Cerebellar Classics

Postnatal Neurogenesis Beyond Rodents: The Groundbreaking Research of Joseph Altman and Gopal Das

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Running title: Postnatal neurogenesis and the cerebellum

This paper is dedicated with gratitude to Professor Shirley A. Bayer, legendary pioneer in developmental neurobiology, whom one of us (L.C.T.) had the good fortune to have as a graduate adviser and colleague around the heydays of her landmark discoveries in neural ontogeny.

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Abstract

An integral component of neural ontogeny and plasticity is the ongoing generation of new neurons from precursor cells throughout the lifespan in virtually all animals with a nervous system. In mammals, postnatal neurogenesis has been documented in the cerebellum, olfactory bulb, hippocampus, striatum, substantia nigra, hypothalamus, and nucleus of the amygdala. Germinal centers of new neuron production in the adult brain have been identified in the neuroepithelium of the subventricular zone and the dentate gyrus. One of the earliest lines of evidence gathered came from studies on the production of cerebellar microneurons in the external germinal layer of rodents and carnivores in the 1960s and 1970s. The undeniable pioneer of that research was the insightful developmental neurobiologist Joseph Altman (1925–2016). This *Cerebellar Classic* is devoted to the groundbreaking work of Altman and his graduate student and, subsequently, fellow faculty member, Gopal Das (1933–1991), on postnatal neurogenesis using tritiated thymidine autoradiography to tag newly formed neurons in the cerebellum of cats. Perseverant to their ideas and patiently working in West Lafayette (Indiana), they were the founders of two fields that brought about paradigm shifts and led to an explosive growth in brain research: adult neurogenesis and neural tissue transplantation.

Keywords

Adult neurogenesis, Cerebellar development, Granule cells, Feline nervous system, Mitotic divisions

The discovery by Joseph Altman (1925–2016) in 1962 of adult neurogenesis as a general phenomenon in the brain of mammals [1] prompted a paradigm shift in ontogenetic and restorative neuroscience. Adult neurogenesis was an incidental discovery during the process of perfecting the complex technique of tritiated (^{3}H) thymidine autoradiography. The production of neurons after birth, an issue of great controversy at that time, was demonstrated by using light microscopic autoradiography, whereby proliferative cells of the cerebellar external germinal layer (EGL) and the cortical subependymal layer of rodents incorporated systemically administered [^{3}H]thymidine, with radioactively labeled cells differentiating into identifiable microneurons. The supporting evidence that was subsequently marshaled by Altman and his coworkers in the 1970s and the 1980s led to his vindication, and a "rediscovery" by others of adult neurogenesis in the 1990s [2].

Altman's systematic studies from the 1960s and the 1970s established in a definitive way that a high proportion of cerebellar, hippocampal, and olfactory bulb microneurons are generated postnatally. Whereas the generation of cerebellar microneurons in the rat is limited to the first three weeks of postnatal life, the production of hippocampal and olfactory bulb microneurons continues through adulthood, albeit at a reduced rate. Postnatal neurogenesis persists in specialized niches within the rostral subventricular zone and the hippocampal dentate gyrus, and, for a limited time, in white matter bundles and the EGL of the cerebellum [3].

Born in Budapest, Hungary, during the interwar period, Altman was forced to emigrate to Australia and then to the United States. He earned his Ph.D. in psychophysiology in 1959 from New York University-Bellevue Medical Center under the supervision of Hans-Lukas Teuber (1916–1977), a neuropsychologist who in 1960 started the Department of Psychology (today the Department of Brain and Cognitive Sciences) of the Massachusetts Institute of Technology (MIT). After postdoctoral training at Columbia University under the renowned neuroanatomist Malcolm Carpenter (1921–1999), Altman became an associate professor at MIT [4]. He joined the faculty at Purdue University as a full professor in 1968, after being denied tenure at MIT [2].

In the early 1960s, Altman discovered that granule cells in the olfactory bulb and in the hippocampal dentate gyrus continue to be generated through adulthood, and he noted that the secondary germinal matrix is far more prominent in young cats than in rats and guinea pigs. Altman's focus on three brain regions with a cortical organization—the cerebellum, the hippocampus, and the cerebrum including the olfactory bulb—showed, in the mid-1960s, that new cells that he identified as granular neurons ("microneurons") continued to be added postnatally to these regions, and such an addition continued into adulthood in the case of the latter two structures [2, 5–8].

Proliferating cells in the rat EGL were consistently labeled with [^{3}H]thymidine up to postnatal day 21. In contrast to postnatal neurogenesis in the cerebellum, hippocampal neurogenesis persisted through adulthood. The subgranular zone containing the proliferating precursors of dentate granule cells was more prominent in the guinea pig than in the rat, and even more so in the cat [5, 6].

Gopal Das was a native of Shikarpur in the Sindh Province (then India, today Pakistan). He earned his Ph.D. in neurobiology in 1965 from Boston University under the supervision of Altman, whom he joined on the faculty at Purdue three years later. One of their collaborative areas of research was the successful intracerebral grafting of cerebellar slabs from donor into recipient animals [9]. Das was able to provide answers to key questions related to the survival and the integration of transplanted nervous tissue, with potential beneficial effects in restoring the injured spinal cord and the degenerating brain [10]. Those early efforts of grafting cerebellar primordia into neonatal hosts [9, 11, 12] set the foundations for a field of research that has since witnessed an exponential growth [13].

Altman gained attention in the minds of many as the first to challenge Ramón y Cajal's doctrine—and that of most neuroanatomists after Cajal—that no new neurons were produced after the completion of embryonic brain development [14]. Altman recalled that it had happened before. In one of his early studies on postnatal neurogenesis [6], he referred to early pioneers of that idea around the *fin du siècle*: Emile Lahousse (1850–1921) of the Department of Physiology at Ghent University [15, 16], Alfred Schaper (1863–1905) of the Anatomical Institute at the University of Zürich [17, 18], and Ramón y Cajal [19, 20] had all recognized in the 1880s and 1890s that granule cells showed a high rate of proliferative activity in the cerebellar cortex of young chicken and teleosts. In addition, Naoki Sugita (1887–1949) of the Wistar Institute of Anatomy and Biology in Philadelphia had obtained quantitative evidence for a continual increase in the total number of neurons in the cerebral cortex of albino rats up to postnatal day 20 [21].

The technique of [^{3}H]thymidine autoradiography was developed in 1956 by the group led by Walter Hughes at the Brookhaven National Laboratory, who found that the tritium was incorporated into the nucleus of newly born leukocytes [22]. Richard Sidman and his coworkers Irene Miale, Ned Feder, and Jay Angevine, working at the National Institute of Neurological Diseases and Blindness and subsequently at Harvard Medical School, injected [^{3}H]thymidine intravenously in mice to study histogenesis and migration patterns in the developing cerebral cortex, cerebellar cortex, and retina [23–26].

Thymidine is incorporated into the nucleus when a cell undergoes mitotic division. Altman and coworkers spent several years to develop a reliable and consistent methodology, including the use of [^{3}H]thymidine of the right specific activity, the optimal dose in relation to the animal's body weight, the proper and consistent dilution of the nuclear emulsion, the drying of the emulsion in the dark before packaging and refrigeration to avoid mechanical artefacts, long exposure periods of 3 months to obtain optimal results, and the use of counterstaining techniques that did not remove part or all of the emulsion [2].

Altman's first major discovery was that new neurons are generated in the brains of adult rats and cats. Despite an outright, almost universal rejection by many in the neuroscience community, Altman persevered, compiling evidence on postnatal neurogenesis in the cerebellar cortex, the olfactory bulb, and the hippocampal dentate gyrus to substantiate his claim [4].

In 1962, after placing experimental lesions in the lateral geniculate nucleus of rats and administering [^{3}H]thymidine intracerebrally, Altman observed labeling in the nuclei of reactive glial cells. He further noted that labeling also occurred in the nuclei of some neuroblasts and neurons sparsely scattered in the cerebral cortex and the thalamus, suggesting the possibility that "new neurons may come into existence in the brain of adult mammals" [1]. He next injected [^{3}H]thymidine intraperitoneally in rats and intracerebroventricularly in cats, and again observed a few labeled neurons in the rat neocortex and hippocampal dentate gyrus, as well as in the midline cortex of cats, reinforcing the idea that new neurons may be formed in adult forebrain structures in both rodents and carnivores [27]. In 1965, Altman and Das [5] reported postnatal neurogenesis of granule cells in several areas of the rat brain. They observed that, besides granule cells of the hippocampal dentate gyrus, the bulk of granule cells in the granular, mitral, and glomerular layers of the olfactory bulb, a large proportion of microneurons in the internal granular layer (IGL) of the cerebellum, and granule cells in the granular layer of the ventral cochlear nucleus were largely formed postnatally [6]. They concluded that in all these areas, postnatal neurogenesis was restricted to short-axon granule cells, or microneurons, whereas long-axon cells, or macroneurons, were overall generated during embryonic life [7].

They extended those studies from altricial rodents (characterized by a relatively brief period of intrauterine development of about 3 weeks) to guinea pigs, a precocial species (with a gestation period of 9 weeks). Whereas postnatal neurogenesis in the hippocampus was pronounced for some time, it was minimal in the cerebellum of the guinea pig [28]. The newborn rat had a pronounced subpial EGL; by contrast, the guinea pig had a much better developed cerebellar cortex at birth, and only a small fraction of its granule cell pool was formed postnatally. It is known from comparative studies that in the rat, the EGL disappears at the end of the third postnatal week, in the cat at the end of the second month, and in humans by 20 months of age.

Altman and Das [28] further suggested that the observed differences in the duration of cerebellar neurogenesis among various mammalian species, inferred from the dissolution of the EGL, correlate with the time period necessary for locomotor and related skills to mature, as well as with the complexity of such skills. Indeed, Lynn Dacey and Robert Wallace of the University of Hartford later found that the postnatal development of the feline cerebellum, with the dissolution of the EGL after postnatal day 15, and a concomitant increase in the thickness of the IGL until 60 days of age, correlated with the development of balance, the acquisition of locomotor patterns, and the elaboration of such behaviors [29].

In 1969, Altman [8] analyzed the cytoarchitectonics of the cerebellar cortex based on the onset of neuronal subset differentiation; he demonstrated that a proportion of the basket and the stellate cells were produced postnatally. He further showed that an extension of the anterior cortical subependymal layer, the "rostral migratory stream," was the source of a large stream of cells that migrated and settled in the granular and periglomerular layers of the olfactory bulb.

In computerized volumetric studies published in 1982, Bayer [30, 31] estimated the total number of granule cells in the rat dentate gyrus and discovered that these increased linearly by approximately 35%–43% between one month and one year of age. This finding provided unequivocal proof that new neurons in the dentate granular layer are actually added to the adult population. Bayer made the distinction that, while in the dentate gyrus there is a net increase in the number of neurons with age, and hippocampal neurogenesis in rodents is additive [31], the genesis of new neurons in the olfactory bulb is involved in the turnover and replacement of decaying granule cells [32]. Indeed, newborn neurons generated from neural stem cells in the subventricular zone of the lateral ventricle and migrating to the olfactory bulb through the rostral migratory stream differentiate into inhibitory interneurons, that is, granule and periglomerular cells. The prolonged supply of newborn neurons leads to continuous addition/turnover of the interneuron population, whereby the dynamic turnover of old granule cells and newly generated granule cells occurs continuously, contributing to plasticity in the olfactory bulb circuit [33].

In the present *Cerebellar Classic*, published 50 years ago, Das and Altman [34] took their studies a step up the evolutionary ladder from the rat and the guinea pig and documented the postnatal neurogenesis of microneurons in the cerebellum of the cat by means of [^{3}H]thymidine autoradiography. Because cats were widely used in neuroanatomical, neurophysiological, and behavioral experiments, the authors explored factors that might explain why, contrary to rodents, the standard dose of [^{3}H]thymidine (10 μCi/g body weight), when administered intraperitoneally to young kittens, did not label neuron precursors in the central nervous system (CNS). Histologically, they distinguished two zones in the EGL, an outer (proliferative) zone typically composed of round cells, and an inner (migratory) zone, composed of spindle-shaped cells. After intracisternal administration of [^{3}H]thymidine in kittens, the average number of labeled cells was higher than that following intraperitoneal injection, but is was restricted to the vicinity of the injection site. Increasing the amount of the radiochemical led to a relatively small increase in labeled cells. When the dose was increased to 40 μCi/g body weight, an almost 300% increase was observed in the number of labeled granule cells. The complete contributions of Altman and Bayer to the comprehensive analysis of cortical and cerebellar ontogeny as well as the development of precerebellar brainstem nuclei from the 1960s to the 1980s [35, 36] led to a series of superb atlases of brain development [37–40]. The cerebellar circuitry now appears to be an excellent model to investigate neural development, specification, and neurogenesis, as the cerebellum is composed of a limited number of phenotypes within a highly organized pattern, and key mechanisms of cerebellar ontogeny have been clarified for glutamatergic, GABAergic, and Purkinje neuron specifications [41]. Regulation of transcription plays a major role in controlling progenitor cell proliferation and differentiation during neurogenesis; this is also true for the cerebellum, and it is currently a field of active research [42].

While at Tulane University in the early 1970s, Michael Kaplan observed neurogenesis with light microscopic autoradiography in layer IV of the visual cortex of adult rats raised in an enriched

environment. Although that was the first evidence that environmental factors may increase the rate of neurogenesis in the visual cortex of an adult mammal, the study was not accepted for publication [43], and subsequent experiments were only published 7 years later [44]. In 1977, Kaplan and James Hinds of Boston University provided electron microscopic evidence for adult neurogenesis in the olfactory bulb [45], and in 1984, Kaplan [46] presented his work before the conference *Hope for a New Neurology*, organized by Fernando Nottebohm at the New York Academy of Sciences, where Bayer [32] also presented her work on adult neurogenesis in the hippocampal dentate gyrus.

In the cerebral cortex, adult neurogenesis was later documented in prefrontal, inferior temporal, and posterior parietal regions of the macaque, with new neurons, originating in the subventricular neuroepithelium, being continually added to these association areas [47]. Adult neurogenesis has also been documented in the hypothalamus, substantia nigra, and amygdala [48]. Stroke/ischemia and neurodegenerative diseases seem to impact neurogenesis in several brain areas, bringing up potential clinical applications by stimulating neurogenesis in these areas [49].

Mammalian neurogenesis gained popularity when the non-radioactive compound 5-bromo-3′-deoxyuridine (BrdU) was introduced as a thymidine analogue and marker of DNA synthesis in dividing cells during the S-phase of the cell cycle [50]. It became the most used method for studying adult neurogenesis *in situ*. Nonetheless, the BrdU method has pitfalls and limitations, including mutagenicity and toxicity, cell death, structural and behavioral abnormalities, and the formation of teratomas. BrdU may also alter the development of the cerebellum [51, 52] and reduce its size by producing defects in proliferation, migration, and patterning in adult progenies [53]. On several occasions, controls were overlooked and findings regarding the new production of neurons in the adult brain have been misinterpreted [50].

Moreover, the development of the cerebellum in mice prenatally exposed to BrdU, compared to [^{3}H]thymidine, and collected at three months of age, showed that several parameters—including the length of the cerebellar cortex, the area of the molecular layer, the number of Purkinje cells, the area of the cerebellar nuclei, and the number of cerebellar nuclei neurons—were lower in the BrdU-injected group, whereas no consequences were observed with the administration of [^{3}H]thymidine. Systematic differences were found between mice injected with BrdU and [^{3}H]thymidine in the pattern of neurogenesis and the spatial location of cerebellar neurons [54].

Neural progenitors, precursors, and stem cells in the adult CNS are capable of generating new neurons and glia. Experimental models are used to investigate the behavior of neural precursor cells *in vitro*, as well as to assess the potential of intracerebral grafting of precursor cells to repair damaged neuronal circuits or by manipulating neurogenesis *in situ* at the cellular and molecular levels without transplantation [55, 56].

The detection of adult neurogenesis in several areas of the CNS indicated that the adult brain exhibits more plasticity than previously thought, and this has implications for concepts of self, memory, and the pathogenesis of neurodegenerative diseases. Moreover, environmental experiences

appear to have consequences for the behavior of animals, and they directly correlate with the rate and extent of adult neurogenesis [57]. Adult neurogenesis was confirmed in the human species in 1998 [58]. Furthermore, with the use of histochemical and ^{14}C-birth-dating methods combined with transcriptome data and western blot analyses, new interneurons were found to integrate in the corpus striatum of the human brain, adjacent to the subventricular neurogenic zone in the wall of the lateral ventricle. Such postnatally generated striatal neurons and their neuroblasts are vulnerable to the pathological processes associated with Huntington disease [59, 60].

Ramón y Cajal [61] had argued that the CNS ceased to add new neurons past fetal life. The dogma since Ramón y Cajal [62] had been "no new neurons in the adult brain" [63–65]: "We must acknowledge that, in the adult centers, the nervous pathways are fixed, completed, immutable. Anything can die, nothing can be reborn…Everything leads one to think, therefore, that in mammals nervous restoration is a purely expansional act, in which the cellular body never participates" [62]. Altman received criticism for a scientific claim that counters an established dogma. It took almost three decades for the neuroscience community to abandon the old doctrine of "no postnatal neurogenesis." Altman was the first, in modern times, to challenge it. Despite the strong opposition, he persevered with this claim and he was right [14]. Das and Altman's groundbreaking work was eventually vindicated and generally accepted [66]. The blooming of the field of early postnatal and adult neurogenesis opened up new vistas at both the technical and the conceptual level. Emerging principles have pertinence to stem cell biology, ontogeny and plasticity, and disease pathogenesis [67].

In 2011, Joseph Altman jointly received the Prince of Asturias Award for Technical and Scientific Research in Spain with Arturo Álvarez-Buylla and Giacomo Rizzolatti, for having provided solid proof of the regeneration of neurons in adult brains (neurogenesis) and for the discovery of what are known as mirror neurons [4, 68]. In 2012, Altman was the recipient of the International Prize for Biology from the Emperor of Japan for the discovery of neurogenesis in the adult mammalian brain. Through a donation, Shirley Bayer, Altman's widow and long-time collaborator, instituted in 2016 the Joseph Altman Award in Developmental Neuroscience, which is awarded each year by the Japan Neuroscience Society to outstanding contributors to the field.

Much about the physiological role of adult neurogenesis in humans remains in the realm of speculation and is solely based on observations from experimental animals [69]. An early speculation by Altman [70] on the functional significance of adult neurogenesis in learning and memory has since received experimental support, as the number of newly generated neurons in the adult hippocampus often correlates with the learning of hippocampal-dependent behavioral tasks [66]. Kaplan, the other underappreciated pioneer of adult neurogenesis, ventured to make the bold proposition that neurogenesis could be involved in humans exposed to culture, who flourish in the enriched environment provided by a supportive family or excellent teachers [43, 71].

Factors such as stress and environmental stimulation in relation to adult neurogenesis may be involved in classic hippocampal behaviors, including context learning, spatial memory, and emotional

behaviors, as well as in other presumed behaviors like decision-making, temporal association memory, and addiction. Moreover, neurogenesis could be experimentally manipulated to offset age- and disease-related brain abiotrophies [72].

Neural stem cells are found in the subventricular zone, the olfactory bulb, and the hippocampal neuroepithelium, and their proliferation and differentiation are modulated in part by the epigenetic regulation of gene expression, including DNA methylation and histone modification. MicroRNAs (small noncoding RNA molecules that post-transcriptionally regulate gene expression) may play an important role in such epigenetic regulation. Dysfunctions of the microRNA machinery have been linked to neurodegeneration, and thus a better understanding of how microRNAs influence neurogenesis and differentiation may offer novel therapeutic applications [73].

The evolutionary dimension of the production of new neurons did not escape the acumen of Ramón y Cajal, who wrote: "We do not, however, deny the facts, apparently well established, of neuronal division in the lower vertebrates, especially amphibians and reptiles" [62]. Adult neurogenesis is conserved throughout evolution across many vertebrate species, underscoring the value of comparative studies toward a better deciphering of this phenomenon [3]. A cross-species comparative perspective presents special interest, because a better understanding of the mechanisms of adult neurogenesis across taxa may shed new light onto physiological plasticity, learning, and the adaptation to external environments. Furthermore, cross-species studies can provide a basis to formulate novel concepts on the phylogenetic and biological significance of adult neurogenesis in the intact and injured CNS [74, 75].

Although adult neurogenesis in mammals is limited to select brain areas, in non-mammalian vertebrates, new neurons are continuously generated in many regions of the adult CNS. Such a difference makes the study of adult neurogenesis from a comparative viewpoint worthwhile: It could yield insight into the evolution of the phenomenon. Furthermore, central sites may be identified that, although quiescent *in vivo*, retain an intrinsic potential to produce new cells during adulthood. Teleost fish exhibit an enormous potential to replace damaged neurons by newly generated ones. In *Apteronotus leptorhynchus*, an average of 100,000 cells are in the S-phase at any 2-hour period. In the cerebellum, about 75% of all neurons are produced during adulthood; upon settling in their target areas from their proliferation zones, about one half of the young cerebellar cells undergo apoptosis, while the remaining cells survive for the rest of the fish's life, thus contributing to permanent brain growth. These events are the basis for the profound regenerative capability of the CNS of *Apteronotus*. In addition, adult neurogenesis in teleost fish appears to have a role in providing central neurons to match the growing number of peripheral sensory and motor elements, and to establish the organic substratum of neurobehavioral plasticity [76]. Zupanc [77] formulated the hypothesis that, during the course of mammalian evolution, a major shift in the growth pattern from hyperplasia to hypertrophy occurred, and the number of neurogenic brain regions and new neurons markedly decreased. Consequently, the regenerative potential was substantially reduced, although remnant neurogenic areas have persisted in

the form of quiescent stem cells in the adult mammalian brain. In reptiles, such as the crocodile (*Crocodylus niloticus*), the brain allometrically gains in mass throughout life. The addition of new neurons appears to be crucial for the increase in brain mass. Similarly to other reptiles, potential newly born neurons immunopositive for doublecortin, a marker for potential adult neurogenesis, have been detected throughout the telencephalon, the main and accessory olfactory bulbs and the olfactory tract, and the cerebellar cortex. With increasing brain mass, there is an apparent moderate decrease in the density of labeled neurons in the cerebellar cortex. At least part of the gain in brain mass over time is conceivably accounted for by the addition of new neurons and their integration into the existing neural circuits, including the telencephalon, the olfactory system, and the cerebellar cortex [78]. In the avian forebrain, the now classical studies of Nottebohm and his coworkers at Rockefeller University elegantly demonstrated seasonal neurogenesis and neuronal replacement in specialized regions in adult canaries [14]. In mammals, the most striking prototype of protracted neurogenesis is the cerebellum, in which granule cells are generated in the secondary surface germinative layer, or EGL. On the other hand, parenchymal neurogenesis has been described in lagomorphs; in the rabbit, newly generated neurons are spontaneously produced in two main regions of the adult CNS, namely the forebrain striatum and the cerebellum. In the New Zealand albino rabbit, protracted cerebellar neurogenesis extends around and beyond puberty [79] and persists, to a lesser extent, during adulthood, in the absence of germinal layers [80, 81]. Unlike other mammals, an overlapping of protracted (germinal-layer derived) and adult (parenchymal) cerebellar neurogenesis occurs in lagomorphs [82].

Scholastic science has divided, on technical grounds, the life of organisms into phases such as infancy, childhood, adulthood, and old age. In actuality, every individual life is a continuum, and development only ends with death. This is even more so true in the case of the nervous system, in which neurons of very different birthdates coexist, and parts of which, owing to sustained or incessant neurogenesis, always contain some young cellular elements.

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