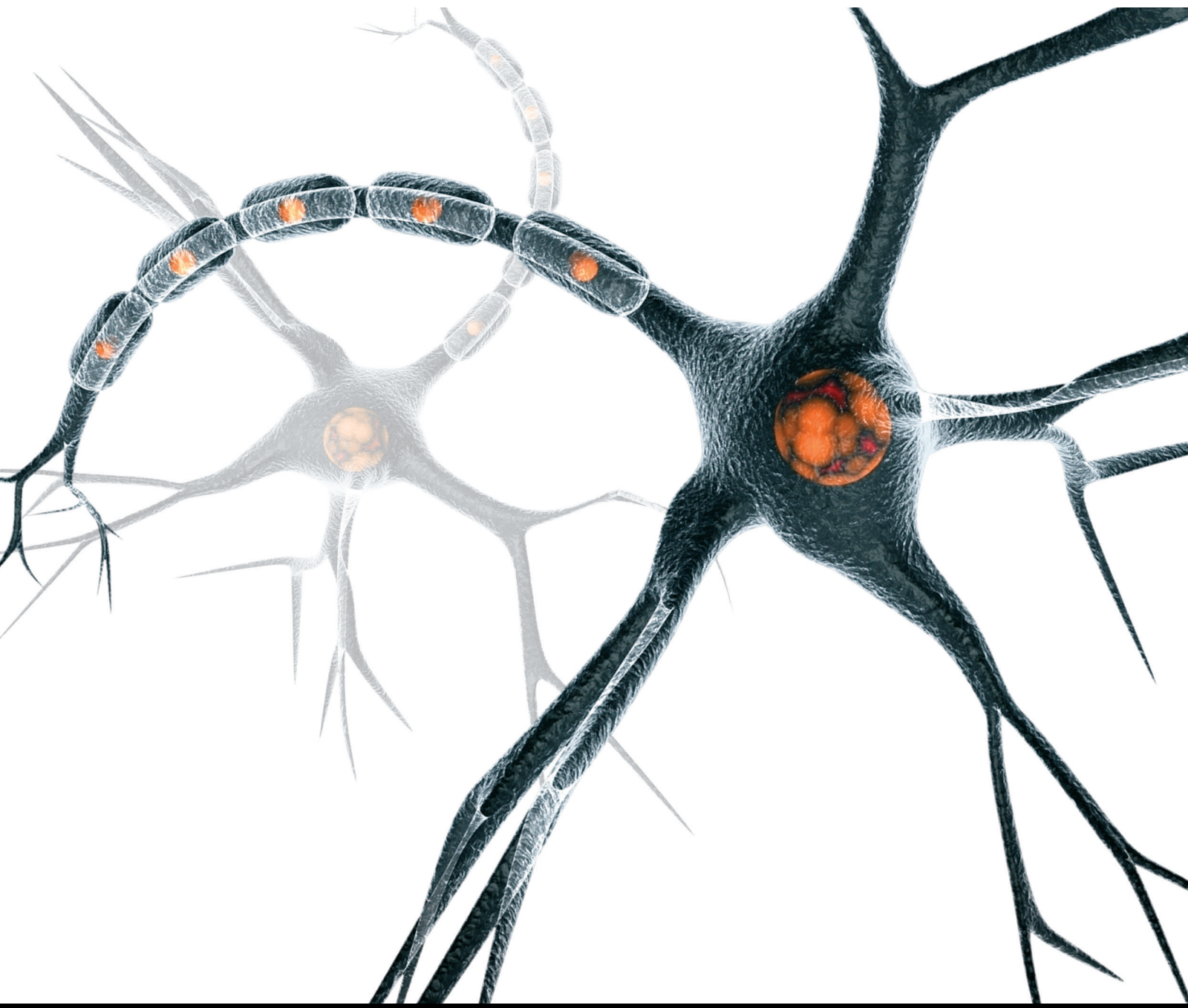
Neural Plasticity

Neural Plasticity and Neurogenesis in Mental Disorders

Guest Editors: Graham Cocks, Mauro G. Carta, Oscar Arias-Carrión, and Antonio E. Nardi



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Editorial Board

Contents

Neural Plasticity and Neurogenesis in Mental Disorders
Graham Cocks, Mauro G. Carta, Oscar Arias-Carrión, and Antonio E. Nardi
Volume 2016, Article ID 3738015, 2 pages

Developmental Dynamics of Rett Syndrome
Danielle Feldman, Abhishek Banerjee, and Mriganka Sur
Volume 2016, Article ID 6154080, 9 pages

Gender Differences in the Neurobiology of Anxiety: Focus on Adult Hippocampal Neurogenesis
Alessandra Aparecida Marques, Mário Cesar do Nascimento Bevilaqua, Alberto Morais Pinto da Fonseca, Antonio Egidio Nardi, Sandrine Thuret, and Gisele Pereira Dias
Volume 2016, Article ID 5026713, 14 pages

N100 Repetition Suppression Indexes Neuroplastic Defects in Clinical High Risk and Psychotic Youth
Joseph Gonzalez-Heydrich, Michelle Bosquet Enlow, Eugene D'Angelo, Larry J. Seidman, Sarah Gumlak, April Kim, Kristen A. Woodberry, Ashley Rober, Sahil Tembulkar, Kyle O'Donnell, Hesham M. Hamoda, Kara Kimball, Alexander Rotenberg, Lindsay M. Oberman, Alvaro Pascual-Leone, Matcheri S. Keshavan, and Frank H. Duffy
Volume 2016, Article ID 4209831, 11 pages

Are Anxiety Disorders Associated with Accelerated Aging? A Focus on Neuroprogression
Giampaolo Perna, Giuseppe Iannone, Alessandra Alciati, and Daniela Caldirola
Volume 2016, Article ID 8457612, 19 pages

Andrographolide Stimulates Neurogenesis in the Adult Hippocampus
Lorena Varela-Nallar, Sebastian B. Arredondo, Cheril Tapia-Rojas, Juan Hancke, and Nibaldo C. Inestrosa
Volume 2015, Article ID 935403, 13 pages

Hindawi Publishing Corporation
Neural Plasticity
Volume 2016, Article ID 3738015, 2 pages
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Editorial

Neural Plasticity and Neurogenesis in Mental Disorders

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Adult neurogenesis, the continuous generation of newborn neurons in discrete regions of the brain throughout life, is now widely regarded as a fundamental mechanism of neural plasticity. This phenomenon, and in particular the integration of new neurons into the dentate gyrus of the hippocampus, has been associated with the regulation of important but quite subtle and complex aspects of cognition and memory formation.

In addition to its important role in the healthy adult brain, adult neurogenesis has also been of considerable interest to the research community because of a growing body of literature implicating its deregulation in mental disorders. Perhaps the most high-profile example of this is the putative role of reduced neurogenesis in the adult hippocampus in the pathogenesis of major depression.

However, despite the fact that adult neurogenesis is confined to very discrete regions of the brain, and the established role of adult neurogenesis in major depression notwithstanding, it is becoming increasingly apparent that the deregulation of neurogenesis may impact a much wider range of mental disorders. In this special issue on neural plasticity and neurogenesis in mental disorders, we are pleased to present a series of articles that reflect the broad scope of psychiatric and neurological conditions that are potentially impacted by abnormalities in neurogenesis and neuroplasticity.

L. Varela-Nallar et al. ("Andrographolide Stimulates Neurogenesis in the Adult Hippocampus") report new data in this issue demonstrating the effects of Andrographolide (ANDRO) on adult hippocampal neurogenesis, a compound the authors have previously identified as a GSK3beta inhibitor. The regulation of beta-catenin by GSK3beta, which is modulated by the Wnt signalling pathway, is well established in playing an important role in regulating neural stem cell proliferation. The authors demonstrate that ANDRO increases adult hippocampal neurogenesis in young and aged mice and in a transgenic mouse model of Alzheimer's disease. Impaired neurogenesis has been associated with early pathological changes in Alzheimer's disease and is also reduced in normal aging. Novel small molecules such as ANDRO that can upregulate adult neurogenesis are therefore of potentially important therapeutic interest in reducing cognitive decline.

D. Feldman et al. ("Developmental Dynamics of Rett Syndrome") review the role of MeCP2 in abnormal developmental neurogenesis and neural plasticity in Rett syndrome across the lifespan. Rett syndrome is a disorder that has until recently been largely characterized as arising from abnormalities in neural plasticity in postnatal development. In their review article in this issue, D. Feldman et al. also provide insight into the more recent identification of earlier pathological events involving developmental neurogenesis. These earlier effects of MeCP2 loss of function on the generation and integration of neurons in the developing brain are also in line with recent research on other closely related neurodevelopmental conditions such as Autistic Spectrum Disorder where a growing body of literature has also begun to identify aberrant developmental neurogenesis to be an important pathological process, in addition to abnormalities in activity-dependent synaptic plasticity postnatally. Such insights will be important for developing therapeutic strategies to target abnormalities in different aspects of neuronal dysfunction in Rett syndrome arising from these different stages of development.

Defects in synaptic plasticity have been implicated in the pathogenesis of Schizophrenia. J. Gonzalez-Heydrich et al. ("N100 Repetition Suppression Indexes Neuroplastic Defects in Clinical High Risk and Psychotic Youth") present original data to begin to validate auditory N100 adaptation as a biomarker of clinical high risk and progression to psychosis individuals. Developing biomarkers such as this as an indication of abnormal neural plasticity, particularly in the prodromal stage of psychosis, will be of great value in assessing therapeutic strategies in clinical trial settings in the future.

Despite the considerable progress being made in understanding the role of adult neurogenesis and neuroplasticity in mental disorders, in some areas, there is also a great deal of uncertainty about the nature of such associations. These articles therefore reflect upon and elucidate what is currently known but also often highlight the uncertainty that exists and the continuing work that needs to be done to understand these associations. In this respect, G. Perna et al. ("Are Anxiety Disorders Associated with Accelerated Aging? A Focus on Neuroprogression") present a valuable novel review of the literature looking at the association of anxiety disorders and aging. This review examines a wide range of potential consequences of anxiety disorder from reduced adult neurogenesis and altered neuroplasticity through to increased beta-amyloid production, telomere shortening, oxidative stress, and chronic inflammation. The paper highlights the need for more work to be undertaken to establish these potentially very serious consequences of the already debilitating condition of anxiety disorder.

Finally, A. A. Marques et al. ("Gender Differences in the Neurobiology of Anxiety: Focus on Adult Hippocampal Neurogenesis") present an insightful review of gender differences in anxiety and potential differences in adult hippocampal neurogenesis between the sexes. This review provides a useful insight into the neurobiological processes that influence these differences and the important implications for the use of animal models of anxiety disorders.

The contributors to this special issue provide a valuable snapshot of the range of mental disorders associated with abnormalities in neural plasticity and neurogenesis. These articles provide important insights into our current understanding of the role of neural plasticity and neurogenesis in mental disorders, highlighting the current gaps in our knowledge and providing a valuable perspective on the future directions of the field.

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Review Article

Developmental Dynamics of Rett Syndrome

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Rett Syndrome was long considered to be simply a disorder of postnatal development, with phenotypes that manifest only late in development and into adulthood. A variety of recent evidence demonstrates that the phenotypes of Rett Syndrome are present at the earliest stages of brain development, including developmental stages that define neurogenesis, migration, and patterning in addition to stages of synaptic and circuit development and plasticity. These phenotypes arise from the pleotropic effects of MeCP2, which is expressed very early in neuronal progenitors and continues to be expressed into adulthood. The effects of MeCP2 are mediated by diverse signaling, transcriptional, and epigenetic mechanisms. Attempts to reverse the effects of Rett Syndrome need to take into account the developmental dynamics and temporal impact of MeCP2 loss.

1. Introduction

Rett Syndrome (RTT) is a developmental neurological disorder that affects 1 in every 10,000–15,000 live births in the US [1]. The genetic origin of RTT, in ~90% of patients, has been traced to sporadic loss-of-function mutations in the X-linked gene *MECP2* coding for methyl CpG-binding protein 2, mainly localized to methylated pericentric heterochromatin [2]. Clinical features of the disorder involve marked developmental regression, progressive loss of acquired motor and language skills, the acquisition of stereotyped repetitive hand movements, muscle hypotonia, autonomic dysfunctions, and severe cognitive impairment.

MeCP2 is an epigenetic modulator of gene expression. It acts as both a transcriptional activator and repressor [3, 4], in addition to regulating gene expression posttranscriptionally via microRNA- (miRNA-) processing machinery [5] and in an activity-dependent manner to regulate synaptic activity [6]. The binding interaction between MeCP2 and DNA is governed by a variety of genetic and epigenetic factors such as the length of the DNA, nearby sequences, and methylation patterns [3, 7, 8]. MeCP2 is a known binding partner of 5-methylcytosine (5mC) at CpG dinucleotides throughout the genome, resulting in transcriptional repression in these regions [9]. However, MeCP2 is also the predominant 5-hydroxymethylcytosine- (5hmC-) binding protein in the brain. Enrichment of 5hmC is linked to highly expressed genes [10, 11] in the absence of 5mC, suggesting that, in the context of this binding interaction, MeCP2 facilitates transcription [11]. Of note, MeCP2 is itself subject to methylation-dependent regulation, disruptions which have been linked to autism [12]. Thus, epigenetic modifications can regulate both the expression of MeCP2 and its downstream binding partners.

Alternative splicing of *Mecp2*/*MECP2* generates two main isoforms that differ exclusively at the N-terminus [13, 14]: MeCP2_e1, the predominant isoform in the brain [13–16], and MeCP2_e2, which displays a later expression onset during mouse brain development [17]. The two isoforms exhibit differential temporal and region-specific differences in their expression profiles in the brain and both contribute to neurological function and gene expression patterns [18–20]. The ratio of splice variants differs in a temporal- and cell type-specific manner, suggesting dynamic regulation of their expression and nonredundant functionality in the distinct stages of neurogenesis and adulthood [15, 17, 18, 21]. Whereas

MeCP2_e1 has been shown to be the isoform most relevant to RTT pathogenesis [20], MeCP2_e2 interacts with forkhead protein FoxG1, which promotes neuronal survival and maturation and in which mutations can also cause RTT [22]. The physiological significance of these two isoforms is not fully understood. Manipulations of independent isoforms in a cell type-specific manner are required in order to reveal their respective contributions to activity-dependent functions of MeCP2.

MeCP2 expression affects successive stages of brain development including prenatal neurogenesis, postnatal development of synaptic connections and function, experience-dependent synaptic plasticity, and maintenance of adult neural function including sensory integration [1, 23, 24]. MeCP2 critically maintains synaptic excitation (E) and inhibition (I), which are fundamental to the function of brain circuits and are often disrupted in neurological disorders including RTT [23, 25]. Additionally, MeCP2 has a remarkably diverse pool of binding partners and downstream targets [26, 27]. This functional and binding complexity, in combination with the domain-specific functionality of the MeCP2 protein [28, 29], confers a pleiotropic effect across age- and cell type-specific backgrounds [7]. Accordingly, different mutations in MeCP2 result in a wide range of phenotypic variability and severity in RTT patients [30], necessitating context-dependent mechanistic insights into MeCP2 function.

Transgenic mouse models that harbor cell type-specific mutations in MeCP2 have shed light on our understanding of RTT pathogenesis in the brain. Expression of MeCP2 under the CamKII or neuron-specific enolase promoter does not prevent the appearance of most RTT phenotypes, suggesting a more complex network of involvement for MeCP2 [31]. Interestingly, mice lacking MeCP2 exclusively in GABAergic neurons recapitulate many RTT features [32], and deletion of MeCP2 in the parvalbumin- (PV-) expressing subset of GABAergic neurons abolishes experience-dependent critical period plasticity in the absence of most RTT phenotypes [33]. The restoration of MeCP2 exclusively in astrocytes results in a non-cell-autonomous ameliorative effect on neurons *in vivo* [34], whereas RTT microglia exhibit adverse non-cell-autonomous effects on WT neurons *in vitro* [35]. In spite of their differing roles and effects on downstream gene regulation [18], transgenic expression of either the MeCP2_e1 or MeCP2_e2 splice variant has been shown to prevent the development of a number of RTT phenotypes in a mouse model lacking MeCP2. However, many abnormalities were only partially prevented, negating the notion that both transcripts are capable of acting independently to fulfill all of the roles of MeCP2 [36]. Accordingly, another study demonstrated that a point mutation in the MeCP2_e1 splice variant is sufficient to recapitulate many RTT phenotypes observed in MeCP2 KO mice [20]. Whereas the complexity that underlies the roles of MeCP2 will not be resolved with a single mouse model, each contributes a piece to the larger puzzle that represents MeCP2 functionality.

Similar to loss-of-function mutations in *MECP2*, the duplication of *MECP2* also results in a progressive neurological disorder that includes stereotypic and repetitive hand or body movements, epilepsy, spasticity, and a severe syndromic form of intellectual disability in male patients [37]. Recent studies show that the neurological dysfunctions in *MECP2* duplication syndrome are reversible in adult symptomatic mice and correction of MeCP2 levels genetically or by using antisense oligonucleotides largely restores molecular, electrophysiological, and behavioral deficits [38].

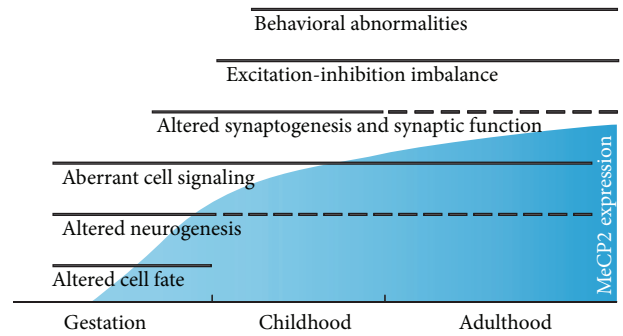

Figure 1: MeCP2 influences multiple features of brain development and function, at a variety of time points as its expression increases and is maintained. Thus, prenatal and postnatal brain development, as well as adult function, are all potentially affected in Rett Syndrome.

Traditionally, the dynamic time-course of RTT is thought to involve a period of apparently normal early development followed by profound neurological regression—a defining feature of RTT—and subsequent stabilization or partial recovery. However, our understanding of the disease initiation and progression and the ways in which *MECP2* impacts distinct phases of neurodevelopment is gradually evolving. In recent years, there has been a gradual shift in our understanding of atypical regression in RTT patients, with growing evidence of prenatal and early postnatal developmental abnormalities resulting in defects in the establishment and refinement of early neural circuits and, later, cortical plasticity (Figure 1). In this review, we aim to summarize recent findings and argue that *MECP2* serves distinct, discrete functions throughout developmental and adult stages, integrating genomic and environmental signals in a context-dependent manner.

2. Deficits in Early Neurogenesis

Early work in mouse models on the function of MeCP2 reported a pattern of expression limited to the neural lineage, with low expression in neuroblasts and a progressive increase during embryonic and postnatal development. Such findings led to the belief that MeCP2 is predominantly involved in the maturation and maintenance of neuronal function, as opposed to early cell fate decisions, and were further supported by a lack of phenotype observed with respect to differentiation in MeCP2-null neural progenitor cells (NPCs) [39].

Evidence has since demonstrated that, whereas MeCP2 expression increases postmitotically, both mRNA and protein can be detected throughout the majority of the mouse and human lifespan, including embryonic stages during which

neurogenesis occurs [21, 40–44]. MeCP2 protein expression does indeed increase after neuronal differentiation, when the vast majority of RTT phenotypes have been described. Experiments designed to determine isoform-specific expression have detected MeCP2_e1 protein in the mouse hippocampus as early as E14, whereas MeCP2_e2 was first detected at E18 [17]. Samples younger than E14 were not analyzed in this study, and thus these results do not preclude the possibility of expression at earlier embryonic stages. MeCP2_e1 expression increased until it reached a plateau at P7–P21. MeCP2_e2 expression overlapped with MeCP2_e1 after E18, albeit at a decreased level. As the gestational period of synaptogenesis overlaps with that of neuronal migration in human development [45], it stands to reason that MeCP2, known to regulate synaptic development [46–48] and cell guidance and laminar organization in the olfactory system [49], may contribute to the processes of cell fate specification and migration in the developing brain, especially the cortex. Accordingly, the landscape of clinical literature has been shifting to suggest an earlier onset of symptoms in RTT patients [50–54].

3. Cell Fate and Signaling Pathways

Expression of MeCP2 during early neurogenesis suggests a consequent role for the protein during this critical developmental time point. Neurogenic functions of MeCP2 have indeed since been demonstrated in mouse, whereby embryonic NPCs overexpressing MeCP2 exhibited a heightened neural identity *in vitro* [55]. Conversely, embryonic NPCs extracted from mice lacking MeCP2 exhibited a more proliferative—as opposed to late postmitotic—identity and revealed morphological alterations as early as 3 days *in vitro* (DIV) [44]. A human patient-derived induced pluripotent stem cell (iPSC) model of Rett Syndrome expressing a *de novo* frame-shift mutation in exon 4 (c.806delG) illustrated a parallel role for MeCP2 in the promotion of neural identity in which neural stem cells lacking MeCP2 exhibited increased astrocytic differentiation *in vitro* [56]. Mesenchymal stem cells (MSCs) isolated from a Rett patient harboring a different *de novo* mutation (del 1164-1207) also demonstrated impaired neural differentiation *in vitro*, which resulted in a reduced percentage of NeuN-expressing cells and increased senescence [57]. Roles for MeCP2 in determining neurogenic potential have been reported in *Xenopus* [58], zebrafish [59, 60], and chick [61] embryos.

Mechanisms underlying the function of MeCP2 with respect to early cell fate decisions are largely unknown. Neurogenic signaling cascades such as the *Notch-Delta* and PI3K-Akt pathways have been demonstrated to coordinate with MeCP2 throughout various time points including neurogenesis. Phosphorylation of MeCP2 at Serine421 (S421)—known to regulate gene transcription and synaptic development in an activity-dependent manner [62]—has since been shown to modulate the balance between proliferation and differentiation in NPCs isolated from the adult mouse hippocampus. Evidence suggests that the *Notch-Delta* signaling pathway, mediated via MeCP2-S421 phosphorylation, may serve as the hub linking MeCP2 to neural cell fate decisions in adult NPCs [63]. Experiments performed in *Xenopus* embryos, in which MeCP2 is expressed and is critical for neurogenesis, have demonstrated that the *Notch-Delta* signaling pathway regulates the patterning of primary neuronal differentiation in conjunction with MeCP2 binding. A complete lack of MeCP2 protein resulted in a decrease in the number of neuronal precursors, whereas expression of a truncated form of MeCP2 often found in Rett Syndrome patients—R168X—resulted in an increase in the number of neuronal precursors relative to WT embryos [58]. This phenotypic variety observed as a result of varying dosages and mutations of MeCP2 is echoed throughout the experimental and clinical literature [30, 52, 53, 64].

The PI3K-Akt signaling pathway is implicated in a wide range of cellular functions including cell cycle and transcriptional regulation [65]. The pathway has also been shown to regulate key neurological processes such as synaptic transmission [66] and neurodegeneration [67, 68] and is implicated in a range of neurological diseases and disorders such as spinocerebellar ataxia type 1 [67], Huntington's disease [68], amyotrophic lateral sclerosis (ALS) [69], and RTT [4, 70–72]. The majority of studies performed in RTT models, including those listed above, have examined the contribution of the PI3K-Akt pathway to disease effects and rescue in mature neurons. Whereas PI3K-Akt signaling has been shown to promote adult neurogenesis in the context of exercise enrichment [73], traumatic brain injury recovery [74], and surgical denervation [75], roles for PI3K-Akt signaling have also been demonstrated throughout embryonic neurogenesis in mouse [76], *Xenopus* [77], and zebrafish [78]. However, the precise roles of PI3K-Akt signaling in embryonic neurogenesis in the context of RTT have yet to be elucidated.

4. microRNAs and MeCP2

microRNAs (miRNAs) finely regulate genetic networks throughout the course of brain development and, with astounding complexity, act as critical determinants of early neurogenic activities such as cortical patterning and activity development, cellular subtype specification, and neuronal differentiation [79–81]. They are themselves subject to upstream epigenetic regulation; many are indeed targets of MeCP2 [82] or, as in the case of miR-132, act in a feedback loop as both target and regulator to maintain MeCP2 levels [83]. miR-132 has in turn been shown to promote postnatal neurogenesis and synaptic integration in neurons of the olfactory bulb [84]. Another brain-enriched miRNA target of MeCP2, miR-137, has been shown to regulate neuronal maturation and dendritogenesis in the postnatal hippocampus [85] and to modulate proliferation and differentiation in adult neurogenesis [82]. Moreover, miR-137 has been shown to negatively regulate neural stem cell proliferation and promote differentiation in the embryonic mouse brain [86]. miR-199a has been demonstrated as a link between MeCP2 and the mTOR pathway [87], previously implicated in RTT [72]. MeCP2 facilitates the postprocessing of miR-199a, which positively regulates mTOR signaling. Notably, exogenous miR-199a ameliorates several impairments in RTT neurons

and the genetic deletion of miR-199a-2 results in decreased mTOR activity in the brain and the recapitulation of several RTT phenotypes [87]. MeCP2 is known to influence the production of growth factors such as BDNF and IGF1—the latter via a miRNA-mediated pathway downstream of BDNF [88]. Many pathways and loops that determine the process of neurogenesis are maintained by the concerted regulation of miRNAs and MeCP2. As such, they provide insight into potential avenues by which MeCP2—or the lack thereof—can influence the developing cortex.

5. Deficits in Neuronal Migration and Cortical Patterning

Functions of MeCP2 during early neurogenesis result in immediate and long-term effects on neuronal migration and cortical patterning. Migration begins at gestational week 8 in humans and at E11 in mouse, at which point neural progenitors proliferating within the ventricular zone that lines the cerebral ventricles begin to differentiate to form the cortical laminae [45, 89]. Postmitotic cells migrate over radial glial scaffolds to form the discrete layers of the cerebral cortex in an inside-first, outside-last temporal pattern. Deep-layer cortical neurons are born first and passed by newly born neurons migrating to upper layers. This process is spatiotemporally governed by a variety of signaling, transcriptional, and epigenetic mechanisms [90–92]. Aberrant regulation of the proliferation and differentiation of neural stem cells results in a range of cortical dysplasias and is associated with many neurological and neuropsychiatric disorders including Alzheimer's disease, schizophrenia, and ASDs [93].

Early work demonstrated morphological cortical deficits in 8-week-old MeCP2$^{-/Y}$ mice including reduced thickness and increased cell density in neocortical layers; due in part to the belief that MeCP2 was not expressed early on, these alterations were believed to be a result of reduced cell size and complexity as opposed to deficits in corticogenesis [39]. Cerebellar expression profiling performed alongside chromatin immunoprecipitation in MeCP2-deficient mice has since revealed increased expression of *Reln*—encoding the extracellular signaling protein Reelin, known to be essential for proper neuronal lamination [94]. Accordingly, recent evidence has demonstrated that mouse NPCs lacking MeCP2 exhibit delayed corticogenesis with respect to migration from the subventricular and ventricular zones into the cortical plate [44]. These findings suggest a need for a thorough evaluation of the role of MeCP2 in cortical migration and lamination, as layering deficits observed at postnatal time points in RTT may result from combinatorial deficits in cortical development and maintenance.

6. Deficits in Synaptic Transmission and Plasticity during Postnatal Development

Along with deficits in early neurogenesis and cortical patterning, *MECP2* has been shown to play a key role in synaptic maturation and plasticity. Mutant mouse models have been generated with a global deletion of MeCP2 (MeCP2$^{-/Y}$) from all neurons and selectively from specific cellular subtypes including various neuronal subtypes and astrocytes. These models have served as a robust starting point in which to study the common principles underlying synaptic defects in RTT. They provide unique insight into the genetics that determine cell type-specific contributions to pathogenesis.

Functional defects in synaptic transmission have been investigated in an *Mecp2* global deletion model in which cortical connections were found to have weaker excitatory synaptic transmission and lower levels of basal activity [1, 95–98], reminiscent of an immature circuit. Cellular mechanisms of long-term plasticity, considered the functional basis of learning and memory, have also been found to be impaired in *Mecp2* mutant animals [2, 99, 100]. The majority of these early studies have used brain slice preparations, recording synaptic transmission including miniature synaptic currents and synaptic plasticity deficits. Similar to deficits in excitatory transmission, deletion of *Mecp2* from all forebrain GABAergic interneurons also recapitulates key features of RTT, suggesting that inhibition plays a crucial role in RTT pathophysiology. This includes reduced GABA synthesis, Gad1 and Gad2 levels, reduced miniature inhibitory postsynaptic currents (mIPSCs), and an array of behavioral deficits including EEG hyperexcitability and severe respiratory dysrhythmias [3, 4, 32]. Anatomical studies have reported enhanced PV+ neuronal puncta and hyperconnected PV+ circuitry in mouse visual cortex, suggesting that these microcircuits contribute to enhanced inhibition in MeCP2$^{-/Y}$ mice [5, 101]. This altered inhibition mediated by PV+ neurons, which regulates the initiation and termination of the critical period, has been proposed to alter the timing of critical period plasticity in RTT [6, 102]. Functional studies, however, have consistently reported decreased inhibitory function including reduced mIPSCs in CA3 pyramidal neurons of MeCP2$^{-/Y}$ mice [3, 7, 8, 103]. Although the density and intrinsic membrane properties of PV+ and somatostatin (SST)+ interneurons were not affected in MeCP2$^{-/Y}$ mice, miniature excitatory postsynaptic currents (mEPSCs) were found to be smaller and less frequent in fast-spiking PV+ neurons, suggesting impaired glutamatergic drive specifically onto this interneuron population compared to SST+ neurons [103]. Studies in slices have also reported a reduction in mEPSC amplitudes and a deficit in excitatory pathways, in the absence of change in mIPSC amplitude or frequency [95, 104]. These results are consistent with the decreased visually evoked responses found in PV+ interneurons in mouse visual cortex *in vivo* [33]. Interestingly, recent studies have highlighted the differential effects of subtype-specific *Mecp2* deletion on GABAergic inhibition regulating nonoverlapping neurological symptoms: mice lacking MeCP2 in PV+ neurons showed sensory, motor, memory, and social deficits, whereas those lacking in SOM+ neurons exhibited seizures and stereotypies [105], further elucidating the complex regulation of inhibition and their disruption in RTT [23].

Taken together, these features indicate that RTT is a complex disorder that arises from an imbalance of excitation and inhibition and a failure of brain circuitry to attain a mature state [9, 23]. Many of these defects can have a strong early

developmental, even prenatal, component (Figure 1) when the brain fails to attain "phenotypic checkpoint" signatures and in turn provides faulty functional feedback that influences gene expression [106] and network malfunction [107]. A coherent set of physiological measurements using *in vivo* awake animal models of global and neuronal subtype-specific *Mecp2* deletion remains necessary to measure and evaluate functional defects in the synaptic balance of excitation and inhibition. Another important consideration in this regard is to extend findings of cell-specific and synaptic defects in mouse models to identify biomarkers of RTT in human patients. Several recent studies are bridging this gap. Visual evoked potential (VEP) recordings in response to high-contrast oriented gratings have previously revealed loss of visual acuity in adult *Mecp2* mutant mice at the onset of RTT-like symptoms during critical periods of mouse visual cortex development [101], strongly suggesting that vision may serve as a biomarker of altered cortical function in RTT. Recent work has demonstrated that RTT patients exhibit a similar decrease in VEP amplitude and a reduction in visual spatial acuity that is impacted by *MECP2* mutation type [108].

7. Deficits in Adult Maintenance and Function

The onset of symptoms during early life in RTT patients, in conjunction with findings from mouse models suggesting neurodevelopmental abnormalities in RTT, has raised the question whether *Mecp2* function is necessary for integrative function in the adult brain. One study used an inducible knockout approach to delete *Mecp2* by crossing a floxed *Mecp2* allele mice with a tamoxifen-inducible Cre-ER expressing allele in adult mice (P60 or older) following normal development [109]. This late-deletion of *Mecp2* recapitulated key germline knockout phenotypes including abnormal gait, hind-limb clasping, motor abnormalities, impaired nesting ability, and impaired learning and memory, further underscoring the importance of *Mecp2* in adult neurological function [109]. Interestingly, this adult deletion recapitulated an epigenetic memory clock, suggesting a mechanism that extends—or is independent from—its early global genetic regulation [110].

Similar to behavioral deficits, the physiological response features of adult *Mecp2*-deleted neurons have also been characterized *in vivo* [111]. CRISPR-associated endonuclease (Cas) 9 has been used to introduce frame-shifting, insertion/deletion (INDEL) mutations that are targeted to the *Mecp2* locus using specific guide RNAs (gRNAs) via adeno-associated viral (AAV) vectors [112]. *In vivo* genome-editing resulted in ~68% of targeted cells containing INDEL mutations with a >60% reduction in MeCP2 protein levels [111]. Stereotactic injection of AAV-SpCas9 and gRNA targeting *Mecp2* into the superficial layers of mouse primary visual cortex followed by two-photon guided targeted electrophysiological recordings from genome-edited neurons revealed altered integrative visual responses, further emphasizing the maintenance role of *Mecp2* in the adult brain after normal developmental milestones have been achieved.

8. Reversal of Functional and Behavioral Deficits in RTT

One of the key discoveries in RTT has been the recovery of function following reactivation of endogenous *Mecp2* [113, 114]. This striking finding, an important feature not only of RTT but perhaps also of neurodevelopmental disorders in general, suggests that the neurodevelopmental pathology is reversible.

The phenotypic reversibility of advanced neurological phenotypes in both immature and mature adult animals shows that reactivation of the MeCP2 protein even at late stages of the disorder can partially rescue the mutant phenotype [113, 115]. Systemic delivery of MeCP2 cDNA via AAV9, under control of a fragment of its own promoter (scAAV9/MeCP2), has been shown to significantly rescue behavioral and cellular deficits when administered systemically into female RTT mice [116]. Proposed as a model for gene therapy, the retroviral-mediated overexpression of the MeCP2_e1 isoform in neural stem cells taken from *Mecp2* heterozygous mice was shown to promote dendritic branching *in vitro* [117]. Perhaps more practically, pharmacological manipulations, such as the treatment of *Mecp2* null mice with recombinant human IGF1 (rhIGF1) or a peptide fragment of IGF1, also resulted in a partial rescue of synaptic defects and cortical excitatory synaptic transmission, in addition to restoring activation of signaling pathway proteins [70, 71]. These studies argue that the brain circuits involved in neural processing may not functionally decline but rather remain in a labile, immature state; their subsequent activation by the reintroduction of *Mecp2* [113, 115] or by pharmacological manipulations to activate downstream signaling pathways [70, 71] is an important measure to ameliorate the syndrome's consequences.

9. Conclusions

The fluidity with which MeCP2 regulates the genomic landscape renders a uniquely moving target that has proven difficult to fully understand. Amongst many factors to be taken into account when attempting to attribute mechanistic function to MeCP2 (e.g., cell type, mutation and associated functional domain, and range of downstream targets), it is crucial to consider the time point in question. Deletion of MeCP2 results in a wide and temporally varied range of phenotypes. A complete picture of the MeCP2 protein includes its roles at various life stages, so as to inform our evolving concept of RTT progression in patients and potential phenotypic reversibility.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Danielle Feldman and Abhishek Banerjee contributed equally.

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Review Article

Gender Differences in the Neurobiology of Anxiety: Focus on Adult Hippocampal Neurogenesis

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Although the literature reports a higher incidence of anxiety disorders in women, the majority of basic research has focused on male rodents, thus resulting in a lack of knowledge on the neurobiology of anxiety in females. Bridging this gap is crucial for the design of effective translational interventions in women. One of the key brain mechanisms likely to regulate anxious behavior is adult hippocampal neurogenesis (AHN). This review paper aims to discuss the evidence on the differences between male and female rodents with regard to anxiety-related behavior and physiology, with a special focus on AHN. The differences between male and female physiologies are greatly influenced by hormonal differences. Gonadal hormones and their fluctuations during the estrous cycle have often been identified as agents responsible for sexual dimorphism in behavior and AHN. During sexual maturity, hormone levels fluctuate cyclically in females more than in males, increasing the stress response and the susceptibility to anxiety. It is therefore of great importance that future research investigates anxiety and other neurophysiological aspects in the female model, so that results can be more accurately applicable to the female population.

Dedicated to the memory of Dr. Anna Claudia Domingos da Silveira da Luz, whose efforts and dedication will always be a legacy for our lab

1. Introduction

Anxiety and fear are adaptive emotional reactions to both innate and conditioned stimuli perceived as dangerous. They have likely been conserved throughout evolution for their adaptive value in the survival of species by warning the individual of potential dangers through the triggering of a series of neurochemical, neuroendocrine, and behavioral responses. However, when these reactions become constant and intense, with prolonged or inadequate responses to neutral stimuli or even in the absence of stressors, it may be indicative of pathological anxiety [1, 2].

Anxiety disorders cause great suffering and loss of quality of life [3, 4]. A substantial literature suggests that women may be more vulnerable than men to developing anxiety [3–6]. Anxiety disorders are diagnosed at least twice as often in women than in men, and the prevalence in women increases with age, with the gradual decline of estrogen E2 secretion from the ovaries at menopause [7, 8]. Generalized anxiety disorder (GAD), for example, occurs in approximately 5%

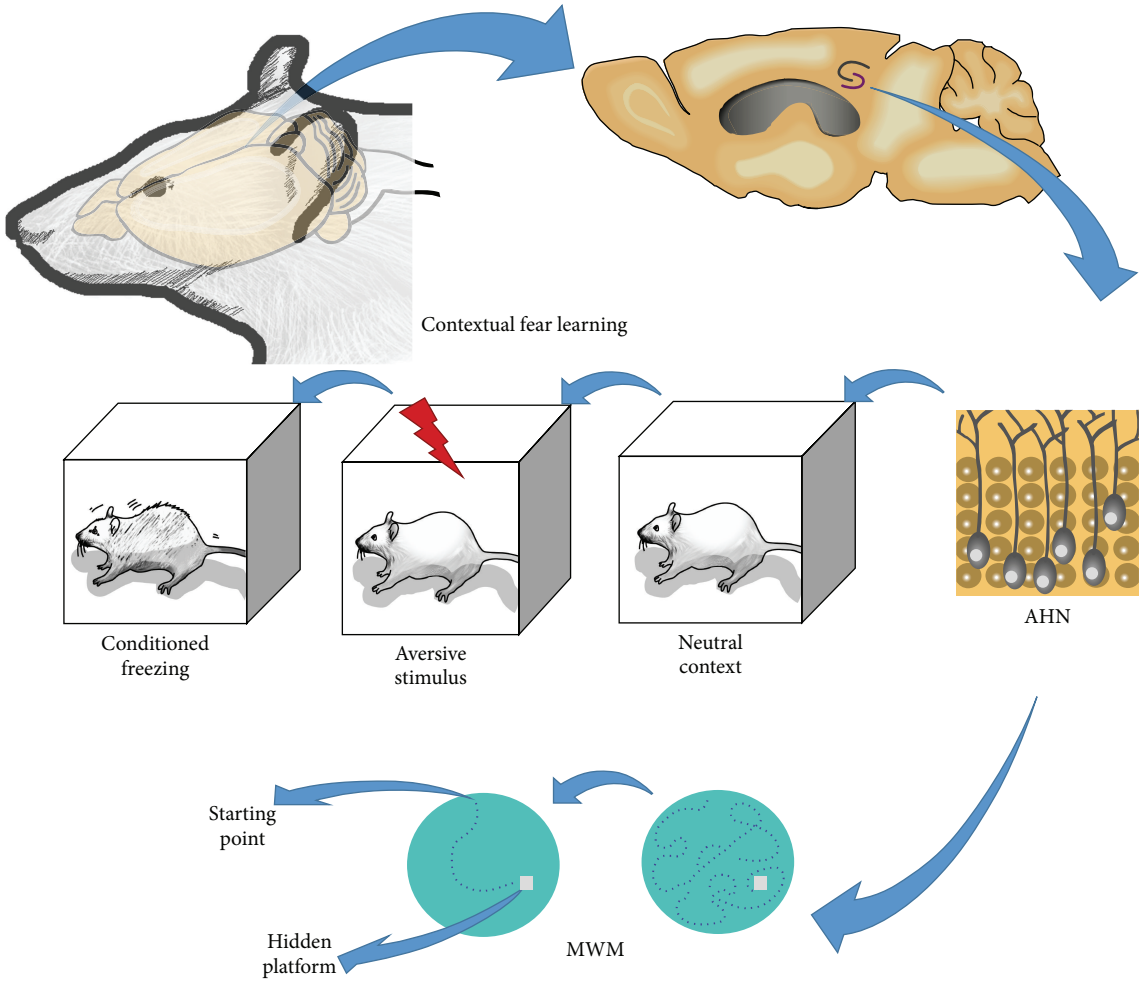

Figure 1: AHN is important for cognitive and emotional learning. The newly born neurons continuously generated in the postnatal hippocampus are believed to regulate cognitive and emotional tasks, as occurs in the contextual fear learning paradigm and the spatial learning assessed in the MWM. In contextual fear learning, the hippocampus is thought to be essential for the association between a previously neutral context and an aversive stimulus (in this case, a mild footshock) leading to a fear response (conditioned freezing) when the individual is reexposed to the context where the fear learning occurred. In the case of spatial learning, as assessed by the MWM, hippocampal cells are believed to play an important role in the cued spatial navigation strategies that make it possible for the rodent to more quickly find the hidden platform across the test trials. AHN = adult hippocampal neurogenesis; MWM = Morris water maze.

of the population; however, the incidence doubles in postmenopausal women [9].

At the neurobiological level, the basis of anxiety can be conceived of as a disruption in the fundamental mechanism of fight and flight responses regulated by the hypothalamic-pituitary-adrenal (HPA) axis. Fear and anxiety, therefore, involve brain structures participating in the regulation of the HPA axis, such as the amygdala, the hypothalamus, the periaqueductal grey, and the hippocampus [10]. Here, we highlight this latter structure and its remarkable ability to generate newly functional neurons throughout life, a phenomenon called adult hippocampal neurogenesis (AHN). Besides their well-known functions in regulating cognitive processes, these newly generated neurons have also been implicated in the regulation of fear and anxiety [11, 12] (Figure 1).

In addition, evidence suggests that both progesterone [13] and estrogens [14, 15] play an important role in enhancing the proliferation and survival of new neurons in the hippocampus of adult females, with ovariectomized (OVX) rats displaying impaired AHN [16]. Estrogens, particularly E2, play an important role in brain development, functioning, and aging; in addition, they exert important antioxidant [17], anxiolytic, and antidepressant-like effects [9, 18–20] besides modulating the dopaminergic [21, 22], serotonergic [23], and cholinergic neurotransmitter systems [24]. The cyclical nature of the secretion of estrogen until menopause, when women experience its almost total withdrawal, supports the role of hormones in gender differences and may contribute to the greater vulnerability of women to anxiety disorders at this age. However, in animal models, both at the behavioral and at the neurogenic levels males and females may respond differently, depending on the treatment, age, or exposure to stressors used, as will be seen in this review. This leads to a lack of a clear understanding on whether males and

The estrous cycle consists of the reproductive cycle of females. It lasts four to five days and is divided into four phases: proestrus, estrous, metaestrus, and diestrus [103]. It can be identified by cytological analysis according to the proportion and cell types in the vaginal secretion [104].

Each phase of the cycle lasts around 24 hours and is mainly linked to fluctuations in estradiol levels, which begins at puberty and ends in senescence (about 12 months in female rats). The metaestrus and diestrus phases have low level concentrations of serum estradiol. The proestrus is characterized by the highest level of estradiol followed by the estrous phase, where levels of the hormone start to decrease, coinciding with ovulation and corpus luteum formation [105].

Box 1: Estrous cycle.

females display different neurogenic profiles either at baseline or under different experimental conditions. Such possible differences in neurogenesis could account at least in part for the differences in anxiety observed between genders in clinical practice. Therefore, considering the importance of understanding gender differences in the context of anxiety, so that more tailored interventions may be delineated for the women population, it is essential to discuss the evidence on the possible biological differences between males and females; here, a special focus is given to AHN and anxious behavior.

2. Neurobiology of Anxiety: Differences between Males and Females

Overall, women and men are physiologically very similar, except for the time, pace, and schedule of the production and secretion of certain hormones. Both genders are undifferentiated until the sixth week of gestation, when the testicles develop in males and the production of androgens begins, while in females a substantial increase in follicle stimulating hormones (FSH) takes place around 12 to 20 weeks. After this period, this process of sexual differentiation is terminated, and the hormonal environment of the brain is again very similar in males and females until puberty [7].

Estrogen is a crucial hormone for the regular functioning of the brain, and its exhaustion at menopause may contribute to the higher probability of development of pathological anxiety [25]. OVX rat models have been widely used to investigate the effects of reduced estrogen levels at menopause, although the fact that it induces a drastic decline in estrogen secretion, whereas at menopause this process occurs gradually, is an important limitation of the model. The aged rodent is another useful model, but less used for this purpose. Aged female mice have very low levels of E2 and experience increased anxiety that is associated with the decline in ovarian function [9].

Hormonal differences also play a role in levels of stress markers. Females present elevated HPA axis markers at both resting and stressed states [26], as well as higher baseline plasma corticosterone (CORT) levels in comparison to males [27]. In addition, greater CORT response has been demonstrated in females than males in some anxiety tests such as the elevated plus-maze (EPM) and the defensive prod-burying test even after treatment with diazepam [28].

Additionally, CORT levels in females are influenced by the estrous cycle peaking in the proestrus phase and declining during the estrous phase, which at least partly explains why males and females respond differently to stressful situations [29] (for more details on the estrous cycle, please see Box 1).

Although clearly important, the influence of sex hormones is not the only mechanism involved in the development of sexual dimorphism. Genetic mechanisms, regardless of hormone action, may trigger the sexual differentiation of the brain and behavior [5]. The environment also appears to have an important impact on the dimorphism and differentiation of the central nervous system (CNS), thus also affecting behavior [5, 30]. In addition, sex differences in AHN (as will be later discussed) can also differently influence sexual dimorphisms.

Growing evidence also points to a role of gastrointestinal (GI) activity in leading to differential behavioral and neuroendocrine changes in males and females. Research data show that gastric inflammation induced by iodoacetamide (IAA) leads to anxiety behavior in female rats by a neuroendocrine pathway (the HPA axis), but not in male rats [27]. The reduction in circulating CORT, in the mRNA expression of glucocorticoid receptors (GR), and the increase in corticotrophin-releasing factor (CRF) mRNA in the hypothalamus of IAA-treated females suggest that GI activity has a gender impact on the HPA axis, being associated with its hyperactivity in females. This hyperactivity, in turn, is likely due to different sensitivities in the negative feedback of the HPA axis by CORT [27].

Gender differences were also found in the maternal separation model in rats, where separation during the lactation period resulted in decreased anxiety in females in comparison to males, despite the decreased expression of gamma-aminobutyric acid- (GABA-) A receptors in both sexes [31].

Significant gender differences were also reported in the prelimbic (PL) cortex activity during fear extinction and extinction recall [32]. Males presented PL activity decreased in the safe context, while females displayed increased PL activity in the same context; however, both showed increased infralimbic (IL) cortex activity before extinction recall compared to before extinction. These results suggest that female rats show increased expression of learned fear involving the persistent activation of PL.

According to the authors, this result may be related to possible disruptions in hippocampal connectivity to the PL, considering the role of the hippocampus in mediating contextual processing. In particular, the input of the ventral hippocampus (VH) to PL appears to be linked to the regulation of the extinction process [33]. In this sense, it has been shown that the temporary inactivation of the VH increases PL activity and expression of learned fear after extinction [34]. The authors highlight that females could therefore display changes in hippocampal-mediated inhibition of mPFC function, in agreement with findings in female patients with posttraumatic stress disorder (PTSD) [32, 35]. Furthermore, anxiety has been linked with impaired hippocampal neurogenesis [12], raising the possibility that altered AHN could also participate in the regulation of the fear circuitry. Whether this could be related to differential rates of AHN between males and females is a question that will be further discussed next.

2.1. Adult Hippocampal Neurogenesis and Anxiety. Adult neurogenesis, a phenomenon first described by Altman during the 60s [36], refers to a mechanism of continuous formation of newly functional neurons throughout life, a process that takes place only in specific regions of the adult brain [37].

In this regard, although also identified in structures such as the hypothalamus and the amygdala [38], neurogenic niches in the adult brain are mainly considered to reside in the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ) adjacent to the lateral ventricles [39, 40].

Due to its influence on mental health-related behaviors, including anxiety, we will focus this review on hippocampal neurogenesis.

The hippocampus is a region extremely sensitive to stress, and neurogenesis has been proposed to be linked to the development of pathological anxiety [41]. Growing evidence supports this idea, as chronic stress has been shown to reduce hippocampal neurogenesis, besides changing the activity of the HPA axis, thereby undermining the ability of the hippocampus to modulate the brain areas involved in stress and anxiety responses [42, 43].

AHN is a dynamic and highly regulated process, comprised of the stages of proliferation, differentiation, migration, integration, and survival [37, 40]. The subgranular zone (SGZ) of the DG is known to contain a large number of neural progenitor cells (NPCs) that retain the ability to divide resulting in cells that have the potential to become mature granule cells [44]. Proliferation is an expansion of the pool of NPCs, followed by a selection process where about half of these new cells will undergo apoptosis [45], while the surviving neuroblasts migrate into the granular zone of the DG [46]. Mice studies have shown that around four weeks these new cells already begin to express neuronal markers and are functionally incorporated into the preexisting circuit [47].

This mechanism of generation of new neurons can be affected by external stimuli, such as learning and memory [48], exercise [49], diet [50], environment [51], stress [52], and aging [53], as well as pharmacological agents [54] and internal stimuli. Among the internal stimuli known to upregulate the AHN process, we can highlight the microenvironmental factors, such as trophic factors [55], growth factors [56], increased vasculature [48], chemical and electrical changes such as excitatory stimuli [57, 58], and gonadal hormones (estradiol in females and testosterone in males) [59]. Moreover, there are also microenvironmental factors that negatively regulate this process, such as immune responses [60], glucocorticoids [42], and physiological changes associated with aging [61].

In addition, recent studies have attributed to AHN a regulatory role in various cognitive processes such as memory [42, 62], learning [61, 63, 64], besides a significant influence on affective disorders [65], anxiety [12], and emotional behavior [66]. Evidence for the role of AHN in the regulation of learning and memory is based on electrophysiological findings showing that new neurons in the hippocampus exhibit enhanced long-term potentiation (LTP), an important cellular mechanism underlying learning processes and memory [67]. In accordance with this, studies have shown that ablation of neurogenesis using genetic manipulation or irradiation techniques results in behavioral changes, such as cognitive deficits [68], including impaired performance acquired in the water maze by runners mice [69].

Despite the classic role attributed to the hippocampus and AHN on cognition, several researchers have suggested a link between neurogenesis and anxiety-related behaviors. One of the most convincing studies demonstrating this association was published by Revest et al. [12]. In this study, the authors used an inducible transgenic strategy that enabled the specific ablation of newborn neurons in the adult DG to demonstrate that deficits in hippocampal neurogenesis lead to an increase in anxious behavior. Furthermore, Dias and colleagues have found a decreased number of immature neurons in the DG of a rodent model for the study of generalized anxiety [70]. In addition, it has been suggested that the birth of new neurons in the hippocampus may be involved in the ability of the DG to distinguish contexts, and deficits in this ability could be an important factor in the etiology of anxiety disorders [11, 71]. The promotion of AHN has therefore been discussed as a potential target for the treatment of anxiety disorders [65, 71, 72].

2.2. Adult Hippocampal Neurogenesis: Differences between Males and Females. The differences between sexes with regard to AHN are derived largely from the singular physiology of females that allows pregnancy, parturition, and lactation [73]. This particular physiology makes females undergo profound hormonal changes throughout life. In addition, hormonal fluctuations experienced by females during biological processes such as the estrous cycle, pregnancy, and maternity are associated with a number of changes in the brain and behavior [74].

Gonadal hormones, besides exerting a powerful anxiolytic role, are also known by their effects in directly affecting hippocampal neurogenesis in females, via regulation of the proliferation and survival of new neurons [59]. This effect is possible due to the presence of estrogen receptors, ERβ and ERα in the DG [59, 75]. Evidence has shown that both ERα and ERβ receptors are involved in the improvement

of AHN in rats [26, 76]. However, ERβ agonists result in a greater neurogenic response, suggesting that ERβ may be more strongly associated with AHN than ERα [75]. Conversely, the ER antagonist ICI 182,780 could partially block the rise in estradiol-induced cell proliferation in the DG of rats [38]. Luteinizing hormone (LH), known for its important role during pregnancy and sexual behavior, also exerts influence on neurogenesis in females. Studies have shown that exposure of females to male pheromone or subcutaneous administration of a high dose of LH induced increased proliferation of new neurons both in the SVZ and in the DG [77]. Induced sexual experience was also reported to increase neurogenesis and reduce anxiety in females [78].

Although other female gonadal hormones like progesterone [73, 79] and LH [77] can also lead to improvements in AHN, estrogens are the most widely studied class of gonadal hormones, especially estradiol, the most potent form of estrogen [75]. Several articles have shown significant increases in the proliferation and survival of newly born neurons in the hippocampus after treatment with estradiol [13, 59, 73, 76, 80, 81], but not with other estrogens, such as estrone [14, 75]. In addition, during the proestrus phase of the estrous cycle, where estradiol levels are higher, rats exhibit about 50% higher proliferation than rats during diestrus and estrus stages when estradiol is low [82]. In OVX rats, proliferation of new neurons is significantly reduced, while estrogen replacement reverses the effect of ovariectomy [38, 83]. Additionally, another study has shown increased proliferation of new DG cells in OVX rats after estradiol injection or addition of soy extract in drinking water [84]. Furthermore, estrogen can also mediate AHN via growth factors and/or neurotransmitter systems.

Evidence suggests that estrogen regulates the expression of BDNF which in turn promotes the survival of newborn neurons in the hippocampus [85]. Moreover, it is known that estrogen plays an important role in modulating the levels of serotonin (5-hydroxytryptamine; 5-HT) synthesis via the regulation of tryptophan hydroxylase [74]. It has been shown that an increase in 5-HT in the DG increases cell proliferation while a reduction of 5-HT reduces proliferative activity [38]. In addition, serotonin antagonists can block the effect of estradiol to enhance cell proliferation [59, 86]. Also, it is well established that animal models of anxiety have exaggerated HPA axis responses to stress. In this context, it has been shown that E2 administration to rats with low endogenous levels of this hormone can alter this response, but its effects are previous experience- and regimen-dependent [9].

Estrogen levels have, therefore, been positively correlated with cell proliferation and negatively correlated with cell death [83]. During pregnancy and *postpartum* period, for example, estrogen is related with an increase of neuroplasticity [83]. During the proestrus phase, which is characterized by high levels of circulating estrogen, females exhibit greater cell proliferation in the DG than males, or females during the other phases of the estrous cycle [87, 88]. Moreover, several studies have shown that females and males respond differently to gonadal hormones administration in the context of hippocampal neurogenesis (see Table 1).

Treatment with estradiol or estradiol benzoate produced different behavioral effects in both genders [80, 89]. With regard to neurogenesis, females after estradiol benzoate treatment exhibited an increase in cell proliferation, and a decrease in both overall cell death and neuron survival in the DG, but males are minimally affected [80]. On the other hand, males injected with estradiol for 30 days presented no change in neurogenesis; however, they showed significant increased AHN via cell survival after treatment with both testosterone and dihydrotestosterone (DHT), one of the main metabolites of testosterone [90]. In addition, rats treated with the organochlorine insecticide methoxychlor (MXC), a synthetic compound known for their xenoestrogens properties able to cause disruption of the endocrine system, exhibited higher density of surviving cells in males than in females [91].

Sex differences in regard to AHN with respect to the stress response have also been widely reported. Exposure to stress increases levels of glucocorticoids, and when occurring during the prenatal period, this increase can cause substantial changes in neuroplasticity, reducing the capacity for cell proliferation in adults [92]. Accordingly, analysis of the brain tissue of adult rats whose dams were subjected to restraint stress 3 times per day during the last 10 days of pregnancy showed decreased survival of new neurons in the DG and increases of hippocampal BDNF levels in males, but not in females [93]. Besides, the type of stress and the duration (whether acute or chronic) are also an important factor in influencing AHN. Both acute stress caused by foot-shock [94] as caused by acute exposure to a predator odor have been reported to be associated with reduced cell proliferation in the male, but not in the female hippocampus [95]. Conversely, individually housed female rats which underwent chronic stress (daily foot-shocks for 3 weeks) showed increased BrdU labeling in comparison to males [82].

The age of animals is another relevant variable for the understanding of gender differences in the context of AHN. Postnatal neurogenesis during puberty occurs in young animals (between postnatal days (PND) 21 and 28; PND21–28) who have not yet reached sexual maturity [96]. Investigations of neurogenesis at this stage of development are important for the understanding of the mechanisms underlying neurogenesis and evaluation of their possible changes and particularities throughout life. Hodes et al. compared the effects of chronic fluoxetine treatment in adult and peripubescent rats of both sexes and found an increase in cell proliferation in adult males but not in adolescent males or females, as well as reduced cell survival in females but not in males [97]. Investigations about the effects of maternal deprivation in rats in early life reported increased proliferation in the DG in males and decreased in females at PND21 [98]. However, in an experiment in which male mice were subjected to early life stress (maternal separation) it was found that rats tested during peripubescence (PND21) exhibited increased neurogenesis, whereas when tested during adulthood (2 months old) no differences were found, and at middle-age (15 months old) AHN was decreased [99]. Moreover, using a different type of early life stressor (limited nesting and bedding material from PND2–9), Naninck and colleagues showed that both sexes exhibited significantly increased proliferation at PND9, but

Table 1: Differences between males and females with regard to AHN.

Model	Intervention/behavioral paradigm	Differences in AHN between males and females	Differences in anxious behavior between males and females	Differences in other types of behavior between males and females	Reference
PND70–90 Sprague-Dawley rats	Eyeblink conditioning	Twice more new cells (mainly neuroblasts) survived in the female than in the male hippocampus	—	Females learned to time the conditioned response faster than males	[63]
380 g (male) and 240 g (female) Wistar rats	Exposure to stressors	↑ proliferation in the DG of females compared to males; ↑ DCX in the DG of males compared to females	—	Females showed ↑ basal and stress-induced HPA axis activity compared to males	[87]
PND80–90 Sprague-Dawley rats	Treatment with E2 or sesame oil	↑ proliferation, ↓ cell death, and ↓ survival in the DG of females; males were affected minimally	—	Female rats froze less than males after contextual fear conditioning	[106]
PND80–90 Sprague-Dawley rats	Treatment with E2 benzoate	↓ survival, ↑ proliferation, ↓ cell death in the DG of females, and no effect in males	—	—	[80]
3-month-old Wistar rats	Chronic stress	↓ BrdU labelling in males, but ↑ in females	—	—	[82]
250–300 g Sprague-Dawley rats	Acute stress (exposure to a predator odor)	↓ proliferation, ↓ cell death in males but not in females	—	—	[95]
PND58–62 Sprague-Dawley rats	Spatial task	↑ BrdU-labeled cells in males, ↑ cell activation in females but not in males	—	Males performed better in the spatial but not cue task than females	[107]
2-3-month-old Sprague-Dawley rats	Acute stress	↓ proliferation in male hippocampus but not in female	—	Exposure to stress significantly ↓ learning ability in females but ↑ in males; males expressed more helplessness behavior than females	[94]
2-3-month-old Swiss CD1 mice	Treatment with MXC	↑ survival in males compared to females		Male mice exhibited ↓ contextual conditioned freezing compared to females	[91]
3-month-old Sprague-Dawley rats	PRS	↓ number of new neurons in the DG and ↑ BDNF levels in males but not in females	Males showed ↑ anxiety, while females displayed ↓ anxiety in the EPM	—	[93]
PND63–65; PND24–26 (peripubescent) Sprague-Dawley rats	Fluoxetine treatment	↑ cell proliferation in males but not in females ↓ cell survival in females but not in males	—	—	[97]
C57Bl/6J mice	ES (limited nesting/bedding material)	↓ cell survival only in males	—	Males showed impaired cognitive performance in the ORT, OLT, and MWM, compared to females	[100]

AHN = adult hippocampal neurogenesis; BDNF = brain-derived neurotrophic factor; BrdU = bromodeoxyuridine; DG = dentate gyrus; DHT = dihydrotestosterone; DCX = doublecortin; EPM = elevated plus maze; ES = early life stress; E2 = estradiol; MXC = methoxychlor (organochlorine insecticide); PND = postnatal day; PRS = prenatal restraint stress; SVZ = subventricular zone; T = testosterone; ES = early life stress; ORT = object recognition task; OLT = object location task; MWM = Morris water maze.

in adulthood males presented reduced long-term survival of newly generated cells, while females were not affected [100].

The effects of early life stress thus appear to be sex- and age-dependent, with different responses depending on the age at which the hippocampus is under analysis. Increased neurogenesis in young rodents which were subject to early stress may be explained as a compensatory mechanism enabling the survival of the organism under adverse conditions. However, this early improvement is not always observed in females. In addition, it may be interesting to note that the decreased neurogenesis reported in some studies in females using early life stress occurs during a critical period of brain development, which could increase the vulnerability of females to the development of psychiatric disorders such as depression [101], a condition often found to be comorbid with anxiety. A reduction in hippocampal volume in women who experienced early childhood trauma has been reported, suggesting that stress early in life can alter the structure and function of the hippocampus in humans also [102]. Whether this reduced hippocampal volume is also associated with reductions in AHN is a question yet to be investigated.

3. Potential Interventions for the Enhancement of AHN and Alleviation of Anxiety

Several external factors can induce physiological changes in the organism, thus exerting influence over AHN rates [38, 108, 109]. Based on this knowledge, different interventions have been proposed as possible enhancers of this mechanism [9, 38, 45, 110]. Here, we highlight some of them as possible ways to help overcome anxious symptoms, although it is clear that further studies are needed in order not only to clarify the participation of AHN as a pivotal mechanism underlying the higher anxiety observed in the women population but also to ascertain the effectiveness of these interventions among females.

Exercise is cited as one of the most powerful stimulants of neurogenesis [111–114]. It is believed that this effect is due to the increased oxygenation, metabolism, and blood flow favored by exercise, which could result in an increased nutrient delivery, providing increased synthesis and release of growth factors, such as brain-derived neurotrophic factor (BDNF), and neurotransmitters [115–117]. Furthermore, physical exercise has been reported to reduce anxiety. Corroborating this idea, studies have shown that voluntary wheel running produced anxiolytic effects in rats and mice [118–120], while the cessation of voluntary wheel running increased anxiety and impacted AHN negatively [121]. This strongly indicates that the improvement of neurogenesis afforded by exercise is associated with reduced anxious symptoms [121], although excessive physical exercise, at least in male mice, has been shown to improve neurogenesis but also anxiety-like behavior [122]. Furthermore, depending on the context, voluntary exercise can accentuate anxiety in females, as is the case with treatment with androgenic anabolic steroids [123]. Further studies are therefore needed in order to unravel the optimal conditions where females can benefit from physical exercise as an intervention to reduce anxiety.

Another factor with great positive impact over AHN is diet. Dietary interventions can comprise nutrient content, quantity, frequency, and texture [50, 124, 125]. Caloric restriction (CR), for instance, has been cited as an effective intervention in the expansion of neurogenesis [50]. Studies showed that CR rats displayed an increase in AHN rates when compared to animals fed *ad libitum* [126]. In addition, CR is also aimed to improve some cognitive processes generally impaired in anxious patients, such as fear extinction learning and retention [127]. As shown by Riddle and colleagues, after CR treatment for 7 days, significant effects on the enhancement of fear extinction and retention were found only in females but not in male mice [127].

Also pointing for anxiolytic properties of CR, a study showed that male rats under a 25% CR regimen entered more in the open arms of the EPM and spent more time in the center of the arena in the open-field test (OFT), indicating reduced anxiety [128].

With respect to the frequency of food intake, studies in rodents subjected to intermittent fasting (IF), where animals are fed on alternate days, also showed improvement in brain plasticity by increasing the survival of new neurons generated [129] as well as improvement in the ability to integrate and consolidate information compared to mice fed *ad libitum* [130]. However, the literature still lacks data on mechanisms underlying the effects of IF specifically in females and its possible role in anxiety disorders. Further studies are therefore required for a deeper understanding of this intervention, especially in the female population.

With regard to the quality of nutrients, some of the beneficial compounds more thoroughly investigated as brain plasticity enhancers are the polyphenols and the n-3 polyunsaturated acids (PUFA). Polyphenols (such as curcumin and resveratrol) are bioactive compounds found in a number of plants and spices present in the human diet, such as turmeric (in the case of curcumin) and red berries, the skin of red grapes, red wine, and nuts (in the case of resveratrol). These compounds are known for their neuroprotective and antioxidants actions [131] in addition to their anxiolytic and antidepressant properties [110, 132]. The improvement of AHN as a result of polyphenolic treatment has been shown both *in vitro* and *in vivo*, with curcumin-treated mice exhibiting increased cell differentiation [133].

OVX rat models have been used to investigate the protective role of grape powder on anxiety in estrogen-deficient females. OVX rats treated with grape powder for 3 weeks showed decreased anxiety [134]. In addition, a study in OVX mice suggested that resveratrol could act as an ER agonist mimicking the effects of estrogen [135], which potentially could be used as a safe alternative to protect the brain against the effects of estrogen deficits that occur in menopause.

As well as treatment with polyphenols, the consumption of foods rich in PUFAs has also been shown to exert positive effects on neurogenesis. A study by Venna et al. found an increase in cell proliferation in the DG after 5 and 6 weeks of PUFA supplementation [131], highlighting the important role of diet in brain plasticity, and AHN in particular.

A number of studies have suggested that an enriched environment (EE) is a promising intervention for the improvement of AHN [136–138]. EE consists of a larger habitat where animals are housed in groups so that social interaction is facilitated. This environment is also characterized by the presence of stimulating toys, tunnels, and platforms. These stimuli are changed regularly in order to promote curiosity and exploration, as well as to provide sensorial experience, and motor and cognitive stimulation to the animals. With regard to AHN, a study by Leal-Galicia et al. showed increased proliferation, survival, and differentiation of new neurons in the DG of rats submitted to EE, as well as reduced anxiety in several behavioral tests accompanied by higher rates of BrdU positive-cells in the hippocampus of animals with a reduced anxiety response [139]. In accordance with these findings, several studies have found an improvement in neuroplasticity accompanied by reduced anxiety in rodents subjected to EE paradigm [140–142]. However, findings suggest that the duration of exposure to an EE seems to influence anxiety-like behaviors. For example, reduced anxiety in the EPM was observed in mice exposed to an EE for 3 weeks, but not in the group exposed for 24 hours, 1 week or in animals subjected to more prolonged periods of exposure, such as five weeks [143]. However, not all these interventional studies were undertaken with female rodents. This highlights the need for future investigations into the effects of these potential interventions on reducing anxiety-like behavior in this population both in AHN-dependent and independent manners.

Another possible category of intervention could be hormonal therapy. E2-based therapies have been used for many years to treat physiological symptoms associated with menopause such as hot flushes, sweating, genital dryness, and mental symptoms, such as cognitive deficits and increased anxiety [20]. Several studies in menopausal women have shown that E2 replacement therapy attenuates the loss of cognitive performance associated with the end of the reproductive cycle [9]. Women who received E2 after menopause demonstrate verbal improvement, and improvement in short and long-term memory, as well as logical reasoning, compared to controls [144, 145]. In animal models, it has been shown that cognitive deficits related to reduction of circulating E2 levels after menopause coincided with the reduction of cell proliferation in the hippocampus [83]. On the other hand, increasing levels of estradiol have been suggested to improve neurogenesis and cognitive aspects. As shown by a study by Frye et al., E2 administration to mice reverses the cognitive deficits caused by aging, significantly improving spatial learning performance and reference memory in the water maze task [146].

However, the limitations of hormone replacement therapies due to their proliferative effects on breast and uterus curbed the enthusiasm of the use of E2 as treatments for anxiety disorders [147]. Moreover, not all individuals respond favorably to E2. Some women with anxiety disorders report less anxiety when E2 levels are low and/or relatively stable, which suggests that some individuals may be more sensitive than others to E2 [9, 148]. For these individuals, other strategies such as diet based on phytoestrogens, compounds present, for example, in soy extract with weak estrogenic or antiestrogenic activity, might be useful considering their neuroprotective effect, and their ability to increase the production of new cells in the hippocampus [84]. Another phytoestrogen, α-zearalanol (α-ZAL), has been used as a safe alternative for estrogen, due to reduced side effects on the uterus and breast. Studies showed that both α-ZAL and 17β-E2 improved neurogenesis and learning and memory deficits [149].

Data from a recent study, though, showed that treatment with 10b, 17b-dihydroxyestra-1,4-dien-3-one (DHED) was effective in reducing symptoms associated with estrogen deficits in the brain and in promoting neuroprotective effects in rats [150]. These are promising findings, considering that DHED is a small-molecule bioprecursor prodrug which is converted to 17b-estradiol in the brain but remains inactive in the rest of the body. This, therefore, prevents the adverse side effects normally found in the periphery and for which reason estrogen replacement therapies cannot be used safely.

Besides, as previously discussed, it could be hypothesized that cases unresponsive to E2 could still benefit from other practices such as those related to diet, exercise, and a stimulating environment. Novel studies are, however, still needed to assess their efficacy in terms of onset, duration, and synergistic effects with other interventions and conditions in humans, with a special focus here on the female population.

4. Discussion

Animal models can help to elucidate some aspects of neuropsychiatric disorders, but their establishment implies some important principles. Behavioral models can be appropriate for one sex and inappropriate for another, and generalizations on the findings in one sex to another seem to be a biased process at best. Therefore, caution is needed when interpreting data, as it may be possible that certain behavioral paradigms and interventions are not interchangeable in males and females [5]. In addition to behavioral differences, there are outstanding physiological differences between genders, as indicated throughout the paper. The reproductive process results in important functional changes in the female brain, mainly due to gonadal hormones. AHN, for example, is regulated in females and males by both gonadal and adrenal hormones, but are sex- and experience-dependent [73]. This shows that it cannot be affirmed that males and females have each a certain established neurogenic profile, as this changes in accordance with a number of variables. Moreover, results of behavioral or physiological analysis in females depend on the stage of the estrous cycle, that is, the hormone levels that are circulating at that moment [83, 89]. Finally, there is a very small number of studies in the literature assessing gender differences in behavioral tests of anxiety. Therefore, the hypothesis raised in this review will only be fully answered by future studies on the possible mechanisms underlying the gender gap with regard to stress or threat responses, as well as using AHN markers as one of their neurobiological readouts. These will be an invaluable source to help us better understand the differential vulnerability for mood and anxiety disorders between men and women [7].

Clinical studies showed that due to the sudden drop of estradiol levels that occurs during pregnancy, postpartum women have a higher reactivity of the HPA axis to stressors [27, 151]. Furthermore, estradiol is pointed to modulate neurotransmission, synaptic plasticity, and neurogenesis [75, 152], besides cognitive functions and emotional responses through the hippocampus, a structure known as one of the key regions of the so-called emotional brain due to its role in modulating anxiety states [12, 153]. There is growing evidence showing that deficits in neurogenesis are related to increased anxiety-like behavior [12, 154–157]; on that account, investigating the mechanisms of action and effects of E2 on the modulation of AHN and anxiety disorders has become a goal of potentially great importance and clinical impact. In recent years, a significant increase in life expectancy of women has been observed; however, the age of onset of menopause has remained relatively constant, resulting in a larger portion of life, about one-third, where women live with low endogenous levels of E2. It is therefore likely that more women make use of therapies based on E2 to relieve symptoms of menopause, necessitating an intensification of research into the possible benefits and risks of hormone replacement therapy [20, 32, 158].

Functional neuroimaging techniques also appear to be a promising tool to reveal the neural mechanisms underlying anxiety disorders, leading to the development of more effective therapeutic options, as they can help us understand how new pharmacological treatment options may work and predict if the patient is likely to respond to a particular intervention or not [159]. Despite the extensive amount of research in animals, little is known about the mechanisms of neurogenesis in the adult human brain, which is limited by *postmortem* histological studies [160, 161]. Thus, the future development of more sensitive and specific techniques of molecular neuroimaging for the investigation of human AHN is of great importance, as they hold unprecedented potential for the design of more effective treatment, with less side effects and improved life expectancy and quality of life.

5. Conclusion

Consistent evidence in the literature points to important differences between males and females with regard to anxious behavior and a number of biological mechanisms, including AHN, with different interventions bringing both sex- and age-dependent differential regulation of the ability of the hippocampus to generate newly functional neurons throughout life. As a whole, studies with animal models support the overall idea that increased levels of AHN are associated with decreased levels of cognitive deficits and anxiety. Therefore, interventions that are able to promote AHN are hypothesized as potentially effective to improve anxiety-like behavior, although further testing in female rodents and in the human population at different ages is still needed.

Of special note, disrupted levels of estrogen at menopause may contribute to the development of pathological anxiety. In addition, with increasing life expectancy, it is likely that more women will make use of estrogen-based therapies to relieve symptoms of menopause, making it necessary that research in females be undertaken into the possible benefits and risks of hormone replacement therapy, as well as on interventions that may enhance AHN and alleviate symptoms of anxiety.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

N100 Repetition Suppression Indexes Neuroplastic Defects in Clinical High Risk and Psychotic Youth

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Highly penetrant mutations leading to schizophrenia are enriched for genes coding for N-methyl-D-aspartate receptor signaling complex (NMDAR-SC), implicating plasticity defects in the disease's pathogenesis. The importance of plasticity in neurodevelopment implies a role for therapies that target these mechanisms in early life to prevent schizophrenia. Testing such therapies requires noninvasive methods that can assess engagement of target mechanisms. The auditory N100 is an obligatory cortical response whose amplitude decreases with tone repetition. This adaptation may index the health of plasticity mechanisms required for normal development. We exposed participants aged 5 to 17 years with psychosis ($n = 22$), at clinical high risk (CHR) for psychosis ($n = 29$), and healthy controls ($n = 17$) to an auditory tone repeated 450 times and measured N100 adaptation (mean amplitude during first 150 tones − mean amplitude during last 150 tones). N100 adaptation was reduced in CHR and psychosis, particularly among participants <13 years old. Initial N100 blunting partially accounted for differences. Decreased change in the N100 amplitude with tone repetition may be a useful marker of defects in neuroplastic mechanisms measurable early in life.

1. Introduction

Schizophrenia (SZ) is a progressive disorder, with the prodromal or clinical high risk (CHR) phase evolving into full psychosis within the first three years of ascertainment in approximately one-third of affected individuals and remitting or remaining stably symptomatic in the remaining two-thirds [1, 2]. Although only a minority of patients in the CHR phase develop SZ, most have marked limitations in mental, cognitive, and emotional functioning that lead to clinical referral. Moreover, these deficits often accrue over time, regardless of ultimate diagnosis, resulting in significant functional impairment [3]. Discovering biomarkers sensitive to the prodromal phase may improve treatment by assisting in the identification of at-risk individuals so that interventions may be applied early, thereby delaying

or preventing the emergence of psychosis and minimizing functional impairments, including among those who do not progress to a psychotic disorder [4, 5].

Accumulating evidence suggests that defects in the molecular pathways subserving synaptic plasticity, including long-term potentiation (LTP) and long-term depression (LTD) processes, play an etiological role in SZ and thus, by extension, in the CHR phase [6]. Postmortem samples from patients with SZ show low spine densities on the basilar dendrites of pyramidal neurons in various cortical regions and altered levels of mRNA for proteins in the LTP pathways in dendritic boutons, including genes in the CDC42 signaling pathways and Neuregulin 1 and its receptor [7, 8]. Additionally, large genomic studies of both common and rare mutations associated with SZ have independently implicated glutamatergic neurotransmission and synaptic plasticity [9]. These genes include components of the N-methyl-D-aspartate receptor signaling complex (NMDAR-SC) as well as genes with effects on plasticity presynaptically, thus pointing to a broad association of risk with synaptic regulation [10].

Defects in brain plasticity mechanisms could impact neurodevelopment via synaptic pruning processes. Current views on the mechanisms by which synapses are eliminated during brain development hypothesize that malfunction in pathways leading from NMDA-type glutamate receptors could lead to excessive synaptic pruning in adolescence [11–14]. Notably, neuroimaging studies of adolescent and young adult patients with first-episode SZ have implicated enhanced synaptic pruning in the development of psychosis [15, 16].

Before much of these genetic and neuroimaging data were available, acute administration of NMDAR antagonists was observed to induce symptoms that closely resemble those of SZ, including negative symptoms [17]. This observation led to the testing in preclinical models of compounds that enhance NMDAR activity, including metabotropic glutamate receptor agonists, glycine receptor agonists, and glycine reuptake inhibitors. Most studies of these compounds were performed in patients already afflicted with SZ with the aim to increase NMDAR dependent activity and, consequently, decrease SZ symptoms, especially negative symptoms, which are not alleviated by conventional antipsychotics. After demonstrating some promise in pilot studies, these agents failed in subsequent large placebo controlled trials [18]. Because earlier stages of SZ might be more amenable to treatment, attempts to use NMDAR modulators in the CHR period have also been undertaken, with some success in pilot studies. For example, D-serine treatment has been shown to decrease negative symptoms in CHR patients [19]. Larger, placebo controlled trials are needed to test these agents in CHR patients, as establishing the efficacy of therapies meant to enhance NMDAR functioning has proven more difficult than expected.

Noninvasive measures of neural plasticity could facilitate this process in several ways. For example, plasticity measures could signal if patients to be included in clinical trials of these agents have measurable deficits in neural plasticity. These measures could also signal if experimental therapies are improving neural plasticity as predicted by preclinical studies. Additionally, since plasticity pathways are active early in brain development, measures of their dysfunction could identify individuals in early life who are at particularly high risk for developing CHR or PS (e.g., among patients at high genetic risk). Finally, these measures could be utilized to evaluate the ability of experimental treatments to reverse plasticity deficits that emerge prior to the onset of CHR or PS symptoms [20].

Methods for measuring neural plasticity are in development, and several have been used to demonstrate neural plasticity deficits in patients with CHR or SZ [21]. Such methods include measuring changes in cortical response after transcranial magnetic stimulation (TMS) [22, 23] and in cortical evoked response potentials (ERPs) after high frequency repetitive sensory stimulation [24, 25]. Notably, ERP-based measures of automatic memory formation and deviance detection have been studied extensively, are plasticity dependent, and are abnormal in CHR and SZ. Moreover, they have shown promise as predictive biomarkers for transition from CHR to psychosis. Specific ERP waveforms that have demonstrated utility in this context include the P300 [26] and the mismatch negativity (MMN) [27]. Both of these responses are elicited by exposing the participant to a repetitive standard sensory stimulus and to a randomly interspersed rare stimulus (the "oddball" stimulus) that violates the regularity of the standard stimulus. To elicit the P300, the participant is asked to attend to the stimulus; no such instruction is given when eliciting the MMN [28]. While its application to pediatric age patients with CHR or SZ has been limited, MMN has advantages over the P300 as a translational measure of plasticity. It does not require participant cooperation, has been studied in newborns, children, and adults, and has an analog measureable in rodents [29, 30]. However, the MMN is hypothesized to sum two different plasticity mechanisms, that is, sensory specific adaptation and deviance detection. Furthermore, in rodent models, NMDAR antagonists differentially affect the amplitude of the response to the standard stimulus and to deviance detection, depending on the dose of NMDA antagonist administered [30]. These findings argue for the potential utility of measuring sensory specific adaptation separately from deviance detection.

Another ERP measure of cortical auditory response, the N100, shows promise as a biomarker of neural plasticity that can be measured very early in development. The N100 response is generated in the auditory cortex approximately 100 milliseconds after an auditory signal. It occurs prior to the MMN and represents an earlier and simpler aspect of sensory processing. Moreover, the amplitude of the N100 decreases with repetition of a tone [28]. This decrease reflects sensory specific adaptation that is not confounded by deviance detection and thus provides a purer measure of plasticity mechanisms subserving this phenomenon than MMN [30]. Furthermore, numerous studies have established that the N100 amplitude is decreased in SZ [31–37]. Deficits in the N100 and in its adaptation with repetitive presentation of an auditory stimulus in CHR and SZ have also been described previously, though to our knowledge not in exclusively pediatric age samples [4, 28, 34, 38]. Importantly, the auditory

N100 can be measured beginning in early childhood [39] and thus may be useful in therapeutic trials aiming to reverse processes leading to SZ very early in development. Moreover, the N100 is measurable in rodents and thus could provide a translational bridge between preclinical studies in rodents and human studies of potential therapeutics [40–42].

The mechanisms of N100 repetition suppression are only partially understood. Several prefrontal, cingulate, and parietal lobe regions exhibit stronger N100 repetition suppression than the auditory cortex, implying that neural networks underlying repetition suppression include these regions and that the initial N100 response to stimulus and its suppression may involve separate mechanisms [43]. Notably, N100 repetition suppression is dependent on baseline N100 amplitude [31–33, 36]. Administration of the NMDR antagonist phencyclidine (PCP) induces SZ-like deficits in the N100 amplitude and dependence on stimulus repetition rate that parallel those observed in SZ [28, 44]. Thus, the deficits in sensory adaptation in SZ and CHR indexed by N100 repetition suppression may be due to weaknesses of synaptic plasticity mechanisms in frontal, cingulate, and/or parietal-temporal connections, low baseline N100 amplitude, or both.

Together, these data suggest that N100 repetition suppression may be a useful biomarker of target engagement by experimental therapies aimed at improving NMDAR functioning [45]. Determining whether N100 repetition suppression is altered in CHR and PS present in early life is critical for evaluating the measure's usefulness as a clinical tool. The goal of the current study was to test whether N100 repetition suppression shows a gradient of deficit that parallels the gradient of clinical severity of psychotic symptoms by comparing healthy control (HC) participants to patients with CHR or PS in a fully pediatric sample. Additionally, clinical group differences in the N100 repetition suppression response were considered separately in subsamples of participants between 5 and 12 years and 13 and 17 years of age to explore whether very early presentation of CHR or PS is accompanied by more marked plasticity deficits. Research suggests that very early onset psychosis (i.e., emerging before the age of 13 years) shows more severe premorbid neurodevelopmental abnormalities and poorer treatment response and outcomes than later onset psychosis [46–48]. We hypothesized a progression in plasticity dysfunction from HC to CHR to PS groups. We further hypothesized that the plasticity dysfunction would be more robust in the CHR and PS groups within the younger subgroup than in the older subgroup of participants.

2. Methods

2.1. Participants. Patients with PS ($n = 22$), patients with CHR ($n = 29$), and healthy controls (HCs; $n = 17$) between 5 and 17 years of age were recruited for this study. PS and CHR participants were drawn from three sources in the Boston area: (1) the psychiatry service at Boston Children's Hospital; (2) the Commonwealth Research Center (CRC, PI L. J. Seidman); and (3) the Social Neuroscience and Psychopathology Laboratory (SNAP Lab, PI C. Hooker) at Harvard University. The Structured Interview for Psychosis-risk Syndromes (SIPS; described in Section 2.2.1(2)) was used to determine whether PS or CHR syndrome criteria were met [49]. For each potential PS participant, the Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version (K-SADS-PL) [50] (described in Section 2.2.1(1)) was utilized along with clinical reports from the participant's treating psychiatrist to determine a specific diagnosis using DSM-IV criteria. The 22 PS participants met criteria for the following psychotic diagnoses: schizophrenia ($n = 7$), schizoaffective disorder ($n = 8$), schizophreniform disorder ($n = 3$), bipolar disorder with psychotic features ($n = 2$), and major depression with psychotic features ($n = 2$). HCs were identified through advertisements and word of mouth. To qualify as HCs for the study, participants could not meet CHR criteria or have a current or past Axis I diagnosis. They also could not have any first-, second-, or third-degree biological relative with a psychotic disorder. Exclusion criteria for all participants included a lifetime diagnosis of substance abuse or dependence, neurological disease (e.g., epilepsy) or head injury, medical illness with cognitive sequelae, sensory impairments, or intellectual disability. Figure 1 displays the results of the screening process (described in Section 2.2.1).

2.2. Procedures and Measures. A screening assessment was administered to confirm study eligibility and determine clinical group assignment. Eligible participants were then invited to complete demographic and clinical interviews/questionnaires and an auditory ERP paradigm. Boston Children's Hospital's Institutional Review Board approved all procedures. Participants provided assent, and a parent or legal guardian provided written informed consent.

2.2.1. Screening Assessment. To determine study eligibility and group status, participants were administered a screening assessment, which consisted of the K-SADS-PL [50], the SIPS [49], and the Scale of Prodromal Symptoms (SOPS) [49]. If the ERP paradigm visit occurred more than one month after the screening assessment, the SIPS/SOPS were readministered to confirm clinical group assignment (i.e., to determine if any CHR participants had progressed to psychosis and to ensure that no HC had developed CHR or PS symptoms). No participant was reclassified based upon reassessment.

(1) Schedule for Affective Disorders and Schizophrenia for School-Age Children-Present and Lifetime Version (K-SADS-PL) [50]. The K-SADS-PL is a reliable and validated semistructured interview widely used to diagnose mood disorders, anxiety disorders, substance abuse disorders, and psychotic disorders in individuals under the age of 18 years [50]. Participants and their parents/guardians were individually administered the K-SADS-PL by trained raters under the supervision of a board certified child and adolescent psychiatrist (JGH). Following standard procedures, children were asked to rate their symptoms, and parents/guardians were asked to rate their child's symptoms. For each participant, final diagnostic ratings were derived that considered both the child and parent/guardian scores [50].

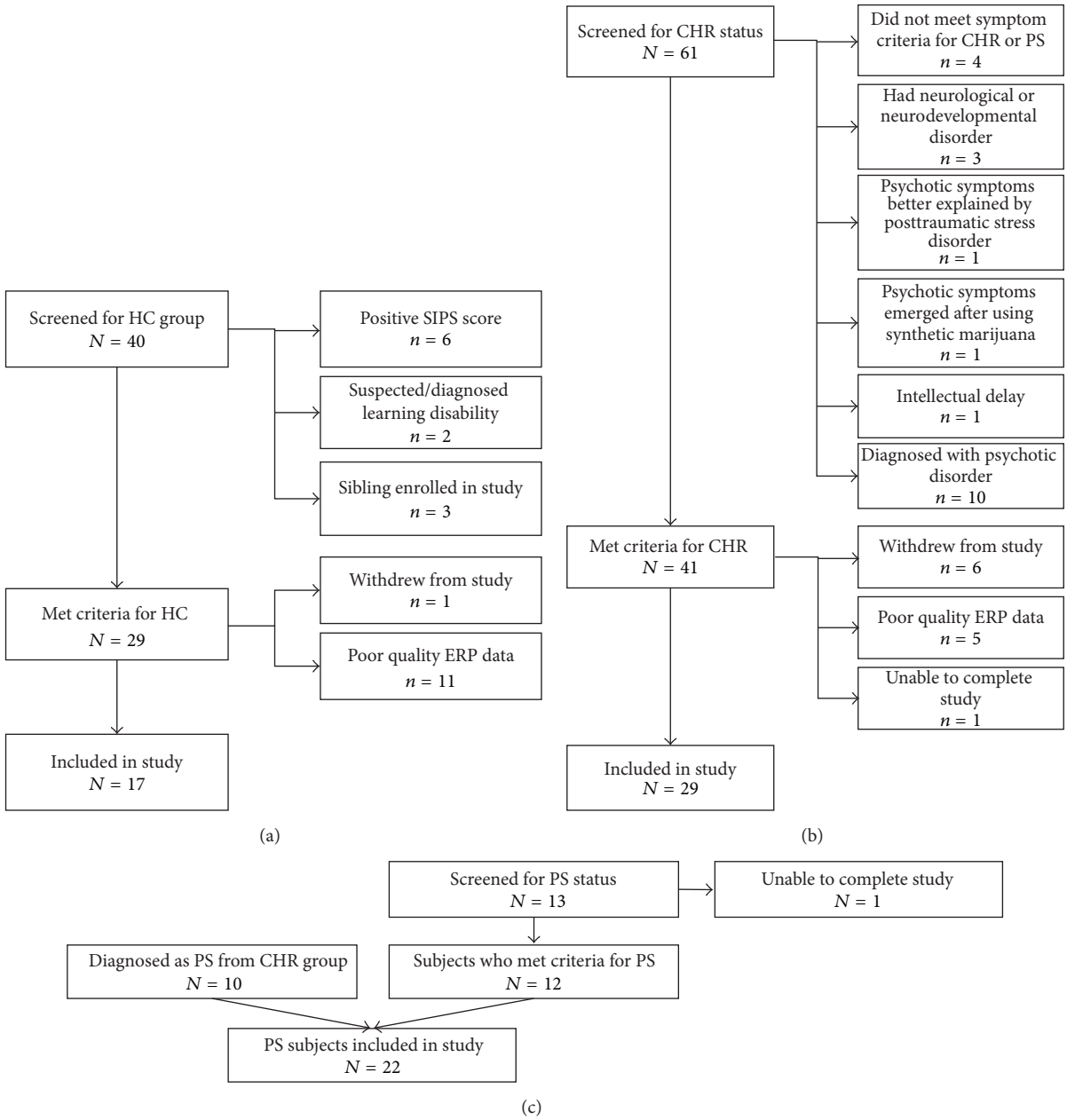

Figure 1: Flow charts depicting participants screened, reasons for exclusion, and number of participants retained for each clinical group. (a): healthy controls (HCs); (b): clinical high risk (CHR); (c): psychosis (PS).

(2) Structured Interview for Psychosis-Risk Syndromes (SIPS) and the Scale of Prodromal Symptoms (SOPS) [49]. The SIPS is an assessment instrument developed to operationally define psychosis disorder diagnoses, and the SOPS qualitatively rates symptom severity for positive and negative symptoms for patients prodromal for psychosis. Both measures have established predictive validity and excellent interrater reliability. Participants were administered the SIPS and the SOPS. Ratings were used to assess current or past psychosis and CHR status and to rate positive and negative symptom severity. SIPS/SOPS raters were trained and certified by Yale University's PRIME Research Clinic, and several attended North American Prodromal Longitudinal Study (NAPLS-2) SIPS interview reviews for 9 months. Sixty-six participants were administered the SIPS/SOPS by study staff; SIPS/SOPS scores for two participants were provided by their referral source (CRC or SNAP Lab), as they had been obtained within 30 days of the ERP paradigm visit.

2.2.2. Demographic and Clinical Assessment. Race, ethnicity, date of birth, medical and psychiatric history, medication usage, and school functioning were determined from parent/guardian interview and record review. Intellectual disability was ruled out if (a) previous IQ testing results

were >70 for full scale or verbal or performance IQ, (b) school functioning was at grade level without special education services, or (c) study staff administration of the Scales of Independent Behavior-Revised (SIB-R) [51], a comprehensive, norm-referenced assessment of functional level, indicated normal functioning.

2.2.3. Auditory ERP Paradigm. Following a 10-minute baseline, EEG recordings were collected with an EGI 128-channel Geodesic Net System (Electrical Geodesics Inc., Eugene, OR) while the participant was seated in a quiet, electrically shielded room with eyes closed to reduce eye-movement artifact. Auditory stimuli were presented with TDH-49P headphones. To facilitate state stabilization, all participants viewed an age-appropriate video with the sound muted during presentation of the auditory stimuli.

The auditory stimuli were 450 identical sinusoidal tones of 1000 Hz constructed digitally using a sine wave function at 44,000 samples per second. Each tone was 50 ms long with a 0.005-second onset and offset ramp. After digital-to-analog conversion, the waveforms were reduced to within audible range (70 dB SPL) and routed to ear inserts, played binaurally with a randomly determined variable 1800–2600 ms interstimulus interval (Noesis software) to avoid rhythm artifact.

Trained staff visually edited data for movement and electrode artifact, eyeblink storms, state changes, and muscle activity. Automatic eyeblink and eye movement artifact removal procedures were then implemented using BESA Research 6.0 software (BESA GmbH, Gräfelfing, Germany). For each participant, the average N100 amplitude to the first 150 sinusoidal tones and to the last 150 sinusoidal tones was calculated. The N100 response was measured at the left frontal EEG position Fc1, which incorporates the left temporal lobe dipole (between Fc1 and Tp9 electrode positions) to provide the maximum N100 signal [52]. For each tone, only data with frequencies between 0.53 Hz and 50 Hz that fell within the averaging epoch (−500 ms to 500 ms) and passed BESA's amplitude filter (set from 150 μV to 250 μV) were used. To calculate the plasticity of participants' N100 generating mechanism, the mean amplitude of each participant's N100 during the last 150 tones was subtracted from the mean amplitude of the N100 during the first 150 tones, providing a measure of the extent of N100 attenuation.

2.3. Data Analysis Plan. Differences among clinical groups on demographic and clinical characteristics were tested using the Freeman-Halton extension of Fisher's exact tests for categorical variables and ANOVAs for continuous variables. Significant differences were followed by 2 × 2 Fisher's exact tests with Bonferroni corrected p threshold for categorical variables and Student-Newman-Keuls (SNK) tests for continuous variables.

Linear regression models were used, consistent with methods of similar analyses [33], to examine the effect of clinical group status on the plasticity measure (i.e., the mean amplitude of the N100 during the last 150 tones subtracted from the mean amplitude of the N100 during the first 150 tones) in the full sample and then separately in the younger (5 to 12 years old) and older (13 to 17 years old) subgroups. Prior to conducting these analyses, potential covariates, including age, gender, handedness, first-degree family history of mental illness (psychosis, nonpsychotic major depression, and nonpsychotic bipolar disorder), and medication usage (antipsychotics, antidepressants, mood stabilizers, benzodiazepines, and stimulants), were individually regressed against the plasticity measure in the full sample. Each variable that reached a significance level of $p < .10$ in its individual regression was included in the linear regression model testing the effect of clinical group status on plasticity. Variables were then removed using backward elimination with a threshold of $p \geq .10$ to produce the final linear regression model. In the subsequent models stratified by participant age, only covariates that achieved significance ($p < .05$) in the final linear regression model on the full sample were included to minimize the likelihood of chance spurious results.

Following testing of the effects of clinical group on the plasticity measure, linear regression analyses were run that added the mean initial N100 amplitude (i.e., the mean N100 amplitude during the first 150 tones) as a covariate, as research in adults suggests that the N100 response is blunted in CHR and SZ [31–36]. This process minimized the risk of capitalizing on floor effects by testing whether any clinical group differences in plasticity were driven by initial N100 blunting in the CHR and/or PS groups, reducing the potential for attenuation over repeated presentations of the stimulus.

For all linear regression models in which clinical group emerged as a significant predictor, we confirmed the appropriateness of using a linear model by running ANOVAs to test deviation from linearity and by examining the residuals to ensure homoscedasticity and normal distributions (results not presented). Follow-up pairwise comparisons specified clinical group differences. For all analyses, $p < .05$ was considered statistically significant except where Bonferroni correction was used, as indicated below.

3. Results

3.1. Descriptive Data. Table 1 depicts the distributions of study variables across clinical groups. ANOVAs revealed significant differences between groups on age [$F(2, 65) = 4.493$, $p < .015$] and SIB-R scaled scores [$F(2, 54) = 8.366$, $p = .001$]. Fisher's exact tests revealed group differences on gender ($p = .007$) and usage of antipsychotics ($p < .001$) and antidepressants ($p = .003$). Table 1 specifies the significant pairwise group differences. Clinical groups did not differ on the remaining variables (ps ≥ .12).

3.2. Plasticity by Clinical Group. In linear regression analyses in which potential covariates were individually regressed on the plasticity measure, antipsychotic usage and first-degree family history of nonpsychotic major depression met the $p < .10$ threshold and were therefore included in the linear regression analyses testing the effect of clincial group status on plasticity. In a linear regression analysis including clinical group status, antipsychotic usage, and first-degree family history of nonpsychotic major depression, backward elimination

Table 1: Demographic and clinical characteristics of clinical groups.

Variable	Clinical group								
	PS (n = 22)			CHR (n = 29)			HC (n = 17)		
	%	M	SD	%	M	SD	%	M	SD
Demographics									
Male (% male)	86.4[a]			44.8[b]			52.9		
Age (years)		11.4[a]	2.8		13.5[b]	2.7		11.0[a]	4.2
Race/ethnicity (% non-Hispanic White)	95.2			79.3			94.1		
Handedness (% left-handed)	13.6			6.9			5.9		
SIB-R SS[c]		75.6[a]	27.9		89.6[a]	13.0		107.1[b]	20.9
First-degree family mental health history[d]									
Psychosis	14.3			4.4			0		
Nonpsychotic major depression	14.3			21.7			0		
Nonpsychotic bipolar disorder	4.8			17.4			11.8		
Medication use at assessment									
Antipsychotic(s)	50.0[a]			27.6			0[b]		
Antidepressant(s)	45.5[a]			31.1			0[b]		
Mood stabilizer(s)	18.2			6.9			0		
Benzodiazepine(s)	4.5			3.5			0		
Stimulant(s)	4.5			0			0		

Note: PS, psychosis; CHR, clinical high risk; HC, healthy control; SIB-R SS, Scales of Independent Behavior-Revised scaled scores.
[a,b]Groups noted by different superscripted letters were significantly different in post hoc pairwise Student-Newman-Keuls (SNK) tests for continuous variables and Bonferroni corrected ($p < .017$) Fisher's exact tests for categorical variables. [c]The SIB-R was administered to 20 PS participants, 21 CHR participants, and 16 HC participants. [d]One PS participant and six CHR participants were unable to provide information about family history of mental illness because they had limited contact with their biological parents or their adoptive parents were unsure of mental illness history among first-degree biological relatives.

procedures resulted in antipsychotic usage dropping out; the overall model was significant, $R^2 = .22$, $F(2, 58) = 7.93$, and $p = .001$, with a significant effect for clinical group status, $\beta = -0.38$, $t = -3.22$, and $p = .002$, and a marginal effect for family history of nonpsychotic major depression, $\beta = -0.22$, $t = -1.82$, and $p = .073$. From the first 150 tones to the last 150 tones, the N100 decreased by a mean of 2.32 μV (2.45) among the HC group, 0.69 μV (1.17) among the CHR group, and 0.42 μV (1.60) among the PS group (Figure 2). Follow-up pairwise analyses revealed that clinical group status was a significant predictor of plasticity when comparing the HC and CHR groups, $\beta = -0.46$, $t = -3.42$, and $p = .001$, and the HC and PS groups, $\beta = -0.47$, $t = -3.23$, and $p = .003$, but not when comparing the CHR and PS groups, $\beta = -0.10$, $t = -0.69$, and $p = .491$.

Secondary analyses added the initial N100 amplitude (i.e., the mean N100 amplitude for the first 150 tones) to determine if initial blunting of the N100 response accounted for clinical group differences in plasticity. When initial N100 amplitude, clinical group status, antipsychotic usage, and family history of nonpsychotic major depression were included in the linear regression model, only initial N100 amplitude survived the backward elimininations procedures, $\beta = 0.60$, $t = 5.76$, and $p < .001$. Initial N100 amplitude explained a large percentage of the variance in plasticity, $R^2 = .32$, $F(1, 66) = 30.98$, and $p < .001$. Follow-up pairwise analyses revealed that, when comparing the HC and CHR groups, both initial N100 amplitude, $\beta = 0.49$, $t = 4.13$, and $p < .001$, and clinical group status, $\beta = -0.33$, $t = -2.80$, and $p = .008$, were significant in predicting plasticity, $R^2 = .43$, $F(2, 43) = 16.48$, and $p < .001$. When comparing the HC and PS groups, initial N100 amplitude, $\beta = 0.58$, $t = 4.07$, and $p < .001$, was significant in predicting plasticity, but clinical group status was not, $\beta = -0.17$, $t = -1.22$, and $p = .231$; $R^2 = .44$, $F(2, 36) = 15.67$, and $p < .001$. Similarly, when comparing the CHR and PS groups, initial N100 amplitude, $\beta = 0.38$, $t = 2.58$, and $p = .013$, was significant in predicting plasticity, but clinical group status was not, $\beta = 0.64$, $t = 0.43$, and $p = .667$; $R^2 = .13$, $F(2, 48) = 3.60$, and $p = .035$.

3.3. Plasticity by Clinical Group and Age. The interaction between age (as a continuous measure) and clinical group status was not significant in predicting the plasticity measure, $p = .583$.

3.3.1. Plasticity by Clinical Group among Younger Participants (5 to 12 Years). In analyses conducted separately for participants between 5 and 12 years of age, there was a significant decrease in the plasticity measure across clinicial groups: From the first 150 tones to the last 150 tones, the N100 decreased by a mean of 2.16 μV (0.68) among the HC group ($n = 10$), 0.58 μV (1.30) among the CHR group ($n = 9$), and 0.15 μV (1.65) among the PS group ($n = 15$), $R^2 = .20$, $F(1, 32) = 7.98$, and $p = .008$. Follow-up pairwise analyses revealed that clinical group status approached signficance as a predictor of plasticity when comparing the HC and CHR groups, $\beta = -0.43$, $t = -1.95$, and $p = .068$, and

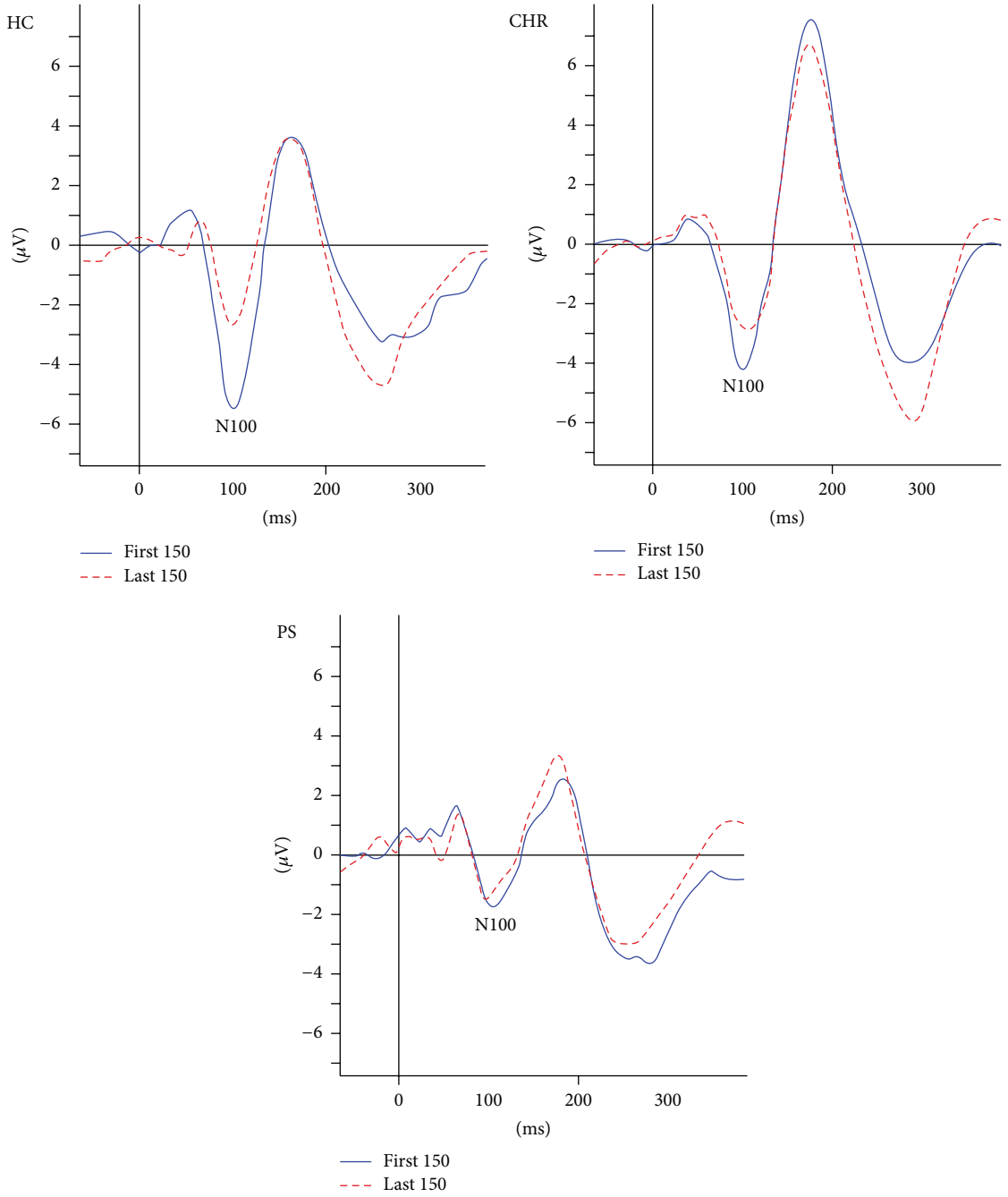

Figure 2: Average auditory N100 response for first and last 150 tones for full sample by clinical group. Auditory stimulus administered at 0 milliseconds. HC: healthy control; CHR: clinical high risk; PS: psychosis. HC > CHR, PS on the difference in the average N100 response to first versus last 150 tones.

was a significant predictor when comparing the HC and PS groups, $\beta = -0.49$, $t = -2.69$, and $p = .013$. Clinicial group status was not a significant predictor when comparing the CHR and PS groups, $\beta = -0.14$, $t = -0.67$, and $p = .513$.

When initial N100 amplitude was added to the model predicting plasticity, it was significant, $\beta = 0.35$, $t = 2.06$, and $p = .048$, and clinical group status approached significance, $\beta = -0.29$, $t = -1.72$, and $p = .095$. In follow-up pairwise analyses comparing the HC and CHR groups, initial N100 amplitude was significant, $\beta = 0.45$, $t = 2.26$, and $p = .038$, and clinical group status approached significance, $\beta = -0.36$, $t = -1.83$, and $p = .085$. When comparing the HC and PS groups, initial N100 amplitude approached significance, $\beta = 0.39$, $t = 1.94$, and $p = .065$, and clinical group status was not significant, $\beta = -0.29$, $t = -1.47$, and $p = .156$; when clinical group status was removed, initial N100 amplitude emerged as a significant predictor, $\beta = 0.54$, $t = 3.05$, and $p = .006$. When comparing the CHR and PS groups, neither clinical

group, $\beta = -0.03$, $t = -0.15$, and $p = .885$, nor initial N100 amplitude, $\beta = 0.28$, $t = 1.22$, and $p = .237$, was predictive of plasticity.

3.3.2. Plasticity by Clinical Group among Older Participants (13 to 17 Years). Among participants (13 to 17 years of age), the plasticity measure was greatest in the HC group but did not decrease in a linear fashion across clinicial groups: From the first 150 tones to the last 150 tones, the N100 decreased by a mean of 2.49 μV (2.15) among the HC group ($n = 7$), 0.73 μV (1.13) among the CHR group ($n = 20$), and 1.00 μV (1.40) among the PS group ($n = 7$), $R^2 = .10$, $F(1, 32) = 3.47$, and $p = .072$. Therefore, follow-up analyses were not conducted for the older subgroup.

4. Discussion

The goal of this study was to examine changes in the N100 response to repeated auditory stimulation as a potential biomarker sensitive to the neurological dysfunction that underlies clinical high risk (CHR) and progression to psychosis (PS). To the best of our knowledge, this is the first study to examine changes in the N100 response among a pediatric sample of patients at risk for or with PS. The findings suggest that impaired plasticity in the N100 is evident among both CHR and PS patients, particularly among younger (i.e., aged 5 to 12 years) patients, and is largely determined by decreased initial N100 amplitude in these patient groups. When analyses were conducted using the full sample, results showed a decrease in plasticity across clinical groups, with the HC group showing the greatest change in the N100 response across trials, followed by the CHR group, and then the PS group. Follow-up analyses showed significant differences in plasticity between the HC and CHR groups and the HC and PS groups, though these differences were diminished to nonsignificance for the latter comparison when the initial N100 response was considered. Only the initial N100 response accounted for differences between the CHR and PS groups.

When considered separately, the younger subgroup (5 to 12 years) demonstrated a decrease in plasticity across clinical groups from HC to CHR to PS. In follow-up analyses, the difference in the plasticity measure was significant between the HC and PS groups and approached significance between the HC and CHR groups. When initial N100 amplitude was considered, the plasticity difference between the HC and PS groups was partially explained by the decreased initial N100 amplitude of the PS patients. In the older subgroup, plasticity did not decrease in a linear fashion across clinical groups. Overall, the findings showed greater attenuation in the N100 repetition suppression response among the CHR and PS groups compared to the HC group, particularly among the younger subgroup. Notably, the CHR and PS groups did not differ in any of the pairwise analyses, suggesting that plasticity deficits associated with psychosis that are detectable via this measure are present early, during the CHR stage.

The findings also suggest that plasticity impairments among CHR and PS participants were largely driven by a decreased initial N100 response. Evidence for a blunted N100 response is consistent with studies in adults [4, 28, 31–36]. Possible explanations for this pattern of results include that the plasticity mechanisms measured by N100 repetition suppression were saturated before the repetition of the auditory stimulus, that these plasticity mechanisms are defective, whether due to NMDAR receptor mediated dysfunction or some other mechanism, that there is decreased sensory responsiveness that leads to underactivation of the plasticity mechanisms, and/or that there is a decreased number of synapses available to be altered by repetition of the sensory response, that is, a floor effect. The final hypothesis is consistent with the observation of low spine densities on the basilar dendrites of pyramidal neurons and decreased neuropil in postmortem cortical samples from patients with SZ, especially in cortical layers subserving corticocortical and thalamocortical connectivity [53]. This hypothesis is also consistent with findings in animal models of SZ (e.g., the NLHV rat model) that have demonstrated reduced numbers of neurons in the auditory cortex [41].

This study has limitations. Its small sample size restricted statistical power, particularly for subgroup analyses. Several of the tests for pairwise clinical group differences approached significance and may have achieved significance in a larger sample. The sample was almost exclusively non-Hispanic White, and the PS subsample was largely male. We attempted to minimize the role of intelligence in contributing to group differences on N100 measures by excluding individuals with intellectual disability. However, future studies should match participants on IQ and/or control for IQ in analyses, as intellectual capabilities are associated with N100 responses [54]. The CHR group was older than the HC and PS groups; however, age was not a significant predictor of the plasticity measure. Although we considered medication usage as a covariate in analyses, we cannot rule out medication effects given that a significant proportion of the PS and CHR patients were taking medication and no HC participants were medicated. Another limitation relates to the assessment of CHR in pediatric populations. Although childhood onset of prodromal symptoms is not rare [55], identification of CHR in pediatric populations is less reliable than in adults [56]. Furthermore, the predictive validity of the SIPS, particularly in children under the age of 10 years, is not established. Finally, the PS group was not limited to DSM-IV SZ due to (a) difficulty in determining if patients with early psychosis would settle into a categorical diagnosis of SZ or an affective psychosis [57, 58] and (b) increasing biological evidence for heterogeneity among patients with SZ and for pleiotropy in the phenotypic expressions of SZ risk alleles [59]. Consequently, we employed a Research Domain Criteria (RDoC) approach to participant selection, as recently advocated by the National Institute of Mental Health [60].

5. Conclusions

The current findings offer evidence of deficits in repetition suppression of the N100 response in early onset (<18 years old) and very early onset (<13 years old) CHR and PS. These findings warrant further study to determine the usefulness of

repetition suppression of the N100 response as a biomarker of deficits in brain plasticity in CHR and PS. Furthermore, these cross-sectional findings indicate the need to follow participants longitudinally to assess the stability of the N100 plasticity measure in CHR and PS patients and to determine if more pronounced sensory adaptation deficits predict which CHR patients progress to psychosis. Additional study is also needed to determine whether this measure is responsive to target engagement by therapies meant to reverse plasticity deficits that may underlie the development of psychosis. If supported by longitudinal studies, measurements of sensory adaptation deficits may be useful outcomes for early clinical trials of proposed therapies for CHR and PS.

Disclaimer

None of the funding agencies had any role in the study design, the collection, analysis or interpretation of data, the writing of the paper, or the decision to submit the paper for publication. The content is solely the responsibility of the authors and does not represent the official views of any funding entity.

Conflict of Interests

In the past 3 years, Joseph Gonzalez-Heydrich has received grant support from the Tommy Fuss Fund, the Al Rashed Family, and GlaxoSmithKline. In previous years, he served as a consultant to Abbott Laboratories, Pfizer Inc., Johnson & Johnson (Janssen, McNeil Consumer Health), Novartis, Parke-Davis, GlaxoSmithKline, AstraZeneca, and Seaside Therapeutics; was a speaker for Abbott Laboratories, Pfizer Inc., Novartis, and Bristol-Meyers Squibb; and received grant support from Abbott Laboratories, Pfizer Inc., Johnson & Johnson (Janssen, McNeil Consumer Health), and Akzo-Nobel/Organon. He is a founder, equity holder, and consultant to Neuro'motion Labs and is an inventor on a patent pending for technologies to enhance the development of emotional regulation. Alvaro Pascual-Leone serves on the scientific advisory boards for Nexstim, Neuronix, Starlab Neuroscience, Neuroelectrics, and Neosync and is listed as an inventor on several issued and pending patents on the real-time integration of transcranial magnetic stimulation (TMS) with electroencephalography (EEG) and magnetic resonance imaging (MRI). Alexander Rotenberg has served as an advisor for Nexstim Inc., Sage Therapeutics Inc., and NeuroRex Inc. and has joint grants with Brainsway Inc. and Vivonics Inc. He is a founder, equity holder, and consultant to Neuro'motion Labs and is an inventor on a patent pending for technologies to enhance the development of emotional regulation.

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Review Article

Are Anxiety Disorders Associated with Accelerated Aging? A Focus on Neuroprogression

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Anxiety disorders (AnxDs) are highly prevalent throughout the lifespan, with detrimental effects on daily-life functioning, somatic health, and quality of life. An emerging perspective suggested that AnxDs may be associated with accelerated aging. In this paper, we explored the association between AnxDs and hallmarks of accelerated aging, with a specific focus on neuroprogression. We reviewed animal and human findings that suggest an overlap between processes of impaired neurogenesis, neurodegeneration, structural, functional, molecular, and cellular modifications in AnxDs, and aging. Although this research is at an early stage, our review suggests a link between anxiety and accelerated aging across multiple processes involved in neuroprogression. Brain structural and functional changes that accompany normal aging were more pronounced in subjects with AnxDs than in coevals without AnxDs, including reduced grey matter density, white matter alterations, impaired functional connectivity of large-scale brain networks, and poorer cognitive performance. Similarly, molecular correlates of brain aging, including telomere shortening, Aβ accumulation, and immune-inflammatory and oxidative/nitrosative stress, were overrepresented in anxious subjects. No conclusions about causality or directionality between anxiety and accelerated aging can be drawn. Potential mechanisms of this association, limitations of the current research, and implications for treatments and future studies are discussed.

1. Introduction

Anxiety disorders (AnxDs) are highly prevalent across the lifespan in the general population. Pooled 1-year and lifetime prevalence have been estimated at around 11% and 17%, respectively [1]. Different AnxDs are more prevalent at specific lifespan stages. Phobias predominate in childhood, panic disorder (PD) predominates in adulthood, and generalized anxiety disorder (GAD) and agoraphobia (AG) predominate in adulthood and older age. AnxDs can also have a late onset, with an incidence of 3-4% after 55–60 years of age [2–4].

AnxDs are chronic and stressful conditions that can negatively affect quality of life, somatic health, and cognitive performance. Several studies documented that anxiety is a risk factor for many age-related medical conditions, such as coronary heart disease, diabetes, and disability, as well as for global mortality [5–7]. Recent findings showed an association between AnxDs or anxiety symptoms and reduced verbal memory, language, and executive functions in older individuals without dementia [8–11].

An emerging perspective suggested that in people with AnxDs decreased somatic health or cognition may partly result from accelerated cellular aging and neuroprogression. Neuroprogression is pathological reorganization of the central nervous system (CNS), along the course of severe mental disorders, leading to cerebral structural changes and functional alterations. It is a combination of increased neurodegeneration, neuronal apoptosis or neurotoxic susceptibility, and lowered neuroplasticity [12]. Neuroplasticity refers to the ability of the brain to modify itself in response

to environmental demands and it plays an important role in optimizing brain functionality. It encompasses neurogenesis, structural and functional brain reorganization, cellular and molecular changes, and cognitive plasticity [13]. These processes occur throughout the lifespan in response to a wide array of genetic and environmental factors. Neuroplasticity is downregulated in adulthood and old age and its impairment can negatively impact successful aging [14] and cognitive performance [15]. Neuroprogression has been extensively investigated in major depressive disorder (MDD)/bipolar disorder (BD) and several potential mechanisms of neuroprogression have been proposed, including immune-inflammatory and oxidative/nitrosative stress with its concomitants and sequels, dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis, autonomic nervous system (ANS), and immune system or neurotransmitters' functioning (for detailed reviews, see [12, 16–19]). This research on AnxDs is at the early stage. However, some neuroprogressive pathways found in MDD/BD may be present also in subjects with AnxDs and contribute to accelerated aging and neuroprogression in this population [20].

In this paper, we reviewed evidence of an association between AnxDs, according to DSM-5 criteria [21], and hallmarks of accelerated aging, with a focus on neuroprogression. Thus, we explored, in animal and human studies, the overlap between processes of neurogenesis, neurodegeneration, structural, functional, molecular, and cellular modifications in AnxDs, and aging. To the best of our knowledge, no reviews on this issue have been published.

2. Materials and Methods

This is a nonsystematic review. Data were sourced from PubMed electronic database and were not limited by date of publication. Only articles written in English language were considered.

3. Neurogenesis

Neurogenesis refers to the formation, growth, and development of new neurons from neural stem cells and progenitor cells. Adult neurogenesis in humans is restricted to the hippocampus (subgranular and subventricular zones of the dentate gyrus) [22–24].

3.1. Impaired Neurogenesis in Anxiety. Adult hippocampal neurogenesis (AHN) is impaired in rodent models of anxiety, including chronic unpredictable mild stress, repeat restraint stress, social defeat stress, and corticosterone administration, as well as in models of social stress in nonhuman primates, such as the intruder stress and social isolation models. These paradigms trigger anxiety- and depression-like behaviors in animals, suggesting a possible association between both anxiety and depression and altered AHN [25]. In rodent models of childhood neglect (which is a risk factor for future anxiety and mood disorders in humans), young rats separated from their mothers exhibited both increased anxiety and decreased AHN in adulthood [26]. Recently, decreased hippocampal number of neuroblasts and dendritic arborization related to high corticosterone were found in Carioca High-Conditioned Freezing rats, an animal model of generalized anxiety disorder (GAD) [27]. Transgenic mice in which AHN was impaired exhibited significant increased anxiety-like behavior [28, 29]. Finally, in rodents, disrupting AHN negatively affected pattern separation, which is the learning process by which similar experiences are transformed into distinct, nonoverlapping representations [30]. Since pattern separation impairment seems to be implicated in overgeneralization of conditioned fear in AnxDs, an association between reduced AHN and anxiety may exist in humans [31]. Both in stressed rodents [32] and in nonhuman primates, antidepressants, which are the first-line treatment for AnxDs, increase AHN which, in turn, can diminish anxiety-like behavior [33–35].

No published postmortem brains studies have directly or indirectly measured AHN in humans with AnxDs. High-resolution MRI volumetric studies showed smaller dentate gyrus size in subjects with anxiety [25], but the extent to which this may be related to changes in AHN or to other forms of structural plasticity remains to be determined. Finally, no studies are available on the relationship between medications and AHN in individuals with AnxDs.

In summary, animal models showed that altered neurogenesis may be associated with anxiety, but whether accelerated AHN impairment is also related to human anxiety remains an open issue. In clinical samples, direct AHN assessment is needed, and new noninvasive measurements of AHN in humans, such as by SPECT or MRI, are emerging [36, 37]. In light of preclinical data and given that multiple biological alterations in people with AnxDs, including higher levels of corticosteroids [38], and proinflammatory factors [39] and/or lower levels of growth factors [40] have well-known detrimental effects on AHN [41, 42], this field is worth of being further investigated.

3.2. Impaired Neurogenesis in Aging. In animal studies, aging has been associated with significant decline in adult hippocampal neurogenesis (AHN) in rodents [43, 44], canines [45], and marmosets [46]. Several studies showed that AHN in rats decreases by 80% by about one-two years of age [47–49]. Also, in humans, the formation of new neurons is abundant during infancy and adolescence and dramatically decreases during adulthood and especially in old age. Although decreased neurogenesis may exert important protective effects, such as tumor prevention [50], it seems also to be linked to cognitive flexibility impairment in mice [51] and age-related cognitive deficits in humans [52].

In conclusion, preliminary evidence suggests that anxiety may be associated with decreased neurogenesis, similar to what has been observed during aging.

4. Brain Structural Changes

4.1. Brain Structural Changes in Anxiety. In murine models, hippocampal volume and trait anxiety were inversely related [53], and stress-related hypercortisolemia or chronic treatment with corticosterone resulted in hippocampal atrophy and anxiety-like behaviors [54, 55]. In nonhuman primates,

high trait-like anxiety has been associated with smaller volume of the dorsal anterior cingulate cortex (dACC), which is a portion of the prefrontal cortex (PFC) [56]. In humans, several structural neuroimaging studies compared people with AnxDs to healthy controls. In subjects with panic disorder (PD), reduced volume of the temporal lobe, as well as reduced gray matter (GM) density in the amygdala and hippocampus, was found. GM abnormalities have also been found in the bilateral putamen, left orbitofrontal cortex, inferior frontal cortex, superior temporal gyrus, right insula, and anterior cingulate cortex [57, 58]. In GAD, decreased structural connectivity between the amygdala, the anterior cingulate cortex (ACC), and the PFC was found. Other studies showed reduced hippocampal volume, decreased white matter (WM) in the ACC and middle cingulated cortex integrity, and decreased GM volumes in the precentral gyrus, precuneus, orbitofrontal gyrus, and posterior cingulate gyrus [59]. Disrupted WM microstructure coherence of the right splenium and right parietal cortex was also found [60]. Finally, preliminary investigation showed altered structural brain connectivity in patients with social anxiety disorder (SAD) suggesting frontal WM alteration in or near the uncinate fasciculus, a structure that connects anterior temporal areas with prefrontal/orbitofrontal cortices [61].

4.2. Brain Structural Changes in Aging. Brain structural alterations accompany normal aging. SAMP10 mice, a strain of inbred mice developed to study human aging, exhibited age-related cortical atrophy in the frontal cortex, occipital lobes, olfactory bulbs, amygdala, and entorhinal cortex [62]. In humans, postmortem and structural neuroimaging findings showed age-related brain atrophy (0.4–0.5% brain tissue loss per year), as indicated by reduced brain volume and weight, ventricular expansion, and sulcal enlargement [63]. Prominent age-related GM loss has been demonstrated both cross-sectionally and longitudinally in the frontal and prefrontal areas, hippocampus, temporal and parietal cortices, amygdala, and cerebellum and was accompanied by shrinkage and dysmorphology of neurons and deafferentation and reduction in synaptic density [64–69]. Structural WM degeneration occurs in the entire brain and mainly in the frontal cortex [70, 71]. Both GM and WM structural alterations are likely to impair communication within and between brain areas and lead to age-related cognitive decline [72].

In conclusion, anxiety has been associated with several brain structural changes, some of which are similar to those observed during aging.

5. Brain Functional Changes

Functional connectivity reflects the quality of information transfer and functional communication between brain areas that increase or decrease their activity synchronically. Among these, a network of brain regions plays a relevant role during resting states: the default-mode network (DMN) (i.e., the "task-negative" network) that consists of the precuneus/posterior cingulate cortex (PCC), medial prefrontal cortex, medial temporal regions, medial, lateral, and inferior parietal cortex, and portions of the ACC, and it is active during internally directed mental states, such as introspective states, remembering, planning, and related cognitive functions, and emotion regulation. The DMN is connected with the "task-positive" network that consists of the dorsolateral prefrontal cortex, inferior parietal cortex, and supplementary motor area and it is associated with task-related patterns of increased attentional orientation and response preparation [73].

5.1. Brain Functional Changes in Anxiety. Impaired functioning of several brain networks involved in cognition and motivation has been found in subjects with anxiety. Individuals with different AnxDs (in particular GAD and SAD) or with high trait anxiety presented decreased functional connectivity among areas of the cinguloopercular and frontoparietal networks compared to controls, resulting in impaired detecting errors/conflicts and cognitive control to resolve future conflicts. Functional changes within the frontoparietal network and between the cinguloopercular and frontoparietal networks and the amygdala were also found [74]. Decreased functioning of the DMN [74] and its functional connectivity with the amygdala has been observed in subjects with AnxDs compared to healthy controls [75, 76]. A recent study comparing subjects with GAD and healthy controls indicated that the presence of GAD, longer duration of illness, and symptoms severity exacerbated the effects of age on decreased functional connectivity in the DMN, in particular between the posterior cingulate and the medial prefrontal cortex and between the PCC and the medial prefrontal cortex [77].

5.2. Brain Functional Changes in Aging. Normal aging is characterized by disrupted coordination of these large-scale brain systems, which may be partly responsible for the cognitive decline during aging. These brain regions are particularly vulnerable to atrophy and amyloid deposition [78]. Poorer cognitive performance in the elderly seems to be a consequence of both increased lateralized intranetwork and decreased internetwork connectivity, which may result in more diffuse and less specialized patterns of functional connections that negatively impact cognition [79–82]. Indeed, in healthy older individuals, several brain imaging studies showed decreased functional connectivity across several regions of the DMN, both at rest and during cognitive tasks, which was associated with impaired performance in processing speed, memory, and executive functions [83–86].

In conclusion, preliminary evidence suggests an association between anxiety and impaired functional connectivity, similar to what has been found during aging.

6. Cognitive Decline

6.1. Cognitive Decline in Anxiety. Both animal and human studies suggested that anxiety may be associated with cognitive changes similar to those observed during normal aging. In mutant mice, anxiety correlated with impaired spatial learning and memory [87]. Transgenic mice with higher levels of corticosterone (an animal model reproducing

hyperactivity of the HPA axis, which is often seen in AnxDs) exhibited learning and memory plasticity deficits [88]. In tree shrews stressful experiences increasing cortisol levels resulted in declarative memory deficits that persisted several weeks afterward despite rebound cortisol levels [89]. Neuropsychological studies on individuals with AnxDs yielded mixed results, probably because of different sampling, methodology, neuropsychological test batteries, and lack of control for confounding variables, such as pharmacological treatments. Preliminary findings suggested that subjects with GAD have poorer performance in processing speed, verbal memory, working memory, cognitive flexibility, and executive functions compared to healthy controls [90–92]. Individuals with PD or SAD exhibited poorer verbal memory, attention, learning, and executive functions [93–98]. In late-life major depressive disorder comorbid AnxDs were associated with greater memory decline at 4-year follow-up [99]. In a sample of older individuals without dementia, anxiety symptoms were associated with memory loss and predicted both cognitive decline and daily-life functioning impairment after 3 years [100]. Anxiety symptoms occurred more frequently in persons with mild cognitive impairment (MCI) than in cognitively intact elderly individuals from the general population and significantly increased risk of progression from MCI to Alzheimer's disease at 3-year follow-up [101]. Finally, in a prospective cohort study of individuals aged 65 to 96 years, incident cognitive impairment was associated with baseline AnxDs in men and with anxiety symptoms in women, independently of depression [102].

6.2. Cognitive Decline in Aging. Advanced aging is accompanied by cognitive decline that is related to structural and functional changes [103]. In healthy older individuals, maintenance of both higher cortical volume and WM complexity has been associated with successful cognitive performance. In the elderly, strong correlations emerged between hippocampal volume and global cognition and memory, between frontal areas volume and executive function [104, 105], and between WM complexity and information processing speed, auditory-verbal learning, and reasoning [106]. Reduced mental speed [107], executive function [108], and episodic memory [109] were found whereas verbal ability and word knowledge were often maintained [110]. As previously described, diminished functional connectivity across several regions of DMN during aging is associated with progressive cognitive decline in several cognitive domains, including attention, concentration, processing speed, memory, and executive functioning [83–85]. The age-related reduced ability to decrease DMN activity when attention is required seems particularly relevant to cognitive and goal-directed activity impairment [111].

In conclusion, AnxDs and aging seem to share reduced cognitive abilities that may be related to the similar structural and/or functional brain changes described above.

7. Beta-Amyloids

Beta-amyloids (Aβ) are protein fragments implicated in neurodegeneration, cellular aging, and cognitive deterioration [112, 113]. At high concentrations, Aβ can negatively influence AHN [114], synaptic functions, and monoaminergic transmission and can have cytotoxic effects and functional antagonism with brain-derived neurotrophic factor (BDNF) [112, 113].

7.1. Beta-Amyloids in Anxiety. In animal studies, a relationship between anxiety and Aβ levels was found. Stress-level glucocorticoids administration in mice increased Aβ production and augmented tau accumulation, suggesting that glucocorticoids, which are also implicated in human AnxDs, may be related to Aβ pathology and development of neurofibrillary tangles (i.e., two neuropathological hallmarks of Alzheimer's disease and severe cognitive decline) [115]. Similarly, behavioral stressors (social isolation over 3 months or acute restraint stress) increased Aβ levels in the brain interstitial fluid, hippocampus, and cortex of mice via corticotropin-releasing factor [116]. Cerebral injection of Aβ fragment in rats exerted profound negative effects on the hippocampus and amygdala and induced both anxiety-like behaviors and memory impairment [117, 118].

Human research on this topic is scant. In middle-aged and older nondemented adults, a PET study found significant associations between trait anxiety symptoms and amyloid senile plaques and tau neurofibrillary tangles in the posterior cingulate of subjects with mild cognitive impairment (MCI) and in the medial temporal and frontal areas of subjects with no cognitive deficits [119]. In subjects with MCI, a significant association was found between Aβ42 and t-tau abnormal concentrations in the cerebrospinal fluid and anxiety symptoms severity [120]. Finally, in a prospective cohort of healthy older adults, anxiety symptoms seem to moderate, with a dose-effect relationship, the negative effects of Aβ on global cognition, resulting in more rapid decline in several cognitive domains [121, 122].

7.2. Beta-Amyloids in Aging. In rhesus monkeys, a significant age-related Aβ increase was found in the basal forebrain cholinergic neurons [123]. Human PET studies showed that about one-third of healthy elderly individuals manifested elevated levels of Aβ deposition in the frontal, cingulated, and parietal areas and in the DMN, even years before clinical cognitive deficits [124, 125]. While some studies failed to report significant associations between amyloid deposition and cognitive decline [126], others found that greater amyloid deposition was negatively related to episodic memory performance and decline in healthy older adults [127–131]. A very recent study [132] showed that normal elderly individuals with high Aβ plasma levels presented lower cognitive performance and thinner cortex than those with low Aβ levels.

In conclusion, the available findings suggest an association between anxiety and Aβ pathology and indicate that this is a critical topic worth of future investigation.

8. Telomere Shortening

Telomeres are specialized DNA-protein complexes found at the ends of chromosomes. Small portions of telomeric DNA are normally lost with time and cell division: when telomeres

get too short, the cell can no longer divide and eventually dies. Telomere shortening is progressive with age and is considered a biomarker of cellular aging/damage and disease [133].

8.1. Telomere Shortening in Anxiety. Both animal and human findings showed an association between anxiety and telomere shortening. Deficiency of telomerase (i.e., an enzyme that preserves telomere length by adding telomeric DNA) resulted in increased anxiety-like behavior in aged transgenic mice when compared with wild-type mice [134]. In human nonpsychiatric samples, associations were found between exposure to chronic stress (e.g., childhood adverse experiences/stressful caregiving status) or high phobic anxiety and accelerated telomere shortening, which may be related to dysregulation of inflammatory markers, HPA axis, and autonomic system function [135–138]. Longitudinal findings demonstrated that AnxDs predicted shorter leukocytes telomeres at 2 years of follow-up in the general population, whereas depressive disorders did not [139], and persistence of internalizing psychiatric disorders, including GAD, from adolescence to adulthood, predicted shorter telomere length at age 38 [140]. Patients with current AnxDs, but not remitted, had shorter leukocyte telomeres compared to healthy controls [20], suggesting that telomere shortening may be partly reversible. Furthermore, anxiety symptoms severity was associated with telomere shortening in the whole sample, suggesting a dose-response association, similar to what was found by Okereke and coworkers [138]. Young women with GAD or PD had shorter telomeres than women with no GAD or PD [141] and older subjects with AnxDs had significantly shorter telomeres than coeval healthy controls [142], suggesting that anxiety may accelerate age-related telomere shortening.

8.2. Telomere Shortening in Aging. Telomere shortening increases with age [133]. Preclinical studies demonstrated that insufficient telomerase activity impairs telomere length restoration, enhancing susceptibility to cellular senescence and death [143]. In adult and old mice with critically short telomeres, dietary supplementation of the telomerase activator TA-65 increased average telomere length and improved many health-span indicators [144].

Human studies also point to a causal relationship between telomere shortening and increased risk of age-related disease, including cancer, diabetes, and coronary heart disease [143, 145, 146]. Since cell or tissue dysfunction is triggered by severe telomere shortening, telomerase activation may promote health maintenance. In humans telomere shortening can be also delayed by telomerase activator dietary supplementation [147] which enhanced several indicators of metabolic, bone, and cardiovascular health (e.g., glycemia, cholesterol, and blood pressure) at 5-year follow-up [148]. Telomeres seem to be involved in neurodegeneration and neurodegenerative diseases as well. Molecular mechanisms of neurodegeneration, such as abnormal levels of Aβ, may accelerate neuronal senescence through telomere attrition [149]. An association between shorter telomeres and poorer cognitive performance has been observed in general elderly populations, suggesting that telomere length may serve as a biomarker of cognitive aging [150, 151]. Telomere shortening is modulated by both genetic and nongenetic factors, including oxidative stress, inflammation, physical activity, and lifestyle [150, 151].

In conclusion, anxiety may be related to shorter telomeres which also characterizes aging and age-related diseases and cognitive decline.

9. Activated Immune-Inflammatory Pathways

Activated immune-inflammatory pathways are considered "core" components of neuroprogressive changes [17]. Cell-mediated immunity (CMI) involves activation of T cells that produce cytokines such as IFN-γ and IL-2, which activate monocytes/macrophages. In turn, monocytes/macrophages produce several cytokines such as IL-1β (exerting a positive feedback loop on T cells), IL-12 (triggering T cells to produce more IFN-γ), TNF-α, IL-6, and IL-8. Inflammation consists of cellular, cytokine, and complement cascades and an acute phase response. Macrophage-derived cytokines, known as proinflammatory cytokines (PICs), mediate inflammation by enhancing the positive acute phase proteins (APPs), for example, C-reactive protein (CRP) and haptoglobin, and lowering the negative APPs, for example, albumin and transferrin. During inflammation, also counter-anti-inflammatory mechanisms become active (e.g., increased production of the IL-1 receptor antagonist) to dampen the primary inflammatory response [12, 152]. Activated immune-inflammatory pathways increase oxidative/nitrosative processes [153] (Figure 1).

9.1. Activated Immune-Inflammatory Pathways in Anxiety. Preclinical and human studies suggested that anxiety is associated with CMI activation and inflammation. Although results are mixed [154], some animal studies showed a relationship between increased proinflammatory cytokines levels including interleukin-6 [155] and IL-1β [156] and anxiety-like behaviors. In mice, sustained inflammatory pain, with concomitant TNF-α increase in basolateral amygdala, was associated with anxiety-like behaviours which was reversed by local infusion of infliximab, a TNF-α neutralizing antibody [157]. In humans, significantly increased levels of proinflammatory cytokines have been detected in patients with AnxDs compared to nonanxious subjects, independently of sociodemographic features and depressive symptoms [39, 158]. Higher inflammatory dysregulation was especially found in persons with late-onset AnxDs [159]. Recently, PD has been associated with lower levels of mannan-binding lectin (MBL), an important arm of the innate immune system, the deficiency of which may result in infections or autoimmune diseases [160]. Plasma anti-serotonin and serotonin anti-idiotypic antibodies are elevated in PD compared to healthy controls, suggesting a link between autoimmune mechanisms and AnxDs [161]. In the general population, anxiety symptoms were associated with increase of several inflammation markers, including C-reactive protein, TNF-α, and IL-6, even after adjusting for multiple confounding factors [162].

Activated immune inflammation may be related to anxiety also through its influence on serotoninergic pathways.

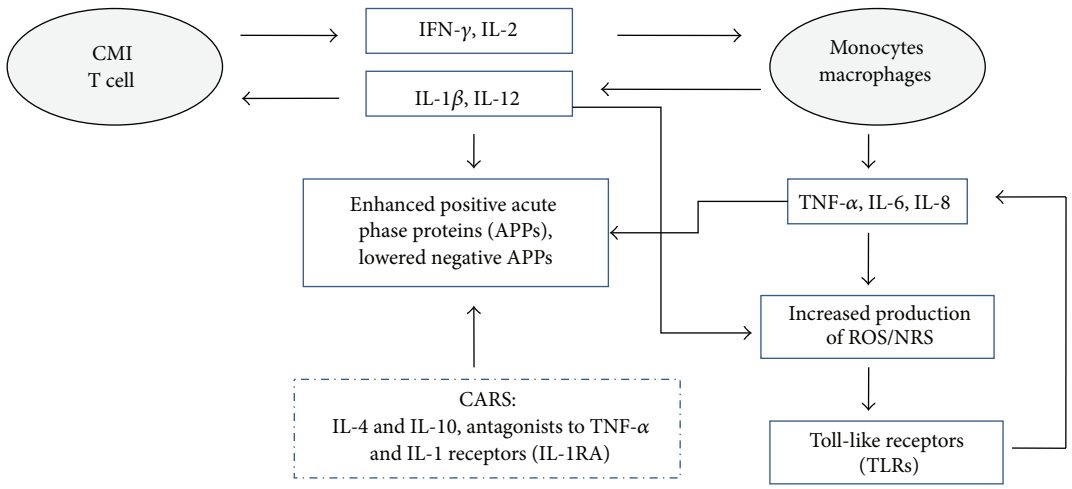

Figure 1: Relationship between activated immune-inflammatory pathways and oxidative/nitrosative stress (O&NS). CMI involves activation of T cells that produce cytokines such as IFN-γ and IL-2, which activate monocytes/macrophages. Monocytes/macrophages produce IL-1β and IL-12 (that exert a positive feedback loop on T cells), as well as TNF-α, IL-6, and IL-8. Proinflammatory macrophage-derived cytokines (PICs) mediate inflammation enhancing the positive acute phase proteins (APPs), for example, C-reactive protein, and lowering the negative APPs, for example, albumin. The counterinflammatory response syndrome (CARS) tends to dampen the acute inflammatory response producing IL-4 and IL-10 (responsible for decreasing TNF-α, IL-1, IL-6, and IL-8) and the antagonists to TNF-α and IL-1 receptors (IL-1RA), which inactivate the cytokine or block the receptors. Immune inflammation and O&NS influence each other. Inflammatory and CMI responses are accompanied by increased production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) while oxidative stress maintains inflammation mainly through the activation of toll-like receptors (TLRs). Damaged macromolecules released during condition of oxidative stress can activate TLRs which produce an inflammatory response whose key mediators are IL-1, IL6, and TNF-α. CMI: cell-mediated immune; IL-6: interleukin-6; IL-1β: interleukin-1β; IL-12: interleukin-12; TNF-α: tumor necrosis factor-α; IFN-γ: interferon-γ; APPs: acute phase proteins; TLRs: toll-like receptors.

During CMI activation, cytokines, mainly IFN-γ, induce indoleamine 2,3-dioxygenase (IDO) [163] which, in turn, stimulates the catabolism of tryptophan leading to its plasma depletion and synthesis of tryptophan catabolites (TRYCATs). The TRYCATs kynurenine and quinolinic acid induced anxiety-like behaviours in animal models [164]. In humans, a correlation between plasma kynurenine concentration and caffeine-induced anxiety has been found [165]. Several studies suggested a relationship between activated immune- inflammatory pathways and increased intestinal permeability, called leaky gut [166]. It is characterized by the weakening of the tight junctions' barrier, formed by epithelial cells, which segregates the luminal bacteria in the gut, and can be produced by inflammatory processes [167] and/or by oxidative stress [168]. When leaky gut is present, Gram-negative bacteria or lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria, is translocated from the gut to mesenteric lymphonodes and, consequently, CMI activation with cytokines release may be elicited. Gut-derived bacterial products, such as LPS, can induce anxiety-related behaviors (e.g., reduced exploratory behavior and social interactions) when administered to rodents [169–171] and cause acute anxiety and cognitive deficits in healthy male volunteers [172]. In line with this, recent animal studies showed that diet-induced changes in the gut microbiota influence long- and short-term memory and cognitive flexibility in mice [173]. LPS effects could partly be related to the LPS-induced elevation of peripheral cytokine that, in turn, may affect amygdala activity [174].

Moreover, LPS-induced inflammation can enhance the IDO activity and the availability of kynurenine, which has been shown to increase anxiety when administered peripherally to mice [175]. Data on TRYCATs and leaky gut/LPS pathways in subjects with AnxDs are still lacking and future studies are warranted.

9.2. Activated Immune-Inflammatory Pathways in Aging. "Inflammaging" refers to the chronic progressive inflammatory status of the brain during aging [176]. In mice, TREM2 expression (an immune receptor involved in suppressing inflammatory responses) increased during aging [177] and protected against aging-related neuroinflammation, neuronal losses, and cognitive impairment [178]. Human investigations showed that elderly people exhibit chronically increased levels of proinflammatory cytokines and reduced levels of anti-inflammatory cytokines [179, 180], which correlated with memory impairment [180] and general cognitive decline [181]. In older nondemented people, MRI studies demonstrated macrostructural brain abnormalities linked to inflammation, including reduced hippocampal and GM volume, global brain atrophy, cortical thinning, and WM hyperintensity, which may partly explain the age-related cognitive decline [182–184]. Recently, an association between reduced microstructural integrity of WM pathways and higher circulating inflammatory markers (i.e., C-reactive protein and tumor necrosis factor-alpha, TNF-α) was found in middle-aged and elderly people, which correlated with higher-order cognitive functions impairment [182]. Neuroinflammation

can be a cause (by generating reactive oxygen and nitrogen species) or a consequence of chronic oxidative stress (OS). Over time, OS triggers a self-perpetuating cycle of chronic neuroinflammation inducing even more OS, leading to neuronal degeneration and cell death [185]. Finally, aging effects on gut microbiota may induce a higher propensity to develop the *Clostridium difficile* infection which enhances local and systemic proinflammatory markers (IL-1β, TNF-α, and CRP) and increases the permeability of gut barrier [186].

In conclusion, anxiety seems to be associated with activated immune-inflammatory pathways, which are also a characteristic of aging.

9.3. Mechanisms by Which Activated Immune-Inflammatory Pathways May Contribute to Accelerated Aging and Neuroprogression. The immune-inflammatory pathways may contribute to accelerated aging and neuroprogression by several mechanisms. In rats, elevated IL-2 levels are associated with neurocognitive impairments, microglial activation, reactive astrogliosis, myelin damage, neuronal loss, and changes in several receptors, such as cholinergic and/or dopaminergic receptors in frontoparietal cortex and hippocampal regions [187, 188]. By inducing IDO activation, elevated IFN-γ may lower serotonin levels (5-HT) and increase TRYCATs, with negative effects on neuronal survival. Indeed, lower 5-HT may negatively affect neurogenesis and BDNF expression in adult mammals [189]. TRYCATs, especially quinolinic acid, may increase production of reactive oxygen species (ROS), induce mitochondrial dysfunctions, exert neurotoxic effects by acting as NMDA-receptor agonists, and cause hippocampal cell death and reduction in cerebral cholinergic circuits in rodents [190–192]. Recently, activation of the kynurenine pathway has been shown to affect hippocampal neurogenesis in humans [193]. Increased levels of IL-6 may have neurodegenerative effects in mice [194] and IL-1β may exert neurotoxic effects with neuronal death [195, 196], impair hippocampal neurogenesis [197], and reduce BDNF expression [198]. TNF-α may potentiate glutamate neurotoxicity and silence cell survival signals [199]. LPS can cause cell death by inducing apoptosis and increasing levels of ROS and reactive nitrogen species (RNS) [200]. Finally, activated immune-inflammatory pathways are implicated in Aβ formation [201], telomere shortening [202, 203], and increasing of O&NS [153] that, in turn, is highly implicated in aging and neuroprogression (see the following sections) (Figure 2).

10. Oxidative/Nitrosative Stress

Oxidative/nitrosative stress (O&NS) may come from free radicals (FR) (superoxide, hydroxyl radical) or nonradical molecules, like hydrogen peroxide, and their derivatives, that is, reactive oxygen species (ROS) and reactive nitrogen species (RNS). Inflammation and mitochondrial processes are sources of ROS and RNS. Under normal conditions, the potentially damaging effects of increased ROS and RNS are counterbalanced by enzymatic and nonenzymatic antioxidant defense systems [16]. Activation of O&NS occurs when excess of ROS/RNS and/or compromised antioxidant mechanisms are present. Consequently, O&NS may damage cellular structures such as DNA, lipids (including omega-3 PUFAs), proteins, mitochondria, and cell membranes, up to cellular death [204]. These processes alter the endogenous fatty acids and proteins and may render them immunogenic, inducing autoimmune responses against these modified antigenic determinants (neoepitopes) that lead to a vicious circle resulting in additional cell dysfunctions or death [205]. Finally, O&NS activates immune-inflammatory pathways [153] (Figure 1).

10.1. Oxidative/Nitrosative Stress in Anxiety. O&NS seems to be involved in the pathogenesis of AnxDs [206]. In murine models, several paradigms inducing distress and anxiety-like behaviors resulted in decreased activity of antioxidant enzymes with increased oxidative damage to lipids, proteins, and DNA in multiple brain areas, such as the hippocampus, prefrontal cortex, and cerebellum. Gene expression and proteomic studies in various mice models of anxiety also showed connections between high anxiety and dysregulated expression of several proteins related to oxidative stress metabolisms [207]. Direct induction of high oxidative stress in rats or knockout mice models induced increased anxiety-like behaviors [207, 208], while antioxidant treatments reduced both oxidative stress markers and anxiety-like behaviors [208]. Similarly, indirect induction of oxidative stress via acute sleep deprivation caused anxiety-like behaviors and memory impairment in rats [209]. In mice, deficiency of the antioxidant vitamin E increased oxidative stress and anxiogenic behaviors [210]. A diet rich in ω3 eicosapentaenoic acid (EPA) (an omega-3 PUFA that is located in cellular membranes, has anti-inflammatory properties, and is damaged by O&NS [211]) reduces the development of anxiety-like behaviors in rats as well as normalizing dopamine levels in their ventral striatum [212]. Changes in mitochondrial energy metabolism and function related to O&NS have been associated with anxiety in preclinical studies. In a trait anxiety mouse model, high anxiety-related behaviors were associated with mitochondrial dysfunction in cingulated cortex with enhanced oxidative stress lipid peroxidation and cell death [213]. Finally, anxiety-like behaviors exhibited by rodents during aging may be partly due to increased oxidative stress levels [214].

Findings in humans with AnxDs are mixed, probably due to methodological differences among studies and several confounding factors that may influence oxidative markers and pathways [207]. However, most studies supported the hypothesis of a connection between anxiety and increased oxidative stress. Individuals with lower serum ω3 or with a higher ω6/ω3 ratio (ω6 has proinflammatory effects) have significantly higher stress-induced anxiety levels and TNF-α and IFN-γ responses compared to those with higher serum ω3 and a lower ω6/ω3 ratio [211, 215]. In line with this, ω3 supplementation reduced inflammation and anxiety among healthy young adults who faced stressful major examination [216]. In patients with SAD [217, 218] and PD [219], increased levels of blood lipid peroxidation (a marker of oxidative stress-related cellular damage) were found. Adult subjects with PD [220], with PD, and with agoraphobia [221]

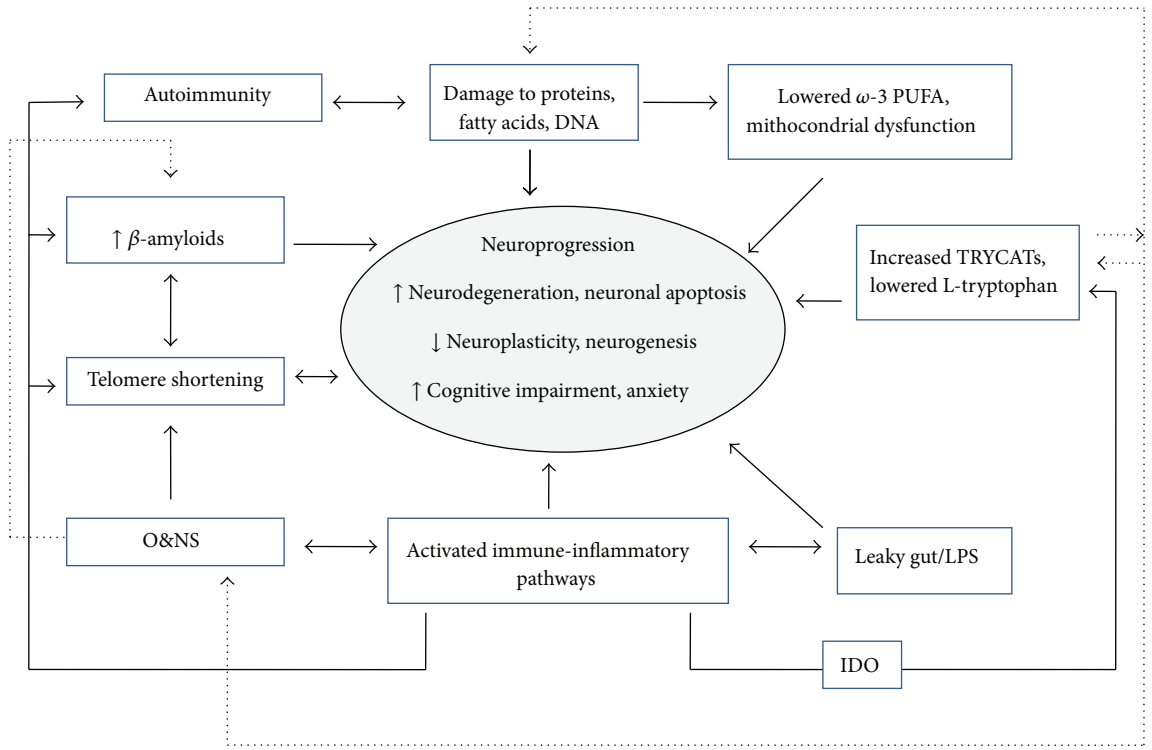

Figure 2: A global model of neuroprogressive changes in aging and anxiety. CMI and inflammatory responses are accompanied by activation of O&NS with production of increased ROS/RNS. ROS/RNS can react with proteins, fatty acids (including ω-3 PUFAs), and DNA and change their chemical structure which became immunogenic and produce an autoimmune response. Both O&NS and CMI inflammation are implicated in beta-amyloid formation and telomeres shortening. Moreover, increased O&NS impairs mitochondrial function with further production of ROS and macromolecular damage. PICs activate IDO which causes depletion of tryptophan/5-HT and the synthesis of tryptophan catabolites (TRYCATs). Some of these TRYCATs (kynurenine and quinolinic acid) are anxiogenic and neurotoxic. The lipopolysaccharide (LPS), caused by bacterial translocation from the gut, may aggravate existing inflammation and O&NS or trigger a primary inflammatory response. LPS and lowered ω3 are associated with decreased neurogenesis. CMI: cell-mediated immune; PICs: proinflammatory cytokines; IL-1β: interleukin-1β; IL-6: interleukin-6; TNF-α: tumor necrosis factor-α; IFN-γ: interferon-gamma; O&NS: oxidative and nitrosative stress; ROS: reactive oxygen species; RNS: reactive nitrogen species; IDO: indoleamine 2,3-dioxygenase; TRYCATs: L-tryptophan catabolites; LPS: lipopolysaccharide (a component of the outer membrane of Gram-negative bacteria); ω-3 PUFAs: omega-3 polyunsaturated fatty acids.

and children and adolescents with AnxDs [222] exhibited impaired oxidative balance and higher oxidative stress. In patients with GAD, decreased levels of the antioxidant serum free sulphydryl were found, which negatively correlated with disease duration [223]. Finally, preliminary, non-placebo-controlled studies showed that selective serotonin reuptake inhibitors (SSRIs) treatment (the first-line drug therapy for AnxDs) decreased both oxidative stress and clinical symptoms in subjects with PD and SAD [217, 220], suggesting that oxidative stress may be a state condition related to the anxious symptomatology.

10.2. Oxidative/Nitrosative Stress in Aging. O&NS contributes consistently to aging. Animal studies showed that reducing oxidative stress/damage increased the healthy period of life [224–226], while a chronic deficit of the antioxidant vitamin C accelerated oxidative stress and amyloid deposition during normal aging [227]. In humans, oxidative stress has been associated with cognitive dysfunction during normal aging [228], in mood disorders [229, 230], and in schizophrenia [231]. The O&NS-induced damage of membrane ω3 is thought to be implicated in aging. Indeed, the ω3 eicosapentaenoic acid (EPA) has a protective effect on neurons [232] and experimental evidence indicated that ω3 docosahexaenoic acid- (DHA-) enriched diet can protect the brain from cognitive decline in aged rats [233]. In humans, a recent meta-analysis exploring the association between ω3 and risk of cognitive decline in elderly individuals has shown that daily doses from 400 to 1800 mg (for 3–40 months) may significantly decrease the cognitive decline [234].

Finally, oxidative damage to mitochondrial functions and macromolecules are thought to play key roles in aging processes. According to the mitochondrial theory of aging [235], ROS-induced mutations of mitochondrial DNA increase over the lifespan and alter mitochondrial respiratory function leading to further increasing of ROS and damage to DNA as well as to other macromolecules, up to irreversible cellular senescence.

In conclusion, anxiety seems to be associated with increased O&NS, which is also implicated in processes of aging.

10.3. Mechanisms by Which Oxidative/Nitrosative Stress May Contribute to Accelerated Aging and Neuroprogression. O&NS may contribute to accelerated aging and neuroprogression by multiple mechanisms. Damage by O&NS involves lipid peroxidation, oxidatively induced protein and DNA alterations, altered neuronal signaling, and neuronal apoptosis [236]. O&NS-induced lower ω3 PUFAs may be associated with decreased neurogenesis, since ω3 PUFAs have beneficial effects on serotonin metabolism stimulating neurogenesis, increase BDNF expression, and exert anti-inflammatory activity [232, 237]. O&NS processes may also cause damage to mitochondria, which play a central role in energy production (in form of adenosine triphosphatase, ATP), are involved in metabolism of amino acids, lipids, and steroids, and regulate free radicals' levels, intracellular calcium concentration, and processes implicated in synaptic development and cell death [238]. Mitochondrial dysfunction impairs neural progenitor cell function [239] and may affect several brain functions by decreasing ATP production. Indeed, high levels of energy are needed for brain activities, including synaptic remodelling, signal transduction, and maintenance of transmembrane potential [240], and deficiency of ATP may lead to activation of the apoptotic cell death program [241]. Disrupted mitochondrial function also provokes mitochondrial-derived hyperproduction of ROS that causes a self-perpetuating cycle of O&NS, inducing even more mitochondrial and macromolecule damage, up to neuronal degeneration and cell death [242]. Finally, O&NS activates immune-inflammatory pathways [153], can induce accelerated telomere shortening and reduce telomerase activity [243, 244], and may be implicated in Aβ formation [245, 246] (Figure 2).

11. Discussion

In this paper, we explored the association between AnxDs and hallmarks of accelerated aging, with a focus on neuroprogression. We reviewed animal and human findings that suggest an overlap between processes of impaired neurogenesis, neurodegeneration, structural, functional, molecular, and cellular modifications in AnxDs, and aging. A putative global model of neuroprogressive changes in aging and anxiety is summarized in Figure 2. Although several studies pointed to a model of accelerated aging and neuroprogression for depression and bipolar disorder [12, 16, 19, 247–249], this research on AnxDs is at an early stage and some caveats should be taken into account. First, since the available data are very limited, we considered AnxDs as a group and reported findings also from nonclinical populations. However, neurobiological mechanisms implicated in the different AnxDs or in subjects with anxiety symptoms but without full-blown disorders do not completely overlap, and AnxDs differ in their incidence across lifespan [3]. Future studies are needed to investigate the specific association of each anxiety disorder with accelerated aging and whether differences exist between clinical and nonclinical populations. Second, brain imaging studies on AnxDs yielded mixed findings due to sampling, methodology, and AnxDs heterogeneity. All the same, a detailed description of these inconsistencies was beyond the scope of this paper. In line with our aim, we only reported evidence common to both anxiety and aging. Third, available animal models of anxiety are based on different theoretical constructs and to date their translational validity is still debated [250]. Thus, parallelisms between animal and human studies should be considered with caution. Fourth, multiple genetic, environmental, and individual factors may influence the biological processes involved in aging and neuroprogression, such as immune-inflammatory pathways, O&NS, telomere shortening, and Aβ generation [149]. Since the role of these confounding factors has not been exhaustively investigated, the findings of an association between these processes and the AnxDs should be considered with prudence. Finally, most studies were cross-sectional; thus, it was not possible to clarify any causal path between anxiety and aging. Considering these limitations, our review suggests a link between anxiety and accelerated aging across multiple processes involved in neuroprogression. Several brain structural and functional changes that accompany normal aging were more pronounced in subjects with AnxDs than in coevals without AnxDs, including reduced GM density, WM alterations, impaired functional connectivity of large-scale brain networks (in particular the DMN), and poorer cognitive performance. Preliminary prospective findings suggested that, in older individuals, anxiety symptoms are risk factor for accelerated cognitive decline, independently of depression. Similarly, molecular correlates of brain aging, such as telomere shortening, Aβ accumulation, immune-inflammatory pathways, and O&NS, were overrepresented in anxious subjects compared with coeval nonanxious subjects, especially when anxiety was severe and long-lasting. These preliminary results do not allow drawing any conclusion about causality or directionality between anxiety and accelerated aging, and future longitudinal studies are needed to shed some light on this issue. Several scenarios are possible: for example, (1) AnxDs may accelerate age-related molecular processes resulting in precocious brain structural changes and functional decline; (2) age-related processes may lead to AnxDs over time; (3) aging and AnxDs may reciprocally influence each other and/or may share some genetic and/or environmental factors which may increase vulnerability to both AnxDs and accelerated aging with neuroprogression. According to the first hypothesis, the sustained arousal and neurobiological sensitivity to different threats in anxious subjects might cause the prolonged activation of HPA axis and ANS, which, in turn, may result in increased immune-inflammatory and oxidative/nitrosative stress (IO&NS) with a self-perpetuating chronic cycle leading to telomere shortening, precocious cellular aging, neurodegeneration, and impaired neuroplasticity [137, 251, 252]. On the contrary, according to the second hypothesis, age-related molecular changes and aging of the human brain may engage biological mechanisms similar to those implicated in anxiety, such as dysregulation of HPA axis, increased IO&NS, and impaired limbic-frontal areas connectivity. Thus, age-related processes,

in combination with environmental/genetic factors, may promote the development of at least some AnxDs in vulnerable individuals [253]. This hypothesis fits with the idea that AnxDs may be neurodevelopmental disorders occurring at different lifespan stages and with the higher prevalence of some AnxDs, such as GAD, in adulthood and older age [3]. Finally, according to the third hypothesis, mutual amplifications are likely implicated in the biological processes of anxiety and aging. Indeed, both conditions are accompanied by activation of IO&NS pathways, which exhibit reciprocal reinforcement and, in turn, contribute to telomere shortening, accelerated aging, and neuroprogression [203, 207, 254]. Immune-inflammatory pathways are involved in both anxiety and cognitive decline, also by stimulating the HPA-axis function with cortisol release that modulates anxiety behavior and exerts detrimental effects on cognition [255].

In conclusion, preliminary evidence indicated an association between AnxDs and hallmarks of accelerated aging with phenomena of neuroprogression. Withal additional animal and human research is needed to satisfactorily elucidate these questions.

11.1. Implications for Treatment and Future Research. AnxDs are complex diseases which tend to be chronic when not adequately treated. Unfortunately, even evidence-based treatments, such as cognitive-behavioral therapy and SSRIs, are often not able to produce full remission and the rate of relapses after drug discontinuation is significant [256]. The theoretical framework of an association between accelerated senescence, neuroprogression, and anxiety may suggest some implications and strategies to fill these gaps. In addition to clinical symptoms of AnxDs, the use of biomarkers (such as inflammatory, oxidative, and telomere length markers) and cognitive assessment may help to better characterize the patients' profiles and clinical stages and allow more personalized treatments. The modifications of these markers during treatments may render the treatments more efficacious and represent reliable treatment-outcome predictors. Moreover, treatments specifically targeting these mechanisms, including both pharmacological and nonpharmacological "anti-aging" interventions, may increase the rate of favorable outcomes. Indeed, preclinical studies suggested that some drugs currently used for AnxDs normalize some hallmarks of accelerated aging and exert a neuroprotective effect. In mice, alprazolam, zolpidem, and buspirone ameliorated the oxidant/antioxidant balance decreasing nitrite concentration and lipid peroxidation in the brain [257] and the SSRI fluoxetine reversed the decreased activity of telomerase in the hippocampus induced by chronic mild stress [258]. SSRIs promoted synaptic plasticity and neurogenesis in mice, probably by increasing BDNF, improved spatial memory learning [259], and facilitated learning and memory during aging [260]. In rats, treatments with antioxidants reduced both oxidative stress and anxiety-like behaviors [261] and in older animals increased serotonin levels [262]. In humans, preliminary data showed that SSRIs reversed high oxidative stress in patients with depression, PD, or SAD [217, 220, 263], promoted hippocampal neurogenesis [264] in depressed subjects, and decreased Aβ production in the cerebrospinal fluid of healthy individuals [265]. Finally, successful pharmacological treatment with the SSRI escitalopram in late-life GAD was associated with episodic memory and executive functioning improvement [90]. Nonpharmacological treatments may include physical activity and nutritional interventions. In mice, physical activity increased telomerase activity and cognitive performance [266, 267] and decreased both oxidative stress and anxiety-like behaviors [208]. In humans, physical activity increased brain volume [268] and preserved cognitive functions in healthy older adults [269], improved comorbid anxiety and executive functioning impairment [270], and has been proposed as a neuroprotective strategy with antioxidant properties [271]. Recent investigations suggested that Mediterranean dietary pattern slowed cognitive decline and improved cognitive performance [272, 273] by reducing inflammation markers [274] and oxidative damage [275]. Higher intake of processed and unhealthy foods was associated with increased anxiety in a population-based study [276], while a healthier dietary pattern was associated with a reduced likelihood of anxiety or depressive disorders [277]. Finally, improvement in both cognition and anxiety was exerted by resveratrol, a component of grapes with important antioxidant properties [278]. Future studies should investigate whether treatments with "antiaging" properties may be beneficial to patients with AnxDs with hallmarks of accelerated aging and neuroprogression.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Andrographolide Stimulates Neurogenesis in the Adult Hippocampus

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Andrographolide (ANDRO) is a labdane diterpenoid component of *Andrographis paniculata* widely used for its anti-inflammatory properties. We have recently determined that ANDRO is a competitive inhibitor of glycogen synthase kinase-3β (GSK-3β), a key enzyme of the Wnt/β-catenin signaling cascade. Since this signaling pathway regulates neurogenesis in the adult hippocampus, we evaluated whether ANDRO stimulates this process. Treatment with ANDRO increased neural progenitor cell proliferation and the number of immature neurons in the hippocampus of 2- and 10-month-old mice compared to age-matched control mice. Moreover, ANDRO stimulated neurogenesis increasing the number of newborn dentate granule neurons. Also, the effect of ANDRO was evaluated in the APPswe/PS1ΔE9 transgenic mouse model of Alzheimer's disease. In these mice, ANDRO increased cell proliferation and the density of immature neurons in the dentate gyrus. Concomitantly with the increase in neurogenesis, ANDRO induced the activation of the Wnt signaling pathway in the hippocampus of wild-type and APPswe/PS1ΔE9 mice determined by increased levels of β-catenin, the inactive form of GSK-3β, and NeuroD1, a Wnt target gene involved in neurogenesis. Our findings indicate that ANDRO stimulates neurogenesis in the adult hippocampus suggesting that this drug could be used as a therapy in diseases in which neurogenesis is affected.

1. Introduction

Andrographolide (ANDRO) is a labdane diterpenoid that is one of the main constituents of *Andrographis paniculata* [1, 2], a well-known medicinal plant widely used in Asia. ANDRO possesses a wide range of biological activities including anti-inflammatory action [3–5] and has also shown neuroprotective properties [6, 7]. More recently, it was also determined that ANDRO prevents neuropathological changes and improves spatial memory in the APPswe/PS1ΔE9 mouse model of Alzheimer's disease (AD) [8].

Regarding the mechanism of action of ANDRO, we recently determined that it directly inhibits the enzyme glycogen synthase kinase-3β (GSK-3β) [9], a key enzyme of the Wnt/β-catenin signaling pathway. The Wnt signaling pathway is activated by the binding of a Wnt ligand to seven-pass transmembrane receptors Frizzled, which may activate the canonical Wnt/β-catenin signaling pathway or noncanonical β-catenin-independent signaling cascades [10]. The Wnt signaling cascade regulates the development of the nervous system but also is an important modulator of adult nervous system regulating the formation of synaptic contacts,

neurotransmission, and plasticity [11–13]. In addition, it has been more recently demonstrated that the Wnt signaling pathway regulates the formation of new neurons in the adult hippocampus (reviewed in [14]), a process known as adult neurogenesis.

Adult neurogenesis occurs mainly in two brain regions, the subventricular zone in the wall of the lateral ventricles and the subgranular zone (SGZ) in the hippocampal dentate gyrus [15, 16]. In the SGZ, neural progenitor cells located between the granule cell layer (GCL) and the hilus proliferate and give rise to neuroblasts that mature into granule cells that integrate into the hippocampal circuitry. These newborn neurons are relevant for hippocampal plasticity, learning, and memory [17–19]. Neurogenesis in the SGZ is controlled by the Wnt/β-catenin signaling pathway, which regulates proliferation, differentiation, and maturation of newborn neurons [20–23], effects mediated by the expression of Wnt target genes [24, 25]. Considering that ANDRO activates the Wnt signaling pathway and the transcription of Wnt target genes [9], in the present study we investigated the effect of ANDRO treatment on neurogenesis in the adult hippocampus. We determined that ANDRO increased cell proliferation and the generation of newborn mature granule cells in young and aged wild-type mice. In addition, ANDRO increased the proliferation and the density of immature neurons in APPswe/PS1ΔE9 mice. In both, wild-type and APPswe/PS1ΔE9 mice, ANDRO increased the activation of the Wnt signaling pathway in the hippocampus and increased the expression of the proneural Wnt target gene NeuroD1.

2. Materials and Methods

2.1. Animals and Treatment. Two- and ten-month-old wild-type C57BL/6 mice were injected intraperitoneally (i.p.) with 0.2% DMSO in saline solution (vehicle) alone or vehicle with 2 mg kg^{-1} ANDRO (Sigma-Aldrich) 3 times a week for 2, 4, or 6 weeks. Seven-month-old APPswe/PSEN1ΔE9 (stock #004462, The Jackson Laboratory) were injected i.p. with 2 mg kg^{-1} ANDRO or vehicle 3 times a week for 4 weeks. 5-Bromo-2′-deoxyuridine (BrdU, Sigma-Aldrich) was injected i.p. at 100 mg kg^{-1} for 1 or 3 days. All animals had access to water and food *ad libitum*, in a 12 : 12 h light/dark cycle. The Bioethical Committee of Pontificia Universidad Católica de Chile approved all procedures involving experimentation on animal subjects.

2.2. Perfusion and Postfixation. Animals were anesthetized (100 μg ketamine + 10 μg xylazine in 10 μL saline/g) and then transcardially perfused with saline, followed by 4% paraformaldehyde (PFA, Sigma-Aldrich) in PBS. The brain was removed and placed in a vial with 4% PFA in PBS for 24 h at room temperature, dehydrated in 30% sucrose, and kept at 4°C until analysis.

2.3. Tissue Sectioning. After dehydration, brains were sectioned on a cryostat in 12 sets of serial coronal slices of 40 μm thickness (Leica Microsystems) and collected in ice-cold PBS in multiwell dishes as previously described [26]. Each set contained 5–7 slices covering the entire length of the hippocampus and therefore corresponds to a representative sampling of the whole hippocampus.

2.4. Immunofluorescence. Immunodetection of BrdU and neuronal markers in tissue sections was carried out as previously described in [26]. Primary antibodies used were rat anti-BrdU (Abcam), rabbit anti-doublecortin (Cell Signaling Technology Inc.), monoclonal anti-NeuN (Millipore, Billerica, MA, USA), rabbit anti-Ki67 (Abcam), goat anti-NeuroD1 (Santa Cruz Biotechnology, Inc.), monoclonal anti-Nestin (Millipore), Alexa (Molecular Probes, Life Technologies), and DyLight (Abcam) conjugated secondary antibodies were used. Slices were mounted on gelatin-coated slides with Fluoromount-G (Electron Microscopy Sciences).

2.5. Image Analysis. For quantification, BrdU, Ki67, or DCX-positive cells were counted using a fluorescence microscope (Olympus BX51, Tokyo, Japan) as described in [26]. Briefly, total numbers of cells counted in all sections of 1 set of brain tissues (see tissue sectioning) were multiplied by the total number of sets to estimate the total number of BrdU, Ki67, or DCX-positive cells in the complete SGZ (BrdU and Ki67) or GCL (DCX). Double-labeled sections were analyzed by confocal laser microscopy (Olympus FV 1000). Image analysis and z-projections were made with ImageJ software (NIH, USA).

In APPswe/PSEN1ΔE9, the density of DCX-positive cells was estimated as previously described by Lie et al. [20] with some modifications. Briefly, DCX-positive cells in the GCL were counted in series of 5-6 random sections. NeuN immunoreactivity was used to measure the GCL volume. The area of the GCL was traced by using ImageJ software and a 10x objective. The density of immature neurons was expressed as DCX cells per volume (mm^3) of dentate GCL.

2.6. Immunoblotting. Hippocampi were dissected on ice and either immediately frozen in liquid nitrogen or processed as previously described [27]. Briefly, hippocampi were homogenized in RIPA buffer (10 mM Tris/HCl pH 7.4, 5 mM EDTA, 1% NP-40, 1% sodium deoxycholate, and 1% SDS) supplemented with a protease inhibitor mixture (1 mM PMSF, 2 μg/mL aprotinin, 1 μg/mL pepstatin, and 10 μg/mL benzamidine) and phosphatase inhibitors (25 mM NaF, 100 mM Na_3VO_4, 1 mM EDTA, and 30 μM $Na_4P_2O_7$), maintained on ice for 30 min before centrifugation at 20,000 g for 15 min at 4°C. Protein concentration in supernatants was determined using the BCA Protein Assay Kit (Pierce). Proteins were resolved in 10% SDS/PAGE, transferred to a PVDF membrane, reacted with primary antibodies overnight at 4°C, and then incubated with peroxidase-conjugated secondary antibodies (Pierce) and developed using the ECL technique (Western Lightning Plus ECL, PerkinElmer). Primary antibodies used were mouse anti-β-catenin, rabbit anti-GSK-3β, goat anti-NeuroD1, rabbit anti-β-tubulin (all from Santa Cruz

Biotechnology, Inc.), and rabbit anti-GSK-3β pSer9 (Cell Signalling).

2.7. Statistical Analysis. Statistical analysis was performed using Prism 5 software (GraphPad Software Inc.). Statistical significance of differences was assessed using the unpaired Student's t-test; nonnormally distributed data was analyzed using the Mann-Whitney test. The number of animals per group in each experiment is indicated in the figure legends.

3. Results

3.1. ANDRO Stimulates Cell Proliferation in the SGZ of Adult Mice. To evaluate the effect of ANDRO on adult hippocampal neurogenesis, we first analyzed the effect of the drug on cell proliferation in the SGZ. Two-month-old mice were injected i.p. with 2 mg kg^{-1} ANDRO or vehicle as control, 3 times a week for 4 weeks. In the last day of the treatment, animals also received in the i.p. injection a single dose of the nucleotide analog BrdU (100 mg kg^{-1}) and were sacrificed 24 h after the injection. The effect in cell proliferation was investigated by nuclear incorporation of BrdU (Figure 1(a)) and Ki67 staining (Figure 1(b)) used as a mitotic marker. BrdU immunoreactivity revealed that there was a significant increase in the total number of BrdU-positive cells in the SGZ of ANDRO-treated mice (Figure 1(a)) compared to control mice injected with the vehicle (control: 1,726 ± 196, ANDRO: 2,554 ± 275; $P = 0.027$). Also, there was a significant increase in the total number of Ki67-positive cells in the SGZ of mice treated with ANDRO compared to control mice (Figure 1(c), control: 2,341 ± 86, ANDRO: 3,711 ± 344; $P = 0.001$). These results indicate that ANDRO stimulates proliferation in the SGZ of adult mice.

Also, we evaluated the effect of ANDRO on cell proliferation in mice aged 10 months which show reduced levels of neurogenesis due to an age-related decline in neurogenesis observed in several species [28–31]. These mice were injected i.p. with 2 mg kg^{-1} ANDRO or vehicle as control, 3 times a week for 4 weeks; proliferation in the SGZ was evaluated by Ki67 staining. As expected, there was a strong decrease in Ki67-positive cells in 10-month-old mice compared with 2-month-old-mice (Figures 1(c) and 1(d)), and there was a significant increase in total number of Ki67-positive cells in mice treated with ANDRO (Figure 1(d), control: 269 ± 12, ANDRO: 470 ± 54; $P = 0.011$). Altogether, these results indicate that ANDRO increases cell proliferation in the dentate gyrus of young and aged mice.

3.2. ANDRO Increases Proliferation of Neural Progenitor Cells in the Adult Mouse Hippocampus. The increased number of BrdU- and Ki67-positive cells may result from increased proliferation of neural progenitor cells or neuroblasts. To evaluate whether quiescent neural progenitor cells are cellular targets of ANDRO, we evaluated Ki67 staining in Nestin-positive cells within the SGZ (Figure 2). Nestin is an intermediate filament expressed in neural progenitors but not in neuroblasts [32, 33]. Mice treated with 2 mg kg^{-1} ANDRO for 4 weeks showed a significant increase of Nestin+Ki67+ cells compared to vehicle-treated animals (Figure 2, control: 584 ± 112, ANDRO: 1,956 ± 343; $P = 0.0191$), indicating that the drug induced the activation of neural progenitors in the SGZ.

3.3. ANDRO Increases Neurogenesis in the Dentate Gyrus of Adult Mice. To evaluate whether ANDRO treatment induced the generation of new neurons in the adult dentate gyrus, first we carried out immunodetection of the immature neuronal marker doublecortin (DCX). A strong increase in the density of immature neurons positive for DCX was observed in the dentate gyrus of 2-month-old mice treated for 4 weeks with 2 mg kg^{-1} ANDRO compared with control mice injected with the vehicle (Figures 3(a) and 3(b)). Immunodetection of DCX was also carried out in 10-month-old mice that received the same treatment (Figure 3(c)). In agreement with the age-dependent decline in neurogenesis, there was an evident decrease in the density of immature DCX-positive neurons in mice aged 10 months compared with 2-month-old animals (compare Figures 3(a) and 3(c)). In 10-month-old mice, ANDRO treatment induced a significant increase in the total number of DCX-positive cells in the GCL compared with control mice injected with vehicle solution (Figure 3(c), control: 1,246 ± 108, ANDRO: 2,141 ± 250; $P = 0.008$).

To further evaluate whether ANDRO induced a net increase in neurogenesis, 2-month-old mice were injected i.p. with 2 mg kg^{-1} ANDRO or vehicle as control, 3 times a week for 2 weeks, and then received a daily i.p. injection of 100 mg kg^{-1} BrdU for 3 consecutive days and then continued with the injections of 2 mg kg^{-1} ANDRO or vehicle 3 times a week for 4 additional weeks (Figure 4(a)). As it was observed in the treatment of 4 weeks (Figure 1(c)), after 6 weeks of treatment there was a significant increase in the total number of Ki67-positive cells in the SGZ of mice treated with ANDRO compared with control mice (Figure 4(b); control: 1,784 ± 86, ANDRO: 2,888±152; $P = 0.0049$), supporting the effect of the drug on cell proliferation. To assess the effect in neurogenesis, we evaluated the total number of newborn granule cells by analyzing the total number of BrdU-positive cells that were also positive for the mature neuronal marker NeuN. This was evaluated by confocal microscopy using z-plane sections to assess NeuN staining in each BrdU-positive cell (Figure 4(c), insets) and the total number of BrdU+NeuN+ cells was estimated in the whole dentate gyrus [26]. A significant increase was observed in the total number of BrdU+NeuN+ cells in the GCL of mice treated with ANDRO compared with control mice (Figure 4(d), control: 477 ± 84, ANDRO: 1,023 ± 108, $P = 0.0286$), indicating that ANDRO increased the generation of new granule neurons in the dentate gyrus. Altogether, these findings indicate that ANDRO increases neurogenesis in the adult hippocampus.

3.4. ANDRO Stimulates the Wnt/β-Catenin Signaling Pathway in the Hippocampus of Adult Mice. We previously showed that ANDRO inhibits the activity of GSK-3β [9], a component of the Wnt/β-catenin signaling pathway that upon activation of the pathway is inhibited, thereby preventing the phosphorylation of its target β-catenin which is translocated into the nucleus to activate the transcription of Wnt target

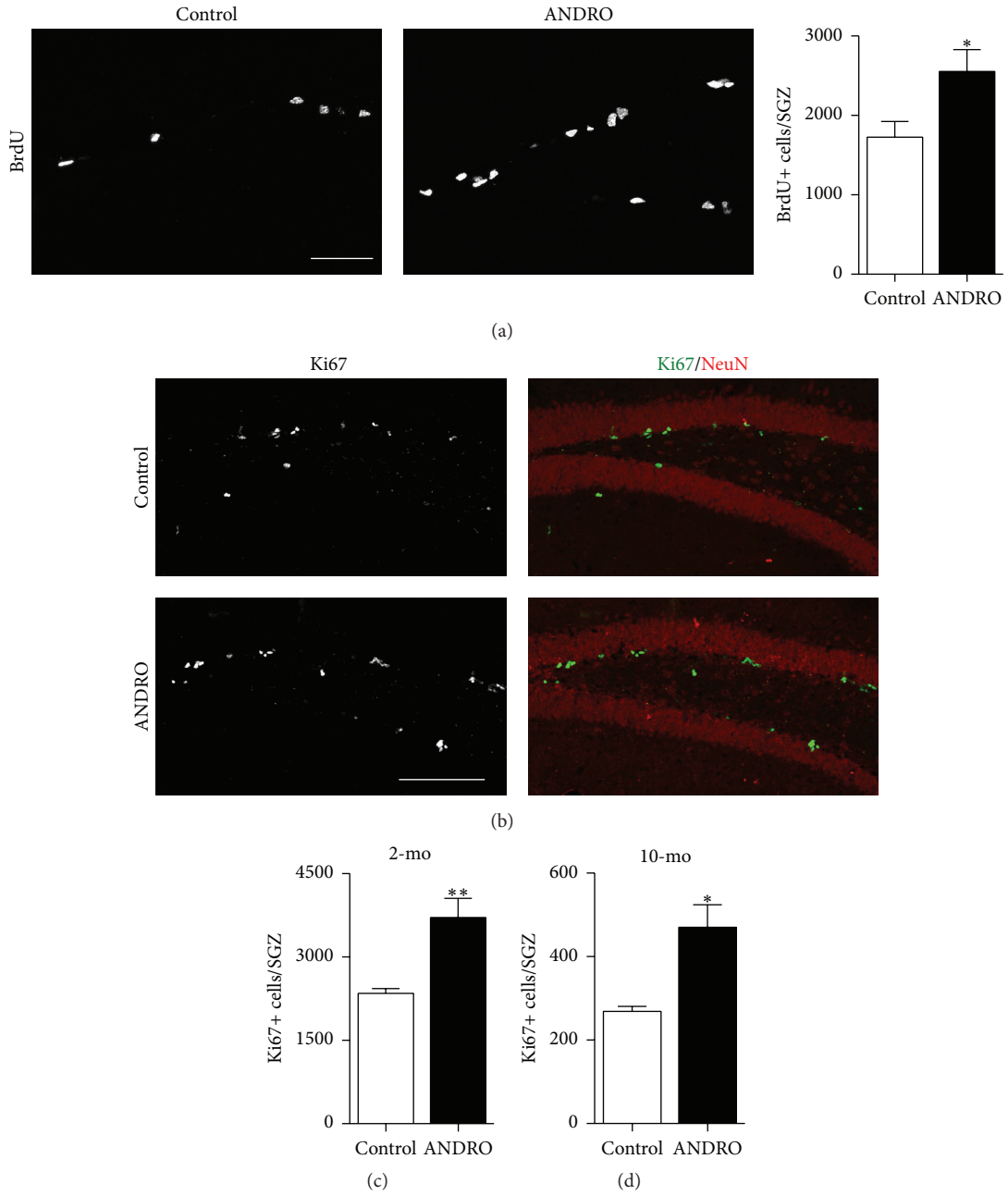

Figure 1: ANDRO induces proliferation in the dentate gyrus of adult mice. Two-month-old mice were injected i.p. with 2 mg kg^{-1} ANDRO or vehicle as control 3 times a week for 4 weeks, received a single dose of 100 mg kg^{-1} BrdU the last day of treatment, and were sacrificed 24 h after BrdU injection. (a) Images show representative immunostaining of BrdU. Scale bar: 50 μm. The graph shows total number of BrdU-positive (BrdU+) cells in the whole SGZ of control and ANDRO-treated mice. Bars represent mean ± S.E. (n = 7 mice). (b) Representative immunostaining of Ki67 and NeuN. Scale bar: 100 μm. (c, d) Total number of Ki67-positive (Ki67+) cells in the SGZ of control and ANDRO-treated 2-month-old (c) or 10-month-old (d) mice. Bars represent mean ± S.E. (n = 7 (c) and n = 5 (d) mice). $^{*}P < 0.05$, $^{**}P < 0.01$, Student's t-test.

genes [34]. We evaluated the activation of the Wnt signaling pathway in the hippocampus of 2-month-old mice injected i.p. with 2 mg kg^{-1} ANDRO or vehicle as control, 3 times a week for 4 weeks. As expected, we observed a significant increase in β-catenin level in the hippocampus of ANDRO-treated animals compared with control mice (Figure 5(a)), concomitantly with an increase in the level of the inactive form of GSK-3β phosphorylated in serine-9 residue (Figure 5(a)). In addition, we evaluated the levels of NeuroD1 which is a transcription factor involved in neurogenesis in the embryonic and adult brain [35] that was previously identified as a Wnt target gene [24]. A significant increase of

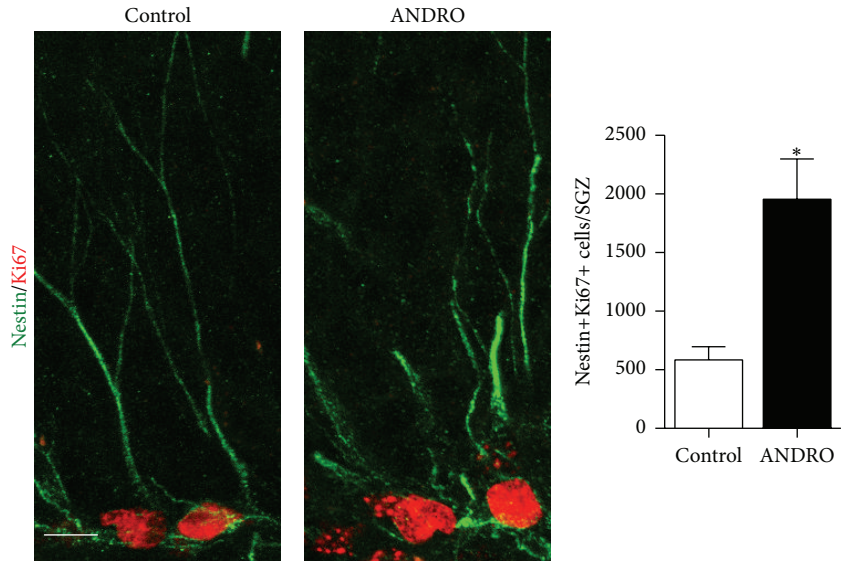

Figure 2: ANDRO induce proliferation of neural progenitors in the SGZ. Representative immunostaining of Ki67 and Nestin in 2-month old mice treated per 4 weeks with 2 mg kg^{-1} ANDRO or saline solution as control. Scale bar: 10 μm. The graph represents quantification of the total number of Ki67-positive/Nestin-positive (Ki67+Nestin+) cells in the SGZ. Bars represent mean ± S.E. (n = 3 mice). $^{*}P < 0.05$, Student's t-test.

NeuroD1 level was observed in the hippocampus of ANDRO-treated mice (Figure 5(b)). This increase was also observed by immunostaining (Figure 5(c)), in which NeuroD1 was mainly observed in DCX-positive cells, as previously described [35]. Altogether, these findings indicate that ANDRO induces the activation of the Wnt/β-catenin signaling pathway in the hippocampus of adult mice.

3.5. ANDRO Increases Neurogenesis in the Dentate Gyrus of APPswe/PSEN1ΔE9 Mice. Finally, we evaluated the effect of ANDRO on neurogenesis in APPswe/PSEN1ΔE9 transgenic mouse model of AD that shows an impaired neurogenesis [26]. These double transgenic animals express the human amyloid precursor protein (APP) with the Swedish mutation (K595N/M596L) and presenilin 1 with the deletion of exon 9 and show histopathological hallmarks of AD (e.g., Aβ deposition, amyloid plaques, astrogliosis, and tau pathology) and cognitive impairment by 7 months of age [27]. We determined that at this age animals show a significant reduction in cell proliferation in the SGZ and show an impaired differentiation of newborn cells into DCX-positive neuroblasts [26]. APPswe/PSEN1ΔE9 mice at 7 months were injected i.p. with 2 mg kg^{-1} ANDRO or vehicle as control, 3 times a week for 4 weeks. Proliferation at the SGZ was evaluated by Ki67 staining (Figure 6(a)). Treatment with ANDRO strongly induced proliferation as determined by the increased number of Ki67-positive cells (APPswe/PSEN1ΔE9 control: 96 ± 37, APPswe/PSEN1ΔE9 ANDRO 256 ± 32; $P = 0.05$). In addition, an increased density of immature DCX-positive neurons was observed in mice treated with ANDRO compared to control transgenics that received vehicle injections (Figure 6(b)), indicating that the drug induced neurogenesis in APPswe/PSEN1ΔE9.

In these mice, we also evaluated the Wnt target gene NeuroD1 (Figure 7(a)). As we previously observed in 12-month-old transgenic mice [8], increased levels of β-catenin and GSK-3β phosphorylated in serine-9 were observed in APPswe/PSEN1ΔE9 mice treated with ANDRO compared with control mice (Figure 7(b)). In addition, and as observed in wild-type animals (Figure 5(b)), ANDRO treatment induced a significant increase of NeuroD1 level at the hippocampus of APPswe/PSEN1ΔE9 mice (Figure 7(c)).

4. Discussion

The generation of new neurons in the hippocampus during adulthood has been determined in several species and has shown to contribute to the plasticity of the hippocampus and to some hippocampal processes including spatial learning and memory [17, 19]. This process is compromised in several pathologies affecting the central nervous system (e.g., AD, schizophrenia, and mood disorders) and also is affected during normal aging [30, 36]; the decreased neurogenesis has been linked, for example, to cognitive deficits associated with some of these conditions; therefore, it is tempting to search for new approaches to stimulate this process in normal and diseased brain. Here, we evaluated the ability of ANDRO, one of the active components of the medicinal plant *Andrographis paniculata*, to stimulate neurogenesis, and demonstrated that ANDRO induces proliferation and the generation of new neurons in the dentate gyrus of the adult hippocampus in wild-type mice and in a mouse model of AD.

In the adult dentate gyrus, new granule cells are continuously being generated from neural progenitor cells that are located between the hilus and the GCL. After activation, these cells give rise to transit-amplifying progenitors or

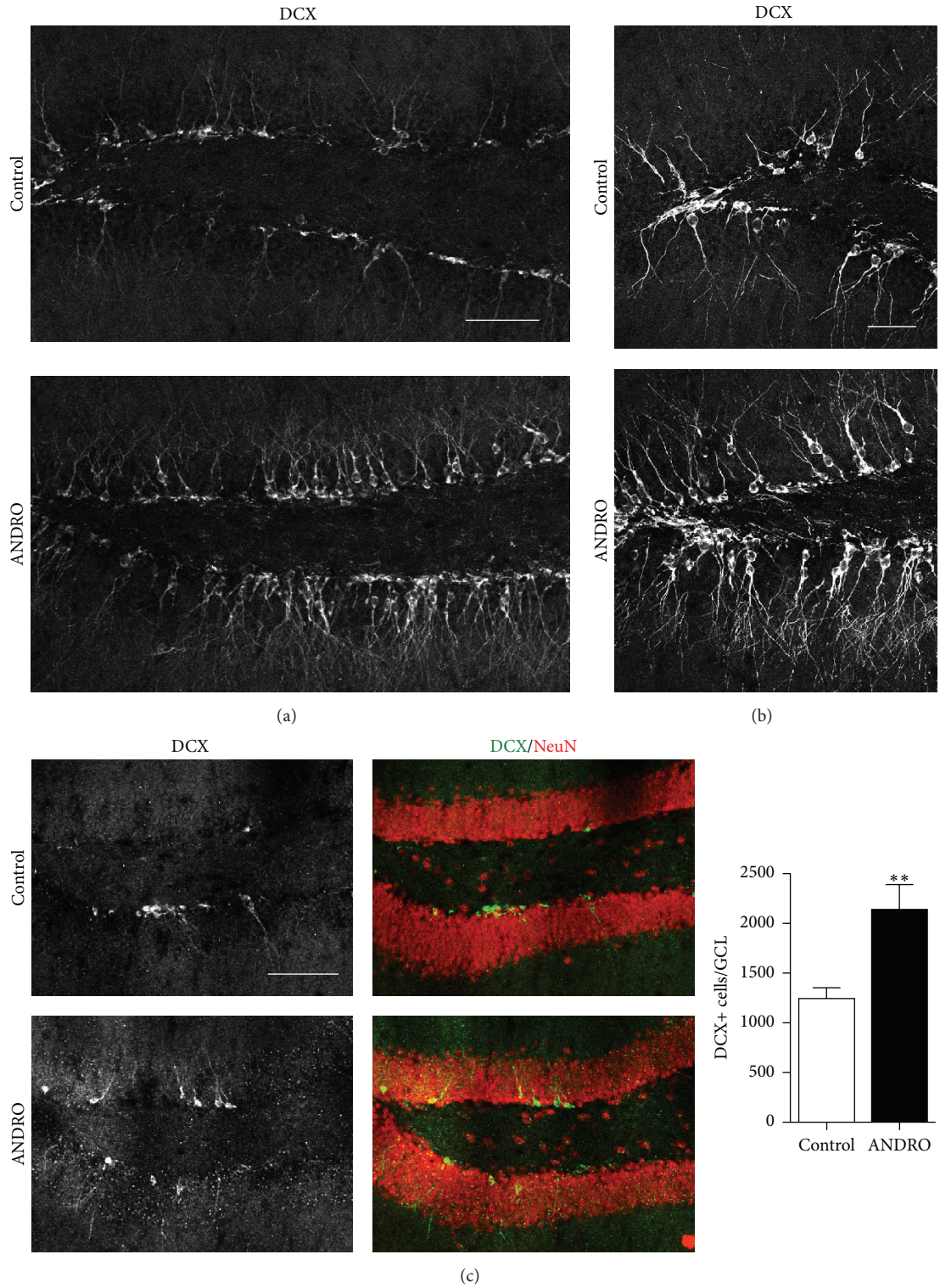

Figure 3: ANDRO increases the density of immature neurons in the dentate gyrus of adult mice. (a) Representative immunostaining of DCX in 2-month-old mice treated with vehicle or ANDRO for 4 weeks. Scale bar: 50 μm. (b) Higher magnification of representative DCX immunostaining in control and ANDRO-treated mice. Scale bar: 30 μm. (c) Representative images of double immunostaining for DCX and NeuN in 10-month-old mice treated with vehicle or ANDRO for 4 weeks. Scale bar: 100 μm. Graph quantification of total number of DCX-positive (DCX+) cells in the GCL 10-month-old mice. Bars represent mean ± S.E. ($n = 5$ mice). $^{**}P < 0.01$, Student's t-test.

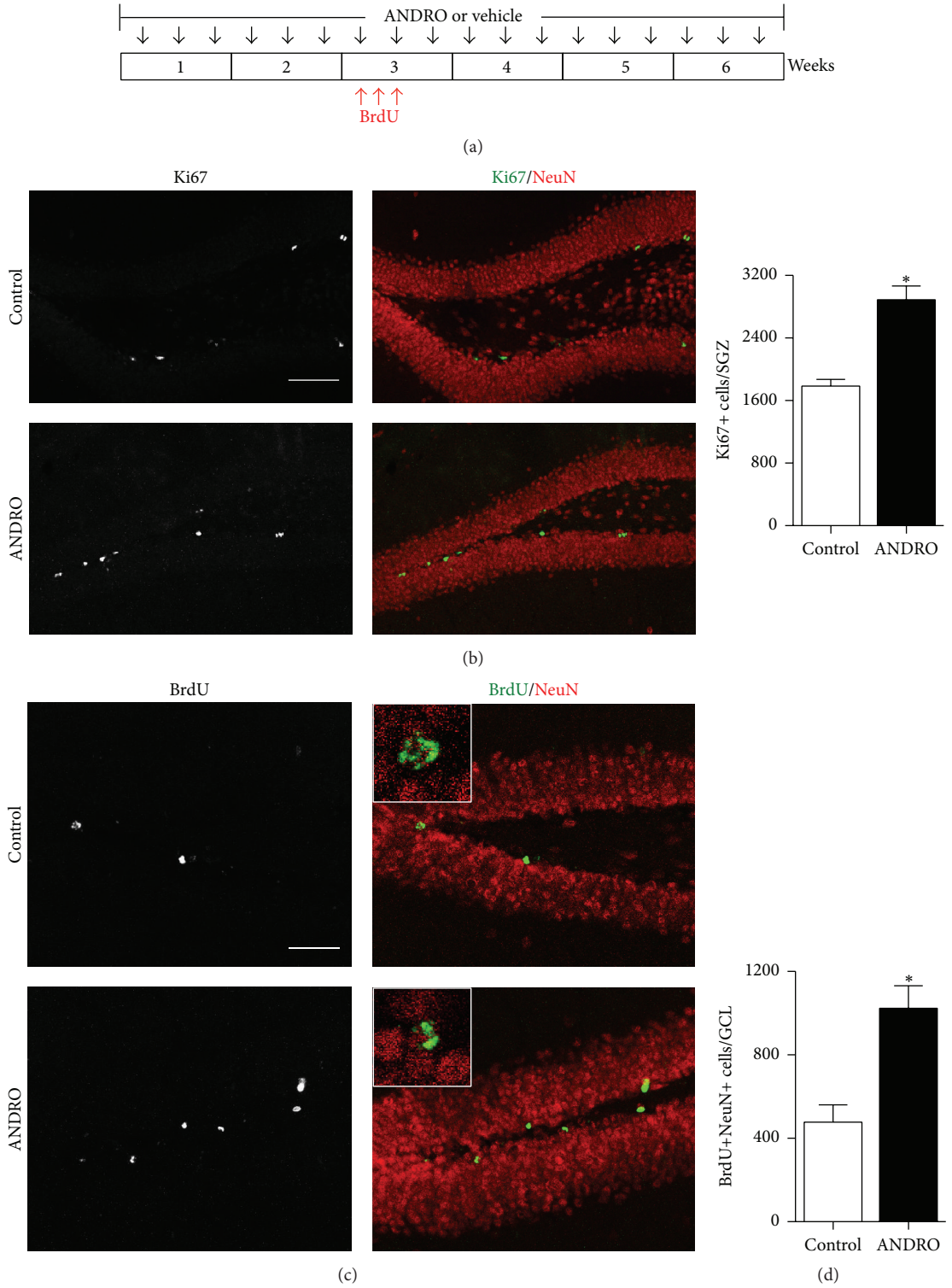

Figure 4: ANDRO increases the generation of newborn granule cells in the hippocampus of adult mice. (a) Schematic representation of the experimental procedure. Two-month-old mice were injected i.p. with 2 mg kg^{-1} ANDRO or vehicle as control 3 times a week for 2 weeks and then received a daily i.p. injection of 100 mg kg^{-1} BrdU for 3 consecutive days and continued with the treatments for 4 weeks. (b) Representative immunostaining of Ki67 and NeuN after 6 weeks of treatment. Scale bar: 100 μm. The graph represents quantification of the total number of Ki67-positive (Ki67+) cells in the SGZ. (c) Representative immunostaining of BrdU and NeuN. Insets show higher magnifications of double-positive cells. Scale bar: 50 μm (d) Quantification of the total number of double-positive (BrdU+NeuN+) cells in the GCL of control and ANDRO-treated mice. Bars represent mean ± S.E. ($n = 4$ mice). $^{*}P < 0.05$, Student's t-test.

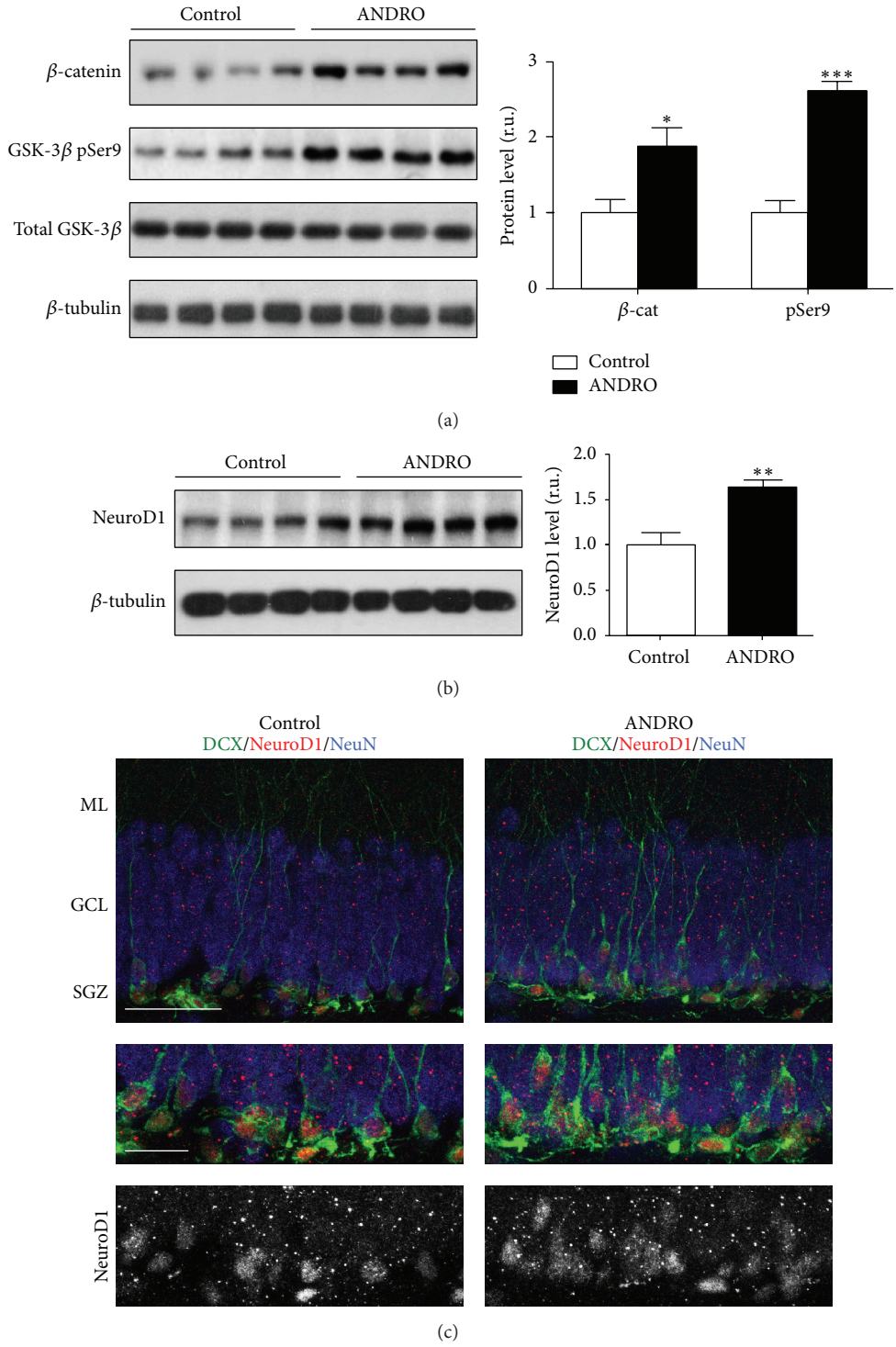

FIGURE 5: ANDRO induces the activation of the Wnt signaling pathway in the hippocampus of adult mice. (a) Immunoblots of β-catenin, inactive form of GSK-3β (phosphorylated in serine-9, pSer9), total GSK-3β, and β-tubulin used as loading control in total protein extracts obtained from the hippocampus of 2-month-old mice injected i.p. with 2 mg kg^{-1} ANDRO or vehicle as control 3 times a week for 4 weeks. The graph corresponds to the densitometric analysis of β-catenin and GSK-3β pSer9 normalized to β-tubulin and total GSK-3β, respectively. (b) Immunoblot of NeuroD1 and β-tubulin. The graph corresponds to the densitometric analysis of NeuroD1 normalized to β-tubulin level. (c) Representative immunostaining of NeuroD1, DCX, and NeuN in mice treated with vehicle or ANDRO for 4 weeks. Scale bar: 20 μm. Bottom, higher magnifications of the images. Scale bar: 20 μm. Bars represent mean ± S.E. ($n = 4$ mice). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, Student's t-test.

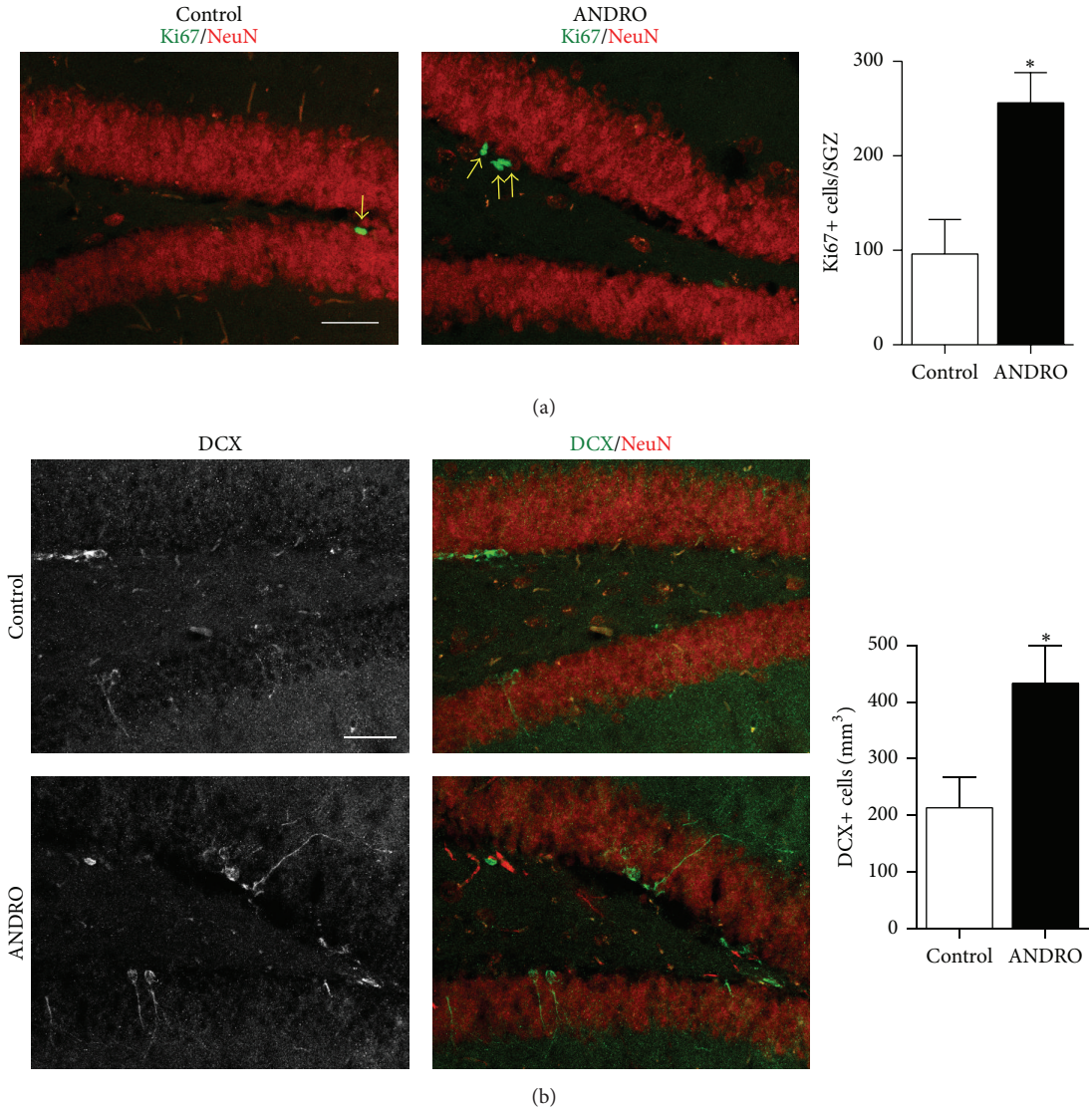

Figure 6: ANDRO stimulates proliferation and the density of immature neurons in the dentate gyrus of APPswe/PSEN1ΔE9. Seven-month-old APPswe/PSEN1ΔE9 mice were injected i.p. with 2 mg kg^{-1} ANDRO or vehicle as control 3 times a week per 4 weeks. (a) Representative images of double immunostaining for Ki67 and NeuN. The graph shows quantification of the total number of Ki67-positive (Ki67+) cells (yellow arrows) in the SGZ of mice treated with vehicle or ANDRO. Scale bar: 20 μm. (b) Images show representative double immunostaining for DCX and NeuN. Scale bar: 20 μm. Graph differences in the density of immature DCX-positive (DCX+) neurons in control and ANDRO-treated APPswe/PSEN1ΔE9 mice. Bars represent mean ± S.E. ($n = 3$ (a) or $n = 5$ (b) mice). $^{*}P < 0.05$, Student's t-test.

intermediate progenitor cells that then commit to the neuronal fate generating neuroblasts that develop into immature neurons that extend dendrites to the GCL and molecular layer and project their axons to the CA3 region [16]. These newborn neurons will mature during several weeks into functional dentate granule neurons that form synaptic connections and become integrated into the hippocampal circuitry [37, 38]. We determined that ANDRO treatment for 4 weeks stimulated proliferation in the SGZ and increased the density of immature neurons in the GCL. Our results showed that ANDRO increased the activation of quiescent neural progenitor cells, strongly suggesting that these are the cellular target of ANDRO activity. Interestingly, the effects of ANDRO on proliferation and immature neurons were observed in mice aged 2 months at the beginning of the treatment and also in mice at 10 months of age where neurogenesis is significantly reduced. An age-dependent decline in hippocampal neurogenesis has been evidenced in different species including humans [28–31]; however, studies reveal that neurogenesis can be stimulated even at advanced stages of aging. The effect of ANDRO in 10-month-old mice indicates that the drug is able to stimulate neurogenesis in aged mice. Moreover, we determined that ANDRO induced a net increase in neurogenesis. The expression of mature neuronal markers by newborn neurons takes about four weeks [16]; we determined that ANDRO increased the total number of newborn cells expressing the mature neuronal marker NeuN (evaluated four weeks after administration of

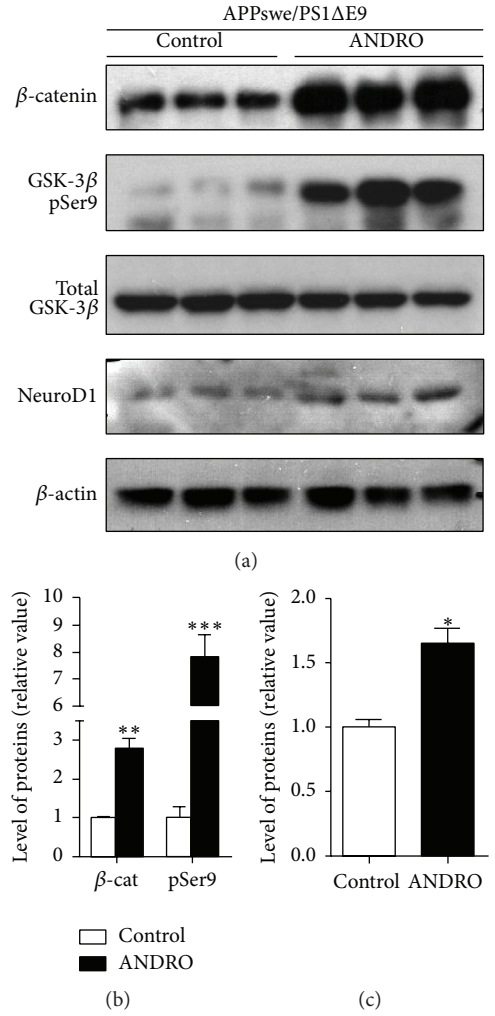

Figure 7: ANDRO increases NeuroD1 in the hippocampus of APPswe/PSEN1ΔE9. Seven-month-old APPswe/PSEN1ΔE9 mice were injected i.p. with 2 mg kg^{-1} ANDRO or vehicle as control 3 times a week per 4 weeks. (a) Immunoblots of β-catenin, inactive form of GSK-3β (phosphorylated in serine-9, pSer9), total GSK-3β, NeuroD1, and β-actin in total protein extracts obtained from the hippocampus of control and ANDRO-treated APPswe/PSEN1ΔE9 mice. (b) Densitometric analysis of β-catenin and GSK-3β pSer9 normalized to β-tubulin and total GSK-3β, respectively. (c) Densitometric analysis of NeuroD1 normalized to β-tubulin. Bars represent mean ± S.E. (n = 3 mice). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$, Student's t-test.

BrdU), indicating that the drug induced a net increase in the total number of newly born granule neurons. Altogether, these findings indicate that ANDRO stimulates neurogenesis in the adult hippocampus.

The effect of ANDRO on neurogenesis may involve the inhibition of GSK-3β. We recently determined that ANDRO inhibits GSK-3β by a substrate-competitive mode of action [9]. Interestingly, ANDRO shows high selectivity for GSK-3β, since it had no effect on cyclin-dependent kinase 5 (Cdk5), Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), protein kinase C (PKC), Akt, casein kinase (CK), and S6 kinase [9]. Stimulation of neurogenesis by GSK-3β inhibition has been reported *in vitro* and *in vivo*. Treatment of cultured adult hippocampal progenitors with the GSK-3β inhibitor lithium induced proliferation [39] and *in vivo* treatment with lithium induced proliferation and neuronal fate specification in the hippocampus of a mouse model of AD [40]. In addition, an impaired neurogenesis was observed in a GSK-3 knock-in mouse carrying mutations to block inhibitory phosphorylation of the kinase [41] and in mice overexpressing GSK-3β [42] which also show morphological alterations in newborn neurons [43]. Therefore, it is likely that ANDRO may stimulate neurogenesis via inhibition of GSK-3β. Other mechanisms that have been associated with the biological effects of ANDRO include inhibition of PI3K/Akt and NF-$\kappa\beta$ pathways [44–46]. As mentioned, we previously determined that ANDRO has no effect on Akt activity [9], and since inhibition of NF-$\kappa\beta$ signaling pathway is associated with an impaired adult hippocampal neurogenesis [47, 48], it is unlikely that the positive effect of ANDRO on neurogenesis may involve these cascades.

As previously mentioned, GSK-3β is a key component of the Wnt/β-catenin signaling pathway, which has been

shown in several *in vivo* studies to regulate proliferation, differentiation, and maturation of adult-born granule neurons [20–22, 24]. Through the inhibition of GSK-3β, ANDRO is a potent activator of the Wnt signaling pathway [9]. Here, we determined that concomitantly with the increase in neurogenesis ANDRO treatment induced the Wnt/β-catenin signaling pathway in the hippocampus of adult mice, as determined by the increase in β-catenin protein and the increase in the level of the inactive form of GSK-3β. In addition, we observed increased level of NeuroD1, a previously described Wnt target gene [24] that is needed for the survival and maturation of adult-born neurons. Therefore, it might be suggested that the effects of ANDRO may be mediated by the activation of the Wnt signaling pathway and the expression of proneural Wnt target genes such as NeuroD1. Interestingly, increased level of NeuroD1 was also observed in ANDRO-treated APPswe/PS1ΔE9 mice. In this mouse model of AD, which shows reduced levels of proliferation in the SGZ and decreased differentiation of neural progenitor cells into neurons compared with age-matched wild-type mice [26, 49], we determined that ANDRO treatment induced almost 3-fold increase in cell proliferation in the SGZ. We had observed such a strong effect in cell proliferation in this transgenic mouse by exposure to voluntary wheel running [50], which is a well-known potent inductor of neurogenesis in young and aged mice [51, 52]. Also, we determined that ANDRO treatment increased the density of immature neurons in APPswe/PS1ΔE9 mice, indicating that the drug induced neurogenesis.

5. Conclusions

In the present study, we have demonstrated that ANDRO, the active component of the medicinal plant *Andrographis paniculata*, stimulates adult hippocampal neurogenesis. Previously, ANDRO showed neuroprotective effects in a rat model of permanent cerebral ischaemia [6] and against oxidative damage induced by nicotine [7]; in addition, in the APPswe/PS1ΔE9 mouse model of AD, ANDRO prevented neuropathological changes associated with the disease and improved spatial memory [8]. Here, we showed that ANDRO induces proliferation and the generation of new neurons in the adult hippocampus of wild-type and APPswe/PS1ΔE9 mice, providing new evidence to suggest ANDRO as a potential therapeutic drug for the treatment of brain diseases.

Conflict of Interests

All authors declare nonfinancial competing interests.

Acknowledgments

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