INTRODUCTION

Neuronal survival in the developing vertebrate nervous system is determined by afferent (presynaptic) and efferent (postsynaptic) contact with other cell populations. In the case of efferent contact, the survival of developing neurons appears to depend on the ability of their terminal arborizations to obtain access to trophic molecules that are synthesized by postsynaptic or target cell populations (reviewed by Oppenheim, 1991). However, since overproduction of neurons is a common feature of early nervous system development, the quantity of trophic support that a target can provide often appears to be insufficient for the survival of all of the neurons that might normally survive. Therefore, perinatal development is characterized by a wave of naturally occurring neuronal cell death that presumably represents the demise of neurons that failed to successfully compete for adequate quantities of trophic molecules. This death is believed to serve either a matching function whereby the numeric size of innervating- and target-cell populations is made commensurate, or as an error-correction mechanism whereby neurons that have made inappropriate efferent connections are eliminated (Cunningham, 1982; Cowan et al., 1984; O'Leary et al., 1986; Oppenheim, 1991; Raff, 1992; but cf. Sohal, 1992).

By comparison, less is known regarding the mechanisms and significance of afferent contributions to neuronal survival. While there have been numerous demonstrations that afferent input is necessary for the survival of developing neurons (e.g., Levi-Montalcini, 1949; Jackson and Rubel, 1976; Clarke, 1985; Furber et al., 1987), in some cases deafferentation induces cell death via an active, protein synthetic mechanism (Catsicas et al., 1992), whereas in others the induced death...
involves a pronounced decrease or loss of protein synthetic activity (Steward and Rubel, 1985; Rubel et al., 1990; Garden et al., 1991). Moreover, specific molecules that are released presynaptically and which enhance the survival of developing neurons have yet to be identified, although there is evidence that neurotransmitters may serve as trophic factors via binding to specific postsynaptic receptor populations. For example, activation of N-methyl-D-aspartate (NMDA) receptors has been shown to promote the survival of developing cerebellar granule cells and spinal neurons in vitro (Balazs et al., 1989; Brenneman et al., 1990). Finally, it is not known whether afferent control of neuronal survival plays a role in population matching, error correction, or some other function (for review, see Clarke, 1992).

The vast majority of studies on neuronal cell death in vertebrates have involved autonomic ganglia, somatic motor neuron pools, or mid- or hindbrain nuclei (but cf. Finlay, 1992). In order to compare and extend the results of these studies to cortical regions involved in learned behavior, we have begun an investigation of efferent and afferent influences on the survival of forebrain neurons that control learned vocalizations in songbirds (see Fig. 1; Johnson and Bottjer, 1993). Our studies involve the zebra finch, a songbird that learns the sounds used for vocal communication during a restricted phase of juvenile development and early adulthood (20-90 days of age; see Bottjer and Johnson, 1992). We are particularly interested in neural pathways that are specialized for early phases of vocal learning. For example, lesions of a cortical premotor area (the lateral magnocellular nucleus of the anterior neostriatum; IMAN) in juvenile birds prevent normal vocal development, but lesions of IMAN abruptly fail to disrupt vocal behavior as adult-like vocal patterns begin to emerge (around 60 days of age; Bottjer et al., 1984; Bottjer and Arnold, 1986; Scharff and Nottebohm, 1991). IMAN is strategically positioned for a role in vocal learning since IMAN projection neurons form a direct pathway to the cortical motor region for song control (the robust nucleus of the archistriatum; RA; Fig. 1). RA contains a population of projection neurons that form the descending motor pathway from cortical song-control regions to the brainstem motor neurons (nXIIIs) that innervate the vocal organ (syrinx; Nottebohm et al., 1976, 1982; Bottjer et al., 1989; Vicario, 1991).

Interestingly, much of the morphological development of the neural song-control system appears to be timed so as to coincide with vocal learning (reviewed by Bottjer and Johnson, 1992). For example, RA undergoes dynamic changes in volume and axonal connectivity as young birds learn to sing and the development of RA (and other song-control regions) is therefore delayed with respect to the maturation of brain structures not specifically involved with song (Bottjer et al., 1985; Herrmann and Bischof, 1986). Although RA neuron number is stable by 20 days of age, a subsequent phase of dramatic volumetric growth occurs during early phases of vocal learning due to an increase in the spacing between RA neurons (i.e., a decrease in neuron density; Gurney, 1981; Konishi and Akutagawa, 1985; Bottjer et al., 1986; Kim and DeVoogd, 1989; Burek et al. 1991). Particularly striking is the late ingrowth of axons that project to RA from another cortical region, the higher vocal center (HVC; Fig. 1). That is, whereas IMAN axons make synaptic contact with RA neurons by 15 days of age, the ingrowth of most HVC axons into RA is delayed until approximately 35 days of age (Konishi and Akutagawa, 1985; Mooney, 1992; cf. Nordeen and Nordeen, 1988). Although IMAN terminals arrive in RA first (presumably a competitive advantage), there follows a naturally occurring loss of both neurons from IMAN and IMAN synaptic input to RA neurons that roughly precedes or coincides with the ingrowth and addition of synaptic terminals from HVC (Bottjer et al., 1985; Bottjer and Sengelaub, 1989; Korsia and Bottjer, 1989; Herrmann and Arnold, 1991a; Mooney, 1992).

These dramatic changes in forebrain structures underlying vocal behavior suggest that the song-control system provides an unusual opportunity to address basic questions relating to the regulation of neuronal survival, mechanisms of cell death, remodeling of neuronal morphology and connections, and neural changes that accompany the development of learned behaviors. In this study, lesions were made in IMAN in birds of various ages to ask whether the survival of RA neurons depends on the presence of IMAN afferent input, and if so to ask whether such dependency changes over the course of vocal development. We report here that IMAN lesions in birds that are in early stages of vocal learning (20 days of age) resulted in the loss of over 40% of RA neurons by 6 days post-lesion. However, removal of IMAN afferent input did not induce RA neuron death among birds that were in later stages of vocal learning (40 and 60 days of age) nor among adult birds.

Fig. 1. Schematic sagittal view of major song-control nuclei and their axonal projections (arrows). Note that RA receives two sources of afferent input, one from HVC and the other from IMAN. However, whereas IMAN afferents establish synaptic contact with RA neurons by 15 days of age (Mooney, 1991), HVC afferents do not begin to grow into RA until approx. 35 days of age (Konishi and Akutagawa, 1985). RA contains two distinct populations of projection neurons, a large population that projects to cranial motoneurons (nXIIIs) that innervate the vocal organ (syrinx), and a smaller population that projects to a midbrain nucleus (ICo, a nucleus that also projects to nXIIIs). Abbreviations: Area X, Area X of the avian striatum; DLM, medial portion of the dorsolateral nucleus of the anterior thalamus; HVC, higher vocal center; ICo, nucleus intercollicularis; IMAN, lateral magnocellular nucleus of the anterior neostriatum; nXIIIs, tracheosyringeal portion of the hypoglossal nucleus; RA, robust nucleus of the archistriatum, SN, avian substantia nigra (from Nottebohm et al., 1982; Bottjer et al., 1989; Vicario, 1991; Bottjer, 1993).
producing already learned vocal patterns. Somewhat to our surprise, IMAN lesions in 20-day-old birds also dramatically increased the incidence of mitotic figures in RA at 2 days post-lesion.

MATERIALS AND METHODS

Vocal-control nuclei are distributed bilaterally throughout major subdivisions of the zebra finch brain (telencephalon, diencephalon, mesencephalon, myelencephalon) and previous work has shown that axonal pathways between these nuclei in the left and right hemispheres of the songbird forebrain are strictly unilateral (Nottebohm et al., 1976, 1982; Bottjer et al., 1989; see Fig. 1). We have made unilateral electrolytic lesions of IMAN, a cortical premotor area that sends an efferent output to a cortical motor region, RA (Nottebohm et al., 1982; Bottjer et al., 1989; Korsia and Bottjer, 1989; Nordeen et al., 1992). Because the intracortical pathway connecting IMAN and RA is completely unilateral (i.e., no commissural pathways connect these nuclei bilaterally; cf. Bottjer et al., 1989), contralateral RA is an appropriate within-subject control for the effects of a unilateral IMAN lesion. Brain tissue from the birds used in the present study was analyzed previously to examine the effects of IMAN lesions on the survival of neurons in the medial portion of the dorsolateral nucleus of the anterior thalamus, DLM (see Fig. 1, Johnson and Bottjer, 1993).

Male zebra finches were removed from our breeding colony at 20, 40, 60 days of age or as adults (>90 days of age). Each bird was deeply anesthetized with Equithesin, secured in a stereotaxic apparatus, and unilateral electrolytic lesions of left IMAN were made using predetermined stereotaxic coordinates (lesion parameters: 100 µA anodal current for 2.5 minutes). The electrode was then repositioned and lowered into right IMAN, but no current was passed and the electrode was withdrawn after 2.5 minutes. An example of a unilateral IMAN lesion in a 20-day-old bird is shown in Fig. 2A. Following surgery, all 20-day-old birds were returned to their home aviaries to be fed by their parents (a necessity at this age), whereas older birds were housed singly or in same-age pairs in cages in which food and water were freely available.

The 20-day-old birds were assigned randomly to one of three post-lesion survival groups; they were allowed to survive either 2 (20-2, n=6), 4 (20-4, n=6), or 6 (20-6, n=4) days following surgery. Because preliminary data showed that the effects of IMAN lesions in 20-day-old birds were maximal by 4 days post-lesion, all 40- and 60-day-old birds were killed 4 days after the lesion (40-4; 60-4; n=5 for both groups). To make direct comparisons with 20-day-old birds, adult birds were assigned randomly to one of three post-lesion survival groups and were allowed to survive either 2 (A-2, n=3), 4 (A-4, n=4), or 6 (A-6, n=4) days after the lesion.

All birds were killed with an overdose of Equithesin and were exsanguinated with bird saline (0.75% NaCl in H2O) and perfused with buffered formalin. Brains were dissected out and placed in buffered formalin for 7-10 days of additional fixation. One day following immersion in buffered sucrose (for cryo-protection), brains were sectioned in the coronal plane on a cryostat and alternate sections (30 µm thick) were mounted onto gelatin-subbed glass slides. Sections were then stained with thionin and the slides coverslipped with Permount. Slides were coded so that data analysis for each bird took place without knowledge of age or post-lesion survival time.

Using a light microscope, cell counts were made at high magnification (1250×) with the aid of an ocular grid (volume of a single ocular grid = 0.0002 mm³). In each bird, densities of neurons, degenerating cells, and cells undergoing mitosis were determined for ipsi- and contralateral RA (with respect to the unilateral IMAN lesion). RA neurons typically possess one spherical, basophilic nucleolus within a large transparent nucleus that is encompassed by a fringe of stained cytoplasm. Because glial cells in RA show little or no cytoplasmic staining and have an irregularly shaped nucleus that contains

Fig. 2. Unilateral IMAN lesions produce a dramatic loss of neurons from ipsilateral RA in juvenile male zebra finches. (A,a) Low power photomicrographs illustrate a unilateral electrolytic lesion of IMAN in a 20-day-old bird (A) and the normal appearance of the nucleus in the contralateral hemisphere (a; arrow). (B,b) Low power photomicrographs of RA in a 20-day-old bird that survived for 6 days following a unilateral IMAN lesion illustrate the volumetric decline and loss of neurons in ipsilateral RA (B) and the normal appearance of contralateral RA at the same rostrocaudal level (b). Photomicrographs were taken from 30 µm-thick coronal sections of IMAN and RA. Scale bars, (A,a) 400 µm; (B,b) 200 µm.
numerous darkly stained granular nucleoli, neurons and glia in RA are easily distinguished from each other (see Fig. 3C,D; cf. Gurney, 1981; Konishi and Akutagawa, 1985; Bottjer et al., 1986; Herrmann and Bischof, 1986; Krim and DeVoogd, 1989; Burek et al., 1991). The nucleolus was used as the unit of count for neurons (cf. Bottjer et al., 1986; Bottjer and Sengelaub, 1989; Johnson and Bottjer, 1992). Careful examination showed that the size of neuronal nucleoli in RA did not differ among experimental groups (or between left and right RA) and a correction factor for changes in nucleolar size was therefore unnecessary (see Fig. 3C,D). Neurons containing two nucleoli were counted as a single cell, although the percentage of neurons with two nucleoli was low and did not vary among groups. No correction factor for split nucleoli was applied because the diameter of nucleoli was very small (1-2 \( \mu \)m) in relation to section thickness (30 \( \mu \)m; see Konigsmark, 1970).

Degenerating RA cells were characterized by a single large sphere or several smaller globules of intensely stained, blue-black condensed chromatin. In some cases, the darkly stained chromatoid globules were enclosed by a nuclear membrane and surrounded by a fringe of cytoplasm (Fig. 3A), but clusters of chromatoid globules that lacked a limiting nuclear membrane were also present (if no nuclear membrane was present, a minimum of 5 globules of condensed chromatin were required for a cell to be counted as degenerating; see Fig. 3B). Because the chromatoid globules of dying RA cells were much larger than the darkly stained granular nucleoli of glial cells, the two cell types were easily distinguished from each other. It is important to note that we identified cells as ‘degenerating’ on the basis of whether they showed evidence of chromatin condensation (i.e., pyknosis); deafferentation has also been found to induce a mode of neuronal death in which cells rapidly down-regulate protein synthesis, become ghost-like in Nissl-stained tissue, and display no evidence of intranuclear chromatin condensation (Born and Rubel, 1985; Rubel et al., 1990). Therefore, our estimates of the number of degenerating cells in RA reflect only the demise of cells that undergo pyknotic death, and we do not rule out the possibility that additional RA cells may have underwent other, non-pyknotic forms of death.

RA cells undergoing mitosis were easily identified by intensely stained, dark-blue strands of chromatin that were present in various stages of mitosis (prophase, metaphase, anaphase, telophase). To reduce the possibility of double counting, an antipode and telophase cells were counted only if the two groups of chromosomes could be clearly visualized. Examples of mitotic figures are shown in Fig. 4.

RA contains two populations of projection neurons: a large population occupies anterior, ventral and medial aspects of RA and projects exclusively to nXIIIs, and a smaller dorsocaudal population projects exclusively to IO (Fig. 1; Vicario, 1991). However, it was not possible to determine whether there was selective neuronal loss from either projection population since neurons in Nissl-stained sections of RA appear as a completely homogeneous population distributed evenly throughout the nucleus (see Fig. 2B,b). Within each age and survival-time group, careful qualitative examination of RA along the three ordinal axes indicated that the densities of neurons, pyknotic cells (when present), and mitotic figures (when present) were uniform throughout all regions of the nucleus (cf. Gurney, 1981). To quantify

![Fig. 3. Unilateral IMAN lesions in 20-day-old male zebra finches induce pyknotic cell death in ipsilateral RA.](image)
these observations, we sampled the densities of neurons, pyknotic cells, and mitotic figures within central, dorsal, ventral, medial, lateral, rostral, and caudal regions of ipsi- and contralateral RA (with respect to the unilateral IMAN lesion) in a 20-day-old bird and in an adult. In five sections that spanned the rostrocaudal axis of both ipsi- and contralateral RA, the ocular grid was randomly positioned within each of five regions (central, dorsal, ventral, medial, and lateral RA) and the number of neuronal nucleoli, pyknotic cells, and mitotic figures that fell within the boundaries of the grid were counted (total number of grids counted per RA = 25, total number of grids per bird = 50). Rostral and caudal values for cell density were obtained by taking the average of the two most rostral grids and the two most caudal grids, respectively, out of the five grids counted in each of the five regions. Thus, a total of 35 samples of cell density per RA were generated for neurons, pyknotic cells, and mitotic figures (five samples each for central, dorsal, ventral, medial, lateral, rostral and caudal RA). In the juvenile (a bird from the 20-2 group), statistical comparison of cell densities indicated that neurons, pyknotic cells, and mitotic figures were distributed evenly throughout ipsilateral RA (neuronal density: F<1; pyknotic cell density: F<1; mitotic figure density: F<1) and that neurons were distributed evenly throughout contralateral RA (neuron density: F<1; pyknotic cells and mitotic figures were not observed in contralateral RA). In the adult (an A-4 bird), statistical comparison of neuron density indicated that neurons were distributed evenly throughout ipsi- and contralateral RA (neuron density of left RA: F<1; neuron density of right RA: F(6,28) = 1.52, P = 0.2; pyknotic cells and mitotic figures were not observed in the adult).

Given the uniformity of cell density throughout all regions of ipsi- and contralateral RA, estimates of cell density for each bird were generated by counting 2 grids positioned randomly within the central portion of the nucleus in 5 sections (i.e., 10 grids were counted per RA, 20 grids per bird). Selection of the 5 sections was made so that the full rostrocaudal dimension of RA was represented. For every grid, the number of neuronal nucleoli, pyknotic cells, and mitotic figures present within the boundaries of the grid were entered into a microcomputer database. The absolute number of each class of cells was divided by the total volume of 10 grids to obtain the density of neurons, pyknotic cells, and mitotic figures per RA. The adequacy of our sampling procedure was verified by recalculating results for each bird from 5 grids that were selected randomly from the 10 grids that were counted per RA (i.e., the sample size for each individual was randomly reduced by half). On average, cell density estimates calculated from the 5 grid samples showed a less than 1% difference from estimates calculated from the 10 grid samples. Thus, a sample of 10 grids per RA is more than adequate to generate statistically reliable estimates of cell density. To estimate the error associated with neuron counting, the mean and 95% confidence interval (CI95) were calculated for the neuron density of each group. CI95 values were then divided by the group means to obtain a percentage measure of error associated with each group mean. This analysis indicated that our sampling procedure produced a mean neuron density for each group with a 4-6% error at a 95% level of confidence (cf. Gurney, 1981). Because the significant within- and between-group differences in neuron density that we report ranged from 14-116% (see below and Table 1), it is reasonable to conclude that the 4-6% error associated with neuron counting did not account for any of these differences.

RA volumes were calculated for each bird by making camera lucida tracings of the Nissl-defined borders of the nucleus, measuring the area of the tracings with a digitizing tablet, and multiplying the combined areal values by the sampling interval (60 µm). Given the uniform distribution of the various cell types throughout RA (see above and Gurney, 1981), we multiplied the estimates of cell density by the volume of RA to calculate the total number of neurons, pyknotic cells, and mitotic figures per ipsi- and contralateral RA in each bird.

Analyses of variance (ANOVA) were used to compare RA data for groups of 20-day-old birds, where ipsi- versus contralateral RA (with respect to the unilateral IMAN lesion) served as a within-subjects factor, and survival time (20-2, 20-4, 20-6) served as a between-groups factor. Data for age groups (20-4, 40-4, 60-4, A-2,4,6) were analyzed in the same way, except that age at time of unilateral IMAN lesion was the between-groups factor (data from 20-2 and 20-6 birds were not included in the age-group comparisons).

RESULTS

20-day-old birds

Preliminary data indicated that the effects of unilateral IMAN

![Fig. 4. Unilateral IMAN lesions dramatically increased the incidence of mitotic figures in ipsilateral RA (nearly 1700 mitotic figures/mm³ were observed), but only among 20-day-old males that survived for 2 days post-lesion. (A-D) Examples of m-phase cells in ipsilateral RA of a 20-day-old bird that survived for 2 days post-lesion; dividing cells are shown in prophase (A), metaphase (B), anaphase (C), and telophase (D). Mitotic figures were not observed at later survival times in 20-day-old birds (see Fig. 6), nor in older birds at any survival time. Photomicrographs were taken from 30 µm-thick coronal sections of RA. Scale bar, 10 µm.](image-url)
lesions were most pronounced among 20-day-old birds. Therefore, statistical comparisons were made among the three survival-time groups (20-2, 20-4, 20-6) in order to analyze the time course of IMAN lesion-induced changes in the cellular composition of RA. In the results that follow, however, it is important to recognize that neurons continue to be born and migrate into RA between 20 and 30 days of age in male zebra finches (Kirk and DeVoogd, 1989). Consequently, the magnitude of any IMAN lesion-induced loss of RA neurons could be at least partially masked by a post-lesion migration of newly generated neurons into RA.

Unilateral removal of IMAN afferent input reduced the volume of ipsilateral RA

Unilateral IMAN lesions produced a rapid and sustained decline in the volume of ipsilateral RA in 20-day-old birds (see Fig. 2B,b; Table 1). By comparison, the volume of contralateral RA was not different among the three survival-time groups. Thus, the main effect of ipsi- versus contralateral RA [F(2,13)=4.36, \( P<0.03 \)] and the interaction with post-lesion survival time [F(1,13)=76.01, \( P<0.0001 \)] were significant, whereas the main effect of survival time was not (F<1). The volume of ipsilateral RA was smaller than that of contralateral RA in each survival-time group [20-2: F(1,13)=8.68, \( P<0.01 \); 20-4: F(1,13)=37.25, \( P<0.0001 \); 20-6: F(1,13)=35.15, \( P<0.0001 \)]. Although there appeared to be a trend of progressive volumetric decline as a function of survival time, there were no significant differences in the volume of ipsilateral RA among the three survival-time groups (20-2 versus 20-4: F(1,13)=3.23, \( P=0.09 \); 20-2 versus 20-6: F(1,13)=2.89, \( P=0.11 \); 20-4 versus 20-6: F<1). These data show that removal of IMAN afferent input significantly reduced the volume of RA by 2 days post-lesion, and that the volumetric decline was sustained at 4 and 6 days post-lesion.

Unilateral removal of IMAN afferent input reduced neuron number in ipsilateral RA

Although the data in Table 1 suggest an increase in neuron density in ipsilateral RA of 20-4 birds, there were no significant effects of unilateral IMAN lesions on RA neuron density when the three groups of 20-day-old birds were compared (i.e., no effect on the spacing between RA neurons; \( P \) of ipsi- versus contralateral RA, survival time, and the interaction of these two factors always \( >0.16 \); but see age-group comparisons below). However, because unilateral IMAN lesions substantially reduced the volume of ipsilateral RA in 20-day-old birds (see above), there was a decrease in the number of neurons in ipsilateral RA (i.e., neuron number = volume \( \times \) neuron density; see Table 1, upper panel of Fig. 5). Across survival-time groups, there were fewer neurons in ipsilateral RA than in contralateral RA [F(1,13)=44.92, \( P<0.0001 \)]. However, while the data presented in the upper panel of Fig. 5 suggest a trend of progressive neuronal loss following the lesion, the main effect of survival time (F<1) and the interaction [F(2,13)=1.71, \( P=0.22 \)] were not significant. Planned comparisons showed that there were fewer neurons in ipsilateral RA than in contralateral RA at each survival time [20-2: \( F(1,13)=8.67, P<0.01 \); 20-4: \( F(1,13)=14.09, P<0.003 \); 20-6: \( F(1,13)=22.74, P<0.0006 \)]. Thus, removal of IMAN afferent input induced significant neuronal loss in RA by 2 days post-lesion, and this decline in RA neuron number was sustained at 4 and 6 days post-lesion.

### Table 1. Summary of the effects of unilateral IMAN lesions on RA morphology (ipsi- versus contralateral) in male zebra finches at various stages of vocal development

<table>
<thead>
<tr>
<th>Group</th>
<th>Ipsilateral Volume $^a$</th>
<th>Contralateral Volume</th>
<th>Ipsilateral Neuron Density $^b$</th>
<th>Contralateral Neuron Density</th>
<th>Ipsilateral Neuron Number</th>
<th>Contralateral Neuron Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>20-2</td>
<td>0.10±0.02</td>
<td>0.12±0.02</td>
<td>108.36±9.86</td>
<td>113.51±11.04</td>
<td>10.93±4.3</td>
<td>14.17±7.4</td>
</tr>
<tr>
<td>20-4</td>
<td>0.07±0.02</td>
<td>0.12±0.02</td>
<td>131.20±23.76</td>
<td>115.43±12.81</td>
<td>9.48±4.7</td>
<td>13.57±2.7</td>
</tr>
<tr>
<td>20-6</td>
<td>0.07±0.01</td>
<td>0.13±0.01</td>
<td>113.58±15.85</td>
<td>114.40±14.70</td>
<td>8.04±2.9</td>
<td>14.47±1.7</td>
</tr>
<tr>
<td>40-4</td>
<td>0.21±0.02</td>
<td>0.24±0.02</td>
<td>68.59±9.08</td>
<td>59.04±26.26</td>
<td>13.99±1.2</td>
<td>14.37±1.6</td>
</tr>
<tr>
<td>60-4</td>
<td>0.27±0.03</td>
<td>0.27±0.02</td>
<td>60.16±49.56</td>
<td>53.33±3.79</td>
<td>16.15±4.0</td>
<td>14.53±3.6</td>
</tr>
<tr>
<td>A-2,4,6</td>
<td>0.20±0.07</td>
<td>0.22±0.07</td>
<td>64.97±11.53</td>
<td>62.26±10.64</td>
<td>14.87±3.3</td>
<td>15.90±3.0</td>
</tr>
</tbody>
</table>

$^a$Volume is mm$^3$

$^b$Neuron Density is neurons / mm$^3$

All data are presented as means ± s.d.
Because RA continues to add new neurons between 20-30 days of age (Kirn and DeVoogd, 1989), it is possible that these data represent an underestimate of the actual magnitude of neuronal loss from RA.

Unilateral removal of IMAN afferent input increased the number of pyknotic cells in ipsilateral RA

The lower panel of Fig. 5 shows that unilateral removal of IMAN afferent input in 20-day-old birds increased the number of pyknotic cells in RA as a function of survival time $[F(2,13)=5.67, P<0.02]$ and ipsi- versus contralateral RA $[F(1,13)=40.82, P<0.0001]$. The interaction of these two factors was not significant $[F(2,13)=2.34, P=0.13]$. Although there were more pyknotic cells in ipsilateral than in contralateral RA at each survival time $[20-2$ versus $20-4: F(1,13)=9.53, P<0.03; 20-4$ versus $20-6: F(1,13)=29.08, P<0.0003; 20-6: F(1,13)=11.80, P<0.005]$, a significant increase in the number of pyknotic cells in ipsilateral RA occurred between 2 and 4 days post-lesion $[20-2$ versus $20-4: F(1,13)=10.11, P<0.007]$. The fact that a significant decrease in the number of neurons was not observed between 2 and 4 days post-lesion (see above) may be due to the addition of new neurons into RA (Kirn and DeVoogd, 1989). Other between-group comparisons of the number of pyknotic cells in ipsilateral RA were not significant $[20-2$ versus $20-6: F(1,13)=2.84, P=0.11; 20-4$ versus $20-6: F(1,13)=1.34, P=0.27]$, and there were no group differences in the zero or low number of pyknotic cells in contralateral RA ($P$ always $≥0.07$). To summarize, neuronal loss in ipsilateral RA was accompanied by the appearance of significant numbers of pyknotic cells (compare upper and lower panels of Fig. 5). The morphology of many of the pyknotic cells was also clearly neuronal (see Fig. 3). Together, these findings suggest that the IMAN lesion-induced loss of RA neurons in 20-day-old birds is due to neuronal cell death.

Unilateral removal of IMAN afferent input induced the appearance of mitotic figures in ipsilateral RA

Unilateral IMAN lesions dramatically increased the incidence of mitotic figures throughout ipsilateral RA of 20-day-old birds that survived for 2 days (see Figs 4, 6). This effect was early and acute in the sense that few, if any, mitotic figures were seen in RA of birds that survived for 4 or 6 days. We do not know at present whether the appearance of the mitotic figures was due to an increase in the rate of cell proliferation or a profound slowing of m-phase (thereby increasing the chances of observing mitotic figures) in a constitutively dividing cell population. Although neither the main effect of survival time $[F(2,13)=3.24, P=0.07]$ nor ipsi- versus contralateral RA $[F(1,13)=2.46, P=0.14]$ were significant, the interaction of these two factors was significant $[F(2,13)=5.17, P<0.02]$. Planned comparisons showed that the interaction occurred because the 2-day survival group had significantly more mitotic figures in ipsilateral RA than did the 4- or 6-day survival groups $[20-2$ versus $20-4: F(1,13)=7.12, P<0.01; 20-4$ versus $20-6: F(1,13)=5.94, P<0.02]$, whereas there were no group differences in the zero or low incidence of mitotic figures in contralateral RA ($F$ always $<1$; note: because mitotic figures were never observed within RA in older groups of birds, age-group comparisons of mitotic figures were not performed).

Age-group comparisons

Given the ability of IMAN lesions in 20-day-old birds to induce substantial cell death and neuronal loss in RA, comparisons were made with older groups of birds to determine whether this effect was developmentally regulated. Among 20-day-old birds, the 4-day survival group (20-4) was selected for these comparisons because the effect of IMAN lesions on the incidence of pyknosis within RA was maximal in this group of 20-day-old birds. Although groups of adult birds were sacrificed at 2, 4, or 6 days post-lesion (for direct comparison to the 20-day-old birds), data from all adult birds were combined for statistical analysis since results from the three survival-time groups were not significantly different $(A-2,4,6;n=11)$.

The normative morphological development of RA has been characterized previously. That is, the volume of RA more than doubles during development (and then declines slightly), but a corresponding decrease in neuron density (i.e., an increase in the spacing between neurons) results in no net change in the total number of RA neurons (Bottjer et al., 1985, 1986; Konishi and Akutagawa, 1985; Kirn and DeVoogd, 1989; Burek et al., 1991). Because the IMAN lesion was unilateral, morphological changes in contralateral RA should reflect normative development, while changes in ipsilateral RA will reveal age-related differences in the ability of IMAN lesions to induce cell death in RA.

Unilateral removal of IMAN afferent input reduced the volume of ipsilateral RA in all but 60-day-old birds

Results from the present study replicate previous findings of dramatic developmental growth in the volume of RA followed by a slight decline (Konishi and Akutagawa, 1985; Herrmann and Bischof, 1986). Table 1 shows that the volume of both ipsi- and contralateral RA exhibited a sizable developmental increase and then declined somewhat $[F(3,23)=45.29, P<0.0001]$. Across age groups, however, unilateral removal of
IMAN afferent input significantly reduced the volume of ipsilateral RA at 4 days post-lesion \(|F(1,23)=61.72, P<0.0001|\). Planned comparisons of the volume of contralateral RA (indicative of normative development) revealed a substantial increase between 20 and 40 days of age \([20-4 \text{ versus } 40-4: F(1,23)=54.01, P<0.0001]\), no change between 40 and 60 days of age \([40-4 \text{ versus } 60-4: F(1,23)=2.89, P=0.09]\), and a decrease between 60 days of age and adulthood \([60-4 \text{ versus } A-2,4,6: F(1,23)=4.32, P=0.05]\). Thus, RA exhibited an initial phase of dramatic volumetric growth between 20 and 40 days of age, and then showed a modest decline between 60 days of age and adulthood.

The interaction between age groups and ipsi- versus contralateral RA was also significant \(|F(3,23)=5.83, P<0.004|\), indicating that IMAN lesions did not reduce the volume of ipsilateral RA in all age groups. Planned comparisons showed that the volume of ipsilateral RA was not different from that of contralateral RA in 60-day-old birds \((F<1)\), but was significantly smaller in all other age groups \([20-4: F(1,23)=42.97, P<0.0001; 40-4: F(1,23)=22.14, P=0.0002; A-2,4,6: F(1,23)=17.42, P=0.0006]\). These data show that removal of IMAN afferent input significantly reduced the volume of RA in 20- and 40-day-old and adult birds, but had no effect on RA volume in 60-day-old birds.

Unilateral removal of IMAN afferent input increased neuron density in ipsilateral RA among 20-day-old birds only

Table 1 shows that neuron density in both ipsi- and contralateral RA generally decreased as a function of age \(|F(3,23)=55.09, P=0.0001|\). However, unilateral IMAN lesions significantly increased neuron density in ipsilateral RA across all age groups \(|F(1,23)=10.83, P=0.003|\). The interaction of these two factors was not significant \(|F(3,23)=1.21, P=0.33|\). Normative changes in RA neuron density as a function of age were analyzed by making planned comparisons of neuron density in contralateral RA. Consistent with previous work (Konishi and Akutagawa, 1985; Bottjer et al., 1986; Burek et al., 1991), these comparisons showed that neuron density in contralateral RA decreased significantly between 20 and 40 days of age and did not change thereafter \([20-4 \text{ versus } 40-4: F(1,23)=91.80, P<0.0001; 40-4 \text{ versus } 60-4: F<1; 60-4 \text{ versus } A-2,4,6: F(1,23)=2.09, P=0.16]\).

Superimposed on the normative age-related decrease in RA neuron density, unilateral IMAN lesions increased neuron density in ipsilateral RA across age groups. However, planned comparisons showed that 20-day-old birds were the only age group in which neuron density in ipsilateral RA was greater than that of contralateral RA \([20-4: F(1,23)=8.56, P<0.007; 40-4: F(1,23)=2.56, P=0.12; 60-4: F(1,23)=1.34, P=0.26; A-2,4,6: F<1]\). It should be recalled that an ANOVA comparing neuron density in ipsi- versus contralateral RA collapsed across the three groups of 20-day-old birds yielded no significant effect of unilateral IMAN lesions (see above). Table 1 shows that this occurred because 20-4, 40-4, 60-4, and A-2,4,6 groups all tended to exhibit an increased neuron density in ipsilateral RA (thereby contributing to the significant main effect of ipsi-versus contralateral RA across age groups), whereas 20-2 and 20-6 groups did not exhibit an increase in ipsilateral RA and masked the increased neuron density in ipsilateral RA of the 20-4 group.

Unilateral removal of IMAN afferent input reduced neuron number in ipsilateral RA among 20-day-old birds only

Although RA shows a sizable increase in volume during vocal development, an equally substantial increase in the spacing between RA neurons (i.e., a decrease in neuron density) results in no net change in the number of RA neurons (Bottjer et al., 1986; Konishi and Akutagawa, 1985; Kim and DeVoogd, 1989; Burek et al., 1991). We also found that RA neuron number did not change during vocal development (see Table 1, and ‘RA Contralateral to the Unilateral IMAN Lesion’ in the upper panel of Fig. 7). Moreover, the present results show that unilateral IMAN lesions reduced the number of neurons in ipsilateral RA in 20-day-old birds only. Thus, the main effects of age \(|F(3,23)=2.69, P=0.07|\) and ipsi- versus contralateral RA \(|F(1,23)=3.14, P=0.09|\) were not significant, but the interaction of these two effects was highly significant \(|F(3,23)=5.00, P<0.008|\). Planned comparisons showed that the interaction occurred because 20-day-old birds had significantly fewer neurons in ipsilateral RA than did older groups of birds \([20-4 \text{ versus } 40-4: F(1,23)=5.63, P<0.02; 20-4 \text{ versus } 60-4: F(1,23)=12.27, P<0.002; 20-4 \text{ versus } A-2,4,6: F(1,23)=7.91, P<0.009]\), whereas there were no group differences in the number of neurons in contralateral RA \((P \text{ always } \geq 0.48)\). Moreover, the number of neurons in ipsilateral RA was not different among 40- and 60-day-old and adult birds \((P \text{ always } \geq 0.21)\). These data indicate that the ability of IMAN lesions to induce neuronal loss in RA is highly age-dependent. That is, although removal of IMAN afferent input induced a substan-
tial loss of RA neurons in 20-day-old birds, the same lesion had no effect on RA neuron number in birds 40 days of age and older.

Unilateral removal of IMAN afferent input increased the number of pyknotic cells in ipsilateral RA among 20-day-old birds only

The lower panel of Fig. 7 shows that unilateral IMAN lesions increased the number of pyknotic cells in RA as a function of age [F(3,23)=4.95, P<0.008] and ipsi- versus contralateral RA [F(1,23)=13.35, P<0.002]. The interaction of these two factors was also significant [F(3,23)=5.40, P<0.006]. The interaction was attributable to the fact that 20-day-old birds were the only age group in which there were more pyknotic cells in ipsilateral RA than in contralateral RA [20-4: F(1,23)=24.78, P<0.0001; 40-4: F<1; 60-4: F(1,23)=2.91, P=0.10; A-2,4,6: F<1]. In addition, 20-day-old birds had significantly more pyknotic cells in ipsilateral RA than did older groups of birds [20-4 versus 40-4: F(1,23)=15.03, P<0.001; 20-4 versus 60-4: F(1,23)=7.49, P<0.01; 20-4 versus A-2,4,6: F(1,23)=14.90, P<0.011], whereas there were no group differences in the zero or low incidence of pyknotic cells in contralateral RA (P always ≥0.16). Thus, removal of IMAN afferent input induced the appearance of pyknotic cells in 20-day-old birds only; significant numbers of pyknotic cells were not observed among older groups of birds. Because 20-day-old birds were also the only age group in which IMAN lesions reduced the number of neurons in RA (compare upper and lower panels of Fig. 7), these data suggest that neuronal cell death accounts for the reduction in neuron number.

DISCUSSION

The results of this study show that afferent input from IMAN is required for the survival of a substantial portion of RA neurons during a restricted period of early vocal development. If IMAN is lesioned in birds that are 20-days old, numerous pyknotic cells appear and over 40% of RA neurons are lost within 6 days of the lesion, despite the fact that some new neurons are migrating into RA during this period (Kirm and DeVoogd, 1989). Axons from IMAN arrive in RA and ramify throughout its extent prior to the earliest onset of song-related vocalizations (by 15 days of age; Mooney, 1992; Johnson and Bottjer, unpublished data), but the number of IMAN synapses on RA neurons decreases 4-fold between 25 and 53 days of age (Herrmann and Arnold, 1991a). Although the time course of this synaptic regression is not known, it probably correlates with a phase of naturally occurring cell death in IMAN that appears to be over by approximately 40 days of age (see Bottjer and Johnson, 1992). Thus, RA neurons apparently require IMAN afferent input at a time when the number of IMAN synapses on RA neurons is at or near peak levels (and HVC input is low or absent).

At the survival times we examined, removal of IMAN afferent input induced neither significant cell death nor neuron loss in RA by the time birds were 40 days of age or older. Although we cannot completely rule out the possibility that IMAN lesions induce a much slower time course of RA neuronal death in older birds (but see below), absence of an short-term effect of deafferentation on RA neuron survival in older birds is consistent with the generally held view that the requirement for afferent input declines with age (e.g., Cowan, 1970; Rubel et al., 1990; Oppenheim, 1991; Clark, 1992). However, the observation that RA neurons lose susceptibility to IMAN lesion-induced death by 40 days of age suggests an alternative interpretation. Because additional sources of afferent input often mitigate the extent of neuronal death produced by deafferentation or target-removal (Clarke, 1985; Furber et al., 1987; Lindon and Pinon, 1987; but cf. Clarke and Egloff, 1988), the late ingrowth of HVC afferent input at approx. 35 days of age could account for the early vulnerability and later insensitivity of RA neurons to the loss of IMAN afferent input (as opposed to a change in the intrinsic properties of RA neurons). That is, we found that removal of IMAN afferent input induced substantial RA neuron death at an age when IMAN is the primary or only source of afferent input to RA neurons (20 days of age; Mooney, 1992), but IMAN lesions were no longer effective by the time HVC axons have elaborated their terminal arbors within RA (40 days of age; Konishi and Akutagawa, 1985; Mooney, 1992). The idea of a transfer of afferent support from IMAN to HVC is particularly attractive in light of evidence suggesting that some adult neuronal populations remain dependent on cell-cell interactions for their survival (Ruit et al., 1990). In summary, RA neurons may lose their dependency on IMAN afferent input because of the availability of a secondary source of afferent input from HVC, or they may undergo intrinsic developmental changes that eliminate the requirement for afferent input altogether. However, the decrease in overall volume (but not neuron number) seen in older birds following IMAN lesions does indicate a continuing role for IMAN afferent input in maintaining the normal morphology of RA.

Interestingly, although RA does not undergo a naturally occurring decline in neuron number during post-hatch development, the overall volume of the nucleus doubles between 20 and 40 days of age (this study; Konishi and Akutagawa, 1985; Bottjer et al., 1986; Herrmann and Bischof, 1986). The growth of RA is primarily attributable to an increase in the spacing of neurons, which along with a substantial increase in somal size suggests that the dendritic arbor of RA neurons may grow dramatically at this time (Konishi and Akutagawa, 1985; Bottjer et al., 1986; Burek et al., 1991). Whereas theories of ‘target matching’ have traditionally emphasized the number of cells in projection and target populations (e.g., Cunningham, 1982; Hamburger and Oppenheim, 1982; Oppenheim, 1991; Sohal, 1992), the total amount of synaptic space in RA probably increases substantially (in terms of dendritic arbor) in spite of the fact that neuron number does not change. It is difficult to know whether regulation of neuron number and total target space in RA is dependent on afferent input from IMAN, HVC or some combination thereof (cf. Konishi and Akutagawa, 1987; Herrmann and Arnold, 1991b). However, if afferent input contributes to the stabilization of neuron number and the subsequent increase in total target space in RA, it seems likely that IMAN terminals could play an important role in this process since the present results show that a large portion of RA neurons require IMAN afferent input for their survival in 20-day-old birds (neuron number in RA is normally stable by this age; Konishi and Akutagawa, 1985; Kirn and DeVoogd, 1989; Burek et al., 1991).

We also found that IMAN lesions in 20-day-old birds...
produced an early, acute influence on cell proliferation in RA. That is, while removal of IMAN afferent input elicited a dramatic increase in the incidence of mitotic figures among 20-day-old birds that survived for 2 days post-lesion, significant numbers of mitotic figures were not observed in RA at later survival times (4 or 6 days). The fact that mitotic figures were seen only at 2 days post-lesion suggests that cell division in RA may be a specific response to the loss of IMAN afferent input in 20-day-old birds. Cell division could also be regulated by a signal produced by dying RA neurons, although pyknotic cells were observed at 2, 4 and 6 days post-lesion and mitotic figures were observed only at 2 days post-lesion. In either case, it is important to recognize that the appearance of mitotic figures could have been due either to an increase in the number of cells that were actively dividing within RA, or to a slowdown in the mitotic cycle of some constitutively dividing cell population within RA (thereby increasing the likelihood that mitotic figures would be observed). In the former case, removal of IMAN afferent input would induce cells to enter mitosis, whereas in the latter the mitotic cycle of constitutively dividing cells would be interrupted or delayed by the removal of IMAN afferent input. This distinction is of interest because there is evidence of a dramatic naturally occurring loss of afferent input from IMAN to RA during normal development (Herrmann and Arnold, 1991a). Therefore, either by increasing the likelihood that a cell will enter the mitotic cycle or by retarding the rate at which a cell divides, it seems possible that the naturally occurring loss of IMAN afferent input might selectively influence the size of the population of one or more cell types in RA. An intriguing question for future study is whether IMAN afferent regulation of the size of particular cell populations could in turn direct other features of RA development (e.g., increased glial proliferation could create a permissive environment for the ingrowth of HVC axons; see David et al., 1990).

The presence of mitotic figures raises the question of the eventual phenotypic identity of these cells. Although the possibility that the dividing cells have neuronal potential cannot be ruled out, this seems unlikely since cells that are neuronal precursors in the avian telencephalon appear to divide only within the ventricular zone, and never within a differentiated brain structure (Alvarez-Buylla and Nottebohm, 1988; Alvarez-Buylla et al., 1990; Brown et al., 1993). In contrast, there is abundant evidence that glial populations respond to local changes in neural activity and/or the loss of neurons with in situ cell growth and division (Graeber et al., 1988; Canady et al., 1990; Canady and Rubel, 1992; Thomas, 1992; Barres and Raff, 1993). These findings are consistent with the idea that the cells seen proliferating within RA will become glia (e.g., astrocytes, oligodendrocytes, or microglia). Interestingly, depending on whether the dividing cells differentiate into astrocytes or microglia, recent data suggest opposing effects on neuronal survival. Whereas cultured astrocytes are capable of synthesizing and releasing a factor that promotes the survival of neurons (Vaca and Wendt, 1992; Giulian et al., 1993), cultured microglia synthesized and release a molecule that induces neuronal death (Vaca and Wendt, 1992; Giulian et al., 1993; see Thanos et al., 1993).

Mechanisms of induced RA cell death
Given the potent survival effects of target-derived neurotrophic factors, one might expect afferents to influence neuronal survival by anterogradely transporting and releasing the same or similar molecules (Patterson and Nawa, 1993). For example, there is evidence that basic fibroblast growth factor (bFGF) can be anterogradely transported by retinal ganglion cells (RGC; Ferguson et al., 1990; cf. Catsicas et al., 1992), although it is not known whether bFGF actually promotes the survival of neuronal populations that receive RGC afferent input. An alternative to anterograde transport and presynaptic release of neurotrophins is the idea that molecules typically associated with chemical neurotransmission may also possess neurotrophic properties. In this context, recent evidence that activation of NMDA receptors can promote the survival of developing neurons (Balazs et al., 1989; Brenneman et al., 1990) has implications for the mechanism of IMAN afferent regulation of RA neuronal survival. Neurophysiological and pharmacological studies of RA suggest that the synaptic terminals of both IMAN and HVC neurons release glutamate as a neurotransmitter, but IMAN and HVC terminals interact with pharmacologically distinct classes of glutamate receptors on RA neurons. That is, excitatory post-synaptic potentials (epsps) in RA neurons evoked by stimulation of IMAN axons are mediated by NMDA receptors, whereas epsps evoked by stimulation of HVC axons are mediated by non-NMDA receptors (Kubota and Saito, 1991; Mooney and Konishi, 1991; Mooney, 1992; cf. Aamodt et al., 1992). Therefore, the ability of IMAN lesions to induce RA cell death in 20-day-old birds suggests the possibility that the survival of some developing RA neurons requires activation of NMDA receptors via IMAN afferent input, although the fact that more than 50% of RA neurons survive following an IMAN lesion indicates that dependency on IMAN afferent input is not shared by all RA neurons. Surviving RA neurons may not require cell-cell interactions or perhaps they obtain access to trophic factors from their post-synaptic targets in nXIIIts or ICo.

A related question concerns the role of protein synthesis as RA neurons die following the removal of IMAN afferent input. For example, Fig. 3 shows that RA neurons in the initial stages of induced death retained cytoplasmic Nissl-staining. Because stained Nissl substance is a general indicator of the presence of protein synthetic machinery (i.e., ribosomes), it seems probable that RA neurons retained the capacity to synthesize proteins as they died. If so, the mechanisms of RA neuron death would appear to be quite different from those involved in a well-characterized instance of deafferentation-induced neuronal death in the chick auditory brainstem. As shown by Rubel and co-workers, deafferentation of nucleus magnocellularis (NM) via removal of the cochlea induces a form of neuronal cell death in which protein synthetic activity is virtually abolished within a few hours of cochlea removal (reviewed by Rubel et al., 1990). Thereafter, dying NM neurons appear as ‘ghost’ cells in Nissl-stained tissue (i.e., these cells show little if any cytoplasmic staining; Born and Rubel, 1985).

Relation of induced RA cell death to behavioral development
An advantage of studying neuronal cell death in brain regions involved with song is the opportunity to analyze possible relationships between mechanisms that control the survival of developing neurons and the plastic neural changes that underlie
the acquisition of learned vocal behavior. For example, we have shown that the survival of a large portion of RA neurons depends on afferent input from lMAN in 20-day-old birds, an age that corresponds to the initial stages of vocal learning in zebra finches (Immelm ann, 1969; Arnold, 1975; reviewed by Bottjer and Johnson, 1992). Because lMAN lesions in 20-day-old birds also severely disrupt vocal development (John t and Bottjer, unpublished data), the mechanisms by which lMAN afferents regulate the survival of RA neurons may be involved in early stages of vocal learning.

The finding that lMAN lesions fail to induce RA neuron death in 40-day-old birds is particularly interesting in light of the fact that lesions of lMAN rapidly (within 48 hours) disrupt vocal development at this age (Bottjer et al., 1984). For example, if the ability of lMAN lesions to produce behavioral disruption was consistently associated with RA neuron death, then the behavioral deficit could be attributed to neuronal death in the descending motor pathway from RA to the motoneurons that control the vocal organ (syninx; see Fig. 1). However, because we found that lMAN lesions induce no RA neuron death in 40-day-old birds at 4 days post-lesion, the rapid behavioral effects of lMAN lesions at this age can be attributed to the absence of lMAN, and not to a secondary effect of neuronal loss from RA. Although removal of lMAN afferent input did produce a significant volumetric decrease in RA among 40-day-old birds, lMAN lesions also produced a volumetric decrease in adults. Since lMAN lesions have neither short- nor long-term effects on vocal behavior in adult birds (Bottjer et al., 1984; Nordeen and Nordeen, 1993), it appears that a slight decrease in volume without neuronal loss will not necessarily interfere with the role of RA in vocal production (the fact that lMAN lesions have no long-term effects on adult vocal behavior also argues against a slow rate of lMAN lesion-induced RA neuron death in older birds, see above). The present results suggest that the functional change that occurs as lMAN lesions lose the ability to disrupt vocal development (at approx. 60 days of age) does not involve factors that influence the survival of RA neurons or the volume of RA, and may instead be related to changes within lMAN itself.

The authors wish to thank Kenneth M. Soderstrom for the design and development of a graphical-interface computer program that greatly enhanced the ease and reliability of cell counting. The expert statistical advice of Dr Susan D. Brown is also gratefully acknowledged.

REFERENCES


Herrmann, K. and Bischof, H.-J. (1986). Delayed development of song control nuclei in the zebra finch is related to behavioral development. J. Comp. Neurol. 245, 167-175.


(accepted 29 September 1993)