Spatiotemporal analysis of electrically evoked activity in the chicken optic tectum: a VSDI study

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Weigel S, Luksch H. Spatiotemporal analysis of electrically evoked activity in the chicken optic tectum: a VSDI study. J Neurophysiol 107: 640–648, 2012. First published October 26, 2011; doi:10.1152/jn.00541.2011.—The midbrain is an important processing area for sensory information in vertebrates. The optic tectum and its mammalian counterpart, the superior colliculus, receive multimodal, topographic information and contain a sensory map that plays a role in spatial attention and orientation movements. Many studies have investigated the tectal circuitry by cytochemistry and by characterization of particular cell types. However, only a few studies have investigated network activation throughout the depth of the tectum. Our study provides the first data on spatiotemporal activity profiles in the depth and width of the avian optic tectum. We used an optical imaging approach with voltage-sensitive dyes to investigate population responses at a high temporal and spatial resolution. With the necessary caution due to cell extension across several layers, we can thus link our findings tentatively with the general layout of the avian optic tectum. Single electrical stimuli in the retinorecipient layers 1–4 evoked a complex optical response pattern with two components: a short, strong transient response and a weaker persistent response that lasted several hundred milliseconds. The response started in layer 5 and spread within this layer before it propagated into deeper layers. This is in line with neuroanatomical and earlier physiological data. Analysis of temporal sequence and pharmacological manipulations revealed that these responses were mainly driven by postsynaptic activation. Thus tectal network responses to patterned input can be studied by voltage-sensitive dye imaging.

The avian optic tectum (TeO) is involved in the processing of topographic, multimodal sensory information. Comparable to its mammalian counterpart, the superior colliculus (SC), the TeO receives topographically organized sensory information and integrates it into a multisensory map of space, dominated by the visual modality (Knudsen 1982; Meredith and Stein 1986; Witten and Knudsen 2005). The tectal space map is involved in many behaviors, including aspects of bottom-up spatial attention and orienting movements (Gruberg et al. 2006; Luksch 2009; Stein et al. 2009).

Because of its importance in the visual pathway and in behavior, many studies have investigated the physiological characteristics of avian tectal cells in vivo (Frost et al. 1988; Frost and DiFranco 1976; Hardy et al. 1984; Knudsen et al. 1994; Marin et al. 2007; Mysore et al. 2010; Wang et al. 2000). Field-potential studies (Cragg et al. 1954; Dye and Karten 1996; Holden 1968; Letelier et al. 2000; Stone and Freeman 1971) and a recent optical imaging study of intrinsic signals (Keary et al. 2010) have gathered information on spatiotemporal activity profiles. Apart from a single study that analyzed responses along the surface of the TeO (Keary et al. 2010), these approaches only gathered activity profiles along the depth. They cannot align spatiotemporal activity patterns with the tectal cytoarchitecture into more than one spatial dimension, which is necessary to understand the functional neuronal circuitry.

Recent work in other vertebrate tecta and in the mammalian SC addressed this issue by using electrode arrays or voltage-sensitive dye imaging (VSDI) (Isa and Hall 2009; Kinoshita et al. 2002; Phongphanphanee et al. 2008; Vokoun et al. 2010). Compared with the SC, the avian TeO has experimental advantages such as a distinct lamination and clearly separated input and output layers (Kanaseki and Sprague 1974; Luksch 2003), whereas the overall functional architecture appears to be matchable (Luksch 2009).

The avian TeO consists of 15 layers (Fig. 1; Cajal 1909), with each layer containing specific cell types with identifiable morphology (Luksch 2003). Cells in layers 4, 5, and 7 are horizontally organized (Luksch and Gollz 2003; Sebesteny et al. 2002; Tömölö 1998), whereas layers 8–12 consist mostly of radial neurons (Hardy et al. 1985; LaVail and Cowan 1971; Scicolone et al. 2006; Sebesteny et al. 2002). Retinal fibers enter the TeO at their respective topographic position and make synapses with dendrites in layers 2–5 and 7 (Yamagata and Sanes 1995; Yamagata et al. 2006). The major ascending output is constituted by neurons of the deep layer 13, where several cell types have specific projection zones in the thalamic nucleus rotundus (Hellmann and Güntürkün 2001; Luksch et al. 1998). In addition, cells throughout the tectal layers form the descending tectobulbar and tectopontine tracts (Reiner and Karten 1982; Wylie et al. 2009).

For the present study, we used a VSDI approach in a midbrain slice preparation to image response profiles across and within the tectal layers. By electrical stimulation of the retinorecipient layers 1–4, we elicited two-component neuronal responses, which could be aligned with particular layers. Pharmacological manipulations indicate that signals were mostly of postsynaptic origin. The inter- and intralaminar spread combined with disinhibition is discussed on the background of the tectal circuitry.

MATERIALS AND METHODS

Slice preparation. White Leghorn chick hatchlings (Gallus gallus) between 1 and 6 days of age were used in this study. All procedures were approved by the local authorities and conform to the guidelines of the National Institutes of Health on the Care and Use of Laboratory Animals.
Brain slices of the midbrain were prepared following published protocols (Dye and Karten 1996; Khanbabaie et al. 2007; Luksch et al. 2004; Meyer et al. 2008). Animals were anesthetized with a mixture of ketamine (50 mg/ml; Inresa Arzneimittel) and Rompun (2%; Bayer) at 37.5 and 5 mg/kg body wt. Preparations were done in ice-cooled, oxygenated, and sucrose-substituted saline (240 mM sucrose, 3 mM KCl, 5 mM MgCl₂, 1.2 mM NaH₂PO₄, 23 mM NaHCO₃, and 11 mM d-glucose). After decapitation, brains were removed from the skull, and forebrain, cerebellum, and medulla oblongata were discarded. A midsagittal cut separated the tectal hemispheres that were subsequently embedded in agar (1.5% in HEPES solution: 290 mM sucrose, 3 mM KCl, 3 mM MgCl₂, and 5 mM HEPES). Tectal hemispheres were sectioned at 500 μm; Biozol, Eching, Germany) in saline for 5–10 min. The dye was freshly prepared from a stock solution (50 mM in distilled water). Slices were transferred to a custom-made recording chamber. The chamber was perfused with Carbogen (95% oxygen, 5% CO₂) and continuously with oxygenated saline at room temperature (37°C). For staining, single slices were incubated with 50 μM 4′,6-diamidino-2-phenylindole (DAPI) and 10 μM propidium iodide (PI) for 15–30 min. The slices were then washed three times in ice-cold saline and left for 15 min to allow the dye to penetrate the cell bodies. The slices were then transferred to 30% sucrose solution in phosphate buffer solution (PB: 0.023 mM NaH₂PO₄ and 0.08 mM Na₂HPO₄) for at least 1 h, cut to 75–100 μm, coated with a cryotome. Layer boundaries were evaluated from microscopy images and associated DIC images. Histology. An important point in this study is the correlation of optical signals with their position in space, in particular with the tectal lamination. A midsagittal cut separated the tectal hemispheres that were subsequently embedded in agar (1.5% in HEPES solution: 290 mM sucrose, 3 mM KCl, 3 mM MgCl₂, and 5 mM HEPES). Tectal hemispheres were sectioned at 500 μm on a tissue slicer (VF-200; Precisionary Instruments) in the horizontal plane (Fig. 1). Slices were collected in oxygenated saline (120 mM NaCl, 3 mM KCl, 2 mM CaCl₂, 1.2 mM NaH₂PO₄, 23 mM NaHCO₃, and 11 mM d-glucose) and kept submerged in a chamber that was bubbled continuously with Carbogen (95% oxygen, 5% CO₂) at room temperature for at least 30 min.

For staining, single slices were incubated with 50 μM RH795 (0.03 mg/ml; Biozol, Eching, Germany) in saline for 5–10 min. The dye was freshly prepared from a stock solution (50 mM in distilled water). Slices were mounted on a poly-D-lysine-coated coverslip and transferred to a custom-made recording chamber. The chamber was perfused with Carbogen (95% oxygen, 5% CO₂) at room temperature (37°C). In the case of Ca²⁺-free experiments, CaCl₂ was substituted by MgCl₂ in the saline. The AMPA receptor blocker 2,3-dihydroxy-6-nitro-7-sulfamoylbenzof[1,4]oxazinone-2,3-dione (NBQX; Ascent Scientific) and the GABA_A receptor blocker bicuculline methiodide (no. 14343; Sigma-Aldrich Germany) were prepared as stock solutions (5 and 10 mM in distilled water) and diluted freshly in saline (1:1,000).

Recording setup. Recordings were performed using a ×10 magnification of a fixed-stage upright microscope (Examiner A1; Zeiss) equipped with differential interference contrast (DIC) optics and fluorescence filters (525/45-nm excitation filter, 560-nm beam splitter, 675/67-nm emission filter; all from AHF Analysentechnik, Tübingen, Germany). Slices were illuminated by a projection LED with a peak wavelength of 525 nm (Ostar LET A2A; OSRAM Optoelectronics).

Local electrostimulation was achieved according to the method of Luksch et al. (2001) by inserting bipolar electrodes under visual control into the upper tectal retinorecipient layers (2 to 4) with a three-axis micromanipulator (U-31CF; Narishige). As recording area, a similar position lateral in prolongation to the basis of the ventricle was always chosen. Electrodes were custom-built from Formvar-coated Nichrom wires (uncoated diameter 51 μm, coated diameter 66 μm; ScienceProducts, Berlin, Germany) that were mounted in glass microcapillaries for stabilization. The wires protruded several hundred micrometers from the capillaries, and the tips were cut at an angle.

Stimulus isolators (Isolated Pulse Stimulator 2100; AM Systems) generated biphasic current pulses (100 μA, 500 μs).

Data recording and analysis. Recordings were obtained with a NeuroSMQ imaging system (RedShirt Imaging, Decatur, GA) with a frame rate of 2 kHz by 80 × 80 pixels. With the use of a ×10 objective, 1 pixel represents ~19.25 × 19.25 μm² in the TeO. All data were averages of 5 trials with 10-s intervals. Data were first preprocessed in Neuroplex (RedShirt Imaging), divided by the resting light intensity, and pixels were spatially binned (2 × 2). Afterwards, data were exported as ASCII for further processing in Matlab. Data were low-pass filtered (200 Hz), and a noise level for each pixel was calculated as the mean ± 4SD in a 250-ms interval before stimulus onset. Pixels with a noise level higher than 10 × 10⁻³ ΔF/F were excluded. To remove drift resulting from bleaching of the dye, we fitted the baseline of data traces (data before stimulation to >750 ms after stimulation) to a biexponential function and subtracted the fit from the traces.

The curvature of the TeO as well as the stimulus position in the camera differed from experiment to experiment. To pool the data, we composed a flat "standard" tectum without curvature for each individual recording, where the stimulus electrode locations were aligned. For this, a mask of the tectal surface was generated and the edge was flattened by shifting the recording pixels. Data were normalized by the maximal signal amplitude under control conditions. Maximal values were automatically chosen from pixels in a retinotopic axis to the stimulation electrode in a time interval 20 ms after stimulus onset. We distinguish signal from noise by calculating a threshold from the noise level (see Figs. 6 and 7); only values beyond the mean plus the noise level were considered.

Intralaminar propagation speeds were calculated from the slope of a fitted line in Fig. 6. Interlaminar propagation speed represents the mean of the time to maximal amplitude in the first 20 ms after stimulation, divided by the distance to the stimulation electrode.

Data from pharmacological tests were tested for normal distribution by using the Lilliefors test and for significances by a two-sided Wilcoxon rank sum test. All data are means ± SE.

Histology. An important point in this study is the correlation of optical signals with their position in space, in particular with the tectal lamination. However, fluorescence images and associated DIC images allowed only a guess of the boundaries. To be able to indicate the borders of the layers, we measured them in histological slices (n = 14) of animals of relevant age (postnatal days 1–5). For this, brain slices were fixed with 4% paraformaldehyde in phosphate buffer solution (PB: 0.023 mM NaH₂PO₄ and 0.08 mM Na₂HPO₄) for at least 1 h, transferred to 30% sucrose solution (in PB), and cut to 75–100 μm thick slices with a cryotome. Layer boundaries were evaluated from microscopy images of sections comparable to the recording locations with ImageJ.

Distances between the outer surface of the TeO and the outermost contour of each of the layers were averaged over three measurements at three different positions per layer and slice. Derived mean values were as follows: layer 5, 112 ± 18 μm; layer 6, 205 ± 34 μm; layer 9, 254 ± 34 μm; layer 10, 357 ± 37 μm; layer 12, 513 ± 48 μm;
and layer 13, 632 ± 55 μm. We divided these values by a pixel size (38.5 μm) and used them to indicate the borders of individual layers (Fig. 2A). The accuracy was about 0.5 (for layer 5) to 1.5 pixels (for layer 13), caused by individual and age-dependent variations.

RESULTS

Characteristics of voltage imaging response. After incubation of single slices with the voltage-sensitive dye RH795, electrical stimulation in layers 1–4 altered the fluorescent signal. The RH795 yields a negative optical signal on membrane depolarization that is inverted in our figures due to data presentation conventions. All figures show the mean data gathered in several slices.

Electrical stimulation of the external layers (layer 1–4) of the TeO led to a complex, highly characteristic response throughout the tectal layers (Fig. 2B). It consisted of two components: a transient component starting after a few milliseconds and lasting around 10 ms, and a persistent second component lasting several hundred milliseconds.

In layer 5 the transient component reached an average maximal amplitude of 5.1 ± 0.3 × 10⁻³ ΔF/F at 5.6 ± 0.5 ms. The persistent component reached about one-half this amplitude (2.6 ± 0.1 × 10⁻³ ΔF/F). In deeper layers, this transient amplitude was lower (2.3 ± 0.2 × 10⁻³ ΔF/F in layers 10 to 11 and 0.9 ± 0.1 × 10⁻³ ΔF/F in layer 13) and nearly indistinguishable from the persistent signal (1.9 ± 0.1 × 10⁻³ ΔF/F in layers 10 to 11 and 1.0 ± 0.1 × 10⁻³ ΔF/F in layer 13). Likewise, the latency of the first peak was increased (11.8 ± 1.0 ms in layers 10 to 11 and 16.1 ± 1.0 ms in layer 13). The peak of the persistent part was first reached in layers 10 to 11 (73.8 ± 8.7 ms), followed by layer 13 (80.8 ± 11.4 ms). In layer 5 the maximum response was observed after 79.5 ± 6.7 ms.

Variation of stimulus amplitude (100 and 250 μA) and frequencies (100 μA applied for 20 ms at 250, 500, and 1,000 Hz) did not change the overall spatial pattern. Lower currents (25 μA) were not sufficient to evoke responses reliably.

Application of Ca²⁺-free saline and NBQX eliminated most optical signals in layers other than layer 5. A transient component and a residual persistent part were visible only in this layer. However, the amplitude of the transient component was drastically reduced (Ca²⁺ free: 38 ± 3%, gray traces in Fig. 2B; NBQX: 45 ± 5%, gray traces in Fig. 2C), indicating that 1) most of the optical signal recorded was postsynaptic and 2) glutamate is the neurotransmitter for this first synapse.

Spatial pattern of maximal activity. To analyze the spatial distribution of neuronal activity, we plotted maps in which the maximal amplitude per pixel was encoded as colors. Since the transient and the persistent response had different dynamics, we analyzed them separately. In the first 20 ms after stimulation (Fig. 3A), the signal spread both between the laminae (interlaminally) and along the laminae (intralaminally). Highest response amplitudes were detected in an area ranging in depth from the spot of stimulation to layer 9 and in width 250 μm laterally to both sides (see also Fig. 7D). The response amplitudes decreased more laterally as well as in deeper layers. Compared with the map for the transient signal, the spatial pattern was different during persistent activity (Fig. 3B). Again, we found the area of maximal activity near the side of stimulation in layers 5–9. The persistent response amplitude, which is generally lower than the transient one, also became smaller in deeper layers. However, the lateral spread was now largest in layers 9–11 (about 300 μm in each direction), whereas it reached only ~150 μm in layers 5–8 (see also Fig. 7D and Supplemental Videos 1–3).

Ca²⁺-free saline and block of AMPA receptors limited the transient response to an area next to the stimulation electrode and abolished most of intra- as well as interlaminar spread (Fig. 3, C and D, for Ca²⁺-free saline and Fig. 3, E and F, for NBQX; see also Fig. 4, B and C).

Interlaminar response. The interlaminar temporal dynamics are depicted in Fig. 4A. We plotted the mean amplitude of pixels (77-μm width × 38.5-μm depth) in the radial dimension of the tectum (equal to retinotopic prolongation of the stimulation electrode) over the whole range of the captured section from 100 ms before to 800 ms after stimulation. In this
Bicuculline methiodide (10 μM) by bath application. In general, block of the GABA<sub>A</sub> receptor enhanced the neuronal response: the GABA-mediated inhibition. Previous in vitro work has shown that neuronal responses in the TO and the SC are highly regulated by GABA release. Disinhibition by GABA<sub>A</sub> receptor blockers usually enhanced signal amplitudes in whole cell or extracellular recordings (Dye and Karten 1996; Özen et al. 2000; Phongphanphane et al. 2008; Saito and Is a 2003) and in optical imaging recordings of the SC (Vokoun et al. 2010). In the SC, it also altered the spatial distribution (Phongphanphan et al. 2008; Vokoun et al. 2010).

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**Intralaminar spatiotemporal pattern.** Because our preliminary analysis pointed toward layer-specific spatiotemporal dynamics, we analyzed the pixels corresponding to each layer separately. Figure 5A shows the response in layers 5, 10 to 11, and 13 plotted against time and lateral extent for each layer, respectively. The insets display an enlargement from -5 ms before to 20 ms after stimulation.

In layer 5, activity spread laterally and reached its full extent of ~250 μm on one side within 10 ms (Fig. 5A; see Fig. 7D). The highest amplitudes are visible 5–10 ms after stimulation up to ~100 μm to each side. After a short depression (~10 ms), persistent activity can be seen. The amplitude was highest between 50 and 200 ms (about 50% of the maximal transient amplitude) and faded slowly in the next 300 ms. Similar to the transient response, the amplitude of the persistent response was maximal up to 100 μm to each side and then decreased at more lateral points. The overall width of the responsive area was slightly higher for the transient than for the persistent response (see Fig. 7D).

The spatial extent of activity was comparable in layers 10 to 11 (Fig. 5A; see Fig. 7D). The amplitude at locations perpendicular to the stimulation electrode was also slightly higher than at more lateral pixels. The maximal amplitude in the first 20 ms is similar to the maximal amplitude of the persistent activity phase (43 ± 3 vs. 37 ± 2%; P = 0.1).

The activation of neurons in layer 13 differed from the pattern seen in layer 5 or layer 10 (Fig. 5A). In layer 13, the overall response amplitude was low and diffuse (16 ± 2 and 19 ± 2%). The response did not have a particular peak perpendicular to the stimulation site. It also was not possible to divide the response into a transient and a persistent part.

To show the temporal sequence of activation, we plotted the time to maximal activity (Fig. 6). In layer 5, the response reached its maximum first perpendicular to the stimulation electrode and spread with almost constant velocity to the side (2.0 ± 0.2 m/s). Maxima in layer 10 were delayed by 8.1 ± 2.2 ms compared with layer 5 over the entire lateral extent, and the signal spread with a velocity of 2.0 ± 0.2 m/s, indicating that activity first spreads laterally in layer 5 before synaptic transmission to layer 10 neurons occurs. The maximal amplitudes in layer 13 were reached at 16.8 ± 0.7 ms independently of lateral position (Fig. 6), and calculated conducting velocity was 17.2 ± 10.6 m/s, indicating a more complex synaptic interaction.

**GABA-mediated inhibition.** Previous in vitro work has shown that neuronal responses in the TO and the SC are highly regulated by GABA release. Disinhibition by GABA<sub>A</sub> receptor blockers usually enhanced signal amplitudes in whole cell or extracellular recordings (Dye and Karten 1996; Özen et al. 2000; Phongphanphane et al. 2008; Saito and Is a 2003) and in optical imaging recordings of the SC (Vokoun et al. 2010). In the SC, it also altered the spatial distribution (Phongphanphan et al. 2008; Vokoun et al. 2010).

To test whether we could observe similar effects in the avian TO, we applied the GABA<sub>A</sub> receptor blocker bicuculline methiodide (10 μM) by bath application. In general, block of the GABA<sub>A</sub> receptor enhanced the neuronal response: the signal amplitude, the duration of the signal, and the spatial extent (Figs. 3–5). The effect on the persistent signal was more pronounced than the effect on the transient response. The amplitude of the neuronal response in retinotopic organization was comparable for the first few milliseconds with and without visualisation, both components of the fluorescence signal can readily be observed. The transient component started in layer 5 after a delay of 0.8 ± 0.1 ms and spread to the deeper layer of the TO with constant speed (1.9 ± 0.4 m/s; see also Fig. 4A, inset). The amplitude was maximal in layers 5–9. At the border to layer 10, the signal decreased to 43 ± 3% of the maximal amplitude and further diminished in deeper layers.

The persistent component was structured less clearly, with long-lasting activity in nearly all layers. However, the duration was longest in layers 5–8 (610.5 ± 33.1 ms in layer 5), whereas it got shorter in deeper layers (452.3 ± 29.5 ms in layer 10; 254.9 ± 26.3 ms in layer 13). In layer 13, we observed a more variable fluorescence change but on a low amplitude level.
Fig. 4. A complex spatiotemporal activity pattern is triggered by a single electrical stimulation. The amplitude of the response over time is color coded for all pixels in retinotopic prolongation of the stimulation electrode. The stimulation pulse was applied at t = 0 ms (represented by the vertical line). Horizontal lines depict the distal borders of the layers of the TeO calculated from histological preparations. Note: some pixels of the imaging camera correspond to more than one layer. A: control (n = 21). Application of Ca²⁺-free Ringer solution (B; n = 7) and NBQX (5 μM; C; n = 8) resulted in a reduced and locally restricted response, whereas the GABA_A receptor blocker bicuculline methiodide (10 μM; D) enhanced the spatiotemporal activity pattern (n = 8). Insets: enlargement from 5 ms before until 20 ms after stimulation.

drug administration (Fig. 4D). The signal started in layer 5 and extended to the deep layers with a peak activity in layers 5–8. However, under GABA block, the signal deprivation after ~10 ms was mostly missing (Fig. 4D, inset). The amplitude was higher in layers 5–12 during the persistent part. In addition, the system exhibited prolonged activity in these layers with a stronger effect on layers 9–11.

To quantify the effects of GABA_A receptor block, we measured the maximal signal amplitude of pixels in prolongation to the stimulation spot, the mean lateral extent of activity, and the duration for layers 5, 10 to 11, and 13 (Fig. 7). The maximal signal amplitude was not altered in the first 10 ms in layers 5 and 13. In layers 10 to 11, the value was enhanced by about 23% (45 ± 3 vs. 58 ± 7%; Fig. 7B). During the persistent period (30–200 ms after stimulation), the signal was significantly increased in layer 5 (by 49%; 49 ± 3 vs. 73 ± 6%) and in layers 10 to 11 (by 84%; 37 ± 3 vs. 68 ± 9%).

The duration of neuronal activity was defined as the period starting at the time point the signal first crossed the noise level till the last time point the signal exceeded this level. Block of

Fig. 5. Color-coded plot of the fluorescence changes over the lateral extent of particular layers under control conditions (A; n = 21) and after perfusion with bicuculline methiodide (B; n = 8). Pixels representing layers were chosen corresponding to mean layer positions in histological slices. If a layer was present in more than 1 pixel perpendicular to the stimulation electrode, pixels were averaged. Stimuli were applied at median lateral position (lateral extent = 0 μm) at t = 0 ms. Insets: enlargement from 5 ms before to 20 ms after stimulation. Conventions as described in Fig. 4.
the GABA_A receptor prolonged the activity in layer 5 by 19% (527.6 ± 32.2 vs. 630 ± 48.8 ms) and that in layers 10 to 11 and 13 by 64% (485.9 ± 16.5 vs. 797.4 ± 78.4 ms) and 77% (320.7 ± 26.4 vs. 566.6 ± 82.2 ms), respectively (Fig. 7C).

Next, we analyzed the intralaminar spread of the signal by the mean number of pixels with signal above noise level times pixel width (Fig. 7D; see also Fig. 5B). The activity spread slightly wider in layer 5 during the first 10 ms (426.0 ± 62.0 vs. 642.2 ± 40.9 μm), whereas other layers were unaffected (layers 10 to 11: 546.0 ± 38.3 vs. 578.0 ± 51.3 μm; layer 13: 204.1 ± 54.1 vs. 218.2 ± 53.1 μm). For later time points, the lateral extent was significantly broader in layer 5 (308.6 ± 44.3 vs. 811.5 ± 88.1 μm) and in layers 10 to 11 (623.2 ± 59.7 vs. 1,027.0 ± 96.9 μm) and nearly four times broader in layer 13 (207.5 ± 63.0 vs. 725.4 ± 146.6 μm).

**DISCUSSION**

We have examined the spatiotemporal dynamics in the chicken TeO with VSDI and present the first analysis of activity patterns across two spatial dimensions of the TeO with high temporal precision and high spatial resolution (~38.5 × 38.5 μm²). This technique allows us to image changes in membrane potential and to align this neuronal activity with the well-known cytoarchitecture of the chicken TeO.

We found that electrical stimulation led to a complex temporal network response with a strong transient and a weaker persistent component (Fig. 2). The transient component was located more distally in layers 5–8, whereas the persistent component was more pronounced in the intermediate layers 9–11 (Figs. 3 and 4). Evoked signals started in layer 5, spread laterally, and then reached deeper layers, indicating first an activation of horizontally oriented neurons followed by postsynaptic signals in radially neurons. The amplitude and the spread were reduced by Ca²⁺-free saline and NBQX and enhanced by blocking of GABA_A receptors. In the following, we discuss our results in relation to previous physiological and anatomical work in the TeO and the mammalian counterpart, the SC.

**Excitatory synaptic connectivity.** Electrical stimulation led to a neuronal response that started in retinorecipient layers and spread into deeper layers. Because this signal was absent in Ca²⁺-free experiments and when AMPA receptors were blocked, we assume it to be mostly postsynaptic. Glutamate is the main neurotransmitter used in retinotectal transmission (Dye and Karten 1996; Pires and Britto 1997). We focused on three particular layers for detailed inspection: layer 5, which consists of horizontal cells; layers 10 to 11, with radial neurons and mostly small dendritic fields; and layer 13, with wide-field projection neurons. From the stimulation side, the signal spread first to elements in layer 5, up to 500 μm laterally, and subsequently activated radially oriented neurons in layers 10 to 11 with a constant delay (Fig. 6). The circuitry activated in this lateral propagation is ambiguous. The most likely candidates to spread the excitation laterally are the horizontal cells located in

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**Fig. 6.** Dot plot of the time necessary to reach the maximal amplitude in the first 20 ms after stimulation. Filled triangles represent data of layer 5, filled diamonds layers 10 to 11, and open squares layer 13. Only if pixel values were higher than the noise level in at least one-half of all experiments (n = 21) were data points taken into account.

**Fig. 7.** Comparison of neuronal response under control condition and GABA_A receptor block. A: traces from layer 5, layers 10 to 11, and layer 13 in retinotopic projection of the stimulation electrode. Black traces were recorded under control conditions and gray traces after 15 min of perfusion of the same slices with 10 μM bicuculline methiodide. Data (n = 8) were binned from an area of 77 × 38.5 μm (width × depth). Electrical stimulation started at t = 0 ms. Scale bar: fractional amplitude 0.2. B–D: bar graphs of maximal amplitude (B), signal duration (C), and lateral extent of activity (D) for layers 5, 10 to 11, and 13. Black bars represent data under control condition and gray bars after bicuculline application. B: the maximal amplitude in the first 10 ms (left) and 30–200 ms after stimulation (right). The maximal amplitudes were gathered from pixels radial to the stimulation electrode. C: the duration of neuronal activity was calculated from data used in A. The time point at which the signal amplitude first crossed the noise level was used as starting time. The last time point exceeding this level indicated the stop of the signal. D: the mean lateral extent of the VSDI signal for the first 10 ms (left) and 30–200 ms after stimulation (right). The lateral extent was determined as the mean number of pixels extending the noise level for the respective period multiplied by the size of a pixel (38.5 μm). Data are means ± SE. *P < 0.05 (2-sided Wilcoxon test).
various layers that, on activation, excite downstream radial neurons. This sequence is in agreement with previous studies that showed radial voltage gradients over the whole tectal depth with peak currents in retinorecipient layers indicating synaptic currents at distal dendrites of radial neurons, which then propagated along the radial organization (Cragg et al. 1954; Dye and Karten 1996; Holden 1968; Letelier et al. 2000; Stone and Freeman 1971). Cragg et al. (1954) additionally observed tangential voltage gradients in areas that correspond to layers 4, 5, and 13. Horizontal neurons in layers 4 and 5 have a suitable extent to provide for the lateral spread. Although this interpretation concurs with the finding that layer 5 showed the initial lateral spread, it is however challenged by reports that these neurons are GABAergic (Luksch and Golz 2003; Tömböl 1998). On the other hand, immunohistochemistry suggested that not all horizontal cells in this layer are indeed GABAergic (Veeman and Reiner 1994). Thus a subpopulation of neurons in layer 5 might be the substrate for the lateral signal propagation.

In layer 13, the signal amplitude was weak and constant over the width of the lamina. Furthermore, the intralaminar temporal activation pattern was diffuse, the intralaminar propagation speed three times higher than in the other layers, and we did not observe a constant latency in respect to layer 5 activities as seen in layers 10 to 11. Intracellular studies of stratum griseum centrale (SGC) neurons, located in layer 13, have shown that some subtypes have dendrites that reach up toward the retinorecipient layer, whereas others only reach up to layer 8 (Hellmann and Gündüzüm 2001; Luksch et al. 1998; Tömböl and Nemeth 1999). The former cells have large dendritic fields and receive direct input from retinal afferents that is likely modulated by horizontal cells (Luksch and Golz 2003; Tömböl 1998). The second cell type has comparable dendritic fields but receives only polysynaptic input from the retina. Thus activation of the SGC might be expected to have a temporal blur, which was found in the broad and fuzzy activation recorded with VSDI.

Inhibitory synaptic connectivity. The TeO receives GABAergic input from various sources including the stratum, the pallium via the substantia nigra, the lateral spinoform nucleus (Veeman and Reiner 1994), and the mesencephalic magnocellular isthmic nucleus (IMC) (Wang et al. 2004). In our slice preparations, most of the connections to GABAergic external nuclei were severed, with the exception of the IMC. IMC neurons receive excitatory input from the TeO and constitute a wide-field GABAergic projection back to the TeO, which presumably spares the input region in the TeO (Wang et al. 2004). Thus this connection should not influence the neuronal response recorded in this study.

In addition to these afferents, GABAergic neurons (Domenici et al. 1988; Veeman and Reiner 1994) and neurons expressing GABAAR receptors (Glencorse et al. 1991; Veeman et al. 1994) are located in most layers of the TeO. Hence, we suppose that most of the inhibition in our system is based on intrinsic tectal networks.

The massive GABAergic innervation of the TeO by afferent sources and intrinsic neurons indicates the importance of inhibitory regulation in the TeO. In the SC of the rat, Binns and Salt (1997) showed that GABA reduces responses of superficial gray neurons to visual stimuli and is important for surround inhibition in vivo. In vitro, disinhibition enhanced response amplitudes in the avian TeO (Dye and Karten 1996) or the SC (Özen et al. 2000; Phongphanphanee et al. 2008; Saito and Isa 2003; Vokoun et al. 2010) and increased the active area (Phongphanphanee et al. 2008; Vokoun et al. 2010).

In our experiments, disinhibition led to complex changes in the response amplitudes and spatiotemporal profiles in the different layers of the TeO. In general, response amplitudes, response durations, and lateral expansion were increased. The transient activity in the first 10 ms was less affected than subsequent neuronal responses. In detail, in the first 10 ms, only the lateral extent of activity in layer 5 and the signal amplitude in layers 10 to 11 were enhanced. Stronger effects were observed during the persistent part of the signal. There, amplitudes were significantly higher in layers 5 and 10 to 11 and slightly enhanced in layer 13. In addition, responses in all layers lasted longer, and lateral spread was drastically enhanced in all layers.

Inhibition is presumably mediated by GABAergic horizontal neurons in layer 5 (Hunt and Brecha 1984; Hunt and Künzle 1976; Luksch and Golz 2003), which are in contact with dendrites of neurons in more distal layers and layer 10. Disinhibition should thus lead to 1) an enhanced transmitter release in the retinorecipient layers, leading to a stronger postsynaptic response, and 2) a broader response due to missing surround inhibition (Binns and Salt 1997), a prediction that could be demonstrated in our experiments. Layer 5 neurons are furthermore supposed to modulate the signal transmission between optic fibers and particular neurons in layer 13 cells (Luksch and Golz 2003; Tömböl and Nemeth 1999) by a transient block of retino-SCG transmission (Luksch et al. 2004). Disinhibition should thus result in enhanced and less defined activity in layer 13, which is supported by our data. However, the overall amplitude in layer 13 is only slightly and not significantly increased. Neurons in this layer are sparsely distributed, which impedes a strong increase of population response amplitude.

Functional relevance. Our overall aim is to understand the processing of sensory signals in the vertebrate midbrain circuitry and the functional significance for behavior. A short electrical pulse elicited a two-component response with a transient and a persistent component that lasted for several hundred milliseconds. A similar response pattern was previously demonstrated in an imaging study in the rat SC (Vokoun et al. 2010) and was explained by either intrinsic biophysical properties or network activity. The authors interpreted their results as population response in analogy to in vivo activity related to saccade generation and saccade selection. In vivo, neurons respond to a stimulus usually with an initial peak activity followed by ongoing tonic activity or just tonic activity, which ends after the saccades (Edelman and Keller 1996; Glimcher and Sparks 1992; Li and Basso 2005, 2008; Munoz and Guitton 1991; Munoz and Wurtz 1995). Thus the electrical stimulation in vitro might signify the onset of a visual stimulus, and the two-component response could then reflect the population response of the activated neurons (Vokoun et al. 2010).

The vertebrate midbrain is a model system to align neuronal function and behavioral significance with cytoarchitecture and connectivity. Work on isolated tissue can extend this analysis to the biophysical level of cellular computation (Isa and Hall 2009). Compared with the mammalian SC, the avian TeO offers advantages in the strict separation of (visual) input and output layers, which might facilitate the analysis of the underlying neuronal computation. VSDI adds a further spatial di-
mension on neuronal activity and connectivity, which allow conclusions on the synaptic organization of the ToE. Albeit desirable, we were, however, not able to assess the activation of identified cell types, e.g., the ascending or descending output neurons of the optic tectum (Hellmann et al. 2004). We plan to address these issues in the future by selective imaging of identified cell types.

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS
Author contributions: S.W. and H.L. conceived and designed of research; S.W. performed experiments; S.W. and H.L. interpreted results of experiments; S.W. and H.L. drafted manuscript; S.W. and H.L. edited and revised manuscript; S.W. and H.L. approved final version of manuscript.

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