

# Evolutionary Conservation of the Egr-1 Immediate-Early Gene Response in a Teleost

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## ABSTRACT

Immediate-early gene expression is a key part of a neuron's response to behaviorally relevant stimuli and, as a result, localization of immediate-early gene expression can be a useful marker for neural activity. We characterized the immediate-early gene *egr-1* (also called *zif268*, *NGFI-A*, *krox-24*, *ZENK*) in the teleost *Astatotilapia (Haplochromis) burtoni*. We compared the *A. burtoni egr-1* predicted protein sequence to that of other vertebrates, characterized its gene expression time course, and localized its induced expression throughout the brain. The *A. burtoni egr-1* predicted protein shared putative functional domains with *egr-1* of other vertebrates and shared 81% sequence similarity with zebrafish and 66% with mouse. We identified distinct mammalian and teleost inserts rich in serine residues within one activation domain, suggesting convergent responses to selection pressures to increase the number of serine residues in this region. Functionally, we found that *A. burtoni egr-1* gene expression peaked near 30 minutes after pharmacological stimulation and thereby displayed the transient expression above basal levels characteristic of *egr-1* expression in birds and mammals. Finally, we observed distinct patterns of *egr-1* gene induction in the brain by natural and pharmacological stimuli. Unstimulated males had very low expression levels of *egr-1*, whereas males stimulated by their normal environment showed higher levels of expression specific to particular brain regions. Males injected with a glutamate receptor agonist also had region-specific induction of *egr-1* expression. We conclude that the *egr-1* immediate-early gene response is evolutionarily conserved and will, therefore, be useful for identifying functional neural responses in nontraditional model species. *J. Comp. Neurol.* 481:220–232, 2005. © 2004 Wiley-Liss, Inc.

**Indexing terms:** ZENK; *krox-24*; *zif268*; *NGFI-A*; *Astatotilapia (Haplochromis) burtoni*; cichlid

*Egr-1* (also known as *zif268*, *NGFI-A*, *krox-24*, *ZENK*) is an inducible transcription factor that functions as part of the immediate-early gene response, the first wave of gene expression induced in a neuron by stimulation. *Egr-1* expression is induced by a variety of natural experiences, sensory stimuli, and during the production of behaviors. For this reason, accumulation of *egr-1* mRNA or protein is often used to identify functional neural activity. For example, *egr-1* is induced in the suprachiasmatic nucleus of rodents by phase-shifting pulses of light (Rusak et al., 1990), in the limbic system of rats by vaginocervical stimuli (Polston and Erskine, 1995), in the auditory forebrain of songbirds listening to songs (Gentner et al., 2001; Mello et al., 1992), and in the basal ganglia when a male bird vocalizes (Jarvis and Mello, 2000; Jarvis et al., 1998, 2000). Although *egr-1* functional mapping studies have proved an invaluable tool in mammalian and avian model systems, similar studies have thus far not been done in

other taxa. *Egr-1* mapping techniques would prove widely applicable if the basic characteristics of *egr-1* expression are evolutionarily conserved. With this in mind, we characterized the *egr-1* response in a teleost, *Astatotilapia (Haplochromis) burtoni*, within the context of the evolu-

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tionary conservation of the *egr-1* immediate-early response.

Broadly conceived, the role of *egr-1* in a neuron is to link membrane depolarization to expression of late response target genes that, in turn, shape aspects of neuronal function. The consequences of *egr-1* expression are mediated by the subsequent expression of *egr-1* target genes which likely vary with neural phenotype and with the cellular context at the time of *egr-1* induction. Neuronal *egr-1* targets regulated by physiological stimuli *in vivo* have yet to be identified, but candidate targets include synapsins (Petersohn et al., 1995; Thiel et al., 1994) and neurofilaments, among others (Mello, 2004). However, knowledge about the downstream effects of *egr-1* expression is unnecessary for using *egr-1* and other immediate-early genes in functional mapping studies.

Three features of *egr-1* expression in mammals and birds have made it a valuable marker for neuronal activity. First, *egr-1* expression in the brain is specific to neurons and is regulated by synaptic activity (Worley et al., 1991). Membrane depolarization activates multiple convergent signaling cascades that lead to phosphorylation of the constitutively expressed transcription factors responsible for expression of *egr-1* (Bozon et al., 2003; Buchwalter et al., 2004; Cheng and Clayton, 2004; Harada et al., 2001; Murphy et al., 1991; Sweatt, 2001; Treisman, 1996; Whitmarsh et al., 1995). Second, although the exact relationship between *egr-1* expression and electrophysiological activation remains uncertain, the emerging view is that the threshold for *egr-1* expression exceeds that for an action potential (Clayton, 2000; Mello, 2004). This view results, in part, from the observation that action potentials are not always accompanied by *egr-1* expression (Mello et al., 1992, 1995; Stripling et al., 1997), but *egr-1* expression appears to depend on depolarization. This trait

is an advantage in mapping studies because one hopes to identify brain regions for which the stimulus is of *particular* significance rather than every neuron that may have recently produced an action potential. Third, the transient expression of *egr-1* following its induction allows one to infer a connection between elevated levels of *egr-1* with immediately preceding events.

The broader use of *egr-1* mapping techniques in nontraditional model systems depends on the evolutionary conservation of *egr-1*. *Egr-1* is a transcription factor that is a member of a four-gene family of *egr*'s characterized by a highly conserved DNA-binding domain composed of three zinc-finger motifs (O'Donovan et al., 1999). *Egr-1* has been partially or fully cloned in several mammal and bird species but published sequences from other vertebrates is limited. An *egr*-like gene has been identified in the non-vertebrate chordate amphioxus that is thought to represent the ancestral *egr* gene that gave rise to all four vertebrate *egr*'s through multiple gene duplication events (Knight et al., 2000; Schilling and Knight, 2001). The domains of mammalian *egr-1* have been defined using functional assays (Gashler et al., 1993; Fig. 1) and the high degree of sequence similarity of *egr-1* among mammals, zebrafish, and *Xenopus* (Drummond et al., 1994; Panitz et al., 1998) suggests that the functional domains of *egr-1* are evolutionarily conserved. Furthermore, the promoter elements of the zebrafish (Drummond et al., 1994) and *Xenopus* (Panitz et al., 1998) *egr-1* gene are very similar to that of mammals, suggesting that *egr-1* may have retained its place within cellular signaling cascades during vertebrate evolution. The time course of *egr-1* gene expression is a product of the cellular signaling cascades controlling it and, hence, is a characteristic trait. For birds and mammals, *egr-1* expression is detectable within minutes of stimulation and mRNA levels peak about 30 min-

#### Abbreviations

3	layer three	MB	mammillary body
ac	anterior commissure	MPn	magnocellular preoptic nucleus
An	anterior nucleus	nMLF	nucleus of the medial longitudinal fascicle
aPPn	anterior part of PPn	NT	nucleus taenia
BS	brain stem	nTe	nucleus of the thalamic eminence
C	central zone	OB	olfactory bulb
CC	corpus of the cerebellum	ON	optic nerve
Cn	central nucleus of IL	OT	optic tectum
CTn	central thalamic nucleus	P	Purkinje cell layer
D	dorsal telencephalon	PG	periventricular gray zone
Dc	central zone of D	PGCn	preglomerular commissural nucleus
Dd	dorsal zone of D	PGn	preglomerular nucleus
DH	dorsal hypothalamus	Pn	posterior nucleus
DI	lateral zone of D	pnPT	periventricular nucleus of the posterior tuberculum
Dld	dorsal part of lateral zone of D	PO	paraventricular organ
Dlv	ventral part of lateral zone of D	PPn	parvocellular preoptic nucleus
Dm	medial zone of D	PPTn	periventricular pretectal nucleus
Dm1,3,4	parts of the medial zone of D	pTn	posterior tuberal nucleus
Dn	diffuse nucleus of IL	S	superficial white and gray zone
Dp	posterior zone of D	Sn	suprachiasmatic nucleus
DW	deep white zone	Tn	tuberal nucleus
E	entopeduncular nucleus	V	ventral telencephalon
ECL	external cell layer	Vc	commissural nucleus of V
G	granular cell layer	Vd	dorsal nucleus of V
Gn	glomerular nucleus	VH	ventral hypothalamus
H	habenula	VI	lateral nucleus of V
ICL	internal cell layer	VLn	ventrolateral nucleus
IL	inferior lobe	VMn	ventromedial nucleus
In	intermediate nucleus	Vp	postcommissural nucleus of V
LH	lateral hypothalamus	Vs	supracommissural nucleus of V
M	molecular cell layer	Vv	ventral nucleus of V



TABLE 1. Domain-Specific Percent Shared Similarity (and Identity) of *A. burtoni* *egr-1* with Other Vertebrates and the Nonvertebrate Chordate Amphioxus<sup>1</sup>

	Full	SAD	R	NLS	DBD	WAD
Zebrafish	81 (76)	74 (68)	93 (93)	94 (94)	100 (99)	84 (75)
<i>Xenopus</i>	70 (61)	61 (50)	85 (73)	94 (94)	100 (97)	65 (51)
Chicken	71 (62)	62 (51)	91 (82)	94 (94)	100 (98)	66 (54)
Mouse	66 (58)	57 (48)	88 (82)	94 (94)	100 (98)	57 (47)
Amphioxus	35 (30)	25 (17)	22 (14)	69 (69)	98 (96)	n/a

<sup>1</sup>For amphioxus, the only functional domains identifiable by sequence similarity were NLS and DBD; therefore, for the purposes of the table, R was defined as 35 residues upstream of the NLS, SAD was defined as the remaining upstream residues, and WAD was excluded because the amphioxus sequence is not complete in this region.

Full, full length *egr-1*; SAD, strong activation domain; R, repression domain; NLS, nuclear localization site; DBD, DNA-binding domain; WAD, weak activation domain.

utes later (Mello and Clayton, 1994; Zangenehpour and Chaudhuri, 2002). A time course of expression of *egr-1* has yet to be established for other classes of vertebrates.

To understand the evolutionary conservation of *egr-1* generally, and *A. burtoni* *egr-1* specifically, we cloned *A. burtoni* *egr-1* and compared its predicted protein sequence to available representatives of other vertebrate groups and to the putative ancestral *egr* of amphioxus. To characterize the *A. burtoni* *egr-1* response, we determined its gene expression time course and described its inducible expression in the brain. To our knowledge, this is the only such characterization of *egr-1* in a vertebrate other than a mammal or bird, and is, therefore, important for understanding the evolutionary conservation of the *egr-1* immediate-early response.

## MATERIALS AND METHODS

### Animal care

We used *A. burtoni* males from a laboratory stock derived from a wild population in Lake Tanganyika, Africa. Fish were housed in aquaria with water at 28°C and pH 8, a daily cycle of 12 hours light and 12 hours dark, with an additional 10 minutes of simulated twilight at each transition. Fish were fed once per day. In all cases, animals were sacrificed by rapid decapitation. All work was performed in compliance with the animal care and use guidelines at Stanford University and was approved by the Stanford University Administrative Panel on Laboratory Animal Care committee.

### Identification of *A. burtoni* *egr-1* sequence

We isolated total RNA from brain homogenates (Ultraspec-II, Biotecx Laboratories, Houston, TX) and synthesized cDNA using an anchored poly-dT primer and Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA). To identify the *A. burtoni* specific *egr-1* gene transcript, we used degenerate PCR with primers designed using Codehop (<http://blocks.fhcrc.org/blocks/codehop.html>) followed by rapid amplification of cDNA ends (RACE) using SmartRACE (ClonTech, Palo Alto, CA) with *A. burtoni* gene-specific primers as follows.

For degenerate PCR, we designed the following primers: forward, 5'-CCT TCC AGG TGC CCA TGA THC CNG A-3'; reverse, 5'-GCC GGT CGC AGG TCT CNA CNG GRC A-3'. To amplify the *egr-1* fragment, we used Klen-taq1 DNA polymerase (AB Peptides, St. Louis, MO) pre-bound with TaqStart Antibody (ClonTech), a magnesium concentration of 3 mM, primers at 0.5 μM each, and a capillary tube Rapidcycler (Idaho Technologies, Idaho Falls, ID); note that the rapid heat transfer in a capillary tube thermal cycler does not require hold times for dena-

turing or annealing steps. We used a cycling program with an initial 2-minute step at 94°C followed by 40 cycles as follows: denaturing for 0 seconds at 94°C, annealing for 0 seconds at 55°C, 54°C, 53°C, 52°C, 51°C (3 cycles at each temperature), or 50°C (25 cycles), and extension for 30 seconds at 72°C, concluding with a final extension for 3 minutes at 72°C. The amplified band was purified (QIAquick, Qiagen, Valencia CA), subcloned (pCR-II-Topo, Invitrogen), and commercially sequenced. We then designed the following *egr-1* *A. burtoni*-specific primers for RACE: forward, 5'-GTA CGC TTG CCC TGT TGA GAC CTG-3'; reverse, 5'-CAG GTC TCA ACA GGG CAA GCG TAC-3'. We used reagents as recommended and a Hybrid PCR Express thermal cycler (Franklin, MA). For 5' RACE, we used a cycling program with an initial 30-second step at 94°C followed by 35 cycles as follows: denaturing for 5 seconds at 94°C, annealing for 10 seconds at 72°C (5 cycles), 70°C (5 cycles), or 68°C (25 cycles), and extension for 3 minutes at 72°. For 3' RACE, we used a cycling program with an initial 1-minute step at 94°C followed by 30 cycles as follows: denaturing for 30 seconds at 94°C, annealing for 30 seconds at 65°C (4 cycles), 64.5–55.5°C (1 cycle at each temperature, decreasing by 0.5°C each cycle), or 55°C (8 cycles), extension for 3 minutes at 72°C, and concluding with a final extension for 5 minutes at 72°. Subclones of these additional fragments were then sequenced and the full-length transcript was determined.

For comparison with *A. burtoni* *egr-1* (GenBank accession No. AY493348; this study), we used *egr-1* sequence from two teleosts, zebrafish (*Danio rerio*, U12895; Drummond et al., 1994) and catfish (*Clarias gariepinus*, AY029282.1; J. Bogerd and A.C.C. Teves, unpubl.), one amphibian (*Xenopus laevis*, AF250345.1; Panitz et al., 1998), one bird (*Gallus gallus*, AY034140.1; V. Cermak and M. Dvorak, unpubl.), and two mammals, mouse (*Mus musculus*, M22326.1; Christy et al., 1988; Sukhatme et al., 1988) and human (X52541.1; Suggs et al., 1990). For the sequence alignment in Figure 1B, we also included the conserved portions of amphioxus *egr* (*Branchiostoma floridae*, AF465940; Jackman and Kimmel, 2002). We aligned the *egr-1* sequences using ClustalX Multiple Sequence Alignment Program (v. 1.81), and we used MacBoxshade (v. 2.15) to construct Figure 1B and determine the shared identity and similarity reported in Table 1.

### Time course of *egr-1* gene expression

To determine the time course of gene expression following depolarization, we injected fish intraperitoneally with 10 mg per kg body mass kainic acid, a glutamate receptor agonist. Fish were removed from home tanks, injected, and then temporarily housed in a small container for 30, 60, 90, or 120 minutes until sacrifice (n = 3 per time

point). To serve as a preinjection baseline, we sacrificed three additional fish immediately upon capture. We chose these time points to include peaks predicted from time courses for *egr-1* in mammals and birds (near 30 minutes; Mello and Clayton, 1994; Zangenehpour and Chaudhuri, 2002) and for a similar transcription factor immediately-early gene, *c-fos*, in trout (near 120 minutes; Matsuoka et al., 1998).

Following sacrifice, we isolated total RNA from whole brains and synthesized cDNA as above. We used quantitative real-time PCR to determine the level of expression of *egr-1* relative to a reference gene, glyceraldehyde 3-phosphodehydrogenase (GAPDH; AF123727). By measuring *egr-1* expression relative to GAPDH, we account for among-sample variation introduced during cDNA synthesis and reaction preparation. We designed primers for real-time PCR as described in detail in Greenwood et al. (2003). Primers for GAPDH were as follows: forward, 5'-CAC ACA AGC CCA ACC CAT AGT CAT-3'; reverse, 5'-AAA CAC ACT GCT GCT GCC TAC ATA-3'; Primers for *egr-1* were as follows: forward, 5'-CTC TGG GCT GAT AGG CAA TGT T-3'; reverse, 5'-TGA GAT GAG GAC GAG GAG GTA GAA-3'.

For the real-time PCR reaction, we combined iQ Supermix reaction solution (Bio-Rad Laboratories, Hercules, CA), SYBR Green 1 (1:75,000, Molecular Probes, Eugene, OR), 10 nM fluorescein (Bio-Rad), 0.5  $\mu$ M of each primer, and 10 ng cDNA (RNA equivalent). The cycling parameters were 3 minutes at 95°C followed by 40 cycles of 95°C, 60°C, and 72°C for 30 seconds each. In order to calculate reaction efficiencies, we first calculated a slope from a dilution series of cDNA by plotting cycle threshold number against the log of relative cDNA concentration. We calculated reaction efficiency using the formula  $E = 10^{(-1/\text{slope})}$ ; reaction efficiencies were 1.99 (GAPDH) and 1.96 (*egr-1*). We defined cycle threshold number as the cycle at which the sample PCR reaction entered the linear phase of amplification. For each sample, we calculated a mean cycle threshold of three reactions. We calculated the expression of *egr-1* relative to GAPDH using the equation: relative *egr-1* expression =  $100 \times E(\text{GAPDH})^{[\text{CT}(\text{GAPDH})]} / E(\text{egr-1})^{[\text{CT}(\text{egr-1})]}$ , where E was reaction efficiency and CT was cycle threshold (Pfaffl, 2001).

### Localization of *egr-1* gene expression in situ

To examine the general neuroanatomical distribution of *egr-1* in animals that are stimulated by natural and pharmacological stimuli, we chose three groups to span the range of *egr-1* expression levels: unstimulated males (sacrificed during the dark phase of the daily cycle), males stimulated by their normal social and abiotic environment (sacrificed during the light phase of the daily cycle), and males with pharmacologically elevated levels of neural activity (sacrificed 30 minutes after injection with kainic acid). Our neuroanatomical analysis included all major divisions of the brain (forebrain, midbrain, hindbrain) and focused, in particular, on brain areas that we expected to be stimulated (e.g., the optic tectum should be stimulated by onset of lights) or that were observed to be notably stimulated in one of the groups. For telencephalic divisions and nomenclature we followed Northcutt and Braford (1980), for the diencephalon we followed Fernald and Shelton (1985), for the optic tectum we followed Northcutt (1983), and for the cerebellum we followed Finger (1983).

After males were sacrificed by rapid decapitation, we promptly removed and froze the brains and stored them at -80°C. We sectioned brains in three alternate series at 14  $\mu$ m in the transverse plane. For in situ hybridization, all steps were carried out at room temperature unless otherwise stated. Sections were fixed on the slides for 5 minutes in 3.7% formaldehyde, washed twice for 3 minutes each in phosphate-buffered saline (PBS, pH = 7.4), equilibrated for 3 minutes in 0.1 M triethanolamine (TEA) followed by acetylation for 9 minutes in 0.25% acetic anhydride in TEA, washed twice for 3 minutes each in 2 $\times$  sodium chloride sodium citrate buffer (SSC, pH = 7.0), followed by dehydration in an ethanol series. We made sense and antisense riboprobes using in vitro transcription (MAXIScript, Ambion, Austin, TX) with <sup>35</sup>S-UTP and diluted the probe in hybridization buffer (Sigma-Aldrich, Milwaukee, WI) supplemented with 0.1 M dithiothreitol (DTT) to a final concentration of 3,000 cpm per  $\mu$ l. The probes were transcribed from the 3' RACE subclone corresponding to nucleotides 1449..3336 of the full-length mRNA. Thus, the 1888 nucleotide probes included sequence coding for part of the DNA binding domain, the weak activation domain (see Results and Fig. 1), and the entire 3' untranslated region. We added 100  $\mu$ l of hybridization solution with probe to each slide, sealed it with a coverslip, and immersed the slides in a mineral oil bath for hybridization at 65°C overnight. At the end of hybridization, the slides were washed in two changes of chloroform, two 10-minute washes in 4 $\times$  SSC to remove coverslips, equilibrated for 5 minutes in 2 $\times$  SSC with 1 mM DTT before a 30-minute incubation in RNase A (5  $\mu$ g/ml in 2 $\times$  SSC) at 37°C followed by a second 5-minute wash in 2 $\times$  SSC with 1 mM DTT. Slides were then washed at 65°C in 50% formamide (in 2 $\times$  SSC with 1 mM DTT) for 1.25 hours and twice in 0.1 $\times$  SSC with 1 mM DTT for 30 minutes each. Slides were then brought to room temperature in 0.1 $\times$  SSC with 1 mM DTT for 5 minutes before dehydration in an ethanol series. We then dipped slides in NTB2 emulsion (Kodak, New Haven, CT) diluted 1:1 with deionized water and stored them for 3 days at 4°C before development. Slides were counterstained with cresyl violet, dehydrated, cleared in xylenes, and sealed with Permount (Sigma-Aldrich) and a coverslip.

We produced photomicrographs (Figs. 3–5) using a digital camera (Spot, Diagnostic Instruments, Sterling Heights MI), PhotoShop (Adobe Systems, San Jose CA), and Illustrator (Adobe Systems). We sharpened images and adjusted their contrast using PhotoShop.

## RESULTS

### Egr-1 sequence comparisons

We identified the *A. burtoni* *egr-1* gene as a 3336 bp transcript coding for a predicted 514 amino acid protein (coding region 505..2049). Comparison of the predicted *A. burtoni* *egr-1* protein sequence with published sequences (zebrafish, catfish, *Xenopus*, chicken, mouse, human, and amphioxus) showed that among vertebrates *egr-1* is conserved throughout its extent (Fig. 1B), with domain-specific variation in percent similarity shared (Table 1). The amphioxus *egr* protein clearly contained a nuclear localization site and DNA-binding domain based on sequence similarity, although no repression domain or strong activation domain of the vertebrate type were ap-

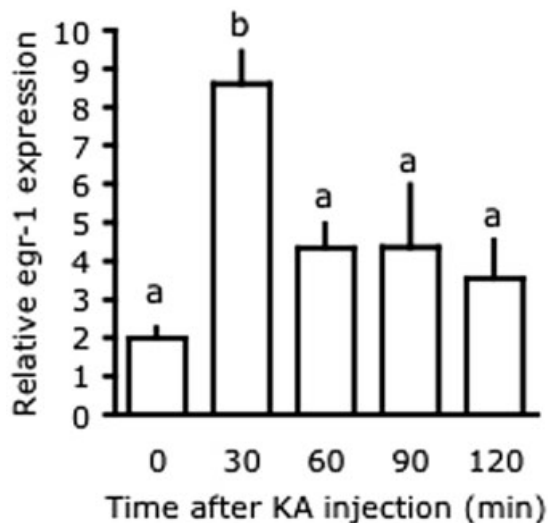


Fig. 2. Expression of *egr-1* gene relative to a control gene GAPDH (mean  $\pm$  SEM) following injection of kainic acid (KA). Noninjected controls are depicted as 0 minutes postinjection. Groups that were deemed to be reliably different are indicated by different letters.

parent (Table 1). A putative weak activation domain of amphioxus was not analyzed, as the amphioxus sequence does not extend into this region.

Among vertebrates, the *egr-1* regions with the highest percent shared similarity were the repression domain, nuclear localization site, and DNA binding domain (Table 1; Fig. 1B). Within the repression domain, there is a notable 5-residue region of divergence within which there is a 3-residue gap in teleosts. Within the DNA binding domain, there is extremely high conservation among distantly related vertebrate taxa that is shared with the ancestral *egr* gene of amphioxus (Knight et al., 2000), and the observed variation that occurs among vertebrates is conservative. There is only one residue difference within the DNA binding domain that distinguishes teleosts from tetrapods. Based on a comparison with the amphioxus *egr* gene, the tetrapod residue appears to be derived. Within the zinc fingers, *A. burtoni* shows only one deviation from tetrapods which is also shared by zebrafish, and this substitution is most likely derived.

*Egr-1* regions with the highest divergence among vertebrates were the putative strong and weak activation domains (Table 1; Fig. 1B). Of particular note are two distinct inserts occurring in mammals and teleosts within the strong activation domain (Fig. 1B). The mammalian insert (~23 residues, also found in rat) was recognized by Drummond et al. (1994) to be common among mammals and lacking in zebrafish, although without additional non-mammalian sequences unavailable at the time, it was not possible to determine whether this was an insert characteristic of mammals or a deletion characterizing zebrafish. Similarly, teleosts have an insert of ~16 residues that is distinct from the mammalian insert. Based on the location of the intron–exon boundaries in the *egr-1* gene (Drummond et al., 1994; Sukhatme et al., 1988), neither the mammalian insert nor the teleost insert can be the product of a splice variant, indicating that the inserts are the result of evolved changes in their corresponding genes.

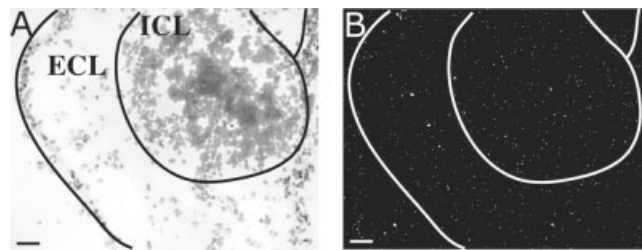


Fig. 3. An example of sense riboprobe binding in the olfactory bulb. A brightfield image (A) shows the internal (ICL) and external (ECL) cell layers of the olfactory bulb stained by cresyl violet. The corresponding darkfield image (B) shows distribution of silver grains after hybridization with the sense probe. See Figure 4C–E for antisense binding in the olfactory bulb. Images were contrast-enhanced to make the cell bodies and silver grains easily visible. Scale bars = 50  $\mu$ m.

The mammalian insert is most probably the result of an insertion in the ancestral mammalian *egr-1* gene since it is not shared with any other vertebrate group (see Drummond et al. (1994) for a hypothesis for how the mammalian gene evolved this insert). However, given the pattern of available sequences, it is not possible to conclude whether the teleost insert was the product of a sequence insertion in the ancestral teleost *egr-1* gene or whether this sequence was deleted from the ancestral tetrapod *egr-1* gene.

#### Time course of *egr-1* gene expression

*Egr-1* gene expression in whole brains showed a distinct temporal pattern following pharmacological stimulation with kainic acid (ANOVA:  $F(4,20) = 6.2, P = 0.009$ ; Fig. 2). We found a 4-fold peak increase of *egr-1* mRNA near 30 minutes followed by a rapid decline to noninjected levels by 60 minutes. Post-hoc statistical analyses showed that mRNA expression at 30 minutes was higher than all other groups, whereas none of the other groups differed from one another. At 60 and 90 minutes, *egr-1* expression was twice as high as the noninjected controls, which, although not statistically significant ( $P = 0.125$  in both cases), suggests that additional stimulation due to postinjection housing contributed to the modest elevation of *egr-1*.

#### Inducible *egr-1* gene expression in situ

Brain sections hybridized with the *egr-1* antisense probe showed distinct regional expression patterns of sil-

Fig. 4 (Overleaf). Photomicrographs of in situ labeled *egr-1* expression in the olfactory bulb (A–E), ventral nucleus of the ventral telencephalon (F–J), ventral part of the lateral zone of the dorsal telencephalon and the central zone of the dorsal telencephalon (K–O), and the anterior part of the parvocellular preoptic nucleus (P–T). The top row shows low-magnification images of the region of the brain depicted in the remaining rows (indicated with a box) with corresponding diagrams of the major cell groups. The second row shows brightfield images corresponding with the darkfield images in the remaining rows. The remaining rows show *egr-1* induction in males stimulated by kainic acid (C,H,M,R), stimulated by their normal environment (D,I,N,S), and unstimulated males sacrificed during the dark phase of the light cycle (E,J,O,T). Images were contrast-enhanced to make cell bodies and silver grains easily visible. For abbreviations, see list. Scale bars = 100  $\mu$ m in A,F,K,P; 50  $\mu$ m in all others.

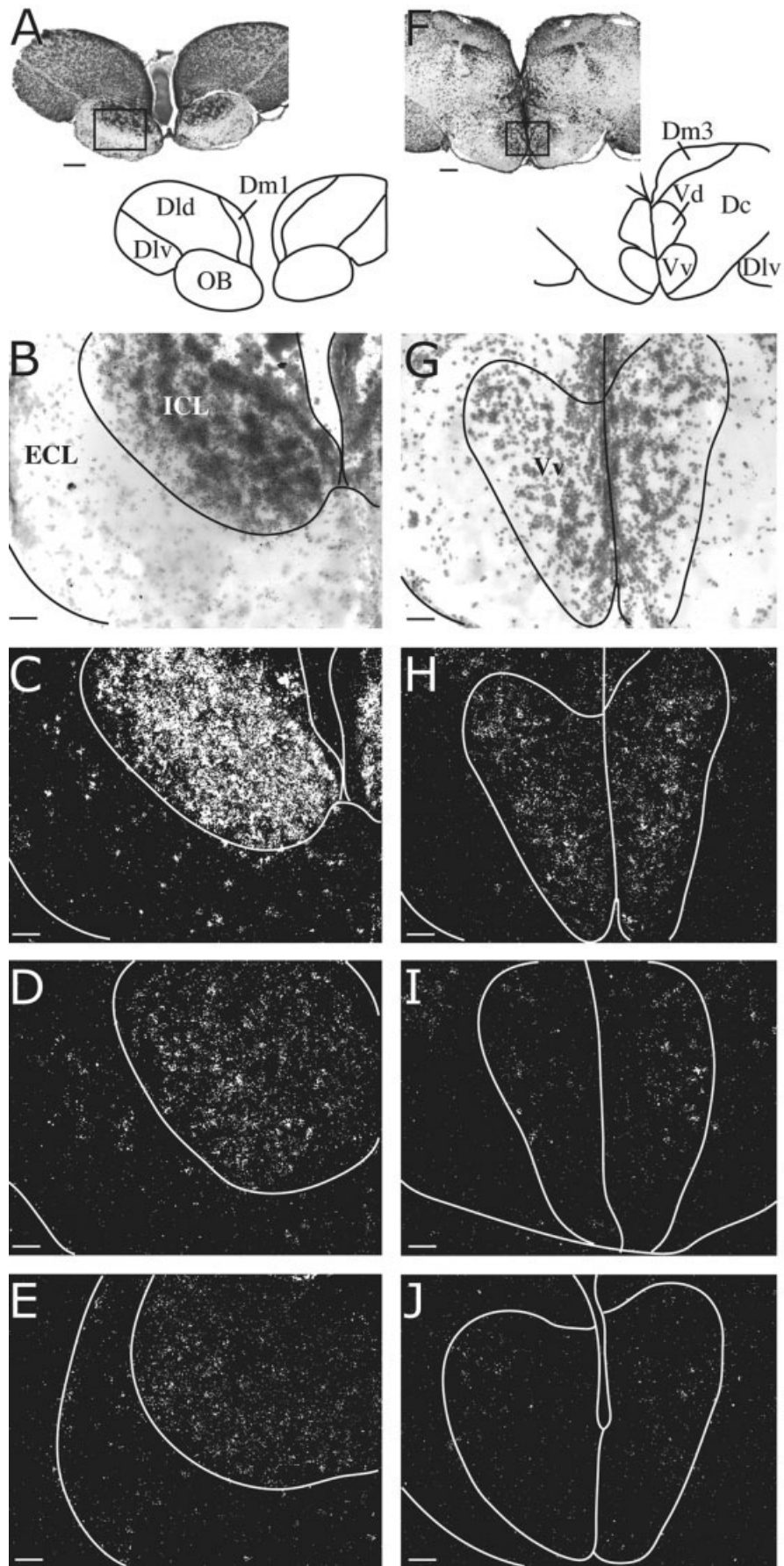


Figure 4

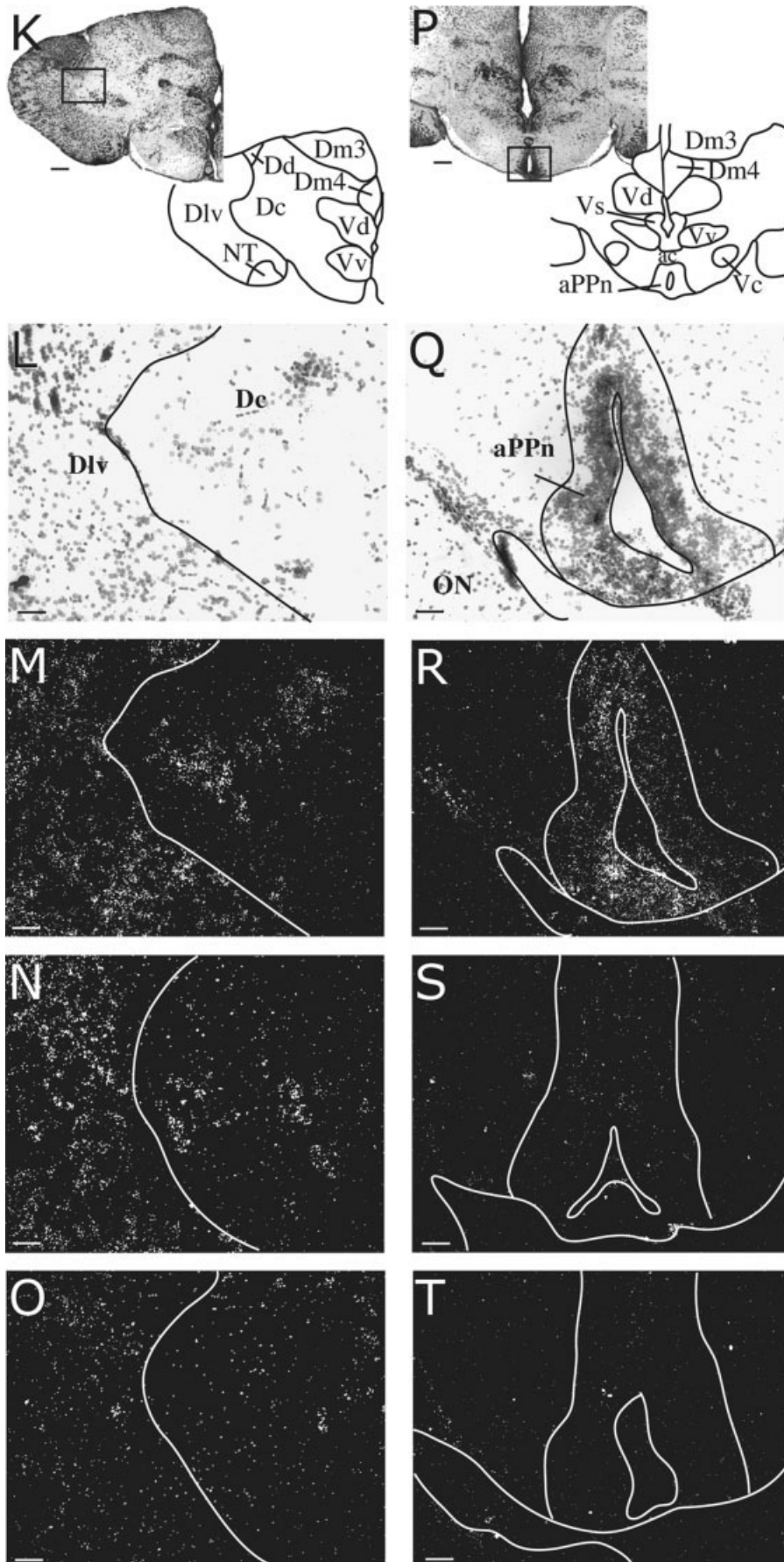


Figure 4 (Continued)



ver grains clustered around individual cells, whereas labeling with *egr-1* sense probe yielded no signal detectable above background (Fig. 3). In general, *egr-1* expression was low or absent in all brain regions of unstimulated males. *Egr-1* expression in the brains of males stimulated by their normal environment or kainic acid varied with brain region (Figs. 4, 5; Table 2). In no case did we observe detectable *egr-1* expression in areas known to lack neuronal cell bodies (e.g., the neuropil of the optic tectum, see Fig. 5C–E), suggesting that the expression of *egr-1* was confined to neurons.

Within the telencephalon, *egr-1* was expressed in the olfactory bulb, all of the major divisions of the dorsal telencephalon (D; generally viewed as the pallium; Wullmann and Mueller, 2004), including the medial (Dm), dorsal (Dd), lateral (Dl), posterior (Dp), and central (Dc) zones of D, and most divisions of the ventral telencephalon (V; generally viewed as the subpallium; Wullmann and Mueller, 2004), including the dorsal (Vd), ventral (Vv), supracommissural (Vs), and postcommissural (Vp) nuclei of V (summarized in Table 2). In normal males, the highest expression level among these regions was in the olfactory bulbs (Fig. 4A–E), Dl, and Dc (Fig. 4K–O). Regions showing relatively lower expression in normal males included Dm (not shown) and Vv (Fig. 4F–J). The only regions of the telencephalon showing an apparent absence of *egr-1* expression in normal males were the commissural (Vc) and lateral (Vl) nuclei of V and the entopeduncular nucleus (not shown). Kainic acid had strong effects on *egr-1* expression in the olfactory bulb (Fig. 4A–E) and Vv (Fig. 4F–J), but more modest effects on Dl and Dc (Fig. 4K–O). Some regions of the telencephalon, by contrast, were not evidently induced by either natural stimuli or kainic acid; for example, Vc, Vl, and the entopeduncular nucleus did not show detectable levels of *egr-1* in any treatment (not shown).

In the diencephalon of awake behaving males, *egr-1* was expressed in nuclei of the thalamus, habenula, preoptic area, and hypothalamus (summarized in Table 2). The nuclei of the synencephalon did not apparently express *egr-1* under any condition. In general, expression was lower in the diencephalon compared to the telencephalon, with the exception of the habenula and several divisions of the hypothalamus, including the tuberal nucleus (Tn) and the dorsal (DH), ventral (VH), and lateral hypothalamus (LH), which all showed moderate to high levels of expression. Subdivisions of the posterior tuberculum of the thalamus showed relatively low levels of *egr-1* expression, and several showed no *egr-1* expression under any condition, including the preglomerular nuclear complex (PGn) and the glomerular nucleus (Gn). Most dorsal and ventral thalamic nuclei showed low to undetectable levels of *egr-1* expression unless stimulated by kainic acid. Kainic acid had robust effects on *egr-1* expression in some regions of the diencephalon, including the anterior part of the parvocellular preoptic nucleus (aPPn; Fig. 4P–T), magnocellular preoptic nuclei (MPn), and the anterior nucleus of the thalamus (An). In the midbrain, *egr-1* expression in the optic tectum was relatively high in normal males and somewhat higher in those injected with kainic acid (Fig. 5A–E), and relatively lower in the torus semicircularis. In the hindbrain, *egr-1* was highly expressed in the granular cell layer but not the Purkinje cell layer of the cerebellum in both the normal males and kainic acid-injected males (Fig. 5F–J), with low to undetectable levels in other areas.

## DISCUSSION

Ultimately, the value of immediate-early genes for mapping behavioral circuits in neuroethological model species depends on the evolutionary conservation of immediate-early gene structure and function. To put our understanding of the immediate-early gene *egr-1* of *Astatotilapia (Haplochromis) burtoni* into this broader evolutionary context, we compared its predicted protein sequence to representatives of other vertebrate groups and to the putative ancestral *egr* of amphioxus. To characterize the function of the *A. burtoni* *egr-1* immediate-early gene response, we determined its expression time course and inducible expression in the brain. We found that the *A. burtoni* *egr-1* predicted protein shared putative functional domains with other vertebrates, suggesting that *egr-1* plays a similar role across diverse vertebrate species. Further support for the evolutionary conservation of *egr-1* function is our demonstration that both the time course of *egr-1* gene expression and its inducible expression in the brain was similar to that found in other species.

The predicted amino acid sequence of *egr-1* across vertebrates was highly conserved (Fig. 2; Table 1; see also Drummond et al., 1994; Panitz et al., 1998). This high degree of sequence similarity suggests that the functional domains defined for mammalian *egr-1* using transient transfection assays (Gashler et al., 1993) are likely to be common to nonmammalian *egr-1* proteins. As expected, the domains responsible for nuclear localization and DNA binding have the highest level of sequence similarity (near identity) among vertebrates, and are very similar to the nonvertebrate chordate amphioxus. This suggests that the ancestral *egr* possessed transcriptional activity. However, amphioxus *egr* lacks activation and repression domains of the vertebrate type, suggesting that it is very unlikely to participate in a vertebrate-like immediate-early response. This suggests that the role of *egr* in transcription is ancient, whereas its role within the immediate-early response is a derived vertebrate characteristic.

Cross-species comparisons of *egr-1* protein sequences can contribute to our understanding of *egr-1* function, particularly in notable cases of similarity or divergence. Divergence among vertebrate *egr-1* proteins is greatest in the activation domains. In their functional characterization of mouse *egr-1*, Gashler et al. (1993) describe a region of the strong activation domain that has a high proportion (30%) of serine, threonine, and tyrosine (S/T/Y) residues, and they speculate that the serine and threonine residues are the site of phosphorylation of *egr-1* (Day et al., 1990; Waters et al., 1990) and, as such, function as acidic activators. Furthermore, Gashler et al. (1993) show that the strong activation domain is relatively impervious to mutation, since substantial deletions in this extensive domain reduce, but do not eliminate, transcriptional activity. This suggests that a preponderance of S/T/Y residues, rather than a particular sequence of residues, may promote activation of *egr-1*. We identified a derived mammalian insert rich in serines (Fig. 1B; in mouse, 43% serine) within the S/T/Y-rich region described by Gashler et al. (1993). The teleost insert we identified also resides within this S/T/Y-rich region of the strong activation domain (Fig. 1B), and it, too, has very high proportion of serine residues (e.g., 69% for *A. burtoni*). Taken together, these data suggest that additional serine residues influence the activation of *egr-1*, and that the mammalian and teleost inserts

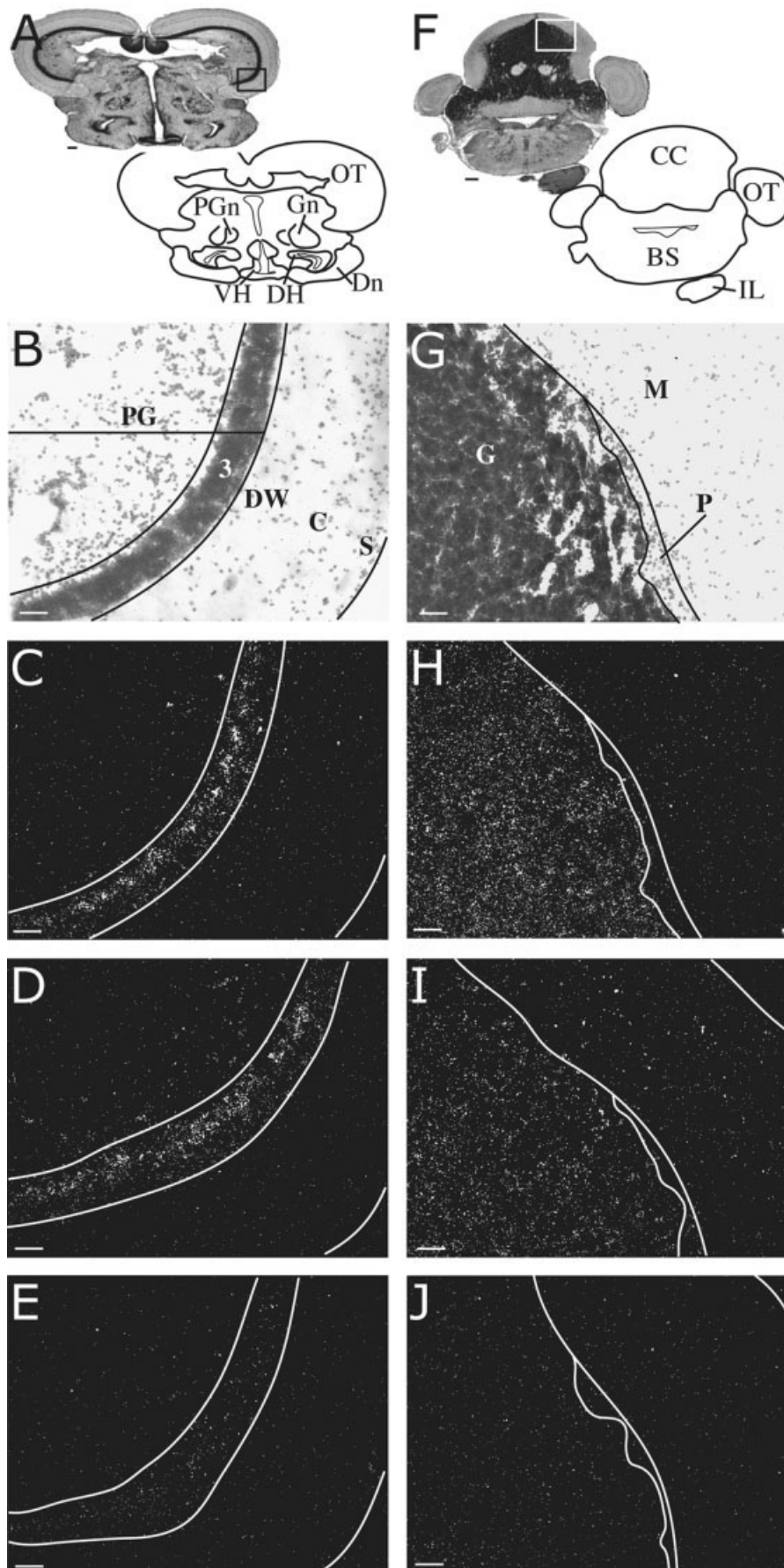


Fig. 5. Photomicrographs of in situ labeled *egr-1* expression in the optic tectum (A-E) and the corpus of the cerebellum (F-J). The top row (A,F) shows low-magnification images of the region of the brain depicted in the remaining rows (indicated with a box) with corresponding diagrams of the major cell groups. The second row (B,G) shows brightfield images corresponding with the darkfield images in

the remaining rows. The remaining rows show *egr-1* induction in males stimulated by kainic acid (C,H), stimulated by their normal environment (D,I), and unstimulated males sacrificed during the dark phase of the light cycle (E,J). Images were contrast-enhanced to make cell bodies and silver grains easily visible. For abbreviations, see list. Scale bars = 100  $\mu\text{m}$  in A,F; 50  $\mu\text{m}$  in all others.

reflect convergent evolutionary solutions to similar selection pressures. This hypothesis is consistent with the teleost insert resulting from a sequence insertion in the ancestral *egr-1* gene of teleosts; however, as noted, it is also possible that the observed sequence patterns are the result of the deletion of the teleost insert from the ancestral tetrapod *egr-1* gene. Additional analyses of *egr-1* sequences from organisms that better represent the common ancestor of tetrapods and teleosts may resolve this issue. Such comparisons of vertebrate *egr-1* protein sequences may be useful for predicting functional traits of *egr-1*.

The peak expression of the *egr-1* gene near 30 minutes postinduction is common to mammals (Zangenehpour and Chaudhuri, 2002), birds (Mello and Clayton, 1994), and at least one teleost (this study). This level of similarity in expression kinetics is remarkable in light of the rather different metabolic resting temperatures of the vertebrates studied to date (mammals, 37°C; birds, 39°C; fish, 28°C), and suggests that this is a common feature of *egr-1* expression kinetics. The expression time course of *egr-1* (rapid onset, early peak, and subsequent decline) confers temporal information to the immediate-early gene response and may be critical to a neuron's ability to link membrane depolarization with subsequent gene expression. This temporally defined pulse of expression is also a key characteristic for mapping studies, since it allows one to infer a connection between elevated expression to immediately preceding events.

The behavioral and pharmacological induction of *egr-1* gene expression in the *A. burtoni* brain suggests that synaptic activity induces expression of *egr-1* in *A. burtoni* in a similar manner to that in mammals and birds. In *A. burtoni*, *egr-1* expression was low during inactive periods (i.e., dark phase of the light cycle) and high during active periods (i.e., light phase), a pattern reminiscent of the sleep-wake cycle of *egr-1* expression in mammals (Cirelli and Tononi, 2000). In *A. burtoni*, divisions of the dorsal telencephalon showed ongoing *egr-1* expression induced by stimuli in the normal environment, a feature shared with *egr-1* expression in the cortex of mammals (Herdegen et al., 1995; Kaczmarek and Chaudhuri, 1997). This ongoing expression of *egr-1* in at least some brain regions of animals stimulated by their normal environment appears to be common to *A. burtoni* and mammals (Herdegen et al., 1995; Kaczmarek and Chaudhuri, 1997), although whether this feature is shared with birds is unclear since, to our knowledge, no studies have addressed this issue in birds. The ability of *egr-1* to be continually induced by synaptic activity is not a general feature of immediate-early genes since, in mammals, expression of the similar inducible transcription factor *c-fos* declines after initial stimulation (Herdegen et al., 1995; Kaczmarek and Chaudhuri, 1997). This suggests that the continuing induction of *egr-1* by ongoing synaptic activity is a general feature of *egr-1*. We note, however, that some brain regions capable of expressing *egr-1*, such as the preoptic area, did not show ongoing induction in awake behaving animals. Therefore, although *egr-1* is capable of being continually induced, whether it is depends on the brain region and, presumably, on the level of synaptic activity in that brain region.

We have shown that *egr-1* in *A. burtoni* is highly conserved in structure and function and that the *A. burtoni* *egr-1* immediate-early gene response is markedly similar to that of mammals and birds. These results provide a

TABLE 2. Summary of the Relative Expression of *egr-1* in the Forebrain

Brain region	KA	Normal	Dark
Telencephalon			
Dc	H	H	M
Dd	M	M	L
Dl	H	H	M
Dm	M	L	L
Dp	H	M	L-M
E	—	—	—
OB	H	H	M
Vc	—	—	—
Vd	H	M	L
Vl	—	—	—
Vp	H	M	L
Vs	H	L	L
Vv	H	L	L
Preoptic area			
MPn	M-H	L	—
PPn	M-H	L	—
Hypothalamus			
Cn	L	L	—
DH	M-H	M-H	L
Dn	L-M	L	L
LH	M-H	M	L
Sn	M	L-M	—
Tn	M-H	M-H	L
VH	M-H	M	L
Epithalamus			
H	H	H	M
Thalamus			
An	H	L	—
CTn	L	—	—
Gn	—	—	—
In	L	—	—
MB	L-M	M	—
nTe	L	—	—
PGCn	L	L	—
PGn	—	—	—
Pn	L	—	—
pnPT	L-M	L	—
PO	M	L	L
pTn	—	—	—
VLn	M	L	—
VMn	M	L	—
Synencephalon			
nMLF	—	—	—
PPtn	—	—	—

H, high; M, moderate; L, low; —, undetectable; KA, males stimulated with kainic acid; normal, males sacrificed during the light phase of the daily cycle; dark, males sacrificed during the dark phase. See list for abbreviations.

framework for the application of *egr-1* as an activity marker in *A. burtoni* as well as other vertebrates. The strength of immediate-early gene mapping studies is that they simultaneously assess the response of multiple brain regions that are participating in natural behaviors in awake and free-moving, or even free-living (Jarvis et al., 2000), animals. As a result, immediate-early gene mapping studies should prove useful in ethological model systems. However, there are two features of *egr-1* expression one must consider when designing mapping studies using *egr-1*. First, since *egr-1*, like other immediate-early genes, is expressed only in a subset of neurons it is important to establish whether a particular brain region or neural phenotype of interest is capable of expressing *egr-1*. Otherwise, a lack of *egr-1* expression does not allow one to conclude that the brain region was not activated. This is a constraint common to all immediate-early gene mapping studies (Chaudhuri, 1997). Second, the high sensitivity of *egr-1* to ongoing synaptic activity is both an advantage and disadvantage for its use in mapping studies. The sensitivity of *egr-1* means that one has a good chance of detecting an *egr-1* response to the stimuli of interest. Potential problems arise, however, if *egr-1* induction is simultaneously caused by nonintentional stimuli, such as handling by the experimenter or the experience of a new

testing environment. Therefore, careful experimental design is necessary to reduce baseline induction of *egr-1*, such as including long periods of quiescence before stimulus presentation and the inclusion of unstimulated controls for comparison.

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