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

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Key words: optical imaging, avian, optic tectum,
Abstract

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 which 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 few studies have investigated network activation throughout the depth of the tectum. Our study provides the first data on spatio-temporal activity profiles in the depth and the 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 VSDI.
Introduction

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 them 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).

Due to 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 spatio-temporal 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 spatio-temporal 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 electrode arrays or voltage-sensitive dye imaging (VSDI; Isa and Hall 2009; Kinoshita et al. 2002; Phongphanphanee et al. 2008; Vokoun et al. 2010). In comparison to 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), while the overall functional architecture appears to be matchable (Luksch 2009).
The avian TeO consists of fifteen 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 Gölz 2003; Sebestény et al. 2002; Tömböl 1998), while layers 8 to 12 consist mostly of radial neurons (Hardy et al. 1985; LaVail and Cowan 1971; Scicolone et al. 2006; Sebestény et al. 2002). Retinal fibers enter the TeO at their respective topographic position and make synapses with dendrites in layers 2 to 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 to 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 & 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 (3:1) of Ketamine (50 mg/ml, Inresa Arzneimittel GmbH) and Rompun (2%, Bayer) at 37.5 mg and 5 mg per kg body weight (i.m.). Preparations were done in ice-cooled, oxygenated, and sucrose-substituted saline (240 mM sucrose, 3 mM KCl, 5 mM MgCl₂, 0.5 mM CaCl₂, 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 Hepsolution; 290 mM sucrose, 3 mM KCl, 3mM MgCl₂, and 5mM Heps). 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, 1 mM MgCl₂, 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 minutes.

For staining, single slices were incubated with 50 µM RH795 (0.03 mg/ml; Biozol, Germany) in saline for 5 to 10 minutes. The dye was freshly prepared from a stock solution (50 mM distilled water).

Slices were mounted on a Poly-D-Lysine coated cover slip and transferred to a custom-made recording chamber. The chamber was perfused continuously with oxygenated saline at room temperature.
temperature (~20 to 25°C). In case of Ca^{2+} free experiments, CaCl_2 was substituted by MgCl_2 in the saline. AMPA receptor blocker NBQX (Ascent scientific) and the GABA_A receptor blocker bicuculline methiodide (#14343 Sigma-Aldrich, Germany) were prepared as stock solution (5mM and 10mM in distilled water) and diluted freshly in saline (1:1000).

**Recording setup**

Recordings were performed using a 10x magnification of a fixed stage upright microscope (Examiner A1, Zeiss) equipped with DIC optics and fluorescence filters (525/45 nm excitation filter, 560 nm beam splitter & 675/67 nm emission filter; all AHF Analysentechnik, Germany). Slices were illuminated by a projection LED with a peak wave length of 525 nm (Ostar LET A2A, OSRAM Optosemiconductors).

Local electrostimulation was achieved according to Luksch et al. (2001) by inserting bipolar tungsten electrodes under visual control into the upper tectal retinorecipient layers (2 to 4) with a three-axis micromanipulator (U-31CF, Narishige). As recording area always a similar position lateral in prolongation to the basis of the ventricle was chosen. Electrodes were custom-built from Formvar coated Nichrom wires (uncoated diameter 51µm, coated diameter 66 µm, ScienceProducts, Germany) that were mounted in glass microcapillaries for stabilization. The wires protruded several hundred µm 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, USA) with a frame rate of 2 KHz by 80x80 pixels. Using a 10x objective one pixel represents approximately 19.25 x
19.25 µm² in the TeO. All data were averages of five trials with 10 s intervals. Data were first preprocessed in Neuroplex (Redshirt Imaging), divided by the resting light intensity, and pixels were spatially binned (2x2). Afterwards data were exported as ASCII for further processing in Matlab. Here, data were low-pass filtered (200Hz) and a noise level for each pixel was calculated by the mean ± 4x standard deviation in a 250 ms interval before stimulus onset. Pixels with a noise level higher than $10 \times 10^{-3}$ ΔF/F were excluded. To remove drift resulting of bleaching of the dye we fitted the baseline of data traces (data before stimulation and past 750 ms after stimulation) by a mono-exponential 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 stimulation electrode locations were aligned. For this, a mask of the tectal surface was generated and the edge was flattened by shifting the according 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 (Fig 6 & 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 of pharmacological test were tested for normal distribution by the Lilliefors test and for significances by a two-sided Wilcoxon ranksum test. All data are shown as mean ± standard error of mean.

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 (P1 - P5). For this, brain slices were fixed with 4% paraformaldehyde in phosphate buffer solution (PB, 0.023 mM NaH$_2$PO$_4$ & 0.08 mM Na$_2$HPO$_4$) for at least 1h, transferred to 30% sucrose solution (in PB) and cut to 75 µm 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 are: 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 & 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 2 A). 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 VSD RH795, electrical stimulation in the layers 1 - 4 altered the fluorescent signal. The voltage sensitive dye RH795 yields a negative optical signal upon 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 2 B). 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 $5.1 \pm 0.3 \times 10^{-3} \Delta F/F$ at 5.6 ± 0.5 ms. The persistent component reached about half of this amplitude ($2.6 \pm 0.1 \times 10^{-3} \Delta F/F$). In deeper layers, this transient amplitude was lower ($2.3 \pm 0.2 \times 10^{-3} \Delta F/F$ in layer 10 - 11 and $0.9 \pm 0.1 \times 10^{-3} \Delta F/F$ in layer 13) and nearly not distinguishable from the persistent signal ($1.9 \pm 0.1 \times 10^{-3} \Delta F/F$ in layer 10 - 11 and $1.0 \pm 0.1 \times 10^{-3} \Delta F/F$ in layer 13). Likewise, the latency of the first peak was increased (11.8 ± 1.0 ms in layer 10 - 11 and 16.1 ± 1.0 ms in layer 13). The peak of the persistent part was first reached in layer 10 – 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 ± 6.7ms.

Variation of stimulus amplitude (100 µA & 250 µA) and frequencies (100 µA applied for 20ms at 250 Hz, 500 Hz & 1000 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 (w/o Ca²⁺: 38 % ± 3%; gray
traces in Fig 2 B; NBQX: 45 % ± 5%; gray traces in Fig 2 C) indicating that (i) most of the optical signal recorded was postsynaptic and (ii) 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 simulation (Fig 3 A), the signal spreads both between the laminae (interlaminarly) and along the laminae (intralaminarly). 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 7 D). The response amplitudes decreased more laterally as well as in deeper layers. Compared to the map for the transient signal, the spatial pattern is different during persistent activity (Fig 3 B). Again, we find the area of maximal activity near the side of stimulation in layers 5 to 9. The persistent response amplitude, which is generally lower than the transient one, also becomes smaller in deeper layers. However, the lateral spread is now largest in layers 9 to 11 (about 300 µm in each direction), while it reaches only ~150 µm in layers 5 to 8 (see also Fig 7 D).

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 & D for Ca²⁺ free saline and Fig 3 E & F for NBQX; see also Fig 4 B & C).

Interlaminar response

The interlaminar temporal dynamics are depicted in Fig 4 A. We plotted the mean amplitude of pixels (77 µm width x 38.5 µm depth) in the radial dimension of the tectum (= retinotopic prolongation of the stimulation electrode) over the whole range of the captured section for 100
ms before and 800 ms after stimulation. In this visualization, both components of the fluorescence signal can readily be observed. The transient component starts in layer 5 after a delay of 0.8 ± 0.1 ms and spreads to the deeper layer of the TeO with constant speed (1.9 ± 0.4 m/s; see also Fig 4 A inset). The amplitude is maximal in layer 5 to layer 9. At the border to layer 10, the signal decreases to 43% ± 3% of the maximal amplitude, and further diminishes in deeper layers. The persistent component is structured less clearly, with long lasting activity in nearly all layers. However, the duration is longest in layer 5 to layer 8 (610.5 ± 33.1 ms in layer 5), while it gets shorter in deeper layers (452.3 ± 29.5 ms in layer 10; 254.9 ± 26.3 ms in layer 13). In layer 13 we observe a more variable fluorescence change but on a low amplitude level.

Intralaminar spatio-temporal pattern

Since our preliminary analysis pointed towards layer-specific spatio-temporal dynamics, we analyzed the pixels corresponding to each layer separately. Fig 5 A shows the response in layer 5, layer 10 - 11 and layer 13 plotted against time and lateral extent for each layers, respectively. The insets display an enlargement of -5 ms before and 20 ms after stimulation. In layer 5, activity spreads laterally and reaches its full extent of ~250 µm on one side within 10 ms (Fig 5 A & Fig 7 D). The highest amplitudes are visible five to ten ms after stimulation up to approximately 100 µm to each side. After a short depression (~10ms), persistent activity can be seen. The amplitude is highest between 50 and 200 ms (about 50% of the maximal transient amplitude) and fades slowly in the next 300 ms. Similar to the transient response, the amplitude of the persistent response is maximal up to 100 µm to each side and then decreases at more lateral points. The overall width of the responsive area is slightly higher for the transient than for the persistent response (Fig 7 D).

The spatial extent of activity was comparable in layers 10 - 11 (Fig 5 A Fig 7 D). 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 5 A). In layer 13, the overall response amplitude is low and diffuse (16% ± 2% & 19% ± 2%). The response does not have a particular peak perpendicularly to the stimulation site. It is also not possible to divide the response in 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 their maximum first perpendicular to the stimulation electrode and spreads with almost constant velocity to the side (2.0 ± 0.2 m/s). Maxima in layer 10 are delayed by 8.1 ± 2.2 ms compared to layer 5 over the entire lateral extent and the signal spreads 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 independent on 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 optic tectum and the superior colliculus are highly regulated by GABA release. Disinhibition by GABA_A receptor blockers usually enhanced signal amplitudes in whole cell or extracellular recordings (Dye and Karten 1996; Özen et al. 2000; Phongphanphanee et al. 2008; Saito and Isa 2003) and optical imaging recordings of the SC (Vokoun et al. 2010). In the SC, it also altered the spatial distribution (Phongphanphanee et al. 2008; Vokoun et al. 2010).
To test whether we observe similar effects in the avian TeO, we applied the GABA_A receptor blocker bicuculline methiodide (10µM) by bath application. In general, block of the GABA_A receptor enhanced the neuronal response: the signal amplitude, the duration of the signal as well as the spatial extension (Fig 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 is comparable for the first few milliseconds with and without drug administration (Fig 4 C). The signal starts in layer 5 and extends to the deep layers with a peak activity in layer 5 to 8. However under GABA block, the signal deprivation after ~10 ms is mostly missing (Fig 4C inset). The amplitude is higher in layer 5 to 12 during the persistent part. In addition, the system exhibits prolonged activity in these layers with a stronger effect on layers 9 to 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 is not altered in the first 10 ms in layer 5 and 13. In layer 10 -11, the value is enhanced by about 23 % (45% ± 3% vs. 58% ± 7%; Fig 7 B). During the persistent period (30 – 200ms after stimulation) the signal is significantly increased in layer 5 (by 49%; 49% ± 3% vs. 73% ± 6%) and layer 10 – 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 extended this level. Block of the GABA_A receptor prolonged the activity in layer 5 by 19% (527.6 ± 32.2 ms vs. 630 ± 48.8 ms), in layer 10 – 11 and 13 by 64% (485.9 ± 16.5 ms vs. 797.4 ± 78.4 ms) and 77% (320.7 ± 26.4 ms vs. 566.6 ± 82.2 ms), respectively (Fig 7 C).

Next, we analyzed the intralaminar spread of the signal by the mean number of pixels with signal above noise level times pixel width (Fig 7 D; see also Fig 5 C). The activity spread slightly wider in
layer 5 during the first 10 ms (426.0 ± 62.0 µm vs. 642.2 ± 40.9 µm), while other layers were unaffected (layer 10-11: 546.0 ± 38.3 µm vs. 578.0 ± 51.3 µm; layer 13: 204.1 ± 54.1 µm vs. 218.2 ± 53.1 µm). For later time points, the lateral extent was significantly broader in layer 5 (308.6 ±
44.3 µm vs. 811.5 ± 88.1 µm) and 10-11 (623.2 ± 59.7 µm vs. 1027.0 ± 96.9 µm) and nearly fourfold broader in layer 13 (207.5 ± 63.0 µm vs. 725.4 ± 146.6 µm).
We examined the spatio-temporal dynamics in the chicken optic tectum with voltage-sensitive dye imaging 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 x 38.5 µm²). This technique allows 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 to 8, while the persistent component was more pronounced in the intermediate layers 9 to 11 (Fig 3 & 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 radial 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. As this signal was absent in Ca²⁺-free experiments and by blocking AMPA receptors, we assume it to be mostly postsynaptic. Glutamate is the main neurotransmitter used in retina-tectal 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, layer 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 layer 10 – 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 various layers that, upon 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 and coworkers (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. While 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 (Veenman 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 layer 10 – 11. Intracellular studies of SGC neurons, located in layer 13, have shown that some subtypes have dendrites that reach up towards the retinorecipient layer, while others only reach up to layer 8 (Hellmann and Güntürkün 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 striatum, the pallium via the substantia nigra, the lateral spiriform nucleus (Veenman 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; Veenman and Reiner 1994) and neurons expressing GABA_A receptors (Glencorse et al. 1991; Veenman 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 superior colliculus of the rat, Binns and Salt showed in the rat SC that GABA reduces responses of superficial gray neurons to visual stimuli and is important for surround inhibition in vivo (Binns and Salt 1997). In vitro, disinhibition enhanced response amplitudes in the avian optic tectum (Dye and Karten 1996) or the superior colliculus (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 spatio-temporal 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 layer 10 – 11 were enhanced. Stronger effects were
observed during the persistent part of the signal. Here, amplitudes were significantly higher in
layer 5 and 10 – 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 (i) an enhanced
transmitter release in the retinorecipient layers leading to a stronger postsynaptic response and
(ii) to 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-SGC 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 impede 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 Guitten 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 to 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 dimension on neuronal activity and connectivity, which allow conclusions on the synaptic organization of the TeO. 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.
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**Marin G, Salas C, Sentis E, Rojas X, Letelier JC, and Mpodozis J.** A cholinergic gating mechanism controlled


Figure 1. Chicken midbrain slice preparation. A: Schematic view of the location of midbrain in the chicken brain. The midbrain is dyed in gray. The square indicated the slice orientation. B: Horizontal Midbrain slice, Nissl counterstain, scale bar = 1 mm. C: Overview of tectal lamination. The numbers represent the nomenclature according to Cajal (Cajal 1909).

Figure 2. Electrical stimulation evokes different response patterns in distinct layers of the TeO. A. CCD image of a midbrain slice showing the layered structure of the TeO. The layers are labeled and their borders are indicated by white lines. The image window is equivalent to the entire field of the imaging camera. Scale bar = 250 µm. B. Normalized voltage imaging traces from layer 5, layer 10 - 11 and layer 13. Traces were chosen in retinotopic projection of the stimulation electrode and binned from an area of 77 x 38.5 µm (width x depth). Data were normalized by the maximal signal amplitude under control conditions in a time interval 20 ms after stimulus onset. They show the VSDI signal 100 ms before and 800 ms after electrical stimulation (applied at t = 0ms). Data represent the mean of 21 experiments. The gray traces in B and C show VSDI traces under Ca2+ free conditions (n = 7) and NBQX application (5 µM; n = 8). Scale bar, fractional amplitude = 0.2.

Figure 3. Color-coded plot of maximal fluorescence change at different time frames after stimulation. For this plot the curvature of the TeO was eliminated and the positions of the stimulation electrodes of different experiments were aligned. Stimuli were applied at median lateral position (lateral extent = 0 µm) and in between the borders of layers 1 and 5. The maximal fluorescence change for transient activity (0 - 20 ms after stimulation) is displayed in A, C, E & G, while B, D, F & H present the data for the persistent activity (30 to 200 ms after stimulation). A & B shows the spatial distribution under control conditions (n = 21), C & D after application of Ca2+ free saline (n = 7), E &F with bath applied NBQX (5 µM; n = 8) and G & H after perfusion with bicuculline methiodide (10 µM; n = 8).

Figure 4. A complex spatio-temporal 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 (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. Inset: Enlargement of 5ms before until 20 ms after stimulation (n = 21). Application of Ca^{2+} free ringer solution (B; n = 7) and NBQX (C; 5 µM; n = 8) resulted in a reduced and locally restricted response, while the GABA<sub>A</sub> receptor blocker bicuculline methiodide (10µM) (C) enhanced the spatio-temporal activity pattern (n = 8).

Figure 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 one pixel perpendicular to the stimulation electrode, pixels were averaged. Stimuli were applied at median lateral position (lateral extent = 0 µm) at t=0ms. The insets show an enlargement of 5ms before and 20 ms after stimulation. Figure conventions as before.

Figure 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 of layer 10 – 11 and open squares of layer 13. Only if in at least half of all experiments (n = 21) pixel values were higher than the noise level data points were taken into account.

Figure 7. Comparison of neuronal response under control condition and GABA<sub>A</sub> receptor block. A shows traces from layer 5, layer 10 - 11 and layer 13 in retinotopic projection of the stimulation electrode. Black traces were recorded under control conditions and gray traces after 15 minute perfusion of the same slices with 10 µM bicuculline methiodide. Data were binned from an area of 77 x 38.5 µm (width x depth). Electrical stimulation started at t = 0 ms. Scale bar: fractional amplitude 0.2; n = 8.

B – D Bar graphs of maximal amplitude (B), the duration of the signal (C) and the lateral extent of activity (D) for layer 5, layers 10 -11 and layer13. Black bars represent data under control condition and gray bars after bicuculline application. B. Bar graphs of the maximal amplitude in the first 10 ms (left) and 30 – 200 ms after stimulation (right). The maximal amplitudes were gathered of pixels radial to the stimulation electrode. C. The duration of neuronal activity was calculated of data used in A. The time point, at which the signal amplitude crossed first the noise level, was used as starting time. The last time point exceeding this level indicated the stop of the signal. D shows the mean lateral extent of the VSDI signal for the first 10 (left) and 30 – 200 ms after stimulation (right). The lateral extent was determined by 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 plotted as mean ± SE. Asterisks indicate significant differences (p < 0.05; two-sided
Wilcoxon test).

Supplemental videos 1-3: The spatio-temporal response pattern was sampled at 1 Hz over 370 ms (20ms prior to electrical stimulation and 350 ms after stimulation). Videos display normalized mean responses over the entire field of the imaging camera at 25 frames per seconds. The curvature of the TeO was eliminated and the positions of the stimulation electrodes of different experiments were aligned. Stimuli were applied at median lateral position (lateral extent = 0 µm) and in between the borders of layers 1 and 5.

Supplemental video 1 shows the response under control conditions. Electrical stimulation elicited a neuronal response spreading laterally in layer 5 and into deeper layers within the first 10 ms (transient activity) with a peak activity in layer 5 to 9. After 20 ms most activity can be seen in layer 9 and 10 (see also Fig 3 A & B; n= 21).

Supplemental video 2 shows the response in Ca²⁺ free saline. The neuronal response lasts only a few milliseconds and is restricted to layer 1-5 (see also Fig 3 C & D; n = 7). The response pattern indicates that most of the recorded signal under control conditions is of postsynaptic origin.

Supplemental video 3 displays the spatio-temporal pattern after application of bicuculline methiodide. Electrical stimulation led to a changed response pattern compared to control. While the transient response was only slightly affected, the amplitude, the duration and the lateral extend of the persistent response was significantly increased (see also Fig 3 G & H and Fig 7; n = 8).
layer 5
layer 10
layer 13

20
15
10
5
0

500 250 0 250 500

lateral extent [μm]

time to maxima [ms]

layer 5
layer 10
layer 13