

Long-Term Recognition Memory of Individual Conspecifics Is Associated With Telencephalic Expression of Egr-1 in the Electric Fish *Apteronotus leptorhynchus*

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ABSTRACT

Primates and songbirds can learn to recognize individual conspecifics based on complex sensory cues; this requires a large, highly differentiated dorsal telencephalon. Here we show that the electric fish *Apteronotus leptorhynchus* can learn to recognize individual conspecifics based on a simple cue, the beat frequency of their summed sinusoidal electric organ discharges (EOD). Male fish produce transient communication signals (chirps) in response to mimic EODs. The chirp response habituates over repeated stimulus presentations within one experimental session, continues to habituate over successive daily sessions and is nearly extinguished after 5–7 days. Habituation of the chirp response was specific to the presented beat frequency. The conversion of short- to long-term habituation could be disrupted by cooling the head 30 minutes after the daily habituation trials. Consolidation of long-term mem-

ory in mammals is thought to involve induced expression of an immediate early gene, Egr-1. We cloned the Apteronotid homolog of the Egr-1 gene and found that chirp-evoking stimuli induced strong expression of its mRNA within the dorsal (Dd), central (DC), and lateral (DL) subdivisions of the dorsal telencephalon. Interestingly, the dorsolateral region is hypothesized to be homologous to the amniote hippocampal formation. We conclude that *A. leptorhynchus* can learn to identify individual conspecifics based on their EOD frequency and can remember these frequencies for several days. We hypothesize that this form of learning, as in primates and songbirds, requires a subset of dorsal telencephalic areas and involves a consolidation-like process that includes the expression of the transcription factor AptEgr-1. *J. Comp. Neurol.* 518:2666–2692, 2010.

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INDEXING TERMS: Egr-1; telencephalon; *Apteronotus leptorhynchus*; memory

Intraspecies communication signals are essential for mating and territorial behaviors. A number of senses, including vision (Pascalis and Bachevalier, 1998), audition (Stoddard et al., 1991), and olfaction (Johnston and Bullock, 2001), can be used for communication. In amphibians, songbirds, primates, and other mammals, such signals can serve to identify individuals (Bee and Gerhardt, 2002; Gentner, 2004; Gothard et al., 2009; Johnston and Bullock, 2001; Pascalis and Bachevalier, 1998; Petkov et al., 2008; Stoddard et al., 1991). Individual recognition involves learning idiosyncratic signal features or configurations of such features (Bee and Gerhardt, 2002; Gentner, 2004; Petkov et al., 2008). The physiological basis of this

process is not well understood, but, in amniotes, it appears to be implemented by the elaborate neural networks of the avian and mammalian dorsal telencephalon (Gentner, 2004; Ghazanfar and Hauser, 2001; Petkov et al., 2008).

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The morphotype of the gnathostome telencephalon consists of pallial and subpallial portions with several conserved subregions in both divisions (Northcutt, 1995). In amniotes, the rostral neural tube evaginates so that its fundamental topology is maintained, although the pallial division is greatly expanded and comprises distinct hippocampal, isocortical, and olfactory cortical territories; in contrast, the telencephalon of bony fish is formed by eversion (Nieuwenhuys and Meek, 1990). Nevertheless, many studies have confirmed the presence, in fishes, of both a subpallium (Sas et al., 1993; Wullimann and Mueller, 2004) and a pallium homologous to at least part of the medial pallium of amniotes (Nieuwenhuys, 2009; Northcutt, 2008; Yamamoto et al., 2007). It is, however, not known whether the small pallium of fish can support the sophisticated computations associated with learning and remembering the signals that identify individual conspecifics.

Apteronotus leptorhynchus is a gymnotiform electric fish with a sinusoidal electric organ discharge (EOD); EOD frequencies range from ~600 to 1,000 Hz, but individual fish maintain near-constant EOD frequencies (Moortgat et al., 1998). Mature males generally having higher frequencies (>800 Hz) than females (<800 Hz; Meyer et al., 1987). The EOD is detected by cutaneous electroreceptors (P units); amplitude modulations (AMs) of the EOD are caused by the presence of environmental features (e.g., rocks, prey; Bastian, 1981; Gussin et al., 2007). AMs are also caused by the summation of the fish's own EOD with that of a neighboring fish [beats with AM frequency equal to the difference frequency (DF) of their EODs; Maller, 2007]. In either case, the electroreceptors encode these AMs by modulating their firing rates and synchrony (Bastian, 1981; Benda et al., 2005, 2006; Gussin et al., 2007; Nelson et al., 1997). This information is

sequentially transmitted to central targets in the hindbrain [electrosensory lateral line lobe (ELL); Krahe et al., 2008; Marsat et al., 2009], midbrain (torus semicircularis; Fortune, 2006; Rose and Fortune, 1999), and diencephalon (nucleus electrosensorius; Heiligenberg et al., 1991; Keller, 1988; Keller and Heiligenberg, 1989; Keller et al., 1990). The large sexual dimorphism of EOD frequency can be used for sex discrimination (Bastian et al., 2001; Engler and Zupanc, 2001; Triefenbach and Zakon, 2003); for example, female fish tend to choose male mates according to their EOD frequency (Bargelletti, Gogarten, and Krahe, personal communication). It is not known, however, whether these fish can recognize individual conspecifics based on beat frequency.

A. leptorhynchus male fish, in response to EOD frequencies similar to their own (small DFs and thus low frequency beats), emit small chirps, transient (~20 msec, ~100 Hz) increases of their EOD frequency (Bastian et al., 2001; Engler and Zupanc, 2001; Triefenbach and Zakon, 2003; Zupanc and Maller, 1993); chirps are traded during agonistic encounters (Hupe and Lewis, 2008; Zupanc et al., 2006). On the motor side, the diencephalic prepacemaker nucleus (PPn) controls the initiation of chirps (Kawasaki et al., 1988; Zupanc and Maller, 1997). On the sensory side, small chirps are encoded by electroreceptors (Benda et al., 2005, 2006) and are detected by neurons in the ELL (Marsat et al., 2009) and up to the diencephalon (Heiligenberg et al., 1991).

The immediate early gene (IEG) Egr-1 (early growth response-1; also called *Zif268*, *krox24*, *NGFI-A*, and *ZENK*) encodes for a transcription factor and has been widely used as a marker of neuronal plasticity in the context of learning (Davis et al., 2003; Knapska and Kaczmarek, 2004). Egr-1 mRNAs and proteins are transiently expressed in several brain regions subsequent to many types of

Abbreviations

AC	anterior commissure	IL	inferior lobe
AptEgr-1	Apteronotid homolog of the early growth response gene 1	I	lateral
CE	central nucleus of the inferior lobe	m	medial
CL1/2	carbon recording electrodes, left side	murEgr-1	murine early growth response gene 1
CR1/2	carbon recording electrodes, right side	NLS	nuclear localization site
d	dorsal	Pit	pituitary
DBD	DNA binding domain	r	Pearson correlation coefficient
DC	dorsocentral telencephalon	R	repressor domain
DDi	intermediate component of the dorsal telencephalon, dorsal subdivision	R1/2	recording dipole, electrodes 1 and 2
DDmg	magnocellular component of the dorsal telencephalon, dorsal subdivision	RL	recessus lateralis
DFL	nucleus diffusus lateralis of the inferior lobe	SAD	strong activation domain
DFm	nucleus diffusus medialis of the inferior lobe	S1/2	stimulating dipole, electrodes 1 and 2
DLD	dorsal-lateral telencephalon, dorsal subdivision	SD1/2	stimulating dipoles 1 and 2
DM	dorsomedial telencephalon	Stim	stimulus
Egr-1	early growth response gene 1	TA	nucleus tuberis anterior
G	glomerular nucleus	TC	trunk cool
Hab	habituated	TeO	optic tectum
HC	head cool	TS	torus semicircularis
HI	lateral hypothalamus	v	ventral
Hv	ventral hypothalamus	Vv	ventral telencephalon, ventral subdivision
		WAD	weak activation domain

perturbations, including those associated with learning (Beckmann and Wilce, 1997; Davis et al., 2003). Knockout mice lacking Egr-1 have intact short-term memory but show an inability to establish hippocampal late long-term potentiation (LTP) and to form long-term memories for spatial navigation, conditioned taste aversion, and olfactory discriminations (Jones et al., 2001; Renaudineau et al., 2009). Egr-1 has now also been shown to be important for consolidation and/or reconsolidation of a variety of memory tasks associated with neural activity in the hippocampus, isocortex and amygdala (Bozon et al., 2002, 2003a; Davis et al., 2003; Frankland et al., 2004; Jones et al., 2001; Lee et al., 2004; Malkani et al., 2004; Poirier et al., 2008). In addition, enhanced strength of the memory trace was recently reported in transgenic mice with forebrain overexpression of Egr-1 (Baumgartel et al., 2008).

Here we used habituation of the chirp response to demonstrate that fish can learn to recognize specific beat frequencies (DFs) and that this memory persists for at least 3 days. Furthermore, we demonstrated a temperature-dependent conversion of immediate (~ 90 minutes) to long-lasting (~ 24 hours) habituation and demonstrated that this conversion occurs between 0.5 and 6 hours after habituation. Finally, we have cloned the *A. leptorhynchus* homolog of the IEG transcription factor Egr-1 (AptEgr-1); we then used *in situ* hybridization to reveal that the initial presentation of chirp-evoking stimuli induces AptEgr-1 expression primarily in neurons of the dorsal telencephalon (pallium). We propose that *A. leptorhynchus*, like some birds and mammals, can learn to recognize individual conspecifics by remembering their EOD frequencies and that these memories might, in part, be stored in pallial neural networks. We further hypothesize that AptEgr-1 is part of a consolidation process that converts (Renaudineau et al., 2009) short- to long-term memory of the conspecific's EOD frequency.

MATERIALS AND METHODS

General setup for chirp habituation studies

Studies of habituation of the chirp response were performed in a “chirp chamber” within a test aquarium (Zupanc and Maler, 1993) under quiet conditions (dim or normal lighting was used, and no differences were seen) and using mature male fish (12–20 cm). The fish had arrived from a supplier at least 1 week before testing and were then housed in large common tanks within our animal care facility (kept at $\sim 27^\circ\text{C}$). We selected test fish by first examining them to ascertain that 1) they appeared healthy, 2) they were male (EOD rate >800 Hz; this was confirmed by dissection after the experiment had ended), and 3) gave an adequate chirp response to appropriate stimulation. We note that many critical variables related

to the test fish’s condition had no control. On the behavioral side, these include 1) the EOD frequencies of the other fish in the common tanks and specifically whether these were near the fish’s own EOD frequency and 2) whether the fish had agonistic encounters (with evoked chirping) in the common tanks that it lost or won (Hupe and Lewis, 2008). We were also not able to test for the hormonal status of the fish, in particular for testosterone (Dulka et al., 1995) and cortisol (Dunlap et al., 2002), two important modulators of the chirp response.

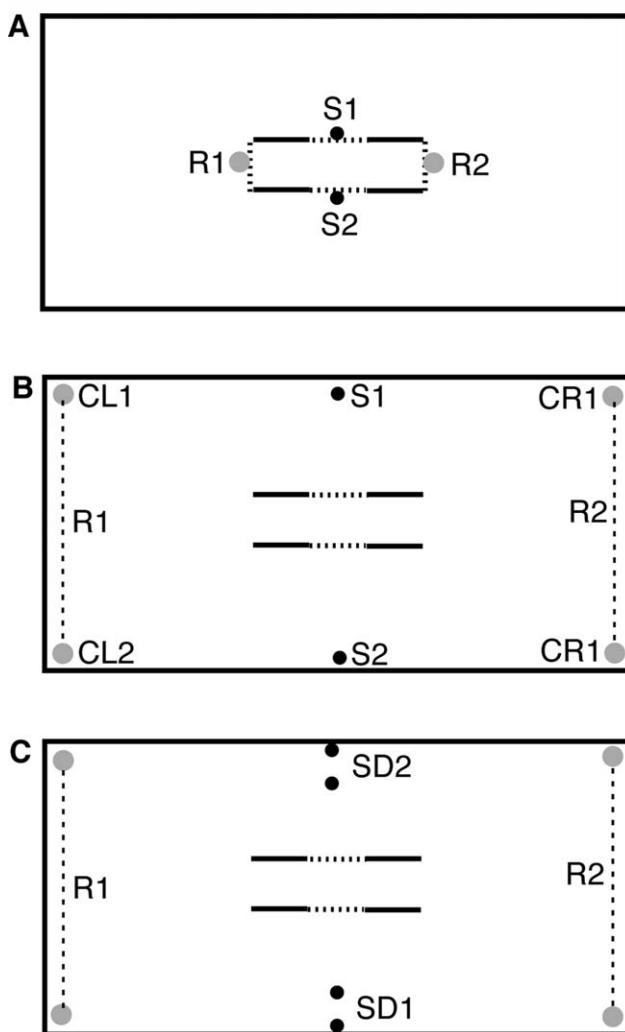
The fish were brought up to the test aquarium and allowed to adapt to the new environment for at least 2 days before experiments commenced (typically Thursday to Monday); it is important to note that this was done for all the behavioral experiments, because a fish’s chirp response is often suppressed immediately after it has been transferred from the common tank (unpublished observations). The aquaria were kept at $\sim 27^\circ\text{C}$ and also contained a corner filter and bubbler for aeration as well as a heater (these are not shown in Fig. 1 for the sake of clarity). The chirp chamber was located in a 57-liter test aquarium (61 cm long \times 31 cm wide \times 32 cm high) and consisted of a nylon home tube placed at the bottom of the aquarium’s center; the tube had removable electrodes (carbon rods) placed outside of mesh-covered cutouts on each of its sides to provide stimulation transversely across the fish’s body (Fig. 1A). During the experiment, the ends of the tubes were also covered with mesh so that the fish could not leave the chirp chamber; this meant that we were able always to provide a precise stimulus amplitude (calibrated to the middle of the chirp chamber). Recording electrodes (chloridized silver wires) for the fish’s EOD were placed at the mesh-sealed ends of the chamber. Stimuli (sine waves) were generated using a Wavetek Function Generator (model 396) and isolation unit (WPI, A395). Recordings were made with an Intronic preamplifier (model 2015), and the EOD frequency estimated with a custom frequency counter and observed on an oscilloscope and audio amplifier. Experimental protocols were based on preliminary experiments to maximize the evoked chirp number for effective statistical comparisons while minimizing response saturation and stress on the fish. Stimulus intensity was chosen, for each fish, to give a strong but nonsaturating chirp response (0.5 or 1 mV/cm; Engler and Zupanc, 2001).

Protocols for behavioral experiments

Standard chirp habituation protocol

We first present a detailed description of the protocol used to evaluate short and long-term chirp habituation. A1: The fish swam freely in the test aquarium during the adaptation period (minimum of 2 days). *Apteronotus* is nocturnal and stayed in the chirp chamber (the only

available “hiding place” within the test aquarium) during the dim lighting conditions of experimental trials. Before each daily experimental trial commenced, the fish were confined to the chirp chamber by blocking the exits with mesh in order to maintain a constant stimulus amplitude for all fish. At this point, the stimulating and recording electrodes were also put in place (Fig. 1A). A2: On each day an experimental trial consisted of 36 stimulus presentations of 60 seconds (chosen to generate a sufficient number of chirps for statistical analysis) with a 120-second rest period between stimuli (preliminary trials with different rest periods suggested that this long rest minimized stress). The stimulus was a sine wave whose frequency was always +10 Hz above the fish’s own EOD frequency. This creates an amplitude modulation or beat whose frequency is equal to the difference between the stimulus and EOD frequency; in the terminology of this field, the effective stimulus would be described as a beat with DF = +10 Hz. The stimulus was standardized in this way to allow for statistical comparisons across animals.



The evoked chirps were identified on the audio monitor and manually counted for each stimulus presentation. In many cases, the fish’s chirp response had completely habituated with <30 stimulus presentations. We nevertheless continued stimulation in order to keep the presentation number constant across fish; we do not know whether stimulus presentations without a chirp response induce long-term habituation but did not want to potentially confound our results by varying the number of presentations. The chirps were summed over all presentations to produce a total chirp count for that day’s experimental trial.

A3: After the trial was over, the mesh blocker and stimulating and recording electrodes were removed so that the fish were able to again move freely within the aquarium. A4: This protocol was then repeated on successive days (24-hour rest period) until the fish had entirely ceased to chirp or gave fewer than five chirps over the 36 stimulus presentations. This generally took <8 days.

Tests for consolidation protocol

The standard protocol was used (A1–A4) except that, 30 minutes after the end of step A2, we gently removed the

Figure 1. Schematic diagram of the aquarium and chirp chamber used for behavioral experiments and the induction of AptEgr-1 mRNA. A: Setup used for all behavioral experiments (protocols A, B). A nylon tube in the center of the aquarium serves as the fish’s resting place. Cutouts at the sides (dashed lines) allow current flow across the fish; the stimulating dipole carbon electrodes (S1, S2) are indicated by the black circles immediately adjacent to the mesh. During an experiment, the ends of the home tube are closed with mesh (dashed lines), and recording dipole (gray circles: silver electrodes R1, R2) placed on the mesh are used to record the fish’s EOD. B: Setup used for most AptEgr-1 induction experiments (protocols C, D, E). The ends of the home tube are now left open (no mesh). The stimulating dipole carbon electrodes (S1, S2) are now placed at the edges of the aquarium opposite the mesh covering the tube cut outs. The recording electrodes (gray circles) are now carbon rods. One pair is placed on the left corners of the aquarium (CL1, CL2) and joined by a wire (dashed line) to form one (R1) of a pair of recording electrodes. The other recording element pair is constructed in the same way (R2). This arrangement allows recording of the fish’s EOD in any part of the aquarium but is far enough away from the fish to prevent acting as a stimulus that induces AptEgr-1 expression. C: Setup used for the experiments that probed the induction of AptEgr-1 expression by spatially novel stimuli (protocol F). The recording electrodes (R1, R2) are as in B. The stimulus electrodes now come in two pairs (SD1, SD2). The initial chirp habituation protocol is run using either of the pairs. After habituation is complete, the second pair (not used for habituation) is now used (same stimulus frequency and intensity) to determine whether the fish now chirps to the spatially novel stimulus and whether AptEgr-1 is induced under these circumstances.

fish from the water and cooled its head until respiration ceased (~ 3 minutes). The fish were subsequently artificially respirated and recovered completely within < 5 minutes. Two types of control experiments were used for this protocol. To control for the stress of handling, fish were removed (again after the standard protocol) and had their trunks cooled 30 minutes after the last habituation trial of each day (end of step A2; no artificial respiration was necessary). In the second control, the fish's heads were again cooled each day but at 6 hours posthabituation (after completion of step A2). This served to control for the possibility that cooling the head caused some nonspecific degradation of neural function unrelated to habituation per se.

Tests for stimulus specificity of chirp habituation protocol

B1: The fish was confined to the chirp chamber as in the standard protocol. B2: The fish were then stimulated with sine waves that resulted in beats with DF = ± 5 Hz, ± 10 Hz, ± 20 Hz, ± 40 Hz, ± 60 Hz, ± 80 Hz, and ± 120 Hz. These stimuli were presented for 60 seconds, only once, and in randomized order (chosen with the IgorPro random digit generator). The evoked chirps were counted for each stimulus presentation. We would have preferred to use two presentations per DF and average the chirp counts (per DF), but preliminary experiments showed that this resulted in strong, long-lasting (> 5 days) habituation across all stimulus frequencies (unpublished observations; N = 4). B3: After a 24-hour rest period, we again ran the complete steps A1–A4 of the standard protocol. In this case, stimulation was not confined to DF = +10 Hz; rather, we habituated to one DF = ± 5 Hz, ± 10 Hz, ± 20 Hz, ± 40 Hz (two fish each per DF), and ± 60 Hz (one fish each per DF). Note that we tested over a broader range of DFs than that used for habituation. This was done to allow better investigation of the DF specificity of chirp habituation. B4: Three days after the end of the standard habituation protocol, we again stimulated the fish as in B2.

Chirp habituation protocol for AptEgr-1 expression

In preliminary experiments, we found that simply trapping the fish within the chirp chamber or allowing it to come in contact with the recording (silver wire) and stimulating (carbon rods) electrodes was sufficient to induce robust expression of AptEgr-1 mRNA within one dorsal telencephalic region (dorsal division, magnocellular component, DDmg; unpublished observations). We therefore modified the experimental design as follows (Fig. 1B). The mesh over the cut out sides of the chirp chamber were retained, but no mesh was placed over its ends; the fish was therefore not confined to the chirp chamber during

the stimulation protocol. The stimulating carbon rod electrodes were placed at the edge of the aquarium in line with the cut out sides of the chamber. We recalibrated the stimulus intensity so that we delivered the same stimulus amplitude as described above within the chirp chamber. The recording electrodes were now four carbon rods that were placed at the top corners of the aquarium and protruded about 7 cm into the water. They were linked as indicated in Figure 1B to permit recording of the fish's EOD from any location within the aquarium. The fish did not approach these electrodes at any time. It is important to note that, although we were able to minimize uncontrolled stimulation (confinement, proximity of electrodes) on the fish, this protocol also meant that we were not able to control the actual stimulus amplitude. On any given experimental trial, the fish might stay in the chamber (same stimulus as for the behavioral protocols), exit and attempt to bite the stimulating electrodes (far stronger stimulation) or exit and go to the edge of the aquarium at a variable orientation with respect to the stimulating dipole (weaker stimulation). We kept a record of this behavior and attempted to find a correlation with the expression levels of AptEgr-1 mRNA; we did not see any obvious correlation, but our sample size was insufficient for statistical analysis. This chirp chamber design was used for protocols C, D, and E described below.

In some experiments, we attempted to determine whether changing the location of the stimulus could induce AptEgr-1 (protocol F below). As shown in Figure 1C, we modified only the design of the stimulating electrodes from that described above. We placed two stimulus dipoles (3 cm between carbon rods) at each end of the aquarium, in line with the mesh cutouts and perpendicular to the chirp chamber. The stimulus intensity was calibrated (for both pairs) so that it was equal to the value for the standard behavioral protocol. We used one of the pairs (randomly chosen) for the initial habituation protocol and the other pair for the subsequent stimulation as described below.

Preliminary experiments revealed that even apparently minor acoustic stimulation (mechanical apparatus, speech outside the laboratory) was enough to induce AptEgr-1 mRNA expression in pallium, especially DDmg (unpublished observations). We therefore were careful to prevent such acoustic stimulation. Unfortunately, we were not able to prevent occasional low-amplitude and frequency acoustic stimulation emanating from distant machinery associated with building maintenance. These stimuli represent another potential basis for the variability of our *in situ* hybridization results.

Finally, we were not able to perform all the AptEgr-1 *in situ* hybridization experiments within a short time frame. Our experimental schedule was completely determined by the availability of healthy male fish that chirped. These

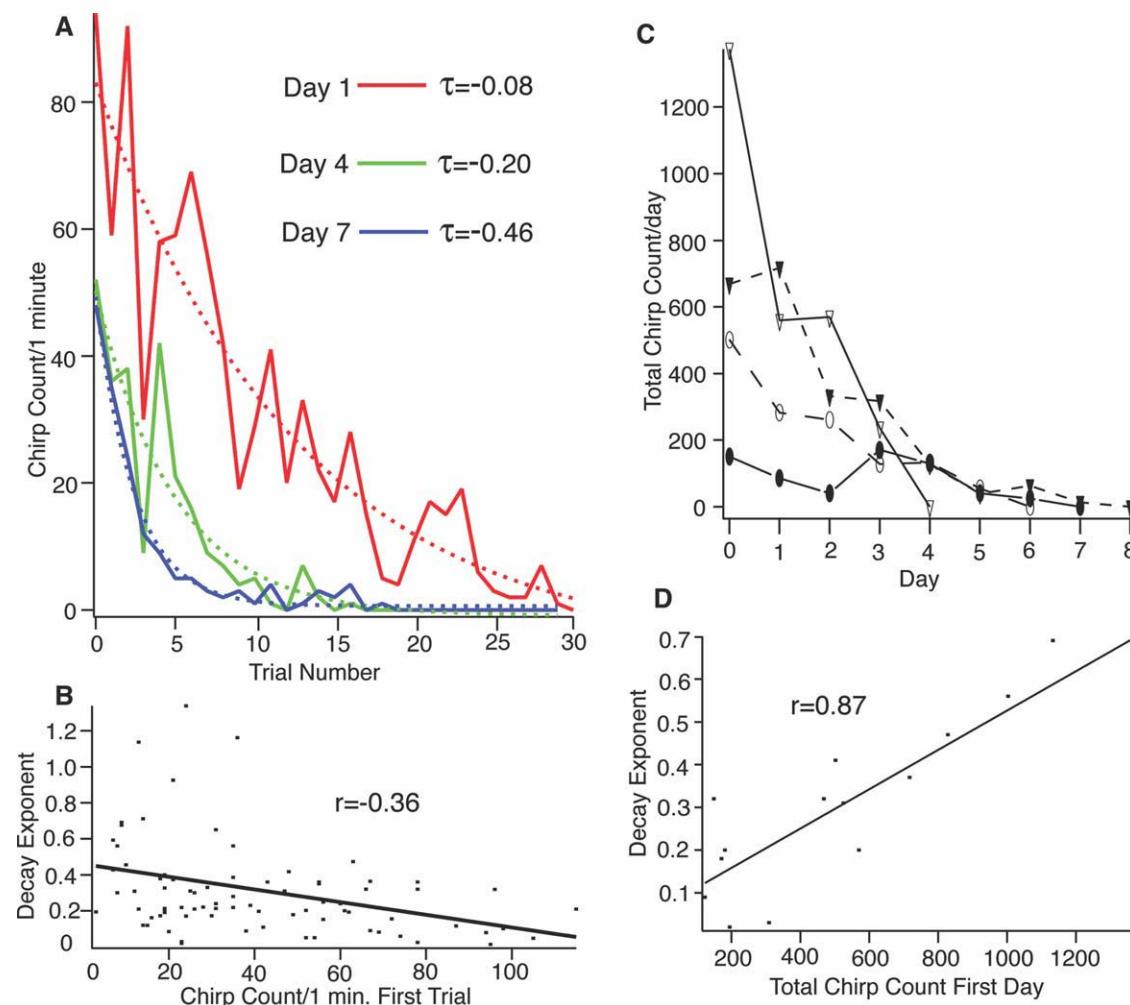


Figure 2. Short- and long-term habituation kinetics. **A:** Typical short-term habituation (~90 minutes) of chirping in one fish on the first, fourth, and seventh days; dashed lines are exponential fits. Note that higher initial chirp rates are associated with slower decay. **B:** The rate of short-term chirp rate decline is negatively correlated with the initial chirp rate (chirp number in first trial) over all days and DFs presented. **C:** Long-term habituation of chirping (four fish that span the range of initial chirp rates); note that the fish that chirps most on the first day also habituates most rapidly over successive days. **D:** The rate of long-term chirp rate decline is positively correlated with the initial chirp rate (summed chirps over all trials on the first day).

experiments were therefore conducted over a period of over 2 years and used different shipments of fish and different AptEgr-1 probes (although we attempted to standardize the probes; see below). There was, as a result, unavoidable, uncontrolled variability in the AptEgr-1 *in situ* hybridization experiments described below, and this might also account for at least some of the variability in the results we report.

Standard AptEgr-1 mRNA induction protocol

C1: As in A1, the fish were brought up from the common fish tanks and allowed to swim freely in the test aquarium during the adaptation period (minimum of 2 days) before experimentation began. Control fish were simply captured (after the adaptation period) and processed as described below. Thus the controls had never

been stimulated or deliberately perturbed in any way but were fully adapted to a stable environment. The control fish were therefore treated identically to the stimulated fish (see below) except that the stimulated fish were, as described below, immediately stimulated in some manner immediately after the adaptation period. **C2:** Fish were stimulated 20 times (30 seconds stimulation, 120 seconds rest) with the +10 Hz DF stimulus (48 minutes total). The animals were killed by intracardiac perfusion (see below) immediately after last stimulus presentation. This protocol was chosen because the maximal decline in chirp production typically occurred by the twelfth stimulation presentation (see Fig. 2A), approximately 30 minutes before death (see below), and preliminary experiments suggested that the 48-minute wait resulted in strong expression of AptEgr-1 mRNA.

Habituated AptEgr-1 mRNA induction protocol

D1: We first ran the complete standard habituation protocol, A1–A4. The habituation protocol was terminated when the fish's chirp rate declined to <10% of its initial value. D2: The induction protocol, C2, was then run on the habituated fish. It should be noted that, because the same stimulus was used ($DF = +10$ Hz), the fish chirped very little (<10% of initial chirp rate) or not at all during this protocol.

Novel DF AptEgr-1 mRNA induction protocol

E1: We again ran the complete standard habituation protocol, A1–A4. The habituation protocol was terminated when the fish's chirp rate declined to <10% of its initial value. E2: The induction protocol, C2, was then run on the habituated fish except that, in this case, we used a novel stimulus with $DF = -10$ Hz (instead of the standard +10 Hz).

Novel spatial location AptEgr-1 mRNA induction protocol

We wished to investigate whether the fish would recognize a stimulus coming from a new spatial location and therefore modified the location of the stimulating dipoles (Fig. 1C). F1: We again ran the complete standard habituation protocol, A1–A4. In this case, we presented the stimulus ($DF = +10$ Hz) from only one of the stimulus dipoles. The habituation protocol was terminated when the fish's chirp rate declined to <10% of its initial value. F2: The induction protocol, C2, was then run on the habituated fish. In this case, we presented the same stimulus ($DF = +10$ Hz) but via the stimulus dipole on the opposite side of the aquarium (not the one used for habituation).

Isolation of the AptEgr-1 cDNA sequence

We were not initially successful in isolating sufficient AptEgr-1 mRNA for cloning. In rats, injection of convulsants can induce the expression of IEG mRNAs (Saffan et al., 1988). We therefore injected fish with pentylenetetrazol (intraperitoneally, 100 mg/1 ml). The fish became highly agitated within 30 seconds after the injection. After 30 minutes postinjection, sufficient mRNA could be extracted to clone the fish IEGs.

One *A. leptorhynchus* fish was sacrificed in ice-cold water, and its brain was removed and homogenized in Trizol (Sigma, St. Louis, MO) to purify total RNA. Reverse transcription was performed with the RevertAid H Minus First Strand cDNA Synthesis kit (Fermentas) according to the manufacturer's recommendations. A 1.5-kb-long cDNA sequence of Apterontotid Egr-1 was obtained by degenerate PCR with the primers: 5'-CMC YTG AGC TTC

CCT CAC TC-3' (HLSFPHS) and 5'-TCA GCA GAT NTC AYG CTG TCC-3' (RTADIC*), where M = A or C, Y = C or T, and N could be any nucleotide. Amplification was performed with 0.2 μl Amplitaq polymerase (Applied Biosystems, Foster City, CA) in a 100-μl reaction volume containing 0.25 mM dNTPs, 4 mM MgCl₂, PCR Buffer II (Applied Biosystems), and primer concentration of 0.25 μM. PCR cycling was as following: an initial step of 2 minutes at 95°C; followed by 40 repetitions of 30 seconds at 95°C, 30 seconds at 56°C, and 72°C for 150 seconds; a final step at 72°C. The PCR fragments were then subcloned in the pGemT vector (Promega, Madison, WI). The AptEgr-1 cDNA clones were sequenced. The AptEgr-1 sequence was published in GeneBank with the access No. FJ666212.

In situ hybridization RNA probes

An 883-bp cDNA fragment of Apterontotid Egr-1 spanning the 5'-end (from HLSFP to RPSKT in amino acid sequence) was obtained by PCR (forward primer: 5'-CMC YTG AGC TTC CCT CAC TC-3'; and reverse primer: 5'-GT CTT ACT TGG CCG GTT AGG-3') under conditions similar to those previously mentioned. This PCR product was inserted in pGemT vector (Promega) to prepare the RNA probes. AptEgr-1 DNA templates were linearized by restriction endonucleases (Fermentas; for antisense probe with PspOM I, for sense probe with Spe I), separated by ethidium bromide-stained agarose gel electrophoresis, and purified with the Gel Purification Kit (Qiagen Germany).

We chose to use the nonradioactive method of in situ hybridization based on our previous experience with both radioactive (Bottai et al., 1997, 1998; Lee et al., 2000) and nonradioactive (Ellis et al., 2007, 2008; Harvey-Girard et al., 2007) methods. We found the nonradioactive method to be better suited, in our electric fish material, for precisely identifying the labeled neurons (Bottai et al., 1997; Harvey-Girard et al., 2007). It was also very sensitive (because of its amplification step), with relatively low noise. These features made it very suitable for detecting increases in expression of AptEgr-1 mRNA (Ellis et al., 2007), although the nonlinear amplification step does imply that our quantitative results should not be considered as scaling simply with mRNA concentration (there will likely be saturation at higher levels of expression).

Digoxigenin-labeled RNA probes were prepared with the DIG RNA Labeling Kit (Sp6/T7; Roche Applied Science, Indianapolis, IN) according to the manufacturer's recommendations. The RNA probe samples were migrated on an ethidium bromide-stained agarose gel beside an RNA standard to estimate its size (about 900 nucleotide long). DIG-labeled RNA probes were quantified with Test Strips (Roche Applied Science). Typically, DIG

TABLE 1.

Summary Statistics (Mean and SD) of Optical Density Values for the Sense Probe and for the Control (Unstimulated) Fish¹

	DDmg	DLd	DC	DDi	CE	TA
Sense probe: control (N)	33.1 ± 12.4 (81)	14.3 ± 20.3 (130)	26.0 ± 16.4 (106)	26.8 ± 14.2 (66)	34.9 ± 13.7 (74)	18.1 ± 16.2 (89)
Antisense probe: control fish (N)	44.7 ± 16.7 (529)	28.0 ± 18.3 (454)	30.4 ± 18.4 (461)	38.4 ± 19.2 (585)	46.7 ± 20.0 (204)	25.4 ± 14.3 (525)

¹Binding of the sense probe was low but varied across brain regions. The mean values of sense probe optical density were subtracted from all subsequent measurements. The optical densities of the control fish appear to vary across brain regions, but, after subtraction of sense probe values, this difference is greatly reduced (see first row of Table 2). There were N = 2 fish sense controls and N = 2 antisense unstimulated fish done at the same time to maintain constant experimental conditions. "N" refers to the number of individual cells analyzed. Additional unstimulated control fish were used at later stages of the experiment for quality assurance; the numbers of cells are therefore lower here than for the analyses of the effects of experimental perturbations and the mean optical density values slightly different. The antisense values in DDmg, DLd, DDi, CE, and TA are greater than the sense values (*t*-test, $P < 0.001$), whereas those in DC are only slightly greater ($P < 0.05$). The control fish therefore have significant baseline activation of aptEgr-1 in these pallial and diencephalic brain regions.

RNA probe concentrations were between 1.5 and 5 ng/ μ l. RNA probes were ethanol precipitated and conserved at -20°C .

In situ hybridization protocols

Nonradioactive in situ hybridization was performed as previously described in detail (Harvey-Girard et al., 2007). Briefly, fish were deeply anesthetized (MS-222) and perfused with 4% paraformaldehyde in PBS (pH 7.2), removed, and incubated overnight in the same fixative solution but now also containing 10% sucrose (at 4°C). Brain cryosections (20 μm) were prepared, mounted on slides, heated, dehydrated in alcohol, and stored at -20°C for less than 1 week before use after the behavioral protocols to avoid mRNA degradation. Slices were then permeabilized with Triton X-100 and proteinase K and neutralized by triethanolamine. Hybridization was performed at 60°C with 10 ng of AptEgr-1 RNA probe in 100 μl of solution for each slide. The next day, slides were washed at 60°C in several salt solutions (Harvey-Girard et al., 2007), blocked, incubated with anti-DIG-AP antibody, and incubated overnight for coloration with NBT/BCIP.

Quantification of AptEgr-1 mRNA expression

To control for variations in staining during the DIG in situ hybridization, small numbers of sections (always including ones containing DDmg) from a control fish (protocol C1; see above) and a stimulated fish (protocol C2; see above) were routinely included among the test fish sections (habituated or stimulated with a novel stimulus). We compared staining results for these two sets of control fish (C1 and C2) and accepted the new test fish data only if these cases had gray level values similar to the equivalent original data. On occasion, we found lower levels of staining in these "control" sections, presumably because the probe concentration was too low; we discarded these experimental runs.

Sections were photographed on an Axioskop 2 microscope (Zeiss, Thornwood, NY) under identical illumination conditions and acquired with Northern Eclipse 6.0 software. The raw images were used for estimating the gray levels of cells expressing AptEgr-1 in ImageJ software (NIH). Exactly the same procedure was used in all cases, including sense probes, control fish, and fish stimulated as described above.

Brain regions were identified according to an atlas of the Apteronotid brain (Maler et al., 1991). We attempted to control for local variations of staining background. We first estimated the gray level of the neuropil within an identified brain region; this gave an estimate of the local background. We then estimated the gray level of the cells within this region; we subtracted the neuropil gray level from that of the cell's gray level. This was done for each section (of each fish) through that brain region.

We selected cells for measurements based on one criteria: that we could identify an unlabeled nucleus within the neuron. For most brain regions, we sampled all cells. In the case of DLd, there were too many cells to sample efficiently. In this case, we sampled only the central sections and therefore avoided any contamination from adjacent brain regions such as dorsomedial or dorsal posterior telencephalon.

In situ hybridization with sense probes served as a control for specificity of the probe and for the probe's non-specific binding within each brain region. The sense probe produced low levels of staining (N = 2 fish; see Fig. 5A), and these differed across brain regions (Table 1), so we subtracted the region-specific mean sense gray level values (of cells and corrected for local background as described above) from those of the in situ antisense estimates for that region to provide a normalized measure of AptEgr-1 mRNA expression. This was done for all the antisense measurements in controls and various experimental groups. In summary, we first subtracted the local neuropil gray levels from all cells sampled (including sense

TABLE 2. Summary Statistics (Mean and SD) of Optical Density Values in Brain Regions That Showed Consistent Increases in AptEgr-1 mRNA Expression With Electrosensory Stimulation¹

	DDmg	Dld	DC	DDi	CE	TA
Control #fish = 8 (N)	11.3 ± 16.2 (748)	12.2 ± 18.4 (595)	4.7 ± 19.4 (719)	11.1 ± 18.7 (753)	8.5 ± 18.8 (324)	6.0 ± 14.1 (675)
Stimulated (+10 Hz) #fish = 9 (N)	53.6 ± 29.9 ² (1,052)	34.5 ± 24.3 ² (728)	34.5 ± 31.3 ² (603)	38.7 ± 25.3 ² (1,088)	37.8 ± 29.1 ² (375)	25.1 ± 19.4 ² (1,122)
Habituated #fish = 5 (N)	16.5 ± 18.7 (456)	23.1 ± 21.5 (352)	15.0 ± 21.2 (279)	21.9 ± 19.7 (514)	25.2 ± 24.4 (179)	15.4 ± 17.9 (598)
Stimulated (-10 Hz) posthabituation #fish = 8 (N)	49.1 ± 31.9 ² (703)	23.9 ± 25.4 (773)	18.9 ± 23.7 (629)	24.7 ± 22.6 (477)	23.0 ± 25.5 (354)	11.2 ± 20.3 (610)
Stimulated opposite side posthabituation #fish = 7 (N)	27.3 ± 26.5 ² (531)	15.6 ± 22.9 (763)	13.4 ± 25.7 (532)	20.4 ± 24.4 (391)	7.7 ± 17.3 (244)	10.9 ± 20.9 (726)

¹The mean values of the sense controls for each region are subtracted. "N" refers to the number of fish analyzed; the number of fish is also shown and mentioned in the text as well. Statistical analysis used ANOVA with the Tukey HSD post hoc test. DDmg: Cells from stimulated fish have significantly higher levels of AptEgr-1 expression compared with both control and habituated fish ($P < 0.01$). Posthabituated fish stimulated with a negative DF or on the opposite side of the body had significantly elevated levels of AptEgr-1 in comparison to the habituated fish ($P < 0.01$); the effect of stimulation with DF = -10 Hz was also significantly greater than for the change in spatial location ($P < 0.01$). Dld: Cells from stimulated fish have significantly higher levels of AptEgr-1 expression compared with both control and habituated fish ($P < 0.01$). Stimulation with a DF = -10 Hz does not increase expression above habituated values; stimulation on the opposite side causes a significant decrease of expression compared with habituated fish ($P < 0.01$). DC: Cells from stimulated fish have significantly higher levels of AptEgr-1 expression compared with habituated values. Ddi: Cells from stimulated fish have significantly higher levels of AptEgr-1 expression compared with habituated values. CE: Cells from stimulated fish have significantly higher levels of AptEgr-1 expression compared with both control and habituated fish ($P < 0.01$). Stimulation with -10 Hz or on the opposite side do not change AptEgr-1 expression compared with habituated values; stimulation on the opposite side causes a significant decrease of expression compared with habituated values ($P < 0.01$). TA: Cells from stimulated fish have significantly higher levels of AptEgr-1 expression compared with both control and habituated fish ($P < 0.01$). Cells from fish stimulated with either DF = -10 Hz or on the opposite side had slightly but significantly lower levels of AptEgr-1 expression compared with the habituated fish ($P < 0.05$).
² $P < 0.01$.

probes samples) and then subtracted the adjusted mean sense gray levels from all control and experimental values.

Statistical comparisons were done (ANOVA) in two ways. First, they were done across all fish (of a specific treatment condition) and across all cells within a given brain region of one fish. This method is, strictly speaking, not entirely rigorous, because the cells within one fish are not independent samples. There was a great deal of variability of AptEgr-1 mRNA expression within all brain areas even within one section of one fish brain, suggesting that the telencephalic neural networks might have effectively decorrelated the expression of AptEgr-1 mRNA. The use of all cells of all fish therefore included all the variability, that within as well as across fish.

We also performed statistical analysis on a per-fish basis, thus guaranteeing completely independent samples. This was done by first calculating the mean of the gray level values for all the cells of each brain region (for example, DDmg) within one fish. This resulted in a sample size of N = 8 (number of fish) for the control, N = 9 for the stimulated fish, etc. (see Table 2; sample size is now the #Fish value). The statistical analysis (ANOVA) then compared these estimates (for DDmg in this example) across control and various experimental groups; the variance in this case is due only to across-fish variability. This latter method is certainly on independent samples (individual fish), but it also removes all the within fish variability. We reasoned that the most secure conclusions would be those supported by both kinds of statistical analysis.

Standard cresyl violet sections

We used the negatives, originally prepared from Nissl-stained sections for an *Apteronotus* brain atlas (Maler et al., 1991), to create low-magnification images of the relevant brain regions (see Fig. 6). The contrast and brightness of these images were adjusted in Photoshop to enhance visualization of the brain regions important for this study; the final figure was prepared in Adobe Illustrator. All protocols were approved by the University of Ottawa Animal Care Committee.

Preparation of figures

Figures were made in Adobe Photoshop and Illustrator. In cases when the original image was unequally illuminated, a Photoshop CS Plug-In from Reindeer Graphics (<http://www.reindeergraphics.com>) was used to produce a flatter background illumination; this plugin (Adaptation equalization) can be found in the "Free Plug-In" drop down menu of the Reindeer Graphics website. The algorithms used are described in some detail in the documentation provided with the Adaptation equalization plugin. Briefly, a weighted brightness histogram is constructed

for a circular neighborhood around every pixel of the image; this is a graph of the number of pixels having a particular brightness value with a weighting that diminishes the contributions of pixels distant from the central pixel. If an image is unevenly illuminated, there will be regions whose mean brightness level is far above or below that of other regions; the means of their brightness histograms will be very different. New histograms are then computed to equalize the mean levels across all brightness histograms, and these are used to create an adjusted image that is effectively brighter in large areas that were darker (mean value over a large region) in the original image and vice versa. This adjusted image can then be combined with the original image to equalize the brightness over the entire extent of the image, without changing the local variations in brightness that represent labeled neurons. Effectively, this process removes smooth variations of brightness across the image without interfering with local contrast.

The *in situ* material is inherently low contrast, especially in cases in which the levels of AptEgr-1 mRNA are low. We therefore, in the original image acquisition, did a through-focus series and chose (EH-G, LM, JT) the sharpest images for presentation. In some case, we also made minor adjustments to the contrast and brightness of the images to define better the key labeled cell groups; we were careful, in these cases, to preserve the relative gray levels of labeled and unlabeled cells. As mentioned above, the actual gray level measurements were taken from the raw images and were therefore not subjected to adaptive equalization or contrast/brightness optimization.

RESULTS

Time course of chirp habituation

Short-term habituation

Fish chirp strongly in response to the $DF = +10$ Hz stimulation; visual inspection of a small subset of evoked chirps showed that they were, as expected, small chirps (Bastian et al., 2001; Engler and Zupanc, 2001; Triefenbach and Zakon, 2003). The chirp response rapidly habituated over the course of several stimulus presentations ($N = 17$ fish) and usually declined to <10% of initial rates after <30 presentations. Although the time course of habituation of individual fish was variable, they were generally (estimated from all trials) well fit by an exponential function (Fig. 2A,B) with a mean exponent of $-0.31/\text{presentation}$ ($N = 82$, $SD = 0.28$, range -0.01 to -1.34). These results suggest that the $+10$ Hz beat stimulus (1 minute) induced a longer lasting process that extends over the interpresentation period (2 minutes) and attenuates the subsequent chirp responses. Fish that chirped

more on the first presentation generally took longer to habituate so that the value of the fitted exponent was negatively correlated with the initial chirp rate on that trial (Pearson correlation coefficient = -0.36 ; this is within the confidence interval of -0.535 to -0.156 with probability $P = 0.95$; Fig. 2B).

For these experiments, we always presented a stimulus frequency that produced a beat with $DF = +10$ Hz (to permit statistical comparison across fish). Possible habituation to other, nonpresented beat frequencies was not tested, because they might have perturbed estimates of habituation to the $+10$ Hz DF . It is therefore possible that the habituation we observed was not specific to $+10$ Hz stimulus but represents a general decline in evoked chirping to all beat frequencies, perhaps as a result of release of a neuromodulator such as serotonin (Maler and Ellis, 1987). In this interpretation, our results would not be due to a short-term memory of the $DF = +10$ Hz stimulus but rather to a nonspecific reduction of the fish's propensity to chirp. We therefore, in a subset of subsequent experiments not related to the behavioral analyses (see AptEgr-1 section below), inserted probe trials with different beat frequencies ($DF = -10$ Hz or $+20$ Hz) after the fish had already greatly reduced its chirp rate (~ 0 chirps) to the standard beat frequency ($DF = +10$ Hz). These probe trials always induced strong chirp responses, suggesting that the reduction in chirp rate is due to stimulus-specific habituation and can therefore be considered a simple form of short-term memory. A more statistically rigorous analysis of the stimulus specificity of evoked chirping is presented below.

Long-term habituation

The short-term habituation of chirp rate seen over the course of up to ~ 36 presentations (up to ~ 90 minutes) also resulted in a long-term chirp habituation. The total number of chirps emitted over each daily trial decreased over successive days, and the decrease was, in all cases, well fitted by an exponential function (Fig. 2C,D) with a mean exponent of $-0.32/\text{trial}$ ($N = 15$, $SD = 0.21$, range 0.02 – 0.69). This result suggests a long-term memory process that extends for >24 hours. For long-term habituation, there was a strong positive correlation between the decay exponent and total number of chirps emitted on the first day ($r = 0.87$; this is within the confidence interval 0.646 to 0.956 with probability $P = 0.95$). Thus, fish that initially chirped at a high rate also habituated more rapidly (Fig. 2D). The striking difference in the correlation of decay exponents with initial chirp rates (-0.36 for short-term and 0.87 for long-term habituation) suggests that different processes might underlie short- vs. long-term habituation.

Consolidation of chirp habituation

Consolidation refers to the process that converts short- to long-term memory storage. Evidence for such a process generally comes from experiments in which neural activity is disrupted after a learning experience and, as a result, long-term memories are not formed. To test for a consolidation process, we attempted to disrupt the conversion of short-term to long-term habituation by cooling the head 30 minutes after the last habituation trial of each day. This approach was adopted because these fish did not tolerate inhibitors of transcription and translation (often used in studies of consolidation), and hypothermia has previously been used to disrupt consolidation in rats (Mactutus et al., 1979). Cooling was done until the fish stopped respiring (~ 3 minutes); the fish were subsequently artificially respiration and recovered completely within <5 minutes. Handling the fish caused greater variability in long-term chirp habituation, so linear fits were used to estimate the decay rates of evoked chirping. Cooling the head had no effect on short-term habituation on the next day's trials (data not shown) but completely prevented long-term habituation (Fig. 3); the mean slope of the best fit line was 0.84 chirps/day ($N = 5$, $SD = 45.5$, range -48 to 67). Two types of control experiments were carried out. Fish had their trunks cooled 30 minutes after the last habituation trial of each day to control for the stress of handling. Habituation was within the normal range in this case, with a mean slope of -190 chirps/day ($N = 5$, $SD = 90$, range -128 to -374). The second control was to determine whether there was a time window for the establishment of long-term habituation. The fish's head was again cooled each day but at 6 hours posthabituation. Again, there was normal habituation, with a mean slope of -331 chirps/day ($N = 5$, $SD = 147$, range -138 to -543). The slopes of the habituation curves of 30-minute-posthabituation head-cooled fish were significantly different from those of both the trunk-cooled and the 6-hour-posthabituation head-cooled fish (ANOVA, $P < 0.05$ post hoc comparisons in both cases); the slopes of the two controls were not significantly different. The apparent difference in slopes of the two control cases likely was due to differences in their mean initial rates of chirping, insofar as normalizing the responses showed that the relative rate of habituation was identical (Fig. 3). The conversion of short-term to long-term habituation is therefore a temperature-dependent consolidation-like process operating between 0.5 and 6 hours after stimulation. We use the term *consolidation* to refer to the fact that long-term habituation of evoked chirping can be prevented by disruption of neural activity. We have not investigated whether the underlying molecular, cellular, and network mechanisms of consolidation of chirp habituation are

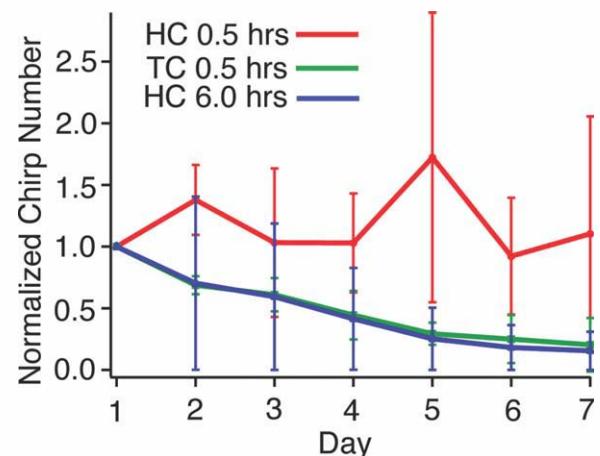


Figure 3. Effect of head cooling on habituation; because the mean chirp rates of the experimental groups were not identical, the chirp counts were normalized as fractions of the total chirp count on the first day. Cooling the head after the last habituation trial of each day completely prevents habituation (red, HC 0.5 hours). Cooling the trunk at the same time (green, TC 0.5 hours) or the head six hours after the last habituation trial (blue, HC 6.0 hours) each day results in normal chirp habituation.

similar to the consolidation processes of rodents or primates.

Specificity of chirp habituation

We next sought to determine whether habituation of evoked chirping was specific to the magnitude and sign of the test beat frequency. Before the habituation trials, a different set of fish was tested for their chirp response to DFs ranging from -120 Hz to $+120$ Hz. There was a great deal of variability in the DF range that evoked chirping; however, as expected (Bastian et al., 2001; Engler and Zupanc, 2001; Triefenbach and Zakon, 2003), maximal chirp rates always occurred at beat frequencies of ± 5 or ± 10 Hz (Fig. 4). Fitting the chirp vs. DF curves with a Gaussian (after smoothing) showed that the DF range (SD of the fitted Gaussian) was strongly correlated with maximal chirp rate ($r = 0.81$, data not shown); that is, fish that chirped more also chirped to a wider range of DFs. The fish were then habituated to a specific DF in a manner identical to that described above; the habituation protocol was terminated when the daily evoked chirp number had dropped to ~ 0 (typically <7 days). Three days after the last habituation trial, the DF-dependent chirp profile was again tested. In all cases, the chirp rate at the test DF was reduced ($N = 18$; 2 cases at $DFs = \pm 5, \pm 10, \pm 20$, and ± 40 Hz and 1 case at each of $DF = \pm 60$ Hz). A greater decrease in chirp rate to the test DF in comparison with the response to the DF of opposite sign was invariably observed (Fig. 4); the response to the opposite

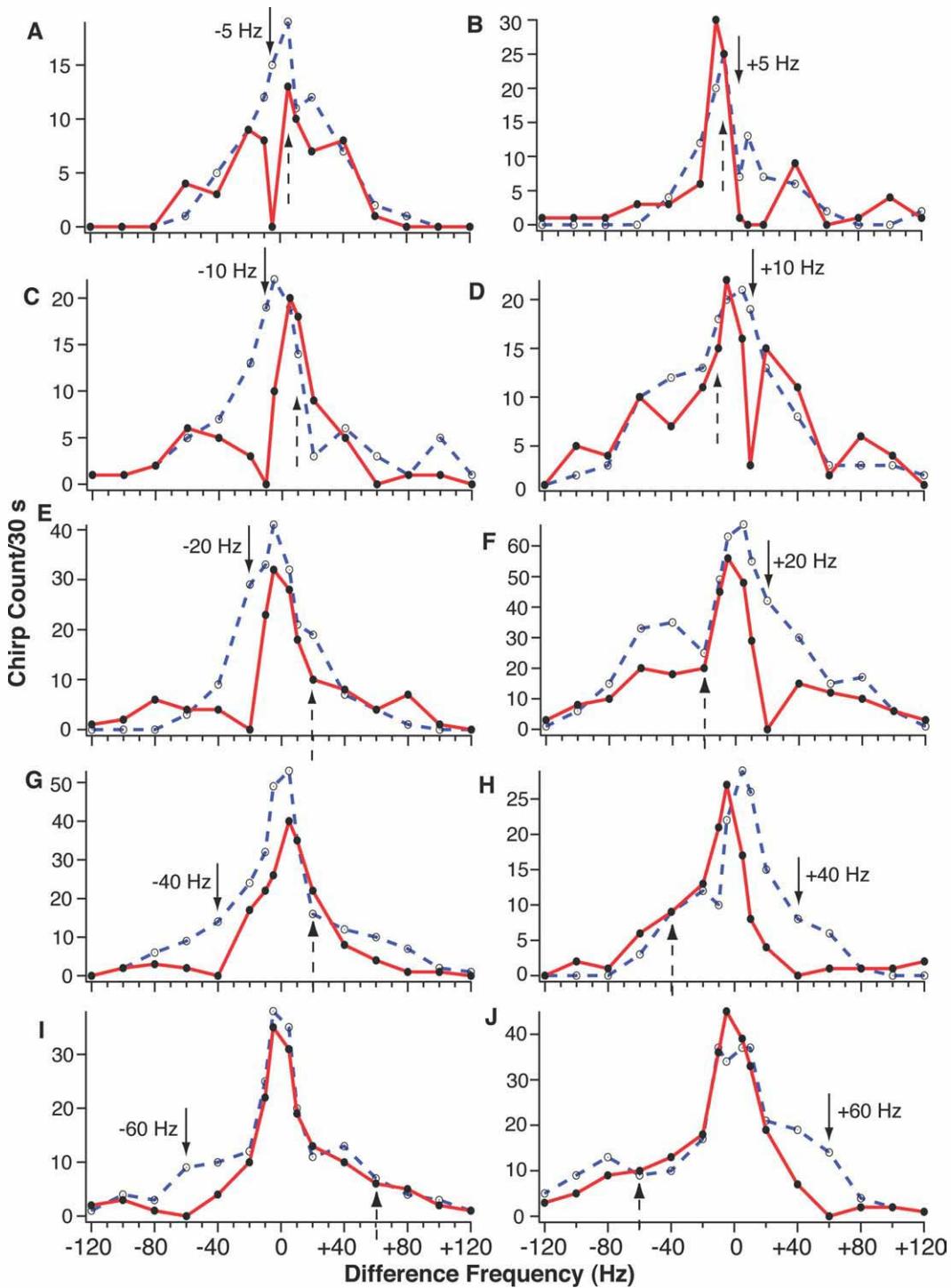


Figure 4. A–J: Habituation of the chirp response is specific to the DF presented. Prestimulation (control) fish chirp response curves are blue (dashed lines) and posthabituation curves (3 days) are red (solid lines); the solid arrow points to the DF used for habituation and the dashed arrow to the DF with the same magnitude but opposite sign; the value of the latter served as a control for statistical comparisons. That is, the chirp count of the DF used for habituation was compared (paired *t*-test; see Materials and Methods) with the chirp count to the DF of opposite sign.

sign DF could decrease ($N = 12$), increase ($N = 4$), or not change ($N = 2$). The change in chirp rate for the opposite sign DF was -2.4 chirps ($SD = 3.8$), whereas for the test DF it was -19.3 chirps ($SD = 16$); this dif-

ference is highly significant (paired *t*-test, $P < 0.0002$). The decrease in chirping was typically not limited to the test DF but spread to some extent to neighboring DFs (Fig. 4); it appeared that habituation might be

A

AptEGR-1	MAAAKTE-LLP-ALQNSEPL-SFPHSP-MDSYPKLEEMIMLNSAGTPFL-NASAPEGGAG-----	55	
A.burtoniMI.-.....-R.S.....VM..S.....-S.....-	55	
M.muscu.A.MQ.MSP..I.D.FG.....T.N.....ML.SNGAPQ..GA.GTP..SGGNSSSTSSGGGGGSNSGS	80	
	SAD		
AptEGR-1	--FGS-GEPGEQ-YDHLVGDTLPEIALNCEKSLAEQSFTQ--RLPPISYTGRFTLEPATNCNSNLWAELFSLVSGLVG	129	
A.burtoni	-----.D.-....A....D.PF....PVV..TY...--...S.....T.....ILG.F...I.	129	
M.muscu.	SA.NPQ...S..P.E..TTESFSD....N..AMVET.Y.S.TT.....T.....S....P.SG.T..P.....S	160	
	R		
AptEGR-1	VNPPPTSISSSASAVAQTPSSAASSSVPSSSSSSSSPSISCSVHQSEPNPIYSSAAPTYSSASPDIIFPEP-GPSF	208	
A.burtoni	-.VA.S.S....ASQTSS..SSVP.SS.....TS...SQ.S.L.S.I.H.....SNS....D-Q.QA.	207	
M.muscu.	MTNP.T.....SAP.PAA.SS..ASQ..PL..A.PSNDDSS.....FPTPNNT.....QSQA.	223	
	NLS	DBD	
AptEGR-1	PSAVGGSLPYPPPAPSSKACGASFVPMIPDYLFPQQQSEISLVPPDQKPFQTPAG--QQPSLTPLSTIKAFAKQGTGSQ	286	
A.burtoni	.TSA.-TVQ.....N..T.ST.....G.....NQS--S.....T.....	283	
M.muscu.	.GSA.TA.Q.....AT.GG---Q.....GDL..GT.....GLENRT.....T.S...	300	
	WAD		
AptEGR-1	DLKSV---YQSQLIKPSRVRKYPNRPSKTPPHERPYACPVETCDRRFSRSDELTRHIRIHTGQKPFQCRICMRNFSDH	363	
A.burtoni-.....TR.....	360	
M.muscu.	...ALNTT.....M.....S.....	380	
	TADIC*		
AptEGR-1	LTHIRHTGEKPFACEICGRKFACSDERKRHTKIHRLQKDCKNAAVAAAAVQGAIAPVSIKVSPVSSYPSP-I	442	
A.burtoniR.....E.VG..V.T.TP.S-----AA..P.....S.	430	
M.muscu.D.....R.....SVVASP..SSLS---S-YPSPVAT.....AT	453	
AptEGR-1	ASYPSPVS-SFPSPVASCYSSPVHNSYPSP-----ATSGTFQTPVAASFPTSVGSSIYSSPVTTPLADMQASLSPR	512	
A.burtoni	T.....-Y.....T.....T.....SIATTYPSVSM.S...SQ..S...S.A.N.....P...P...TT....	509	
M.muscu.	T.F....PT.YS..GS.T.P..A.SGF...SVATTFASV--PPA.P.Q.S...SAGV..SF.T--S.G.S..T.TF...	528	
AptEGR-1	TADIC*	517	
A.burtoni	.IE..*	514	
M.muscu.	.IE..*	533	

B

	aptEGR-1	270	299	314	403	517	
		SAD	R	NLS	DBD	WAD	
A.burtoni		266	295	310	399	514	
	73%		97%	87%	99%	59%	
murEGR-1		283	315	331	419	533	
	42%		84%	94%	97%	46%	

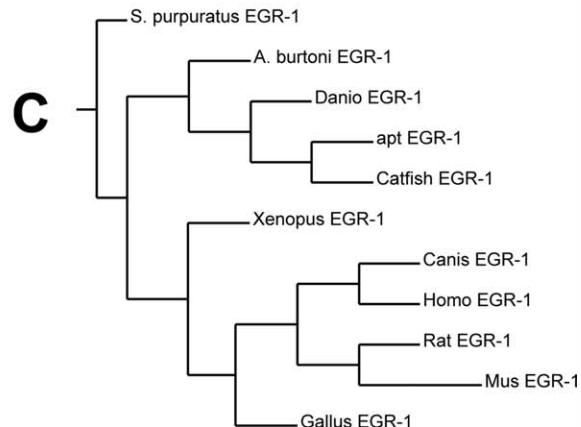


Figure 5. Sequence comparison of the *A. leptorhynchus*, *A. burtoni*, and murine Egr-1. **A:** Amino acid sequence of Apteronotid Egr-1 was compared with that of *A. burtoni* and murine Egr-1 with the ClustalW method in the Megalign program (DNAStar, Madison,WI). Amino acids in the murEgr-1 and *A. burtoni* sequences that match residues in AptEgr-1 are shown as dots. Gaps in the sequences are identified by dashes. Mammal and teleost sequence inserts are identified by a box. Domains are shown by bars overlying the sequences. For abbreviations see list. **B:** Schematic representation of the relative sequence conservation within functional domains of AptEgr-1 displayed as in A. Amino acid residue numbers are on the top for Apteronotid isoform, on the bottom for *A. burtoni* and the murine Egr-1. Percentage values calculate the amino acid identity for each domain. Overall amino acid identity is 59%. **C:** Phylogenetic tree comparing 11 Egr-1 sequences from *A. leptorhynchus*, human (NM_001275.2), rat (NM_012551.1), mouse (M20157), dog (XM_846145), frog (BC072770.1), chicken (AY034140.1), zebrafish (XM_688016.1), catfish (AY029282.1), *A. burtoni* (AY493348.1), and purple urchin (XM_782145). Calculations of phylogenetic distances using the maximum likelihood estimates based on the Dayhoff PAM matrix was performed in the PROTDIST program in the Phylogeny Inference Package (PHYLIP; Felsenstein 1989). The optimal phylogenetic tree was determined by the UPGMA method using the NEIGHBOR program (PHYLIP) and analyzed by bootstrap resampling ($\times 1,000$) in SEQBOOT (PHYLIP).

more DF specific for low-frequency DFs (5 or 10 Hz), but data were not sufficient to test this possibility rigorously. Habituation specific to the beat frequency and sign demonstrates that these fish have established a

long-term memory of the presented DF and that this requires a consolidation process that converts short-term habituation to habituation that lasts for at least 3 days.

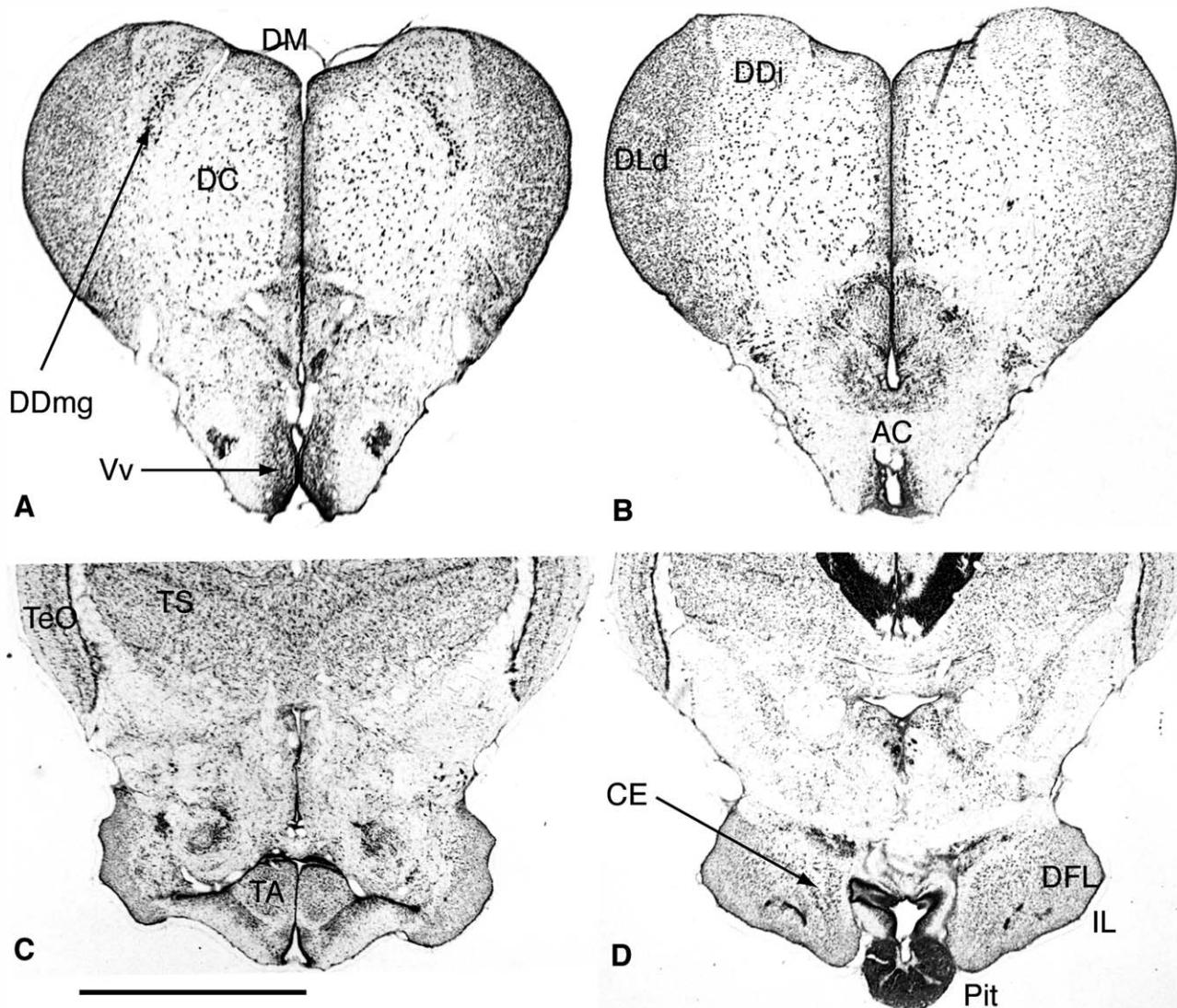


Figure 6. Cresyl violet-stained transverse sections illustrating the cell groups where stimulation with EOD mimics consistently induced expression of AptEgr-1. For clarity of presentation, only those cell groups and other landmark structures are labeled in this figure; the reader is referred to the *Apteranotus* brain atlas of Maler et al. (1991) for identification of the unlabeled cell groups. A,B: Mid-telencephalon illustrating the relative location of dorsolateral telencephalon, dorsal subdivision (DLd); dorsocentral telencephalon (DC), dorsal telencephalon, intermediate subdivision (DDi); and dorsal telencephalon, magnocellular component of the intermediate subdivision (DDmg). The level at B is slightly caudal to that at A and both are between T30 and T33 of the *Apteranotus* brain atlas. Note that DDmg is actually embedded within DDi, which is found both rostral and caudal to it. The dorsomedial telencephalon (DM), anterior commissure (AC), and ventral subdivision of ventral telencephalon (Vv) are indicated for general orientation. C: A section through mid-hypothalamus showing the nucleus tuberis anterior (TA). The optic tectum (TeO) and torus semicircularis (TS) are indicated for general orientation. This level corresponds to T16 of the *Apteranotus* brain atlas. D: A section through caudal hypothalamus (corresponding to T13 of the *Apteranotus* brain atlas) illustrating the location of the central nucleus (CE) of the inferior lobe (IL) of the hypothalamus. The nucleus diffusus lateralis (DFL) of the inferior lobe and the pituitary (Pit) are also indicated for general orientation. Scale bar = 1 mm.

Cloning of the transcription factor AptEgr-1

Learning and memory formation as well as late long-term synaptic plasticity have been associated with the rapid expression of IEGs such as c-Fos, Egr-1 (transcription factors), and Arc (Davis et al., 2003; Guzowski et al., 2001; Jones et al., 2001; Knapska and Kaczmarek, 2004). To identify possible brain regions associated with DF-spe-

cific long-term habituation, we therefore sought to clone IEGs from *A. leptorhynchus* brain. We succeeded in cloning an *A. leptorhynchus* homolog to Egr-1. We isolated partial AptEgr-1 cDNA fragment by degenerate RT-PCR prepared from *A. leptorhynchus* brain mRNA. The PCR product was a cDNA fragment of 1.6 kb and coded for an open reading frame sequence of 517 amino acids

(Fig. 5A; GeneBank access No. FJ666212). The phylogenetic comparison with 11 complete Egr-1 protein sequences from several species confirmed that we had found apteronotid Egr-1 (Fig. 5B). As already noted for *Astatotilapia burtoni* (Burmeister and Fernald, 2005), the highest similarities were found in the three central domains: the repressor domain (97% amino acid similarity to *A. burtoni* Egr-1), nuclear localization site (87%), and the DNA-binding domain (99%), whereas lower homology levels were found in the strong (73%) and weak (59%) activation domains (Fig. 5C). Catfish Egr-1 is the closest Egr-1 isoform, with an amino acid homology of 83%.

Induction of AptEgr-1 expression

To localize the possible neuronal expression of AptEgr-1 mRNA associated with electrosensory stimulation by EOD mimics ($DF = +10\text{ Hz}$), we performed *in situ* hybridization for AptEgr-1. To facilitate the reader's visualization of the anatomical context of our results, we include cresyl violet-stained brain sections through the relevant brain regions; these illustrate the pallial and diencephalic structures that reliably express AptEgr-1 subsequent to stimulation as well as additional structures useful for general orientation (Fig. 6). The sense probe ($N = 2$ fish) produced barely detectable labeling (Fig. 7A) whose intensity varied across brain regions (Table 1); the mean gray level values measured for cells in each brain region were therefore subtracted from the antisense measurements (see Materials and Methods) of the same region. Neurons of the control (unstimulated, antisense probe) fish were, except in the brain regions described below, at near-background levels. Labeling of neurons in some divisions of the dorsal pallium of control fish ($N = 8$ fish) with the antisense probe was slightly but consistently greater than that of the sense probe (Figs. 7B, Table 1, and see below).

Below we first present qualitative and quantitative descriptions of the brain regions that consistently expressed AptEgr-1 after stimulation using the standard induction protocol (C1-C2); by "consistent" we mean that AptEgr-1 mRNA expression was increased in every stimulated fish. In this section, we also mention some negative results; these are for brain regions where, based on physiological studies, we might have expected AptEgr-1 expression but failed to find it. Second, we present a qualitative description of brain regions that inconsistently expressed AptEgr-1 and for which statistical analysis was therefore not carried out; by "inconsistent" we mean that less than one-third of the stimulated fish (three or fewer) showed increases of AptEgr-1 mRNA expression in those areas.

Finally, we quantitatively analyze the effects of habituation and protocols involving novel stimuli on AptEgr-1

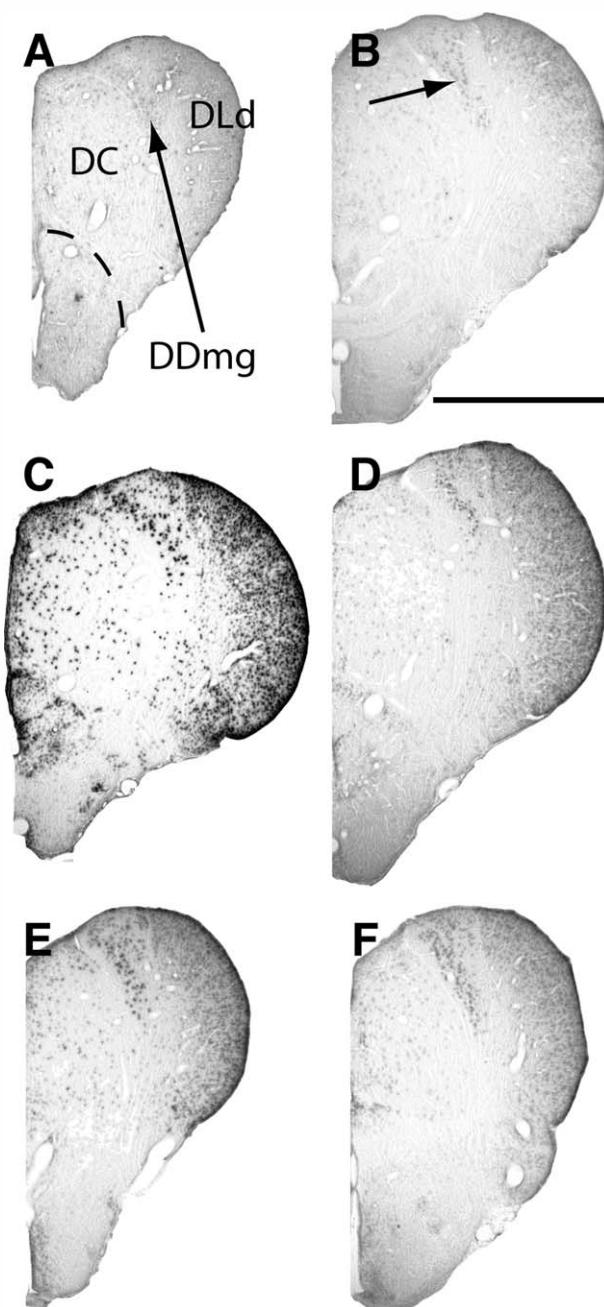


Figure 7. Expression of AptEgr-1 mRNA transcript in the telencephalon. A: Sense control. Dashed line marks the separation of ventral (below dashed line) and dorsal telencephalon. B: Unstimulated control with antisense probe. Weak labeling is seen in DDmg (arrow). C: Stimulated fish. Strong expression of AptEgr-1 is seen in DC, DDmg, and DLd. D: Habituated fish. Expression of AptEgr-1 mRNA is greatly reduced in all regions of the dorsal telencephalon. E: Fish stimulated with $DF = -10\text{ Hz}$ after it has been habituated ($DF = +10\text{ Hz}$). Expression of AptEgr-1 mRNA in DDmg is increased compared with the habituated fish. F: Stimulation with $DF = +10\text{ Hz}$ on the opposite side of the tank (after habituation) increased expression of AptEgr-1 mRNA in DDmg compared with the habituated fish. For abbreviations see list. Scale bar = 1 mm.

mRNA expression; this analysis was carried out only for brain regions that consistently expressed AptEgr-1 mRNA after the standard induction protocol. We note that, in the zebra finch, there are many genes (in addition to the avian Egr-1) whose expression is up- or down-regulated subsequent to presentation of appropriate stimuli (Dong et al., 2009). Therefore, our negative or inconclusive results with respect to AptEgr-1 induction within a brain region do not mean that expression of other IEGs is not altered within neurons of this region after stimulation by EOD mimics or that this brain region is not involved in habituation of the chirp response.

Here we present a brief qualitative summary of our most salient results, those that can be readily observed and do not require statistical analysis. This, in conjunction with Figure 6, should provide an overview that will make the detailed description below easier to follow. An initial qualitative examination of the brain sections labeled by *in situ* hybridization for expression of AptEgr-1 mRNA immediately revealed telencephalic and diencephalic regions that were clearly positive, whereas most of the brain was obviously negative. Quantitative analysis was performed only on the brain regions that showed qualitative, even if weak, evidence of AptEgr-1 mRNA expression. We also examined very carefully (qualitatively) all brain regions known, through prior anatomical or physiological studies, to be involved in electroreception or the production of communication signals such as chirps, to be certain that no trace of AptEgr-1 mRNA expression was evident by inspection.

Chirp-inducing stimulation ($DF = +10\text{Hz}$) caused, in all cases, strong increases in AptEgr-1 mRNA expression in the following dorsal telencephalic regions only: dorsolateral telencephalon, dorsal subdivision (DLd), dorsal magnocellular telencephalon (DDmg), dorsal intermediate telencephalon (DDi), and central telencephalon (DC). It also caused such increases in two diencephalic regions, the medial portion of central nucleus of the hypothalamus (CE) and the nucleus tuberis anterior (TA).

The same stimulus presented after the fish had been habituated (to that stimulus, $DF = +10\text{ Hz}$) produced almost no AptEgr-1 mRNA expression above control levels. However, when the fish was presented with a novel stimulus ($DF = -10\text{ Hz}$) after habituation to $DF = +10\text{ Hz}$, a strong increase in AptEgr-1 mRNA expression was observed in DDmg (only).

AptEgr-1 expression after electrosensory stimulation

Brain regions with consistent expression of AptEgr-1 mRNA

Chirp-inducing stimulation ($N = 9$ fish, $DF = +10\text{ Hz}$) caused strong and consistent increases in the expression of AptEgr-1 transcripts in nuclei confined to the dorsal

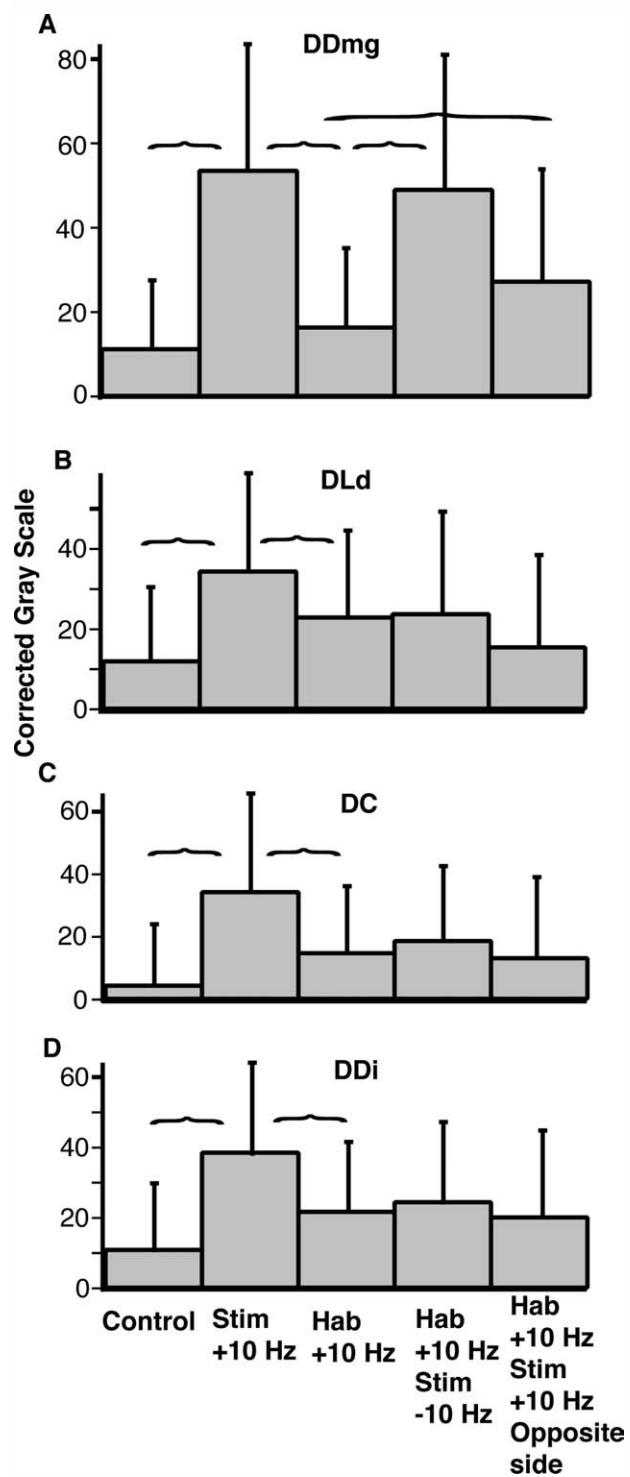


Figure 8. Bar graphs illustrating the corrected gray levels (nonradioactive *in situ* hybridization) of cells within the dorsal telencephalic brain regions where stimulation induced the expression of AptEgr-1 mRNA; the various experimental conditions are shown on the x axis. The braces indicate significant differences. The statistical values corresponding to this figure are completely reported in Table 2.

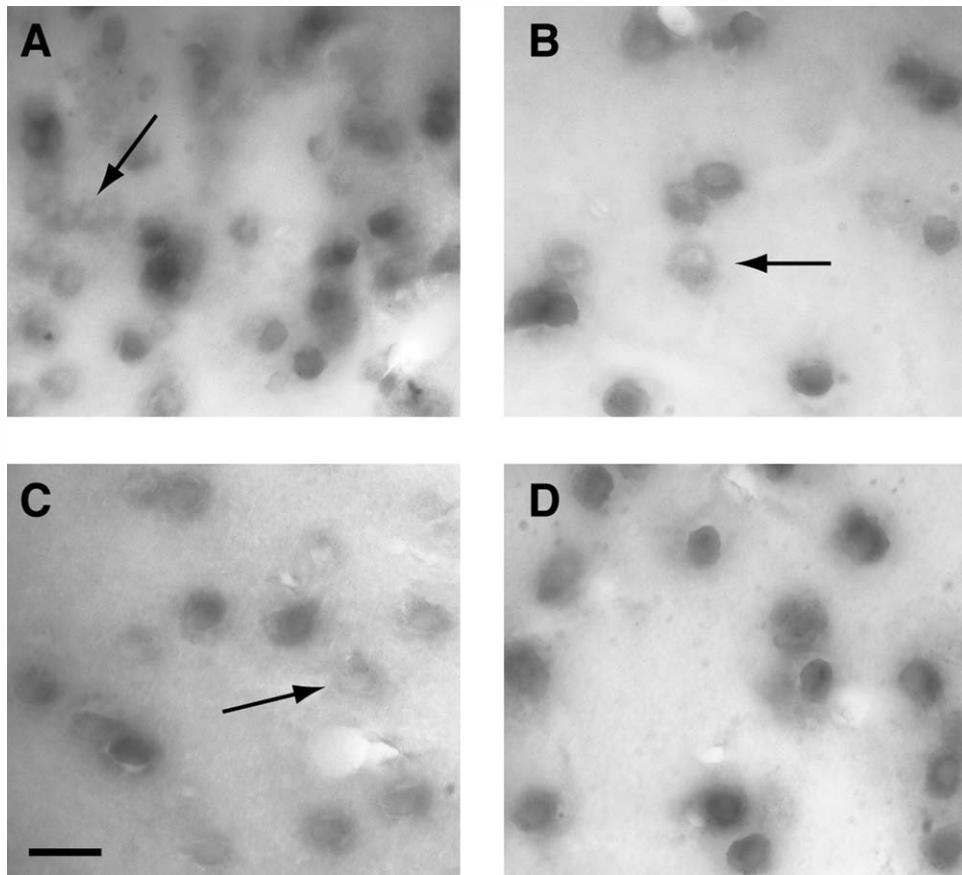


Figure 9. Higher magnification of pallial neurons labeled for AptEgr-1 after stimulation (corresponding to Fig. 4C). A: Neurons in DLd are organized into clusters, and labeling for AptEgr-1 is heterogeneous both within and across clusters. Arrow points to a cluster containing lightly labeled cells. B: Both strongly labeled and nearly unlabeled (arrow) cells are seen in DC. C: The intermediate component of the dorsal telencephalon, dorsal division (DDi), contains both moderately labeled and unlabeled (arrow) cells. D: Cells in DDmg are strongly and fairly homogenously labeled. Scale bar = 20 μ m.

telencephalon (pallium, Figs. 7C, 9) and diencephalon (Figs. 10, 11); Figures 8 and 12 and Table 2 show summary statistics and significant differences. Activated pallial regions (Figs. 7–9) were the dorsolateral telencephalon, dorsal subdivision (DLd), dorsal magnocellular telencephalon (DDmg), dorsal intermediate telencephalon (DDi), and central telencephalon (DC). In contrast, the pallial recipients of direct olfactory bulb input (Sas et al., 1993) had only low levels of AptEgr-1 mRNA expression, unchanged from those of control fish (data not shown). The highest levels of expression were seen in DDmg, where all cells appeared to be labeled (Figs. 9, 13C); in contrast, labeling was less intense and heterogeneous in the other pallial regions (Figs. 7C, 9; see Fig. 8 and Table 2 for summary statistics). The precise delineation of these pallial regions is presented in Figure 6A,B.

A small number of diencephalic cells with stimulus-induced increases in AptEgr-1 mRNA expression were located within the medial portion of central nucleus of the hypothalamus (CE, Figs. 10C, 12) and the nucleus tuberis

anterior (TA; Figs. 11C, 12; Fig. 12 and Table 2 show summary statistics and significant differences). The precise delineation of these diencephalic regions is presented in Figure 6C,D.

A simple hypothesis regarding the cause of AptEgr-1 mRNA induction is that it is due to the increased spiking of neurons. The complete lack of AptEgr-1 induction in the ELL, prepacemaker nucleus (PPn), and nucleus electrosensorius contradict this simple idea. Neurons in the ELL (first-order electrosensory processing) are known to respond strongly to chirp-evoking stimuli (Krahe et al., 2008; Marsat et al., 2009) yet failed to show any increase in AptEgr-1 mRNA expression (data not shown). Premotor neurons (PPn) that initiate chirping (Kawasaki et al., 1988) were also never labeled for AptEgr-1 transcript (data not shown). These are primary sensory and motor structures, respectively, so it might not be surprising that they failed to express AptEgr-1 after sensory stimulation and evoked chirping.

The n. electrosensorius (within the diencephalon) is believed to be specifically involved in electrocommunication

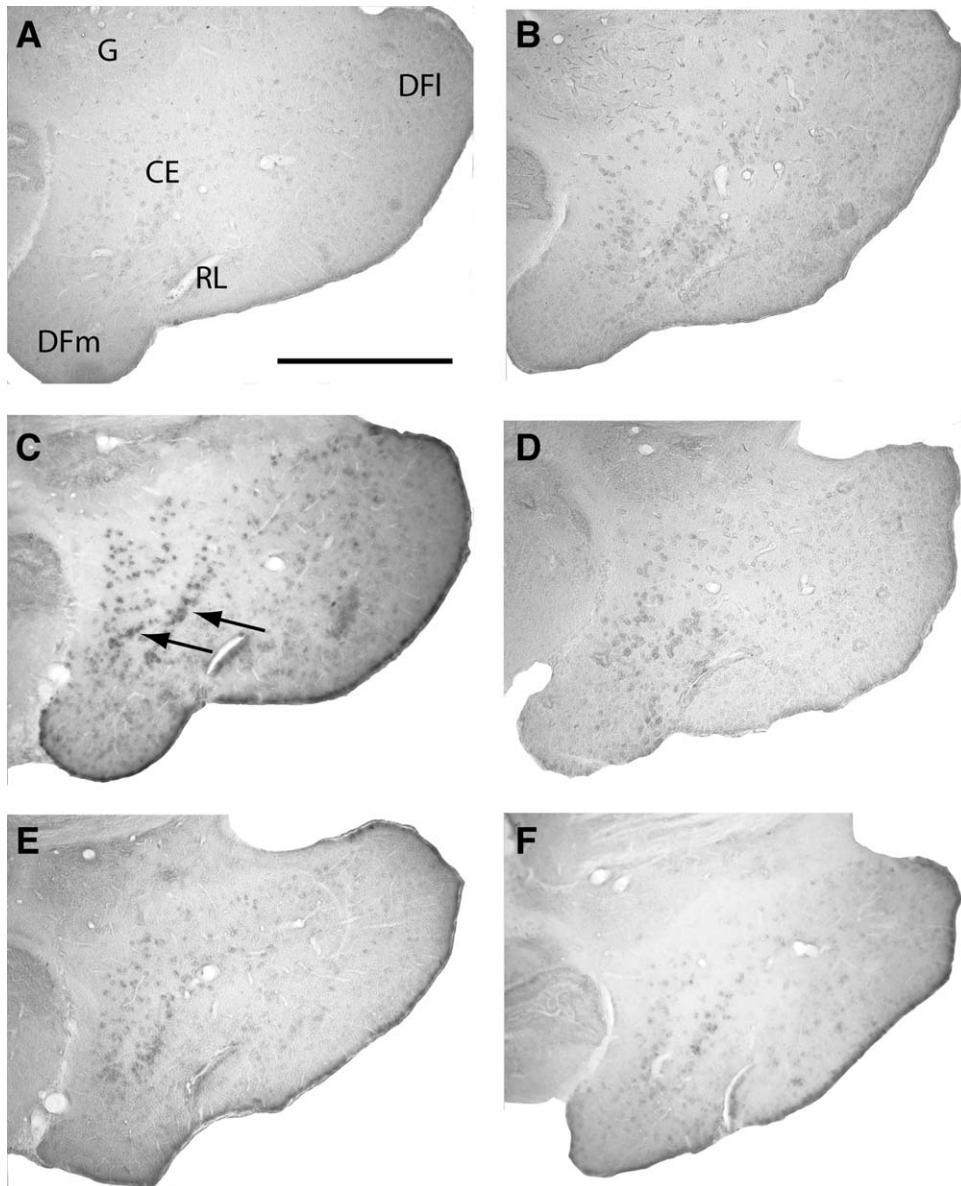


Figure 10. Expression of AptEgr-1 mRNA transcript in the hypothalamus. **A:** Sense control. **B:** Unstimulated control. Weak labeling is seen in cells within the medial aspect of the central nucleus of the hypothalamus (CE). **C:** Stimulated fish. Expression of Apt-Egr-1 mRNA is confined to two strands of cells within medial CE (arrows). **D:** Habituated fish. Expression of AptEgr-1 mRNA in CE is greatly reduced in comparison with the stimulated fish. **E:** Fish stimulated with $DF = -10$ Hz after it has been habituated. Expression of AptEgr-1 mRNA in CE is similar to that of the habituated fish. **F:** Stimulation with $DF = +10$ Hz on the opposite side of the tank (after habituation) fails to increase expression of AptEgr-1 mRNA over levels seen in the control fish. For abbreviations see list. Scale bar = 500 μ m.

(Heiligenberg et al., 1991; Keller, 1988; Keller and Heiligenberg, 1989; Keller et al., 1990), and strong electrophysiological responses to low-frequency beats have been reported for this nucleus (Keller, 1988). We therefore anticipated that there would be AptEgr-1 induction within n. electrosensorius and diligently searched for it. Labeling with the AptEgr-1 probe (under any experimental condition) was, however, never seen in nucleus electrosensorius (data not shown).

We conclude, based on the negative findings described above, that the induction of AptEgr-1 in pallium and diencephalons is unlikely to be due merely to high rates of spike discharge. This is completely consistent with a large body of earlier work as summarized by Clayton (2000).

Brain regions with inconsistent expression of AptEgr-1 mRNA

Although expression of AptEgr-1 mRNA was sometimes observed in scattered neurons within ventral

telencephalon and preoptic area, this was very inconsistent. The brain areas that contained sporadic labeled cells were identified as (see the Maler et al., 1992, Apterontid brain atlas for identification) ventral telencephalon dorsal, intermediate and central divisions, and ventral telencephalon, ventral division plus anterior preoptic area (the demarcation between the latter regions were difficult to distinguish in the *in situ* hybridization material). Neurons in the central posterior nucleus of the diencephalons were also occasionally labeled. There are two important points regarding this inconsistent labeling. First, labeling occurred only in stimulated fish (standard protocol C1–C2) and never in control animals, suggesting that it was associated with stimulation. Second, in cases in which labeling was not seen (the majority of cases), AptEgr-1 was still strongly expressed (in the same tissue sections) within cells of the pallial or diencephalic nuclei described above; this implies that technical failure of the *in situ* hybridization method could not account for the negative results.

Although this sporadic labeling could not be used for statistical analysis, there is reason to believe that it might be of interest. Anatomical studies of the ventral telencephalon and central posterior nucleus suggest connectivity with both AptEgr-1-labeled pallial areas (A. Giassi and L. Maler, unpublished observations) and the prepacemaker nucleus responsible for chirping (Wong, 1997a,b; Zupanc and Maler, 1997). Electrical stimulation of the preoptic region of a related electric fish (*Eigenmannia*) has also been shown to cause modulations of the EOD frequency (Wong, 2000). We therefore suspect that the variability of AptEgr-1 induction in these brain areas relates to the variability of the prior behavioral history and uncontrolled endocrine status of our experimental fish sample (see Materials and Methods). The degree of specificity that might be required for consistent induction of Egr-1 is indicated by a recent paper on a cichlid fish (Burmeister et al., 2005). These authors demonstrated that expression of mRNA of the cichlid homolog of Egr-1 was induced in the preoptic area of a particular subset of males during a very specific type of social encounter. Experiments using Apterontid fish during more naturalistic social interactions (Hupe and Lewis, 2008) and whose endocrine status is better controlled will be required to resolve this important issue.

Labeling with the AptEgr-1 probe was also sometimes seen in the electrosensory dorsal torus semicircularis (TS) and occurred predominantly in its deeper layers (layers 8, 9; Carr and Maler, 1981, 1985). This was of some interest, in that induction of Egr-1 mRNA has been reported to occur in the torus semicircularis of the frog (an auditory region in frogs) in response to conspecific mating calls (Hoke et al., 2004); in addition, Hoke et al. also describe Egr-1 expression in some toral subdivisions

even in unstimulated animals. However, the induction of AptEgr-1 in TS of *Apteronotus* was not in any obvious way driven by the stimulation protocol, because it was also seen in some of the control fish and was not seen in the majority of stimulated fish. Electrophysiological studies of the TS have been confined mostly to its dorsal layers (Fortune and Rose, 2000; Rose and Fortune, 1999); these are the layers in receipt of direct input from ELL (Carr and Maler, 1981). The Apterontid TS is a complex structure with about 50 cell types (Carr and Maler, 1985), and more detailed electrophysiological studies of its deeper layers will be required before suitable stimulation protocols can be developed for examining AptEgr-1 induction within this structure. It will be especially important to examine whether expression of AptEgr-1 is induced in TS in response to specific Apterontid communication signals such as beats and/or chirps. In the sections below, we analyze the effect of various behavioral manipulations on the induction of AptEgr-1 expression only in the consistently labeled regions of pallium (DLd, DC, DDmg, DDi) and diencephalons (TA, CE).

Effects of habituation on AptEgr-1 mRNA expression

If the stimulus-induced increase in AptEgr-1 mRNA expression is linked to learning the DF of the stimulus presented, then it should be reduced in a fish that has already habituated to a specific DF. To test this idea, fish ($N = 5$) were habituated to a final chirp rate $<10\%$ of their initial value (mean = 6.6%, SD = 6.5%). Stimulation of habituated fish (with the same DF = +10 Hz; for details see Materials and Methods) induced only modest but statistically significant increases of AptEgr-1 mRNA expression above control levels in all pallial and diencephalic regions (Figs. 7D, 8, 10D, 11D, 12, Table 2). Of greater interest is that the level of expression attained in habituated fish is far below that seen in fish after the initial stimulation. The extent of reduction in expression (compared with stimulated fish) was similar in DLd, DC, DDi, and the diencephalic nuclei (CE, TA); a significantly greater reduction was observed in DDmg (the region with the highest level of initial activation), where the habituated levels of AptEgr-1 expression were barely above control (Figs. 7D, 8, 13, Tables 2, 3). We can therefore conclude that AptEgr-1 induction is greatest for the initial learning process that occurs during exposure to a mimic EOD and declines greatly after habituation.

Effects of habituation followed by a novel beat frequency on AptEgr-1 mRNA expression

We next tested whether a novel DF stimulus could increase AptEgr-1 mRNA expression in the pallium and

TABLE 3.

Changes in mRNA Expression of AptEgr-1 (Computed From Table 2) Upon Stimulation, Habituation, and Stimulation With a Novel Stimulus After Habituation¹

	DDmg	DLD	DC	DDi	CE	TA
Stimulated, control	42.3 ²	22.4	29.8	27.6	29.1	19.1
Stimulated, habituated	36.8 ²	11.4	19.5	16.8	12.5	9.7
DF = -10 Hz, habituated	32.7 ²	0.8	3.9	2.7	-2.2	-3.9
Stimulated on opposite side, habituated	10.9 ²	-7.5	-1.6	-1.5	-17.5	-4.6
DF = -10 Hz, control	37.8 ²	11.7	14.2	13.6	14.5	5.2
Stimulated on opposite side, control	16.0 ²	3.4	8.7	9.3	-0.8	4.9

¹Note that AptEgr-1 mRNA expression is most increased in DDmg after the initial stimulation (row 1) as well as after the presentation of novel EOD mimic (-10 Hz, rows 3, 5) or novel location (rows 4, 6). AptEgr-1 mRNA expression also decreases maximally in DDmg upon habituation (row 2). The differences between DDmg and the other brain regions were significant ($P < 0.05$ in all cases; Wilcoxon sum rank test). There differences across the other brain regions were not significant.

² $P < 0.05$.

diencephalon of habituated fish. The fish were first habituated so that their chirp count ($N = 8$, $DF = +10$ Hz) was reduced to $<10\%$ of its initial value ($5.8\% \pm 3.2\%$). Stimulation with $DF = -10$ Hz caused, as expected (Fig. 4), recovery to the initial chirp rate ($103.7\% \pm 78.2\%$). In all pallial and diencephalic regions, the novel DF induced AptEgr-1 mRNA expression levels above those of control fish, with the greatest increase in DDmg (Figs. 7E, 8, 10E, 11E, 12, 13, Tables 2, 3), although the expression levels were lower than those resulting from stimulation of naïve fish (Tables 2, 3). However, the novel DF significantly increased AptEgr-1 mRNA expression above that of the habituated fish only within DDmg (Figs. 7D,E, 8, 13, Tables 2, 3); in the other regions (pallium and diencephalon), the expression levels were not different from those of the habituated fish (Figs. 7D, 8, 10D,E, 11D,E, 12, Tables 2, 3). We can therefore conclude that a novel stimulus frequency will induce AptEgr-1 induction beyond that seen in habituated fish only in DDmg.

Effects of habituation followed by a novel spatial location on AptEgr-1 mRNA expression

Work on a different gymnotiform species has suggested that they can discriminate the spatial location of a conspecific (McGregor and Westby, 1992) and induction of Egr-1 expression has been shown to be required for spatial memory in mice (Jones et al., 2001). To determine whether spatial novelty could also induce AptEgr-1 mRNA expression, we used a spatial habituation protocol (see Materials and Methods) and first habituated the fish to a $DF = 10$ Hz presented via a dipole on one side of its tank. After habituation ($8.7\% \pm 8.3\%$ of initial chirp count), we presented the same $DF = 10$ Hz stimulus via a dipole on the contralateral side of the habituated fish ($N = 7$). Although all fish increased their chirp count over their habituated values, the response was quite variable: four

fish did not reach their initial chirp count, one hardly changed, and two substantially increased their chirp count (mean change over habituated values = $125.1\% \pm 134\%$). The fish typically changed their orientation during the course of this experiment, either between trials or between days; this suggests that the increase of chirping was due to the change in stimulus location rather than merely the change in which side of the body was in receipt of the strongest input. AptEgr-1 mRNA expression was significantly increased over habituated values only in DDmg (Figs. 7F, 8, 10F, 11F, 12, 13, Tables 2, 3); in the other brain regions, expression either did not change in comparison with the habituated fish (DC, DDi) or decreased (DLD, CE, TA). We can therefore conclude that a stimulus novel with respect to spatial location will induce AptEgr-1 induction beyond that seen in habituated fish only in DDmg.

Comparison of AptEgr-1 mRNA induction across brain regions

Comparison across activated brain regions (Figs. 8, 12, 13, Tables 2, 3) showed that DDmg had the greatest increase in AptEgr-1 mRNA transcript expression following initial stimulation and the greatest reduction in expression after habituation. After habituation, DDmg also showed the greatest increase in expression subsequent to either stimulation with a different stimulus ($DF = -10$ Hz) or stimulation with the same stimulus ($DF = +10$ Hz) on the opposite side of the body. These differences were significant ($P < 0.05$, Wilcoxon summed rank test for all comparisons), although comparisons across the other brain regions did not show significant differences. Figure 13 summarizes, at a higher magnification, the levels of AptEgr-1 mRNA expression in DDmg across the various behavioral protocols.

We also compared AptEgr-1 expression levels across fish after first pooling all the measurements (separately

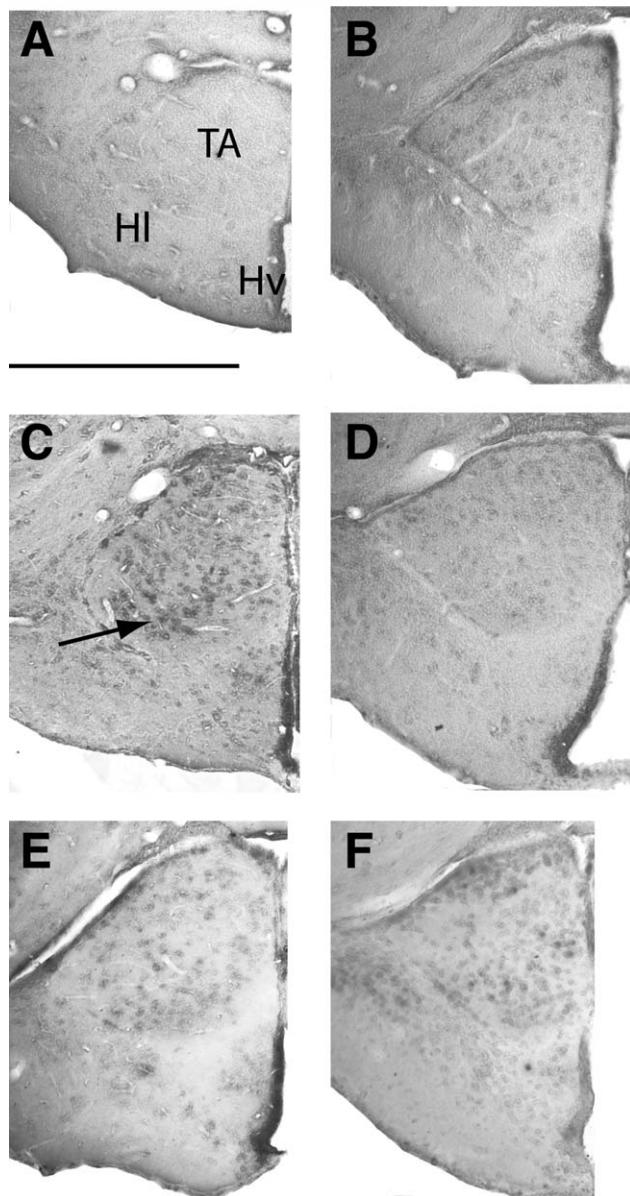


Figure 11. Expression of AptEgr-1 mRNA transcript in the diencephalic nucleus tuberis anterior (TA). A: Sense control. B: Unstimulated control. C: Stimulated fish. Expression of AptEgr-1 mRNA is seen within cells mainly in the ventrolateral aspect of TA (arrow). D: Habituated fish. Expression of AptEgr-1 mRNA is greatly reduced in TA compared with the stimulated fish. E: Fish stimulated with $DF = -10$ Hz after it has been habituated. Expression of AptEgr-1 mRNA in TA is similar to that of the habituated fish. F: Stimulation with $DF = +10$ Hz on the opposite side of the tank (after habituation) fails to increase expression of AptEgr-1 mRNA over levels seen in the control fish. For abbreviations see list. Scale bar = 500 μ m.

for the various brain regions) within each fish. The mean values were, as expected, very similar to those reported in Table 2 (data not shown), so the major difference for this analysis was the large reduction in effective sample

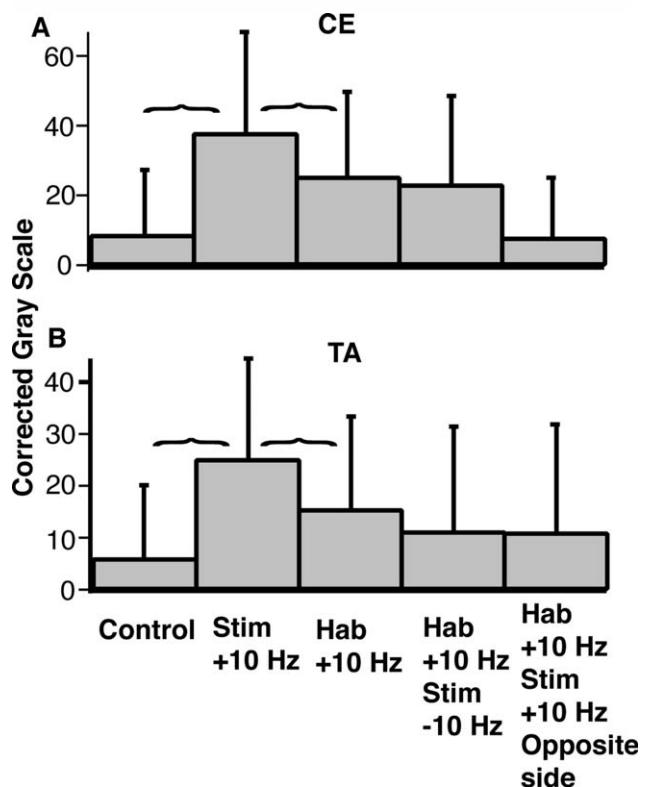


Figure 12. Bar graphs illustrating the corrected gray levels (non-radioactive in situ hybridization) of cells within the diencephalon (central nucleus or CE and nucleus tuberis anterior or TA) where stimulation induced the expression of AptEgr-1 mRNA; the various experimental conditions are shown on the x axis. The braces indicate significant differences. The statistical values corresponding to this figure are completely reported in Table 2.

size (the number of fish for each behavioral treatment). Although our major conclusions were upheld, the lower sample size meant that some comparisons no longer reached statistical significance. In all brain regions mentioned above, stimulation ($DF = +10$ Hz) significantly increased AptEgr-1 expression above that of the control fish (at the $P = 0.05$ level, Tukey's post hoc comparison in all cases). However only in DDmg, DC, and TA were the expression levels following stimulation significantly higher than those of the habituated fish. In DDmg (but not the other brain regions) stimulation of a habituated fish with a -10 Hz DF significantly increased AptEgr-1 expression over that of the habituated fish. Presentation of the $+10$ Hz stimulus at a spatial location different from that at which habituation had taken place no longer caused a significant increase in AptEgr-1 expression for any brain region.

We therefore conclude that, even after this much more stringent statistical analysis, stimulation increases AptEgr-1 expression in all the brain regions above control

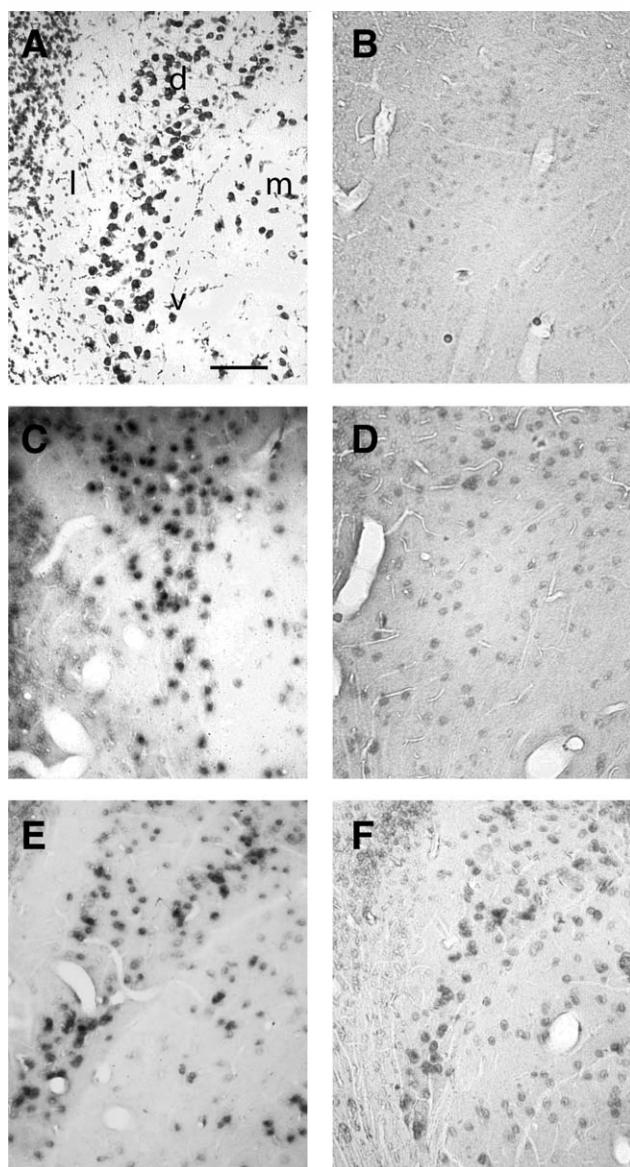


Figure 13. A: DDmg as seen in a cresyl violet-stained transverse section. B–F: Higher magnification views of DDmg under the various experimental conditions. B: Control. C: Stimulated. D: Habituated. E: Habituated to $DF = +10$ Hz and then stimulated with $DF = -10$ Hz. F: Habituated to $DF = +10$ Hz and then given the same stimulus but with a dipole on the opposite side (see Fig. 1C). Note that DDmg cells strongly express AptEgr-1 mRNA after stimulation and that expression is greatly reduced after habituation but is again strong after the presentation of a novel stimulus ($DF = -10$ Hz). A spatially novel stimulus (F) produces a far weaker increase in expression over control or habituated fish DDmg. For abbreviations see list. Scale bar = 100 μ m.

levels, and in some regions (DDmg, DC, and TA) increases it above habituated levels. Furthermore, presentation of a novel stimulus ($DF = -10$ Hz, thus with respect to stimulus frequency) also increases AptEgr-1 expression in DDmg (only). The effect of spatial novelty (in DDmg) was

not upheld in this analysis. This is consistent with the relatively smaller increase in AptEgr-1 expression after this treatment (Figs. 7F, 8, 13, Tables 2, 3) and the highly variable effect of this behavioral protocol on evoked chirping (see above). Further work will be required to determine the conditions under which *Apteronotus* can more robustly learn the location of a conspecific.

DISCUSSION

We used habituation of a stimulus-evoked chirp response to demonstrate that *A. leptorhynchus* can remember a specific mimic EOD frequency and its location over both short (minutes) and long (days) time frames. In an ethological context, our results suggest that these electric fish might be able to identify individual conspecifics based their EOD frequency. Moortgat et al. (1998) have shown that the EOD frequency can remain stable over a period of several hours but that, over longer time frames, can drift due to changes in the water temperature. Over long time periods (days), there may well be consistent temperature drift or even changes in the hormonal/behavioral status of individual fish that cause large differential changes in their EOD frequencies. Further studies will therefore be required to determine the duration of long-term individual recognition under more natural conditions.

Our results also suggest that *Apteronotus* can also remember the usual location of individual conspecifics; the greater variability of this effect also suggests that this form of spatial learning is less robust than is the learning of EOD frequency alone. This conclusion is restricted to the identification of male–male recognition, because our behavioral memory measure was based on evoked small chirps, and these occur at high rates only in male fish (Dulka et al., 1995; Zupanc and Maler, 1993). However, recent studies on mate choice by females suggest that they also can recognize males based on much higher differences in EOD frequencies (Bargelletti, Gogarten and Krahe, personal communication). Earlier work has already suggested that a different gymnotiform fish (*Gymnotus carapo*, a pulse species; Bullock, 1969) can also remember the identity and location of conspecifics based on their EOD shape (McGregor and Westby, 1992). More generally, there is evidence that many teleost species can learn to recognize conspecifics (Griffiths, 2003). Learned recognition of individual conspecifics may therefore not be confined to mammals and songbirds but may be a widespread vertebrate ability; it is tempting to speculate that the main difference between learned recognition in electric fish vs. that in songbirds and primates might lie in the far simpler nature of electric communication signals. Our data on AptEgr-2 mRNA expression

further suggest that the dorsal telencephalon is involved in learned recognition by *Apteronotus*, as appears also to be the case for songbirds and primates (Gentner, 2004; Ghazanfar and Hauser, 2001; Petkov et al., 2008). The possible relationship between pallial structures of electric fish and other vertebrates is discussed below. It will be important, in this regard, to determine whether learned recognition in other electric or nonelectric fish is also associated with pallial structures.

We have demonstrated both short- and long-term habituation of chirping, and our data also strongly suggest that they are dependent on different cellular/molecular mechanisms. First, the kinetics of short-term habituation are negatively correlated with initial chirp rate (fish that chirp more tend to habituate more slowly over a minutes time scale), whereas long-term habituation kinetics are positively correlated with initial chirp rates (fish that chirp more tend to habituate more rapidly over a days time scale). Second, short- and long-term habituation can be dissociated: cooling the head after stimulation has no effect on the next day's short-term habituation but prevents long-term habituation. Finally, stimulation is correlated with a delayed change in expression of AptEgr-1, and this IEG might therefore play a role in long- but not short-term chirp habituation (see below). Short-term habituation is clearly evident by the fourth stimulus presentation (after ~12 minutes; Fig. 2A). AptEgr-1 mRNA is first detected at ~30 minutes after the end of stimulation (unpublished observations), and AptEgr-1 protein will be expressed after even longer times. We therefore conclude that AptEgr-1 is unlikely to be important for the short-term memory of the mimic EOD. It is also conceivable that short- and long-term habituations are implemented by entirely distinct neural structures as well as via different molecular mechanisms. We propose a more parsimonious hypothesis, that short- and long-term habituation utilize different cellular/molecular mechanisms but are both dependent on the same pallial structures.

We have shown that the conversion of short- to long-term memory is a temperature-dependent process and hypothesize that it represents a form of memory consolidation. We then cloned the Apterontid homolog of the Egr-1 gene; the expression of this IEG has been associated with learning and memory in both birds (Bolhuis et al., 2000; Jarvis et al., 1995; Terpstra et al., 2004) and mammals (Jones et al., 2001), and it has been suggested that its induction is important for consolidation and/or reconsolidation (Davis et al., 2003; Knapska and Kaczmarek, 2004). Stimulation that induced chirping also induced extensive expression of AptEgr-1 mRNA in specific pallial regions and in two small diencephalic nuclei. AptEgr-1 expression was not reliably induced in the sen-

sory and motor regions known to be active under these stimulus conditions, suggesting that it is not merely a marker for strongly spiking neurons. The activated diencephalic regions are known to connect to nuclei directly associated with electrocommunication behavior (Giassi et al., 2007; Keller et al., 1990), although their function is not known. There also appear to be several routes by which electrocommunication signal can reach the pallium (Correa and Zupanc, 2002; Keller et al., 1990; Zupanc, 1997; Zupanc and Correa, 2005; Zupanc and Horschke, 1997). Previous studies in other teleosts have implicated the pallium in learning (Portavella et al., 2004; Rodriguez et al., 2002) dependent on N-methyl-D-aspartate receptors (Gomez et al., 2006). We have previously shown that the AptEgr-1-expressing pallial regions of *A. leptorhynchus* have high levels of expression of proteins known to be important for learning and memory (Harvey-Girard et al., 2007; Maler and Hincke, 1999; Maler, 1999a,b). We therefore hypothesize that the pallial regions expressing AptEgr-1 mRNA are the sites for long-term memory storage of the identity (via EOD frequency) and location of conspecifics; it should be noted that these regions make up most of the pallium of *Apteronotus* (Maler et al., 1991).

The association of AptEgr-1 expression and long-term chirp habituation is, at present, merely a correlation. We hypothesize, but have not demonstrated, that AptEgr-1 expression plays a causal role in the conversion of short-term to long-term chirp habituation; we do note, however, that a causal link between Egr-1 expression and learning and memory is strongly supported by studies in rodents (see references above). We also cannot rule out the possibility that lower sensory and/or motor brain regions not expressing stimulus-dependent AptEgr-1 mRNA might also be involved in chirp habituation; these regions might utilize entirely different molecular mechanisms for learning and memory. Evidence for the involvement of both lower and higher brain centers in habituation has been presented in the literature. In *Aplysia*, habituation of the gill/siphon withdrawal reflex is mediated directly by cellular changes in sensory-motor synapses (Cohen et al., 1997; Frost et al., 1997). In contrast, habituation of the crayfish escape response appears to be dependent on "higher centers" as opposed to changes in the efficacy of local sensory-motor synapses (Krasne and Teshiba, 1995). In the rodent, habituation of the orienting reflex appears to be dependent on the retrosplenial cortex (Kwon et al., 1990). For *Apteronotus*, neuroanatomical studies suggest that the neural basis for chirp habituation is likely to be complex. The dorsocentral pallium projects strongly to the torus semicircularis, the midbrain electro-sensory processing region (Correa et al., 1998; A. Giassi and L. Maler, unpublished observations). The TS in turn

projects to and can influence processing in hindbrain electrosensory structures (Bell and Maler, 2005). Therefore, even if induction of AptEgr-1 expression in dorsal telencephalon were causal to long-term chirp habituation, it might still be associated with changes in sensory processing in the ascending electrosensory system.

DDmg shows the highest increase in AptEgr-1 mRNA expression to the initial presentation of the stimulus and the greatest decline after habituation; it also is the only region that shows strong AptEgr-1 mRNA induction to novel electrosensory stimuli. Although we did not study this systematically, we also found that expression of AptEgr-1 mRNA transcripts was induced in DDmg (but not the other regions) after stimulation with acoustic stimuli (unpublished observations). We propose that the small numbers of DDmg neurons function as novelty detectors; recent electrophysiological studies are consistent with this interpretation (B. Elliott and L. Maler, unpublished observations). It is interesting that induction of Egr-1 (and other IEGs) has, in other systems, already been suggested to be a response to novel and significant stimuli (Clayton, 2000). The very simple and controllable stimuli used in electric fish communication may allow for deeper insights into what makes a stimulus “novel” and “significant.”

Novel stimuli (either DF or location; see Tables 2, 3) did not increase the mean expression levels of AptEgr-1 mRNAs in DLd, DDi, or DC. It is possible that the original high rates of AptEgr-1 expression reflect learning about many features of the novel experimental perturbation. We propose that subsequent presentation of a novel stimulus may induce expression of AptEgr-1 in only a small percentage of the large numbers of neurons in these regions; this is especially true of DLd, by far the largest pallial region in *A. leptorhynchus* (Maler et al., 1991). Averaging would prevent us from detecting such activation; more sensitive measures will be required to determine whether networks in DLd (and/or DC, DDi) are the site(s) of long-term memory storage in the Apterontotid brain.

The formation of the teleost telencephalon by eversion of the neural tube has presented great difficulties for comparative analyses of its function. Recent studies have, however, converged on the conclusion that DLd is either in part (Yamamoto et al., 2007) or entirely (Nieuwenhuys, 2009; Northcutt, 2006, 2008; Wullimann and Mueller, 2004) homologous to the medial pallium of tetrapods and therefore the hippocampal formation of mammals; the possible relation of DDi, DDmg, and DC to telencephalic structures of tetrapods remains unclear. Behavioral studies are also consistent with this interpretation (Rodriguez et al., 2002). For mammals, very extensive research has implicated Egr-1 in LTP and various forms of learning associated with many regions of the tel-

encephalon, including the hippocampus (Abraham et al., 1993; Bozon et al., 2002, 2003a,b; Frankland et al., 2004; Guzowski et al., 2001; Jones et al., 2001; Knapska and Kaczmarek, 2004; Lee et al., 2004; Malkani et al., 2004; Poirier et al., 2008). Although Egr-1 clearly plays an important role in learning, its the exact role in consolidation/reconsolidation has not yet been definitively established (Davis et al., 2003). The simple nature of electric communication signals and relatively simple structure of the electric fish telencephalon make it an attractive system for investigating the links among neural activity, Egr-1 expression, and storage of long-term memories in medial pallium.

Our results on AptEgr-1 induction by communication signals are also similar to those reported for other vertebrate classes. In cichlid fish, perception of social opportunity induces expression of Egr-1 mRNA in subpallial regions (Burmeister et al., 2005). In frogs, pallial expression of Egr-1 can also be induced by presentation of species-specific vocalizations (Mangiameli and Burmeister, 2008). The most remarkable similarities are seen in songbirds. Expression of Egr-1 mRNA is induced in songbird pallium upon presentation of a conspecific’s song (Bailey et al., 2002; Liu and Nottebohm, 2005; Mello and Clayton, 1994; Mello et al., 1992). Continued presentation of the same song results in a decline of Egr-1 expression, but presentation of a new song (Mello et al., 1995) or of the same song in a new location (Kruse et al., 2004) causes pallial Egr-1 expression again to increase. It will be interesting to examine whether induction of pallial Egr-1 expression is also associated with long-term memory of communication signals and individual recognition in other vertebrates as well.

LITERATURE CITED

- Abraham WC, Mason SE, Demmer J, Williams JM, Richardson CL, Tate WP, Lawlor PA, Dragunow M. 1993. Correlations between immediate early gene induction and the persistence of long-term potentiation. *Neuroscience* 56:717–727.
- Bailey DJ, Rosebush JC, Wade J. 2002. The hippocampus and caudomedial neostriatum show selective responsiveness to conspecific song in the female zebra finch. *J Neurobiol* 52: 43–51.
- Bastian J. 1981. Electrotlocation I. How the electroreceptors of *Apteronotus albifrons* code for moving objects and other electrical stimuli. *J Comp Physiol A* 144:465–479.
- Bastian J, Schneiderjen S, Nguyenkim J. 2001. Arginine vasotocin modulates a sexually dimorphic communication behavior in the weakly electric fish, *Apteronotus leptorhynchus*. *J Exp Biol* 204:1909–1923.
- Baumgartel K, Genoux D, Welzl H, Tweedie-Cullen RY, Koshibu K, Livingstone-Zatchej M, Mamie C, Mansuy IM. 2008. Control of the establishment of aversive memory by calcineurin and Zif268. *Nat Neurosci* 11:572–578.
- Beckmann AM, Wilce PA. 1997. Egr transcription factors in the nervous system. *Neurochem Int* 31:477–510; discussion 517–476.

- Bee M, Gerhardt H. 2002. Individual voice recognition in a territorial frog (*Rana catesbeiana*). *Proc R Soc Lond B Biol Sci* 269:1443–1448.
- Bell C, Maler L. 2005. Central neuroanatomy of electrosensory systems in fish. In: Bullock TH, Hopkins C, editors. *Electroreception*. New York: Springer. p 68–111.
- Benda J, Longtin A, Maler L. 2005. Spike-frequency adaptation separates transient communication signals from background oscillations. *J Neurosci* 25:2312–2321.
- Benda J, Longtin A, Maler L. 2006. A synchronization-desynchronization code for natural communication signals. *Neuron* 52:347–358.
- Bolhuis JJ, Zijlstra GG, den Boer-Visser AM, Van Der Zee EA. 2000. Localized neuronal activation in the zebra finch brain is related to the strength of song learning. *Proc Natl Acad Sci USA* 97:2282–2285.
- Bottai D, Dunn R, Ellis W, Maler L. 1997. N-methyl-D-aspartate receptor 1 mRNA distribution in the central nervous system of the weakly electric fish *Apteronotus leptorhynchus*. *J Comp Neurol* 389:65–80.
- Bottai D, Maler L, Dunn R. 1998. Alternative RNA splicing of the NMDA receptor NR1 mRNA in the neurons of the teleost electrosensory system. *J Neurosci* 18:5191–5202.
- Bozon B, Davis S, Laroche S. 2002. Regulated transcription of the immediate-early gene Zif268: mechanisms and gene dosage-dependent function in synaptic plasticity and memory formation. *Hippocampus* 12:570–577.
- Bozon B, Davis S, Laroche S. 2003a. A requirement for the immediate early gene zif268 in reconsolidation of recognition memory after retrieval. *Neuron* 40:695–701.
- Bozon B, Kelly A, Josselyn S, Silva A, Davis S, Laroche S. 2003b. MAPK, CREB and ZIF268 are all required for the consolidation of recognition memory. In: Bliss T, Collingridge G, Morris R, editors. *LTP*. Oxford, UK: Oxford University Press. p 329–345.
- Bullock TH. 1969. Species differences in effect of electroreceptor input on electric organ pacemakers and other aspects of behaviour in electric fish. *Brain Behav Evol* 2: 85–118.
- Burmeister SS, Fernald RD. 2005. Evolutionary conservation of the egr-1 immediate-early gene response in a teleost. *J Comp Neurol* 481:220–232.
- Burmeister SS, Jarvis ED, Fernald RD. 2005. Rapid behavioral and genomic responses to social opportunity. *PLoS Biol* 3: e363.
- Carr CE, Maler L. 1981. Laminar organization of the afferent and efferent systems of the torus semicircularis of gymnotiform fish: morphological substrates for parallel processing in the electrosensory system. *J Comp Neurol* 203: 649–670.
- Carr CE, Maler L. 1985. A Golgi study of the cell types of the dorsal torus semicircularis of the electric fish *Eigenmannia*: functional and morphological diversity in the midbrain. *J Comp Neurol* 235:207–240.
- Clayton DF. 2000. The genomic action potential. *Neurobiol Learn Mem* 74:185–216.
- Cohen TE, Kaplan SW, Kandel ER, Hawkins RD. 1997. A simplified preparation for relating cellular events to behavior: mechanisms contributing to habituation, dishabituation, and sensitization of the *Aplysia* gill-withdrawal reflex. *J Neurosci* 17:2886–2899.
- Correa SA, Zupanc GK. 2002. Connections between the central posterior/prepacemaker nucleus and hypothalamic areas in the weakly electric fish *Apteronotus leptorhynchus*: evidence for an indirect, but not a direct, link. *J Comp Neurol* 442:348–364.
- Correa SA, Grant K, Hoffmann A. 1998. Afferent and efferent connections of the dorsocentral telencephalon in an electrosensory teleost, *Gymnotus carapo*. *Brain Behav Evol* 52: 81–98.
- Davis S, Bozon B, Laroche S. 2003. How necessary is the activation of the immediate early gene zif268 in synaptic plasticity and learning? *Behav Brain Res* 142:17–30.
- Dong S, Replinger KL, Hasadsri L, Imai BS, Yau PM, Rodriguez-Zas S, Southey BR, Sweedler JV, Clayton DF. 2009. Discrete molecular states in the brain accompany changing responses to a vocal signal. *Proc Natl Acad Sci USA* 106: 11364–11369.
- Dulka JG, Maler L, Ellis W. 1995. Androgen-induced changes in electrocommunicatory behavior are correlated with changes in substance P-like immunoreactivity in the brain of the electric fish *Apteronotus leptorhynchus*. *J Neurosci* 15:1879–1890.
- Dunlap K, Pelczar P, Knapp R. 2002. Social interactions and cortisol treatment increase the production of aggressive electrocommunication signals in male electric fish *Apteronotus leptorhynchus*. *Horm Behav* 42:97.
- Ellis LD, Mehaffey WH, Harvey-Girard E, Turner RW, Maler L, Dunn RJ. 2007. SK channels provide a novel mechanism for the control of frequency tuning in electrosensory neurons. *J Neurosci* 27:9491–9502.
- Ellis LD, Maler L, Dunn RJ. 2008. Differential distribution of SK channel subtypes in the brain of the weakly electric fish *Apteronotus leptorhynchus*. *J Comp Neurol* 507:1964–1978.
- Engler G, Zupanc GK. 2001. Differential production of chirping behavior evoked by electrical stimulation of the weakly electric fish *Apteronotus leptorhynchus*. *J Comp Physiol A* 187:274–256.
- Fortune ES. 2006. The decoding of electrosensory systems. *Curr Opin Neurobiol* 16:474–480.
- Fortune ES, Rose GJ. 2000. Short-term synaptic plasticity contributes to the temporal filtering of electrosensory information. *J Neurosci* 20:7122–7130.
- Frankland PW, Bontempi B, Talton LE, Kaczmarek L, Silva AJ. 2004. The involvement of the anterior cingulate cortex in remote contextual fear memory. *Science* 304:881–883.
- Frost L, Kaplan SW, Cohen TE, Henzi V, Kandel ER, Hawkins RD. 1997. A simplified preparation for relating cellular events to behavior: contribution of LE and unidentified siphon sensory neurons to mediation and habituation of the *Aplysia* gill- and siphon-withdrawal reflex. *J Neurosci* 17:2900–2913.
- Gentner TQ. 2004. Neural systems for individual song recognition in adult birds. *Ann N Y Acad Sci* 1016:282–302.
- Ghazanfar AA, Hauser MD. 2001. The auditory behaviour of primates: a neuroethological perspective. *Curr Opin Neurobiol* 11:712–720.
- Giassi AC, Correa SA, Hoffmann A. 2007. Fiber connections of the diencephalic nucleus tuberis anterior in the weakly electric fish *Gymnotus cf. carapo*: an in vivo tract-tracing study. *J Comp Neurol* 503:655–667.
- Gomez Y, Vargas JP, Portavella M, Lopez JC. 2006. Spatial learning and goldfish telencephalon NMDA receptors. *Neurobiol Learn Mem* 85:252–262.
- Goethard KM, Brooks KN, Peterson MA. 2009. Multiple perceptual strategies used by macaque monkeys for face recognition. *Anim Cogn* (in press).
- Griffiths S. 2003. Learned recognition of conspecifics by fishes. *Fish Fisheries* 4:256–268.
- Gussin D, Benda J, Maler L. 2007. Limits of linear rate coding of dynamic stimuli by electroreceptor afferents. *J Neurophysiol* 97:2917–2929.
- Guzowski JF, Setlow B, Wagner EK, McGaugh JL. 2001. Experience-dependent gene expression in the rat hippocampus after spatial learning: a comparison of the immediate-early genes Arc, c-fos, and zif268. *J Neurosci* 21:5089–5098.

- Harvey-Girard E, Dunn RJ, Maler L. 2007. Regulated expression of N-methyl-D-aspartate receptors and associated proteins in teleost electrosensory system and telencephalon. *J Comp Neurol* 505:644–668.
- Heiligenberg W, Keller CH, Metzner W, Kawasaki M. 1991. Structure and function of neurons in the complex of the nucleus electrosensorius of the gymnotiform fish *Eigenmannia*: Detection and processing of electric signals in social communication. *J Comp Physiol A Sens Neural Behav Physiol* 169:151–164.
- Hoke KL, Burmeister SS, Fernald RD, Rand AS, Ryan MJ, Wilczynski W. 2004. Functional mapping of the auditory midbrain during mate call reception. *J Neurosci* 24: 11264–11272.
- Hupe GJ, Lewis JE. 2008. Electrocommunication signals in free swimming brown ghost knifefish, *Apteronotus leptorhynchus*. *J Exp Biol* 211:1657–1667.
- Jarvis ED, Mello CV, Nottebohm F. 1995. Associative learning and stimulus novelty influence the song-induced expression of an immediate early gene in the canary forebrain. *Learn Mem* 2:62–80.
- Johnston R, Bullock T. 2001. Individual recognition by the use of odours in golden hamsters: the nature of individual representations. *Anim Behav* 61:545–557.
- Jones MW, Errington ML, French PJ, Fine A, Bliss TV, Garel S, Charnay P, Bozon B, Laroche S, Davis S. 2001. A requirement for the immediate early gene Zif268 in the expression of late LTP and long-term memories. *Nat Neurosci* 4: 289–296.
- Kawasaki M, Maler L, Rose G, Heiligenberg W. 1988. Anatomical and functional organization of the prepacemaker nucleus in gymnotiform electric fish: the accommodation of two behaviors in one nucleus. *J Comp Neurol* 276: 113–131.
- Keller CH. 1988. Stimulus discrimination in the diencephalon of *Eigenmannia*: the emergence and sharpening of a sensory filter. *J Comp Physiol A Sens Neural Behav Physiol* 162:747–757.
- Keller CH, Heiligenberg W. 1989. From distributed sensory processing to discrete motor representations in the diencephalon of the electric fish *Eigenmannia*. *J Comp Physiol A Sens Neural Behav Physiol* 164:565–576.
- Keller CH, Maler L, Heiligenberg W. 1990. Structural and functional organization of a diencephalic sensory-motor interface in the gymnotiform fish *Eigenmannia*. *J Comp Neurol* 293:347–376.
- Knapska E, Kaczmarek L. 2004. A gene for neuronal plasticity in the mammalian brain: Zif268/Egr-1/NGFI-A/Krox-24/TIS8/ZENK? *Prog Neurobiol* 74:183–211.
- Krahe R, Bastian JA, Chacron MJ. 2008. Temporal processing across multiple topographic maps in the electrosensory system. *J Neurophysiol* 100:852–867.
- Krasne FB, Teshiba TM. 1995. Habituation of an invertebrate escape reflex due to modulation by higher centers rather than local events. *Proc Natl Acad Sci USA* 92:3362–3366.
- Kruse AA, Stripling R, Clayton DF. 2004. Context-specific habituation of the zenk gene response to song in adult zebra finches. *Neurobiol Learn Mem* 82:99–108.
- Kwon SE, Nadeau SE, Heilman KM. 1990. Retrosplenial cortex: possible role in habituation of the orienting response. *J Neurosci* 10:3559–3563.
- Lee JL, Everitt BJ, Thomas KL. 2004. Independent cellular processes for hippocampal memory consolidation and reconsolidation. *Science* 304:839–843.
- Lee S, Maler L, Dunn RJ. 2000. Differential expression of the PSD-95 gene family in electrosensory neurons. *J Comp Neurol* 426:429–440.
- Liu WC, Nottebohm F. 2005. Variable rate of singing and variable song duration are associated with high immediate early gene expression in two anterior forebrain song nuclei. *Proc Natl Acad Sci USA* 102:10724–10729.
- Mactutus CF, Riccio DC, Ferek JM. 1979. Retrograde amnesia for old (reactivated) memory: some anomalous characteristics. *Science* 204:1319–1320.
- Maler L. 1999a. The distribution of adenylate cyclase in the brain of *Apteronotus leptorhynchus* as revealed by forskolin binding. *J Comp Neurol* 408:170–176.
- Maler L. 1999b. The distribution of protein kinase C in the brain of *Apteronotus leptorhynchus* as revealed by phorbol ester binding. *J Comp Neurol* 408:161–169.
- Maler L. 2007. Neural strategies for optimal processing of sensory signals. *Prog Brain Res* 165:135–154.
- Maler L, Ellis WG. 1987. Inter-male aggressive signals in weakly electric fish are modulated by monoamines. *Behav Brain Res* 25:75–81.
- Maler L, Hincke M. 1999. The distribution of calcium/calmodulin-dependent kinase 2 in the brain of *Apteronotus leptorhynchus*. *J Comp Neurol* 408:177–203.
- Maler L, Sas E, Johnston S, Ellis W. 1991. An atlas of the brain of the weakly electric fish *Apteronotus leptorhynchus*. *J Chem Neuroanat* 4:1–38.
- Malkani S, Wallace KJ, Donley MP, Rosen JB. 2004. An egr-1 (zif268) antisense oligodeoxynucleotide infused into the amygdala disrupts fear conditioning. *Learn Mem* 11: 617–624.
- Mangiamele LA, Burmeister SS. 2008. Acoustically evoked immediate early gene expression in the pallium of female tungara frogs. *Brain Behav Evol* 72:239–250.
- Marsat G, Provile R, Maler L. 2009. Transient signals trigger synchronous bursts in an identified population of neurons. *J Neurophysiol* 102:714–723.
- McGregor P, Westby G. 1992. Discrimination of individually characteristic electric organ discharge by a weakly electric fish. *Anim Behav* 43:977–986.
- Mello CV, Clayton DF. 1994. Song-induced ZENK gene expression in auditory pathways of songbird brain and its relation to the song control system. *J Neurosci* 14:6652–6666.
- Mello CV, Vicario DS, Clayton DF. 1992. Song presentation induces gene expression in the songbird forebrain. *Proc Natl Acad Sci USA* 89:6818–6822.
- Mello C, Nottebohm F, Clayton D. 1995. Repeated exposure to one song leads to a rapid and persistent decline in an immediate early gene's response to that song in zebra finch telencephalon. *J Neurosci* 15:6919–6925.
- Meyer JH, Leong M, Keller CH. 1987. Hormone-induced and maturational changes in electric organ discharges and electroreceptor tuning in the weakly electric fish *Apteronotus*. *J Comp Physiol A* 160:385–394.
- Moortgat KT, Keller CH, Bullock TH, Sejnowski TJ. 1998. Submicrosecond pacemaker precision is behaviorally modulated: the gymnotiform electromotor pathway. *Proc Natl Acad Sci USA* 95:4684–4689.
- Nelson ME, Xu Z, Payne JR. 1997. Characterization and modeling of P-type electrosensory afferent responses to amplitude modulations in a wave-type electric fish. *J Comp Physiol A Sens Neural Behav Physiol* 181:532–544.
- Nieuwenhuys R. 2009. The forebrain of actinopterygians revisited. *Brain Behav Evol* 73:229–252.
- Nieuwenhuys R, Meek J, editors. 1990. The telencephalon of Actinopterygian fishes. New York: Plenum Press. p 31–73.
- Northcutt R. 1995. The forebrain of Gnathostomes: in search of a morphotype. *Brain Behav Evol* 46:275–318.
- Northcutt RG. 2006. Connections of the lateral and medial divisions of the goldfish telencephalic pallium. *J Comp Neurol* 494:903–943.

- Northcutt RG. 2008. Forebrain evolution in bony fishes. *Brain Res Bull* 75:191–205.
- Pascalis O, Bachevalier J. 1998. Face recognition in primates: a cross-species study. *Behav Process* 43:87–96.
- Petkov CI, Kayser C, Steudel T, Whittingstall K, Augath M, Logothetis NK. 2008. A voice region in the monkey brain. *Nat Neurosci* 11:367–374.
- Poirier R, Cheval H, Mailhes C, Garel S, Charnay P, Davis S, Laroche S. 2008. Distinct functions of egr gene family members in cognitive processes. *Front Neurosci* 2:47–55.
- Portavella M, Torres B, Salas C. 2004. Avoidance response in goldfish: emotional and temporal involvement of medial and lateral telencephalic pallium. *J Neurosci* 24:2335–2342.
- Renaudineau S, Poucet B, Laroche S, Davis S, Save E. 2009. Impaired long-term stability of CA1 place cell representation in mice lacking the transcription factor zif268/egr1. *Proc Natl Acad Sci USA* 106:11771–11775.
- Rodriguez F, Lopez JC, Vargas JP, Gomez Y, Broglie C, Salas C. 2002. Conservation of spatial memory function in the pallial forebrain of reptiles and ray-finned fishes. *J Neurosci* 22:2894–2903.
- Rose GJ, Fortune ES. 1999. Frequency-dependent PSP depression contributes to low-pass temporal filtering in *Eigenmannia*. *J Neurosci* 19:7629–7639.
- Saffan D, Cole A, Worley P, Christy B, Ryder K, Baraban J. 1988. Convulsant-induced increase in transcription factor messenger RNAs in rat brain. *Proc Natl Acad Sci USA* 85: 7795–7799.
- Sas E, Maler L, Weld M. 1993. Connections of the olfactory bulb in the gymnotiform fish, *Apteronotus leptorhynchus*. *J Comp Neurol* 335:486–507.
- Stoddard P, Beecher M, Horning C, Cambell S. 1991. Recognition of individual neighbors by song in the song sparrow, a species with song repertoires. *Behav Ecol Sociobiol* 29: 211–215.
- Terpstra NJ, Bolhuis JJ, den Boer-Visser AM. 2004. An analysis of the neural representation of birdsong memory. *J Neurosci* 24:4971–4977.
- Triefenbach F, Zakon H. 2003. Effects of sex, sensitivity and status on cue recognition in the weakly electric fish *Apteronotus leptorhynchus*. *Anim Behav* 65:19–28.
- Wong CJH. 1997a. Afferent and efferent connections of the diencephalic prepacemaker nucleus in the weakly electric fish, *Eigenmannia viriscens*: interactions between the electromotor system and the neuroendocrine axis. *J Comp Neurol* 383:18–41.
- Wong CJH. 1997b. Connections of the basal forebrain of the weakly electric fish, *Eigenmannia viriscens*. *J Comp Neurol* 389:49–64.
- Wong CJ. 2000. Electrical stimulation of the preoptic area in *Eigenmannia*: evoked interruptions in the electric organ discharge. *J Comp Physiol A* 186:81–93.
- Wullimann MF, Mueller T. 2004. Teleostean and mammalian forebrains contrasted: Evidence from genes to behavior. *J Comp Neurol* 475:143–162.
- Yamamoto N, Ishikawa Y, Yoshimoto M, Xue HG, Bahavar N, Sawai N, Yang CY, Ozawa H, Ito H. 2007. A new interpretation on the homology of the teleostean telencephalon based on hodology and a new eversion model. *Brain Behav Evol* 69:96–104.
- Zupanc GK. 1997. The preglomerular nucleus of gymnotiform fish: relay station for conveying information between telencephalon and diencephalon. *Brain Res* 761:179–191.
- Zupanc GK, Correa SA. 2005. Reciprocal neural connections between the central posterior/prepacemaker nucleus and nucleus G in the gymnotiform fish *Apteronotus leptorhynchus*. *Brain Behav Evol* 65:14–25.
- Zupanc GK, Horschke I. 1997. Reciprocal connections between the preglomerular nucleus and the central posterior/prepacemaker nucleus in the diencephalon of weakly electric fish *Apteronotus leptorhynchus*. *Neuroscience* 80: 653–667.
- Zupanc GK, Maler L. 1993. Evoked chirping in the weakly electric fish *Apteronotus leptorhynchus*: a quantitative biophysical analysis. *Can J Zool* 71:2301–2310.
- Zupanc G, Maler L. 1997. Neuronal control of behavioral plasticity: the prepacemaker nucleus of weakly electric fish. *J Comp Physiol A* 180:99–111.
- Zupanc GK, Sirbulesscu RF, Nichols A, Ilies I. 2006. Electric interactions through chirping behavior in the weakly electric fish *Apteronotus leptorhynchus*. *J Comp Physiol A Neu-roethol Sens Neural Behav Physiol* 192:159–173.