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Response to Comment on "Impact of neurodegenerative diseases on human adult hippocampal neurogenesis"

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Rakic and colleagues challenge the use of extensively validated adult hippocampal neurogenesis (AHN) markers and postulate an alternative interpretation of some of the data included in our study. In Terreros-Roncal *et al*., reconstruction of the main stages encompassed by human AHN revealed enhanced vulnerability of this phenomenon to neurodegenerative diseases. Here, we clarify points and ambiguities raised by these authors.

Rakic and colleagues (*1*) question whether various neural stem cell (NSC) markers are related to adult hippocampal neurogenesis (AHN) in our study (*2*). By applying widely used phenotypic criteria to identify NSCs (*3*), we detected a population of nestin$^+$ cells that express a panel of radial glia–like (RGL) markers [such as SRY-Box transcription factor 2 (Sox2) and vimentin] while lacking S100 calcium-binding protein-B (S100β) expression, which demonstrates that these cells are not astrocytes. Nestin$^+$ S100β$^-$ NSCs differ from nestin$^+$ S100β$^+$ astrocytes by showing distally branched [figure S3E of (*2*)] long apical processes [figure S3, A, C, D, and F, of (*2*)] and predominant location in the subgranular zone (SGZ) [figure S3G of (*2*)]. These features are also evidenced by vimentin staining (Fig. 1A) and are consistent with the morphology of hippocampal NSCs observed in aged rodents (*3*). Rakic and colleagues claim the failure of previous studies to detect human proliferative progenitor cells, overlooking that adult human hippocampal NSCs have been shown to express proliferation markers in vivo and to hold proliferative and neurogenic capacity in vitro (*4*). They also overlooked that antibodies may require distinct immunohistochemistry conditions that preclude their combination in the same assay, such as nestin and phospho-histone 3 (PH3) in our study [see table S1 of (*2*)]. Our quantitative data contest Rakic and colleagues' interpretation that the Sox2$^+$ cells observed in (*2*) are astrocytes. Neither control nor diseased subjects show statistically significant correlations between the densities of Sox2$^+$ cells and S100β$^+$ astrocytes [figure S16F of (*2*)]. Contrary to the homogeneous distribution of S100β$^+$ astrocytes across the granule cell layer (GCL), Sox2$^+$ cells are four times as abundant in the SGZ (Fig. 1B). These data indicate that Sox2$^+$ cells and S100β$^+$ astrocytes are distinct cell populations. Together with the identification of nestin$^+$ S100β$^-$ cells (*2*), the presence of Sox2$^+$ and vimentin$^+$ cells with morphological features of RGL cells provide additional evidence for the persistence of hippocampal NSCs throughout human life.

Rakic and colleagues believe that ~10,000 PH3$^+$ proliferative cells per mm^3 are not sufficient to support the presence of immature neurons, and they suggest that the proliferation detected in (*2*) corresponds to glial and vascular cells. They overlook the presence of proliferative neuroblasts in the human dentate gyrus (DG) revealed by the expression of PH3 in ~5 to 10% doublecortin (DCX)$^+$ dentate granule cells (DGCs) [figure 2, b and i, of (*5*)]. Moreover, in (*2*), the use of ELAV-like proteins HuC/HuD [which are transiently expressed by intermediate progenitors and proliferative neuroblasts immediately after cell division (*6*)] allowed phenotypic characterization of human DG proliferative cells. About 90% of these cells express DCX, and ~85% are located in the SGZ [figure S4 of (*2*)]. Examples of triple-labeled (DCX$^+$ PH3$^+$ HuC/HuD$^+$) cells are shown in figure 1B of (*2*). These data contest Rakic and colleagues' view that proliferation detected in (*2*) corresponds to vascular and glial cells and support the

notion that most proliferative cells in the human DG correspond to transit-amplifying progenitors and neuroblasts located in the SGZ. Not only are the numbers of proliferative cells detected in (*2*) not negligible, but they are compatible with previous estimations of ~700 new neurons being incorporated daily into the human DG (*7*) and with our own determinations of immature neuron numbers.

The intriguing suggestion that DCX protein is expressed in mature neurons goes against experimental evidence (*8*). In (*2*), DCX^+ cells colabeled with calretinin, neuronal nuclei, or calbindin (which identify sequential stages of AHN) show morphologies and positioning [figure 1, F to J, and figure S2H of (*2*)] matching those observed in rodents and paralleling the maturation continuum characteristic of AHN (*9*). Contrary to Rakic and colleagues' claim, the percentage of DGCs positive for DCX reported in (*2*) is consistent with that observed in other studies [>1% in (*10*), 1.1% in (*11*)]. As previously discussed (*12*, *13*), the different methods used to estimate the reference volume and cell numbers in (*14*) and our studies impede direct comparison of cell numbers.

Rakic and colleagues raised an issue regarding the representation of outlier values in figure 1 of (*2*), which gives values obtained from all the control subjects. The two outliers were indicated by crossed-out dots and identified by means of SPSS software using a 1.5×IQR step. As indicated in the supplementary materials of (*2*), outlier values were not included in other figures or statistical comparisons. All the remaining data points are shown in the main and supplementary figures of (*2*), and average values were automatically calculated and plotted by Graphpad 8 software. All the subjects showed positive staining for all the cell markers analyzed. In those exceptional cases in which the signal-to-noise ratio or signal intensity and/or quality were deemed insufficient to fulfill the criteria needed to perform stereological cell counts, individual subjects were excluded from particular cell counts. All these aspects were disclosed in the supplementary materials of (*2*) but were perhaps overlooked by Rakic and colleagues.

Despite questioning the existence of human AHN, Rakic and colleagues suggest that increased densities of DCX^+ immature neurons might reflect augmented AHN in amyotrophic lateral sclerosis (ALS) and Huntington's disease, which would support the notion that human AHN does occur. Moreover, their suggestion overlooks the massive apoptotic cell death, imbalanced proliferation/NSC ratio, and neuronal maturation impairments we observe in the brains of diseased subjects [figures 2 and 3 of (*2*)].

The use of high-quality samples and controlled methods are cornerstones for the study of human AHN. These premises allowed us to overcome previous drawbacks that led to the absence of evidence for human AHN. The alternative interpretations of our results by Rakic and colleagues lack technical grounds and are not supported by the literature. We hope that these clarifications serve to address any putative misinterpretation of our data, and we are confident that our study will contribute to a greater understanding of how the human brain maintains the capacity to generate new neurons throughout life.

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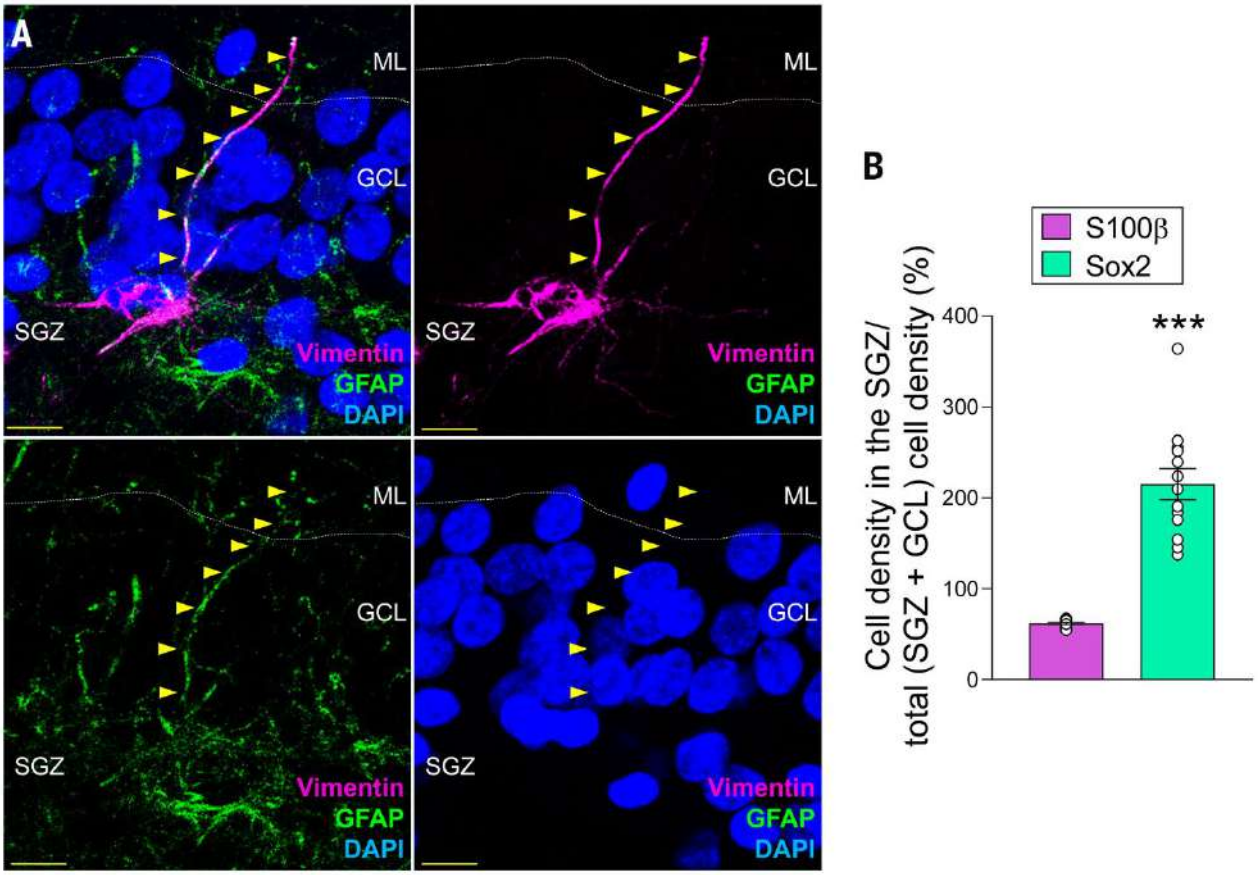

Fig. 1. Vimentin and glial fibrillary acidic protein (GFAP) staining in the adult human dentate gyrus. (**A**) Vimentin staining in the human DG. (**B**) Relative cell density in the SGZ. ML, molecular layer; GCL, granule cell layer; SGZ, subgranular zone; DAPI, 4′,6-diamidino-2-phenylindole. Scale bar, 10 μm. ***$P < 0.001$.

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