“Shepherd's crook” neurons drive and synchronize the enhancing and suppressive mechanisms of the midbrain stimulus selection network

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The optic tectum (TeO), or superior colliculus, is a multisensory midbrain center that organizes spatially orienting responses to relevant stimuli. To define the stimulus with the highest priority at each moment, a network of reciprocal connections between the TeO and the isthmi promotes competition between concurrent tectal inputs. In the avian midbrain, the neurons mediating enhancement and suppression of tectal inputs are located in separate isthmic nuclei, facilitating the analysis of the neural processes that mediate competition. A specific subset of radial neurons in the intermediate tectal layers relay retinal inputs to the isthmi, but at present it is unclear whether separate neurons innervate individual nuclei or a single neural type sends a common input to several of them. In this study, we used in vitro neural tracing and cell-filling experiments in chickens to show that single neurons innervate, via axon collaterals, the three nuclei that comprise the isthmotectal network. This demonstrates that the input signals representing the strength of the incoming stimuli are simultaneously relayed to the mechanisms promoting both enhancement and suppression of the input signals. By performing in vivo recordings in anesthetized chicks, we also show that this common input generates synchrony between both antagonistic mechanisms, demonstrating that activity enhancement and suppression are closely coordinated. From a computational point of view, these results suggest that these tectal neurons constitute integrative nodes that combine inputs from different sources to drive in parallel several concurrent neural processes, each performing complementary functions within the network through different firing patterns and connectivity.

Significance

In a crowded environment, animals must direct their behavior to the stimulus with the highest priority at each moment. Stimulus selection seems to develop from competitive interactions between concurrent neural inputs. In the avian midbrain, a neural network that reciprocally connects the optic tectum to the isthmi forms a simple neural mechanism for stimulus competition, where neurons mediating enhancement and suppression of tectal inputs are located in separate isthmic nuclei. Here, we show that collaterals of the same tectal neurons simultaneously drive these nuclei, synchronizing both antagonistic processes. These results contribute to our understanding of how distinctive neurons integrate inputs from different sources to drive in parallel several concurrent neural processes underlying a complex behavior.

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The superior colliculus (SC) or optic tectum (TeO) organizes attention, orienting, prey-catching maneuvers, and escape reactions in all vertebrates (1–5). Implicit to these functions are neural mechanisms in the TeO that topographically represent the multiple objects composing a visual scene and select the most relevant that will guide the responses. Stimulus selection seems to develop from competitive processes that increase the saliency of the neural representation with the highest priority at each moment (6–9), but how these processes are implemented in the SC or other neural structures is far from clear (10, 11). In birds, a conspicuous midbrain circuit that connects the TeO to the isthmic complex carries out a simple stimulus-selection mechanism, where competition between simultaneous visual as well as auditory inputs takes place (2, 12–16). Exploring the detailed functional connectivity that produces the interplay of excitatory and suppressive interactions underlying competition is crucial to understand how neural networks increase the saliency of specific sensory inputs.

A central node of the avian midbrain selection network is the “shepherd’s-crook” neurons (Shc) (Fig. 1) (17, 18), which relay retinal inputs arriving to the superficial tectal layers to the nuclei isthmi pars parvocellularis (Ipc), pars semilunaris (Slu), and pars magnocellularis (Imc) (16, 19). Neurons from Ipc and Slu send reentrant signals via columnar axons that terminate in the same tectal position from which the visual input originates (16, 20, 21). While the Slu targets in TeO remain unknown, Ipc axon terminals excite tectal ganglion cell (TGC) dendrites (22), boosting the transmission of the retinal inputs impinging on these TGCs to the nucleus rotundus (Rt, or caudal pulvinar) in the thalamus (13). In parallel, Imc neurons suppress the Ipc and Slu reentrant signals at other competing locations, via GABAergic, wide terminal fields that spread antitopographically, upon TeO, Ipc, and Slu (13, 19). This arrangement tends to focalize the enhancement of input activity mediated by the Ipc to those tectal zones receiving stronger stimulation at the expense of locations activated by weaker stimuli.

Despite the Shcs’ crucial role in carrying the competing inputs to this stimulus selection network, it is unclear whether different
Fig. 1. The isthmotectal network. (A) Schematic of the isthmotectal network representing a slab of TeO and its reciprocal connectivity with the isthmic nuclei. A focal retinal input is transmitted by Shc neurons to the isthmic nuclei, activating a zone (in red) in Ipc, Imc, and SLu. The Ipc and SLu return activity back to TeO, affecting a larger area but centered upon the same locus as shown in A, Inset. Imc neurons generate wide-field inhibition across complementary regions of the tectum, Ipc, and SLu. Imc, n. isthmi magnocellularis. It is not clear whether Ipc, SLu, and Imc axons actually terminate on Shc neurons and thus constitute “true” feedback connections; Ipc, n. isthmi parvocellularis; SLu, n. isthmi semilunaris [based on Wang et al. (16)]. (B) Nissl stained coronal section of the chicken midbrain showing the tectum and the isthmic nuclei. L10 and L13, tectal layers 10 and 13. (Scale bar, 500 μm.) (C) Drawing by Ramón y Cajal of a Shc neuron depicting its retinal input and the curved axon originating at the main dendrite above the soma. Arrows suggest the flow of signals through this neuron’s complex bipolar morphology. Reproduced with permission from ref. 17.

Types of Shcs project to individual isthmic nuclei or a single type sends a common input to several of them. A close time synchrony found between Ipc and SLu visual responses suggests that at least these nuclei receive a common input (13). However, experiments performed in chick slices have suggested that several electrophysiological differences between Imc and Ipc neurons reveal specific Shcs’ innervation (23, 24).

Resolving this issue is essential to understanding the operational rules and control mechanisms of this circuit that is becoming paradigmatic of a simple stimulus selection network. Separate Shc populations would permit an independent modulation of the topographic Ipc and SLu reentrant gains, as well as that of the antitopographic suppression mediated by the Imc. Conversely, a common input might be functional to produce a close covariance and even synchrony of the respective excitatory and inhibitory drives.

To assess whether the same or different types of Shcs project to Imc and Ipc, we performed neural tracing and cell-filling experiments in chick slices as well as in vivo recordings in anesthetized chicken. The results show that, through axon collaterals that terminate in each isthmic nuclei, individual Shcs operate as integrative nodes combining multiple inputs to drive, simultaneously, the excitatory and inhibitory mechanisms of the isthmo-tectal network.

Results

In Vitro Neural Tracing and Cell-Filling Experiments. We performed in vitro injections of neural tracers and cell-filling experiments in coronal and horizontal slices containing the isthmo-tectal circuit. Because SLu is more distant from the tectum, neural tracing to and from this structure was difficult to obtain, and only the cell-filling experiments gave consistent information regarding this projection.

Injections in TeO of fluorescent conjugates of biocytin dextran amine (BDA) and biocytin (nine slices, four chicks) labeled a large tract of Shc axons projecting to the isthmi along with dense terminal fields intermingled with retrogradely labeled cells in Imc and Ipc. As observed in in vivo tracing experiments (16), the terminal field and the labeled neurons in Imc were distributed in a wide area, whereas in Ipc they were restricted to a narrower band. In this preparation, it was not easy to follow single axons, especially in Imc, where labeled dendrites, axon terminals, and retrogradely labeled axons from Ipc, en route to TeO, are mixed together. However, in every section, it was common to observe passing axons leaving collaterals in Imc without reducing their diameter, indicating that Shc axons innervate Imc en route to other targets (Fig. 2 A and B).

Consistent with this result, injections in near-homotopic areas of Ipc and Imc with fluorescent tracers of different colors (15 slices, seven chicks) showed that the large majority of retrogradely labeled Shcs were double-labeled (Fig. 2 D–F). Cell counts (four slices), in the tectal area where Shc neurons labeled with each tracer overlapped, revealed that 75% and 67%, from the total of Shcs labeled from Imc and Ipc, respectively, were double-labeled. As presumably many axons course outside the slice section plane, this high double-labeled cell ratio reveals that Imc and Ipc receive a high proportion of common innervation.

Fig. 2. In vitro injections of fluorescent tracers in TeO and the isthmi. (A) Photomicrograph of a chicken midbrain horizontal slice showing an anterograde tracing experiment with an injection site of B-TMR in the intermediate layers of TeO. Inset represents the approximate section plane and position of the isthmic nuclei. (B) Labeled Shc axons leave regularly spaced collaterals (arrows) and continue their course outside the Imc nucleus (asterisks). (C) Retrograde tracing experiment showing the injection sites of B-TMR in Ipc and fluorescein-conjugated BDA (BDA-F) in Imc. (D) Merged channels showing the double-labeled Shcs. (E) Red channel, B-TMR-labeled Shcs in tectal layer 10 from the injections in C. (F) Green channel, BDA-F-labeled Shcs. [Scale bars, 500 μm (A and C); 100 μm (B and D–F).]
Next, to directly resolve the morphology, axonal course, and termination sites of single Shc neurons, we performed cell-filling experiments in thick slices using sharp micropipettes. With this procedure, most impaled Shcs were completely filled, and their remarkable bipolar morphology could be observed with exceptional detail (Fig. 3 A–C). The apical dendrite followed a radial course to the superficial retino-recipient layers, giving rise to the characteristic curved axon at ~40–60 μm above the cell body. The thicker lower dendrite extended toward layer 13 and ramified more profusely than the apical dendrite, forming a wide dendritic field, especially evident below the cell body and in the upper parts of layer 11. The Shc axons descended straight down from their origin in the apical dendrite, keeping a parallel course to the cell axis. Then, they entered the stratum album centrale and continued their course to the isthmi, following either a straight path or bending in a right angle to bypass the tectal ventricle.

Without exception, all Shc axons (n = 15) that were filled well enough to be tracked down to the Imc continued their path toward the other isthmic nuclei. In 12 cases, it was possible to observe their terminal field in Ipc, and in 8 of these cases, we could track their path beyond Ipc; in 3 of the latter we observed their terminals inside the Slu. Conversely, no Shc axon was found with terminals in Slu or Ipc without ramifications in the preceding nuclei.

The Shcs terminal fields in each nucleus were clearly different. In Imc, individual Shc axons spread 8–10 collaterals that branched perpendicularly at intervals of ~12–60 μm. Some of them formed long branches of up to 300 μm, with secondary

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Fig. 3. Shc neurons with axon terminals in the isthmic nuclei. (A) Camera lucida reconstruction and microphotographs of a Shc neuron filled with biocytin. Note the extended divergent terminal in Imc contrasted with the compact terminal in Ipc. (B) Camera lucida reconstruction of a labeled Shc neuron and microphotographs (a–c) of the axon terminals in each isthmic nucleus. (C) Another example of a filled Shc with axon terminals (d–f) in the isthmic nuclei. Note that three axon collaterals arising in different parts of the axon converge upon the same region in Ipc (e). [Scale bars: 100 μm (A–C); 50 μm (a–f).]
ramifications that covered a significant proportion of the cross-section of the nucleus (Fig. 3A). In Ipc, the Shc axon produced a dense terminal field restricted to a narrow column perpendicular to the plane of the nucleus. Remarkably, in two filled Shc, one or two secondary axon terminals originated before the edge of the nucleus formed additional dense terminals that merged with the first in the same loci (Fig. 3C).

In three cases, the biocytin was amplified with the tyramide reaction and streptavidin, and the slice optically cleared instead of being resectioned (Experimental Procedures). This allowed a detailed 3D reconstruction of its microstructure under a confocal microscope, revealing the contrast between the divergent and the convergent morphology of the axon terminal in Imc and Ipc, respectively (SI Appendix, Fig. S1 and Movies S1–S4).

Synchrony Between Imc and Ipc Visual Neuronal Responses. It has been shown in pigeons that Ipc neurons respond to visual stimulation with fast bursting discharges that are tightly synchronized to double spikes fired by SLu neurons (13). As this synchrony is most probably explained by the common innervation that we now report for the three thamic nuclei, we investigated whether Imc and Ipc neurons synchronize their responses as well, despite differences in discharge profiles observed in these neurons both in vivo (12) and in vitro (23).

To that end, we made simultaneous in vivo extracellular recordings of neurons located in nearly topographically corresponding regions in Imc and Ipc, in response to moving visual stimuli that crossed their overlapping visual receptive fields (RFs).

Single and multiunit recordings (n = 20) showed that Imc neurons did not have spontaneous discharges and responded briskly to moving spots that swept their RFs. As in pigeons (12), all Imc units displayed highly elongated RFs (16° ± 2.8° × 116° ± 13°, n = 12) oriented in the vertical axis (Fig. 4A) and, depending on the region of the RF stimulated, responded with either high-frequency barrages of spikes ranging between 500 and 700 Hz, or bursting responses with intraburst frequency, also ranging between 500 and 700 Hz, and interburst peak frequencies ranging between 15 and 45 Hz (Fig. 4B and D). These frequency components were manifested as clear peaks in the respective Fourier transform of the spike timing events (Fig. 4C). Each type of response occurred in patches along the Imc RF, the location of which would vary for consecutive renditions of the stimulus. The bursting firing of Imc neurons was very similar to the bursting responses typically displayed by Ipc neurons, as described in pigeons and owls, and the peak frequency of the interburst interval varied in the same high-beta, low-gamma frequency range (15–50 Hz, unpaired t test, P = 0.98; Fig. 4D). Chicken Ipc neurons possessed round RFs (14° ± 2.5° in diameter, n = 14) and also responded with brisk sequences of spike bursts to static and moving visual stimuli. Simultaneous recordings in both nuclei showed that the interburst peak frequencies of Ipc and Imc units with overlapping RFs indeed coincided (Fig. 5C and D). Moreover, visual inspections of the records and computed cross-correlograms showed that there was a close synchrony between bursting events fired by units in each nucleus (Fig. 5A, B, E, and F).

There was a variable phase shift between the simultaneous Ipc–Imc recordings, presumably caused by the fact that the RFs were only partially overlapping. Usually, there was a phase precession, such that the burst from one record initially lagged the burst from the other, to gradually end up with an inverted phase relation. In cases where the Ipc RF overlapped with Imc RF regions displaying high-frequency responses, the cross-correlation between both responses was very weak or nonexistent.

Electrophysiological and Visual Response Properties of Shc Neurons. The common innervation and synchrony shown by the previous experiments, contrasted with the differences in spike firing profile recorded in each nucleus, raised the question of what would be the visual responses and firing properties of Shc neurons. To answer that, we performed in vivo extracellular recordings of layer 10 neurons, seeking to identify some of the recorded units using the juxtacellular technique.

We recorded from 29 units in 12 chickens, at a range of depths (between 550 and 800 μm) that encompassed tectal layer 10. Although for several technical reasons, including mechanical brain stability and the small size of the cells, juxtacellular labeling became particularly difficult, we successfully recorded and identified four Shc neurons in four chickens. Another three fusiform neurons labeled in layer 10b were not considered Shcs, as the characteristic curved axons could not be seen. Overall, recorded units at this depth responded with trains of single spikes to visual motion, without bursting bouts. The responses of the identified Shcs were rather homogeneous: They did not fire spontaneously and fired reliably to visual motion stimulation, with a moderate accommodation to repeated stimuli, from a RF of 15° ± 6° in diameter (Fig. 6A). When a small bright dot was moved through their RF, they responded with seemingly regular trains of single spikes with modal instantaneous frequencies ranging from 20 to 40 Hz (mean maximum rate 28 ± 10 Hz). Importantly, even though we used the same visual stimuli as those used in the Imc and Ipc recordings (2° spot, 10° to 20° per s), in no occasion did units fire high-frequency barrages like the Ipc (700 Hz) neurons or bursting spikes like the Ipc neurons.

To evaluate the cells’ spike regularity, we first computed the peristimulus time histograms (PSTHs) of spike responses to 10 stimulus trials. Then, we fitted a Gaussian function to the PSTH and defined a response window of ±1 SD. Using that window, we calculated the interspike interval (ISI) distribution and the corresponding coefficient of variation (CV), assuming a stationary condition for the basal firing rate. We then fitted a gamma distribution to the ISI histograms and calculated the...
shape parameter $k$ for the fitted curve. Irregular, Poisson-like firing produce $k$ and CV values that are close to one, whereas regular spiking tend to produce values that are higher and lower than one, respectively (25). For the Shc neurons, we obtained $k$ values between 2.29 and 5.61 ($3.9 \pm 1.4$) and CV values between 0.4 and 0.6 ($0.5 \pm 0.08$) (Fig. 6D), both indicative of a more regular firing than a random Poisson process. The same calculation made for the rest of the neural sample, presumably including numerous non-Shc neurons, gave parameters significantly closer to one than Shcs ($k = 2.2 \pm 1.1$, Mann–Whitney test, $P = 0.01$; CV = $0.7 \pm 0.14$, $P = 0.01$). A related estimation of regularity was obtained by plotting the mean and variance of the spike counts recorded across stimulus repetitions for each cell. Again, points falling along the diagonal indicate mean equal to variance values, as predicted by a Poisson process (25–27). As shown in Fig. 6B, Shcs tended to have points below the diagonal, whereas the points of the rest of the recorded neurons were more evenly distributed. To quantify this difference, we calculated the mean vertical distance from the diagonal (residuals) in both cases, obtaining average residual values of $-0.1 \pm 0.04$ for the Shcs vs. $0.0 \pm 0.1$ for the rest of the sample (Mann–Whitney, $P = 0.02$).

Altogether, these results indicate that Shcs do not fire spike bursts but respond with spike trains of variable rates that are as variable as predicted by a Poisson process. Although the brief response samples obtained produced noisy power-density functions without significant peaks, the average instantaneous firing frequency distribution for the recorded Shcs did show a clear peak at 20 Hz (Fig. 6E).

**Discussion**

In this study, we showed that single Shc neurons innervate via axon collaterals the three nuclei that comprise the isthmotectal circuit of the avian midbrain. This indicates that the input signals representing the strength of the incoming stimuli are simultaneously relayed to all of the elements of this stimulus selection network: to the Ipc and SLu, which provide the topographic reentry to TeO, and to the Imc, which provides the antitopographic suppression of competing inputs. This common innervation explains the synchrony between bursting events in the Ipc and the Imc and also between bursts in the Ipc and spikes in SLu reported in pigeons (13). From a computational point of view, the results suggest that Shc neurons may perform as “operational” hubs (28). While receiving retinal inputs in their apical dendrites as well as other inputs in their deeper dendrites (18), they drive and synchronize three concurrent neural processes, each with different functions and spike-firing profiles. We will argue that this feat could be attained by marked differences in axonal convergence, varying degrees of synchrony between Shc neurons, and postsynaptic mechanisms.

In vitro double injections of fluorescent tracers into the Ipc and Imc double-labeled >60% of the labeled Shcs. Considering that in the slice many axons are severed, this result strongly suggested that most, if not all, Shcs innervate both the Ipc and the Imc. The cell-filling of single cells performed in vitro confirmed this hypothesis, showing in all complete cases that the labeled neurons extended axon terminals into both the Ipc and the Imc and, in some cases, presumably where the reconstruction was not limited by the section plane, also to the SLu. Although occasional Shcs projecting to only individual nuclei may exist,
In the Ipc and TeO suppress the Ipc (and SLu) signals to tectal zones receiving weaker retinal inputs (12, 31).

Previous results in the pigeon (12) and the present results in the chick demonstrate that these concurrent processes have different firing profiles. In addition to episodes of bursting responses, Imc neurons respond to visual input with spike discharges that can reach very high frequencies (500–700 Hz) and ride on fast subthreshold depolarizations. Ipc neurons respond with discrete bursts of spikes that arise from pronounced depolarizations, with interburst frequency ranging between the high-beta and low-gamma range (15–50 Hz). SLu neurons respond with single/double spikes in the same frequency range and in close synchrony to the Ipc bursting firing (13). Electrophysiological studies in chicken brain slices concluded that the gamma frequency bursting in Ipc and the high-frequency spike firing of Imc were induced by separate Shc types: one type firing at high rates that innervate the Imc and a separate type firing with low-frequency periodic discharges that innervates the Ipc (23, 24).

The present results clearly demonstrate that virtually all Shcs projecting to Ipc also project to the Ipc, thus making that hypothesis implausible.

However, the question remains how a single neural type could drive dissimilar firing patterns in each of its separate neural targets. Extracellular recordings in tectal layer 10 of the chicken, in addition to Shcs, contains other neural types (4, 5, 32), have shown that some neurons respond with frequencies within the characteristic gamma range that pervades the isthmotectal circuit (23, 33). Indeed, our sample of identified Shcs indicated that they responded with more regular spike trains than most neurons recorded in and around this layer, with firing frequencies that neither reached the high-frequency levels observed in Imc neurons nor showed bursting episodes like those of Ipc and Imc neurons. The average PSTH calculated for these cells showed a clear peak at 20 Hz, which is about the modal frequency of Ipc neural firing. If Shc neurons synchronize in that frequency range, they would trigger the respective bursting and double-spike discharges of the Ipc and SLu neurons and also the bursting sequences often fired by the Imc neurons. As Ipc and SLu neurons burst intrinsically to sustained current injections (24, 34), they may filter by resonance the gamma frequency component of the Shc input. The high convergence of a single Shc upon Ipc neurons would explain the pronounced postsynaptic depolarization underlying each Ipc’s burst. Imc neurons, on the other hand, are able to follow their synaptic input up to very high frequencies (12, 23), and as they receive a converging input from a large number of Shc, they might fire either in high-frequency trains, if their Shc input is unsynchronized, or fire in bursts, if it is synchronized (Fig. 7).

Therefore, to represent the stimulus strength, the Shc neurons may use synchrony in addition to firing frequency and population recruitment. As the Shc’s synchrony might be dynamically controlled, a strong impact on the operation of the circuit is expected. Increasing levels of synchrony between Shcs responding to a given target would promote the bursting firing in Ipc neurons, which might suppress activity within that frequency band, perhaps allowing the transmission of other retinal inputs receiving Ipc reentry at other frequencies or with interleaving phases. If Shcs respond asynchronously, they will induce high-frequency discharges in Imc neurons, generating a more widespread suppression, presumably inducing a winner-take-all effect. In the slice preparation, it has been shown that acetylcholine entrains the oscillatory circuits of layer 10, seemingly increasing the synchrony between Shc neurons (33). In any case, the extent of synchrony in this system must be assessed under natural conditions.

Presumably, Shc responses do not depend on specific visual features but on a wide variety of visual motion configurations and visual contrasts, as is observed in their target Ipc neurons. In
Fig. 7. Summary model of the connectivity and neural firing of the isthmo-tectal circuit. A single type of Shc neuron innervates the three isthmic nuclei, with high axonal divergence upon Imc neurons and high convergence upon Ipc and SLu neurons. In cases where visually activated Shcs synchronize their firing (1, red) at low gamma rates, Imc neurons would fire bursts of spikes synchronized to bursts in Ipc and double spikes in SLu. Unsynchronized Shc’s spikes (2, blue) would be summed at Imc neurons provoking high firing rates, while intrinsic bursting properties of Ipc and SLu neurons would filter by resonance the gamma components of fewer Shc neurons, keeping the synchrony between both nuclei.

the barn owl, Ipc neurons are not even modality-specific, but respond to both visual and auditory stimuli (35, 36). This sensory integration is a characteristic of selection algorithms in spatial attention models, in which spatial saliency is computed across different sensory values (7, 9, 37, 38). The peculiar bipolar morphology of Shc neurons would facilitate this integration, as their apical dendrites could summate visual inputs from different types of retinal ganglion cell terminals. The deep dendrites could add up auditory inputs arriving on deeper tectal layers (18, 39) and also top-down influences from the arcopallium, which are able to bias the competing interactions within the isthmo-tectal system (31, 40). The arcopallium can induce saccadic eye movements through a direct pathway to brainstem oculomotor centers and has been compared with the mammalian frontal eye fields (41); thus, Shcs may link stimulus selection to saccades, initiated by both pallial and mesencephalic circuits.

More generally, these results illustrate a case in point where a single neural type integrates multiple inputs to drive and synchronize three concurrent neural processes, each performing a different neural function. Neurons with long-range axons carry-
In Vitro Intracellular Filling Revealed with Fluorescence. Slices with biocytin-filled neurons were fixed overnight in 4% PFA and then washed three times for 5 min each in 0.1 M PB (pH 7.4). This solution was then replaced with 0.5% (vol/vol) ABC solution in PBS (4% NaCl)-Tx 0.5% (vol/vol) for 24 h at 4 °C. After washing in PB, slices were incubated with tyramide signal amplification (for details, see Vega-Zuniga et al. (42)). Briefly, sections were incubated in 0.001% biotin-tyramide (catalog no. LS-3500, lot 1407008; IRIS Biotech GmbH) and 0.003% H₂O₂ in 0.05 M borate buffer (pH 8.5) for 24 h at 4 °C. Finally, and after washing in PB, slices were incubated in streptavidin–Alexa 546 (Thermo Fisher Scientific) diluted to a concentration of 1:500 in PBS (4% NaCl)-Tx 0.5% for 24 h at 4 °C.

For optical analysis, slices were cleared with 2,2’-thiodiethanol (TDE; Sigma-Aldrich) dissolved in 0.1 M PB (for details, see Aoyagi et al. (43)). Slices were incubated in an ascending TDE concentration solution for 1 h at 42 °C per treatment (20%, 40%, and 60%). Confocal laser-scanning microscopy was performed with Olympus Fluoview FV1000 BX61 (Olympus). Images were processed with Fiji (44). For neuronal reconstruction, Neuotube (45) was used. Then, SWC files were analyzed and animated with Vaa3D (46) and Photoshop CSS.

**In Vivo Recordings.** Chickens were anesthetized by an intramuscular injection of ketamine (75 mg/kg) and xylazine (5 mg/kg) and placed in a stereotaxic frame in the standard position. Anesthesia was maintained during surgery and the duration of each experiment. Typically, the initial dose every 60 min via an intramuscular cannula. During the experiment, the body temperature of the animal was kept between 38 °C and 42 °C by means of a thermo-regulated blanket (FHC, Inc.). The dorsal-lateral part of the tectum, including the region above the isthmic region, was exposed and the dura was removed, keeping the surface moist with saline solution.

**Physiological recordings in the isthmus.** Simultaneous extracellular recordings in the Ipc and the lmc were obtained by using tungsten microelectrodes (1–6 Mohms) or micropipettes (2–3 μm tip, filled with 2 M sodium acetate) and a multichannel amplifier (model 3600; AM System Inc.). A silver and carbonate paste (model 3600; AM System Inc.) was used. Then, slicing was performed with a standard DC amplifier (IR-83, Neurodata; Cygnus Technologies). Simultaneous extracellular recording of both neurons was performed using the R software. Unpaired t and Mann–Whitney tests were used for two group comparisons for normally and not normally distributed data, respectively. The criterion for significance was defined as P < 0.05. All presented numerical values are given as mean ± SD unless otherwise indicated.

Synchrony between the Ipc and the Imc was assessed by computing discrete cross-correlograms in 200-ms segments of simultaneously recorded traces. These correlograms represented the distribution of 1-ms binned time intervals between each spike in the reference trace and all of the spikes in the target trace. Normally, 10 correlograms were averaged for each recording pair. For each pair of recording traces, a cross-correlogram was computed and normalized by dividing the cross-correlogram by the number of spikes in the reference trace. Accordingly, for a given time delay, a value of 1 indicated that there was on average one spike in the target trace for each spike in the reference trace, at 1-ms resolution. “Shift predictor” correlograms estimating the cumulative production by chance, were computed for alternate recording traces.

Power spectra of recording traces were obtained by either computing a standard fast Fourier transform of the trace’s autocorrelograms using the NumPy package for Python or obtained directly from the spike trains by using Neuro Explorer (Nex Technologies). For each unit, power spectra of 10 individual traces were smoothed by using a Gaussian function with a SD of 1 Hz, normalized to the maximum and averaged.

To evaluate spike regularity, we took the ISI for each cell resulting from 10 stimulus presentations. To that end, we first computed the PSTHs of spike responses to 10 visual stimulus repetitions. Then, after fitting a Gaussian function to the PSTH, we calculated the ISI in a ±1 SD time window. We characterized the ISI distributions by measuring their CV (SD/mean) and by fitting a gamma distribution following the method described in Maimon and Assad (25). The use of gamma was instrumental; it fitted the data better than a lognormal distribution, but other functions may provide better fits. In a Gamma probability density function, the frequency of a value x is defined by:

\[
f(x) = \frac{x^{\alpha-1}e^{-x/\beta}}{\Gamma(\alpha)}\]

where \(\Gamma(\alpha)\) is a complete gamma function; \(\Gamma(x) = (x - 1)!\), and \(\alpha\) and \(\beta\) are the shape and scale parameters of the distribution, respectively. Irregular, Poisson-like firing produced \(\alpha\) and \(\beta\) values that are close to one, whereas regular spiking tended to produce higher and lower values that one, respectively (25). A related estimation of regularity was obtained by plotting the mean and variance of the spike count recorded across stimulus repetitions for each cell (25–27). To that end, spikes were counted in 100 ms response windows in 10 trial repetitions, and the variance vs. mean of that count across trials was represented in a scatter plot. Lastly, unit’s firing rate distribution was obtained by taking the inverse of the ISI values obtained before.

**Histological procedures.** For recordings in the isthmus, in some animals, marking lesions were made at the end of the recordings by passing 5 μA for 5 s through each electrode. The animals were then euthanized with an overdose of anesthetic, and their brains were perfused as described before, cryoprotected (30% sucrose for 3 d), cut in 45–to-60-μm coronal sections, and stained with cresyl violet. Sections were mounted, and the lesions were examined and photographed by using a conventional microscope (BX 60; Olympus), equipped with a Spot digital color camera (Diagnostic Instruments).

For the juxtacellular labeling, after perfusion, the tissue was processed and observed following the same procedures as the in vitro material.

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