

New Neurons: Extraordinary Evidence or Extraordinary Conclusion?

Gould *et al.* (1) reported that in the adult primate new neurons may be added to some neocortical association areas. This remarkable finding explicitly challenges previous work indicating that the production of new neurons in the primate neocortex is limited to the fetal period (2), and, if true, would force a reevaluation of virtually all current conceptual bases for understanding how neuronal circuitries in neocortex develop and are modified (3). But “extraordinary claims require extraordinary evidence” (4)—and the potential impact of these findings demands a close examination of the chain of evidence constructed by Gould *et al.* For the overall conclusions to stand, each link in the chain must be unambiguously correct; plausible alternative explanations must be considered and eliminated. Yet each proffered link contains ambiguity or uncertainty, and plausible alternative explanations remain.

As the first link in the chain, Gould *et al.* (1) used bromodeoxyuridine (BrdU) labeling and immunohistochemical detection as the sole indicator of cell proliferation and new cell generation in the subventricular zone (svz), in contrast to earlier studies (2, 5) that used tritiated thymidine ($^3\text{H-dT}$) labeling and autoradiographic detection. BrdU labeling was introduced as a tool for studying the developing nervous system (6); it is unclear how well that tool functions in adult monkeys or in any other adult animal. The doses reported in (1) were larger than in studies of the developing brain in mouse (6, 7, 8) or monkey (9) fetuses or in a previous study of neurogenesis in adult monkeys (10). The issue of dose is further complicated, because BrdU incorporation when assayed by immunohistochemistry (1) is not stoichiometric (6, 11), and the amount of label detected per cell is not a good indicator of the amount incorporated into the cell. In contrast, $^3\text{H-dT}$ has good (albeit nonlinear) stoichiometry if constant exposure times and development procedures are used (12). This is important because, traditionally, studies of cell proliferation in the developing brain using $^3\text{H-dT}$ have relied on the existence of “heavily” and “lightly” labeled cells (13, 14) to indicate the passage of cells through more than one cell cycle. BrdU and $^3\text{H-dT}$ have comparable availability times and labeling efficiencies when specific doses, fixation, and detection procedures are used in fetal mice (8), but other species, ages, doses, fixation, and detection might yield different results.

In the absence of comparative information in adult monkeys, BrdU labeling might produce false negatives and false positives. Table 1 of Gould *et al.* (1) indicates that 2 weeks after five daily injections of BrdU, approximately the same number of cells are labeled as after a single injection—17.6 cells per mm^3 (mean of 13.2, 13.7, and 26.5) versus 14.4 cells per mm^3 . Multiple daily injections of BrdU should, however, add to the number of labeled cells approximately arithmetically (five daily injections should give about a fivefold increase) because a single injection of BrdU will label cells in the S phase (6), the S phase is a small proportion of the whole cell cycle (6, 7, 9), and the cell cycle probably is not an even multiple of 24 hours (7, 9). The similar number of cells labeled by multiple and single injections suggests that the labeling is not associated primarily with proliferation, and that the BrdU method as used in (1) may overestimate the number of new cells. In particular, the combination of higher doses and enhanced sensitivity of immunohistochemistry may detect DNA repair (15). One simple criterion for differentiating between DNA replication during proliferation and DNA repair would be the existence of a commensurate number of mitotic figures or, better yet, labeled mitotic figures that would appear as the cells labeled in S phase pass through G_2 and enter M, but that would not appear in a population undergoing DNA repair. Unfortunately, Gould *et al.* (1) present no evidence tying the BrdU incorporation unequivocally to cell proliferation.

The second link in the chain of logic involves interpretation of BrdU-labeled cells in white matter as neurons migrating from the svz to neocortex. These labeled cells apparently traverse 6000 to 10,000 μm after BrdU injection [figure 4 of (1)], arriving, with even only a 1-week survival, both in the depths of the principal sulcus and along its walls. BrdU labels cells in S phase, so the labeled cells would be expected to begin migrating out of the svz only after passing through S, G_2 , and M and reaching the restriction checkpoint sometime in G_1 (16)—that is, after a ~ 24 -hour delay, given the cell cycle length in developing monkey neocortex (9). This means that the labeled cells purportedly complete their migration in ~ 6 days and that the labeled cells travel at a putative rate of ~ 1000 to $1600 \mu\text{m}$ per day, or ~ 40 to $70 \mu\text{m}$ per hour—much faster than the migration rate of $\sim 5 \mu\text{m}$ per hour reported for young neu-

rons moving to neocortex in the developing monkey (17) and faster than the rates of 2 to 30 μm per hour reported in developing neocortex, cerebellar cortex, or rostral migratory stream of other mammals (18–24). This unusually rapid migration rate in the adult macaque is present despite the increased complexity of the terrain in the adult brain. Intriguingly, recent data from the rat svz suggest that glial-cell progenitors may migrate at a rate of almost 90 μm per hour (25), much faster than neurons and closer to the rates reported in (1). The migrating cells were identified (1) as young neurons using the antibody TOAD-64, which recognizes rodent CRMP-4 (26). This antibody is uncharacterized in adult animals (and in primates), and it should be noted that CRMP-2, a protein with considerable homology, is found in both astrocytes and oligodendrocytes in adult mice (27).

For the third and final link in the chain of evidence, the labeled cortical cells were identified (1) as neurons using the “neuron-specific” markers NSE, MAP-2, and NeuN. NSE is present in both astrocytes and oligodendrocytes as well as in neurons (28, 29), and MAP-2 labels EGF-responsive precursor cells, a possible stage of astroglial development (30). NeuN is generally accepted to be “neuron specific” but is known to label other cell types, such as cells from the adrenal gland and the intermediate lobe of the pituitary gland (31). Moreover, the NeuN antigen is unknown, and neither it nor MAP-2 nor NSE have been characterized in primates. On the surface, the retrograde transport experiments would seem to define the neuronal phenotype clearly, but under some circumstances glial cells can be labeled by retrograde tracers (32) and after 1 week the effective uptake zone for neuronal transport is unclear.

Quite apart from the weak links in their chain of evidence, Gould *et al.* (1) do not suitably quantify the proliferating population or the number of cells produced, but only speculate that “a considerable number” of new neurons are added “daily.” The “considerable number” corresponds to cells double-labeled by BrdU and NeuN, NSE, or MAP-2 [table 1 of (1)], and suggests a total of ~ 10 to 20 new neurons per day per mm^3 of cortex, or 4000 to 8000 per day in the area of the principal sulcus alone [volume estimate from figure 1 of (1)]. Gould *et al.* (1) suggest that the number would be “much higher” if the short availability time of BrdU were considered. This effect can be estimated (conservatively) to be about fivefold (33), which means that 50 to 100 new neurons per mm^3 per day are allegedly produced, or 20,000 to 40,000 per day in the area of the principal sulcus. Since there are $\sim 133,000$ neurons per mm^3 in this area of monkey neocortex (34), the alleged rate of addition (~ 0.038 to

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0.075% per day) is sufficient to equal the entire population once every 3.6 to 7.3 years.

These calculations imply three specific, experimentally verifiable predictions, none of which have yet been supported. First, the considerable alleged new neuron production in neocortex must either be balanced out by an equivalent amount of cell death or result in an increase in the size of the association cortex. Gould *et al.* (1) suggest that new neurons that might have been labeled by previous studies with ³H-dT (2) “may have died in the interval between injection and perfusion,” which implies that the added neurons have a life-span greater than 2 weeks (1) but less than 35 days (2) and requires evidence for the death of a sufficient number of cells to match the new production. The alternative—that the new neurons are long-lived—should result in a substantial increase in the size of the association neocortex over the course of the decades-long span of a primate’s life. Second, regardless of the life-span of the new neurons, the influx of so many per day would mean that at any given time the neocortical association areas should be replete with young neurons (several hundred per mm³), with their characteristic bipolar appearance; in addition, growth cones on dendrites and axons should be plentiful and perhaps detectable with markers specific for immature neurons. Third, the production of 20,000 to 40,000 new neurons per day would require a substantial population of proliferating cells in the svz—20,000 to 40,000 cells assuming steady-state kinetics and a 24-hour cell cycle, more if the cell cycle is longer. All three predictions are testable with current technology.

In sum, although the case made by Gould *et al.* (1) is intriguing, the “burden of proof” (4) that a “considerable number” of “new neurons” are produced has not been met; doubts remain in each link of the chain of logic, and crucial supportive quantification is missing. Discussion of the functional impact of the addition of new neurons should be tempered until other investigators confirm, or refute, the paper’s findings.

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Response: We agree with Nowakowski and Hayes that for the conclusions in (1) to hold, each link in our chain of logic must be “unambiguously correct,” and that “plausible alternative explanations must be considered.” We find their account of our work inaccurate, however, and their criticism of our inferences unjustified by data, logic, or literature.

First, Nowakowski and Hayes state that BrdU may label cells that are not dividing but instead are undergoing DNA repair. This interpretation is incompatible with our finding of many more BrdU-labeled cells in the neocortex of adult macaques 1 and 2 weeks after BrdU injection than 2 hours after BrdU injection [table 1 in (1) and new data in Fig. 1]. If BrdU were labeling cells that were synthesizing DNA and not dividing, the number of

BrdU-labeled cells would not increase with time following injection. In addition, we have indeed observed evidence of BrdU-labeled mitotic figures in the svz and in the dentate gyrus (Fig. 2), as requested by Nowakowski and Hayes as evidence for cell division. Kaplan (2) had also observed mitotic figures in the svz of the adult monkey, but with ³H-thymidine-labeled cells. These findings strongly suggest that BrdU is labeling cells that are synthesizing DNA in preparation for division and that the cells ultimately do divide. That some animals with multiple BrdU injections did not have more labeled cells in the principal sulcus region likely reflects age, sex, and dosage differences among the monkeys used in our study (1).

Second, the authors claim that the BrdU-labeled cells migrating from the svz toward the neocortex cannot represent immature neurons, because they cover too great a distance in too little time. They estimate that the new cells move at a rate of 40 to 70 μ m per hour, based on the observation that more BrdU-labeled cells are in the neocortex at 1 week than at 2 hours. Most of the BrdU-labeled cells actually remain localized to the white matter at 1 week’s time, which in turn suggests that most cells take longer than that to migrate. Even the exaggerated speed of 40 to 70 μ m per hour suggested by Nowakowski and Hayes, however, lies within the range of previously reported neuronal migrations. Cerebellar granule cells have been shown to migrate as fast as 70 to 120 μ m per hour (3, 4) and olfactory granule cells have been shown to migrate as fast as 70 μ m per hour (5). Also, comparisons between our results and neocortical neurons during development may not be relevant because the migratory substrate is likely to differ.

One additional finding not reported in (1) was our observation of pairs of BrdU-labeled cells with neuronal characteristics in the neocortex. That observation suggests that in situ cell division may occur as well as migration from the svz. These two possibilities are not mutually exclusive: New cells may originate in the svz and continue to divide while migrating and after they reach the neocortex, a possibility compatible with recent observations of progenitor cells with neuronal potential in the rat neocortex (6).

Third, Nowakowski and Hayes maintain that all three markers of mature neurons used in (1), as well as the retrograde tracing method, are inadequate because they can label glia. We are indeed familiar with reports that neuronal markers sometimes label glia, mostly under cell culture conditions (7–9); however, it is unlikely that the expression of three different markers, in combination with retrograde tracing data, would all yield similar evidence. In fact, a number of recent studies, including research on primates (10–12), have

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accepted coexpression of these neuronal markers by newly generated cells as reasonable evidence that new cells attain a neuronal phenotype.

Misreading table 1 in (1), Nowakowski and Hayes write that it suggests the production of “~10 to 20 new neurons per day per mm³ of cortex.” Apparently, they did not realize that the table refers to the total number of BrdU-labeled cells, not the number of BrdU-labeled cells with neuronal characteristics. Looking only at the animals with the fewest and most BrdU-labeled cells produced (animals 6 and 3), the estimated number of cells expressing neuronal markers that were observed after a single BrdU injection (calculated by dividing the number of labeled cells by the number of injections, and multiplying the quotient by the highest and lowest percent expressing a neuronal marker) was 0.8 cells per mm³ (animal 6) and 2.8 cells per mm³ (animal 3). This is actually considerably fewer new BrdU-labeled neurons

than observed in the dentate gyrus of adult humans, macaques (Fig. 1), and rats (10, 11, 13). The volume of the principal sulcus examined in each of these animals after tissue processing (174.7 mm³ for animal 6, 164.8 mm³ for animal 3) implies an estimate of 140.0 to 461.4 BrdU-labeled cells that are positive for a neuronal marker after a single BrdU injection. Even using the larger volume estimate of Nowakowski and Hayes, 400 mm³ [which is based on a line drawing in (1), and which does not account for tissue shrinkage during BrdU processing], these numbers imply 320 to 1120 new neurons in the principal sulcus per injection—considerably fewer than the 4000 to 8000 cited in their comment.

Nowakowski and Hayes next suggest that the daily production of cells would actually be five times higher than our result from BrdU labeling, based on a previous study of the length of the cell cycle in fetal monkeys (14). No relevant inferences can be drawn from this study, however, because it did not use a cumulative labeling procedure that requires BrdU injections every 2 hours until a plateau in the number of labeled cells is reached. Further, the study was in fetal macaques, not mature ones, in which the lengths of S phase and the cell cycle are presumably different. Even if we accept a fivefold multiplication of the number of neurons and the overestimated volume of the principal sulcus cited by Nowakowski and Hayes, our results yield 1600 to 5600 cells per day, far fewer than the 20,000 to 40,000 figure that Nowakowski and Hayes attribute to us.

Finally, Nowakowski and Hayes assert

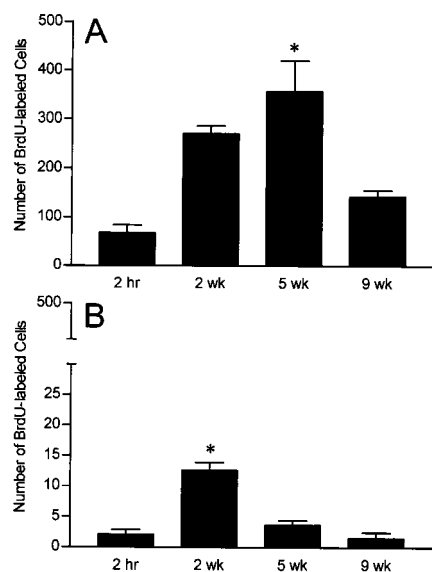


Fig. 1. Density of BrdU-labeled cells (number of BrdU-labeled cells per mm³) in the (A) dentate gyrus and (B) prefrontal cortex of adult male macaques (5 years old) at different survival times after a single injection of BrdU (100 mg/kg). A higher density of BrdU-labeled cells was observed in the dentate gyrus than in the prefrontal cortex at all times. The number of BrdU-labeled cells increased between 2 hours and 2 weeks after BrdU labeling in both brain areas, which suggests expansion of the originally labeled population by mitosis and, potentially, migration of cells into the area. By 9 weeks after BrdU labeling, the number of labeled cells in both structures was substantially diminished, which suggests that many of these new cells ultimately died (**p* < 0.05 compared with 2 hours, 9 weeks; ANOVA followed by Tukey HSD post hoc comparisons; *n* = 2 monkeys per time point).

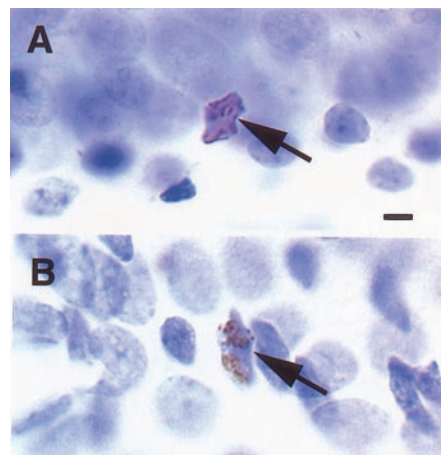


Fig. 2. Photomicrographs of BrdU-labeled mitotic figures (arrows) in the (A) dentate gyrus and (B) subventricular zone of a 5-year-old male macaque. Cells appear to be in anaphase. Scale bar in (A) equals 5 μm and applies to both frames. The images indicate that BrdU is incorporated into cells that subsequently divide.

that “the considerable alleged new neuron production in neocortex must either be balanced out by an equivalent amount of cell death or result in an increase in the size of the association cortex.” In a recent, unpublished study, we have indeed demonstrated that the majority of new cells in the principal sulcus of the adult macaque have a transient existence. The vast majority of the new cells die between 2 and 5 weeks after their birth (Fig. 1), a finding that accounts for the lack of a measurable increase in size or number of cells throughout adulthood. As for the lack of previous suggestions of even transient neuronal growth in the adult neocortex, we point out that although neurogenesis in the dentate gyrus and olfactory bulb of adult rats is now universally accepted, extensive study of these structures for several decades yielded no reports of growth cones or immature neurons in them. Given the relatively low density of new cells produced in the neocortex compared with the dentate gyrus (Fig. 1), it is not surprising that evidence of neuronal growth was not detected in past studies of the adult macaque neocortex.

In conclusion, the results of our study support the view that new neurons are added to the neocortex of adult monkeys—however “extraordinary” this might seem. The function of those neurons, if they indeed have any function, remains to be discovered through a combination of behavioral, selective lesion, ultrastructural, and electrophysiological studies.

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