Pauses during communication release behavioral habituation through recovery from synaptic depression

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SUMMARY

During interactive communication, animals occasionally cease producing communication signals. The behavioral significance of resumed communication signals following a cessation, or silent pause, has been described in human speech: word recognition by listeners is enhanced after silent pauses, and speakers tend to place such pauses prior to words that are contextually unpredictable and that therefore have high information content[1-5]. How central nervous systems process signals following pauses differently from signals during continuous communication has not been studied at a cellular level. Here we studied behavioral and neurophysiological impacts of pauses during electric communication in mormyrid fish. We found that isolated fish produced fewer and shorter pauses than fish housed in pairs, and that fish tended to produce burst displays immediately following pauses. In the electrosensory pathway, sensitivity to pauses first arose in the midbrain posterior exterolateral nucleus (ELp): evoked field potentials were enhanced as pause duration increased, with a time constant of ~1 s. Intracellular recording from single ELp neurons suggested that this increased sensitivity resulted from a pause-associated recovery from synaptic depression that was induced by the preceding stimulation. Behavioral responses were also facilitated by longer pauses, with a similar time constant of ~1 s. Further, during natural electric communication between pairs of fish, the insertion of artificial pauses resulted in increased signaling by the receiving fish immediately following the pause. Thus, our results suggest that pauses during communication release sensory circuits from synaptic depression, thereby maximizing the physiological and behavioral effects of subsequent communication signals.

RESULTS

The mormyrid *Brienomyrus brachyistius* produces electric organ discharges (EODs) with inter-pulse intervals (IPIs) that are typically around 10-500 ms[6]. Fish also occasionally cease discharging for longer durations (Figure 1A). Since mormyrids use EODs not only for communication but also for actively sensing their surroundings[7], we first asked whether long pauses are potentially related to communication between animals, by comparing distributions of IPIs under different social conditions. Figure 1B exemplifies sequences of IPIs recorded from an animal housed in isolation (isolated fish) and an animal housed with another individual (paired fish). There was a highly significant interaction effect between housing condition and the frequency distribution of IPIs. In particular, isolated and paired fish differed in the long tail end of their IPI distributions, with paired fish generating more IPIs >500 ms (Figure 1C).

Using this value as a pause threshold, we quantified pause frequency (number of

pauses/recording duration), pause duration, and pause duty cycle (pause frequency x mean pause duration) for each individual during both day and night recordings. Paired fish generated more pauses of longer duration compared to isolated fish, both during the day (1.2 ± 0.5 pauses/min and 1.5 ± 0.3 seconds/pause vs. 0.5 ± 0.2 pauses/min and 0.9 ± 0.2 seconds/pause) and night (2.0 ± 0.5 pauses/min and 2.0 ± 0.3 seconds/pause vs. 0.7 ± 0.4 pauses/min and 1.4 ± 0.3 seconds/pause). The resulting pause duty cycle was larger in paired fish, though this difference was only significant during the night, when mormyrids are most active (Figure 1D).

We further investigated the temporal dynamics of electric signaling by comparing the timing of pauses and three previously described burst displays called scallops, rasps, and accelerations[8]. A cross-correlation analysis revealed that fish generated all three displays with an increased probability immediately following pause offset (Figure 1E).

Mormyrids have an identified sensory pathway (Knollenorgan, or KO) that is dedicated to processing the electric communication signals of neighboring fish (Figure 2A)[9,10]. We hypothesized that sensitivity to pauses arises in the midbrain posterior exterolateral nucleus (ELp), the first stage in this pathway in which single-neuron tuning to IPI variation has been found[10-14]. To test this hypothesis, we first performed *in vivo* field potential recordings using electrosensory stimuli that mimic the EODs of a neighboring conspecific. We applied two electrosensory stimulus trains that were separated by a pause of varying duration (Figure 2A). Each train consisted of 10 bipolar square pulses with behaviorally relevant duration and intensity, and the pulses were separated by 30 ms IPIs.

Single-pulse electrosensory stimulation elicited field potentials in ELp with a peak latency of ~7 ms, as shown in previous studies[11,15-18] (Figures 2B and S1A). These evoked potentials were attenuated by 45% with a time constant of 42.4 ms during the first stimulus train (Figure S1B). The second stimulus train evoked an attenuated response when the pause duration was short (Figures 2B). However, the amplitude of onset evoked potentials recovered gradually as pause duration increased, with a time constant of ~1 s (Figure 2B). By contrast, in the anterior exterolateral nucleus (ELa), one step earlier in the pathway, evoked field potentials (peak latency: ~3 ms[11,15-18]) showed virtually no change in amplitude during stimulus trains and thus were insensitive to pauses (Figures 2C and S1). These results suggest that ELp is the first region in the KO pathway where pauses affect the sensory processing of electric communication signals.

To obtain insights into cellular mechanisms underlying the recovery of ELp evoked potentials during pauses, we performed whole-cell patch recording from ELp neurons in an *in vitro* whole-brain preparation[14,19,20]. In this preparation, local ELp circuitry remains

intact and IPI selectivity of ELp neurons can be reproduced in a behaviorally relevant way by direct stimulation of afferent inputs from ELa. As exemplified in Figure 2D, ELa stimulation evoked synaptic depolarizations, or excitatory postsynaptic potentials (EPSPs), in ELp neurons (14 cells, 4 fish). These stimulus-evoked EPSPs summated, but were also attenuated throughout the stimulus train in every neuron we tested. We measured EPSP amplitude as the change in membrane potential from the minimum to the maximum that immediately followed each stimulus pulse (Figure S1A). On average, EPSPs were attenuated by 57% (range: 33-87%) with a time constant of 61.9 ms during the first stimulus train (Figure S1B). Stimulus-evoked EPSPs recovered partially after a pause of 200 ms, but remained significantly attenuated (0.63 ± 0.06 relative to the first EPSP, $t_{13} = -6.39$, $p < 10^{-4}$, one-sample t-test against 1.0). Recovery from this attenuation showed a similar time course to the recovery of ELp evoked potentials (Figure 2D).

We next asked whether GABAergic inhibition could be contributing to the suppression of EPSPs after short pauses. Although short-term facilitation has never been observed for excitation or inhibition in ELp neurons[21-23], it is possible that potentiated inhibition following the first stimulus train could suppress responses to the onset of the second stimulus train. However, close inspection of synaptic responses revealed that the earliest depolarizing components of synaptic responses following stimulus pulses were greatly reduced following short pauses (Figure S2A). These short-latency responses are due to monosynaptic excitation from ELa[22,24], whereas all inhibitory inputs to ELp neurons are due to local, polysynaptic pathways[21,23], suggesting that these attenuated responses were due to depression of excitatory inputs from ELa rather than inhibition. Indeed, a single neuron with responses that were dominated by inhibition showed no evidence of potentiated inhibition following short pauses (Figure S2B).

Long-lasting inhibition following the last pulse in the first stimulus train could also suppress responses to the onset of the second stimulus train. However, we saw no evidence for elongated inhibitory responses at the end of the first stimulus train (Figure S2B). Further, there was no correlation between pause duration and the difference between ELp neuron membrane potentials immediately preceding the onset of the first and second stimulus trains (Figure S2C).

To definitively address whether inhibition contributes to the suppression of responses following short pauses, we performed *in vivo* field potential recordings from ELp before and after blocking GABAergic inhibition[25]. The amplitude and waveform of evoked potentials were affected by blocking inhibition (Figure S3A, B), but the attenuation of responses during the first stimulus train and the recovery of responses following pauses both showed very similar dynamics compared to control conditions (Figure S3C,

D). This strongly suggests that inhibition is not responsible for the suppression of responses following short pauses.

The above results demonstrate that pauses allow ELp circuitry to recover from synaptic depression and thereby maximize the responsiveness of ELp neurons to electrosensory stimuli occurring immediately after pauses. We next examined the behavioral consequences of this effect. Mormyrid species including *B. brachyistius* respond to a novel stimulus with a transient increase in EOD rate, called the 'novelty response'[17,26,27]. In response to a single electrosensory stimulus train of ten pulses, as used in the *in vivo* evoked potential experiments, animals exhibited a novelty response in which EOD rate returned to the resting level within a few seconds after the stimulus (Figure 3A, B). When we delivered a second stimulus train after a long pause following the first train, the animals exhibited a second novelty response similar to the first (Figure 3C). However, when pause duration was short, the second response transient summed with the first, but with a smaller amplitude (Figure 3C).

To quantify this behavior, we counted the number of EODs emitted within a two-second window after the onset of both stimulus trains (Figure 3B, C, gray shading), subtracted the single-train response from the double-train response, and then normalized to the single-train response. This analysis revealed that, when pause duration was 200 ms, the response to the 2nd train was significantly smaller than the single-train response (for 0.2 ms pulses: normalized response of 0.28 ± 0.13, t_5 = -5.62, p = 0.002; for 2 ms pulses: 0.30 ± 0.13, t_5 = -5.50, p = 0.003; one-sample t-tests against 1.0). As pause duration increased, the response to the second train increased and approached the single-train response with a time constant of 1.0 s (Figure 3D).

For the following reasons, we concluded that the weak responses to the second train following short pauses were not due to a saturation of EOD rate. First, *B. brachyistius* can generate EODs at a much higher rate than we observed: the shortest IPI previously observed is ~8 ms, corresponding to a frequency of ~125 Hz[8,28] (Fig. 1). Second, fish increased the amplitude of the novelty response as stimulus intensity increased up to 320 mV/cm, and the intensity we used (104 mV/cm) was in the middle of this dynamic range (Figure S4A, B). Third, the number of EODs emitted after the second train, but without subtracting the single-train response, was not larger than the single-train response at any pause duration tested (Figure S4C). Finally, a similar recovery of the second train response with increasing pause duration was observed when the fish were stimulated with shorter pulses (Figure 3D), which are effectively weaker stimuli that reduce the overall activation of electroreceptors[17]. These results suggest that the response specific to the second train was habituated when only a short pause was presented after the first

train. The close match of recovery time constants between the behavioral response and ELp synaptic response further suggests that synaptic depression and its recovery in ELp circuitry is a key mechanism underlying habituation and recovery of behavioral responses.

Finally, we tested the behavioral impact of pauses under more realistic conditions. As illustrated in Figure 4A, we mediated electric communication between two fish in real time, by connecting two sets of the behavioral setup used in Figure 3. In brief, we recorded the EOD times of the two animals simultaneously and each recorded EOD immediately triggered stimulation of the other fish (<0.5 ms delay). Stimulation of one fish was occasionally blanked for two seconds to artificially insert pauses during the ongoing electric communication.

Figure 4B shows exemplary time courses of EOD rate around artificial pauses, recorded from one fish in three different conditions: when the fish was receiving artificial pauses (receiver, top), when the fish's own EODs were blanked (sender, middle), and when the electric communication was not disrupted (bottom). As expected from the previous experiment, receiver fish exhibited a transient increase in EOD rate at pause offset. Interestingly, receiver fish also increased EOD rate upon pause onset. By contrast, sender fish showed no obvious change in EOD rate in response to artificial pauses, even though the sender fish received the pause-induced response of the receiver fish. These results suggest that receiver responses are induced by pause onset and offset, not by response feedback from the sender.

We repeated the same experiment on five fish in total (each fish was paired with four other fish). We quantified behavioral responses as the number of EODs emitted within two-second windows immediately before pause onset (baseline), after pause onset, and after pause offset. Receivers emitted significantly more EODs at pause onset and pause offset compared to baseline, whereas neither sender nor control fish exhibited significant changes in EOD production (Figure 4C). These results suggest that pauses during electric communication facilitate behavioral responses from receivers upon the resumption of signaling.

DISCUSSION

We found that mormyrids actively generate pauses, and that pauses facilitate behavioral responses of receivers to subsequent signals by releasing habituation that occurs during continuous communication. Electrophysiology and pharmacology demonstrated that pauses inserted within a train of afferent sensory inputs allow for recovery from short-term synaptic depression of network activity in the midbrain ELp. The time course of recovery from this depression closely matched that of behavioral habituation, suggesting that this

neurophysiological process is a predominant driver of enhanced behavioral responses to resumed communication signals after pauses.

We observed increased signaling at both the onset and offset of artificially inserted pauses. Signal production of receivers during pausing by senders has also been described in communicating birds[29,30] and frogs[31,32]. Increased signaling at pause onset is similar to the omitted stimulus response that has been described in vertebrate visual, auditory, and somatosensory systems, which has been interpreted as a response to novelty[33]. Increased signaling at pause offset is also likely a form of novelty response, which has been described in both mormyrid and gymnotiform electric fishes[26,34]. Both types of novelty responses might result from deviations of sensory input from an internal template of expected input based on recent experience. Mormyrids may be an excellent system for identifying such a template and determining the underlying mechanisms for novelty detection.

Previous studies have shown that ELp neurons exhibit IPI tuning across intervals ranging from 10 to 100 ms[11], which results from the integration of excitation and GABAergic inhibition that vary in the dynamics of their temporal summation and short-term synaptic depression[21,35]. Short-term depression is ubiquitous in the synaptic responses of ELp neurons recorded both *in vivo* and *in vitro*[21,22,24,35]. For high-pass neurons, inhibition depresses more rapidly than excitation, leading to increased responses at high rates of synaptic input[21]. However, this response is transient; regardless of their IPI tuning, the responses of ELp neurons steadily decrease in response to sustained stimulation over longer timescales due to short-term depression. This may be an adaptation to reduce resources devoted to sensory processing, as an ongoing stream of signals from a neighboring fish provides less information over time. Pauses, then, may be an adaptation of senders to release the sensory system of receivers from depression.

Pauses have been studied in acoustic communication including human speech[5,29-32]. Mormyrid and gymnotiform weakly electric fish are also known to pause during electric communication[6,36-40]. To our knowledge, however, only human studies have payed particular attention to the behavioral, or psycholinguistic, significance of the resumed communication signals after silent pauses. Our finding that burst displays tend to occur immediately after pauses in mormyrids is similar to the finding that human speakers tend to place pauses prior to words with high information content[1]. Interestingly, the relevant timescales for pauses in human speech are roughly similar to those in the electric communication of mormyrids, occurring in the range of hundreds of milliseconds to seconds[4,5]. Neurophysiological recording of brain activities, such as

electroencephalograms[3], have been applied in human studies, but information about cellular mechanisms underlying these responses is lacking. Thus, the present study is the first to propose a cellular model to account for the role of silent pauses in the sensory processing of upcoming signals: continuous speech could depress the activity of sensory circuits in listeners through short-term synaptic depression, and silent pauses would release the depression, thereby maximizing the impact of sensory inputs resulting from the resumed utterance.

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AUTHOR CONTRIBUTIONS

Conceptualization & Methodology, T.K. and B.A.C.; Investigation & Formal Analyses, T.K., A.J.L., J.H.Y., P.S.R-G., and B.A.C.; Writing – Original Draft, T.K.; Writing – Review & Editing, B.A.C.

DECLARATION OF INTERESTS

The authors declare no competing interests.

FIGURE LEGENDS

Figure 1. Paired fish paused more than isolated fish.
(A) Electrical recording from a freely moving mormyrid, *B. brachyistius*. Mormyrid electro-communication consists of a fixed electric organ discharge (EOD, displayed in head-positive polarity) produced with variable interpulse intervals (IPIs). The changes in EOD amplitude are due to movement of the fish relative to the recording electrode, not to changes in EOD amplitude emitted by the fish. Discharging occasionally ceases for longer than typical IPIs (pause). (B) Example sequences of IPIs recorded from individual fish housed in different social conditions. Paired fish tended to generate pauses (IPIs > 500 ms, red circles) more frequently than isolated fish. (C) IPI frequency distributions from 20 social fish and 12 isolated fish are shown as average (± SEM) normalized histograms with a bin size of 0.1 in common logarithm. There was a highly significant interaction effect between social housing condition and the frequency distribution of IPIs (two-way repeated-measures ANOVA: $F_{40, 1200} = 3.79$, $p < 0.001$). A pause threshold of IPIs > 500 ms was used to quantify pauses as this value captures the difference in the tail end of the distributions at long IPIs. (D) Pause duty cycle (pause frequency x mean pause duration) reflects the percentage of time spent pausing during a recording. Paired fish produced significantly higher pause duty cycles than isolated fish during the night (Mann-Whitney $U_{28} = 136$, $p < 0.03$), but not during the day (Mann-Whitney $U_{27} = 82$, $p > 0.75$). Sample sizes reflect the number of fish that generated pauses relative to the total number of fish recorded from. (E) Cross-correlation analysis of the timing of burst display onset relative to pause offset. Insets show an expanded view of the x-axis near the origin. Fish generated scallops, rasps, and accelerations with an increased probability immediately following pause offset.

Figure 2. Neurons in the midbrain ELp responded more strongly to stimuli following longer pauses due to recovery from synaptic depression.
(A) Top, Experimental design for recording evoked field potentials in response to electrosensory stimulation. Field potentials were recorded *in vivo* from the midbrain ELa or ELp in response to electrosensory stimulation applied transversally across the body (arrows). Stimuli consisted of two trains of 10 bipolar square pulses (0.2 ms duration, 104 mV/cm) delivered with a 30 ms IPI, separated by a non-stimulating period of varying duration (pause). Bottom, Anatomy of the Knollenorgan electrosensory pathway. Knollenorgan primary afferents project ipsilaterally to the hindbrain nELL via the posterior lateral line nerve (nPLL). Neurons in the nELL project bilaterally to the ELa

in the midbrain, which projects ipsilaterally to the adjacent ELp. (B, C) Stimulus-evoked field potentials in ELp (B) and ELa (C) recorded from a single fish. Examples are average responses to 20 presentations of a single stimulus pulse (left) or stimulus train with a pause duration of 200 ms (top middle) or 4000 ms (bottom middle). Timing of each stimulus pulse (stim.) is indicated underneath each trace. Plots to the right summarize the amplitude of evoked potentials in response to the eleventh stimulus pulse (i.e. the first stimulus after the pause). Data were normalized to the amplitude of the response to the first pulse, and were then plotted against pause duration. ELp field potentials attenuated rapidly during the first stimulus train and recovered following pauses with a time constant of 1.2 s (magenta line; single-exponential fit). ELa field potentials exhibited minimal attenuation during stimulus trains (magenta line indicates 1.0). Similar results were obtained with a stimulus intensity of 34 mV/cm (time constant of ELp recovery = 1.4 s). (D) Pauses released ELp neurons from synaptic depression. Left and Middle, Whole-cell recording of an ELp neuron in an *in vitro* whole-brain preparation. Postsynaptic potentials were evoked by direct electrical stimulation of ELa, with timings as noted in B and C (left: single-pulse stimulation, middle: pulse trains). Arrowheads indicate stimulus artifact. Right, The EPSPs recovered following pauses with a time constant of 1.3 s (magenta line; single-exponential fit). See Figures S1, S2 and S3 for further additional analyses.

Figure 3. Pauses released habituation of behavioral responses to electrosensory stimuli.

(A) Setup for the behavioral playback experiment. Uniform electrosensory stimuli were presented to the fish using stimulus electrodes spanning the length of both sides of the chamber (thick black lines). EOD timing was determined using a pair of recording electrodes located at each end of the chamber (gray circles). (B, C) Behavioral responses to a single stimulus train (B) or double trains (C) of bipolar square pulses (2 ms duration, 104 mV/cm peak-to-peak amplitude), with pulse timings as in Figure 2. Upper traces represent the time course of instantaneous EOD rates estimated by convolving EOD times with a 300 ms wide Gaussian filter, averaged over 40 repetitions. Lower traces indicate timing of the stimulus trains. Gray bars indicate the response windows in which the number of EODs were used to quantify responses. The windows started with each stimulus train and ended 2 s after the end of each train. (B) In response to a single stimulus train, fish exhibited a transient increase in EOD rate that returned to baseline after ~2 s. (C) The second stimulus train elicited behavioral responses as large as the first train after pauses of 4000 ms (bottom). With shorter pauses of 200 ms (top), the

response to the second train partially overlapped the first response. The additional increase in EODs, however, was smaller than the single-train response, suggesting habituation of behavioral responses to the second train. (D) Behavioral responses to the second stimulus train were normalized by the single-train response and plotted against pause duration. We tested 6 fish with bipolar square pulses of 2 ms (magenta) and 0.2 ms (blue) duration, which is relevant to the extremes of observed conspecific EOD durations[48]. Stimulus intensity was the same as in B and C. Behavioral responses following pauses recovered from habituation with similar time constants to the recovery of ELp synaptic responses from depression (1.0 s, single-exponential fits). See Figure S4 for further additional analyses.

Figure 4. Experimentally inserted pauses during interactive electric communication enhanced behavioral responses to subsequent communication signals.

(A) Experimental setup to mediate real-time electric communication. EOD timings of two fish in different tanks were recorded simultaneously. Each fish was stimulated using the EOD timings and waveform of the other fish (curved arrows). Stimulation from one fish (sender) was occasionally blanked for two seconds to artificially provide the other fish (receiver) with pauses. Fish enclosures are the same as in Figure 3A. (B) Time course of EOD rates around artificial pauses, obtained from one fish under three different conditions. Instantaneous EOD rate was calculated as in Figures 3B, C (100 repetitions). The fish was paired with the same fish in all three conditions. Top, When the fish was provided artificial pauses (receiver), it increased its EOD rate at both pause onset and offset. The gray bar indicates the timing of the artificial pause. Middle, no obvious change in EOD rate was observed when artificial pauses were given to the other fish (sender). Bottom, control EOD rate that was obtained when no artificial pauses were applied to either of the fish (no pauses). (C) Number of EODs emitted within two-second windows after pause onset and pause offset (5 fish). Values were normalized by the EOD number emitted within two seconds prior to pause onset (baseline). There was a significant interaction effect between experimental conditions (receiver, sender, or no pauses) and the time windows ($F_{4,16} = 4.84$, $p = 0.009$, two-way repeated-measures ANOVA). A post-hoc multiple comparison analysis (Holm-Sidak method) further revealed that the receiver emitted significantly more EODs at pause onset and pause offset compared to baseline ($t_4 = 4.40$ and 5.02, respectively, $p < 0.001$, triple asterisks), whereas neither the sender nor the control fish (no pauses) exhibited significant changes ($t < 0.5$, $p > 0.94$).

STAR METHODS

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Bruce A. Carlson (carlson.bruce@wustl.edu).

Materials Availability

This study did not generate new unique reagents, strains, or lines.

Data and Code Availability

The datasets supporting the current study have not been deposited in a public repository but are available from the corresponding author on request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

We used adult individuals of both sexes of the weakly electric mormyrid fish *Brienomyrus brachyistius*, ranging from 6.2 to 22.8 cm in fork length. The fish were obtained through commercial vendors and housed in groups with a 12h:12h light/dark cycle, temperature of 25-28 °C, pH of 6-7, and water conductivity of 200-400 μS/cm. Fish were fed live black worms four times per week. All procedures were in accordance with guidelines established by the National Institutes of Health and were approved by the Animal Care and Use Committee at Washington University in St. Louis.

METHOD DETAILS

EOD recordings and interpulse interval analysis

Recordings of EOD times, which were originally collected in a previous study from 16 mature males and 16 mature females[41], were analyzed to investigate differences in discharge patterns between fish under different social conditions. In brief, the fish were divided into two groups: (1) 'isolated', in which the fish were housed in isolation (6 males and 6 females) and (2) 'paired', in which a single male and a single female were housed together in an aquarium (10 males and 10 females). Fish were acclimated to their housing conditions for several days before recording. Paired fish were briefly separated using a plastic barrier during the recording. Every fish was recorded once during the daytime and once during the nighttime. The duration of each recording session was 10-25 min.

For each recording, we generated an interpulse interval distribution. We then averaged the daytime and nighttime histograms from each fish, yielding one histogram

per individual. We then normalized each individual's histogram to an integral of 1. Finally, we averaged the normalized histograms across individuals to obtain an overall average interval distribution (Figure 1C). We counted all intervals >500ms as a pause, and, for each recording, we determined pause frequency (number of pauses/recording duration), the duration of each pause, and pause duty cycle (100 x pause frequency x mean pause duration = percentage of recording spent pausing).

To analyze the temporal relationship between pauses and three previously defined burst displays[8], we performed a cross-correlation analysis between the timing of pause offset and the timing of display onset in each recording. The resulting cross-correlograms were averaged across all recordings to obtain an overall average cross-correlogram.

Evoked potential recording

Sensory-evoked field potentials were recorded in five fish as described previously[11,17]. In brief, while being anesthetized by respiration with 100 mg/l MS-222, fish were submerged underwater, except for the dorsal surface of the head, in a recording chamber, and ELa and ELp were exposed. Once the surgery was complete, we switched respiration to aerated freshwater to bring the fish out of anesthesia. A pair of electrodes was placed next to the caudal peduncle to monitor EOD command times. The EOD command triggers inhibition of the electrocommunication pathway in the hindbrain[42]. Therefore, any repetition in which the fish emitted an EOD command 2-4 ms before any pulse in the stimulus train was ignored.

Recording electrodes (o.d. = 1.00 mm, i.d. = 0.50 mm; A-M Systems model 626000) were pulled with a Sutter P-97, broken to a tip diameter of ~15 µm and filled with 3 M NaCl. The electrodes were inserted into either ELa or ELp. Evoked field potentials were amplified 1000x, band-pass-filtered from 10 Hz to 5 kHz with a differential AC amplifier (A-M Systems model 1700), and digitized at 97.6 kHz (Tucker-Davis model RX8). Evoked potentials were identified to be from ELa or ELp, based on their characteristic shape and timing[11,15,16].

We delivered transverse electrosensory stimulus pulses (bipolar square pulses with 0.2 ms duration) using electrodes located on the sides of the tank. The pulses were generated at 97.6 kHz (Tucker-Davis model RX8), attenuated (Tucker-Davis model PA5), and isolated from ground (A-M Systems model 2200). The stimuli were either single pulses or two separated pulse trains, each of which consisted of 10 pulses with constant intervals of 30 ms. We chose 30 ms intervals because these reliably elicit synaptic depression in ELp neurons[21,22] and are towards the high-frequency end of interval distributions in *B. brachyistius*, but not at the extreme (the shortest intervals observed are

~10 ms)[8,28,38]. The two pulse trains were separated by a pause of 200-4000 ms. Each stimulus set was repeated 20 times for averaging, with an inter-stimulus interval between repetitions of 4 s. Stimuli were delivered at intensities of 34 and 104 mV/cm as measured from the center of the recording chamber in the absence of a fish. These values approximate stimulus intensities resulting from the EODs of a neighboring fish at different distances, and are within the dynamic range of the knollenorgan sensory pathway[43-46]. Evoked field potential amplitudes were measured as the negative peak of the evoked potential within 15ms following each stimulus, relative to the pre-stimulus baseline. Stimulus generation, data recording, and averaging were performed in MATLAB (Mathworks, Natick, MA, USA).

In 4 fish, we assessed the role of inhibition in suppressing evoked potential responses following short pauses using SR-95531 (gabazine), a high-affinity, competitive inhibitor of $GABA_A$ receptors. After recording baseline responses to all stimuli, we added 15 μl of 5 mM gabazine in Hickman's Ringer to the brain cavity surrounding ELa/ELp[25]. Then, we again obtained responses to all stimuli. In response to gabazine application, the rate of EOD command production increased dramatically, likely due to effects on ELa/ELp as well as the adjacent cerebellum and optic tectum. This made it impractical to ignore repetitions in which the fish emitted an EOD command 2-4 ms before any pulse in the stimulus train. We therefore increased the number of repetitions to 40 to minimize the effect of occasionally blocked responses on average responses. There was no apparent tendency for EOD commands to occur at a specific time during stimulus trains, and thus there was no systematic suppression of responses to particular pulses during trains.

Whole-cell recording from ELp neurons

We used an *in vitro* whole-brain preparation that was developed in previous studies[19,20]. In brief, we anesthetized fish in 300 mg/L MS-222, and then performed a craniotomy in ice-cold, oxygenated artificial cerebrospinal fluid (ACSF; composition in mM: 124 NaCl, 2.0 KCl, 1.25 KH_2PO_4, 24 $NaHCO_3$, 2.6 CaCl2, 1.6 $MgSO_4.7H_2O$, and 20 glucose, pH 7.45; osmolarity: 310 mOsm) containing 1 mM kynurenic acid (KA) to reduce potential excitotoxicity. The valvula cerebellum and dorsal part of the hindbrain were removed by suction while in ACSF, leaving the remainder of the brain intact. After one hour of equilibration at room temperature (23-27 °C), the brain was transferred to a recording chamber (Warner Instruments RC-26GPL) and secured by two slice anchors (Warner Instruments SHD-26GH) placed on the bottom and the top of the brain. The chamber was then placed on a recording platform (Burleigh Gibraltar). On the platform, the brain was continuously perfused (flow rate: approximately 1 ml/min) with oxygenated ASCF at room

temperature for one additional hour before we started recording to wash out KA. We visualized ELp neurons using transmitted light microscopy in an upright microscope (Olympus BX51WI) in combination with a Newvicon tube camera (DAGE-MTI NC-70).

We performed whole-cell intracellular recordings using filamented, borosilicate patch pipettes (1.00 mm outer diameter; 0.58 mm inner diameter) with tip resistances of 4-8 MΩ. The electrode internal solution contained the following (in mM): 130 K gluconate, 5 EGTA, 10 HEPES, 3 KCl, 2 $MgCl_2$, 4 Na_2ATP, 5 Na_2 phosphocreatine, and 0.4 Na2GTP, pH 7.3–7.4 (osmolarity: 280–290 mOsm). Recordings were amplified using a MultiClamp 700B amplifier (Molecular Devices), digitized at a sampling rate of 50 kHz (Molecular Devices Digidata 1440A) and saved to disk (Molecular Devices Clampex v10.2).

To stimulate excitatory inputs to ELp, we placed an array of stimulus electrodes in ELa, just anterior to the ELp border[12,19,20]. The array consisted of four channels of bipolar stimulation (8 electrodes total; FHC models CB and MX). We delivered isolated, biphasic square current pulses (100 μs total duration; less than 200 μA amplitude) through four separate isolated pulse generators (A-M Systems model 2100). We stimulated ELa with single pulses as well as two separated pulse trains, each of which consisted of 10 pulses with constant intervals of 30 ms. The pulse trains were separated by a pause of 200-10,000 ms. Each stimulus set was repeated 5 times for averaging, with an inter-stimulus interval between repetitions of 4 sec (for 200-4000 ms pause duration) or 10 sec (for 10 sec pause duration). Amplitude of the postsynaptic potentials evoked by each pulse during stimulus trains was measured as the maximum membrane potential following each stimulus pulse minus the minimum membrane potential between the stimulus pulse and this maximum.

Modeling

We modeled short-term depression of synaptic responses during stimulus trains according to the following equation:

$$\frac{dP_{rel}}{dt} = \frac{P_0 - P_{rel}}{\tau}$$

$$P_{rel}(t) \rightarrow P_{rel}(t) f_D, \text{ if } t = t^k$$

where P_{rel} is the probability of neurotransmitter release, P_0 is the steady-state release probability, τ is the time constant of recovery in P_{rel}, f_d is a depression factor that ranges

from 0 to 1, and t^k is the last spike-time of the presynaptic input[47]. Thus, every time there is a presynaptic spike, P_{rel} is depressed by the fraction f_d and it recovers towards P_0 with time constant τ. We obtained best-fit parameters to f_d and τ from observed EPSP and evoked potential amplitudes.

We fit the recovery of evoked potentials, synaptic responses, and behavioral responses following pauses according to the following equation:

$$P_{rel} = P_0 - ae^{-t/\tau}$$

where P_{rel} is response amplitude, P_0 is the steady-state response, t is pause duration, a describes the degree of response suppression at short pauses, and τ is the time constant of response recovery.

Behavioral playback

The setup for behavioral playback experiments was described in detail elsewhere[17,27]. In brief, each fish was placed in a rectangular PVC enclosure (3.5 x 3.5 x 20 cm). Uniform electric stimuli were presented to the fish using Ag/AgCl stimulus electrodes spanning the length of both sides of the enclosure, with recording electrodes on each end of the enclosure (Figure 3A). Biphasic square pulses (2 or 0.2 ms in total duration) were delivered using the same equipment as in evoked potential recordings. Stimuli were delivered at an intensity of 104 mV/cm as measured from the center of the enclosure in the absence of a fish. Recorded signals were amplified 100x and band-pass-filtered (A-M Systems model 1700). Recordings were digitized at 97.6 kHz (Tucker-Davis model RX8). MATLAB was used to generate stimulus waveforms and time-stamp the fish's EOD times.

Behavioral responses to each stimulus train were measured as the number of EODs occurring within a time window starting at train onset and ending 2 s after the end of the train. The EODs generated within the overlapping window between the first train and second train were counted only once. We counted the total number of EODs in both windows, then subtracted the response to a single stimulus train. To normalize, the response was further divided by the response to a single stimulus train. Thus, the resulting measure represents how the total response deviated from that expected due to a linear summation of responses to the two trains, in which a value of 1 represents the expected response. We collected responses to 20-40 repetitions of the stimulus for averaging

(inter-stimulus intervals between repetition: 20 sec). To minimize habituation, fish were allowed at least 1 min of rest between stimulus sets.

Interactive communication between two fish

Electric communication between two fish was mediated by the behavioral playback system as if the animals stimulated each other directly with their own EODs, but isolated the electric sense and allowed us to interrupt the communication. Animals, housed separately in different aquarium tanks, were placed in the same PVC enclosures used in the behavioral playback experiments (Figure 4A). EOD times were recorded simultaneously from the two fish and were immediately used to stimulate the other fish with individual EOD waveforms recorded from the same pair of fish. Stimulus intensity was fixed at 320 mV/cm peak-to-peak. Temporal delay between recording and stimulation was minimized by the Tucker-Davis RX8 processor (300-500 µs).

To test behavioral effects of pauses during communication, we artificially inserted pauses into stimulus trains by blanking transmission of EOD times of one fish for 2 seconds once every 10 seconds. Behavioral responses were quantified as the average number of EODs (100 repetitions) within two-second windows immediately before blanking (baseline), after pause onset, and after pause offset.

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analyses were performed using SigmaPlot 12 (Systat Software, San Jose, CA, USA) or SPSS v. 27 (IBM, Armonk, NY, USA). Logarithmic-transformation was applied when a data set failed the Shapiro-Wilk test for normality ($p < 0.01$). Values are reported as the mean ± SEM.

KEY RESOURCES TABLE

This study did not include genetically modified organisms or strains, cell lines, special reagents, unique software, or experimental models.

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