hemisphere had a dominant peak of $\Phi_{\text{max}}$ orientations around 175° and a second, less prominent peak around 135° (Fig. 2E). Neurons with somata in the right hemisphere, in contrast, had a peak of $\Phi_{\text{max}}$ angles around 45° (Fig. 2F). In addition, four neurons had $\Phi_{\text{max}}$ angles between 140° and 180° (Fig. 2F). The mean of the coefficients of determination ($R^2$) for the $\sin^2$-fits was 0.92 (binocular stimulation, $n=48$, SD 0.075).

**LoTu1 neurons**

Recordings from LoTu1 neurons were obtained in 55 experiments. The soma of LoTu1 is located in close proximity to those of the TuTu1 neurons in the inferior lateral protocerebrum and has a diameter of 25-30 μm. Ramifications in both brain hemispheres are in the ventralmost layer 1 of the anterior lobe of the lobula and, via fibers in the anterior optic tracts, in the lower unit of the AOTu. Ipsilateral arborizations have a smooth appearance, while ramifications in the contralateral AOTu and lobula are varicose (Fig. 3, A and B). Small, blebby sidebranches extend from the neurite in the contralateral anterior optic tract (Fig. 3A). The commissural axon of LoTu1 connects both lower units of the AOTu via the intertubercle tract and has a diameter of 6 μm. In 35 preparations the cell bodies were located in the right, in 15 preparations, in the left brain hemisphere, and five neurons were not stained. In all preparations with two stained LoTu1 neurons, their cell bodies were on different sides ($n=6$). More than two LoTu1 cells were never stained in one brain. Only one LoTu1 neuron was recorded from the soma-sided AOTu, and this recording was the only one showing graded potentials in addition to spikes.

LoTu1 neurons had a background activity of 8.1 (SD 5.4) impulses/s and a maximum activity of 43.3 (SD 16.1) impulses/s. In 39 out of the 55 recordings, maximum activity was reached during stimulation with unpolarized light. Unpolarized halogen light from ipsi- or
contralateral had no effect in 20 of 21 recordings. Unpolarized xenon light from ipsilateral caused phasic-tonic excitations, followed in some cases by off inhibitions (Fig. 3F). Zenithal stimulation led to tonic inhibition, in some cases with rebound excitation, and contralateral stimulation did not result in a significant response (Fig. 3F).

Zenithal stimulation with polarized light led to tonic excitation that was sinusoidally modulated in strength by the rotating e-vector; maximum and minimum activity occurred at perpendicular e-vectors (Fig. 3, C and D). Ocular dominance was tested in 12 experiments. When the contralateral eye was shielded from polarized light stimulation by a piece of cardboard, the e-vector response was similar to that of binocular stimulation, but with a slightly reduced activation in some recordings (Fig. 3D). When the ipsilateral eye was shielded and/or painted black, spiking activity remained at background level and was not significantly different from uniformity during e-vector rotation (Rayleigh test). This indicates that the response to polarized light is mediated exclusively by the ipsilateral eye (Fig. 3D).

Depending on the location of the cell body, $\Phi_{\text{max}}$ was at a mean e-vector of $41^\circ$ (soma in the right hemisphere; n=26, angular deviation $21.4^\circ$) or at $134^\circ$ (soma in the left hemisphere; n=20, angular deviation $21.9^\circ$) (Fig. 3E, Rao’s spacing test, $p < 0.01$). The mean of the coefficients of determination ($R^2$) for the $\sin^2$-fits was 0.795 (binocular stimulation, $n = 55$, SD 0.114).

LoTu2 neurons

Recordings from LoTu2 neurons were obtained twice. Their cell bodies (diameter: 20-25 $\mu$m) are located in the inferior lateral protocerebrum more lateral than that of the LoTu1 neuron. Smooth ramifications extend through layer 2 of the anterior lobe of the lobula. The axon enters the median protocerebrum via the anterior optic tract, bypasses the ipsilateral AOTu posteriorly, and crosses the brain midline within the intertubercle tract. Arborizations with small numbers of beaded terminals are present in the upper unit of the contralateral
AOTu and widely in layer 2 of the anterior lobe of the contralateral lobula (Fig. 4A; Homberg et al. 2003).

LoTu2 neurons had a background activity of about 35 impulses/s. Unpolarized light led to weak tonic inhibition when presented from ipsilateral and dorsal, but excited the cells when presented from contralateral (Fig. 4, B and C; xenon arc stimulation with standard glass optics). Zenithal polarized light led to tonic reduction in activity, which was independent of e-vector angle in both recordings (Fig. 4D).

**Neurons of the tubercle-accessory lobe tract**

Three types of neuron connected the AOTu with the lateral accessory lobe through the tubercle-accessory lobe tract. One of these cell types had additional ramifications in the lobula.

**TuLAL1a neurons**

Colabeling of TuLAL1a neurons occurred in many recordings from LoTu1 or TuTu1 (n = 14). However, because of their small neurites only two intracellular recordings were obtained. The somata of TuLAL1a neurons are located close to the anterior surface of the brain near the antennal lobe. Primary neurites enter the AOTu from median and give rise to dense ramifications that are concentrated in the inner, middle, or outer third of the lower unit of the AOTu. The arborizations of the neuron presented in Fig. 5A are confined to the middle third of the lower unit, while the micrograph of Fig. 5B shows a TuLAL1a neuron with a dendritic field in the inner third of the lower unit (black arrowheads). Axonal fibers of TuLAL1a neurons leave the tubercle and project via the tubercle-accessory lobe tract to the lateral accessory lobe. Terminals were always confined to a certain subfield in the lateral accessory lobe, the lateral triangle, and were shaped as large, irregular bulges (up to 15 μm) with tiny filiform appendices (Fig. 5C).
The background activities of the two recorded TuLAL1a neurons ranged from 4 to 6.5 impulses/s. Neuronal activity during $e$-vector-rotation was significantly different from a uniform distribution for both neurons (Rayleigh test, $p < 0.01$). Both TuLAL1a neurons showed polarization opponency with sinusoidal $e$-vector response curves, $\Phi_{\text{max}}$ at 40°, respectively 117°, and total inhibition accompanied by membrane hyperpolarization around $\Phi_{\text{min}}$ (Fig. 5, D and E). Halogen light stimulation with unpolarized light from ventrofrontal led to excitation followed by an off-inhibition of up to 4 s duration in one recording, while it had no effect in the second recording. Ipsilateral unpolarized light had no influence on the spiking activity of the neurons, contralateral stimulation evoked activation in one recording. Dorsal unpolarized light, tested in one experiment, inhibited the neuron.

**TuLAL1b neurons**

Neurons of this type were co-stained in 21 preparations of LoTu1- and TuTu1-recordings, indicating a close spatial relationship between these cell types. Owing to the small axon diameter of TuLAL1b neurons, however, only two intracellular recordings were achieved (Fig. 6). TuLAL1b neurons comprise at least three morphological subtypes. All of them have their somata (15 μm diameter) in the inferior protocerebrum together with the TuLAL1a cell bodies. Fine smooth processes in the lobula and in the lower unit of the AOTu are located in the same areas as those of LoTu1 neurons (Fig. 6B). From the lower unit of the tubercle, axonal fibers run via the tubercle-accessory lobe tract medially through the angle formed by the $\alpha$-lobe and peduncle of the mushroom body and then ventrally into the lateral accessory lobe. The three subtypes of TuLAL1b project to different subcompartments of the lateral accessory lobe. The cells terminate in large knobs of about 10 μm diameter either in the lateral triangle, in the median olive, or in both neuropils of the lateral accessory lobe (Fig. 6B).
Without stimulation one of the recorded TuLAL1b neuron remained silent (Fig. 6A), the other neuron spiked at 2.5 impulses/s. One of the two neurons was excited by contralateral but not by ipsilateral light (halogen light source, Fig. 6A), while the second cell was unresponsive to both stimuli. In both neurons spiking activity during e-vector-rotation was significantly different from a uniform distribution (Rayleigh test, p<0.01). Stimulation with polarized light resulted in an e-vector dependent sinusoidal all-activation response with $\Phi_{max}$ at 79° in one neuron (Fig. 6C) and $\Phi_{max}$ at 15° in the second cell (not shown).

**TuLAL2a neurons**

Two recordings were obtained from TuLAL2a neurons. Their cell bodies are located in the inferior lateral protocerebrum close to the antennal lobe and have diameters from 18-20 $\mu$m. Arborizations in the upper unit of the AOTu have a fine granular structure (Fig. 7B). Axons project through the tubercle-accessory lobe tract and invade large areas of the lateral accessory lobe with varicose processes, but spare the median olive and the lateral triangle (Fig. 7, A and C).

Both recordings showed action potentials as well as graded potentials (Figs. 7E) indicating that the recording sites were in or near the dendritic zones of the neurons. Although the recordings were stable, the activity of both neurons showed strong fluctuations both at background level and during stimulation. Background activity ranged from 10 to 20 impulses/s in one recording and between 50 and 60 impulses/s in the second. During e-vector rotation neuronal activity in one of the two neurons was not significantly different from a uniform distribution (Rayleigh test, p = 0.123; Fig. 7D, xenon arc stimulation, standard glass optics). The second neuron, of high background activity, was polarization-sensitive. The responses to different e-vector orientations were significantly different from a uniform distribution (Rayleigh test, p = 0.006). The neuron showed polarization opponency with $\Phi_{max}$ at 109° (halogen light source, $R^2 = 0.64$; not shown). Both neurons were phasic-tonically
excited by zenithal unpolarized light (Fig. 7E). One neuron stimulated with xenon light (standard glass optics), showed phasic on-inhibition to contralateral stimulation and phasic-tonic excitations to ipsilateral light (Fig. 7E), while the second neuron was unresponsive to lateral halogen stimulation.

*Lobula loop neuron*

This neuron was encountered only once. Its soma (20-25 \( \mu m \) diameter) was in the superior lateral protocerebrum posterior to the calyx. Two collaterals extended from the primary neurite to the lobula and gave rise to a dense meshwork of ramifications in layer 2 of the anterior lobe of the lobula. Another fiber descended to the upper unit of the AOTu and gave rise to dense sidebranches in both lobes of the upper unit. The fiber continued into the anterior optic tract toward the lobula, contributed to the arborizations in layer 2 of the anterior lobe of the lobula, and continued to terminal ramifications in dorsal aspects of the outer lobe of the lobula (Fig. 8A). The processes in all three neuropils were of beaded shape, with those in the AOTu being the most large and dense ones. The neuron had a background activity of 20-30 impulses/s. When stimulated with dorsal polarized light, spiking activity was independent of \( e \)-vector angle (Rayleigh test, \( p = 0.762 \); Fig. 8B).

**DISCUSSION**

The upper and lower units of the AOTu in the locust brain are parts of two parallel pathways that connect peripheral visual neuropils to the central complex (Homberg et al. 2003). Overlapping branching patterns, especially between neurons from the lower unit of the AOTu and polarization-sensitive interneurons (POL neurons) from the central complex, suggested that the lower unit of the AOTu is part of the polarization vision pathway and feeds input into the central complex. Here we describe the morphology and physiological responses, especially to plane-polarized light, of neurons from the AOTu. All neurons from the lower
unit of the AOTu were tuned to distinct $e$-vector orientations of dorsally presented plane-polarized light, substantiating our hypothesis that the AOTu is part of the locust’s sky-compass navigation system. In contrast, only one out of five recorded neurons from the upper unit was weakly polarization-sensitive. During the course of experiments several combinations of light intensities and bandwidths were used, but all stimuli included blue light corresponding to the spectral sensitivity of locust polarization-sensitive photoreceptors (Eggers and Gewecke 1993). Accordingly, neither differences in intensity nor in spectral bandwidth of the stimuli we used affected the properties of polarized-light responses (types of responsive neurons, polarization-opponency, ocular dominance, and $e$-vector tuning) in these neurons. All neurons, however, also showed responses to unpolarized light that strongly depended on the stimulus position within the visual field of the animal. These responses were more pronounced with high intensity xenon arc stimulation.

Orientation to polarized light was first discovered in honeybees by Karl von Frisch over 50 years ago (von Frisch 1949). Subsequently polarization vision was demonstrated behaviorally in several other insect species, including the ant *Cataglyphis bicolor* (Wehner 1994), the cricket *Gryllus campestris* (Brunner and Labhart 1987), the fly *Musca domestica* (von Philipsborn and Labhart 1990), the locusts *Locusta migratoria* and *Schistocerca gregaria* (Eggers and Weber 1993; Mappes and Homberg 2004), the beetle *Scarabaeus zambesianus* (Dacke et al. 2003), and the butterfly *Danaus plexippus* (Reppert et al. 2004). The mechanisms in the central nervous system, however, which accomplish this fascinating skill are still poorly understood. So far, POL neurons have been recorded from the optic lobe (cricket: Labhart 1988; Labhart et al. 2001; desert ant: Labhart 2000; cockroach: Kelly and Mote 1990; Loesel and Homberg 2001; desert locust: Homberg and Würden 1997) and, in the locust and cricket, from an area in the midbrain, the central complex (Vitzthum et al. 2002; Sakura and Labhart 2005). To bridge the gap between both stages of polarized-light processing, we recorded from an intercalated neuropil that receives input from the optic lobe
and sends output toward the central complex. While multiple recordings were archived from bilateral interneurons of the AOTu and from projection neurons to the central complex, no recordings were obtained from the large number of medulla line tangentials with axonal terminals in the AOTu (Homberg et al. 2003). If these neurons, like LoTu1 and TuLAL1b, also provide polarization input to the AOTu, their e-vector tuning might largely account for the broad distribution of $\Phi_{\text{max}}$ orientations found in the central complex (Vitzthum et al. 2002).

$\Phi_{\text{max}}$ tuning

Polarization-sensitive interneurons in the medulla of crickets, termed POL1, exist as three physiological subtypes per brain hemisphere (Labhart and Petzold 1993), and each subtype is tuned to a different e-vector orientation (Labhart et al. 2001). In contrast, in the central complex of the locust and cricket, there is no evidence for the presence of only a few e-vector tuning types, but neurons are tuned to a large variety of e-vector orientations (Vitzthum et al. 2002; Sakura and Labhart 2005). The two types of bilateral POL neurons studied here, LoTu1 and TuTu1, did show distinct $\Phi_{\text{max}}$ orientations. In LoTu1, we found one tuning type per brain hemisphere (Fig. 3). This corresponds with Dextran injections into the AOTu (Homberg et al. 2003) that never revealed more than one LoTu1 neuron per brain hemisphere. The mean $\Phi_{\text{max}}$ orientations of the two neurons (LoTu1 of the right hemisphere: 41°, LoTu1 of the left hemisphere: 134°) are nearly perpendicular to each other and are mirror-symmetric with respect to the longitudinal axis of the animal (Fig. 3E). These properties correspond well with the general bilateral symmetry in the organization of insect brain functions.

In TuTu1 neurons, four classes of $\Phi_{\text{max}}$ orientations appear to be present. Neurons with somata in the left hemisphere show peaks in $\Phi_{\text{max}}$ tuning around 175° and around 135° (Fig. 2E). Most neurons with somata in the right hemisphere, in contrast, are tuned to a $\Phi_{\text{max}}$
around 45°, but a small group of four cells with $\Phi_{\text{max}}$ orientations between 140° and 180° deviate considerably from this population and suggest a second peak in this range. The presence of two $\Phi_{\text{max}}$ classes per hemisphere in TuTu1 neurons fits well with anatomical data. Impalements of pairs of TuTu1 neurons per hemisphere were occasionally encountered in this study, but more than two TuTu1 neurons per hemisphere were never found, even in mass dye injections into the AOTu (U. Homberg and S. Hofer, unpublished). As expected from bilateral symmetry of brain functions, two classes of $\Phi_{\text{max}}$ orientations (135° in right TuTu1 and 45° in left TuTu1) are orthogonal to each other, while the two other tuning types (near 175°) may deviate only slightly from each other.

Due to earth rotation, a sky compass to function properly requires constant time compensation. Neurons that use the solar azimuth to signal directions, therefore, have to shift their $\Phi_{\text{max}}$ tuning as the sun moves across the sky during the day. All experiments in this study were performed between 9:00 a.m. and 10:30 p.m. Since LoTu1 and TuTu1 neurons showed distinct classes of $\Phi_{\text{max}}$ independent of daytime, they appear to signal the orientation of the animal relative to the sky and irrespective of daytime. We therefore assume that time compensation is performed at higher stages of the polarization vision system, possibly in the central complex.

**Ocular dominance**

Ocular dominance for polarized-light sensitivity was tested in LoTu1 and TuTu1 neurons. The responses to ipsilateral and bilateral stimulation were identical in TuTu1 neurons (Fig. 2D), and they were similar in LoTu1 cells (Fig. 3D). When the ipsilateral eye was occluded, visual responses were abolished in LoTu1, indicating that the neuron receives polarization input only through the ipsilateral eye. In TuTu1, polarization sensitivity was strongly reduced but not completely abolished after occlusion of the ipsilateral eye. This indicates that polarization input is dominated but not exclusively mediated by the ipsilateral eye. Both in
TuTu1 and LoTu1, smooth fiber specializations are present on arborizations in the ipsilateral hemisphere, commonly interpreted as synaptic input sites, and beaded or varicose processes on contralateral terminals, generally interpreted as synaptic output sites. This suggests that both neurons receive input largely or exclusively in the ipsilateral lobula and AOTu and provide polarized light input to the contralateral brain hemisphere.

Polarization sensitivity with and without opponency

POL neurons of the locust central complex show polarization opponency and, thus, receive antagonistic input from orthogonally oriented polarization analyzers (Vitzthum et al. 2002). While TuTu1 and TuLAL1a neurons are of the opponency type (Figs. 2, 5), LoTu1 and TuLAL1b are excited by all e-vector orientations, i.e. they show sinusoidal modulation of spiking activity during e-vector rotation but no inhibitory component (Figs. 3, 6). This indicates that LoTu1 and TuLAL1b neurons may receive excitatory input from only one tuning-type of polarization analyzers. Alternatively, they might receive a polarization-opponent input together with a polarization-insensitive excitatory input to light, perhaps through different photoreceptors, as suggested by their strong tonic responses to unpolarized light.

Sensitivity to unpolarized light

In contrast to POL neurons in the cricket optic lobe (Labhart and Petzold 1993) all neurons of the AOTu responded to unpolarized light stimuli, largely with tonic activity changes. Responses were generally stronger and more robust with quarz optics and xenon arc stimulation, which was about 1 log unit below the irradiance of direct sunlight under clear sky conditions in Marburg (personal measurements). Responses were strongly dependent on stimulus position, and the occurrence of inhibitory and excitatory responses in the same neuron (e.g., LoTu1, Fig. 3F) indicates complex receptive field properties with inhibitory and
excitatory subfields. In certain POL neurons like TuTu1, weak tonic responses to unpolarized light might reflect an imbalance in the polarization-opponent inputs, while the strong responses in others, especially LoTu1, argue for the integration of spatially separate polarization and non-polarization inputs. The differences in neuronal responses to halogen-vs. xenon-lamp stimulation are likely to reflect both wavelength-dependency of the response and intensity differences between the light stimuli of the two experimental setups. Preliminary data on the chromatic properties of the LoTu1 and TuTu1 neurons, indeed, revealed eye-region dependent sensitivity to unpolarized UV and green light, in addition to polarization sensitivity mediated by blue receptors of the drosal rim area (M. Kinoshita, K. Pfeiffer and U. Homberg, in preparation).

Behavioral studies, especially in ants and honeybees, showed that, in addition to the sky polarization pattern, intensity and spectral gradients in the sky, solar azimuth, and landmarks are exploited for spatial orientation (Wehner 1997, 2003; Giurfa and Capaldi 1999). How and where in the brain these features are integrated with polarization information is unclear at present. Some aspects of the sky, like solar azimuth and intensity gradients are tightly linked to the sky polarization pattern and might possibly be encoded in the response profiles of neurons of the lower unit of the AOTu. Other aspects like landmarks are more independent from celestial cues and might be funnelled to brain areas supervising spatial orientation through an independent pathway, perhaps via the upper unit of the tubercle. While the biological role of visual projections through the two units of the AOTu is still speculative, it offers some attractive possibilities for further experimentation. If responses to unpolarized light in POL neurons of the AOTu do, indeed, reflect solar azimuth, maximum response to unpolarized light should occur at a meridian orthogonal to $\Phi_{\text{max}}$ orientation in the zenith, reflecting the situation in the sky. First experiments indeed, showed a spatial relationship of about 90° between the azimuthal position of the receptive field for unpolarized light and $\Phi_{\text{max}}$ orientation in LoTu1 neurons (Pfeiffer et al. 2005) and thus support this hypothesis. Such
response profiles may serve to distinguish between the solar and the antisolar azimuth, which is not possible based on the analysis of e-vector orientation alone.

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**FIG. 1.** Diagram of the locust brain and experimental setup for stimulation with polarized light. A: frontal reconstruction of the locust brain, showing the main neuropils. Visual information is primarily processed in the optic lobe, which is formed by the three visual neuropils lamina (La), medulla (Me), and lobula (Lo). The dorsal rim of the lamina (DRL) and medulla (DRM) receive input from polarization-sensitive photoreceptors of the dorsal rim area. Recordings were made from neurons in the anterior optic tubercle (AOTu, gray) of the median protocerebrum. AL, antennal lobe; aL, Cα, α-lobe and calyx of the mushroom body; CB, central body; LAL, lateral accessory lobe. B: ventrolateral view of a locust (L) during polarized-light stimulation. Arrow indicates clockwise (CW) rotation of the polarizer (P). dE, different electrode; iE, ground electrode; H, headstage; MH, metal holder.
FIG. 2. Polarization-sensitive TuTu1 neurons. A: frontal reconstruction. Arborizations of TuTu1 are confined to the lower units of the AOTu (LU) in both hemispheres of the protocerebrum. The axon crosses the midline of the brain within the intertubercle tract. Contralateral terminals are varicose (arrowheads) and more dense than ipsilateral ones. aL, α-lobe of the mushroom body; CB, central body; UU, upper unit of the AOTu. B: relative tonic activity changes in TuTu1 in response to unpolarized light flashes (xenon arc, standard glass
optics) from ipsilateral, dorsal, and contralateral (n = 7); solid line indicates background 
activity; asterisk indicates significant inhibition to dorsal stimulation (Wilcoxon test for 
paired samples, p < 0.05). C: mean activity and intracellular recording trace of a TuTu1 
during stimulation with dorsally presented polarized light, rotating through 360° (halogen 
light source). Lights on is indicated by a step in the stimulus trace (pol dors). D: e-vector 
response plots for stimulation of the ipsilateral eye (open squares), the contralateral eye (open 
circles), and both eyes (solid triangles), same recording as in C. Solid line indicates 
background activity. Each dataset shows means of spike frequencies from two 
counterclockwise revolutions of the polarizer. Neural activity under all stimulus conditions is 
significantly different from a uniform distribution (Rayleigh test, p < 0.01). Sin²-fits (dotted 
lines) revealed $\Phi_{\text{max}}$ orientations at 76° for ipsilateral stimulation (open squares, $R^2 = 0.982$), 
at 66° for contralateral stimulation (open circles, $R^2 = 0.732$), and at 75° for binocular 
stimulation (solid triangles, $R^2 = 0.983$). E,F: distribution of $\Phi_{\text{max}}$ orientations from TuTu1 
neurons with cell bodies in the left ($E$, n = 25) and right ($F$, n = 22) brain hemisphere. Only 
mean $\Phi_{\text{max}}$ angles from experiments with equal numbers (2-6) of clockwise and 
counterclockwise rotations are included.
FIG. 3. Polarization-sensitive LoTu1 neurons. A,B: morphology of LoTu1. A: frontal reconstruction and photomicrographs of ramifications in the right and left AOTu. The neuron ramifies in the lower unit (LU) of the AOTu and in the anterior lobe of the lobula (ALo) of both brain hemispheres. Terminals in the contralateral LU show varicosities (black arrowheads in photomicrograph). Additional varicose sidebranches arise from the neurite in
the contralateral anterior optic tract (gray arrowheads). CB, central body; MB, mushroom body; UU, upper unit of the AOTu. B: photomicrographs of arborizations in the ipsilateral (upper panel) and contralateral (lower panel) ALo. Arrowheads indicate varicosities in the contralateral ALo. C: mean activity and intracellular recording showing the response of a LoTu1 to dorsally presented polarized light, rotating through 360° (halogen light). Lights on and off are indicated by steps in the stimulus trace (pol dors). D: e-vector response plots for stimulation of the ipsilateral eye (open squares), the contralateral eye (open circles), and both eyes (solid triangles), same recording as in C. Solid line indicates background activity. Each dataset shows spike frequencies from a single counterclockwise revolution of the polarizer.

Neural activity during stimulation of the ipsilateral eye and both eyes are significantly different from a uniform distribution (Rayleigh test, p < 0.01). Sin²-fits (dotted lines) revealed Φ_max orientations at 71° for ipsilateral stimulation (open squares, R² = 0.714) and at 73° for binocular stimulation (solid triangles, R² = 0.690). E: e-vectors eliciting maximal spike frequencies (Φ_max) in 46 LoTu1 neurons with somata in the left (gray) or right brain hemisphere (black). Only mean Φ_max angles from experiments with equal numbers (2-6) of clockwise and counterclockwise rotations are included. Arrows show mean Φ_max angles of either group (Rao’s spacing test, p < 0.01). F: relative tonic activity changes in LoTu1 in response to unpolarized light flashes from ipsilateral, dorsal, and contralateral (xenon arc stimulation, standard glass optics); solid line indicates background activity, asterisks indicate significant activity changes to ipsilateral and dorsal stimulation (Wilcoxon test for paired samples, p < 0.05).
FIG. 4. Anatomy and physiology of LoTu2 neurons. A: frontal reconstruction. The neuron connects the anterior lobulae (ALo) of both optic lobes via the intertubercle tract and has beaded terminals in parts of the upper unit (UU) of the contralateral AOTu. CB, central body; LU, lower unit of the AOTu. B,C: intracellular recordings (10 Hz digitally high pass filtered) from a LoTu2. While stimulation with unpolarized light from dorsal (B, xenon arc with standard glass optics) and ipsilateral (C) causes tonic inhibitions followed by phasic off-excitations, stimulation of the contralateral eye (B, contra) leads to excitation. D: e-vector response plot from 180°-rotations (double-plotted, i.e. 180°-360° identical with 0°-180°, means ± SD, n = 4; xenon arc stimulation, standard glass optics). Polarized dorsal light inhibits LoTu2 independent of e-vector angle (Rayleigh test, p = 0.999). Solid line, background activity.
FIG. 5. Polarization-sensitive TuLAL1a neurons. A: frontal reconstruction of a TuLAL1a neuron. The neuron ramifies in the lower unit (LU) of the AOTu and projects to the lateral triangle (LT) of the lateral accessory lobe (LAL). CB, central body; UU, upper unit of the AOTu. B: arborizations of double impaled LoTu1/TuLAL1a neurons. Unlike ramifications of LoTu1 (white arrowheads), those of TuLAL1a (black arrowheads) are restricted to the inner third of the lower unit of the AOTu. C: axon terminals within the lateral triangle form large irregularly shaped knobs that extend to tiny, filiform appendices (arrowheads). D: intracellular recording and mean spiking activity from TuLAL1a depicted in A. Membrane potential and spiking activity of the cell are sinusoidally modulated during a full revolution of the dorsally presented polarizer (pol dors; xenon arc stimulation, quarz optics). E: e-vector response plot (means ± SD from one clockwise and one counterclockwise turn through 360°. The occurrence of spikes during e-vector rotation is significantly different from a uniform distribution (Rayleigh test, p < 0.01). The sin²-fit (dotted line) revealed a Φ_max of 117° (R² = 0.969). Solid line, background activity.
FIG. 6. Polarization-sensitive TuLAL1b neurons. *A*: intracellular recording (10 Hz digitally highpass-filtered) and mean activity of one of the TuLAL1b neurons shown in B. The neuron shows no background activity and remains silent, when the animal is stimulated with unpolarized ipsilateral halogen light (ipsi). Contralateral stimulation with unpolarized light (contra; halogen light source) leads to tonic excitation. *B*: frontal reconstruction. Two TuLAL1b neurons were colabelled. Both neurons connect the most ventral layer of the anterior lobula (ALo) to the lower unit (LU) of the AOTu via the anterior optic tract (AOT). Continuing axons project to the lateral accessory lobe. The few, large terminals are confined to the lateral triangle (LT) and the median olive (MO) or to the MO alone. CB, central body; UU, upper unit of the AOTu. *C*: *e*-vector response plot (means ± SD from two clockwise and two counterclockwise turns through 360° (xenon arc stimulation, quarz optics). The occurrence of spikes during *e*-vector rotation is significantly different from a uniform distribution (Rayleigh test, p < 0.01). The sin²-fit (dotted line) revealed a $\Phi_{max}$ of 79° ($R^2 = 0.664$). Solid line, background activity.
FIG. 7. TuLAL2a neuron from the upper unit (UU) of the AOTu. A: frontal reconstruction. The neuron connects the upper unit of the AOTu to large areas of the lateral accessory lobe (LAL). LU, lower unit of the AOTu; CB, central body. B: ramifications in the AOTu are of fine grainy appearance. C: projections in the LAL bear varicosities (arrowheads). D: e-vector response plot from 180°-rotations (double-plotted, i.e. 180°-360° identical with 0°-180°, means ± SD, n=4; xenon arc stimulation, standard glass optics). Solid line, background activity. Spiking activity during e-vector rotation was not significantly different from uniformity (Rayleigh test, p = 0.123). E: mean activity and original recording trace during
stimulation with unpolarized light from the zenith (dorsal), and at 30° elevation from ipsilateral (ipsi) and contralateral (contra; xenon arc stimulation, standard glass optics).
FIG. 8. Neuron from the upper unit of the AOTu that loops from and to the lobula. A: frontal reconstruction. The neuron has extensive arborizations in the anterior lobe of the lobula and projects to the upper unit (UU) of the AOTu. The axon projects back to the optic lobe and gives rise to another field of ramifications in the outer lobe of the lobula. B: e-vector response plot (means ± SD from 7 counterclockwise turns through 360°; xenon arc stimulation, quarz optics). Neuronal activity during e-vector rotation is not significantly different from uniformity (Rayleigh test, p = 0.762). Solid line, background activity.