Serotonergic modulation of startle-escape plasticity in an African cichlid fish: a single-cell molecular and physiological analysis of a vital neural circuit


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Whitaker KW, Neumeister H, Huffman LS, Kidd CE, Preuss T, Hofmann HA. Serotonergic modulation of startle-escape plasticity in an African cichlid fish: a single-cell molecular and physiological analysis of a vital neural circuit. J Neurophysiol 106: 127–137, 2011. First published March 30, 2011; doi:10.1152/jn.01126.2010.—Social life affects brain function at all levels, including gene expression, neurochemical balance, and neural circuits. We have previously shown that in the cichlid fish Astatotilapia burtoni brightly colored, socially dominant (DOM) males face a trade-off between reproductive opportunities and increased predation risk. Compared with camouflaged subordinate (SUB) males, DOMs exposed to a loud sound pip display higher startle responsiveness and increased excitability of the Mauthner cell (M-cell) circuit that governs this behavior. Using behavioral tests, intracellular recordings, and single-cell molecular analysis, we show here that serotonin (5-HT) modulates this socially regulated plasticity via the 5-HT receptor subtype 2 (5-HTR2). Specifically, SUBs display increased sensitivity to pharmacological manipulation of 5-HTR2 compared with DOMs in both startle-escape behavior and electrophysiological properties of the M-cell. Immunohistochemistry showed serotonergic varicosities around the M-cells, further suggesting that 5-HT impinges directly onto the startle-escape circuitry. To determine whether the effects of 5-HTR2 are pre- or postsynaptic, and whether other 5-HTR subtypes are involved, we harvested the mRNA from single M-cells via cyttoplasmic aspiration and found that 5-HTR subtypes 5A and 6 are expressed in the M-cell. 5-HTR5, however, was absent, suggesting that it affects M-cell excitability through a presynaptic mechanism. These results are consistent with a role for 5-HT in modulating startle plasticity and increase our understanding of the neural and molecular basis of a trade-off between reproduction and predation.

Mauthner cell; single-cell polymerase chain reaction; Astatotilapia burtoni; 5-HT receptor subtype 2; ketanserin

BRAIN AND BEHAVIOR ARE SCULPTED by an intricate and dynamic interplay between genotype, prior experience, and an individual’s current social and physiological state (Hofmann 2003; Robinson et al. 2008; O’Connell and Hofmann 2010). Understanding how social life in particular affects brain function at all levels of biological organization is a fundamental question in modern biology (McClung and Nestler 2008). Social experiences manifest themselves as changes in gene expression, neurochemical balance, neural circuit function, and structural reorganization in various brain regions (Edwards and Kravitz 1997; Feder et al. 2009; Fernald 2002; Hofmann 2003). In vertebrates, much of this work has focused on fore- and midbrain circuits, such as the mesolimbic reward system and the social behavior network, because these systems regulate incentive salience and social behavior, respectively (Deco and Rolls 2005; Goodson et al. 2005; Newman 1999; O’Connell and Hofmann 2010, 2011). Also, their function can vary profoundly either as a consequence of social experience or in relation to sex-typical behavior (e.g., Crews 2003; Krishnan and Nestler 2008).

The genetic and physiological analysis of the consequences of social experience at the level of identified neurons has been invaluable (Dickson 2008; Edwards and Kravitz 1997; Yeh et al. 1996). In vertebrates, natural social behavior and the underlying neural substrates are typically not amenable to detailed in vivo neurophysiological studies. Thus, to reveal the neural mechanisms that link molecular and endocrine processes to social life, it is essential to study distinct and robust behavior patterns associated with the overall phenotypic state of an individual. Invertebrates have proven to be excellent model systems for such an integrative approach (Edwards and Kravitz 1997). In crayfish, for example, social control of escape behavior and subsequent modification of the underlying neural circuitry (such as the lateral giant neuron system) through serotonergic pathways has provided a powerful conceptual framework and important insights into the neurophysiological effects of social experience (Antonsen and Edwards 2007; Edwards et al. 1999, 2003; Spitzer et al. 2005; Yeh et al. 1996, 1997). However, few, if any, vertebrate model systems lend themselves to such an approach.

The African cichlid fish Astatotilapia burtoni has become an important model system in social neuroscience (Hofmann 2003; Robinson et al. 2008; Wong and Hofmann 2010). Males of this species display remarkable behavioral and neural plasticity throughout life (Hofmann 2003; Robinson et al. 2008; Wong and Hofmann 2010), as they repeatedly change status in a social dominance hierarchy (Hofmann and Fernald 2000). When an individual is dominant (DOM) in a social group, he maintains bright body coloration and displays a diverse behavioral repertoire to defend a territory and attract females for mating. In contrast, subordinate males (SUBs) display few distinct behavior patterns, adopt cryptic coloration, and school with conspecifics. These traits make individual SUBs difficult to distinguish from the school (Turner and Pitcher 1986) and from the surrounding environment but severely limit their reproductive opportunities (Fernald and Hirata 1977). Since
DOMs are more conspicuously colored and isolated from the school, they are likely more vulnerable to predation than SUBs (Fernald and Hirata 1977; Maan et al. 2008). We have previously shown (Neumeister et al. 2010) that the trade-off between reproductive opportunities and predation risk leads to a robust difference between DOMs and SUBs in startle-escape performance: behavioral responses to sound clicks of various intensities revealed significantly higher startle-escape responsiveness in DOMs compared with SUBs.

This behavioral plasticity can be explained at least in part by social status-dependent variation in the excitability of the Mauthner cells (M-cells) that triggers the startle-escape behavior (Neumeister et al. 2010). The M-cell is a sensory integrator that initiates an escape response in most teleost fishes and larval amphibians and constitutes a powerful model system for deciphering the cellular mechanisms underlying simple decision-making processes in the nervous system (Preuss et al. 2006; Schlegel and Schuster 2008; for review see Korn and Faber 2005). Moreover, M-cell activity and membrane properties directly control the expression of the behavior (Neumeister et al. 2008; Preuss and Faber 2003; Weiss et al. 2006). M-cell responses to auditory stimuli have been studied in detail even though this circuit can also be activated by visual and lateral line inputs (Canfield 2003; Canfield and Rose 1996; Preuss et al. 2006). In the case of 8th nerve afferents, some axons form mixed electrical-chemical synapses onto the distal portion of each M-cell’s laterally projecting dendrite (Kohno and Noguchi 1986; Pereda et al. 2003) and onto inhibitory interneurons [so-called passive hyperpolarizing potential (PHP) cells], which in turn synapse onto the M-cell soma and dendrites (Furukawa and Furshpan 1963; Satou et al. 2009; Weiss et al. 2008). M-cell axons decussate and form en passant synapses with recurrent and contralateral inhibitory interneurons as well as motoneurons along the contralateral body wall (Faber and Korn 1978). Thus a loud, unexpected sound may trigger a contraction along one body wall of the fish, pulling the head and tail together into a “C” shape (“phase 1” of the startle-escape response according to Eaton et al. 1991) with a latency as short as 8 ms.

Previous studies have shown adaptive changes in M-cell excitability in response to altered environmental conditions (Preuss and Faber 2003; Szabo et al. 2008) and during sensory gating (Neumeister et al. 2008). In addition, we recently demonstrated (Neumeister et al. 2010) that in A. burtoni M-cells of DOMs exhibit larger excitatory responses to sound clicks in a manner consistent with the increase in behavioral responses. We also found that the inhibitory drive mediated by PHP cells presynaptic to the M-cell is significantly reduced in DOMs. This study showed for the first time that escape probability can vary with social status, and that these differences are due to changes in M-cell excitability. More generally, these insights provided an integrative explanation of an ecological and social trade-off at the level of an identifiable decision-making circuit. The neurochemical basis of this plasticity, however, remains to be determined.

The biogenic amine serotonin (5-hydroxytryptamine, 5-HT) is a prominent neuromodulator of social behavior (Edwards and Kravitz 1997; Veenema 2009). In crustaceans, higher 5-HT levels are typically associated with increased aggression and social dominance (Edwards and Kravitz 1997). Moreover, in crayfish 5-HT has been shown to modulate the lateral giant escape reflex differentially in DOMs and SUBs, facilitating and inhibiting, respectively (Krasne et al. 1997; Yeh et al. 1997). These studies also suggested that differential expression of 5-HT receptors (5-HTRs) is controlled by social status (Edwards et al. 2002). In vertebrates, however, higher levels of 5-HT are generally believed to lower aggression. Importantly, Winberg et al. (1997) found that in the brain stem of A. burtoni males 5-HT metabolism is higher in SUBs compared with DOMs. Also, intracranially administered 5-HT inhibits aggressive behavior in the blue Acara cichlid, Aequidens pulcher (Munro 1986). Several physiological and morphological studies have suggested that 5-HT modulates the M-cell system. Local 5-HT administration enhances tonic and evoked inhibitory currents observed in the M-cell (Mintz et al. 1989; Mintz and Korn 1991). Moreover, immunohistochemical studies in goldfish and zebrafish (Gotow et al. 1990; McLean and Fetcho 2004; Petrov et al. 1991) demonstrated at least two types of serotonergic inputs onto the soma and dendrites of the M-cell, underscoring the putative role of this neuromodulator for this system.

Despite extensive pharmacological studies examining M-cell neurotransmitter systems (see above), very little work has focused on the molecular level. The only exception has been the study by Imboden et al. (2001), who cloned and sequenced three glycine receptor subunits from zebrafish and provided evidence that glycine is a major inhibitory neurotransmitter acting on the M-cells. Despite the important neuromodulatory role 5-HT plays in M-cell function, there have been no molecular studies.

In the present study, we pharmacologically perturbed the function of the 5-HTR subtype 2 (5-HTR2) in A. burtoni DOMs and SUBs and showed that 5-HT plays an important role in determining the socially regulated plasticity in startle-escape performance we have described previously for this species (Neumeister et al. 2010). Using transcriptomics, we then demonstrated that high-quality mRNA can be harvested from single M-cells, paving the way for single-neuron molecular analyses of the M-cell system. Finally, we showed that only a subset of 5-HTR subtypes is expressed in the M-cell. Together, these results provide a compelling foundation for the integrative analysis of a socially regulated neural circuit in a highly social vertebrate.

**MATERIALS AND METHODS**

**Animals.** Adult A. burtoni from a laboratory-reared stock were housed in acrylic tanks (30 × 30 × 60 cm) in communities of 8–12 males and 8–12 females under conditions mimicking the natural environment in their native Lake Tanganyika (pH 8.5 ± 0.2; 27 ± 0.2°C; 12:12-h light-dark cycle). Gravel substrate and terracotta pots were provided to allow multiple males to establish territories within each community. For the individuals used in this study, we assessed dominance status at least twice weekly for more than 2 wk to identify stable DOMs and SUBs, using established methods (Renn et al. 2008). All experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committees of Hunter College of CUNY and the University of Texas at Austin.

**Assessing startle behavior.** To evaluate auditory startle responses in male A. burtoni we used a paradigm identical to the one described by Neumeister et al. (2010). Briefly, isolated fish were transferred to a restricted arena (~17 cm in diameter, 22 cm deep) within a larger (110 liter) tank between two underwater speakers (UW30, Electro Voice, Burnsville, MN) and filmed from above with a high-speed camera (resolution 512 × 384 pixels, 1,000 frames/s; Kodak Extapro
1000 HRC, Eastman Kodak, San Diego, CA). Three stimulus intensities (155, 157, and 160 dB) were presented in randomized order within each of four blocks of stimuli with variable intertrial intervals (range: 2–10 min). To account for variation in stimulus intensity as a function of distance from the loudspeaker and other factors, we systematically measured stimulus intensities (at given loudspeaker settings) throughout the test arena. This information enabled us to present a defined stimulus intensity to a fish independent of its respective location. Fish were allowed to acclimate for 50 min before the first 200-Hz sound stimulus was presented. Individual startle probabilities were calculated from the number of C-starts occurring within a given number of trials compared with the total number of trials.

M-cell electrophysiology. The electrophysiological experiments involved the same surgical, stimulation, and recording techniques as described by Neumeister et al. (2010). Fish were initially anesthetized for 3–5 min by bath immersion in MS-222 (120 mg/l), fixed in the recording chamber with two pins, and respirated through the mouth with a steady flow of aerated saline containing the general anesthetic MS-222 at a concentration of 20 mg/ml. Before dissection, a topical anesthetic (20% benzocaine gel, Ultralard) was applied to the incision sites and fish were immobilized with intramuscular injections of d-tubocurarine (1–3 μg/g body wt). A small hole in the cranium exposed the medulla for somatic M-cell recordings. A small lateral incision at the caudal midbody exposed the spinal cord to bipolar electrodes for antidromic activation of the M-axons with small pulses (5–8 V) with an isolated stimulator. Antidromic stimulation produces a negative potential in the M-cell axon cap (typically >15 mV), which unambiguously identifies its axon hillock and allows intracellular recordings from the M-cell soma or along the lateral dendrite at defined distances from the axon hillock (Faber and Korn 1978).

Postsynaptic responses (PSPs) to sound stimuli before and after drug application (see below) were recorded in the M-cell with sharp glass electrodes (10–12 MΩ) filled with 5 M potassium acetate (CH₃CO₂K) with an Axoprobe-1A amplifier in current-clamp mode. Sound stimuli consisted of 200-Hz sound pips produced by a subwoofer with underwater intensities ranging from 126 to 133 dB re. 1 μPa in water. Auditory stimuli were recorded with a microphone positioned above the fish during the experiments and with a hydrophone (SQ01; Sensor Technology, Collingwood, ON, Canada) inside the recording chamber tank for stimulus calibration trials. Throughout the experiments (typically 2–3 h), we monitored the M-cell resting membrane potential (RMP). A small lateral incision at the caudal midbody exposed the spinal cord to bipolar electrodes for antidromic activation of the M-axons with small pulses (5–8 V) with an isolated stimulator. Antidromic stimulation produces a negative potential in the M-cell axon cap (typically >15 mV), which unambiguously identifies its axon hillock and allows intracellular recordings from the M-cell soma or along the lateral dendrite at defined distances from the axon hillock (Faber and Korn 1978).

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Iontophoresis. Iontophoretic application of ketanserin was injected intramuscularly (IM) during the electrophysiological experiments, dissolved either in the saline–ethanol vehicle described above (n = 5) or in 20% DMSO (n = 3). We then examined electrophysiological M-cell properties of four DOMs and four SUBs within 0.5–2 h of drug exposure. No differences were observed in relation to the vehicle used. It should be noted that different routes of administration (e.g., slow vs. fast) might alter the effect of the drug on neuronal functioning (Teshiba et al. 2001), although it seems unlikely that the rate of bioavailability would differ significantly between IM and IP injections (as opposed to, e.g., intracerebroventricular vs. oral administration). In addition, because only a single dose was applied by IM/IP administration, ketanserin likely reached the brain in a single bolus as opposed to multiple peaks.

Immunohistochemistry. We used immunohistochemistry to visualize 5-HT in the A. burtoni hindbrain according to the procedure described by Gotov et al. (1990). Fish were euthanized under anesthesia (200 mg/ml MS-222 for 10 min), and the brain was quickly removed and placed into freshly prepared 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.5) at 4°C overnight. After rinsing with 0.1 M phosphate buffer the brain was cryoprotected in 30% sucrose at 4°C overnight and embedded in OCT compound (Sakura Finetek, Torrance, CA). We then prepared 30-μm transverse sections of the embedded brain stem on a cryostat. Sections were transferred onto precoated SuperFrost Plus slides (Fisher Scientific, Pittsburgh, PA; Erie Scientific, Portsmouth, NH), air-dried, rehydrated with 0.1 M phosphate buffer, quenched with 1% hydrogen peroxide (H1009, Sigma-Aldrich), 1.5% heat-inactivated normal goat serum (Vector Laboratories, Burlingame, CA), and 0.02% saponin (S7900, Sigma-Aldrich) for 1 h at room temperature. After rinsing, slides were incubated overnight with primary rabbit anti-5-HT polyclonal antibody (1:2,000; Sigma S5545). After several rinses, they were then incubated with goat anti-rabbit IgG secondary antibody (1:200) for 1 h at room temperature. Immunolabeling was assessed by adding drops of avidin-biotin-peroxidase complex (Vectastain ABC peroxidase kit, Vector Laboratories) onto each slide. After rinsing, diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories) solution was applied for visualization. Rinsed sections were then air-dried overnight and dehydrated through an alcohol series (2 × 70%, 2 × 95%, 2 × 100%). Slides were counterstained with cresyl violet. Finally, slides were mounted with Permount (SP15, Fisher Scientific) and viewed with an Aristoplan microscope (Leitz). To confirm that background staining did not affect our interpretation of the signal, we conducted controls without primary antibody. Several brains were only stained with cresyl violet for morphological analysis.

RNA isolation, amplification, and quality control. RNA was isolated from whole brain of three A. burtoni individuals (1 DOM, 1 SUB, 1 female) and from single M-cells of three fish. For the whole brain sample, the tissue was homogenized in 500 μl of TRIzol (Invitrogen) and processed according to the manufacturer’s instructions. Pellets were redissolved in RNA Storage Solution (Ambion). For single-cell RNA isolation, we harvested the cytoplasm of one M-cell from each of one DOM, one SUB, and one female at the end of the respective electrophysiological recording session. We aspirated the cytoplasm with a low-resistance (2–4 MΩ) glass electrode pulled from 3-mm glass with a vertical puller (PE-22, Narishige) filled with a 2.5 M KCl solution made up with RNase-free water. After penetration of the M-cell, we applied a small negative pressure for ~15–20 min with a 60-ml syringe and aspirated the cytoplasmic contents into the tip of the electrode. During aspiration we monitored the M-cell RMP and, every 4 s, the amplitude of an antidromically evoked M-cell spike (i.e., input resistance) to verify a good seal. The electrode was
then quickly removed, the tip broken off, immediately frozen in powdered dry ice, and stored at −80°C until RNA isolation and amplification. To avoid contamination from extracellular sources, the electrodes were coated before the experiment with a film of Sigmacote (Sigma; neutral, hydrophobic film on the electrode surface), which repels water, retards clotting, and prevents adsorption of macromolecules. We extracted the RNA by previously established protocols (Baugh et al. 2001; Dufnner et al. 2008).

We subjected the RNA samples obtained from the three whole brains and the three M-cells (2 replicates each) to two rounds of linear amplification. Linear RNA amplification strategies based on in vitro transcription with T7 RNA-polymerase are generally prone to lose sequence information at the 5′ end. This is mainly due to (1) the alignment of the T7-promoter/oligo(dT) primer and the transcription start at the 3′ poly(A)-tail of the original mRNA and (2) the use of random hexamers during the second cDNA synthesis step, causing reduction of fragment length. To facilitate the generation of almost full-length molecules we used the picoRNA amplification system by Oicmum Biosolutions, which is based on Baugh et al. (2001) with the following modifications. For first-round amplification, in place of the First Strand cDNA Synthesis Mix 2 was 2 µl of Primers used for determining Astatotilapia burtoni 5-HTR sequences.

5-HTR microarray (GEO platform GPL6416) constructed from a brain-specific library and a mixed tissue library representing a total of ~14,660 potential genes (Renn et al. 2004; Salzburger et al. 2008). Protocols for cDNA preparation and hybridization were according to Dufnner et al. (2008) with several notable changes: a Qiagen MinElute PCR Purification Kit was used for both cDNA purifications; 1 µl of 5 µg/µl yeast tRNA (Invitrogen) was included in the probe labeling reactions. Hybridization was performed under a coverslip submerged in a humidified chamber at a temperature of 65°C for 15–16 h in the dark. Slides were rinsed at room temperature, first in 0.6× SSC, 0.025% SDS, 0.001 M DTT and then in 0.05× SSC, 0.001 M DTT, before they were centrifuged until dry. The slides were kept in the dark until they were scanned with an Axon 4000B scanner (Molecular Devices) with the software package Genepix 6.0 (Molecular Devices). The data were prepared for analysis in a manner similar to that of Renn et al. (2008) with an additional normalization (“within-array batch normalization”) to correct for the presence of two different cDNA libraries on our array (Dufnner et al. 2008). The normalized intensities were used to calculate ratios of Cy3 to Cy5 intensity for each feature for each array. All raw and processed data are available at the GEO database (www.ncbi.nlm.nih.gov/projects/geo/) under accession number GSE26277.

Between-group (M-cell vs. whole brain) analyses were performed by computing a t-statistic for each feature with the LIMMA package (Smyth et al. 2004; Smyth 2005) in R/Bioconductor. We also did an individual analysis using LIMMA in which we made all possible pairwise comparisons between our six samples and corrected for multiple hypotheses testing according to Benjamini and Hochberg’s (2000) false discovery rate (FDR) correction method. This analysis necessitates that one sample be designated as a reference, which we arbitrarily chose as M-cell 1.

**Sequenceing and classification of 5-HT receptor subtypes.** To determine 5-HT receptor expression in single M-cells, we obtained sequences for multiple A. burtoni 5-HTRs by a PCR-based approach. The PCR fragments obtained for all receptor subtypes were sequenced. Products similar to 5-HTRs in GenBank were cloned into pCRII-TOPO vectors (Invitrogen). The 5A (CN469330) and 6 (CN469925) subtypes had previously been obtained in A. burtoni by expressed sequence tag (EST) analysis (Renn et al. 2004; Salzburger et al. 2008). For the 1A and 1D subtypes, we designed primers based on the orthologous genes in the closely related Nile tilapia, *Oreochromis mossambicus* (GenBank accession nos. AY219038 and AY219039, respectively). To obtain the A. burtoni 2A sequence, we designed degenerate primers with CODEHOP (http://blocks.fhirc.org/codehop.html) based on homologous sequences from stickleback, medaka, and pufferfish obtained by blasting the zebrafish sequence against the genomes of these three species (http://genome.ucsc.edu/). Finally, we obtained the A. burtoni 3B sequence by designing degenerate primers with CODEHOP based on the *Carassius auratus* sequence (EF490968.1) and orthologous sequences from stickleback, medaka, and pufferfish again obtained by blasting the zebrafish sequence against the genomes of these three species (http://genome.ucsc.edu/). Table 1 contains information on fragment lengths, number of amplification rounds, and accession numbers, as well as sequence information for the gene-specific primers we designed for subsequent PCR analysis.

<table>
<thead>
<tr>
<th>5-HTR Subtype</th>
<th>Accession No.</th>
<th>Forward Sequence (5’ to 3’)</th>
<th>Reverse Sequence (5’ to 3’)</th>
<th>Product Size, bp</th>
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<tbody>
<tr>
<td>1A</td>
<td>GQ2218441</td>
<td>AACTTCTCCAGTGCCATCG</td>
<td>GTCCTCAAGATCAGCCCTCAAG</td>
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</tr>
<tr>
<td>1D</td>
<td>GQ227406</td>
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<tr>
<td>2A</td>
<td>GQ227409</td>
<td>GACATGTGCTGGGTCCC</td>
<td>CACCATGATGTTGGCGGG</td>
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<tr>
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<tr>
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<td>450</td>
</tr>
<tr>
<td>6</td>
<td>GQ227403</td>
<td>CTGCTCAAAGCTGTCTCTTG</td>
<td>AGCACAGATGGCCACCAGAG</td>
<td>502</td>
</tr>
</tbody>
</table>

5-HTR, serotonin receptor.
On the basis of the partial mRNA sequences obtained, we determined the respective A. burtoni amino acid sequences. To assess whether our putative 5-HTR sequences indeed encode the expected subtypes, we used the Mega 4 freeware package (http://www.megasoftware.net/m_con_select.html) to align the sequences with ClustalW to the orthologous protein sequences of multiple species (Gasterosteus aculeatus, Homo sapiens, Mus musculus, Oryzias latipes, Rattus norvegicus, Takifugu rubripes, Tetraodon nigroviridis; see Supplemental Table S1 for sequence information) and generate a bootstrapped nearest-neighbor-joining gene tree.

Analysis of 5-HT receptor expression in single M-cells. No 5-HTR sequences are present on the microarray (Renn et al. 2004; Salzburger et al. 2008). To determine which, if any, 5-HTR subtypes are expressed in M-cells we used the amplified RNA samples as templates for PCR assays using the gene-specific primers listed in Table 1. cDNAs of subtypes 5 and 6 were denatured for 5 min at 95°C and then amplified over 35 cycles of 95°C for 45 s, 53.3°C for 20 s, 72°C for 60 s with a final elongation at 72°C for 5 min. For subtypes 1A and 1D, there was one round of amplification, then the same process as above. For the 3B subtype, the template was denatured at 94°C for 6 min and then amplified over 40 cycles of 95°C for 45 s, 52.4°C for 30 s, 72°C for 60 s, and a final elongation of 72°C for 10 min. The PCR conditions for 5-HTR2A comprised a 6-min denaturation step at 95°C, 40 cycles of 95°C for 45 s, 54.6°C for 30 s, 72°C for 60 s, and a final elongation at 72°C for 5 min.

RESULTS

Antagonizing 5-HTR2A startle probability. The presence of ketanserin, a 5-HTR2 antagonist, significantly increased (ANOVA, P = 0.0002) the responsiveness of DOMs (saline: 39.6 ± 7.2%; ketanserin: 70.8 ± 6.9%) and SUBs (saline: 43.8 ± 10.4%; ketanserin: 79.1 ± 8.5%) to short sound pips (Fig. 1). We observed an injection effect that reduced the responsiveness of DOMs and eliminated the difference between males of different status observed in noninjection studies of behavior (Neumeister et al. 2010).

Antagonizing the 5-HT 2A receptor increases M-cell excitability in SUBs. To determine whether 5-HT modulates M-cell excitability via the 2A subtype (5-HTR2A), we quantified in SUBs (n = 4) and DOMs (n = 4) the effect of the 5-HTR2A antagonist ketanserin on sound-evoked M-cell excitatory postsynaptic potentials (EPSPs) in response to sound pips of various intensities (126, 130, and 133 dB re. 1 Pa in water). After measuring baseline values, we administered ketanserin and analyzed its effect on the M-cell within 0.5–2 h.

Ketanserin evoked differential changes in the postsynaptic (PSP) amplitude in SUBs and DOMs (F = 25.80; P = 0.0023; repeated-measures ANOVA). In all SUBs tested, ketanserin increased the synaptic response to sound pips as indicated by an overall increase of the somatically recorded PSP (Fig. 2A). Interestingly, the observed relative increase in peak amplitude was inversely correlated to sound intensity (Fig. 2B, open bars; P = 0.041, GLMM). In contrast, the synaptic response in DOMs remained essentially unchanged after application of ketanserin (Fig. 2B, filled bars; P = 0.296, GLMM).

Consistent with the PSP effects, we also observed differential effects of ketanserin on the M-cell input resistance following activation of the collateral feedback network of inhibitory interneurons in SUBs and DOMs. As discussed previously (Faber and Korn 1978; Neumeister et al. 2010), this powerful inhibition is mediated by a Cl−-dependent change in membrane conductance, which effectively shunts incoming excitatory synaptic currents out of the cell. This inhibition can be quantified as the fractional reduction in peak amplitude (inhibitory shunt) of an evoked test action potential (AP) after activating the feedback inhibitory network with a preceding AP. Systematically changing the interstimulus interval (ISI) between the conditioning AP and the test AP exposes the evoked inhibitory time course. This approach is possible because the M-cell’s soma and dendrites are unexcitable. An increase in the fractional shunt [defined as 100 – (APtest/APcontrol × 100)] thus indicates a decrease in input resistance.

Specifically, in SUBs ketanserin significantly (P = 0.03, paired t-test) attenuated the magnitude of inhibition at onset by 28% [mean inhibitory onset shunt: 60.2% ± 1.17 (SE) and 43.16 ± 1.17 (SE) for control and drug, respectively], but inhibition increased in the presence of ketanserin during the later portion of the response (Fig. 2C). In contrast, DOMs do not show the initial reduction in inhibition [P = 0.80; paired t-test; mean inhibitory onset shunt: 48.31% ± 5.91 (SE) and 47.06 ± 1.93 (SE) for control and drug, respectively], yet they exhibit the later (<10 ms) enhancement seen in SUBs (Fig. 2D). Indeed, under control conditions inhibition in SUBs is ~20% larger compared with DOMs (black traces in Fig. 2, C and D). This difference is essentially eliminated by ketanserin (compare gray trace in Fig. 2C with black trace in Fig. 2D). Thus, as seen with the inhibitory responses, the 5-HTR2 antagonist ketanserin seems to affect SUBs more than DOMs.

5-HT varicosities envelop M-cell soma and dendrites. Figure 3, A and B, show the morphology of the paired M-cell in A. burtoni. They are located in the anterior part of the hindbrain.
beneath the fourth ventricle, ~700 μm below the surface of the medulla and 150–200 μm lateral from the midline. The M-cell is characterized by a large soma and two major dendrites that extend up to 600 μm dorsolaterally (lateral dendrite, LD) and ventrorostrally (ventral dendrite, VD). The axon hillock is surrounded by the so-called “axon cap” (Fig. 3C), a region densely packed with glia cells and nerve fibers that creates a perimeter of high electrical resistance.

Fig. 3. Morphology and 5-HT immunohistochemistry of the M-cell in DOMs. A: cresyl violet stain of both M-cells. B: overview of 5-HT immunolabeling of both M-cells. C: labeled 5-HT-containing type I and type II fiber varicosities at the soma and axon cap, respectively. The M-cell axon is also visible in this micrograph. D: 5-HT labeling at the M-cell ventral dendrite. D1: overview showing distribution of 5-HT labeling along the dendrite. D2 and D3: detailed view of type I fibers characterized by larger terminals, or boutons (>1-μm diameter; black arrowheads, D2) and of type II fibers characterized by smaller beaded varicosities (<1-μm diameter; white arrowheads, D3). E: overview showing few beaded varicosities (type II; white arrowheads) at the lateral dendrite. A, axon; AC, axon cap; LD, lateral dendrite; S, soma; VD, ventral dendrite; SVD, small ventral dendrite.
The latter provides the structural basis for electrical field (ephaptic) inhibition and classical chemical inhibition at the M-cell axon hillock (Bierman et al. 2009; Faber and Korn 1978; Weiss et al. 2008) The axon extends further medially to cross the midline before joining the spinal cord running posterior (Fig. 3C).

We used immunohistochemistry to label 5-HT in the A. burtoni hindbrain in three animals. As can be seen in Fig. 3, B–E, 5-HT-containing profiles are present at the M-cell soma and along the LDs and VDs, with the latter being labeled predominantly (compare Fig. 3, E and D1, respectively). Similar to the situation in goldfish (Gotow et al. 1990), type I and type II fibers can be distinguished. Type I fibers form few fusiform varicosities, and they establish larger terminals (boutons) close to the M-cell membrane (Fig. 3D2, black arrowheads). Type II fibers are thinner and form small beaded varicosities parallel to the membrane (Fig. 5D3, white arrowheads). Larger 5-HT-containing type I boutons terminate also onto the M-cell soma, and chains of beaded small varicosities (type II) run across the axon cap (Fig. 3C).

The M-cell transcriptome shows little variation. The immunohistochemical results encouraged us to examine 5-HTR expression in the M-cell at the molecular level. However, the aspiration technique we adapted to harvest the M-cell cytoplasm (see MATERIALS AND METHODS) could potentially result in a biased and highly variable representation of the mRNA pool, especially if only portions of the cytoplasm are harvested. If this were the case, we would expect a large amount of variation in gene expression across M-cell samples. Therefore, we first assessed the extent of this variation by comparing the transcriptomes of three M-cells and three whole brain samples, which served as an unbiased control. After removing control features as well as those with signal intensities below threshold, we obtained data for 16,892 array features. Using a FDR-adjusted significance threshold of \( P < 0.01 \), we found gene expression to be differentially regulated between M-cells and whole brains for 5,026 (29.8%) of the 16,890 features, indicating the unique properties of this cell type. Specifically, 2,972 (18%) features were upregulated in M-cells and 2,054 (12%) were upregulated in whole brains.

We then examined transcriptome variation across individual samples; 16,090 features remained for this analysis after removal of control and below-threshold features. When using a significance threshold of \( P < 0.01 \), we found that 2,972 (18%) features were upregulated in M-cells and 2,054 (12%) were upregulated in whole brains.

We used Pearson correlation analysis to quantitatively assess the extent to which transcriptomes varied across either M-cells or whole brains. We correlated the normalized intensities for all M-cell and whole brain samples, respectively, against each other and found that overall whole brains were significantly more variable (i.e., yielded smaller correlation coefficients; \( t \)-test, \( P = 0.013 \)) compared with M-cells. Table 2 shows the correlation coefficients for all six pairwise comparisons.

Some 5-HT receptor subtypes are expressed in the M-cell. We cloned a single mRNA transcript from each of the A. burtoni 5-HTR subtypes 1A, 1D, 2A, 3A/B, 5A, and 6. Subtype identities were confirmed by constructing a gene tree with the

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Pearson correlation coefficients are shown for pairwise comparisons between M-cell (left) and whole brain (WB; right) transcriptomes.

Fig. 4. Classifying 5-HTR subtypes. Astatotilapia burtoni 5-HTR sequences cluster with the putative 5-HTR orthologs identified in other species, confirming their subtype identities. Numbers indicate confidence values based on bootstrap analysis.
orthologous 5-HTRs of other vertebrates (Fig. 4). Next, we assed the expression of 5-HTRs in amplified mRNA samples collected from individual M-cells (n = 3) and whole brains (n = 3) by PCR. While all receptors investigated are expressed in whole brain, only 5-HTR2A and 5-HTR6 mRNAs are present in M-cells (Fig. 5). The 5-HTR1A, 5-HTR1D, and 5-HTR3B subtypes are clearly not present in the M-cell. Similarly, the 5-HTR2A subtype is not expressed in the M-cell.

DISCUSSION

We have shown that in A. burtoni males both startle-escape performance and M-cell physiology are modulated by 5-HT. In particular, we found that M-cell excitability in SUBs and DOMs was differentially affected by the specific 5-HTR2 antagonist ketanserin, suggesting a likely role of this subtype in controlling the socially regulated startle-escape plasticity observed in this species (Neumeister et al. 2010). The fact that 5-HTR2A receptors are not expressed in the M-cell suggest that the observed changes in M-cell excitability (i.e., input resistance) are mediated through 5-HTR2A receptors at the PHP interneurons that are part of the M-cell inhibitory system. We have further demonstrated the feasibility of obtaining high-quality and robust mRNA from single M-cells, which enabled us to reveal the 5-HTR expression pattern of the M-cell.

These results constitute a significant step forward in our understanding of social effects on neural circuits and the underlying neurochemical and molecular processes in vertebrates.

The 5-HTR2A receptor subtype has been heavily studied in the context of cognition, mood disorders, and psychosis (Landolt and Wehrle 2009; Maier et al. 2008). Allee et al. (2008) were the first to employ the selective 5-HTR2 antagonist ketanserin in another teleost, the weakly electric fish Brachydanio rerio, in which they demonstrated the role of this receptor subtype in modulating the electric signal waveform. To determine whether 5-HT modulates M-cell excitability via this subtype, we quantified in both DOMs and SUBs the effect of the 5-HTR2 antagonist ketanserin on sound-evoked M-cell EPSPs in response to sound pips. Interestingly, lowering the 5-HT tone in SUBs increased the synaptic response (PSP) only if it was not already saturated by a high-intensity sound, i.e., the effect declined with increasing sound intensities. In contrast, the synaptic response in DOMs remained essentially unchanged after application of ketanserin for the entire range of sound intensities, likely indicating a ceiling effect. In a preceding study, an identical range of sound pips evoked significantly larger PSPs in DOMs compared with SUBs (Neumeister et al. 2010). The synaptic response in DOMs might thus be maximal for a given sound intensity presumably due to the already low 5-HT tone in the M-cell pre- and postsynaptic circuit and cannot be further increased by applying the 5-HTR2 antagonist. Consistent with this notion is the fact that the amount of 5-HT and its principal metabolite in the hindbrain is lower in DOMs compared with SUBs (Winberg et al. 1997). This interpretation is further supported by the differential effects of ketanserin on the magnitude of M-cell feedback inhibition in SUBs vs. DOMs, where ketanserin affected only SUBs.

In principle, the reduction in inhibitory drive and M-cell input resistance in SUBs could be explained by both post- and presynaptic mechanisms. The two social phenotypes might differ in the degree of 5-HTR2-mediated excitation of the inhibitory glycinergic interneurons (Koyama et al. 2011), which provide phasic and tonic inhibition to the M-cell (Faber et al. 1991; Mintz and Korn 1991). The latter hypothesis is supported by our finding that the 5-HTR2A gene is not expressed in the M-cell (see below). In summary, our electrophysiological results provide strong evidence that DOMs and SUBs indeed differ at the level of M-cell physiology and underscore the utility of pharmacological approaches for uncovering the differences in the circuit mechanisms that underlie socially regulated differences in escape performance.

Our behavioral results also indicate an increase in M-cell excitability following ketanserin; however, this increase was independent of social state. How can we reconcile this discrepancy between electrophysiology and behavior? One possibility is that the injection effect described above masks any social status-specific effects. Even though A. burtoni males are overall quite robust when placed in a test arena (Neumeister et al. 2010) or when handled and injected with drugs (e.g., Trainor and Hofmann 2006), used in combination these methods apparently act as stressors that reduce startle responsiveness

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Fig. 5. Expression of 5-HT subtypes in single M-cells. mRNAs extracted from a homogenized whole brain and 3 different M-cells were used as the template for a PCR reaction with primers specific to 5-HT subtypes. All subtypes are present in the brain, as expected, but only subtypes 5A and 6 are present in M-cells, suggesting a postsynaptic role for 5-HT via these receptors. The subtypes 1A, 1D, 2A, and 3B are not expressed in the M-cell. N/A indicates a PCR product for a gene not examined in this study.
SEROTONERGIC MODULATION OF M-CELLS

A. burtoni is comparable to the structures in goldfish (Faber and Korn 1978). In addition, 5-HT immunohistochemistry in the hindbrain indicated a distribution of 5-HT type I and II fibers similar to that of goldfish (described by Gotow et al. 1990), suggesting synaptic and paracrine modulation of the M-cell, respectively.

We found that the morphology of the paired M-cells in A. burtoni is comparable to the structures in goldfish (Faber and Korn 1978). In addition, 5-HT immunohistochemistry in the A. burtoni hindbrain indicated a distribution of 5-HT type I and II fibers similar to that of goldfish (described by Gotow et al. 1990), suggesting synaptic and paracrine modulation of the M-cell, respectively.

While these histological results were encouraging, they did not allow us to conclude which receptor subtypes, if any, might regulate M-cell function. In the absence of A. burtoni 5-HT subtype-specific antibodies, we decided to examine receptor expression at the level of mRNA. Compared with the respective orthologs from other teleost species and representative mammals, our receptor sequences clearly indicated that we were successful in obtaining cDNA fragments for the genes that encode the 1A, 1D, 2A, 5A, and 6 subtypes. Because the identity of the subtype 3 sequence was more ambiguous, we have tentatively labeled it as 3B subtype. It should also be noted that because of the additional whole genome duplication that occurred early on in teleost evolution (Meyer and Schartl 1999), additional subtypes may exist in A. burtoni. Since the sequencing of the A. burtoni genome is currently nearing completion, we will soon have the ability to identify other members of this family.

We found that the cytoplasmic aspiration technique permits a robust and reliable assessment of transcript levels in individual M-cells. Importantly, our correlation analysis showed that M-cell transcriptomes were more similar to each other than were those from whole brains, suggesting that the cytoplasmic aspiration technique does not introduce additional (technical) variation in mRNA content. Also, the correlation coefficients we obtained by comparing the transcriptomes of individual M-cells are comparable to or greater than those reported for single neurons in previous single-neuron studies (e.g., Esumi et al. 2008; Tietjen et al. 2003). There is considerable variation in gene expression throughout the brain of DOMs and SUBs (for a detailed analysis, see Renn et al. 2008). The transcriptome of M-cells is relatively stable in comparison and largely unaffected by the single-cell harvesting procedure. It should be noted that most likely we did not extract cytoplasm beyond the trunks of the major dendrites and thus might have missed mRNA species present in the dendrites, although evidence for local translation in the M-cell is currently limited to the axon (see Koenig 1979; Koenig and Martin 1996). In any case, mRNAs targeted for translocation to the periphery still need to be transcribed in the nucleus and thus would be expected to be present in the cytoplasmic samples. Experiments currently underway aim to determine which 5-HTR mRNAs, if any, might be present in the dendrites.

We then analyzed the molecular substrate of M-cell physiology by assaying the expression of 5-HTRs in M-cells. Of the subtypes we have identified, only 5-HTR5A and 5-HTR6 mRNAs are present in M-cells. These results support a postsynaptic role of 5-HT in the M-cell and provide a great opportunity to learn more about the function of these receptors, given that their role in the vertebrate nervous system is still largely unclear. 5-HTR5A has been associated with schizophrenia (Dubertret et al. 2004) as well as bipolar disorder and major depression (Arias et al. 2001), although its function is still only poorly understood (but see Doly et al. 2004 for its potential role in the suprachiasmatic nucleus and Sprouse et al. 2004 for nociception in the spinal cord). 5-HTR6 has been implicated in anxiety, cognition, and the regulation of food intake (Woolley et al. 2004); however, most of the evidence is still circumstantial. Our finding that the 5-HTR1A and 5-HTR1D subtypes are not present in the M-cell is consistent with the notion that these subtypes play an important role as autoreceptors in serotonergic neurons (Roberts et al. 2001). We also find that the 5-HTR2A and 5-HTR3B subtypes are not expressed in the M-cell. This is important evidence that the effects of the 5-HT2 antagonist ketanserin are indeed presynaptic, likely via the inhibitory interneurons that impinge onto the M-cell, as discussed above.

5-HT is not the only neuromodulator known to affect the M-cell system. In goldfish, for example, dopamine enhances the efficacy of excitatory synapses to the M-cell LD (Pereda et al. 1992, 1994). Also in goldfish, the neuropeptide somatostatin is colocalized with glutamatergic excitatory and GABAergic inhibitory synaptic contacts that impinge onto the M-cell, an arrangement suggesting a modulatory role for this neuropeptide (Sur et al. 1994). Indeed, local application of somatostatin onto the M-cell LD increases excitatory synaptic transmission in the large club endings that provide auditory input to the cell (Pereda et al. 1997). This latter finding is interesting because somatostatin also plays an important role in regulating social behavior in A. burtoni (Hofmann and Fernald 2000; Trainor and Hofmann 2006, 2007).

In conclusion, the present study shows that 5-HT modulates socially regulated startle-escape responsiveness and underlying M-cell excitability, likely via presynaptic effects on inhibitory interneurons. Furthermore, we have established a powerful paradigm that will greatly facilitate the study of these and other neurochemical mechanisms at the level of behavior, electrophysiology, and single-neuron molecular biology within the same individuals.

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SERTONERGIC MODULATION OF M-CELLS


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