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Neuron Production in the Hippocampus and Olfactory Bulb of the Adult Rat Brain: Addition or Replacement?[a]

SHIRLEY A. BAYER
Department of Biology
Indiana-Purdue University
Indianapolis, Indiana 46223

INTRODUCTION

In 1962, Altman[1] proposed that neurogenesis takes place in the cerebral cortex of adult rats. Between 1963 and 1969, Altman and coworkers found adult and juvenile neurogenesis to be especially prominent in the granule cell populations of both the hippocampus and olfactory bulb.[2–6] Kaplan and Hinds[20] subsequently showed that these newly formed cells had the ultrastructural characteristics of neurons. In the meantime, Bayer and Altman[10] obtained circumstantial evidence that the number of granule cells in the hippocampus was increasing in the adult period, while Roselli-Austin and Altman[29] found the number of granule cells in the olfactory bulb to remain constant during adult life. This paper summarizes recent studies[7,8,12] comparing the patterns of adult neurogenesis in these two populations of the rat brain.

METHODS AND RESULTS

Volumetric Analysis of Hippocampal Granule Cells

The total number of granule cells was estimated in the hippocampus of 17 male Purdue-Wistar rats, four animals each at 30, 120, and 200 days, and five at 365 days. After a transcardial perfusion with 10% neutral formalin and Bouin's fixative, the brains were stored in 10% neutral formalin until the block containing the entire right hippocampus was embedded in methacrylate. Serial 3-μm slices were cut in the horizontal plane with a JB-4 microtome (Sorvall) and stained with cresyl violet. A running count was made of all slices containing the granular layer; care was taken to assure that slices were of uniform thickness.

Two previous volumetric estimates of granule cell numbers in rats used modified Abercrombie correction factors to count neuronal nucleoli.[16,30] This method was unworkable in our preparations since the Purdue-Wistar

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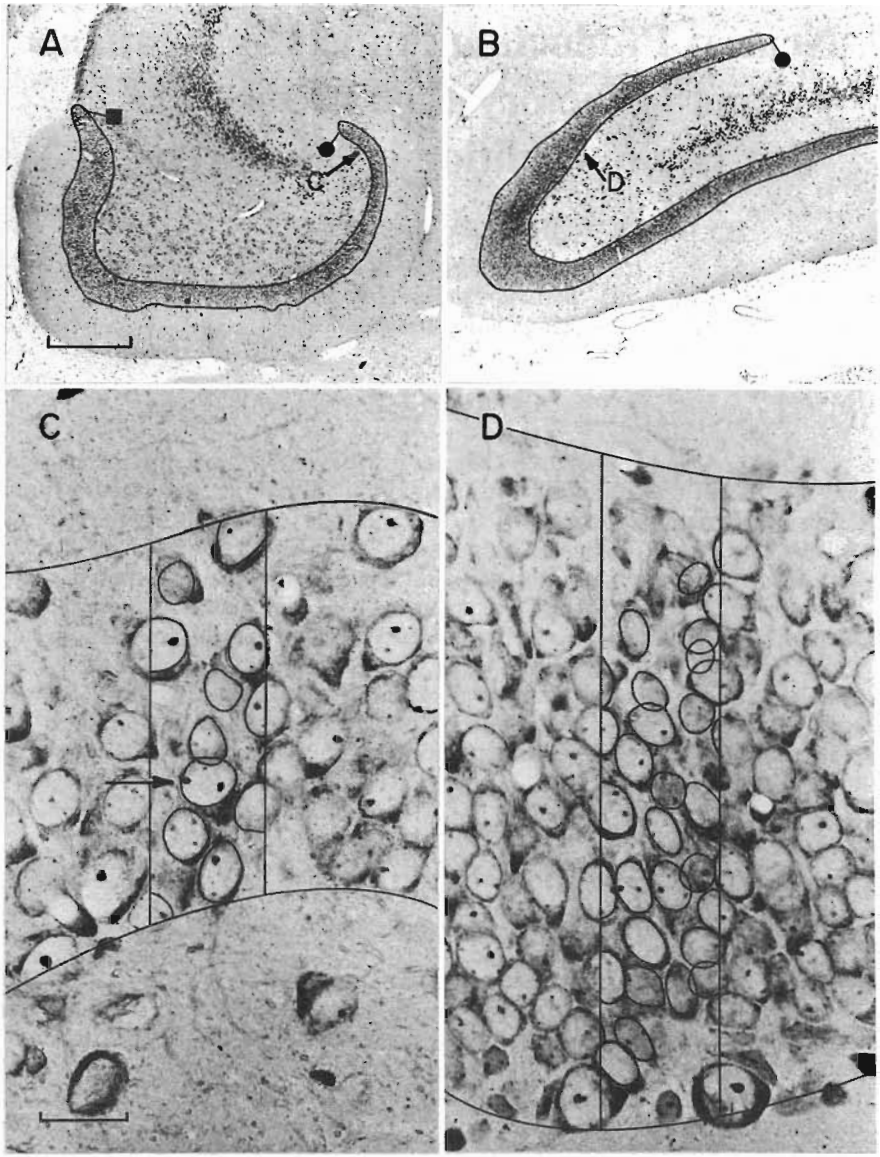

FIGURE 1. (**A** and **B**) Low-magnification (bar = 0.4 mm) views of ventral (**A**) and dorsal (**B**) horizontal sections of the dentate granular layer in a P30 rat; only the posterior part of the granular layer is shown in **B**. The length at the base of the layer was measured starting with the point indicated by the *solid circle*, and ending at the point indicated by the *solid square* to determine the total number of available samples in each section to be quantified. (**C** and **D**) High-magnification views (bar = 20 μm) of samples randomly drawn from either **A** or **B** (the location of **C** is indicated by *arrow* in **A**, while that of **D** is indicated by *arrow* in **B**). The areas of the outlined features in each photograph were measured and formed the data base needed to determine both V_i and V_a. *Arrow* in **C** indicates a granule cell with two nucleolus-like bodies. Notice that the ventral granule cell nuclear profiles (**C**) tend to be larger than the nuclear profiles in the dorsal section (**D**). (3-μm methacrylate sections stained with cresyl violet.) (From Bayer.[7] Reprinted by permission from *Experimental Brain Research*.)

strain consistently shows two or more nucleolus-like bodies in many granule cell nuclei (arrows in FIGURE 1). Consequently, the nucleus was quantified. The method was based on the equation:

$$N = V_t/V_a$$

where N is the total number of cells, V_t the total volume of cell nuclei, and V_a the average nuclear volume. V_t was experimentally determined from low-magnification photomicrographs of dorsal (FIG. 1A) and ventral (FIG. 1B) granular layer slices at regularly spaced intervals. The granular layer was divided into 25-μm wide strips running perpendicular to the length; each strip was a possible sample. Two hundred strips from the total sample pool in all selected slices were randomly chosen for examination at high magnification (FIG. 1C and D). The sampling technique was adjusted so that the experimental error was sensitive to a 10% change in cell number. For each slice examined, the areas of all complete and partial granule cell nuclear profiles (outlined in FIGURE 1C and D) within the samples were measured with a digitizer (Summagraphics) connected to a Wang 2200 computer (accuracy within 0.75%). These measurements provided the basis for the final estimation of V_t (for more details of the method, see Ref. 7).

To estimate V_a in the ventral part of the granular layer, for example, the nuclear profiles completely contained in all ventral samples (between 700 to 1000 in each animal) were used to form empirical distributions of observed ventral granule cell nuclear sizes. Complete profiles contained in all dorsal samples (usually 2000 or more observations for each animal) were similarly processed. In both parts, V_a was estimated by applying a modification[28] of the method of Hendry[18] to the empirical distributions.

Final cell numbers ranged from means of 890,146 at 30 days to 1,276,734 at 365 days (FIG. 2A).[b] Between 1 month and 1 year there is a 43% gain in cell number which the analysis of variance showed to be primarily due to age differences ($F = 34.16$, $p < 0.0001$). There is a high positive correlation with age and cell number (Pearson $r = 0.86$, $p < 0.0001$), which regression analysis shows to be linear (solid line, FIG. 2A; $F = 38.93$, $p < 0.0001$), and cells are added at the approximate rate of 1149 per day between 30 and 365 days. Assuming that the left hippocampus has similar gains, nearly 770,000 granule cells originate between 1 month and 1 year. During this time, the granular layer increased 35% in total volume (FIG. 2B) in an age-related linear pattern (regression analysis: $F = 25.68$, $p < 0.0001$: Pearson $r = 0.80$). There was also an 18% decrease in V_a in the ventral granular layer (FIG. 2C) with a negative correlation between age and cell volume (Pearson $r = -0.64$), and ventral nuclear packing density increased by approximately 50,000 cells

[b] One of the animals (#161) at P365 had an abnormal total cell count 4.74 standard deviations below the mean of the other four animals in this age group. When #161 is included, the mean at P365 is 1,206,209, and the increase between P30 and P365 is 35% (dashed line in FIG. 2A: analysis of variance, $F = 14.66$, $p < 0.0021$; Pearson $r = 0.72$, $p < 0.001$; regression analysis, $F = 16.22$, $p < 0.001$). Since #161 is not representative of the age group at one year, it was eliminated from the data shown in FIG. 2B and C.

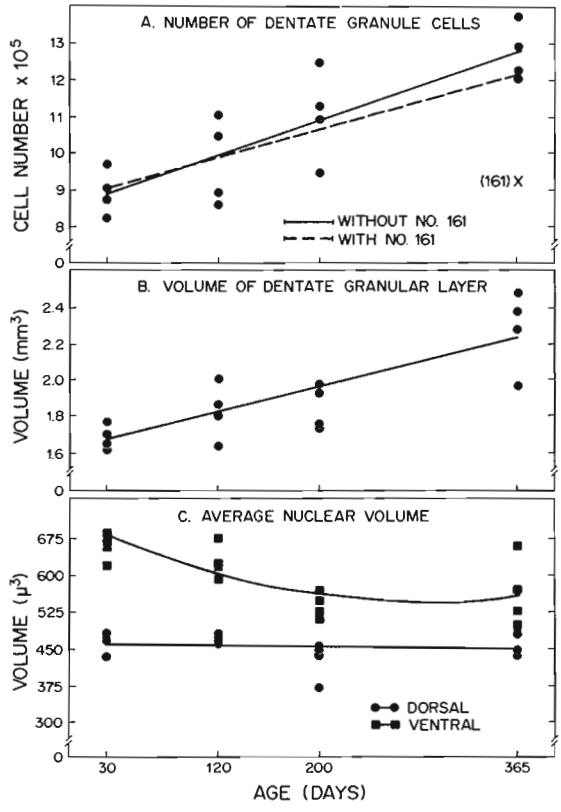

FIGURE 2. Scattergrams with regression lines of best fit showing total number of dentate granule cells (**A**), total volume of the dentate granular layer (**B**), average nuclear volume in dorsal and ventral parts of the granular layer (**C**) in the right hippocampus in rats between 1 month and 1 year of age. (From Bayer.[7] Reprinted by permission from *Experimental Brain Research.*)

per cubic millimeter between 1 month and 1 year. In the dorsal part, V_a was considerably smaller (approximately 465 μm^3) at all ages and remained constant.

Adult Hippocampal Granule Cell Neurogenesis

The proportion of labeled mature granule cells and precursor cells to total cells was determined in groups of Purdue-Wistar male rats after four consecutive daily subcutaneous injections of [^{3}H]thymidine (5 μCi/g body weight) according to the following schedules: postnatal (P) days P30–P33 ($n = 9$), P60–P63 ($n = 5$), P120–P123 ($n = 6$), and P180–P183 ($n = 4$). Three animals from the P30–P33 group were killed on P60; all remaining animals were killed on P200. All animals underwent transcardial perfusion with 10%

neutral formalin. The brains were placed in Bouin's fixative for 24 hr and were then stored in 10% neutral formalin until they were embedded in paraffin. Serial 6-μm sections were cut in the sagittal plane (every 15th section was saved); slides were dipped in Kodak NTB-3 emulsion, exposed for 3 months, developed in Kodak D19, and sections were poststained with hematoxylin and eosin. Anatomically matched sections were selected for cell counts 1.0 and 3.6 mm lateral to the midline. The number of labeled and unlabeled mature granule cells was determined (obvious glial and endothelial cells were eliminated) and the proportion of labeled cells was calculated. A labeled cell had reduced silver grains above background levels in the emulsion layer overlying the nucleus (see FIGURE 5 for examples). Small cells either immediately below the granular layer or embedded into the base were also classed as either labeled or unlabeled, and the proportion of labeled cells was calculated. The analysis of variance was applied to the data to test for age-related changes.

Mature dentate granule cells were routinely labeled after injections of ^{3}H-thymidine in juvenile and adult rats (arrows, FIG. 3B) as were small cells lying beneath the granular layer (asterisk, FIG. 3B). There is evidence that some of these small cells, which cannot be distinguished from glia in paraffin sections, are granule cell precursors,[6,9,30] and mitotic figures have been occasionally observed in this zone in adult animals. FIGURE 3A shows that the

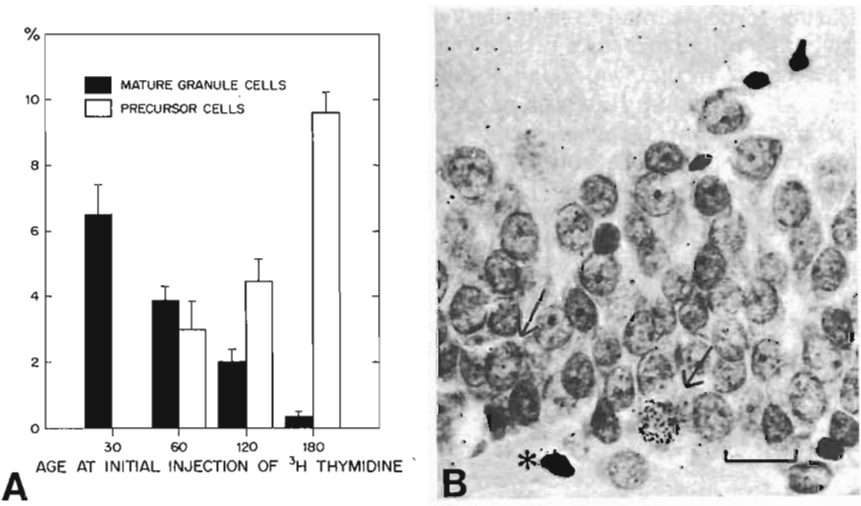

FIGURE 3. (**A**) Means (with standard deviation) of the proportion of labeled mature granule cells (*solid bars*) and labeled precursor cells (*open bars*) in animals injected with [^{3}H]thymidine on four consecutive days beginning with the day indicated. All animals survived to 200 days of age. With a shorter survival after injection (180-day group) there are many precursors and few mature granule cells labeled. Increasing the survival time allows more precursors to mature into granule cells. (**B**) Labeled mature granule cells (*arrows*) and a labeled presumptive granule cell precursor (*asterisk*) in the granular layer of a rat given four injections of [^{3}H]thymidine at 120–123 days of age and killed at 200 days of age. (6-μm paraffin sections; hematoxylin and eosin stain; bar = 20 μm.) (Modified from Bayer.[7]).

proportion of labeled mature granule cells decreases linearly with age (analysis of variance, $F = 61.54$, $p < 0.0001$) while, at the same time, the proportion of labeled precursor cells increases linearly (analysis of variance, $F = 167.55$, $p < 0.00001$). The proportion of labeled precursor cells was not quantified in animals injected with [^{3}H]thymidine on 30–33 days because some glial generation is still taking place at this time.[9] Increasing the survival time in the P30–P33 group from P60 to P200 results in a slight increase (2–3%) in the proportion of mature granule cells; these data are not illustrated in FIGURE 3A.

Adult Olfactory Bulb Granule Cell Neurogenesis

The same groups of Purdue-Wistar male rats used in the preceding experiment were also used to study adult olfactory bulb neurogenesis. The proportion of labeled neurons in the granule cell population in the olfactory bulb was determined in serveral randomly selected unit areas (0.04 mm^2), and the effects of both a change in survival time (P30–P33 group) and in injection time were assessed either with the analysis of variance (serveral groups) or the t test (two groups). While care was taken to eliminate glial cells from the counts, it should also be noted that small granule cell nuclei are difficult to distinguish from those of astrocytes in paraffin sections. Only nuclei appearing to be definitely "neuronal" were counted. Thus, the data reported are conservative measures of adult neurogenesis. FIGURE 4 shows that there is a decrease in the proportion of labeled cells in injection schedules beyond the P30–P33 group (analysis of variance, $F = 10.87$, $p < 0.0003$). There is also a significant decrease in the proportion of labeled cells if the survival time in the P30–P33 group is extended from P60 to P200 ($t = 8.72$, $p < 0.0001$, two-tailed probability). FIGURE 5 illustrates the decrease in the proportion of labeled cells with a lengthening of the survival time.

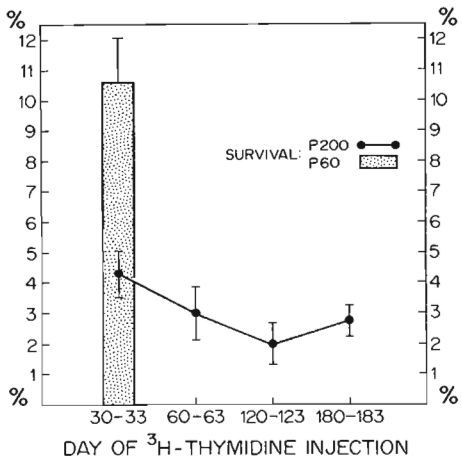

FIGURE 4. The proportion of granule cells in the main olfactory bulb labeled with four consecutive injections of [^{3}H]-thymidine on the days indicated. All *points* are means with standard deviations. *Line graph* is the proportion determined at a P200 survival date; *bar graph* shows that a higher proportion of labeled cells is attained in the P30–P33 group if survival time is decreased to P60. (From Bayer.[8] Reprinted by permission from *Experimental Brain Research.*)

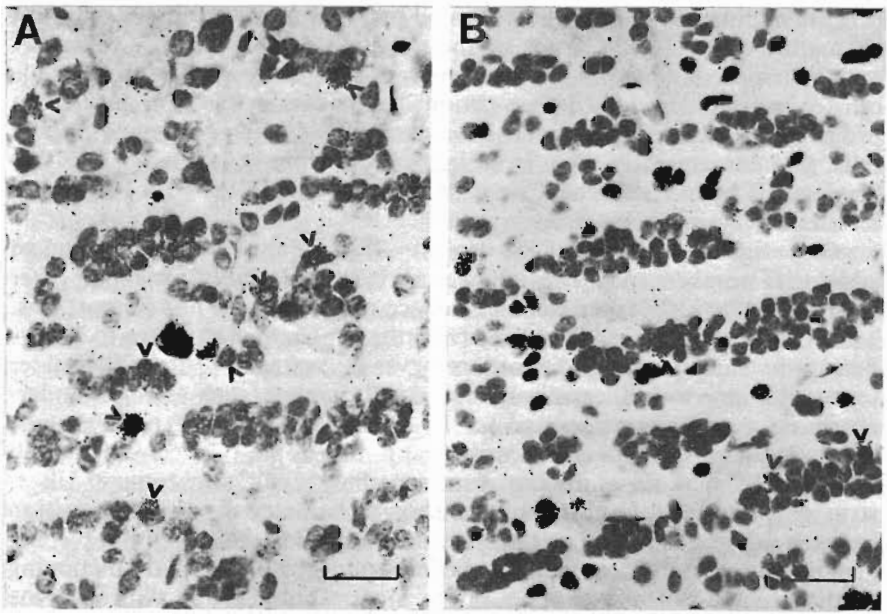

FIGURE 5. Photomicrographs of the main olfactory bulb granule cells in the P30–P33 group with survival times to P60 (**A**) and P200 (**B**). There is a higher proportion of labeled cells (*arrowheads*) in the P60 survival group. (6 μm paraffin sections; hematoxylin and eosin stain; bar = 20 μm.)

DISCUSSION

Hippocampal Granule Cells

It was originally found that approximately 85% of the granule cells in the dentate gyrus originate after birth when rats survive to P60.[9] This must now be considered a conservative estimate since the postnatal part of the granule cell population is continually enlarging. The 43% increase between P30 and P365 (FIG. 2A) is considerable. During this time, two aspects of granular layer morphology are changing, which together reflect the numerical increase. First, new neurons add to total granular layer volume (FIG. 2B); and second, ventral granule cell nuclear volume becomes smaller in older animals (FIG. 2C). So older rats not only have a larger granular layer, but they also tend to have more and smaller cells. Even though morphologic evidence of hippocampal granule cell death, such as pyknotic fragments in the granular layer, is almost never observed, dentate granule cells may also be dying in adult rats. If so, the data of this study indicate that the rate of cell production exceeds the rate of cell death to bring about a net gain.

The volumetric analysis shows that granule cells are added at a constant rate (FIG. 2A), while the autoradiographic analysis shows a sharp decline (FIG. 3B) in the ratio of labeled cells to total cells. However, the proportion of labeled precursor cells increases with age. This is to be expected since

[^{3}H]thymidine will only be incorporated by multiplying precursor cells, not by mature granule cells. Some of the progeny of the precursor cells may remain undifferentiated, while some others may differentiate into granule cells over an undetermined time period. In the earlier injection groups the progeny of the labeled precursors have more time to become mature granule cells, and consequently there is a higher proportion of labeled cells. Confirming this hypothesis, decreasing the survival time in the P30–P33 group resulted in fewer labeled mature granule cells.

One might speculate that the capacity of the granule cell population to add to its numbers may also indicate an increased ability for repair after injury. But the little experimental evidence available does not support this. The losses in granule cells resulting from the exposure of rat pups to X rays during the early postnatal period are never recovered.[10,11] Possibly milder forms of injury with a less severe loss in cell number (such as slight undernutrition) may be compensated for by an increase in adult neurogenesis. To date, there are no data available on this topic. Conceivably, adult neurogenesis may serve another function than repair after injury. Ultrastructural studies of the granular layer show a characteristic direct apposition between adjacent granule cell plasma membranes without any intervening glial processes.[22] This contact may be important for the normal functioning of the granule cell population. As the hippocampus grows with age, the growth pressure will tend to pull adjacent granule cells farther apart. Perhaps adult neurogenesis occurs simply to "fill the gaps" created by increased growth.

Assigning a specific function to the hippocampus is still controversial. Many experiments show it to be involved in short-term memory[19,21,31] especially spatial memory.[26,27] Other studies implicate hippocampal activity in response to inhibition,[13,15,24] which develops in synchrony with the maturation of the dentate gyrus.[14] The growing body of evidence for morphologic and physiological plasticity of the dentate granule cells in both immature and mature animals[23] suggests that the dentate gyrus may be active during the brain's response to changes in the environment. Whatever the function may be, dentate granule cells must play a pivotal role, since their elimination by early postnatal X-irradiation in rats gives behavioral deficits similar to those seen after bilateral hippocampal lesions.[11,17] The continued increase of dentate granule cells in the adult suggests that their influence on total hippocampal function grows with age.

Olfactory Bulb Granule Cells

The level of adult neurogenesis in the granule cells of the olfactory bulb is very prominent, sometimes reaching higher proportions than those found in the dentate granule cell population. If the granule cell populations in both the olfactory bulb and hippocampus are similar, one would predict an even more dramatic increase in the olfactory bulb granule cell population. There are two sets of data that indicate that this is not the case. First, a volumetric determination of total granule cell number in the olfactory bulb showed no

significant increase between the ages of 1 month and 1 year.[29] Second, the data of FIGURE 4 and earlier studies by Altman and coworkers[3,29] show that increasing the survival time in the P30–P33 injection group from P60 to P200 results in a *decrease* in the proportion of labeled neurons, possibly due to death of the new neurons. Since neurons are added for several weeks after the injections in the P30–P33 group, the precursors may eventually dilute their supply of [^{3}H]thymidine by repeated cell divisions to undetectable levels and may generate unlabeled new neurons, thus bringing down the proportion of labeled cells. In contrast, the proportion of labeled dentate granule cells slightly *increases* with a longer survival time. The overall implication of these data is that the rate of granule cell production in the main olfactory bulb during adult life is balanced by an equal rate of granule cell death. There is some histologic evidence for cell death since pyknotic fragments are often observed in the granular layer (Bayer, unpublished observations). Cell turnover and regeneration are shown by neurons in the olfactory epithelium,[25] and this phenomenon may also be found in the granule cell population. Thus, prolonged neurogenesis in the olfactory bulb may be more indicative of cell turnover in a numerically stable population rather than cell accumulation in a growing population.

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