Dual Perspectives

Dual Perspectives Companion Paper: Positive Controls in Adults and Children Support That Very Few, If Any, New Neurons Are Born in the Adult Human Hippocampus, by Shawn F. Sorrells et al.

Evidences for Adult Hippocampal Neurogenesis in Humans

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The rodent hippocampus generates new neurons throughout life. This process, named adult hippocampal neurogenesis (AHN), is a striking form of neural plasticity that occurs in the brains of numerous mammalian species. Direct evidence of adult neurogenesis in humans has remained elusive, although the occurrence of this phenomenon in the human dentate gyrus has been demonstrated in seminal studies and recent research that have applied distinct approaches to birthdate newly generated neurons and to validate markers of adult-born neurons. Our data point to the persistence of AHN until the 10th decade of human life, as well as to marked impairments in this process in patients with Alzheimer's disease. Moreover, our work demonstrates that the methods used to process and analyze postmortem human brain samples can limit the detection of various markers of AHN to the point of making them undetectable. In this Dual Perspectives article, we highlight the critical methodological aspects that should be strictly controlled in human studies and the robust evidence that supports the occurrence of AHN in humans. We also put forward reasons that may account for current discrepancies on this topic. Finally, the unresolved questions and future challenges awaiting the field are highlighted.

Key words: **adult neurogenesis; controversy; human; methodology; hippocampus; immature neuron**

Introduction

Adult neurogenesis is a striking form of plasticity that persists throughout life. The addition of new neurons can potentially rewire an entire neural circuit. How the delicate balance between synaptic plasticity and memory persistence is shaped by an apparently disruptive element, such as an uninstructed newly born neuron, is still an enigma for modern neuroscience. Notably, adult neurogenesis is restricted to selected brain regions, including the neurogenic niches of the hippocampal dentate gyrus (DG) (Altman and Das, 1965) and the ventricular-subventricular zone/olfactory bulb (Lois and Alvarez-Buylla, 1994; Lois et al., 1996; Doetsch et al., 1999). Of these, adult hippocampal neurogenesis (AHN) has attracted particularly great interest because of the involvement of the hippocampus in learning, memory, and mood regulation.

The history of AHN has always been touched by controversy. Indeed, the first reports of AHN by Altman and Das (Altman, 1963; Altman and Das, 1965) were initially disbelieved by the scientific community. In these seminal studies, the authors demonstrated selective incorporation of ^{3}H-thymidine in the adult rat subgranular zone, thereby clearly demonstrating the presence of adult-born proliferating cells in this structure. Subsequent work conducted by the group of Nottebohm (Nottebohm, 1981; Alvarez-Buylla et al., 1988), demonstrating seasonal changes of volume in the song control nuclei of canary birds, provided a physiological perspective to the phenomenon of adult neurogenesis and returned momentum to the field. This was probably the first time that a putative physiological role was attributed to this process (Paton and Nottebohm, 1984). The hypothesis that the addition of new neurons was related to increased plasticity, learning, and memory was embraced with enthusiasm by the field.

The seminal studies by Altman and Das were confirmed by Kaplan and Hinds (1977) using electron microscopy as well as by Stanfield and Trice (1988) using retrograde tracers. Moreover,

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Nowakowski et al. (1989) and Gould, Cameron, and McEwen (Gould et al., 1992; Cameron and Gould, 1994) pioneered the use of thymidine analogs, such as BrdU, combined with the immunolabeling of various specific markers, to demonstrate the neuronal phenotype of newly generated dentate granule cells *in vivo*. Subsequently, the group of Alvarez-Buylla demonstrated that newborn dentate granule cells are originated from a population of resident radial glia-like astrocytes (Seri et al., 2001). Gould and collaborators demonstrated that stress is one of the most potent negative regulators of AHN (Cameron and Gould, 1994). By showing that AHN could be modulated extrinsically, these studies paved the way for further development of the field. For instance, Kuhn et al. (1996) showed an age-dependent reduction of AHN rate in rodents, a finding that was later demonstrated in humans (Spalding et al., 2013) and nonhuman primates (Gould et al., 1999). Subsequent studies showed that physical exercise, environmental enrichment, and learning also regulate AHN by promoting the maturation and survival of newly generated cells in rodents (van Praag et al., 1999; Kempermann et al., 2000; van Praag et al., 2000). Throughout the following years, newly generated dentate granule cells were demonstrated to show long-term survival (Kempermann et al., 2003), and the sequential maturation stages encompassed by AHN were precisely defined using the expression of specific cell markers (Kempermann et al., 1997, 2003, 2004; Brandt et al., 2003; Brown et al., 2003; Filippov et al., 2003; Rhodes et al., 2003; Couillard-Despres et al., 2005; Laplagne et al., 2006).

Pioneering work by Luskin et al. (1988) using retroviruses to label newly generated progenitor cells in the rodent ventricular-subventricular zone was complemented by extensive studies of the DG (Seri et al., 2001; van Praag et al., 2002; Zhao et al., 2006; Toni et al., 2007, 2008; Kelsch et al., 2008), and retroviral labeling was combined with electrophysiological recordings (van Praag et al., 2002) and electron microscopy (Toni et al., 2008). Newly generated neurons were found to be much more plastic than their fully mature counterparts (Schmidt-Hieber et al., 2004; Bischofberger, 2007; Ge et al., 2007; Piatti et al., 2011; Marin-Burgin et al., 2012) and eager to learn (or possibly to forget) (Akers et al., 2014), so to speak. Immature dentate granule cells have unique electrophysiological properties (Schmidt-Hieber et al., 2004; Bischofberger, 2007; Marin-Burgin et al., 2012; Heigele et al., 2016; Li et al., 2017) and a distinct innervation pattern (Overstreet Wadiche et al., 2005; Marin-Burgin et al., 2012; Temprana et al., 2015; Luna et al., 2019). Thanks to these unconventional features, these cells profoundly impact hippocampal functioning (Gu et al., 2012; Nakashiba et al., 2012; McAvoy et al., 2016; Anacker and Hen, 2017). Several fascinating papers showed that immature neurons are instructed by mature granule cells (and, as recently suggested, that they also instruct the latter) (Luna et al., 2019) in memory formation and information processing (Schinder and Gage, 2004). In rodents, newly generated neurons not only play important roles in mood regulation (Revest et al., 2009; Snyder et al., 2011; Hill et al., 2015; Anacker et al., 2018), but also are crucial in learning and memory, particularly in pattern separation (Sahay et al., 2011; Tronel et al., 2015; Ishikawa et al., 2016).

AHN in distinct mammalian species

The outstanding refinement and controlled methodology that the field of AHN has reached in rodent studies have not, for evident (technical and ethical) reasons, been achieved in other species. Despite exhaustive examination of AHN in particular mammalian species, systematic studies across the whole mammalian phylogenetic scale have not been conducted to date. Given that only captured wild specimens can be examined for some species, heterogeneity between such studies may increase, not only because the exact age and life history of the animals may be unknown, but also because capture and manipulation of the animals may alter AHN (Chawana et al., 2014; Wiget et al., 2017). For instance, several studies described interspecies differences in the expression of AHN markers in bats (Amrein et al., 2007; Gatome et al., 2010), but the expression of these markers has been proposed to be influenced by post-capture stress (Chawana et al., 2014). Moreover, a detailed description of tissue processing techniques and the inclusion of large numbers of specimens are required for the quantitative and qualitative comparison of adult neurogenesis data between distinct mammalian species and studies.

Despite these difficulties, AHN has been observed in the brains of most of the mammalian species examined to date, including several species of primates. Seminal work using both thymidine analogs and markers of AHN revealed the persistence of adult neurogenesis in various nonhuman primate species, thereby firmly paving the way toward the study of adult neurogenesis in humans (Gould et al., 1999; Kornack and Rakic, 1999; Jabes et al., 2010; Kordower et al., 2010; Kohler et al., 2011; Ngwenya et al., 2015). These studies have revealed that primate and rodent AHN share similarities, but also show marked differences (Miller et al., 2013; Fasemore et al., 2018). Perhaps one of the most remarkable differences, elegantly brought to light by Kohler et al. (2011), is the slower maturation of newly generated cells in nonhuman primates than in rodents. This finding has profound physiological implications since the period characterized by the highest excitability of these cells may be considerably extended in long-living organisms. This prolonged period of higher excitability has been proposed to confer an important evolutionary advantage to these species by permitting increased cognitive flexibility (Kohler et al., 2011; Yuan et al., 2014).

The particular case of human adult neurogenesis

The first study addressing whether AHN occurs in humans was published by Eriksson et al. (1998). Those authors showed the incorporation of BrdU into the adult human DG. Several months after administration, $BrdU^+$ cells coexpressed several neuronal markers, thus demonstrating the presence of a restricted population of adult-born dentate granule cells in humans. The conclusions of that study were supported by Spalding et al. (2013), who used another innovative approach to quantify the number of cells newly generated in the human brain. The method was based on the measurement of the transient increase in the availability of ^{14}C during nuclear assays, as well as on the incorporation of this isotope by dividing cells. Using this technology, sophisticated mathematical models, and the injection of thymidine analogs as a positive control, they corroborated the existence of neurons with a record of division in the adult human brain. These two studies took advantage of the higher postmortem stability of nucleic acids compared with proteins, paving the way for the future development of the field. Over the following years, studies by other groups, mainly based on the use of immunohistochemistry, contributed to building a large body of evidence supporting the occurrence of AHN in humans (Boldrini et al., 2009; Crews et al., 2010; Knoth et al., 2010; Ernst et al., 2014) (for a full list of these studies, see Table 1).

Despite the evidence described above, the existence of human AHN has been recently challenged. Dennis et al. (2016), Sorrells et al. (2018), and Cipriani et al. (2018)

Table 1. Studies addressing the occurrence of human AHN[a]

Study	Methods	Age	Tissue	Markers	Main results	Tissue processing
Eriksson et al., 1998	IHC	67-72 yr	Postmortem	BrdU, calbindin, NSE, GFAP, NeuN	Incorporation of BrdU into the adult human DG	4% PFA for 24 h, sliding microtome sectioning
Blümcke et al., 2001	IHC	4 GW-46 yr	Biopsy (epilepsy and controls) and postmortem (controls)	Nestin, vimentin, Tuj1, Ki67, MAP1b/5, MAP2a-d, NF-L, NeuN, S100β, GFAP, calbindin, CD68, CD45	Increased number of Nestin$^+$ and Ki67$^+$ cells in epileptic patients	4% PFA for 24 h (biopsy) or formalin for $>$ 2 w (autopsies); vibratome sectioning
Jin et al., 2004	IHC, WB	13-90 yr	Postmortem	DCX, PSA-NCAM, TUC4, NeuroD1	Increased expression levels of markers of immature neurons in AD patients measured by means of WB	Frozen samples (WB), or PFA 4% (unknown fixation time) plus embedding in paraffin
Boekhoorn et al., 2006	IHC	63-70 yr (controls), 63-69 yr (AD)	Postmortem	DCX, Ki67, GFAP	Unaltered AHN in presenile AD cases	10% formalin for 30-646 d; microtome sectioning
Manganas et al., 2007	Functional magnetic spectroscopy	—	Living humans	—	Identification of NSCs in the adult human hippocampus	NA
Monje et al., 2007	IHC	10 mo-63 yr (controls), 10 mo-61 yr (Cancer)	Postmortem	DCX, Ki67, Olig2, CD68, CD20, CD3	AHN is abolished by cancer treatment	Undisclosed fixation protocol and duration; embedding in paraffin
Liu et al., 2008	IHC, WB, RT-PCR	18-78 yr (controls), 13-57 yr (epilepsy)	Biopsy (epilepsy) and postmortem (controls)	DCX, PCNA, MCM2, PSA-NCAM,Tuj1, NeuN, Reelin, calretinin, calbindin	Increased AHN in epilepsy	Perfusion/immersion in 15% formalin (following Waldvogel et al., 2006)
Boldrini et al., 2009	IHC	17-53 yr (controls), 34-62 yr (MDD), 24-61 yr (MDDT-SSRI), 28-61 yr (MDDT-TCA)	Postmortem	Nestin, Ki67, NeuN, GFAP	Decreased number of progenitor cells with age; increased number of progenitor cells in females; antidepressant treatment increased the number of progenitor cells	Frozen tissue, fixed in 4% PFA for 1 wk and freezing
Mattiesen et al., 2009	IHC	35-81 yr (controls), 35-85 yr (HIE)	Postmortem	TUC-4, calretinin, PCNA	Increased AHN and apoptosis after hypoxic-ischemic encephalopathy	Formalin fixation (undisclosed time and concentration) and embedding in paraffin
Geha et al., 2010	IHC	22-35 yr (epileptic), 25-66 yr (control)	Biopsy (epilepsy and controls) and postmortem (controls)	Ki67, MCM2, Tuj1, MAP2, NeuN, calretinin, GFAP, Nestin, vimentin	Fixation affects cycle-related immunostaining; persistence of cycling cells in the adult SGZ; these cells were more abundant in epileptic patients	Formalin-zinc (formol 5%; zinc 3 g/L; sodium chloride 8 g/L) for 3 mo (postmortem) or 16 h (surgical), and embedding in paraffin
Lucassen et al., 2010	IHC	45-85 yr	Postmortem	MCM2, PH3	Reduction in the number of MCM2$^+$ cells in depressed patients	4% formaldehyde for 4-5 wk, and embedding in paraffin
Knoth et al., 2010	IHC, ISH, WB	11 GW-100 yr	Postmortem	DCX, PCNA, Ki67, MCM2, Sox2, Nestin, TUC4, Tuj1, Prox1, PSA-NCAM, NeuroD1, GFAP, calretinin, NeuN	Murine features of human AHN; mild decrease of AHN with age	Formalin fixation (undisclosed fixation protocol) embedding in paraffin
Crews et al., 2010	IHC, qRT-PCR	87 $\pm$ 4.6 yr (controls), 86.1 $\pm$ 1.7 yr (early/moderate AD), 80 $\pm$ 1.9 yr (severe AD)	Postmortem	DCX, Sox2	Reduction in the number of DCX$^+$ and Sox2$^+$ cells in patients with AD	4% PFA (undisclosed duration); vibratome sectioning
D'Alessio et al., 2010	IHC	40.1 $\pm$ 6 yr (epileptic), 45.8 $\pm$ 14 yr (controls)	Biopsy (epilepsy) and postmortem (controls)	DCX	Reduced number of DCX$^+$ cells in patients with epilepsy	Formalin for 5 d (undisclosed concentration), and embedding in paraffin; microtome sectioning
Johnson et al., 2011	IHC	71-101 yr (controls), 75-87 yr (LBD)	Postmortem	PCNA, DCX, GFAP, Musashi	Decrease in the number of Musashi$^+$ and increase in the number of DCX$^+$ cells in the GCL in patients with Lewy body dementia	Undisclosed fixation protocol; embedding in paraffin; microtome sectioning
Perry et al., 2012	IHC	81.2 $\pm$ 7 yr (AD), 80.9 $\pm$ 8.5 yr (controls)	Postmortem	Musashi, Nestin, PSA-NCAM, DCX, Tuj1	Reduced numbers of Musashi$^+$ cells, increased numbers of Nestin$^+$ and PSA-NCAM$^+$ cells, and unchanged numbers of DCX$^+$ and Tuj1$^+$ cells in patients with AD; the expression of DCX was specifically increased in the GCL	4% formaldehyde for 4 wk and embedding in paraffin; microtome sectioning
Boldrini et al., 2012	IHC	41.8 $\pm$ 14.6 yr (Control), 43.6 $\pm$ 13.3 yr (MD), 38.8 $\pm$ 13.8 yr (MD-SSRI), 43.2 $\pm$ 17.1 yr (MD-TCA)	Postmortem	Ki67, Nestin	Decreased number of Nestin$^+$ cells with age; SSRI increased the number of Nestin$^+$ cells in the DG of depressed patients	4% formaldehyde (undisclosed duration) and freezing; freezing microtome sectioning
Epp et al., 2013	IHC	46.8 $\pm$ 3.49 yr (Control), 42.83 $\pm$ 2.92 yr (MD), 41.5 $\pm$ 3.47 yr (MD-Psy)	Postmortem	DCX, p21, NeuN	Increased number of DCX$^+$ cells in depressed patients	Frozen tissue, fixed for 15 min in 4% formaldehyde
Spalding et al., 2013	^{14}C dating, IHC	19-92 yr	Postmortem	—	Mild decrease in the AHN rate with aging	Frozen tissue; undisclosed fixation protocol
Gómez-Nicola et al., 2014	IHC	20-34 yr (Creutzfeldt-Jakob), 58-76 yr (AD), 20-35 yr or 58-79 yr (controls)	Postmortem	Ki67, Sox2, calretinin	Increased number of Ki67$^+$ and calretinin$^+$ cells in patients with Creutzfeldt-Jakob and ADs; decrease in these cell populations in aged controls	Formalin fixation (undisclosed fixation protocol) and embedding in paraffin
D'Alessio et al., 2015	IHC	22-60 yr (controls), 22-51 yr (TLE)	Biopsy (epilepsy) and postmortem (controls)	Nestin	Reduced number of Nestin$^+$ cells in patients with epilepsy	Formalin (undisclosed concentration) for 5 d, and embedding in paraffin; microtome sectioning

(*Table continues.*)

Table 1. Continued

Study	Methods	Age	Tissue	Markers	Main results	Tissue processing
Bayer et al., 2015	IHC	17-45 yr	Postmortem	Musashi, Nestin, Ki67, calretinin, GFAP, NeuN, Tuj1, DCX	Reduced number of Musashi$^+$ and Nestin$^+$ progenitor and proliferating cells in heroin-addict patients	4% formaldehyde for 48 h-5 yr and embedding in paraffin; microtome sectioning
Dennis et al., 2016	IHC	0.2-59 yr	Postmortem	Ki67, DCX, Tuj1, Olig2, EGFR, GFAP, PCNA	Marked decrease in proliferation with age; only microglial cells were identified among the proliferating cell population	15%-20% formalin for 2-3 wk, and embedding in paraffin
Allen et al., 2016	IHC	21-81 yr (controls), 55–75 yr (schizophrenia)	Postmortem	Ki67, NeuN	Reduced number of Ki67$^+$ cells in patients with schizophrenia	Undisclosed fixation protocol
Le Maître et al, 2018	IHC	24-78 yr	Postmortem	NeuN, DCX, Sox2, Ki67	Reduction in AHN markers in alcohol addicts	Flash-frozen tissue, fixed in 4% formaldehyde for 15-30 min
Mathews et al., 2017	qPCR, IHC	18-88 yr	Postmortem	Ki67, DCX, GFAP, Tbr2	Reduced mRNA expression of DCX and Ki67 with aging	Frozen tissue (qPCR); fixation in formalin (undisclosed fixation protocol) and embedding in paraffin (IHC)
Liu et al., 2018	IHC	12 GW-76 yr	Biopsy (epilepsy) and postmortem (epilepsy and controls)	Nestin, DCX, Mushashi, Tuj1, NeuN, GFAP, MAP2, Olig2, MCM2	Increased densities of Nestin$^+$ cells in patients with epilepsy	Formalin fixation (undisclosed fixation protocol) and embedding in paraffin
Cipriani et al., 2018	IHC	13 GW-72 yr (controls), 74-89 yr (AD)	Postmortem	Nestin, GFAP, DCX, Ki67, MCM2, Sox2, Pax6, Tbr2, vimentin, Tuj1	Sharp reduction of radial glia-like, proliferative, and DCX$^+$ cells in the adult human DG	Frozen tissue was fixed in 4% PFA (undisclosed duration); other tissues were fixed in formalin (undisclosed duration) embedded in paraffin
Sorrells et al., 2018	IHC, EM	14 GW-77 yr	Biopsy (epilepsy) and postmortem (controls)	Ascl1, BLBP, DCX, BrdU, GFAP, Hopx, Ki67, MCM2, Nestin, NeuN, NeuroD, Olig2, Pax6, Prox1, PSA-NCAM, Sox1, Sox2, Tbr2, Tuj1, vimentin	Absence of AHN markers in the adult human DG	Perfusion with 4% PFA, immersion in either 4% PFA or 10% formalin (undisclosed duration); additional fixation in 4% PFA for 2 d
Boldrini et al., 2018	IHC	14-79 yr	Postmortem	PSA-NCAM, Sox2, Nestin, Ki67, DCX, NeuN, GFAP, BLBP	Preserved numbers of progenitor and immature cells throughout aging	Flash-frozen tissue, fixed in 4% PFA (undisclosed duration)
Tartt et al., 2018	IHC, RNAscope	19-67 yr	Postmortem	DCX, NF, Sox2, PSA-NCAM, NeuN	Detection of AHN markers by means of RNAscope	Flash-frozen tissue and short fixation (undisclosed fixation protocol)
Stepień et al., 2018	IHC	70 ± 6.03 yr (nonhemorragic stroke), 64.75 ± 12.23 yr (hemorrhagic), 64 ± 10.95 yr (controls)	Postmortem	GFAP, PH3	Increased number of PH3$^+$ cells in patients with hemorrhagic stroke	Undisclosed fixation method and embedding in paraffin; rotary microtome sectioning
Moreno-Jiménez et al., 2019	IHC	43-97 yr	Postmortem	DCX, PH3, GFAP, Prox1, Tau, NeuN, calretinin, calbindin, Tuj1, PSA-NCAM	Persistence of AHN markers in neurologically healthy control subjects, mild decrease in AHN markers with aging, and sharp decrease in AD patients	4% PFA for 24 h and vibratome sectioning
Tobin et al., 2019	IHC	79-99 yr	Postmortem	DCX, PCNA, Nestin, Sox2, Ki67	Persistence of AHN markers during aging and AD patients; correlation between AHN and cognitive scores	Undisclosed fixation method and embedding in paraffin
Seki et al., 2019	IHC	9-49 yr	Biopsy	PSA-NCAM, Ki67, HuB, DCX	Reduced numbers of Ki67$^+$/HuB$^+$/DCX$^+$ cells in the adult DG	4% PFA for 3 d and freezing
Flor-García et al., 2020	IHC	43-97 yr	Postmortem	DCX, NeuN, PSA-NCAM, MAP2, calbindin, Prox1, calretinin, PH3, GFAP, Tuj1, Tau	Reduced expression of NeuN in DCX$^+$ compared with fully mature DGCs	4% PFA for 24 h and vibratome sectioning

[a] For each study, the first author's last name, journal, year of publication, methodological approach, age of the subjects studied, type of samples, markers analyzed, main results obtained, and tissue processing protocol are indicated. As shown, most of these studies used postmortem human hippocampal samples and immunohistochemistry. PFA, Paraformaldehyde; IHC, Immunohistochemistry; ISH, in situ hydridization; RT-PCR, real time-polymerase chain reaction; qPCR, quantitative polymesare chain reaction; WB, Western blot; SGZ, subgranular zone; EM, electron microscopy; GCL, granule cell layer; GW, gestational week; MDD, major depression; MDDT-SSRI, MD patients treated with selective serotonin reuptake inhibitors; MDDT-TCA, MD patients treated with tricyclic antidepressants; MD-Psy, MD patients with psychotic symptoms; HIE, hypoxic-ischemic encephalopathy; LBD, Lewy body dementia; TLE, temporal lobe epilepsy; NSE, neuron-specific enolase; NeuN, neuronal nuclei; PCNA, proliferating cell nuclear antigen; MCM2, minichromosome maintenance complex component 2; Sox2, SRY (sex determining region Y)-box 2; Olig2, oligodendrocyte transcription factor; EGFR, epidermal growth factor receptor; Tbr2, T-box brain protein 2; Pax6, paired box protein 6; Ascl1, achaete-scute family BHLH transcription factor 1; BLBP, brain lipid-binding protein; NF-L, neurofilament light chains; PH3, phosphorylated histone 3; MAP2, microtubule-associated protein 2; and MAP1, microtubule-associated protein 1; NF, Neurofilament; NSE, Neuron-Specific Enolase; GFAP, Glial fibrillary acidic protein; DCX, Doublecortin; PSA-NCAM, Polysialylated-neural cell adhesion molecule; Prox1, Prospero homeobox protein 1; HuB; ELAV-like protein 2..

reported scarce or absent staining with markers of AHN in the adult human DG, starting from the early second decade of life. Seki et al. (2019) described relatively low numbers of proliferative cells and immature neurons in the adult human DG, thus supporting the previous studies. In contrast, contemporaneous work showed robust persistence of markers of AHN in the human DG throughout the lifetime (Boldrini et al., 2018; Moreno-Jiménez et al., 2019; Tobin et al., 2019). Intriguingly, recent studies that applied apparently equivalent immunohistochemistry methods have drawn opposite conclusions. During 2018 and 2019, these discrepancies raised important technical concerns in the field, as reflected by numerous review papers and opinion pieces (Kempermann et al., 2018; Paredes et al., 2018; Tartt et al., 2018; Lucassen et al., 2020). The human issue is probably a unique chapter within the controversial story of AHN. Our experience working with postmortem human brain samples has taught us that this tissue is particularly sensitive to subtle variations in preservation and histologic procedures, and that methodology is critical for the study of human AHN. The next section summarizes our

view of the general limitations of the studies performed on postmortem material of human origin and possible explanations for the discrepancies between human AHN studies.

Limitations of human studies

The most notable limitation that pertains to most research into human AHN is related to the postmortem nature of the samples used. Consequently, information about the past life history of the subjects from whom the samples have been obtained is usually scarce. Digging into the details of drug consumption, medication, and lifestyle habits, including physical activity, sleep patterns, and diet, as well as the existence of either known or unknown comorbidities is not always possible after brain donation. This consideration applies not only to adult neurogenesis studies, but potentially to any analysis performed on postmortem material of human origin.

The second major limitation associated with the use of human samples is the incompatibility between the particular tissue processing methodologies required to perform certain studies and the optimized workflow followed at most brain banks. The methodologies routinely used in these banks, which include either long-term fixation of the samples in formalin and their subsequent embedding into paraffin, or flash-freezing, generate tissues of high quality to perform a great number of biochemical, molecular, and histologic investigations. However, studies of adult neurogenesis require special processing techniques that are not fully compatible with this standard workflow (Moreno-Jiménez et al., 2019; Flor-García et al., 2020). Therefore, researchers working on human AHN are sometimes required to start customized prospective collections of samples that fulfill their needs, and this is not only extremely time- and resource-consuming but not always offered by biobanks.

A third major limitation is related to difficulties in precisely tracking the fixation procedure and duration to which each sample of a single study has been subjected. This is an essential requirement for guaranteeing methodological homogeneity within and between studies, but it is not always fulfilled. Notably, recent work by our group has revealed that subtle differences in the tissue fixation procedure can critically affect the detection of markers of AHN in the human DG (Moreno-Jiménez et al., 2019; Flor-García et al., 2020), as further detailed in the next section.

Another important limitation to human studies concerns the wide variety of specialized technical expertise required by the whole research team to be able to tackle this type of research in a rigorous manner. Using external anatomic cues, neuropathologists performing the autopsies have to be extremely accurate in identifying the brain region intended to be studied to ensure the reliability of the neuropathological examination, accurate classification of the patients, and homogeneity of the brain region analyzed for all the subjects. Moreover, tissue must be extracted with minimal manipulation. Compliance with these requirements not only guarantees the precision and rapidness of the dissection process but also contributes to preserving the macroscopic quality of the tissue. In addition to obtaining properly prepared samples, basic researchers must receive specialized training on performing and interpreting immunohistochemistry on human brain tissue because the evaluation of both positive and negative immunohistochemical signals can be biased by the presence of high autofluorescence and nonspecific staining that characterizes aged human brain tissue. In view of this, studies conducted in humans call for even more restrictive criteria than those used for rodents (Flor-García et al., 2020). The identification of false-positive and -negative results is a crucial task that untrained personnel may not always find straightforward.

A final limitation that applies to any histologic analysis of human tissue is the inherent observational nature of these studies. This includes, but is not limited to, the need to use "proxy markers" for any phenomenon of interest. Given that humans cannot be subjected to interventional studies, careful validation experiments are crucial. Consequently, positive and, in particular, negative results should always be interpreted with caution.

Given the aforementioned limitations of working with postmortem human samples, biopsy specimens from temporal lobe resection surgeries emerge as a potentially promising alternative. These samples have a major advantage over postmortem samples in that they are immediately fixed after surgical dissection. However, the results obtained from these samples must also be interpreted with care as these surgeries are performed almost exclusively on patients with intractable epilepsy or brain tumors. These health conditions have deleterious effects on AHN in rodents (Parent et al., 1997; Jessberger et al., 2005; Overstreet-Wadiche et al., 2006). Although several studies suggest that the consequences of seizures in humans are similar to those observed in mice (Table 1), epilepsy may not affect human AHN in the same way as in mice. Therefore, special emphasis should be placed on not extrapolating the conclusions obtained from patients with these diseases to the general population *a priori*. Moreover, the methodologies used to process tissues obtained from biopsies should be as precisely controlled as for postmortem samples.

Methodological considerations for the study of adult neurogenesis in humans

Figure 1 shows a schematic representation of distinct techniques routinely implemented to process postmortem human brain samples. As shown, these approaches are not only divergent but also mutually incompatible. Our recent work (Moreno-Jiménez et al., 2019; Flor-García et al., 2020) revealed the great extent to which certain tissue processing methods hinder the detection of markers of AHN in the human DG. Of the steps shown in Figure 1, we focused our attention on determining the impact of a seemingly trivial but, indeed, critical factor, namely, tissue fixation, on the qualitative and quantitative detection of markers of AHN in this region of the brain. To this end, we obtained the whole hippocampus from several neurologically healthy control subjects. Subsequently, hippocampi were divided into several fragments, each of which was fixed for a different period (ranging between 1 and 48 h) in freshly prepared 4% PFA. After completing the fixation step, we counted the number of cells expressing doublecortin (DCX^+) or polysialylated neural cell adhesion molecule (PSA-$NCAM^+$), the two gold-standard markers for immature neurons in the adult brain (Rao and Shetty, 2004), in the different fragments from each subject. Fixation for longer than 12 h increased background intensity and nonspecific staining, thus impeding the detection of both DCX^+ and PSA-$NCAM^+$ cells, which were clearly observed with shorter fixation times. When fixation was prolonged to up to 48 h, we found that the loss of the DCX and PSA-NCAM signals was reversible using specific histologic procedures (Moreno-Jiménez et al., 2019; Flor-García et al., 2020). In samples fixed for 24 h in 4% PFA, 5 of 9 anti-DCX antibodies produced reliable staining (Flor-García et al., 2020). In contrast, when fixation with commercial formalin was prolonged for several months, which is a routine method used at most brain banks worldwide, none of those 9 anti-DCX antibodies detected the presence of positive cells in the same patients. Thus, we concluded that samples fixed in formalin for long periods are, at least in our hands, totally inadequate for the

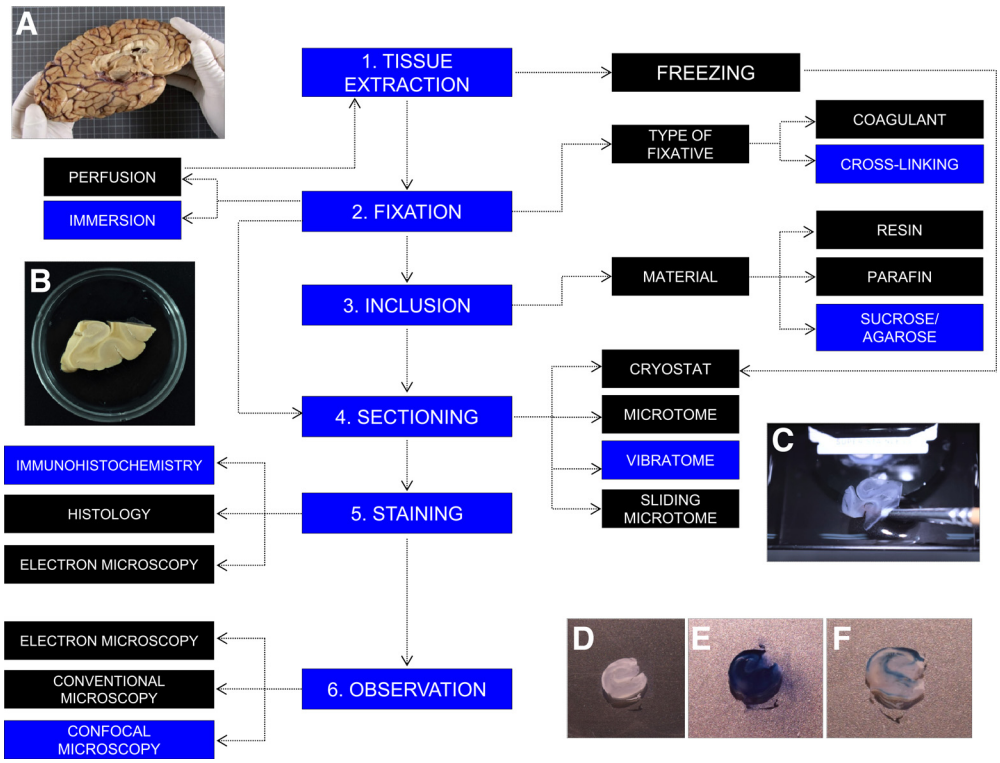

Figure 1. Variety of methods routinely used to process postmortem human brain samples. During autopsy, the brain is removed from the skull (***A***). After dissecting the area of interest, tissue can be either frozen or fixed. Various chemical fixatives can be used. After fixation, samples can be embedded in resin, paraffin, or a mixture of agarose/sucrose (***B***) to favor tissue preservation and sectioning. Depending on the embedding support, a cryostat, microtome, or vibratome (***C***) sectioning method should be used. Staining is performed following previously established immunohistochemical, histologic, or electron immunohistochemical protocols. Human hippocampal sections subjected to immunohistochemistry staining before (***D***), during (***E***), and after (***F***) the final autofluorescence elimination step are shown. Stained sections can be observed under conventional epifluorescence, electron, or confocal microscopes. Blue represents the protocol followed in our laboratory. A complete description of this protocol can be found in Moreno-Jiménez et al. (2019) and Flor-García et al. (2020).

detection of immature neurons in the human DG (Flor-García et al., 2020). Given the clear-cut consequences that the aforementioned subtle changes in tissue processing methods have on the detection of markers of AHN in the human DG, we believe that it should be mandatory for all human histologic studies to report on the following: the fixation procedure and/or duration; whether histologic or nonhistologic pretreatments were performed; and what controls were used to validate the antibody signal.

Interestingly, several reflective Dual Perspectives papers (Kempermann et al., 2018; Lucassen et al., 2020) pointed to prolonged postmortem delay, namely, the time elapsed between death and sample immersion in fixative, as a potential explanation for the absence of staining with distinct markers of AHN in several human studies. We carefully analyzed the impact of variable postmortem delay intervals on the quantitative and qualitative staining of several of these markers. We compared samples obtained from 58 individuals whose postmortem delay intervals fell between 2.5 and 38 h (Moreno-Jiménez et al., 2019). We did not observe variations in the number of DCX^+ or $PSA\text{-}NCAM^+$ cells, staining quality, or cellular distribution of these markers between samples with short and long postmortem delay. Although loss of DCX staining in the distal dendrites and confinement of DCX signal to the nuclear and somatic compartments has been reported in rats after long postmortem delay intervals, we did not qualitatively observe any of these phenomena in our samples. Nevertheless, it should be noted that the postmortem delay of our samples was relatively short compared with those of other studies. Future research should address the impact of longer postmortem delay intervals or other potentially important factors, such as temperature, on the detection of markers of AHN in the human brain.

Due consideration should be given to the apparent disparity in the number of positive cells reported by different studies. Importantly, various recent studies conducted in humans (Knoth et al., 2010; Boldrini et al., 2018; Moreno-Jiménez et al., 2019; Tobin et al., 2019; Flor-García et al., 2020) applied unbiased stereological criteria to perform cell counts in the DG. Of note, not only the microscopy equipment (confocal, conventional, or epifluorescence microscopes), but also the unbiased stereological method used to count cells (i.e., either physical or optical dissector, or optical fractionator) differed between these studies. Moreover, only a few studies have systematically reported total numbers of cells in the entire DG (Boldrini et al., 2018; Tobin et al., 2019). A cautionary note concerns the comparison of total numbers of cells between those studies and others, such as ours (Moreno-Jiménez et al., 2019; Flor-García et al., 2020), in which only cell densities were estimated. We calculated cell densities by dividing the number of cells counted within each stack of confocal images by the reference volume, which was defined as the volume of the granule cell layer included in that stack (Moreno-Jiménez et al., 2019; Flor-García et al., 2020), following the previously described physical dissector stereological method coupled to confocal microscopy (Llorens-Martin et al., 2006). When comparing data from different studies, it should be noted that changes in the structure or substructure used for calculation of the reference volume (e.g., including, or not, the portion of the hilus that appears in the image), or differences in the precision achieved when manually delineating the reference volume, might account for differences of at least one

order of magnitude in the total number of cells reported. Moreover, it should be noted that both Boldrini et al. (2018) and Tobin et al. (2019) examined the whole rostrocaudal extent of the human DG, whereas only the posterior pole of the anterior hippocampus was examined by Moreno-Jiménez et al. (2019). The differences in the precise anatomic regions examined and how the reference volume was calculated in different studies may add disparity to the number of cells reported. Therefore, the results of the aforementioned studies should not be considered contradictory but rather calculated and reported in a dissimilar manner.

A final remark in this section regards the need to apply strict criteria to assign a given cell the attribute of "positive" for a certain marker. For human studies based on histologic determinations, exhaustive control experiments for each type of sample and marker of interest are particularly important. These controls should include systematic consultation of the available literature, the use of several antibodies raised against distinct domains of the same protein (Moreno-Jiménez et al., 2019; Flor-García et al., 2020), specific synthetic blocking peptides to rule out nonspecific staining (Moreno-Jiménez et al., 2019), biological validation of the results using positive and negative controls (i.e., fetal vs adult non-neurogenic regions, respectively), and the careful examination of autofluorescence phenomena (Flor-García et al., 2020). Similarly, when negative results are obtained, each step of the method used should be subjected to exhaustive evaluation. For instance, 4 of 9 anti-DCX antibodies that provide quality staining of murine tissue did not show specificity on high-quality human samples that had been fixed for 24 h in PFA (Flor-García et al., 2020). In contrast, another 5 distinct antibodies worked appropriately in the same samples. In light of these results, the correct interpretation of negative results gains further relevance in the context of research into human AHN.

Human AHN in physiology and pathology

As previously mentioned, most of the studies that have either questioned or supported the persistence of AHN in the human DG throughout lifetime are based on the histologic analysis of proxy markers of AHN (Table 1). The two exceptions were those by Eriksson et al. (1998) and Spalding et al. (2013), which examined cell division through the incorporation of either BrdU or ^{14}C into dividing cells. Importantly, these two robust studies provided key quantitative data strongly supporting the occurrence of AHN in the adult human DG. The latter study determined that ~700 new dentate granule cells are incorporated into the adult human DG per brain hemisphere per day (Spalding et al., 2013). These data are potentially in agreement with the quantification of proliferation markers reported previously (Boldrini et al., 2009, 2012; Knoth et al., 2010). Moreover, the work by Spalding et al. (2013) also showed a mild decrease in the rate of human AHN with aging. We examined markers of AHN in 13 neurologically healthy control subjects aged between 43 and 87 yrs old. Although we did not quantify the absolute number of proliferative cells, we observed a mild decrease in the number of DCX^+ immature dentate granule cells with age (Moreno-Jiménez et al., 2019). All the aforementioned observations are consistent with the findings of numerous studies performed on rodents, which also support a reduction of AHN rate with age (Kempermann et al., 2002). Because of significant differences in the lifetimes of humans and rodents, calibration is required to quantitatively compare AHN between these species (Snyder, 2019). In this regard, although the seminal studies by Eriksson et al. (1998) demonstrated that newly generated dentate granule cells achieve full maturation in humans, there is still no precise information regarding the time required by these cells to complete their maturation in humans. Given the high phylogenetic proximity between humans and nonhuman primates, it has been hypothesized that newly generated dentate granule cells in humans, like those in other primates, mature at a slower rate than those of rodents (Kohler et al., 2011; Sorrells et al., 2018).

Despite not providing information on the timing of this maturation, our data support the notion that the population of DCX^+ cells is not static but instead continuously undergoes a dynamic maturation process. Indeed, DCX^+ cells at different maturation stages are present in the adult human DG, with some subpopulations expressing additional well-characterized cell markers of immature neurons, such as PSA-NCAM and calretinin (Fig. 2*A*), whereas others express markers of more advanced stages of maturation, such as NeuN, calbindin, and Tau (Moreno-Jiménez et al., 2019). For instance, Figure 2*A* illustrates the immature morphology of a triple-labeled DCX^+/calretinin$^+$/PSA-NCAM$^+$ dentate granule cell. In the subpopulation of cells that coexpress DCX and markers of advanced differentiation stages, we carefully analyzed the expression of those markers. We observed that the expression of NeuN in these cells was lower than in fully mature surrounding DCX^- dentate granule cells (Flor-García et al., 2020). Moreover, DCX^+ cells that expressed calbindin, a calcium-binding protein expressed by dentate granule cells at advanced maturation stages, showed slightly smaller somas than fully mature calbindin$^+$ DCX^- dentate granule cells (Flor-García et al., 2020). These data strongly support the notion that DCX^+ dentate granule cells are a dynamic cell population that undergoes progressive maturation and whose features clearly differ from those of fully differentiated mature dentate granule cells.

In addition to the physiological variations in AHN observed throughout human life, solid evidence supporting the dynamic nature of this phenomenon in humans comes from the direct examination of markers of AHN in patients with various diseases (Table 1). As has been systematically reported in animal studies, AHN appears to be altered in patients with epilepsy, ischemic and hemorrhagic stroke, major depression, schizophrenia, ethanol or heroin addiction, cancer, and neurodegenerative conditions, such as Alzheimer's disease (AD), Creutzfeldt-Jakob disease, and Lewy body dementia (for a full list of these studies, see Table 1). Of particular importance are the following observations. First, cancer treatment selectively eliminates cells positive for markers of AHN in humans (Monje et al., 2007). Second, reduced numbers of progenitor and proliferating cells are observed in patients with major depression (Boldrini et al., 2009; Lucassen et al., 2010). Importantly, antidepressants increase AHN in mice (Santarelli et al., 2003) and also in humans (Boldrini et al., 2009, 2012) and nonhuman primates (Perera et al., 2007). Finally, a specific decrease in the number of immature neurons (Moreno-Jiménez et al., 2019), an altered number of progenitor and proliferative cells (Crews et al., 2010; Gomez-Nicola et al., 2014; Tobin et al., 2019), and a blockade in the maturation of neuroblasts (Moreno-Jiménez et al., 2019) have been reported in patients with AD, while numbers of fully mature dentate granule cells are unchanged. Our data obtained from these patients suggest that, in addition to the physiological decrease in AHN rate caused by aging, independent neuropathological mechanisms drive this decrease in AHN in AD (Moreno-Jiménez et al., 2019). This hypothesis is particularly relevant because the decrease in the number of immature neurons was found to start during early-stage AD, although AHN impairments were accentuated as the disease progressed (Moreno-

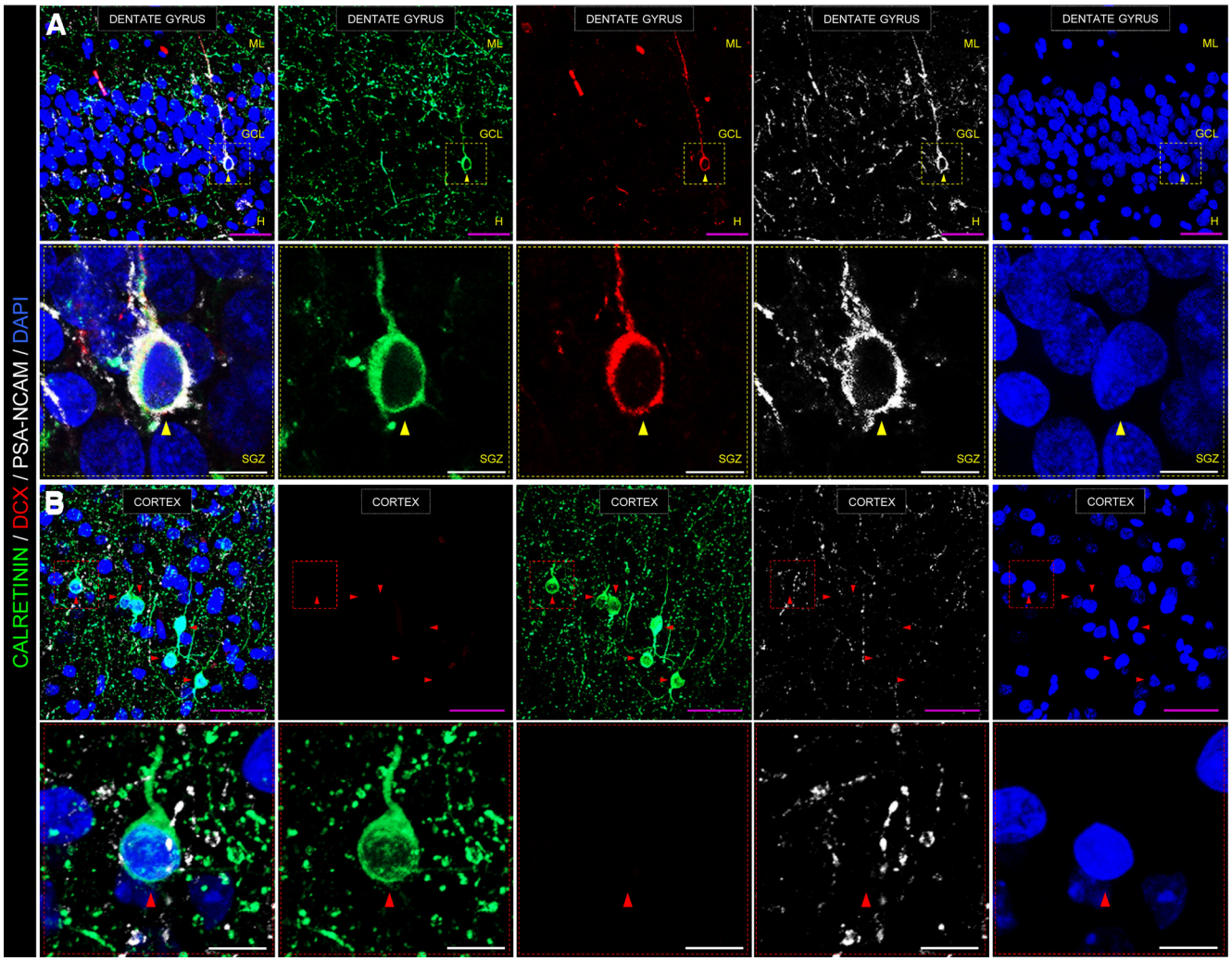

Figure 2. Representative images showing an immunohistochemical staining performed on postmortem human hippocampal samples. ***A***, Low- and high-power magnification images show the anatomic organization of the human DG. In high-power magnification images, a triple-labeled DCX^+/$calretinin^+$/$PSA\text{-}NCAM^+$ immature dentate granule cell is shown. ***B***, Representative image of the human cortex is shown as a negative control for DCX staining. Note the abundance of $calretinin^+$ interneurons, which is in sharp contrast to the absence of DCX^+ cells in this structure. GCL, Granule cell layer; ML, molecular layer; H, hilus; SGZ, subgranular zone. Scale bars: magenta, 50 μm; white, 10 μm. Yellow triangles represent DCX^+/$calretinin^+$/$PSA\text{-}NCAM^+$ immature dentate granule cell. Red triangles represent DCX^-/$calretinin^+$/$PSA\text{-}NCAM^-$ cortical interneuron. Immunohistochemistry was performed following a previously published protocol (Moreno-Jiménez et al., 2019; Flor-García et al., 2020), which included sodium borohydride ($NaBH_4$), heat-mediated citrate antigen retrieval (HC-AR), and autofluorescence elimination steps. Low-power confocal stacks of images were obtained in a LSM800 Carl Zeiss confocal microscope, equipped with three GaAsP detectors and using a 40× immersion oil objective. ***A***, 0.8 zoom, *XY* dimensions: 199.66 µm, *Z* interval: 1.5 µm, pinhole dimensions: 0.9 Airy units. ***B***, 1.1 zoom, *XY* dimensions: 145.2 µm, *Z* interval: 1.5 µm, pinhole dimensions: 0.9 Airy units. High-power magnification images were obtained in the same confocal microscope using a 63× immersion oil objective (2.2 zoom, *XY* dimensions: 46 µm, *Z* interval: 0.5 µm, pinhole dimensions: 0.9 Airy units). All the images shown in this figure correspond to postmortem human samples obtained from a neurologically healthy control subject. Fixation time was limited to 24 h in freshly prepared 4% PFA at 4°C. The epidemiological data of this subject are the following: gender, male; age, 43; postmortem delay, 5 h; Braak-Tau stage, 0; CERAD stage, 0; and Braak α-synuclein stage, 0. Brain tissue donation, processing, and use for research were in compliance with published protocols (Martinez-Martin et al., 2010), which include the obtaining of informed consent for brain tissue donation from living donors, and approval of the whole donation process by the Ethical Committee of the Spanish Research Council (Committee Approval Reference 025-2020#).

Jiménez et al., 2019). Moreover, the expression of proxy markers of AHN correlated with cognitive scores in these patients (Tobin et al., 2019). Together, these data might provide insight into how the neurogenic potential of the human hippocampus contributes to cognition and mood regulation.

Future directions and unsolved questions

Despite the outstanding development of the human AHN research field in recent years, numerous key questions remain unanswered.

First, most human AHN studies published in the last decade have been limited to the postmortem observational analysis of markers of AHN. Despite the extensive validation of these markers in rodents and other mammalian species, the generally poor performance of most antibodies on human tissue has been a major obstacle for the further development of the human AHN research field and has prevented the detailed reconstruction of the whole differentiation process of newly generated neurons. Generally speaking, the efficacy of various markers of immature neurons on human tissue is satisfactory. In contrast, the poor performance of numerous markers that characterize the initial stages of AHN, namely, those in which radial glia-like cells give rise to progenitor cells with a variable commitment to the neuronal lineage, has hindered the detailed reconstruction of these early stages. Alternative techniques to immunohistochemistry, such as those at the single-cell level, are expected to shed light on the field in the coming years. These techniques may help to identify novel markers, or to cluster new subpopulations of cells or

intermediate differentiation stages. Indeed, one of the next key challenges in the field concerns the identification of a stem cell signature in the human DG.

Another crucial piece of information that is currently lacking is the tempo of AHN in humans. Current techniques do not allow the determination of the time required for a new neuron to fully mature. Nonmutagenic approaches to replace the use of BrdU and capable of birthdating new cells are needed to achieve a precise reconstruction of the maturational time course of these cells. We also need to determine whether this time course is modified in response to physiological aging or pathologic conditions. In this regard, Trinchero et al. (2017) demonstrated that newborn dentate granule cells matured more slowly in aged rodents than in young animals. Understanding the fine details of the timing of human AHN throughout physiological and pathologic aging might provide novel ways to preserve and stimulate the survival and maturation of new neurons under certain pathologic conditions.

The field also requires the standardization and exchange of protocols to precisely define the minimal requirements that human samples must fulfill to be considered valid for AHN studies. Of note, the vast majority of samples stored at brain banks worldwide are subjected to several weeks/months fixation in formalin. As discussed above, in our hands and to date, this methodology appears to be incompatible with the detection of markers of AHN. The development of a method that can work with these samples in the context of human AHN studies would be an extremely valuable contribution to the field.

Finally, although postmortem observational studies have an enormous potential to increase our basic knowledge about human AHN, they are of limited therapeutic or diagnostic use. Therefore, great hope is placed on methodologies capable of visualizing AHN in living organisms (Manganas et al., 2007; Sierra et al., 2011) or exploring pattern separation capabilities as a proxy for human AHN (Suwabe et al., 2018; Bernstein and McNally, 2019; Riphagen et al., 2020). Breakthroughs in this regard are likely to be the only way through which the role played by newborn dentate granule cells in the human brain will be unraveled. Demonstration that this cell population in humans plays similar roles to that in rodents may lead to the use of noninvasive methodologies to diagnose (or even to prevent) certain pathologic or neurodegenerative conditions and would turn AHN into a relevant biomarker for these conditions.

In conclusion, evidence discussed here demonstrates that slight differences in either tissue processing or histologic methodologies do limit the detection of several markers of adult neurogenesis in the human hippocampus, to the point of making them undetectable. In contrast, when high-quality tissues are subjected to short fixation times and to the appropriate histologic pretreatments, thousands of immature neurons can be observed in the DG until the 10th decade of human life. These data are in line with previous evidence, including that based on immunohistochemistry, BrdU incorporation, and carbon dating. Moreover, they indicate that adult neurogenesis is a robust phenomenon that occurs in the human hippocampus during physiological and pathologic aging.

AHN is an unquestionable source of plasticity in rodents. Given that a significant number of new neurons seem to be added to the human healthy hippocampus and that AHN sharply decreases in patients with AD, research efforts should be channeled into finding ways to protect these cells before the onset of neurodegeneration. In the long run, unraveling the mechanisms that control newborn neuron maturation and synaptic integration under physiological and pathologic conditions is expected to pave the way toward the promotion of the regenerative potential of the human brain, potentially helping to preserve this unique source of plasticity throughout lifetime.

Response From Dual Perspective Companion Author–Arturo Alvarez-Buylla

The companion Dual Perspectives article argues that markers of adult neurogenesis are detectable in the adult human hippocampus only under specific tissue processing and staining conditions (4% PFA and dual antigen retrieval), implying that the lack of staining for such markers in our study and that of others (e.g., Dennis et al., 2016; Cipriani et al., 2018; Seki et al., 2019) is a technical issue. However, most of the tissue we have evaluated is indeed 4% PFA-fixed, and our analysis of positive controls indicates that DCX^+ cells with immature morphology can be detected, even with variations in fixation, antigen retrieval, and background elimination protocols. In the companion Dual Perspectives article, the presence of $DCX^+PSA\text{-}NCAM^+CR^+$ cells is argued to provide confirmation of newly born neurons in adult human DG. However, compared with newborn cells observed in other species or in our younger samples, these cells are frequently deep in the dentate where older cells are frequently found, and the majority have a large size and mature morphology. They do not consider alternative interpretations or potential caveats, including expression of DCX in adult neurons born earlier in development. For example, DCX is reexpressed in old neurons following fluoxetine administration (Ohira et al., 2019). It is also argued that a patient's clinical history could limit detection of newly born DCX^+ neurons in the dentate. We agree, and we have raised this possibility when discussing our findings. However, clinical history could also increase expression of markers, such as DCX, and this possibility is not discussed.

If only certain sample processing parameters permit detection of immature neurons, why is it possible for us to see immature neurons in the child and adolescent hippocampus, but not in the adult? If prolonged or formalin fixation interferes with sample detection, why are we able to detect positive DCX^+ cells near Ki-67^+ cells in 10% formalin fixed samples at 2 yrs of age in the human dentate, but not at older ages using either formalin or 4% PFA-fixed tissue (our Dual Perspectives article, Fig. 3*B*,*C*)? Why are we able to detect DCX^+ cells (as in other studies) clearly in other adult brain regions (e.g., subventricular zone, parahippocampal gyrus, entorhinal cortex, or amygdala) in the same brains without DCX^+ cells in the hippocampus? If sample degradation is such a concern, why are DCX^+ neurons outside of the hippocampus beautifully preserved with intricate processes still visible (e.g., our Dual Perspectives article, Fig. 3; see also Sorrells et al., 2019)? Why is the specialized proposed staining protocol (Flor-García et al., 2020) not revealing these DCX^+ cells outside the hippocampus which have been observed by others as well? Importantly, if there is indeed a robust population of immature neurons in the

hippocampus, where is the germinal zone giving rise to all these "new neurons?" In Moreno-Jiménez et al. (2019, their Fig. 1*J*), in a single field of a brain sample of unspecified age, there are at least 7 DCX-labeled cells, but Ki-67$^+$ dividing cells are extremely rare. In the companion Dual Perspectives article, multiple previous studies are cited as congruent confirmatory evidence supporting adult hippocampus neurogenesis in humans (their Table 1). However, it is not discussed how previous studies are not consistent with each other: (1) Some studies claim a dramatic decrease in adult neurogenesis, whereas others claim robust persistent neurogenesis into very old age. (2) DCX$^+$ cells in the companion Dual Perspectives article (their Table 1) are not all equivalent; most studies show only examples of small and round DCX-labeled cells, which are quite different from the mature neurons revealed with the fixation and dual antigen retrieval that is suggested to be essential to reveal these cells (such mature DCX$^+$ cells can be detected not only in hippocampus using this approach, but also cortex: see our Dual Perspectives article, Fig. 3*C*,*D*). (3) Glial cells have also been found to express DCX$^+$, and the possibility that studies in their Table 1 might reveal glia instead of neurons is not considered.

We agree that studying adult neurogenesis in rodents provides a way to understand how new neurons can be integrated into adult circuits, for plasticity or repair. We also agree that it should be mandatory to include postmortem, fixation, and staining procedure in any publication investigating adult human neurogenesis. It should also be critical to include evidence of proliferating progenitor cells and evidence of morphologically immature neurons, which are rare or nonexistent in all studies, consistent with our interpretation that, if adult neurogenesis continues into adult humans, it is a rare phenomenon. We hope that this friendly discussion stimulates the development of new approaches to precisely determine the level of neuronal birth in the juvenile and adult human brain.

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