Neuronal replacement occurs in the forebrain of juvenile and adult songbirds. To address the molecular processes that govern this replacement, we cloned the zebra finch insulin-like growth factor II (IGF-II) cDNA, a factor known to regulate neuronal development and survival in other systems, and examined its expression pattern by in situ hybridization and immunocytochemistry in juvenile and adult songbird brains. The highest levels of IGF-II mRNA expression occurred in three nuclei of the song system: in the high vocal center (HVC), in the medial magnocellular nucleus of the neostriatum (mMAN), which projects to HVC, and to a lesser extent in the robust nucleus of the archistriatum (RA), which receives projections from HVC. IGF-II mRNA expression was developmentally regulated in zebra finches. In canary HVC, monthly changes in IGF-II mRNA expression covaried with previously reported monthly differences in neuron incorporation. Combining retrograde tracers with in situ hybridization and immunocytochemistry, we determined that the HVC neurons that project to area X synthesize the IGF-II mRNA, whereas the adjacent RA-projecting neurons accumulate the IGF-II peptide. Our findings raise the possibility that within HVC IGF-II acts as a paracrine signal between nonreplaceable area X-projecting neurons and replaceable RA-projecting neurons, a mode of action that is compatible with the involvement of IGF-II with the replacement of neurons. Additional roles for IGF-II expression in songbird brain are likely, because expression also occurs in some brain areas outside the song system, among them the cerebellar Purkinje cells in which neurogenesis is not known to occur.

**Key words:** adult neurogenesis; Golgi; development; neurotrophins; growth factors

Discrete brain nuclei control the acquisition and production of learned song in songbirds. These nuclei include the high vocal center (HVC), the robust nucleus of the archistriatum (RA), and area X (Fig. 1) (Nottebohm et al., 1976, 1982). Whereas HVC and RA are required for the acquisition and production of learned song, area X is necessary for song acquisition, but not for production (Simpson and Vicario, 1990; Sohrabji et al., 1990; Scharff and Nottebohm, 1991).

There are two major classes of projection neurons in the HVC of male canaries and zebra finches: (1) those that synapse onto RA and continue to be produced and replaced during adulthood, and (2) those that project to area X and are produced only in ovo and not in adulthood (Alvarez-Buylla et al., 1988a; Nordeen and Nordeen, 1988a). Peaks in the recruitment of new RA-projecting neurons occur in adult canaries at times of the year when the birds develop new songs (Alvarez-Buylla et al., 1988a; Alvarez-Buylla, 1992; Kirn et al., 1994; Nottebohm et al., 1994). Thus, processes that are typically seen only in the developing mammalian brain, e.g., production, migration, and differentiation of new neurons, are ongoing events in the adult songbird brain. During development in other systems these processes seem to require the participation of trophic molecules, such as the insulin-like growth factors (IGFs), members of the fibroblast growth factor family (FGFs), and neurotrophins such as neurotrophin (NT) 3, NT4, brain-derived neurotrophic factor (BDNF), and nerve growth factor (NGF). In addition, many of these trophic factors also play a role in the maintenance of the adult nervous system (for review, see Lo, 1995; Thoenen, 1995). Recently, it has been shown that BDNF and NT3 promote the survival of neurons in the developing song system (Johnson et al., 1997).

This paper focuses on one particular trophic factor, IGF-II. The IGFs are secreted growth-promoting peptides that are structurally related to insulin. They bind to specific cell surface receptors that activate second messenger pathways, ultimately regulating cell growth and/or differentiation. Their function has been studied in rodents and chicken; IGF-I and IGF-II are expressed throughout the embryonic rodent brain but are less expressed in adulthood (Bondy, 1991; Marks et al., 1991; Bondy et al., 1992; Bondy and Lee, 1993). IGF knock-out experiments (for review, see Ludwig et al., 1996) have shown that the naturally occurring levels of IGF expression are necessary for normal brain development. Other experiments have shown that IGFs regulate the survival of neurons during development (Quin-Wei et al., 1994; Johnston et al., 1996) and early postnatal life (D'Mello et al., 1993; Galli et al., 1995).

We have cloned the zebra finch IGF-II cDNA and used in situ hybridization (ISH), immunocytochemistry (ICC), and retrograde tracers to show very selective IGF-II expression in the...
Figure 1. Diagram highlighting the song nuclei that express IGF-II mRNA (dark gray) and outlining their relationships (thick black arrows) as well as relationships among other song nuclei (thin black and white arrows). The two identified populations of projection neurons of HVC, those that project to RA (black) and those that project to area X (white), are schematically indicated. The direct descending motor pathway includes HVC, the RA, and the tracheosyringeal portion of the hypoglossal nucleus (nXIIa). The anterior forebrain pathway is necessary for song acquisition and connects (white arrows) HVC, area X the medial portion of the dorsolateral thalamic nucleus (DLM), and the lateral magnoccular nucleus of the anterior neostriatum (lMAN), which contains the song X that project back to RA (Okuhata and Saito, 1987; Bottjer et al., 1989; Nixdorf-Bergweiler et al., 1995; Yates and Nottebohm, 1995). Neurons in RA give rise to two feedback loops. The first one connects RA with the posterior portion of the dorsomedial thalamic nucleus (DMP), which in turn projects to the lMAN that synapses with HVC (Yates et al., 1997). The second feedback loop from RA projects to the thalamic nucleus DLM, which in turn projects to lMAN (Wild, 1993; Yates et al., 1997).

songbird brain. Within the song system, expression was highest in HVC, in which the localization of mRNA and peptide was segregated into two different types of projection neurons, one renewable and the other stable. This paracrine mode of action within HVC suggests that IGF-II is a candidate molecule involved in neuronal turnover. To our knowledge this is the first report of anatomically restricted expression of a growth factor with neurotrophic potential in a subset of song system neurons.

MATERIALS AND METHODS

Animals. Canaries (Serinus canaria) and zebra finches (Taenopygia gutata) bred at the Rockefeller University Field Research Center (Millbrook, NY) were used for this study. Adult male canaries, ranging in age from 2.4 to 2.9 years (n = 14), were used to establish the IGF-II expression patterns throughout the CNS. Additional canaries (total n = 47; 20–32 months old) were killed monthly between April 1995 and March 1996 for seasonal comparisons. Adult male zebra finches (n = 8) were used to establish their general IGF-II expression pattern. Twenty-one male zebra finches, which become sexually mature at ~90 d, were killed at ages ranging from 36 d to 4.7 years to study the developmental mRNA expression of IGF-II in HVC. To establish which cell class in HVC was expressing IGF-II mRNA, we used eight adult canaries and six adult zebra finches. Adult, free-ranging, black-capped chickadee males (Parus atricapillus; n = 5) were captured in October 1995 on the premises of the Rockefeller University Field Research Center and were used to confirm the presence of IGF-II mRNA expression in free-ranging individuals. The IGF-II peptide distribution was studied immunocytochemically in 10 adult canaries and 6 zebra finches. Twenty-one adult canaries were used to establish which HVC neurons accumulated the IGF-II peptide.

cDNA cloning. Total RNA was isolated from zebra finch embryos by the method of Chomczynski and Sacchi (1987). Poly(A+) RNA was selected through oligo-oligoT cellulose columns (Pharmacia Biotech, Piscataway, NJ) and used to construct a size-selected cDNA library using the pSPORT kit of Life Technologies–Bethesda Research Labs (Gaithersburg, MD). Nylon replica filters (Hybond-N; Amersham, Arlington Heights, IL) containing 4 × 10^4 recombinant colonies were prepared and screened with a cDNA fragment containing 0.4 kb of the 5′ end of the chicken IGF-II coding region (Darling and Brickell, 1996; M. Holzenberger and C. Ayer-LeLievre, unpublished observations) using the method of Hanahan and Meselson (1983). A 1.4 kb clone, pIGF-II-ZF, containing the complete open reading frame (ORF) of zebra finch IGF-II was isolated and manually sequenced (Sequenase version 2.0, United States Biochemical, Cleveland, OH) using SP6 and T7 promoter primers (New England Biolabs, Beverly, MA) and IGF-II-specific primers synthesized at The Rockefeller University. The entire IGF-II ORF was sequenced on both strands. Regions containing unresolved compressions or ambiguities were resequenced with appropriate new primers. The final DNA sequence, assembled with GeneWorks (IntelliGenetics, Mountain View, CA), is shown in Figure 2 and was submitted to GenBank. The deduced amino acid primary sequence was compared with prepro-IGF-II from other vertebrates using GeneWorks (IntelliGenetics).

Radioactive ISH. ISHs were done as described previously (Jarvis et al., 1995; Mello and Clayton, 1995) with modifications. 35S-labeled sense and antisense riboprobes were generated from the zebra finch IGF-II plasmid clone pIGF-II-ZF, previously linearized with SacI or NotI, in the presence of SP6 (antisense) or T7 (sense) RNA polymerase, respectively; 1.5 × 10^6 cpm of radiolabeled probe was applied to each slide in 80 μl of hybridization solution. Sections were hybridized and washed at 65°C; a final treatment with 5 μg/ml RNase A in 0.1× SSC for 10 min at room temperature was included to reduce background. Slides were exposed to x-ray film for 5 d to determine signal intensity. Tissue sections were then coated with Kodak NTB2 photographic emulsion and stored in light-tight boxes with a desiccant for 8 weeks. After development, the sections were lightly counterstained with cresyl violet and observed with a microscope under bright- and dark-field illumination. For seasonal and developmental comparisons, sections from all animals were hybridized simultaneously to minimize procedural variability.

Quantification. Emulsion-dipped slides were analyzed using National Institutes of Health Image software. To measure expression levels specific for a given brain region, we measured the optical density of grains in a 100 × 100 μm area in this region and subtracted the equivalent value over an adjacent region of equal size. On average four sections per bird were measured, and the final value represents the means of these sections. Significant differences within seasonal and developmental comparisons were tested with one-way ANOVA; subcomparisons between individual groups were tested with Scheffé’s F tests and post hoc Student’s t tests.

Northern hybridization. Riboprobes for Northern hybridization were synthesized as described for ISH with the substitution of 32P label for 35S label. Twenty micrograms of total RNA or 2 μg of poly(A+) RNA per lane were electrophoresed through 1% agarose gels containing formaldehyde in 1× 3-(N-morpholino)propanesulfonic acid (MOPS) buffer, as described in Sambrook et al. (1989). Gels were soaked for 1 hr in 20× saline-sodium phosphate–EDTA (SSPE), and the RNA was blotted to Hybond-N+ (Amersham). Blots were prehybridized for 15 min and then hybridized overnight at 65°C, as described in Clayton et al. (1988). Filters were washed in four changes of 0.1× SSPE and 0.1% SDS at 65°C for 15 min each and exposed to x-ray film at −80°C with amplifying screens for 2 weeks.

Immunocytochemistry. Animals were perfused under deep anesthesia (Nembutal) with 60 ml of PBS, followed by 60 ml of 4% paraformaldehyde. Brains were removed and kept at 4°C in fixative for 16–20 hr. Sections were cut in the sagittal or frontal plane either on a vibratome (20 or 40 μm) or, after cryoprotection in 30% sucrose, on a freezing sliding microtome (14 or 20 μm). IGF-II-like immunoreactivity was detected using a polyclonal antibody against human IGF-II (Groppe, Australia). Cross-reactivity with chicken IGF-II in a radioimmunoassay was 100%, but cross-reactivity with chicken IGF-I or ovine insulin was <0.1%. Sections were permeabilized with 0.1% Triton X-100 and blocked with 3% skim milk, and endogenous peroxidase activity was quenched with 0.3% H2O2/10% methanol. Slides were then incubated overnight at 4°C with a 1:500 antibody dilution. After PBS washes, a biotinylated second-

Image
of ABC plus DAB reaction only, use of DAB reaction only, and preabsorption of the primary antibody with 40 μM [Gly1] IGF-II (Gropep), a recombinant analog of human insulin-like growth factor.

ICC with anti-mouse glial fibrillary acidic protein GFAP (Sigma) and with antibody 40 E-C, which recognizes avian vimentin (Alvarez-Buylla et al., 1987), was done at dilutions of 1:400 and 1:100, respectively, with all other incubation steps as outlined above.

Double labeling with retrograde tracers, ISH, and ICC. To identify the classes of neurons in HVC that synthesize IGF-II mRNA and IGF-II peptide, we retrogradely labeled the area X-projecting or RA-projecting...
neurons with rhodamine microspheres (Lumafluor, New York, NY) or with Fluorogold (Fluorochrome). This was accomplished by stereotaxically guided pressure injections of 50 nl of tracer through glass micropipettes (30 μm in diameter) into R.A or area X. Five days after tracer injections, brains were processed for ICC as outlined above and for nonradioactive ISH as described below.

Birds were decapitated, and their brains were quickly removed, placed in plastic block molds, surrounded with Tissue Tek (Miles, Elkhart, IN), immediately frozen in a dry ice and ethanol mix, and stored at −80°C. 10 μm frozen sections were cut sagittally or frontally on a cryostat, mounted onto 3-aminopropyl triethoxysilane (Aldrich, Milwaukee, WI) (TESPA)-coated slides, and stored at −80°C. Before hybridization, the sections were fixed in 3% paraformaldehyde in PBS for 5 min at room temperature, washed in three changes of PBS, and acetylated for 10 min in 1.4% triethanolamine with 3% acetic anhydride. The sections were washed in three changes of 2×SSPE, and dehydrated by ethanol (70%, 95%, and 100%, 3× each wash).

Slides were placed in blocking solution containing 2×SSPE, 2 mg/ml tRNA, 1 mg/ml bovine serum albumin, 100 mM Tris, pH 9.5, 100 mM NaCl, and 50 mM MgCl2. Chromogenic detection antibody was removed with four washes of 5 min each in TN buffer, and slides were equilibrated to pH 9.5 by incubating for 1 hr in 100 mM Tris, pH 9.5, 100 mM NaCl, and 50 mM MgCl2. Chromogenic detection was performed in the same buffer containing for every 1 ml: 4.5 mg/ml nitrophenol blue (Fluka, Buchs, Switzerland) in 70% dimethylformamide, 3.5 μl of 50 mg/ml X-phosphate (Fluka) in 100% dimethylformamide, and 10 μl of 24 mg/ml levamisole solution (Vector Laboratories, Burlingame, CA). A bluish-brown precipitate appeared within 30 min to 2 hr over labeled cells. Double-labeled cells were photographed with double exposure, ISH or ICC under bright-field illumination and rhodamine or Fluorogold under UV illumination.

RESULTS

cDNA cloning of songbird IGF-II

A cDNA library was constructed from zebra finch embryos and screened with a chicken IGF-II cDNA. A 1.4 kb cDNA containing the ORF encoding the zebra finch prepro-IGF-II was isolated and sequenced (Fig. 2). There was 88% homology to quail cDNA (M. Holzenberger and C. Ayer-LeLièvre, unpublished observations) within the first 0.3 kb of the 5′-untranslated region (UTR); homology was 87% in the 556 bp coding region but only 65% in the 3′-UTR, where several deletions and insertions were found (data not shown). Compared with quail, the deduced 68 amino acid sequence of the mature peptide from zebra finch showed only one substitution (residue 39, Fig. 3). This residue was located within a stretch of 10 amino acids (residues 31–40) that shows increased sequence variability when mammalian and avian IGF-II
IGF-II mRNA expression pattern in song nuclei

There was a high level of IGF-II mRNA expression in HVC of 100 canaries, zebra finches, and black-capped chickadees inspected (Fig. 5). Comparably high levels were seen only in choroid plexus and leptomeninges (Figs. 6H, 7A). Two other song control nuclei, mMAN and RA, also showed IGF-II expression, but there was no such expression in song nuclei area X and lMAN. In both zebra finches and canaries, the relative expression levels, as judged by density quantification of ISH label, differed significantly among HVC, mMAN, and RA (ANOVA, n = 7; F = 8.3; p = 0.003 for canaries) (n = 8; F = 15.6; p = 0.0001 for zebra finches). In canaries, HVC-specific label density was on average sixfold higher than RA-specific label density but only 1.7-fold higher than mMAN-specific label density. In each canary quantified (n = 7), the relative relationship of label intensity was HVC > mMAN > RA. In individual zebra finches, the relative expression levels among HVC, RA, and mMAN were more variable than in canaries. On average, HVC density in zebra finches was eightfold higher than mMAN density and threefold higher than RA density, but two of the eight adult zebra finches quantified had higher expression levels in RA than in HVC.

High magnification showed that only subsets of cells in HVC, RA, and mMAN expressed IGF-II mRNA (Fig. 6). In all three nuclei, IGF-II-positive cells were evenly distributed throughout the nucleus, and the anatomical borders of the nucleus coincided sharply with the limits of high IGF-II expression. The labeled cells had large cell bodies, a pale cytoplasm, and one or two well defined, darkly staining nucleoli, suggesting a neuronal phenotype. Within HVC, the IGF-II mRNA-positive cells often formed part of cell clusters, which typically contained one and sometimes more IGF-II mRNA-positive cells surrounded by several others that did not express the gene (Fig. 6E). The ventricular zone in which neuronal progenitor cells reside (Goldman and Nottebohm, 1983) did not contain IGF-II mRNA-positive cells (Fig. 6A, C). Isolated cells expressing high levels of IGF-II mRNA were found in the region traversed by axons going from HVC to RA. Some of those cells (data not shown) appeared to be glia, based on their size and Nissl-staining properties.

A crescent-shaped layer of cells expressing high levels of IGF-II was sometimes observed on the caudal border of the caudomedial neostriatum (NCM), just subjacent to the lateral ventricle (Fig. 5C). These cells are either adjacent to or part of the region of NCM that has been shown to be involved in song discrimination (Chew et al., 1995).

IGF-II mRNA expression pattern in other brain areas

We found widespread low-level IGF-II mRNA expression in parts of the neostriatum, hyperstriatum accessorium, and hyperstriatum ventrale as well as in the hippocampus and parahippocampus in all animals of the three species studied (Fig. 5). IGF-II-positive cells in the hippocampal complex were arranged in a layer parallel to the lateral ventricle (Fig. 7A); the majority of these cells was of medium to large size and apparently neurons, judged by their Nissl-staining profile, and more cells were labeled in proximity to the ventricle than toward the brain surface. This latter distribution is reminiscent of that of the new neurons added to the hippocampus of adult black-capped chickadees (Barnea and Nottebohm, 1994). Consistent labeling was also seen in the archifrontal tract, in which small, densely packed, apparently glial cells expressed IGF-II (Fig. 5A, 4). The rest of the telencephalon [e.g., lobus parolfactorius (LPO) including area X and ectostriatum] had no or very low IGF-II mRNA expression.

IGF-II mRNAs were present in several pontomesencephalic
nuclei, including the area ventralis of Tsai (AVT) (Fig. 7E), nucleus tegmenti pedunculopontinus pars compacta (TPc), and nucleus pontis lateralis (PL). Cerebellar Purkinje cells and several deep cerebellar nuclei expressed IGF-II mRNA in an otherwise IGF-II-negative cerebellum (Fig. 7C). There also was some IGF-II expression in the central layer of the olfactory bulb (data not shown). Leptomeninges and the choroid plexus, which are known to be sources of IGF-II in the embryonic and adult vertebrate brain, also expressed IGF-II mRNA in songbirds (Figs. 5C, 7A, G).

Negative control hybridizations included in each hybridization series using the IGF-II sense riboprobe produced uniform low background signals (Figs. 6B, D, F, 7B, D, F, H).ISH using a cRNA from the chicken IGF-II coding region or radiolabeled chicken IGF-II-specific oligonucleotides (M. Holzenberger and C. Ayer-LeLievre, unpublished observations) gave identical results to those observed using the zebra finch probe, confirming the specificity of the expression pattern.

Developmentally regulated IGF-II mRNA expression in zebra finch HVC

We studied the IGF-II mRNA expression levels in HVC during three phases of zebra finch development related to known times of neural (Nordeen and Nordeen, 1988b; Burek et al., 1990) and behavioral (Immelmann, 1969; Price, 1979; Eales, 1985) plasticity: (1) between posthatching days 36 and 54 during the stage that corresponds to early song development, (2) between posthatching days 65 and 75 when juvenile zebra finches are in late plastic song, and (3) in adults, ranging from 93 d to 4.7 years old, a period when song is crystallized and stable. The level of IGF-II expression in HVC differed significantly among younger juveniles (mean age of 42 d), older juveniles (mean age of 68 d), and adults as shown in Figure 8. Older juveniles had significantly higher IGF-II expression than either younger juveniles or adults (p < 0.01 and p < 0.001, respectively). The younger juvenile birds had more IGF-II label than adults, but not significantly more. Other song nuclei, e.g., RA, area X, and IMAN, did not exhibit elevated IGF-II mRNA levels during the developmental time period studied.

Seasonal regulation of IGF-II mRNA expression

Song plasticity, blood testosterone levels, neuronal recruitment, and cell death in HVC change significantly during the annual reproductive cycle of adult male canaries (Nottebohm et al., 1987; Kirn et al., 1994), so we were curious to establish whether IGF-II expression might also be seasonally regulated. We compared IGF-II expression levels in HVC of an average of four canaries (range, 2–6) per month throughout an entire year, starting in April...
Figure 6. Cellular expression of IGF-II mRNA in canary (A, C, E, G) and zebra finch (H) song nuclei and negative controls (B, D, F) hybridized with the sense probe. A, HVC shows a uniform distribution of IGF-II-labeled cells and a homogeneous signal intensity throughout the area. Arrowheads (A–D) indicate the position of the lateral ventricle. C, Higher magnification reveals that not all cells in HVC are labeled and that the ventricular zone is negative. E, Within the cell clusters typical for HVC, IGF-II mRNA is often expressed in a subset of HVC cells (black arrows) surrounded by IGF-II negative cells. Nomarski optics highlight cell nuclei as darker, round profiles surrounded by a lighter-appearing cytoplasmic region, the boundaries of which cannot be seen. The grain distribution of the upper IGF-II-positive cell indicates labeled mRNA both in the cytoplasm overlying the nucleus and in the cytoplasm extending beyond the nucleus to the top right. B, D, F. In control sections hybridized with the sense strand, adjacent to those sections shown in A, C, and E, respectively, label is not different from background. G, IGF-II is strongly expressed in adult canary mMAN. H, IGF-II-positive cells are uniformly distributed in adult zebra finch RA, although this intensity of label was rare. White arrows indicate the anatomical outline of the RA; white arrowheads point to the IGF-II-positive meninges. All photomicrographs were obtained from parasagittal sections (dorsal is at the top, and rostral is to the right) under dark-field (A, B, G, H) or Nomarski illumination (C–F). Scale bars: A, B, 500 μm; C, D, 50 μm; E, F, 10 μm; G, H, 250 μm.

Figure 7. Conspicuous IGF-II mRNA expression was found in the hippocampus (A), cerebellar Purkinje cells (C), brainstem (E), and choroid plexus (A, G). B, D, F, H. Negative controls hybridized with the sense probe show signals not different from background. A, A low-magnification dark-field photomicrograph shows a streak of IGF-II-positive cells (white arrowheads) in the hippocampus of an adult canary. The IGF-II-positive meninges are indicated with a black arrowhead. Note also that the choroid plexus (white arrow) is strongly labeled. C, Cerebellar Purkinje cells, shown here in an adult zebra finch, were strikingly labeled (arrows). Often deep cerebellar nuclei were also positive. E, Several pontomesencephalic nuclei showed IGF-II mRNA expression, among them AVT (arrow) shown here in a dark-field photomicrograph of an adult canary section. G, A high-power light-field photomicrograph shows that IGF-II mRNA is primarily expressed in the epithelium (arrows) of the choroid plexus, shown here in an adult zebra finch. All sections are cut in the parasagittal plane ~500 μm from the midline; dorsal is at the top, and caudal is to the left. Scale bars: A–F, 1 mm; G, H, 10 μm.
and ending in March, and found that the average monthly levels across the 12 month period changed but that these changes were not significantly different, owing partly to high intramonth variability (one-way ANOVA, n = 45; F = 1.2; p > 0.05; Fig. 9B). However, when we compared our observations on IGF-II mRNA levels in HVC with the published data on neuronal recruitment in the same nucleus (Fig. 9A,B) in birds of comparable age, we saw that the month-to-month changes in both curves were in the same direction for 10 of the 11 transitions. In fact, the monthly changes in neuronal recruitment covaried significantly with the monthly changes in IGF-II mRNA expression (Fig. 9C; n = 11; r = 0.595; p < 0.05). No other previously studied seasonally varying parameters (cell death, testosterone or estrogen levels, and syllable addition) covaried with IGF-II expression levels (p > 0.05 in all cases).

**Immunocytochemical localization of IGF-II in the adult brain**

HVC showed conspicuous and specific IGF-II-like immunoreactivity in all brains analyzed (n = 23) (Fig. 10A). The boundaries defined by the IGF-II immunoreactivity coincided with the Nissl-stained boundaries of HVC in adjacent sections. Preincubation of the antibody with recombinant IGF-II peptide abolished the specific IGF-II staining in HVC (Fig. 10B) and in all other parts of the brain (n = 2; data not shown). Often, some cells in a neuronal cluster within HVC would show the antibody label whereas others were negative. The label occurred as numerous oval or curved profiles located in proximity to the nucleus of cells (Fig. 10C). This pattern suggested that the immunoreactivity was localized to the Golgi or late endosomal compartment. Analysis of 1 μm plastic sections supported this interpretation (data not shown). Also mMAN was always immunopositive (Fig. 10D,E), but in this case the label was not Golgi-like but diffusely present throughout mMAN. Interestingly, some canaries (4 of 10) showed Golgi-like staining in mMAN, although of less intensity than in HVC in the same brain. We did not find IGF-II-like immunoreactivity in RA or area X.

In addition to the ICC label seen in HVC and mMAN, the radial cells typical of adult songbird forebrains were always strongly IGF-II-immunoreactive (Fig. 10H, I). Most of these radial cells had their cell bodies in the ventricular zone of the lateral wall of the lateral ventricle and had a long, single, undivided process that reached into the brain parenchyma. The radial cells of adult canaries are vimentin-positive and serve as guides for the initial dispersal of the newly generated neurons (Alvarez-Buylla et al., 1987, 1988b). The ventricular zone throughout the lateral ventricle showed punctate IGF-II immunoreactivity (Fig. 10K). Also immunopositive was a population of stellate cells with a morphology reminiscent of microglia (Fig. 10F, G). Both radial cells and stellate cells often contacted microvasculature. The stellate cells were negative to antibodies against avian GFAP and vimentin, markers for astrocytes (data not shown). Expression of IGF-II mRNA in stellate cells was found throughout the brain, although with varying densities; e.g., they were much more abundant in the LPO than in neostriatum (Fig. 10G). IGF-II-immunoreactive stellate cells were never observed in HVC, and only very few were seen in RA, although in the surrounding brain tissue in both cases this cell type was commonly detected. Cerebellar Purkinje cell bodies had faintly positive cytoplasmic immunoreactivity, but strong varicose immunoreactivity was seen on what appeared to be the Purkinje cell dendrites extending into the molecular layer of the cerebellum (data not shown).

**Retrograde identification of IGF-II-positive neurons in HVC**

To identify which HVC neurons expressed IGF-II mRNA and which accumulated IGF-II peptide, we combined injection of two retrograde tracers, Fluorogold and rhodamine microspheres, with either IGF-II nonradioactive ISH or ICC (total n = 21). The smaller RA-projecting neurons often cluster around area X-projecting neurons (Krn and Nottebohm, 1990). When we injected rhodamine microspheres into area X and performed nonradioactive ISH on sections containing HVC, we found that all of the cells within HVC that were retrogradely filled with rhodamine microspheres also showed IGF-II mRNA label. Thus, area X-projecting neurons accounted for >85% of IGF-II mRNA-positive HVC cells (mean, 87%; range, 75–92%; six animals; three sections per HVC) (Fig. 11A, B). The small proportion of IGF-II mRNA-positive cells that was not retrogradely labeled from area X could be either another cell class such as interneurons, glia, RA-projecting cells, or area X-projecting cells that had not been backfilled by the rhodamine beads. The possibility that these are RA-projecting cells was tested by retrogradely labeling RA-projecting cells with rhodamine microspheres (n = 4). In this case, none of the rhodamine-labeled RA-projecting cells was IGF-II mRNA-positive. Instead, IGF-II mRNA was often detected in the cells immediately adjacent to the RA-projecting, rhodamine-labeled cells (Fig. 11C, D). Thus, within HVC, IGF-II mRNA is likely to be produced mainly and possibly exclusively by the area X-projecting neurons. Interest-
neuron addition were calculated from the original data generated by Kirn et al. (1994). B, HVC-specific IGF-II mRNA was present at varying levels throughout the year, but differences were not statistically significant (ANOVA, $F = 7.3; p = 0.0001$): Reproduced with permission from Kirn et al. (1994) and were used to plot A. Monthly changes in IGF-II expression levels were calculated from the data shown in B. Each point on the scatterplot (C) represents one monthly transition, e.g., June-to-July, July-to-August, etc. The letters on the x-axis for A and B are the first letter for each month of the year, starting on the left with June.

DISCUSSION

Cloning of the zebra finch IGF-II cDNA

In agreement with previous findings in mammals (Lund et al., 1986; Beck et al., 1988) and chicken (Taylor et al., 1991), Northern hybridization of IGF-II total RNA from songbird forebrain revealed multiple transcripts; only one of these mRNA species, the 4.0 kb, was enriched by poly(A$^+$) selection, as was observed in rats (Graham et al., 1986). This suggests that the smaller mRNA species detected in total RNA are not polyadenylated. The different transcript sizes could be attributable to RNA processing, alternative polyadenylation signals leading to different lengths of 3'-UTRs, or initiation of transcription at different promoters resulting in different length 5'-UTRs (Nielsen, 1992). The variability in the 5'-UTRs has been proposed to be correlated with different subcellular localizations. Our results suggest that transcriptional events leading to multiple transcripts may be as complex in birds as in mammals.

The 1.4 kb zebra finch clone we isolated contains the entire coding region of the IGF-II prepropeptide and parts of the 5'- and 3'-UTRs. The more distal portions of the UTRs are not present in our clone, resulting in a cDNA that is smaller in size than the 4.0 kb seen in the poly(A$^+$) Northern hybridizations. Our zebra finch clone was similar in size to a quail IGF-II cDNA also obtained from an oligo-dT-primed embryonic library (M. Holzenberger and C. Ayer-LeLièvre, unpublished observations). The presence of strong secondary structures in the UTRs may lead to inefficient reverse transcription, generally resulting in smaller cDNAs. Sequencing of the more distal 3'-UTR of quail IGF-II (M. Holzenberger and C. Ayer-LeLièvre, unpublished observations) revealed several poly(A$^+$)-rich regions, upstream of the poly(A$^+$)-tail, where oligo-dT-initiated cDNA synthesis could have started, providing another explanation for the shorter 3'-UTR in our cDNA.
IGF-II expression pattern in the songbird brain

Two findings stand out. First, the IGF-II gene is selectively and strongly expressed in neurons of HVC of songbirds. Previously, robust IGF-II mRNA expression in adult vertebrate brain had been seen only in cells of non-neuroepithelial lineage, e.g., choroid plexus, leptomeninges, and vascular endothelial cells (Bondy et al., 1992; Couce et al., 1992; Sullivan and Feldman, 1994), all of which expressed IGF-II in our study also. Second, the HVC neurons that produce the IGF-II mRNA are members of the stable, nonreplaceable population that projects to area X, whereas the replaceable neurons that project to RA (Alvarez-Buylla et al., 1990; Kirn et al., 1991) accumulate the IGF-II peptide in the Golgi/late endosomal compartment. This suggests a paracrine mode of IGF-II action in a particularly interesting brain region that shows remarkable adult plasticity (neuronal replacement) and controls a quantifiable, learned motor behavior (song).

Why does the IGF-II peptide of the RA-projecting cells accumulate in the Golgi/late endosomal compartment? In mammals and birds, IGF-II binds to the IGF type 1 receptor, a tyrosine kinase, activating a second messenger cascade (for review, see LeRoith et al., 1995) that is thought to mediate the mitogenic and metabolic actions of IGF-II (for review, see Nielsen, 1992; Efstratiadis, 1994). In mammals, but not in birds, IGF-II also binds to the IGF-II/mannose 6-phosphate receptor (IGF type 2 receptor/M6PR). This IGF type 2 receptor functions in receptor-mediated endocytosis and cycles between the cell membrane and the Golgi network in a pathway that seems to be involved in rapid internalization and degradation of excess IGF-II (for review, see Nielsen, 1992). Consistent with this, immunoreactive detection of the IGF-II peptide as well as accumulation of radiolabeled IGF-II in the Golgi apparatus has been seen in mammalian tissues (Mossner et al., 1986; Kotani et al., 1993). The IGF type 2 receptor-mediated rapid turnover pathway in mammals is essential for perinatal survival of mice, as has been convincingly argued by combinatorial knock-outs of the IGF type 1 and 2 receptors and of IGF-II itself (for review, see Ludwig et al., 1996). Interestingly, the avian M6PR does not have an IGF-II binding site, and other than the type 1 receptor (Holzenberger et al., 1996), no alternative avian IGF type 2 receptor has yet been identified (Zhou et al., 1995). However, the immunoreactive staining we observe in HVC suggests that a type 2-like receptor-mediated endocytic pathway exists in birds, leading to accumulation of IGF-II in the Golgi/late endosomal compartment.

Functional implications of IGF-II in HVC

Because IGF-II mRNA is produced in the area X-projecting cells of HVC and the peptide is found in the RA-projecting cells of HVC, IGF-II secreted by the area X-projecting neurons may act locally. A more remote, but possible, source of IGF-II peptide in HVC could be the mMAN neurons that project into HVC.
type of orthogradic transport has been observed for IGF-I in the olivary–cerebellar system (Nieto-Bona et al., 1993) and for BDNF and NT3 in songbirds (Johnson et al., 1997). Whatever the source, the presence of IGF-II in HVC could protect a subset of RA-projecting cells from cell death while others die and are replaced. A precedent for the ability of neurotrophins to protect neurons in the song system from apoptosis has recently been described (Johnson et al., 1997). The potential of IGFs to prevent neuronal apoptosis has been demonstrated in cerebellar granule cells (D’Mello et al., 1993; Galli et al., 1995) and in the spinal cord (Quin-Wei et al., 1994). Recently, IGF-II has been demonstrated to prevent apoptosis in a variety of non-neuronal cells in vitro (Polychronakos et al., 1995; Stewart and Rotwein, 1996; Ueda and Ganem, 1996).

Our observation that monthly changes in neuron incorporation covary with monthly changes in IGF-II expression levels in HVC raises the possibility that relatively higher levels of IGF-II facilitate the incorporation or survival of new HVC neurons. However, IGF-II could also act directly on ventricular zone stem cells that give rise to new neurons (Goldman and Nottebohm, 1983), as attested by the punctate immunoreactivity present in the ventricular zone. A direct action of IGF-II on neurogenesis would be in line with the control of cell proliferation in other systems (Edbladh et al., 1994; Zackenfels et al., 1995). It is possible, too, that the amount of singing influences IGF-II expression, as has been reported recently for two immediate early genes (Jarvis and Nottebohm, 1997; Kimpo and Doupe, 1997), and we are currently examining this.

Additional evidence of the potential importance of IGF-II in the life of new neurons in HVC stems from our observation that IGF-II mRNA expression in HVC was higher during development than in adulthood. The connections of HVC to its two efferent targets, RA and area X, are well established by day 35 (Konishi and Akutagawa, 1985; Mooney and Rao, 1994), but significant numbers of new neurons are added to zebra finch HVC until ~50–60 d of age, coinciding with the major sensory–motor integration phase of song learning (Nordeen and Nordeen, 1988a,b). The intriguing peak in IGF-II mRNA expression in older (65–75 d) juvenile zebra finches coincides with the closing of the sensory–motor integration phase and may have something to do with the consolidation of connections that underlie the learned song pattern. This could occur via neurite extension and synapse formation, effects that IGF-II has been shown to facilitate in other systems (Liu and Lauder, 1992; Konishi et al., 1994). In summary, although our data on seasonal and developmental fluctuations of IGF-II mRNA levels in HVC are not conclusive, they suggest a role for IGF-II in the generation, maturation, and survival of new neurons in the song system.

**Functional implications of IGF-II in other areas of the brain**

The proposed role of IGF-II in HVC might not apply to the rest of the brain. It is worth noting that both the hippocampus and NCM, which showed IGF-II mRNA expression, exhibit adult neurogenesis (Barnea and Nottebohm, 1994; F. Nottebohm, unpublished observations) and are implicated in mediating memory formation (Krebs et al., 1989; Chew et al., 1996). We also found IGF-II mRNA expressed in the olfactory bulb, as has been observed in chicken and mammals (Ayer-Le Lièvre et al., 1991; Lee et al., 1993). Again, adult neuronal recruitment is well documented in this area (Altman, 1969; Kaplan et al., 1985; Lois and Alvarez-Buylla, 1994) and seems to be regulated by behavior (Corotto et al., 1994). Conversely, no new neurons are added to the cerebellum of adult songbirds, where we detected IGF-II expression, or to the cerebellum of adult rodents (Altman and Bayer, 1993), where IGF-II is expressed and necessary for the acquisition of a simple learning paradigm (Castro-Alamancos and Torres-Aleman, 1994). An explanation for this coincidence of IGF expression with behavioral plasticity and/or recruitment of new neurons is that IGF-II may be involved in processes that are prominent in areas of active remodeling, whether because of neuronal turnover requiring neurite extension and synaptic remodeling or because of learning-induced changes in neuronal architecture.

Our understanding of regional IGF-II distribution is certainly not complete. For example, we noticed variable presence of IGF-II mRNA, but no peptide, in RA neurons. Transfational discrimination of the various IGF-II mRNA transcripts or lack of immunoreactivity in areas with only moderate or low IGF-II peptide levels are possible explanations for this. Furthermore, peptide levels in mMAN were low and diffuse, whereas the mMAN neurons showed solid mRNA expression. Intriguingly, in some instances lMAN, which was not seen to express IGF-II mRNA, had Golgi-like peptide staining associated with its neurons, raising the possibility that IGF-II secreted by mMAN neurons finds its way to the adjacent IMAN. No anatomical connection between these nuclei so far has been described. Finally, we found strong immunopositive IGF-II staining in some areas that have little if any IGF-II mRNA expression (e.g., thestellate cells in LPO). Similarly, the radial cells, the cell bodies of which abut the lateral ventricle, accumulate peptide along the length of their process but show no mRNA production in their cell body. These cells may take up IGF-II from...


Burek MJ, Nordeen KW, Nordeen EJ (1990) Neuron loss and addition of the cerebrospinal fluid, which has high titers of IGF-II produced by the choroid plexus and leptomeninges. Clearly, more work is necessary to address the functional relationship between sites of IGF-II production and peptide accumulation and before we can decide whether the IGF-II peptide exerts its effect in all cases by acting on the same cellular processes.

**Perspectives**

Our study suggests a correlation between IGF-II expression and neuronal plasticity. More definitive insights should come from interferring with IGF-II expression in the songbird brain through the use of selective lesioning methods (Scharff et al., 1994) as well as from blocking IGF-II at the transcriptional (Castro-Alamancos and Torres-Aleman, 1994) and translational (Nielsen et al., 1995) levels. It is likely that the striking pattern of IGF-II mRNA and peptide distribution that we have described in songbirds is just the first glimpse of a complex system that includes not only the IGFs but also their receptors and the binding proteins that modulate the bioavailability of the IGFs (for review, see Jones and Clemmons, 1995). HVC may be a particularly compelling place to study these events because this nucleus, which shows striking levels of IGF-II expression and adult neuronal replacement, also governs the acquisition and production of a changeable and quantifiable learned behavior.


