

Insulin-Like Growth Factor-1 Is a Radial Cell-Associated Neurotrophin that Promotes Neuronal Recruitment from the Adult Songbird Ependyma/Subependyma

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ABSTRACT: In the adult songbird forebrain, neurons continue to be produced from precursor cells in the forebrain ependymal/subependymal zone (SZ), from which they migrate upon radial guide fibers. The new neurons and their radial cell partners may coderive from a common SZ progenitor, which may be the radial cell itself. On this basis, we asked whether radial cells might provide trophic support for the migration or survival of newly generated neurons. We focused upon the insulin-like growth factors (IGFs) IGF-1 and IGF-2, which have previously been shown to support the survival and differentiation of neural progenitor cells. We found that IGF-1 immunoreactivity was expressed heavily by adult zebra finch radial cells and their fibers, with little expression otherwise. IGF-2, in contrast, was expressed by paren-

chymal astrocytes and exhibited little radial cell expression. Despite their distinct distributions, IGF-1 and IGF-2 exerted similar trophic effects on finch SZ cells *in vitro*; both greatly increased the number of neurons migrating from explants of the adult finch SZ, relative to explants raised in low-insulin, IGF-1-deficient media. However, neither factor extended neuronal survival. These results suggest that in neurogenic regions of the adult avian forebrain, IGF-1 acts as a radial cell-associated neuronal differentiation and/or departure factor, which may serve to regulate neuronal recruitment into the adult brain. © 1998 John Wiley & Sons, Inc. *J Neurobiol* 36: 1–15, 1998

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INTRODUCTION

The avian forebrain exhibits ongoing neurogenesis throughout adulthood, with the production of new neurons from ependymal/subependymal zone (SZ) precursor cells overlying the mediocaudal neostriatum (Goldman and Nottebohm, 1983; Alvarez-Buylla and Nottebohm, 1988). These SZ progenitors

proliferate tonically and give rise to both neurons and radial guide cells, upon which the neurons migrate to infiltrate the neostriatal parenchyma (Alvarez-Buylla et al., 1990; Goldman et al., 1996). We previously noted that in cultures of the adult SZ, new neurons were most abundant in association with ependymal and/or radial cells (Goldman et al., 1993). Neuronal survival was promoted *in vitro*, at least in part through the activation of an NgCAM-driven calcium response experienced by the migrating new neuron (Goldman et al., 1996). *In vivo*, this response might be triggered by radial cell-derived heterophilic ligands for NgCAM (neuron-glia cell adhesion molecule), which include membrane-bound receptor tyrosine phosphatase- β (Grumet et al., 1994) and its soluble form, phosphacan (Milev et al., 1994). Together, these observations suggested that radial cells might be neurotrophic for their neuronal partners.

In this report, we asked whether radial cells might influence neuronal migrants through humoral as well as contact-dependent means, and focused upon one set of likely agents, the insulin-like growth factors (IGFs), IGF-1 and IGF-2. IGF-1 is distributed widely in the brains of both birds and mammals (de la Rosa et al., 1994; Kar et al., 1993), where it is a potent stimulus to both oligodendrocytic (McMorris et al., 1986; Beck et al., 1995) and neuronal differentiation and maturation (Recio-Pinto et al., 1986; Zackenfels et al., 1995; reviewed in de Pablo and de la Rosa, 1995). Disruption of the IGF-1 gene results in reduced brain size and loss of hippocampal granule and striatal parvalbumin-containing neurons (Beck et al., 1995). IGF-1 supports both the production (Nielsen et al., 1991; Lenoir and Honegger, 1983) and survival (Drago et al., 1991) of neural precursors during ontogeny, and its expression persists in the adult brain. Like IGF-1, IGF-2 is heavily and widely expressed in brain development, although its expression falls with maturation, such that in the adult brain, only the choroid plexus, meninges, and blood vessels continue to express significant levels (Valentino et al., 1990).

The role of the IGFs in adult neurogenesis has not yet been explored in avian or mammalian model systems, even though its role in developmental neuronal and oligodendrocytic production makes it a likely participant in postnatal neurogenesis as well. In this study, we therefore asked whether IGF-1 or IGF-2 were specifically localized to neurogenic regions of the adult songbird brain, to what cells IGF expression could be attributed, and whether exogenous IGF proteins were supportive of neu-

ronal production, outgrowth, or survival *in vitro*. We report that in the forebrain of the adult zebra finch (*Poephilia guttata*), IGF-1 is expressed in a distribution that suggests its importance in the recruitment of new neurons into the neostriatum. In particular, IGF-1 may act as a radial cell-associated agent that induces neuronal differentiation within, and departure from, the adult ependymal/subependymal zone.

MATERIALS AND METHODS

Immunocytochemistry

To immunostain IGF-1 and IGF-2, we used a mouse monoclonal anti-human IGF-1 immunoglobulin G (IgG) and a mouse monoclonal anti-rat IGF-2 IgG (Upstate Biotechnology, NY). Adult zebra finches ($n = 10$: four males and six females) were perfused via a transcardiac approach with cold 0.1 M phosphate-buffered saline (PBS), followed by 4% paraformaldehyde; the brains were removed and postfixed for 45 min, then immersed in 6% sucrose in 0.1 M PB for 2 h and 30% sucrose overnight at 4°C. The samples were frozen, cut into 10- μ M coronal sections on an IEC cryostat, and collected on Vectabond-coated slides (Vector Labs). All sections were immunostained by successive incubation in PB, 0.1% Triton X-100 in PB [15 min at room temperature (RT)], 10% normal goat serum in PB (10 min at RT), primary antibody (mouse anti-human IGF-1 or anti-rat IGF-2 IgG, each at 1:25 overnight at 4°C), three washes with 1% goat serum in PB (1 min each at RT), and then biotinylated anti-mouse IgG (1:100; Vector; 1 h at RT). After three additional washes (1 min each), the sections were exposed to horseradish peroxidase-avidin-biotin complex (1 h at RT; Vector), washed again (three times at 1 min each), then developed with diaminobenzidine/ H_2O_2 (5–10 min/section, RT). Cross-reactivity between IGF-1 and IGF-2 was <5% for each antibody, and was assessed for each in western immunoblots of recombinant chicken IGF-1 and IGF-2 (data not shown).

Double Labeling for Radial Cells and IGF-1

To determine the phenotype of both IGF-1- and IGF-2-positive cells, sections were double stained for IGF-1 together with (a) the ependymoglia vimentin-associated filament recognized by monoclonal antibody (mAb) 3A7, a mouse IgM (Lemmon, 1985; a gift of Dr. V. Lemmon); (b) the neuronal RNA-binding protein Hu (Szabo et al., 1991; Barami et al., 1995), stained using human anti-Hu autoimmune antiserum (generously provided by Drs. J. Posner and J. Dalmau); or (c) the neuronal protein β III tubulin (Lee et al., 1990), recognized

by rabbit anti- β III tubulin IgG (a gift of Dr. A. Frankfurter). Each was immunostained using previously described protocols (3A7: Goldman et al., 1993; Hu: Barami et al., 1995; β III tubulin: Menezes and Luskin, 1994).

Assessment of IGF-1 and IGF-2 Effects *in vitro*

Culture Preparation and Media Formulation. Explants were obtained from a total of 22 adult female zebra finches. Cultures were prepared from the neostriatal SZ, both overlying and directly medial to nucleus higher vocal center (HVC), as previously described (Goldman, 1990; Goldman et al., 1992). Briefly, 40 to 50 explants were obtained from each bird and distributed in groups of four into laminin-coated, 35-mm Petri dishes. The plates were then incubated in 5% CO₂/95% air at 37°C in 0.65 mL media. For these experiments, we used a low-insulin formulation that consisted of 1:1 Dulbecco's modified Eagle's medium (DMEM)/F12, free of Phenol red and glutamate, and supplemented with reduced-insulin N2 (Bottenstein and Sato, 1979) (with 20 ng/mL insulin), hydrocortisone (300 ng/mL), progesterone (20 nM = 6.29 ng/mL), and 50 U/mL penicillin–streptomycin–amphotericin. The medium was supplemented with 10% fetal bovine serum (FBS) with an average insulin level of 0.35 ng/mL by radioimmunoassay (RIA) (HyClone Labs). Each culture was given a complete medium change twice weekly in its test medium.

Assignment to Treatment Groups. From each finch, approximately 40 explants were prepared and distributed randomly among three different treatment conditions. These included: (a) 10% FBS only, (b) 10% FBS with 80 ng/mL (10 nM) recombinant chicken (rc) IGF-1 (GroPep, Australia), and (c) 10% FBS with 80 ng/mL (10 nM) rcIGF-2 (GroPep). Once an explant displayed outgrowth, neurons were identified morphologically using established criteria (Goldman, 1990; Goldman et al., 1992) and counted on roughly alternate days from 4 to 24 days *in vitro* (DIV). From each treatment group, the number of neurons per explant was calculated for each day of observation; those explants with five or more neurons per outgrowth by day 8 were followed serially.

The primary outcome measure was the number of neurons per outgrowth at each time point. Several factors were considered for possible association with the outcome: (a) bird, (b) treatment (control, IGF-1, or IGF-2), and (c) DIV. The primary hypothesis was that the number of neurons per outgrowth would be changed by the addition of IGF-1 and/or IGF-2. Ninety-five percent confidence intervals were also constructed for the proportion of explants exhibiting outgrowth in each group.

Statistical Analysis. The mean numbers of neurons per explant were calculated for each treatment at each time point, as were the standard deviations (S.D.) and errors of

the mean (S.E.). Analysis of variance (ANOVA) using a generalized linear model was employed to determine whether treatment was significantly associated with the number of neurons in each outgrowth (Searle, 1987). Higher-level effects were considered as follows: (a) Bird \times Treatment group, (b) Bird \times Day, (c) Treatment group \times Day, and (d) Bird \times Treatment group \times Day. The use of this model was based on an assumption of normally distributed data; the data were therefore plotted to determine their normality, and transformations used as appropriate. Posthoc pairwise comparisons were made using the *t* test with Bonferroni adjustments for multiple comparisons. Three comparisons were tested: (a) control versus IGF-1, (b) control versus IGF-2, and (c) IGF-1 versus IGF-2. A difference was deemed significant if its associated *p* value was $< p = 0.017$ (0.05/3 comparisons), based on a two-sided test. The Bonferroni method was applicable in this case, given the unbalanced sample sizes and small number of tested hypotheses (Miller, 1981).

Three additional comparisons among treatment groups were performed using ranked data, by means of the Kruskal–Wallis test. These included: (a) the initial rates of neuronal outgrowth between days 0 and 4, (b) a day-by-day comparison of neuronal outgrowth in each group, and (c) a comparison of the maximal neuronal accumulation in each group (as reflected in the day 8 neuronal outgrowth).

All analyses were done using SAS/STAT for Windows v.6.12 (SAS Institute, Cary, NC).

[³H]Thymidine Autoradiography. To assess the incidence of mitotic neurogenesis in these cultures, [³H]-thymidine was added to a sample of 16 plates derived from two birds ($n = 112$ explants), which were divided into groups raised in the presence or absence of IGF-1 (80 ng/mL). Cultures were exposed to [³H]thymidine (0.25 μ Ci/mL; 5 Ci/mM; Amersham) continuously for 8 days after preparation, then fixed, immunostained for MAP-2, and autoradiographed as previously described (Goldman et al., 1992).

IGF-1 Radioimmunoassay

Adult female finches ($n = 6$) were anesthetized and their brains were perfused by a transcardiac approach with cold 0.1 M PBS. Brains were removed and dissected on ice, to collect the following regions: (a) HVC and its immediately adjacent neostriatum, (b) the medio-caudal neostriatum (MCN), (c) anterolateral hyperstriatum, (d) lateral neostriatum (at the anteroposterior level of HVC), (e) cerebellum, and (f) optic tectum. To ensure adequate amounts of tissue for analysis, tissues from 2 birds/region were pooled and designated as one sample. Samples were then prepared in triplicate, with six birds contributing to 3 samples/region. Each was weighed and stored at -70°C until RIA. At that point, the tissue IGF-1 concentrations

were determined using a homologous RIA for chicken IGF-1, as previously described (McMurtry et al., 1994). Briefly, frozen samples were thawed and homogenized in 1 M glacial acetic acid, at a 1:2 wt/vol ratio. The samples were then vortexed and maintained on ice for 2 h. Each was then centrifuged at $15,000 \times g$ for 5 min, and 25- μ L aliquots of the supernates were apportioned to polypropylene tubes, then dried under vacuum. Dried pellets were redissolved in RIA diluent [0.03 M sodium phosphate buffer, pH 7.5, with 0.01 M ethylenediamine tetraacetic acid (EDTA), 0.02% protamine sulfate, 0.02% sodium azide, and 0.05% Tween-20]. All samples were assayed in duplicate in a single assay to avoid interassay variation. The intra-assay coefficient of variation was <5%.

RESULTS

IGF-1 Was Expressed by Radial Cells in the MCN

IGF-1 protein was identified throughout the MCN and HVC, with heavy expression by radial cells [Fig. 1(A–D)]. To identify the phenotype of the IGF-1-immunoreactive cells, sections were concurrently stained for both IGF-1 and the ependymogial vimentin-associated filament recognized by mAB 3A7 (Lemmon, 1985; Goldman et al., 1993). Double immunolabeling revealed that essentially all of the neostriatal IGF-1-positive cells expressed 3A7 [Fig. 1(C, D)]. No β III-tubulin or Hu expression was noted by IGF-1-positive cells. No difference was noted in either the immunocytochemical distributions or staining intensities of IGF-1 immunoreactivity in male and female finches (data not shown). In both sexes, the high level of IGF-1 expression by radial cells suggested a role for this protein in radial cell support of neuronal differentiation, migration, and/or survival.

To further validate that IGF-1 immunoreactivity specifically reflected IGF-1 protein, we performed a set of preabsorption controls over and above routine nonimmune sera controls. We found that preabsorption of anti-IGF-1 IgG with recombinant chicken IGF-1 (1 μ g/mL of diluted antibody) completely abolished IGF-1 immunostaining. Furthermore, neither preabsorption of anti-IGF-1 with IGF-2 protein nor that of anti-IGF-2 with IGF-1 protein affected the pattern or intensity of IGF immunostaining. Thus, the anti-IGF-1 and anti-IGF-2 antibodies used were operationally specific for their respective immunogens.

IGF-1 Concentrations Were Highest in HVC and the Adjacent MCN

Radioimmunoassay for IGF-1 was employed to measure and compare the tissue levels of IGF-1 in regions dense in radial cells, the HVC and adjacent MCN, to regions containing lower densities of radial cells and fibers (the lateral neostriatum and anterior hyperstriatum were chosen in the forebrain, and the optic tectum and cerebellum in the hindbrain). RIA for IGF-1 revealed that among the six regions tested, the highest levels of IGF-1 were found in the HVC (Table 1). In fact, HVC, with 276 ± 29 pg IGF-1/g tissue, had more than threefold the IGF-1 concentration of the anterolateral hyperstriatum, a forebrain region with far fewer radial cells (86 ± 16 pg/g) ($p < 0.01$ by Tukey's posthoc t test after one-way ANOVA) (Table 1). Interestingly, the neostriatum immediately lateral to HVC, a region characterized by dense populations of radial cells and neuronal migrants but little mitotic neurogenesis, had relatively high levels of IGF-1 (201 ± 5 pg/g), whereas the adjacent MCN, a region with active neurogenesis but relatively short radial fibers, had less than half of HVC's IGF-1 concentration (133 ± 12 pg/g). Thus, among the sampled regions, which included a large portion of the adult finch brain, IGF-1 levels were highest within the HVC, and appeared to correlate in part with radial cell density.

IGF-2 Was Expressed by Neostriatal Astrocytes in the Adult Songbird

IGF-2 immunostaining was noted by pial cells and surrounding blood vessels, and also by small fiber-bearing cells scattered throughout the neostriatum (Fig. 2). These parenchymal IGF-2-positive cells were astrocytic morphologically, and generally colabeled with the anti-ependymogial antibody 3A7; in contrast, they did not label with antibodies against either of the neuronal proteins Hu or β III tubulin (not shown). Importantly, whereas 3A7 is expressed by both astrocytes and radial cells, IGF-2 expression was noted only by astrocytes: No radial cell or fiber expression of IGF-2 was noted in the finch. This pattern of immunoreactivity was distinct from, and nonoverlapping with, IGF-1's predominant radial cell expression, and suggested distinct functions for IGF-1 and -2 in this system.

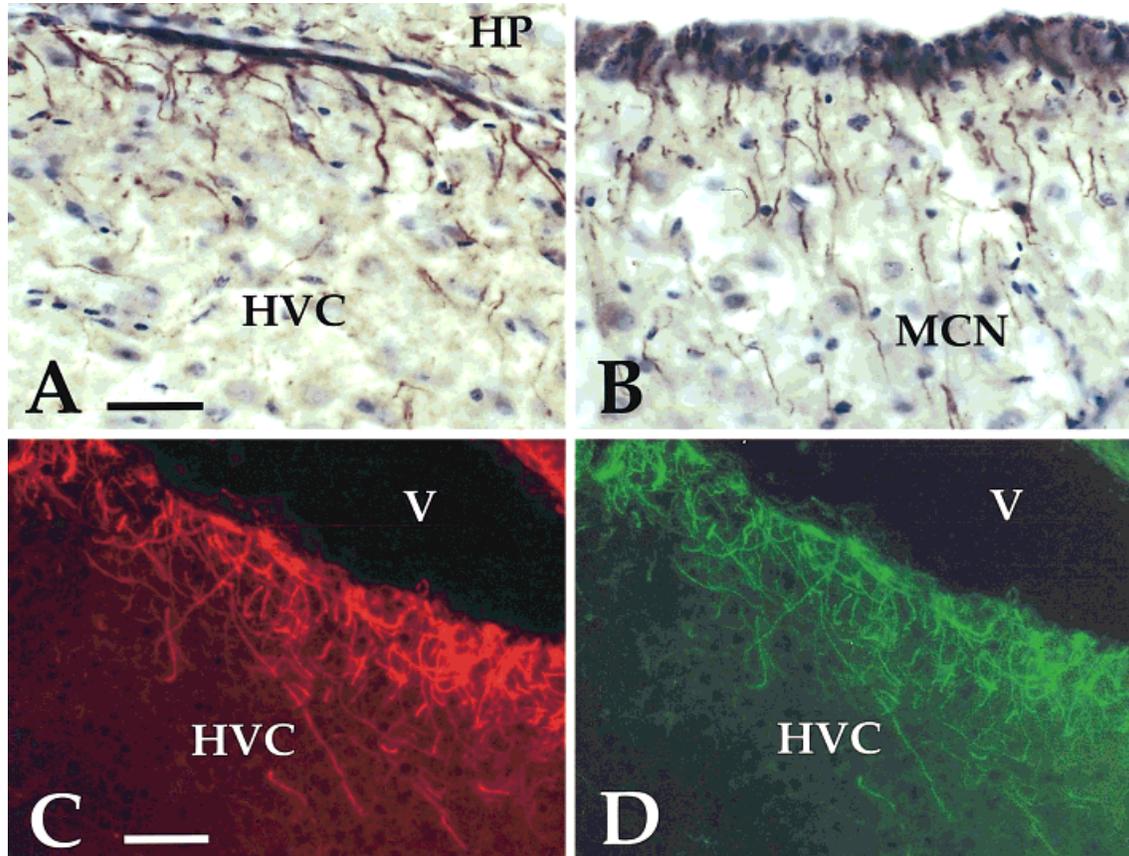


Figure 1 IGF-1 immunoreactivity was expressed by radial cells throughout the neostriatum. (A, B) Heavy expression of IGF-1 by radial cells and fibers within the subventricular parenchyma of (A) the higher vocal center (HVC) and (B) the mediocaudal neostriatum (MCN). Immunoperoxidase stained for IGF-1, cresyl violet counterstained. (C, D) IGF-1-immunoreactive radial cells expressed the ependymoglia vimentin-associated filament recognized by mAB 3A7 (Goldman et al., 1993). The adult finch brain at the level of HVC, double immunostained for both IGF-1 [(C) red] and the ependymoglia antigen 3A7 [(D) green]. Essentially all 3A7-positive radial cells expressed IGF-1. HP = hippocampus; NS = neostriatum; V = lateral ventricle. Scale bar = 50 μ m.

Both IGF-1 and IGF-2 Promoted Neuronal Outgrowth from the Adult Finch HVC SZ

On the basis of these localization studies, we asked whether the IGFs influenced the recruitment of neurons from the adult SZ. We prepared explant cultures derived from adult finch neostriatal SZ and supplemented them with either IGF-1, IGF-2 (each at 80 ng/mL), or vehicle alone (see Methods). Cultures were supplemented beginning with their initial explantation, and IGFs were added to the test cultures with twice weekly media changes thereafter. The number of neurons per explant outgrowth were scored every other day using morphological criteria

that we had previously validated antigenically, ultrastructurally and functionally (Goldman, 1990; Goldman et al., 1992; Goldman and Nedergaard, 1992). This paradigm allowed us to distinguish between factor effects upon neuronal departure, differentiation, or survival.

The proportion of neurogenic explants did not vary by treatment. Neuronal production by these SZ explants was characterized by neuronal outgrowth in the first week (Fig. 3), maturation in the second week, and then death in the third and fourth weeks *in vitro*. By 8 DIV, among 572 explants obtained from the SZ and HVC (derived from 22 birds) pooled across treatments, 214 exhibited neuronal outgrowth: 75 of 224 control explants (31%), 90

Table 1 Regional Variation of IGF-1 Tissue Levels in the Adult Zebra Finch Brain

Region	IGF-1 Immunoreactivity (pg/g)
HVC	276 ± 29*†
Lateral neostriatum	201 ± 5
Mediocaudal neostriatum	133 ± 12
Anterolateral hyperstriatum	86 ± 16
Cerebellum	182 ± 34
Optic tectum	95 ± 11

* One-way ANOVA of IGF-1 level by region demonstrated overall region effect at $p = 0.0002$ ($F = 12.28$; six groups; 17 *df*).

† $p < 0.01$ for each comparison between HVC and every other area, by Tukey's posthoc *t* tests after one-way ANOVA; also, $p < 0.01$ after multiple *t* tests with Bonferroni adjustment for multiple comparisons.

of 244 IGF-1–treated explants (37%), and 49 of 84 IGF-2–treated explants (58%) generated more than 5 neurons each. The 95% confidence intervals for these proportions were $31 \pm 6\%$, $37 \pm 6\%$, and $58 \pm 11\%$, respectively. Thus, no significant difference was noted between the IGF-1–treated and control groups in the number of explants with neuronal outgrowth, although IGF-2 was associated with a higher frequency of neuronal outgrowth than control.

IGF-1 and IGF-2 both promoted net neuronal outgrowth from adult SZ explants. Over the period of maximal treatment effect, which spanned days 6–11 *in vitro*, the mean number of neurons per IGF-1 supplemented explant was 52 ± 8.4 at 6 DIV, 64 ± 9.7 at 8 DIV, and 47 ± 7.2 at 11 DIV ($n = 88$; 84 and 67 explants, respectively). IGF-2 treatment yielded a similar result: 67 ± 13.0 neurons/explant at 6 DIV, 86 ± 16.1 at 8 DIV, and 55 ± 11.3 at 11 DIV ($n = 49$, 48, and 37, respectively). In contrast, their controls had 25 ± 4.0 , 32 ± 5.8 , and 21 ± 5.4 neurons/explant at 6, 8, and 11 DIV respectively ($n = 74$, 68, and 62) (Fig. 4).

By virtue of the nonnormal distribution of our data, a generalized linear model (GLM) was constructed and ANOVA was performed using ranked data. This analysis revealed the following main effects: treatment group [partial *F* test (2, 1341) = 52.30; $p = 0.0001$] and number of DIV [partial *F* test (9, 1341) = 116.22; $p = 0.0001$]. In addition, individual birds were found to differ in outgrowth [partial *F* test (21, 1341) = 9.10; $p = 0.0001$], providing a third main effect. The two-way interac-

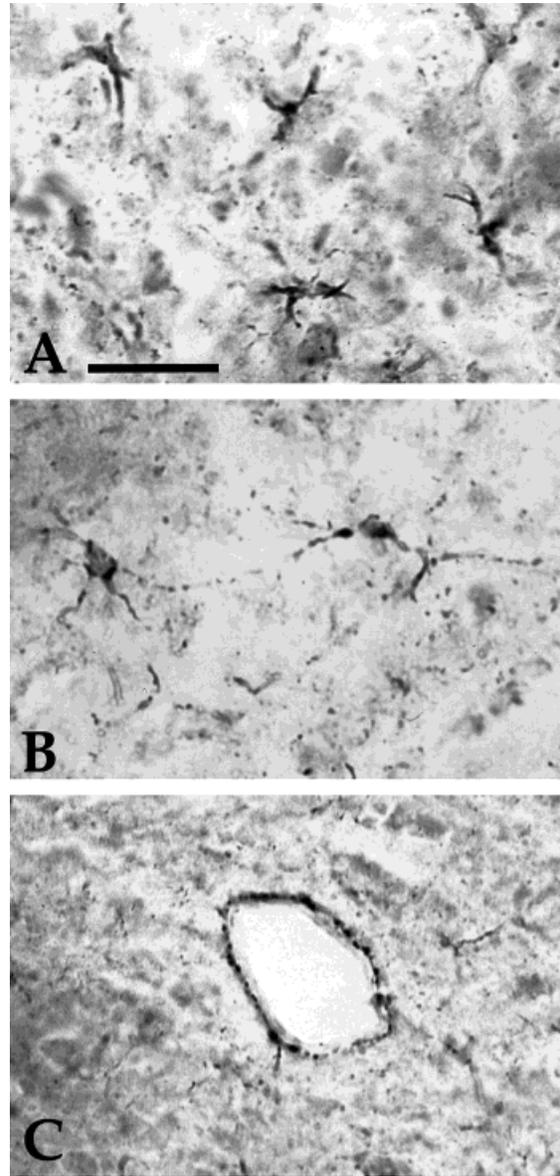


Figure 2 IGF-2 immunoreactivity was expressed by neostriatal astrocytes and their end feet. (A) Neostriatal IGF-2 expression by astrocytes, evident as small fiber-bearing cells scattered throughout the neostriatum. (B) Occasional astrocyte-like IGF-2–positive cell bodies were found in the striatal parenchyma as well. Combined immunostaining for IGF-2 and the neuronal protein Hu failed to reveal double-labeled cells, confirming the predominantly non-neuronal origin of parenchymal IGF-2. (C) Perivascular IGF-2 immunoreactivity was widespread and abundant. Whether this was of pericytic or vascular origin, or associated with astrocytic end feet could not be assessed at the light-microscopic level. Scale bar = 25 μm .

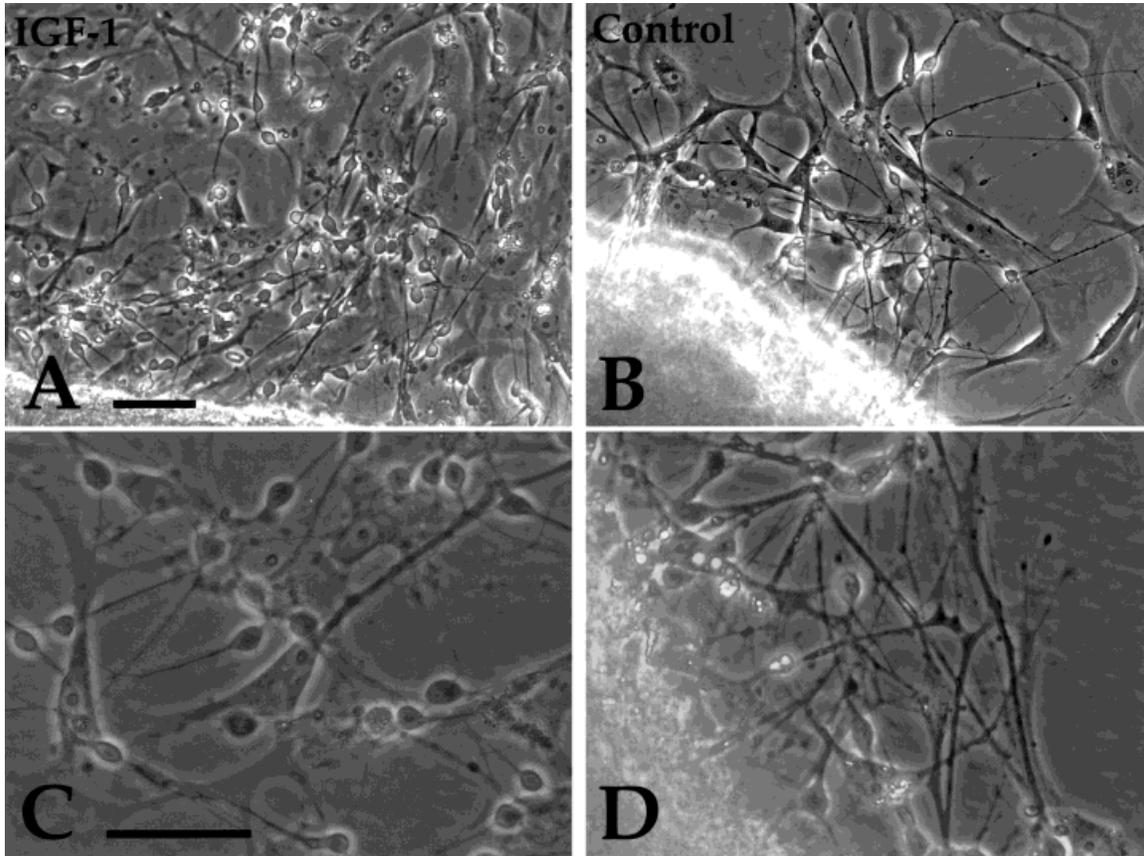


Figure 3 IGF-1 promotes the outgrowth of neurons from the adult finch SZ. Neuronal outgrowth from cultured explants of the adult zebra finch SZ in IGF-1-supplemented [left (A, C)] and control cultures [right (B, D)] after 7 DIV. Neuronal outgrowth was significantly more abundant in cIGF-1-treated cultures than their untreated controls. (A, B) $\times 100$; (C, D) $\times 400$. Scale bar = 50 μm .

tion of Bird \times Treatment group was also significant [partial F test (11, 1341) = 5.09; $p = 0.0001$] and was included in the final model along with the three main effects. In contrast, neither the two-way interactions of Bird \times Day or Group \times Day were significant, nor was the three-way interaction of Bird \times Day \times Group. Thus, IGF-1 and IGF-2 treatments were each associated with significantly greater neuronal outgrowth than their untreated controls ($p < 0.017$ with Bonferroni adjustment for multiple comparisons).

Notably, the IGF-1 and IGF-2 groups did not differ significantly from one another in their onset, rate, or maximal extent of neuronal outgrowth. In fact, the effects of IGF-1 and -2 could not be distinguished from one another in these cultures, despite their nonoverlapping patterns of expression and cell-type association. Individually, IGF-1 and -2

might function to promote either neuronal differentiation, departure, or both; either function would be reflected in greater neuronal outgrowth.

Neither IGF-1 nor IGF-2 accelerated the onset of neuronal migration. Despite the substantial promotion of neuronal outgrowth associated with IGF-1 and IGF-2, no treatment-associated effects were noted on the onset or initial rate of neuronal accumulation in these explant outgrowths. In both treated and untreated plates, neuron-like cells began migrating from the explant borders between 4 and 5 DIV. Using the Kruskal–Wallis test, the slopes of neuronal accumulation for each bird were compared among the three treatments for the period of initial outgrowth spanning days 0–4. No significant differences were noted between the control and treatment groups in these initial rates of neuronal outgrowth ($p \geq 0.10$). Similar results were obtained with the

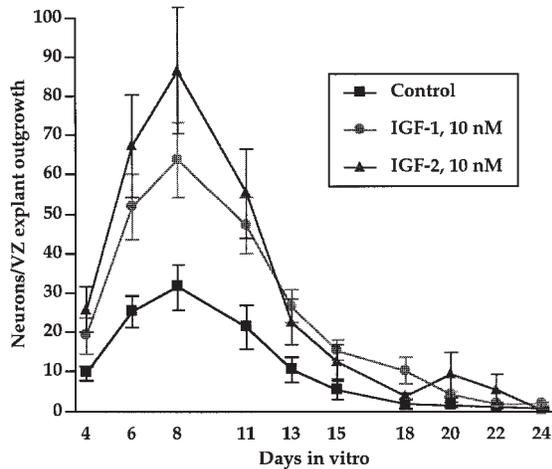


Figure 4 IGF-1 and IGF-2 promote neuronal outgrowth *in vitro*. Explant cultures derived from the adult songbird SZ, with or without 80 ng/mL (10 nM) recombinant chicken IGF-1 or IGF-2. By two-way ANOVA, the number of neurons per explant outgrowth in both IGF-1- and IGF-2-treated groups was significantly higher than that of their unsupplemented controls. Each group achieved its maximal number of neurons at 8 DIV, with progressive decay thereafter, but the IGF-treated outgrowths generated between two- and threefold the number of neurons as untreated explants.

generalized linear model using both the actual values and ranked data.

IGF treatment effects were temporally restricted. Neuronal accumulation became significantly greater among the IGF-1- and -2-treated cultures by day 6 *in vitro*, and remained so through 18 DIV. Yet, no long-term effects of IGF-1 or -2 on neuronal survival were noted relative to controls: Each group achieved its maximal number of neurons by 8 ± 1 DIV, after which neuronal counts fell according to the same time course (Fig. 4). The Kruskal–Wallis test was used to compare treatment groups on a day-by-day basis, and first revealed a significant effect of IGF-1 and IGF-2 on day 6 *in vitro* ($p = 0.0038$); this effect was maintained on days 8 ($p = 0.0005$), 11 ($p = 0.0002$), 13 ($p = 0.0001$), 15 ($p = 0.0001$), and 18 ($p = 0.02$). Thereafter, the advantage accruing to IGF treatment was lost ($p > 0.10$ on days 20–24). Between 6 and 15 DIV, the differences between control and IGF-treated groups survived Bonferroni adjustment for multiple comparisons (which dictated a threshold of $p = 0.05/9$ tests, or 0.0056, achieved at all time points spanning the range of 6–15 DIV).

The IGFs each substantially enhanced maximal neuronal outgrowth. The number of neurons per ex-

plant differed markedly between the IGF-1- or IGF-2-treated and control groups. To confirm that these treatments specifically enhanced maximal neuronal outgrowth, the data for day 8, the time point of maximal neuronal outgrowth, were analyzed separately. At this single time point, the effects of bird, group, and Bird \times Group were considered in the generalized linear model, after log transformation to normalize the data. The Bird \times Group interaction was not significant here [partial F test (21, 155) = 1.03; $p = 0.43$]. The model was therefore reduced to include only the main effects of bird and group; this analysis revealed significant main effects for bird [partial F test (21, 176) = 1.93; $p = 0.01$] and treatment group [partial F test (2, 176) = 5.37; $p = 0.005$]. Thus, both IGF-1 and IGF-2 were associated with significant and substantial increments in maximal neuronal outgrowth arising from explants of the adult songbird SZ.

The IGFs Did Not Act as Neuronal Mitogenic Factors under These Culture Conditions

Mitotic neurogenesis in these cultures was minimal and did not differ between IGF-1-treated and untreated cultures. Among the explants subjected to [^3H]thymidine autoradiography, only sporadic labeled neurons, constituting $<10\%$ of the MAP-2-positive neuronal outgrowth, were noted in either the IGF-1-treated cultures or their controls. Thus, the early difference in neuronal numbers in the IGF-1-treated and untreated cultures reflected IGF's actions on postmitotic daughter cells, rather than on their cycling progenitors.

Neither IGF-1 nor IGF-2 Acted as Survival Factors in this Preparation

After maximum neuronal outgrowth was attained, the rate of decay in the percentage of surviving neurons was similar between the IGF-1- and IGF-2-supplemented cultures and their controls. The number of neurons per IGF-supplemented and control culture fell 95% between 8 and 24 DIV (Fig. 4). By 3 weeks in this low-insulin, charcoal-stripped media, virtually no neurons survived in any group. Thus, under the culture conditions used in this study, neither IGF-1 nor IGF-2 treatment was associated with any improvement in long-term survival.

DISCUSSION

We found that IGF-1 was expressed by radial cells in the adult songbird neostriatum, and was particularly abundant in regions actively engaged in neuronal recruitment. IGF-2, in contrast, was expressed largely by adult neostriatal astrocytes but not by radial cells. In addition, IGF-1 and IGF-2 each promoted neuronal outgrowth from the adult SZ in culture, with a time course of action that suggested their importance early in neuronal postmitotic ontogeny and migration. Together, these observations indicated that in the adult songbird brain, IGF-1 and -2 act postmitotically to promote neuronal specification and departure, and/or viability during migration, without otherwise prolonging cell survival (Fig. 5).

By What Means Does IGF-1 Promote Neuronal Outgrowth?

Although IGF-1 has been described as a neuroblastic mitogen in serum-free media (Nielsen et al., 1991; DiCicco-Bloom and Black, 1988), the culture conditions used here included 10% FBS, a serum level at which neuronal mitogenesis is negligible in these explants, regardless of the insulin concentration (Goldman et al., 1992). Under these culture conditions, no mitogenic effect attributable to IGF-1 was noted; the profound effects of IGF-1 and -2 upon neuronal outgrowth from adult explants therefore reflected the actions of these agents upon SZ daughter cells, and not upon their parental precursors.

We next considered the means by which an agent could act upon ventricular zone cultures to enhance neuronal outgrowth. We had noted in a previous study that the entry of new neurons into the songbird neostriatum is delayed for up to a week postmitotically (Barami et al., 1995). The entry of new neurons into the parenchyma occurs only after they down-regulate expression of N-cadherin, which is heavily expressed by their parental precursors (Barami et al., 1994). Accordingly, antibodies against N-cadherin accelerated neuronal outgrowth from the adult avian SZ. These findings suggested that the down-regulation of N-cadherin might constitute a critical, rate-limiting step in the departure of new neurons from the adult SZ; as such, humoral stimuli that down-regulate N-cadherin might directly promote the

recruitment of new neurons into the adult brain parenchyma.

In this regard, prior studies have shown that insulin is causally associated with the down-regulation of N-cadherin (Roark et al., 1992). In addition, insulin treatment appears to be associated with an elevated level of the soluble 90-kD form of N-cadherin, which itself may serve to inhibit the cadherin-mediated adhesion of neighboring cells (Paradies and Grunwald, 1993). The latter studies were done *in vitro*, using doses of insulin high enough to activate IGF receptors. The IGFs themselves have been implicated as motility factors in a number of settings in which cadherins contribute to histiotypic integrity, again suggesting an antagonistic interaction between the two (El-Badry et al., 1990; LeRoith et al., 1995). Together, these observations suggest the possibility that IGF-1 might act to down-regulate the expression of N-cadherin by SZ daughter cells and thereby initiate neuronal recruitment into the adult parenchyma. Indeed, if IGF-1 acts as an estrogen-induced paracrine agent in the songbird brain, as it does in other systems (Norstedt et al., 1989; Sahlin et al., 1994), then IGF-1-mediated neuronal emigration from the SZ could account for estrogen's described role in supporting neuronal recruitment in the songbird brain (Nordeen and Nordeen, 1989; Hidalgo et al., 1995).

On this basis, we asked whether IGF-1 might induce the departure of new neurons from the adult SZ. We found that IGF-1 clearly supported neuronal outgrowth from the adult SZ *in vitro*. However, the overlap of the IGF-1 treated and control growth curves for the first 4 DIV indicated that IGF-1 did not hasten neuronal departure from the adult SZ. Rather, IGF-1 appeared to increase the peak neuronal outgrowth achieved. Notably, neither IGF-1 nor IGF-2 influenced the long-term survival of newly generated neurons: Although more neurons departed the SZ explants, these died as rapidly thereafter as their untreated controls. This finding may be explained in several ways: First, the proportion of SZ daughter cells committing to neuronal lineage may be increased in response to IGF-1. Alternatively, the fraction of neuronal daughter cells leaving the SZ may be enhanced by IGF-1, as might be the survival of these cells while within the SZ or during initial migration. Thus, IGF-1 may not speed neuronal departure from the SZ, and does not alone support the long-term survival of these cells. Instead, it appears to act as a neuronal specification or differentiative factor, in

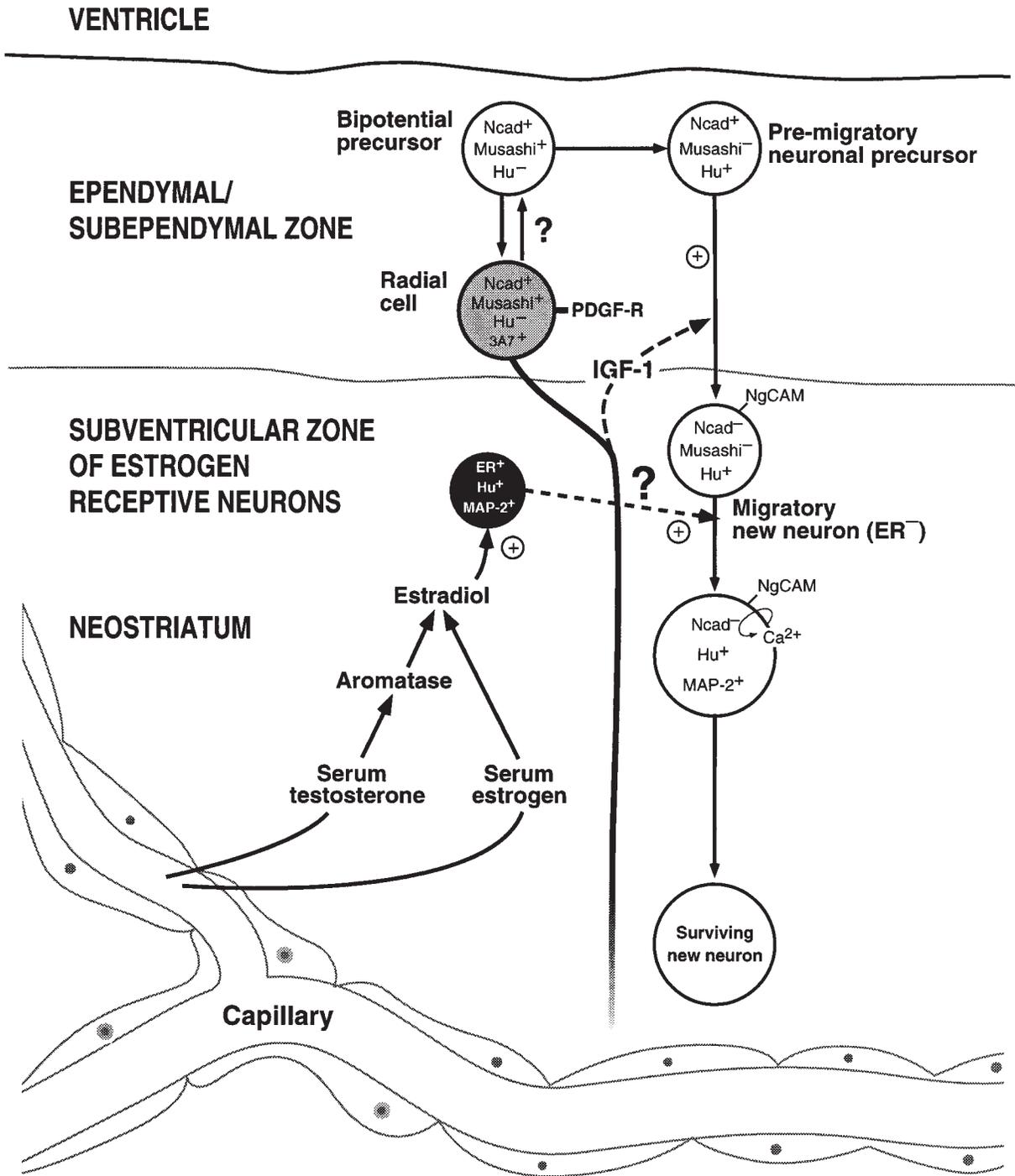


Figure 5 This schematic summarizes much of what we know about neuronal recruitment into the adult songbird HVC. The likely role of IGF-1 in this system, as based upon the present data, is highlighted.

that it increases both the number and proportion of neurons able to leave the adult SZ. As such, IGF-1 actions appear to be directed at new and

potential neuronal migrants, for whom it serves as a permissive factor for recruitment into the adult neostriatal parenchyma.

What Is the Relationship between Gonadal Steroids and Radial Cell-Derived IGF-1?

In both adult canaries and juvenile finches, the *in vivo* survival of new SZ-derived neurons is supported by estrogen, which increases the number of neurons surviving their first month after production (Nordeen and Nordeen, 1989; Hidalgo et al., 1995), without affecting the rate of precursor cell division (Brown et al., 1993). Yet, despite their sensitivity to the effects of gonadal steroids, the new neurons of the adult songbird neostriatum do not express estrogen receptor, at least not at any time during their first postmitotic month. Rather, when the new neuronal migrants enter the brain parenchyma, they initially encounter and must traverse a layer of estrogen-receptive “gatekeeper” neurons (Gahr, 1990; Hidalgo et al., 1995). The estrogen-dependent activation of these gatekeeper cells may therefore modulate the survival of new migrants as they enter the neostriatum. This process might in part be effected by the concurrent coupling of NgCAM to NgCAM-dependent calcium signaling pathways (Goldman et al., 1996). CAM-calcium coupling may result in an elevation in cell calcium during initial migration and is associated with enhanced neuronal survival during this period (Goldman et al., 1996). This process occurs only in the presence of estrogen and fails to develop in estrogen-deficient cultures (Williams et al., 1996). By mediating the coupling of NgCAM to its dependent calcium-signaling pathways, estrogen might then modulate the response of new migrating neurons to their local environment.

On this basis, we postulated that estrogen might induce the release of paracrine humoral agents from the estrogen receptor-expressing (ER-positive) subventricular cells, and that these in turn would favorably influence new neuronal migrants from the adult SZ (Hidalgo et al., 1995). Such estrogen-dependent paracrine neurotrophism has already been demonstrated in the mammalian brain, in which BDNF has been shown to be expressed by estrogen-receptive neurons and to act as a paracrine neurotrophin (Sohrabji et al., 1994, 1995). The insulin-like growth factors were likely candidates for such paracrine intermediaries in the songbird neostriatum, given their inducibility and regulation by both the gonadal steroids and growth hormone (Sahlin et al., 1994; reviewed by LeRoith, 1997). However, we found no evidence here for detectable IGF-1 or -2 expression by the ER-positive subven-

tricular cells of the adult neostriatum. Nonetheless, even though radial cells do not express the principal estrogen receptor- α , their expression of IGF-1 may yet be regulated by estrogen through non-receptor-mediated interactions (Keefe et al., 1991) or by activation of the newly described estrogen β receptors (Kuiper et al., 1996; Mosselman et al., 1996). Alternatively, radial cell IGF-1 expression may be regulated in response to androgens, which also influence the survival of new neurons in HVC and MCN (Rasika et al., 1994). These androgenic effects may be direct, through androgen receptors in HVC (Nastiuk and Clayton, 1995), or indirect through the local aromatase-mediated conversion of testosterone to estrogen (Schlinger and Arnold, 1991). Indeed, despite the well-described androgen sensitivity of this region (Brenowitz and Arnold, 1992), the specific androgen responsiveness of adult radial cells is unknown. Further studies will need to determine whether nonclassical estrogen actions might modulate radial cell IGF-1 production or sequestration.

Where Does the IGF-1 Originate?

IGF-1 immunoreactivity was noted in many, but not all, ventricular zone cell bodies, with contiguous staining of ependymally-derived radial fibers. Little parenchymal IGF-1 expression was noted save for that by radial fibers, which extended throughout the neostriatum. Nonetheless, the localization of IGF-1 immunoreactivity to radial cells need not indicate that IGF-1 is synthesized by those cells; it might instead suggest the sequestration of IGF-1 synthesized elsewhere, by the IGF-binding proteins (IGFBPs) (reviewed in de Pablo and de la Rosa, 1995). Although low-stringency *in situ* hybridization using a chick IGF-1 cDNA probe (generously provided by Dr. Peter Rotwein) revealed IGF-1 mRNA expression in the SZ and scattered parenchymal cells (Jiang and Goldman, unpublished data), high stringency *in situ* must await sequencing of homologous songbird IGF-1.

Indeed, whatever its source, the bioavailability of radial cell-associated IGF-1 to new neuronal migrants depends upon the distribution and activities of the IGFBPs, of which at least six have been identified (Clemmons, 1993; D’Ercole et al., 1994). The extent to which cellular IGF-1 immunoreactivity reflects secreted IGF-1, and the degree to which the latter is made available by the interstitial IGFBPs, are unknown in this system. As a result, we can say little at this point regarding the local

levels of accessible IGF-1 in the radial cell microenvironment.

What Is the Significance of Glial IGF-2?

IGF-2 is abundant in development, but in the adult mammalian brain, its expression is restricted largely to blood vessels and meninges (Sullivan and Feldman, 1994; Stylianopoulou et al., 1993; Beck et al., 1988). We found here that in the finch brain, IGF-2 is expressed widely by parenchymal astrocytes, as well as by perivascular glial endfeet. Although we saw no evidence of IGF-2 expression by radial fibers in these adult finches, Holzenberger et al. (1997) did report IGF-2 immunoreactivity among radial cells in canaries. Nonetheless, they saw no evidence of radial cell IGF-2 mRNA, suggesting local sequestration of IGF-2.

Whatever its cellular source, IGF-2 is clearly a potent neurotrophin in this system: Its addition to cultures of the adult avian SZ substantially increased the extent of neuronal outgrowth, to a level indistinguishable from that elicited by IGF-1. To be sure, in development IGF-2 expression characterizes a period associated with the massive recruitment of new cells. Yet even in development, the respective roles of IGF-1 and IGF-2 have not been clearly differentiated from one another; their cross-activation of one another's receptors makes many of their activities overlapping and their specific functions hard to distinguish (de Pablo et al., 1995). Since IGF-1 and -2 appear to act at the same receptor in birds (de la Rosa et al., 1994), the significance of their differential expression by radial cells and astrocytes in the adult songbird is even harder to understand. Indeed, at the dose level used in this study, IGF-2 activation of the IGF-1 receptor may have been sufficient to promote neuronal outgrowth through entirely IGF-1-dependent pathways.

Our anatomic data suggest that radial cell-derived IGF-1 normally contributes to the recruitment of new neurons from the adult SZ; whether the same can be said for astrocytic IGF-2 is unclear. IGF-2 does appear, however, to act as an interneuronal paracrine agent within HVC: Holzenberger (1997) reported a selective expression of IGF-2 mRNA by X-projecting neurons within the canary HVC, with the sequestration of locally synthesized IGF-2 by neighboring neurons projecting to RA. Johnson and Bottjer (1995) had previously reported that these RA-projecting neurons were largely sensitive to androgens, and X-projecting neurons to estrogen. Together, these data argued that estrogen receptive X-

projecting neurons might be the recipients of IGF-2, expressed in an androgen-dependent fashion by their RA-projecting neighbors. This suggests a steroid-modulated, IGF-2-mediated paracrine interaction between the two HVC neuronal phenotypes. These lines of data suggest a scenario in which radial cell-associated IGF-1 is involved in the recruitment of new neurons to the neostriatal parenchyma, whereas neuronal and glial IGF-2 may be involved more broadly in the steroid-modulated paracrine support of migratory and resident neurons alike.

Overview

Higher vertebrates appear to retain competent neuronal progenitor cells into adulthood, in the subependymal vestige of the embryonic ventricular zone (Morshead et al., 1994; Kirschenbaum et al., 1994; Kirschenbaum and Goldman, 1995; reviewed in Weiss et al., 1996; Goldman, 1997; Luskin et al., 1997; Goldman and Luskin, 1998). The avian brain is distinguished from its mammalian counterparts, however, by its widespread recruitment of new neurons from the SZ, compared to the tight restriction of new neurons in the adult mammalian brain to the olfactory stream (Luskin, 1993; Lois and Alvarez-Buylla, 1993). The songbird neostriatum, with its persistent neuronal recruitment in adulthood, is distinguished from non-neurogenic systems in a number of ways, prominent among which is the persistence of radial guide cells. We have found that IGF-1 is heavily and selectively expressed by these radial cells and directly promotes the specification and/or departure of their neuronal siblings. Together, these observations suggest that in neurogenic regions of the adult avian brain, IGF-1 acts as a radial cell-associated factor that may serve to regulate neuronal differentiation and parenchymal recruitment.

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