filtration and sodium dodecyl sulfate electrophoresis and agree well with the minimum molecular weights obtained from amino acid analysis. Isoelectric points (*p*I) were determined by isoelectric focusing: $CdPB_1$ and $CdPB_3$ both gave single bands, but $CdBP_2$ did not stain with Coomassie blue and its *p*I is based on the NaCl concentration required for elution from the ion exchange column. Metal contents per mole of protein were determined on accurate weights of salt-free protein by flame atomic absorption (five determinations per protein, uncorrected for residual H_2O content and based on the molecular weights in Table 2).

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* To whom requests for reprints should be sent.

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Neurons Generated in the Adult Brain Are Recruited into Functional Circuits

Abstract. *Adult canaries,* Serinus canarius, *received injections of ^{3}H-labeled thymidine, a marker of DNA synthesis. Thirty days after the last injection, intracellular potentials were recorded from neurons in the nucleus hyperstriatum ventralis pars caudalis, a vocal control nucleus in the telencephalon; these same cells were then injected with horseradish peroxidase. Of the 74 neurons labeled with horseradish peroxidase that were recovered, the nuclei of seven were radioactively labeled. Four of these seven neurons had responded to auditory stimuli. These double-labeled neurons were apparently generated during or after the ^{3}H-labeled thymidine treatment (during adulthood) and subsequently incorporated into functional neural circuits.*

The vast majority of neurons in the central nervous system of warm-blooded vertebrates are thought to be generated in prenatal or early postnatal development (*1*). To the extent that this is true, it places limits on the plasticity of the adult brain and on its ability to recover from injury. Thus, examples of neurons generated during adulthood have been of special interest since first being reported by Altman and others in the early 1960's (*2*). In these studies, adult rodents or cats received injections of [^{3}H]thymidine, a specific precursor of DNA, to label newly generated cells (*3*); labeled cells were identified as neurons on the basis of their appearance in paraffin sections. Questions were raised, however, about the interpretation of labeling and the ambiguity of neuronal identification inherent in this material (*4*). In later [^{3}H]thymidine studies, Kaplan and coworkers used 1-μm plastic sections to better locate label and then identified labeled cells as neurons on the basis of their ultrastructure, especially the presence of morphologically defined synapses (*5*). This strictly anatomical identification is important but not definitive, for synapses have been found on glia as well as neurons, particularly during development (*6*). Given this reservation, the only incontrovertible evidence of neuronal identity would seem to be physiological. In this paper we report that cells labeled with [^{3}H]thymidine in adult animals were identified as neurons by intracellular recording of synaptic and action potentials. These same cells received injections of horseradish peroxidase (HRP) and were shown to have neuronal morphology.

Our experiments were done with canaries, *Serinus canarius*, a species in which Goldman and Nottebohm recently reported a robust example of neurogenesis in adult animals (*7*). They treated animals with [^{3}H]thymidine and relied on light microscopic and ultrastructural criteria to identify labeled cells as neurons. More than 1 percent of the morphologically identified neurons within the nucleus hyperstriatum ventralis pars caudalis (HVc), a telencephalic nucleus with a role in vocal control (*8*), were labeled each day of [^{3}H]thymidine treatment. These cells are thought to be generated in the ventricular zone overlying the HVc and then to migrate into the HVc. Although the HVc is remarkable in that it is sensitive to sex hormones and expands and contracts seasonally (*9*), ^{3}H-labeled neurons have also been noted in other, less specialized regions of the telencephalon (*10*).

In our experiments sexually mature, 1-year-old canaries, either males or testosterone-treated females (*11*), received 50-μCi intramuscular injections of [^{3}H]thy-

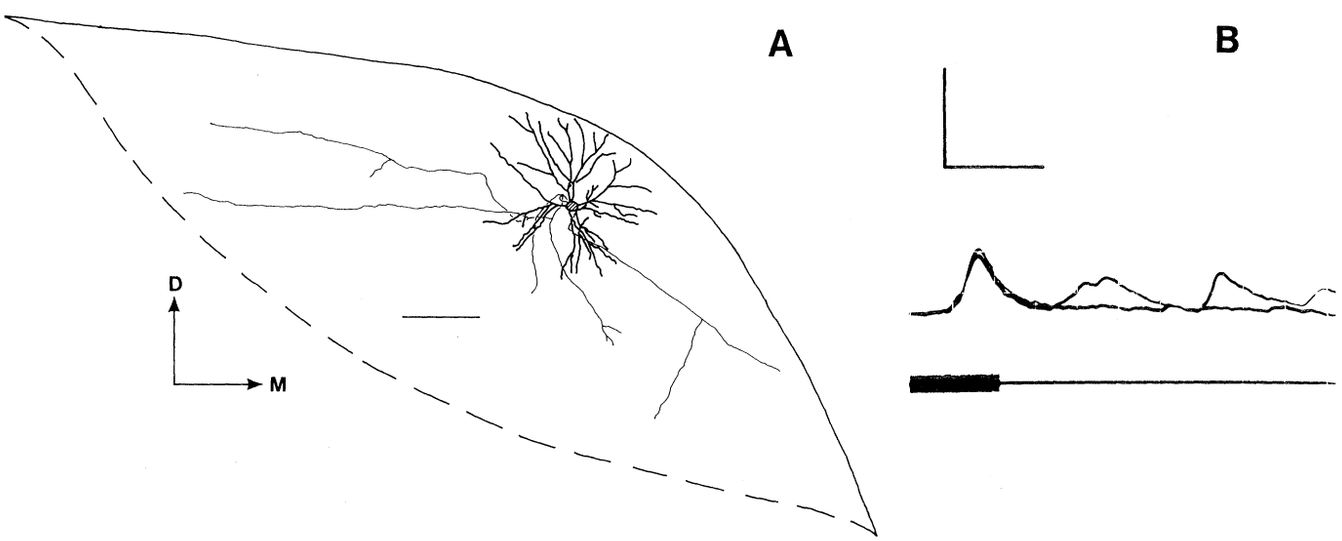

Fig. 1. (A) Camera lucida tracing of dendritic and axonal processes of a cell labeled with both HRP and [^{3}H]thymidine. The dendrites are shown as dark lines, and the axons as light lines. The boundaries of HVc, the ventricle above, and a fibrous lamina below are also shown. The calibration bar is 100 μm. (B) Intracellular recording from the cell whose anatomy is shown in (A). The two responses in the upper trace were recorded in response to a 40-msec 80-dB noise burst. The lower trace is a stimulus marker. The length of the entire trace is 200 msec. The calibration bars are 20 mV (vertical) and 40 msec (horizontal).

midine (New England Nuclear, methyl-[^{3}H]thymidine, 6.7 Ci/mmol) every 12 hours for 14 days. After a delay of 30 days from the last [^{3}H]thymidine injection to allow time for migration and differentiation of new cells, the birds were anesthetized with Chloropent (Fort Dodge Laboratories) to prepare them for physiological recording. The birds were held in a stereotaxic apparatus by a nail glued to the dorsal surface of the skull. Beveled micropipettes containing an HRP solution (Sigma type VI, 25 percent HRP in 0.3*M* KCl, 0.05*M* phosphate buffer, *p*H 7.6) were mounted on a stepping microdrive and advanced into the HVc through a small hole in the skull. Penetrations of cells were accompanied by a negative d-c shift in electrical potential, and cells were identified as neurons by the presence of spontaneous or depolarization-induced action potentials. Many of the neurons in the HVc respond to auditory stimuli (*12*), so noise bursts [80-dB sound pressure level (SPL), 40 msec, one per second, free field] were presented and the latencies of any time-locked synaptic potentials were noted. Then the intracellular label, HRP, was iontophoretically injected (1.0 to 3.0 nA, 400 msec, one per second for 5 to 20 minutes). The stereotaxic coordinates of all cells injected with HRP were recorded.

To prepare brains for histology, birds were killed with an overdose of Chloropent and perfused with 0.9 percent NaCl followed by a fixative solution (1.25 percent glutaraldehyde, 1 percent paraformaldehyde in 0.1*M* phosphate buffer, *p*H 7.6). The brain was removed from the skull, fixed overnight at 4°C, and then cut into 100-μm transverse slices with a Vibratome (Oxford Laboratories). The slices were reacted with hydrogen peroxide and diaminobenzidine to visualize HRP-containing cells (*13*). Slices were cleared in glycerine and examined under a microscope. The locations of labeled cells were marked on camera lucida drawings showing the boundaries of the HVc (Fig. 1A). This information was used to confirm that cells were within the HVc and to determine stereotaxic coordinates to match each cell with its previously recorded physiology. Slices containing HRP-labeled cells were embedded in paraffin, cut into 6-μm sections, and processed for autoradiography (*7*).

Of the 74 neurons containing HRP, seven were also labeled with silver grains. Six were found in females treated with testosterone (6 of 61 cells in 12 animals), and one was found in a male (1 of 13 cells in 5 animals). The grains were specifically concentrated over the nucleus (see Fig. 2). In one large cell whose nucleus extended through three sections, grains were found over the nucleus in two of the three sections, suggesting that the source of radiation was indeed within the cell. In all cases, the number of grains over the nucleus was above ×5 background (see Fig. 2, B and D) and comparable to that seen over labeled cells identified as glia or endothelial cells. Given that these other cell types undergo proliferation in adulthood, it is reasonable to conclude that the seven double-labeled cells were also generated in adulthood, at some time after the first thymidine injection (*14*).

As revealed by HRP staining, these seven cells all had the morphology of multipolar neurons (Fig. 1A). They had somas ranging in size from 7 by 10 μm to 11 by 16 μm (Fig. 2, A and C); thick, spine-covered dendritic trees (Fig. 3B); and smooth, thin axonal processes that arborized within the HVc (Fig. 3A). Although many of the HRP-labeled cells not labeled by thymidine had axons that left the HVc and could be followed several millimeters toward nuclei to which the HVc projects, none of the processes of the seven double-labeled neurons were seen to exit the HVc.

As determined before injection of HRP, all the double labeled cells supported action potentials and showed

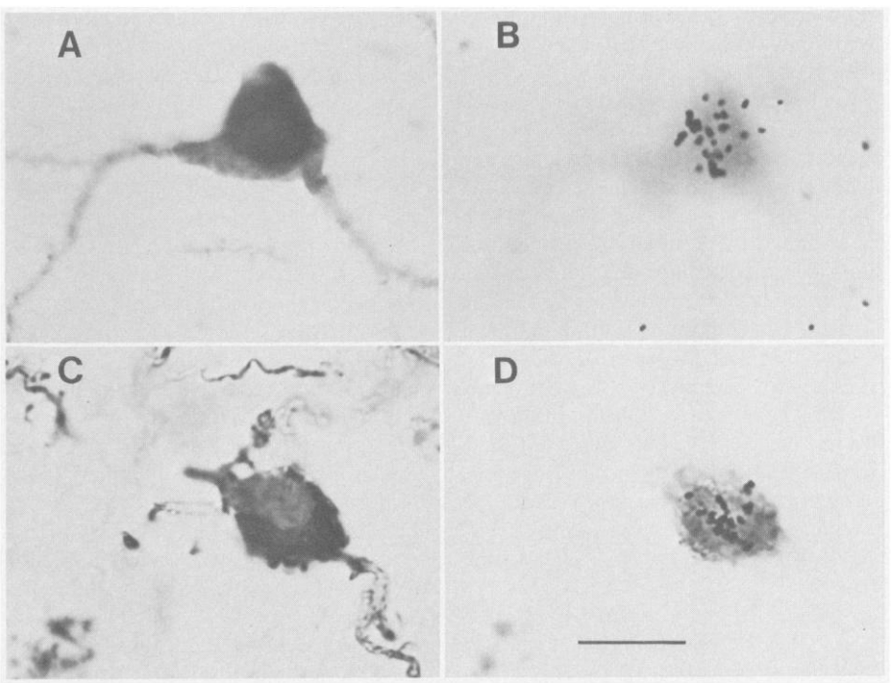

Fig. 2. Anatomy of cell bodies labeled with both HRP and [^{3}H]thymidine. Separate photographs were taken of the same field, but focused first at the level of the cell itself and then at the level of the layer of emulsion above the cell. (A) Soma of cell. (B) Autoradiographic grains over the soma in (A). (C) Soma of another cell. (D) Autoradiographic grains over soma in (C). The calibration bar is 10 μm.

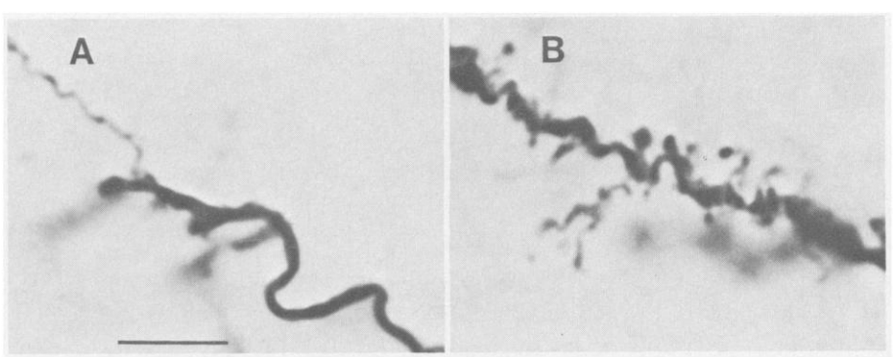

Fig. 3. Processes of cells labeled by both HRP and [^{3}H]thymidine. (A) Axonal process from a double-labeled cell. The thick primary process branches into a number of thinner processes. (B) Dendritic process from a double-labeled cell. Note especially the spines with long narrow necks and bulbous ends. The calibration bar is 5 μm.

slower changes in membrane potential characteristic of synaptic potentials. In addition, four of the seven double-labeled cells responded to the onset or offset of auditory stimuli with depolarizing synaptic potentials at latencies of 26 to 32 msec (Fig. 1B). We have not yet determined whether any of the double-labeled cells form synapses on other HVc cells, though the presence of axonal arbors within HVc suggests this. The double-labeled neurons we have described may constitute a category of local interneurons, some of which are sensitive to acoustic stimuli. This hypothesis is supported by preliminary experiments in which none of the neurons whose axons leave the HVc (identified by retrograde labeling with HRP) were radioactively labeled after [^{3}H]thymidine treatment similar to that used in the present experiments (*15*).

The evidence presented here constitutes a direct demonstration of central nervous system cells that are generated in adulthood, adopt a neuronal morphology, show synaptic and action potentials, and are recruited into functional brain circuits. Though the likelihood of such a series of events has been accepted for fish, whose body and brain continue to grow in adulthood (*16*), the proof offered here establishes that neurogenesis can occur in a vertebrate brain well after it has achieved its full adult size. Thus the question can no longer be whether this phenomenon exists, but rather how it comes about, why is it so rarely found, and what is its significance?

JOHN A. PATON
FERNANDO N. NOTTEBOHM
Rockefeller University, New York 10021

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Flying Squirrels Are Monophyletic

Abstract. *Seven genera of flying squirrels share five characters of wrist anatomy, which form a functional complex associated with the support of the patagium. In these characters, they differ from all genera of tree and ground squirrels examined. Among mammals, gliding membranes have evolved independently in several other groups. The manner of attachment of the patagium to the forelimb is different in each and demonstrates five morphologies differing from that of flying squirrels. This complex wrist anatomy of flying squirrels provides evidence that gliding evolved only once among squirrels and that the flying squirrels are a monophyletic group.*

Several investigators have proposed that the flying squirrels (Petauristinae) are a polyphyletic group (*1*), consisting of genera derived from Southeast Asian tree squirrels (Callosciurini) and from Holarctic tree squirrels (Sciurini). Other investigators have suggested that flying squirrels evolved from a subfamily of paramyid rodents, different from that of tree squirrels (*2*, *3*), and hence that the family Sciuridae is polyphyletic. Although recent classifications ignore these arguments and include all squirrels in a single family, the Sciuridae (*4*), the arguments for polyphyly have gone unchallenged.

The hypothesis that flying squirrels are not derived from tree squirrels is based on the observation that they were as diverse in the Miocene as they are at present and the supposition that this diversification required a long period of evolution after the flying squirrels diverged from the tree squirrel lineage (*2*, *3*). The earliest fossil thought to be a flying squirrel (*Paracitellus* sp. A. Dehm) dates from the Burdigalian, approximately 17 million years ago (*5*). However, the oldest known fossil tree squirrel, *Protosciurus*, dates from the Chadronian Oligocene, about 35 million years ago (*6*), so that there remains a period of almost 20 million years during which flying squirrels could have evolved and radiated from an ancestral tree squirrel. This alternative hypothesis is supported by the presence of several shared derived features in modern tree squirrels and flying squirrels, such as the sciuromorphous jaw musculature, morphology of the ear region, and the presence of a subscapular spine. Sciuromorphy was not fully developed in *Protosciurus*, although the postcranial anatomy is remarkably similar to that of the North American fox squirrel *Sciurus niger* (*6*). Therefore the simplest hypothesis is that flying squirrels evolved from a tree squirrel ancestry sometime after the Chadronian, 35 million years ago (*7*).

All flying squirrels have a patagium supported at the wrist by a styliform cartilage (*8–10*), which attaches to the pisiform bone. The ulnar carpal flexor muscle attaches to the base of the styliform cartilage and functions to fold the cartilage and the gliding membrane against the forearm when the squirrel is not gliding. The styliform cartilage and the gliding membrane are extended by the abductor pollicis muscle, which inserts on the small falciform bone in the palm of the hand, which in turn connects by a ligament to the base of the styliform cartilage. This morphology results in maximum extension of the wing tip of the gliding membrane when the hand is dorsiflexed and medially deviated, as can be seen in photographs of gliding squirrels (*11*, *12*).

The morphology of the wrist joint of flying squirrels (*13*) shows specializations associated with its function in gliding (Fig. 1). The pisiform bone articulates both with the triquetrum and also with the scapholunate. This articulation with the scapholunate, which would appear to function as a stabilizer of the pisiform and hence of the styliform cartilage, was not found in any other squirrels examined (*11*). The joint surface between the ulna and the triquetrum has a