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# Adult neurogenic process in the subventricular zone-olfactory bulb system is regulated by Tau protein under prolonged stress

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## Abstract

**Objectives:** The area of the subventricular zone (SVZ) in the adult brain exhibits the highest number of proliferative cells, which, together with the olfactory bulb (OB), maintains constant brain plasticity through the generation, migration and integration of newly born neurons. Despite Tau and its malfunction is increasingly related to deficits of adult hippocampal neurogenesis and brain plasticity under pathological conditions [e.g. in Alzheimer's disease (AD)], it remains unknown whether Tau plays a role in the neurogenic process of the SVZ and OB system under conditions of chronic stress, a well-known sculptor of brain and risk factor for AD.

**Materials and methods:** Different types of newly born cells in SVZ and OB were analysed in animals that lack *Tau* gene (Tau-KO) and their wild-type littermates (WT) under control or chronic stress conditions.

**Results:** We demonstrate that chronic stress reduced the number of proliferating cells and neuroblasts in the SVZ leading to decreased number of newborn neurons in the OB of adult WT, but not Tau-KO, mice. Interestingly, while stress-evoked changes were not detected in OB granular cell layer, Tau-KO exhibited increased number of mature neurons in this layer indicating altered neuronal migration due to Tau loss.

**Conclusions:** Our findings suggest the critical involvement of Tau in the neurogenesis suppression of SVZ and OB neurogenic niche under stressful conditions highlighting the role of Tau protein as an essential regulator of stress-driven plasticity deficits.

## 1 | INTRODUCTION

The brain is the most adaptive of all organs due to its continuous plasticity in response to a variety of internal and environmental stimuli. A dynamic form of neuronal plasticity in the adult brain is neurogenesis, the process of generation of new, functional neurons from neural stem cells (NSCs) and progenitor cells,<sup>1</sup> which enables the brain to adapt to the constantly evolving interaction between

environmental signals and the brain's internal reaction to these stimuli.<sup>2</sup> The two main neurogenic niches of the adult brain are the hippocampal dentate gyrus (DG) and the subventricular zone (SVZ) of lateral ventricles<sup>3</sup>; the latter exhibits the biggest amount of proliferative cells in the brain.<sup>4,5</sup> Newly born neurons generated in the SVZ of the adult brain migrate through the rostral migratory stream to the olfactory bulb (OB) where they differentiate into local circuit interneurons that are implicated in learning and memory processes

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related to smell sensation in rodents.<sup>6,7</sup> In brief, type B cells are quiescent neural stem cells (NSCs) expressing glial fibrillary acidic protein (GFAP) that give rise to type C cells (also known as transient-amplifying progenitors); type C cells give rise to type A cells, which are neuroblasts expressing doublecortin and migrate to the OB.<sup>8,9</sup> In the OB, these neuroblasts differentiate into interneurons and migrate radially to the outer cell layers, namely granular cell layer (GCL), mitral cell layer (MCL) and glomerular cell layer (GL). Specifically, they differentiate in the GCL and MCL into granule cells (GC) and in the GL into periglomerular cells (PGC).<sup>10,11</sup> Additionally, it has also been described that type B cells generate oligodendrocytes—see also Figure 1.<sup>12,13</sup>

Although the extent and relevance of adult neurogenesis in humans are currently debated,<sup>14,15</sup> accumulating evidence suggests that neurogenesis persists in the adult brain of both humans and rodent animals during the entire lifespan while it drops in Alzheimer's disease (AD)<sup>16-20</sup> and other pathological conditions causally related to AD, such as depression and stress.<sup>21-23</sup> Chronic stress, a major precipitant of depression and AD<sup>24-27</sup> is known to impair brain plasticity, including suppression of neurogenesis.<sup>23,28-32</sup> Recent evidence about stress-driven neurogenic deficits highlights the critical role for the cytoskeletal Tau protein<sup>22,33</sup> a prominent stabilizer of microtubules (MT),<sup>34</sup> which promotes co-organization of MT and actin networks.<sup>35-38</sup> However, our knowledge related to the impact of chronic stress on adult neurogenesis is mainly based on the hippocampus, as the vast majority of studies have neglected the other main neurogenic niche of the adult brain, the SVZ. As a matter of fact, the SVZ area exhibits the highest number of proliferative cells in the adult brain<sup>4,5</sup> and constitutes the origin of the newly born cells/neurons that migrate into the OB under control conditions<sup>39</sup> and to other neocortical sites under injury (eg. after stroke or trauma).<sup>40</sup> Also, hippocampal and SVZ-OB cytogenic areas exhibit essential differences in their anatomical/layer organization and input received from other brain areas as well as the type of newborn cells generated in each of these two neurogenic niches.<sup>41</sup> Moreover, their vulnerability to stress or pharmacological/irradiation treatment may be different, as previous studies suggested.<sup>42,43</sup> Despite that the above findings point towards essential differences between these two neurogenic niches in the adult brain, our knowledge about the cell-type specific impact on chronic stress on SVZ-OB system and the underlying mechanisms remain poor.

In light of the limited and conflicting evidence about whether exposure to stressful conditions affects (or not) the SVZ and OB cytogenesis in the adult brain<sup>42,43</sup> and the selective involvement of Tau in specific types of newborn cells (eg DG newborn neurons, but not glial cells), the current study aims to clarify the effect of stress on different populations of newborn cells in the SVZ-OB system. For that purpose, we have exposed animals lacking Tau protein (Tau-KO) and their wild type (WT) littermates to a chronic unpredictable stress (CUS) paradigm and evaluated differences in the cell population resident in the SVZ and OB neurogenic niches. Our findings suggest that exposure to chronic stress suppresses proliferation as well as

neuronal differentiation and maturation in the SVZ and OB of the adult brain while the absence of Tau protein diminishes these neuroplastic effects of stress highlighting an essential role for Tau in the mechanisms through which prolonged stressful conditions damage brain plasticity.

## 2 | MATERIALS AND METHODS

### 2.1 | Animals

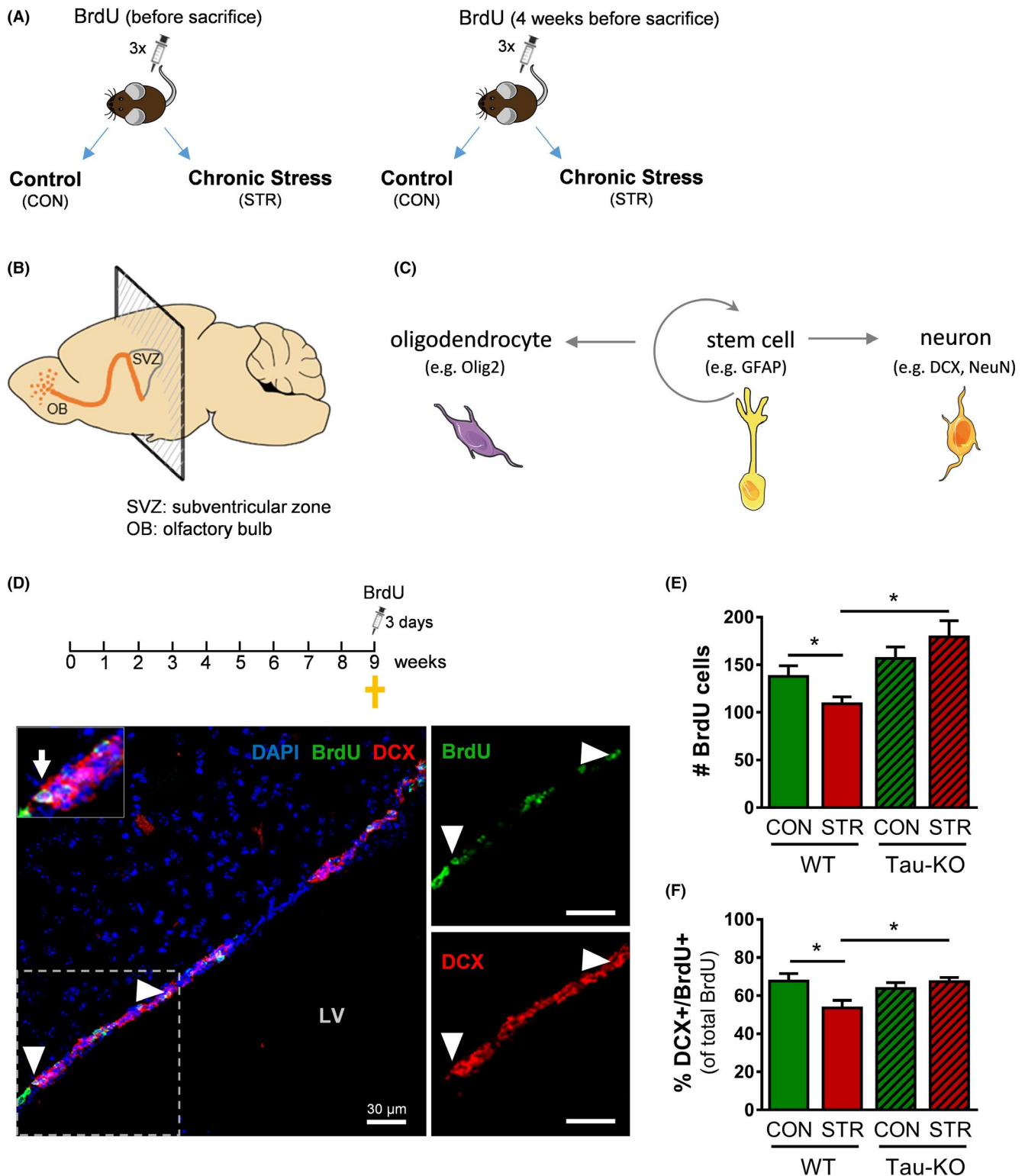
Twenty-eight male mice lacking Tau protein (Tau-KO) and their wild-type littermates (6-7 months old; C57BL/6J background) were used in this study divided into stressed and control groups (7 animals per group per genotype)—see also below. Mice were group-housed under standard environmental conditions (8 AM-8 PM light cycle; 22°C; 55% humidity, ad libitum access to food and water) in accordance with the guidelines for the care and handling of laboratory animals in the Directive 2010/63/EU of the European Parliament and Council. All experiments were conducted in accordance with the Portuguese national authority for animal experimentation, Direção Geral de Alimentação e Veterinária (ID: DGAV9457).

### 2.2 | Chronic stress paradigm

Animals were exposed to a 9-week chronic unpredictable stress (CUS) paradigm during the daily period of light, while control (non-stressed; CON) mice remained undisturbed in their home cages. The CUS protocol included 4 different stressors: restraint, vibrating platform, overcrowding and exposure to a hot air stream. Animals were exposed to one stressor per day for 3 hours (restraint, vibrating platform, overcrowding) or 30 minutes (hot air stream). The order of stressors and the time of the day at which the stressor was applied were randomly chosen and varied among weeks to promote unpredictability, as previously described.<sup>22,25,28,44</sup> At the end of the CUS protocol, mice body weight was measured and blood was collected from all animals. Blood serum was isolated after centrifugation and corticosterone (CORT) levels were measured using a radioimmunoassay kit (R&D Systems) according to the manufacturer's instructions.

### 2.3 | BrdU treatment

For assessment of cell proliferation, a set of control and stressed WT and Tau-KO animals (four animals per group) were injected with 5-bromo-2'-deoxyuridine (BrdU; 50 mg/kg per day) for 3 consecutive days before killing. For cell survival monitoring, another set of control and stressed animals of both genotypes (three animals per group) were injected with BrdU (50 mg/kg per day) for 3 consecutive days, 4 weeks before killing—see also Figure 1A.



**FIGURE 1** Chronic stress suppresses the number of proliferating cells and neuroblasts in the adult subventricular zone of WT, but not Tau-KO, animals. A, Schematic representation of the experimental design where wild-type (WT) and Tau-knockout (Tau-KO) mice were divided into control (CON) and chronic stress (STR) groups. Animals of all groups were randomly divided into two groups receiving 5-bromo-2'-deoxyuridine (BrdU) injections before sacrifice (left panel) and 4 weeks before sacrifice (right panel). B,C, Schematic illustration of the mouse brain (B) highlighting the neurogenic areas of the subventricular zone (SVZ) and olfactory bulb (OB) as well as different types of newly born SVZ cells analysed and the markers used for their monitoring. D-F, Representative microphotograph of BrdU/DCX double-labelled cells (arrow head) in the SVZ (D). Chronic stress evoked a decrease in BrdU-positive cell density (reflecting proliferating cells) in WT, but not Tau-KO, animals. Note that stressed Tau-KO animals present higher number of proliferating cells when compared to stressed WT animals (E). Similarly, stress reduced the percentage of DCX/BrdU double-labelled cells (reflecting neuroblasts) only in WT animals (F). All numerical data are shown as mean  $\pm$  s.e.m (\* $P < .05$ ). CON, control-non-stressed; STR, stressed; BrdU, 5-bromo-2'-deoxyuridine; DCX, doublecortin; WT, wild type; Tau-KO, Tau-knockout

## 2.4 | Tissue preparation

At the end of the CUS protocol, animals were deeply anesthetized (ketamine hydrochloride [150 mg/kg] plus medetomidine [0.3 mg/kg]) and transcardially perfused with saline followed by ice-cold 4% paraformaldehyde perfusion. Brains were removed, post-fixed in 4% paraformaldehyde for 2 hours and then transferred to a 30% sucrose solution until they sunk. Then, brains were included in optimal cutting temperature compound (OCT; Tissue Tek, Sakura FineTek), snap-frozen in liquid nitrogen with 2-methylbutane and sectioned in a cryostat (Leica CM1900) into 20 µm sections.

## 2.5 | Immunofluorescence staining

Coronal brain sections of SVZ and OB were double-stained for BrdU (1:200; Abcam) followed by doublecortin (DCX; for neuroblast; 1:250; Santa Cruz Biotechnology) or GFAP (for neural-stem cells; 1:200; Dako) or Olig2 (for oligodendrocytes progenitor cells; 1:300; Merck Millipore) or NeuN (for mature neurons; 1:100; Cell Signalling, Leiden, The Netherlands) for 1 or 2 overnights. Briefly, sections were first washed in PBS (RT) for 3 minutes. Then the sections were heated for 15 minutes in the microwave in citrate buffer (#C9999; Sigma Aldrich/Merck). After washing in PBS, the sections were incubated in HCl 2 mol/L for 30 minutes. After washing in PBS, cells were permeabilized using PBS-Triton X-100 0.5% (v/v) for 15 minutes and then incubated on blocking solution (10% foetal bovine serum (v/v) and PBS-Triton X-100 0.5% (v/v) at RT for 30 minutes. After incubation with primary antibodies for 1 or 2 overnights (4°C) and PBS washes, sections were incubated with the appropriate secondary antibodies for 2 hours (RT): Alexa Fluor 488 goat anti-rat for BrdU, 1:1000; Alexa Fluor 568 donkey anti-goat for DCX, 1:1000; Alexa Fluor 594 donkey anti-rabbit for GFAP, Olig2 and NeuN, 1:1000; Thermo Fisher Scientific). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (1:1000; Sigma Aldrich), and slides were mounted with PermaFluor Aqueous Mounting Medium (TA-006-FM; Lab Vision, Thermo Fisher Scientific). For cell quantification, confocal images (x40) of SVZ and OB were obtained by the confocal microscope Olympus FluoView FV1000 (Olympus). SVZ and OB images were analysed using the Olympus FluoView™ FV1000 software. Additionally, for the SVZ analysis, images of the dorsolateral and ventral divisions of posterior sections were used.<sup>9</sup> For the OB, we used images of three major areas, namely granular cell layer (GCL), mitral cell layer (MCL) and glomerular cell layer (GL) and their correspondence areas were measured with the ImageJ software (<http://rsb.info.nih.gov/ij/>).

## 2.6 | Behavioural testing

### 2.6.1 | Open field

We used an open-field square arena (43.2 cm × 43.2 cm) surrounded by tall Perspex walls (Med Associates Inc). Each mouse was placed in

the centre and allowed to explore the arena for 10 minutes. Infrared beams and manufacturer's software were used to automatically register animals' movements.

### 2.6.2 | Ultrasonic vocalizations

Measurement of ultrasonic vocalizations (USVs) was performed as previously described with some modifications.<sup>45</sup> Briefly, each animal was placed in a cage for 24 hours. Then, the animal was in close proximity with a female animal, and USVs were recorded for 15 minutes using the Avisoft-Recorder (version 5.1.04) and manually analysed with AvisoftSAS Lab Pro (version 5.1.22, Avisoft Bioacoustics).

### 2.6.3 | Novel object recognition test

The test arena consisted of a white rectangular box (33 cm × 33 cm × 33 cm). Mice were placed for 20 minutes during 3 consecutive days inside the test arena (habituation). On the following day, mice were placed in the test arena, which contained two identical objects equally distant, and returned to their home cage after 10 minutes of exploration. The next day, the animals were presented a novel object (NO) and one old, familiar object (FO) for 10 minutes; both objects were generally similar regarding height and volume but they were different in shape, colour and texture. Animal's behaviour was recorded, and the time spent exploring each object was manually analysed by the Kinoscope software (<http://sourceforge.net/projects/kinoscope/>) by an experimenter blind to the animal group. The preference index % (PI%) was calculated based on the formula  $PI\% = \text{time in NO} * 100 / (\text{time in NO} + \text{time in FO})$ .

## 2.7 | Statistical analysis

Data were analysed using two-way analysis of variance (ANOVA) before the application of appropriate post hoc pair-wise comparisons (GraphPad Prism v.6.01; GraphPad Software). Differences were considered statistically significant when  $P < .05$ . Results are presented as mean ± SEM

## 3 | RESULTS

### 3.1 | Exposure to chronic stress reduces proliferating cells in the subventricular zone of the adult brain while Tau ablation blocks this stress effect

For clarifying the impact of prolonged stress exposure on the neurogenic niche of the subventricular zone (SVZ) - olfactory bulb (OB) system and monitoring the potential role of Tau in the stress-driven

regulation of cytotogenesis, we exposed wild-type (WT) mice and their littermates lacking Tau protein (Tau-KO) to a chronic unpredictable stress (CUS) paradigm for 9 weeks (see Figure 1). For detection of newly generated cells in the SVZ-OB system, we followed the widely used approach of administration of the synthetic nucleotide bromodeoxyuridine (BrdU), which is incorporated into the newly synthesized DNA during the S phase of the cell cycle. To evaluate proliferation in the SVZ, animals were injected with BrdU for three consecutive days before killing (Figure 1A,B). Analysis of BrdU by immunofluorescent staining in the SVZ showed an interaction between *Stress* and *Genotype* in the number of BrdU-positive cells (two-way ANOVA,  $F_{1,84} = 4.129$ ,  $P = .045$ ) (Figure 1D,E). Post hoc analysis revealed a significant reduction in the number of BrdU-positive cells in stressed WT animals when compared to control WTs ( $P = .039$ ) suggesting that chronic stress reduces the proliferation of newly born cells in the SVZ. In contrast, the number of BrdU-positive cells of stressed Tau-KO animals was not different from control Tau-KOs ( $P = .289$ ) (Figure 1E). Additionally, the number of proliferating cells in stressed WT animals was lower when compared with stressed Tau-KO ( $P < .001$ ) (Figure 1E). No difference was found between WT and Tau-KO animals under control conditions. Altogether, the above data suggest that, while the absence of Tau does not regulate SVZ proliferation under control conditions, it blocks the reduction of SVZ proliferation under stress.

### 3.2 | Chronic stress affects neuroblasts in the SVZ in a Tau-dependent manner

Neural stem cells (NSCs) in the SVZ may give rise to neuronal and oligodendrocytes precursors (Figure 1C). To monitor the impact of chronic stress on SVZ neuroblasts, brain sections from mice injected with BrdU before sacrifice (Figure 1D) were double-stained with antibodies against BrdU and DCX; the latter is a cytoskeletal protein expressed in neuroblasts and immature neurons.<sup>46,47</sup> Quantification of BrdU-labelled cells that were co-stained with DCX (Figure 1F—see also Figure S1), revealed a *Stress* × *Genotype* interaction in the percentage of DCX/BrdU double-labelled cells in the SVZ (two-way ANOVA  $F_{1,41} = 6.865$ ,  $P = .0123$ ). Post hoc analysis showed that exposure to CUS reduced the percentage of DCX/BrdU-labelled neuroblasts in the SVZ of WT animals when compared to control WTs ( $P = .024$ ). However, this was not true for Tau-KO animals, where the percentage of DCX/BrdU double-labelled cells in stressed and control Tau-KOs did not differ ( $P = .365$ ) (Figure 1F). Furthermore, the percentage of DCX/BrdU double-labelled cells in stressed WT animals was decreased when compared with stressed Tau-KO ( $P = .006$ ). Again, we did not detect differences in the percentage of DCX/BrdU-labelled cells between WT and Tau-KO animals under control conditions.

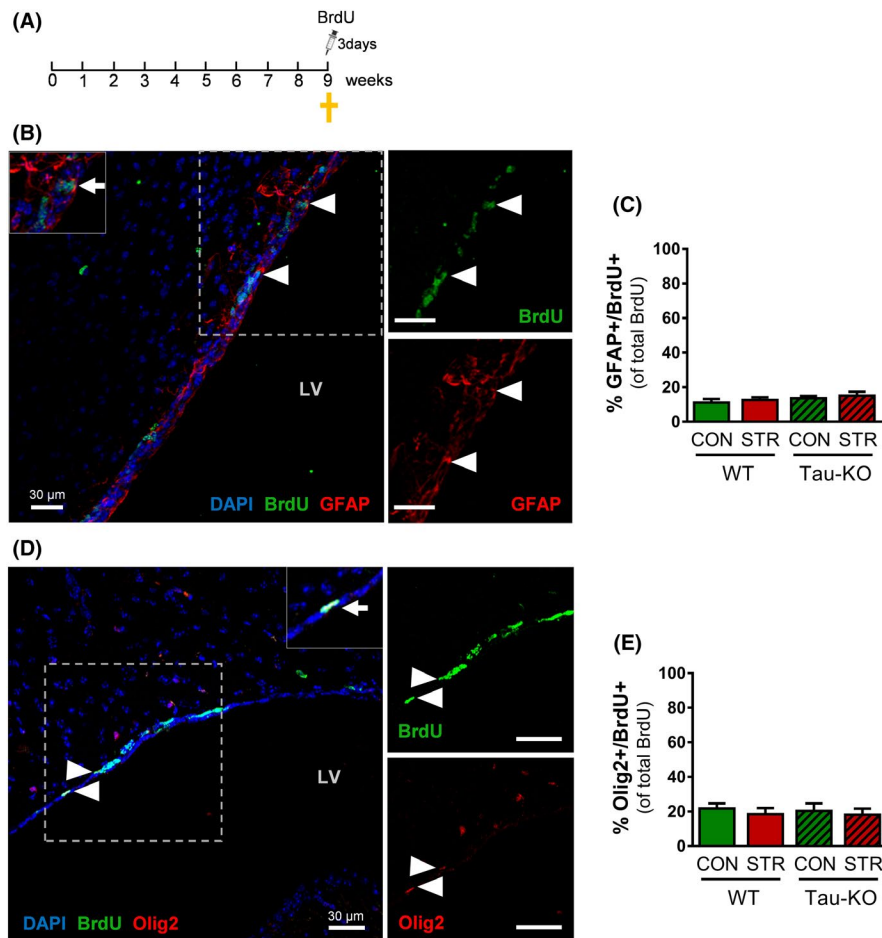
We next monitored the effect of chronic stress and Tau ablation in the pool of NSCs and oligodendrocytes, the two other main cell types present in the SVZ (Figure 1C). To monitor NSCs,

we performed staining with antibodies against BrdU and GFAP, a cytoplasmic marker that identifies NSCs in the SVZ (Figure 2A,B—see also Figure S2). As shown in Figure 2C, we found no differences in the percentage of GFAP/BrdU double-labelled cells in the SVZ among groups, suggesting that chronic stress exposure does not affect this cell population in animals of both genotypes. Furthermore, we have also monitored oligodendrocyte precursors by double labelling with BrdU and Olig2, a nuclear marker that identifies oligodendrocyte progenitors (Figure 2D and Figure S2). Here, we detected no significant differences in Olig2/BrdU-labelled cells among all groups, indicative of an absence of any significant effect of stress or Tau deletion in this cell population of the SVZ (Figure 2E).

### 3.3 | Newly born neurons are differentially regulated by Tau deletion and chronic stress in the different sublayers of the olfactory bulb

As the olfactory bulb (OB) is the brain region where the newly born neurons generated within the SVZ migrate, we next analysed the effect of Tau deletion and chronic stress on another set of animals. In this case, animals had been injected with BrdU 4 weeks before the sacrifice, to give enough time for the newly generated cells to reach the OB (Figure 3A). This allows us to monitor the migration of newborn cells and neurons from the SVZ to OB along the different main layers of the OB, namely granular cell layer (GCL), mitral cell layer (MCL) and glomerular cell layer (GL) (Figure 3B,C). For that purpose, we performed double immunofluorescent staining with BrdU and NeuN, a marker that identifies mature neurons (Figure 3D—see also Figure S3). Quantification of BrdU cells in the GCL of the OB, the first layer that newborn neurons reach after migrating through the RMS and differentiate into interneurons and specifically into granule cells, showed an overall *Genotype* effect (two-way ANOVA,  $F_{1,30} = 4.590$ ,  $P = .04$ ), while post hoc analysis revealed a significant increase in BrdU-labelled cells in control Tau-KO ( $P = .035$ ) animals when compared with control WT (Figure 3E). No clear impact of stress was detected in WT or Tau-KO animals. Additionally, quantification of NeuN/BrdU-labelled cells showed an overall *Genotype* effect (two-way ANOVA,  $F_{1,30} = 4.074$ ,  $P = .05$ ), with post hoc analysis revealing a significant increase in control Tau-KO ( $P = .035$ ) animals when compared with control WT (Figure 3E). However, the percentage of NeuN/BrdU-labelled cells among the total BrdU-labelled cells revealed no statistical differences among groups.

We next monitored the BrdU- and NeuN-positive cells in the MCL of the OB, the second layer of migration and differentiation into granule cells in the OB. Two-way ANOVA analysis of BrdU-labelled cells showed a *Stress* × *Genotype* interaction effect ( $F_{1,30} = 4.814$ ,  $P = .036$ ). Similarly, quantification of NeuN+/BrdU+ cells showed a *Stress* × *Genotype* interaction effect ( $F_{1,30} = 6.565$ ,  $P = .015$ ) accompanied by a decrease in stressed WT animals when compared to control WT ( $P = .037$ ). Additionally, Tau-KO CONs showed



**FIGURE 2** Levels of neural stem cells and oligodendrocytes in the subventricular zone are not altered by chronic stress. A-E, GFAP/BrdU and Olig2/BrdU immunofluorescent staining of SVZ in animals injected with BrdU before sacrifice. B, Representative microphotograph of BrdU/GFAP double-labelled cells (arrow head) in the SVZ. C, Chronic stress does not affect the percentage of GFAP/BrdU double-labelled cells (reflecting neural stem cells) in WT and Tau-KO animals. D, Olig2/BrdU microphotograph of double-labelled cells (arrow head) in the SVZ. E, Exposure to chronic stress does not affect the percentage of Olig2/BrdU double-labelled cells (reflecting oligodendrocyte progenitor cells) in WT and Tau-KO animals. All numerical data are shown as mean  $\pm$  SEM; GFAP, glial fibrillary acidic protein; Olig2, oligodendrocyte transcription factor 2; WT, wild type; Tau-KO, Tau-knockout

decreased number of NeuN+/BrdU+ cells when compared to WT CONs ( $P = .046$ ). When we calculated the percentage of NeuN/BrdU double-labelled cells among the total BrdU-positive cells, we found a similar *Stress*  $\times$  *Genotype* interaction effect ( $F_{1,30} = 4.257$ ,  $P = .047$ ) (Figure 3F).

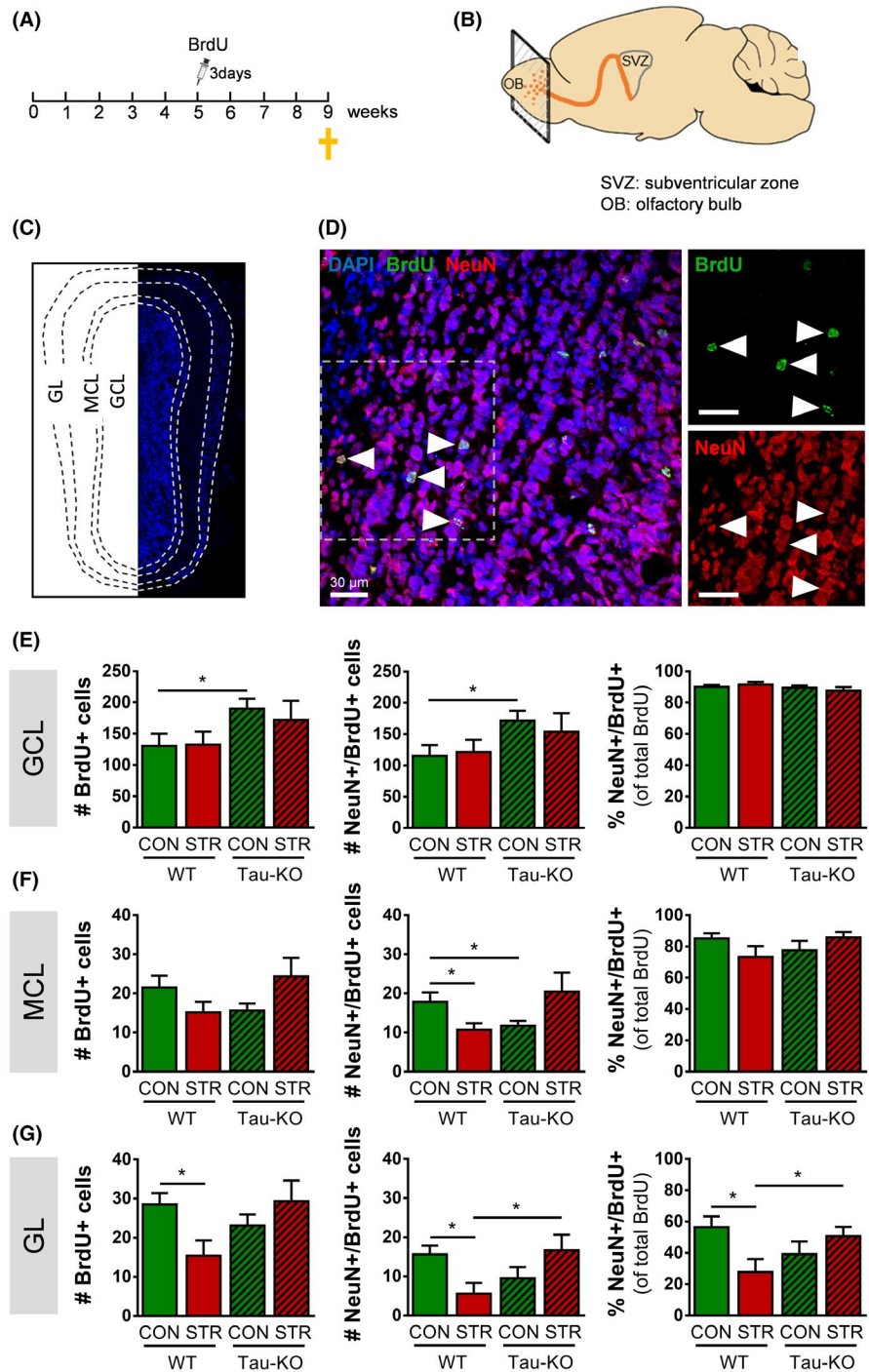
We also monitored the GL of OB, the last layer of OB and where cells differentiate into periglomerular cells. Quantification of BrdU-labelled cells in the GL revealed a *Stress*  $\times$  *Genotype* interaction effect (two-way ANOVA,  $F_{1,30} = 6.071$ ,  $P = .0197$ ). Post hoc analysis revealed a significant decrease in BrdU-labelled cells in stressed WT mice when compared with control WTs ( $P = .014$ ). However, the levels of BrdU-labelled cells in stressed Tau-KO animals were not different from the levels of control Tau-KOs (Figure 3G). Similarly, quantification of NeuN/BrdU-labelled cells showed a *Stress*  $\times$  *Genotype* interaction ( $F_{1,30} = 7.834$ ,  $P = .008$ ). Post hoc analysis showed that exposure to chronic stress reduced the number of NeuN/BrdU-labelled cells in WT animals ( $P = .012$ ), but not in Tau-KO when compared to their corresponding controls (Figure 3G). Moreover, in stressed WT animals, these cells were reduced when compared to Tau-KO stressed ( $P = .048$ ). We next calculated the percentage of NeuN/BrdU double-labelled cells and found a *Stress*  $\times$  *Genotype* interaction effect ( $F_{1,30} = 7.476$ ,  $P = .010$ ). Further analysis showed that stressed WT animals presented a reduced percentage of

NeuN/BrdU-double-labelled cells when compared to control WTs ( $P = .018$ ). However, the percentage of NeuN/BrdU-labelled cells was not altered in stressed Tau-KO in comparison with control Tau-KO ( $P = .265$ ) but was significantly higher when compared to stressed WT ( $P = .036$ ). Altogether, the above data suggest that exposure to chronic stress reduced the newly born neurons found in the MCL and GL of OB, and this decrease was blocked in Tau-KO animals indicating that Tau protein is critical in stress-induced neurogenic suppression.

### 3.4 | Tau deletion does not interfere with the endocrine response to stress but blocks the related behavioural changes

Exposure to chronic stress is known to impact organism's homeostasis with stressed animals exhibiting reduced body weight, elevation of stress hormones and behavioural deficits<sup>31,32,48</sup> For monitoring stress efficacy and impact on behaviour, we performed biometric, biochemical and behavioural analysis. Body weight was measured at the end of the stress period and statistical analysis revealed an overall *Stress* effect (two-way ANOVA,  $F_{1,24} = 24.92$ ,  $P < .0001$ ) (Figure 4A). Specifically, stressed WT animals exhibited reduced body weight when compared to control WT animals

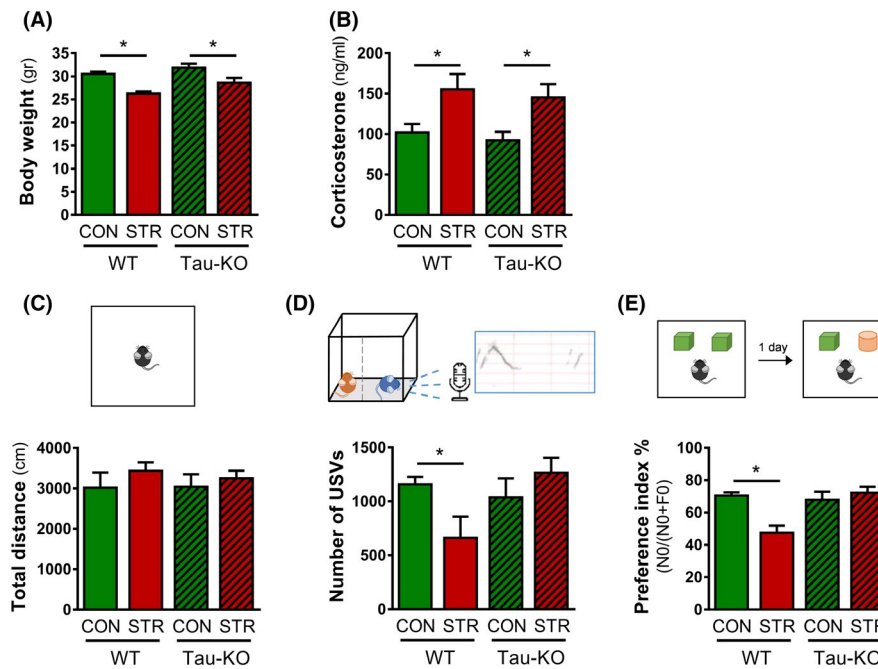
**FIGURE 3** Impact of chronic stress and Tau on newborn neurons in differential sublayers of the olfactory bulb (OB). A, For olfactory bulb (OB) analysis, mice were injected with BrdU 4 weeks before sacrifice. B-C, Schematic illustration of the mouse brain highlighting the olfactory bulb (OB) level of analysis followed by an olfactory bulb coronal section (C) and the different sublayers analysed; granular cell layer (GCL), mitral cell layer (MCL), glomerular cell layer (GL). D, Representative microphotograph of BrdU/NeuN double-labelled cells (arrow head) in the OB. E, Tau-KO animals exhibited increased number of BrdU-positive cells and NeuN/BrdU double-labelled cells in the GCL; no differences were found in the percentage of BrdU-positive cells that are NeuN/BrdU double-labelled. F, In MCL of OB, Tau-KO animals exhibited reduced levels of NeuN/BrdU double-labelled cells compared to WT and chronic stress reduced NeuN/BrdU cells in WT, but not Tau-KO, animals. G, In GL, exposure to chronic stress reduced the density of BrdU-positive cells and NeuN/BrdU double-labelled cells, as well as, the percentage of NeuN/BrdU cells in GL of WT animals; this stress effect was not found in Tau-KO animals. All numerical data are shown as mean  $\pm$  SEM ( $*P < .05$ ). OB, olfactory bulb; GCL, granular cell layer; MCL, mitral cell layer; GL, glomerular cell layer; BrdU, 5-bromo-2'-deoxyuridine; WT, wild type; Tau-KO, Tau-knockout



( $P < .0001$ ). Similar to WT, stressed Tau-KO animals exhibited reduced body weight in comparison with control Tau-KOs ( $P = .03$ ) suggesting that stress affected body weight independently of Tau. Similarly, analysis of the levels of corticosterone, the main stress hormone, showed an overall Stress effect (two-way ANOVA,  $F_{1,24} = 13.48$ ,  $P = .001$ ). Furthermore, stressed animals of both genotypes presented increased levels of blood corticosterone when compared to the corresponding CON group (WT CON vs. WT STR:  $P < .0001$  and Tau-KO CON vs. Tau-KO STR:  $P = .033$ ) (Figure 4B). These results suggest that the absence of Tau does not interfere

with the endocrine response to stress leading to the expected elevation in corticosterone levels. Next, we monitored different behavioural domains starting with locomotion and overall activity of the animals. The Open-field (OF) test showed no differences in the total distance travelled among groups indicating no changes in locomotion under stress conditions in both WT and Tau-KO animals (Figure 4C). Additionally, we monitored ultrasonic vocalizations (USVs) of the animals as an index of their emotional status.<sup>45</sup> Our results showed a Stress  $\times$  Genotype interaction in the number of USVs emitted by each animal (two-way ANOVA  $F_{1,24} = 5.578$ ,





**FIGURE 4** Tau deletion does not interfere with endocrine response to stress but attenuates stress-induced behavioural impairment. A-B, Stressed animals of both WT and Tau-KO genotype exhibited reduced body weight (A) and increased levels of corticosterone, the main stress hormones (B), when compared with their corresponding control animals. C, Total distance travelled in the open-field arena was not different among groups. D, Chronic stress decreased the number of ultrasonic vocalizations (USVs) in WT animals indicating deficits of emotional status; no effect of stress was found in Tau-KO animals. E, Preference index in the Novel Object Recognition test was reduced in stressed WT animals when compared to WT controls; this stress effect was not found in Tau-KOs suggesting a Tau-dependent cognitive impairment caused by chronic stress. All numerical data are shown as mean  $\pm$  SEM (\* $P < .05$ ). USVs, ultrasonic vocalizations; WT, wild type; Tau-KO, Tau-knockout

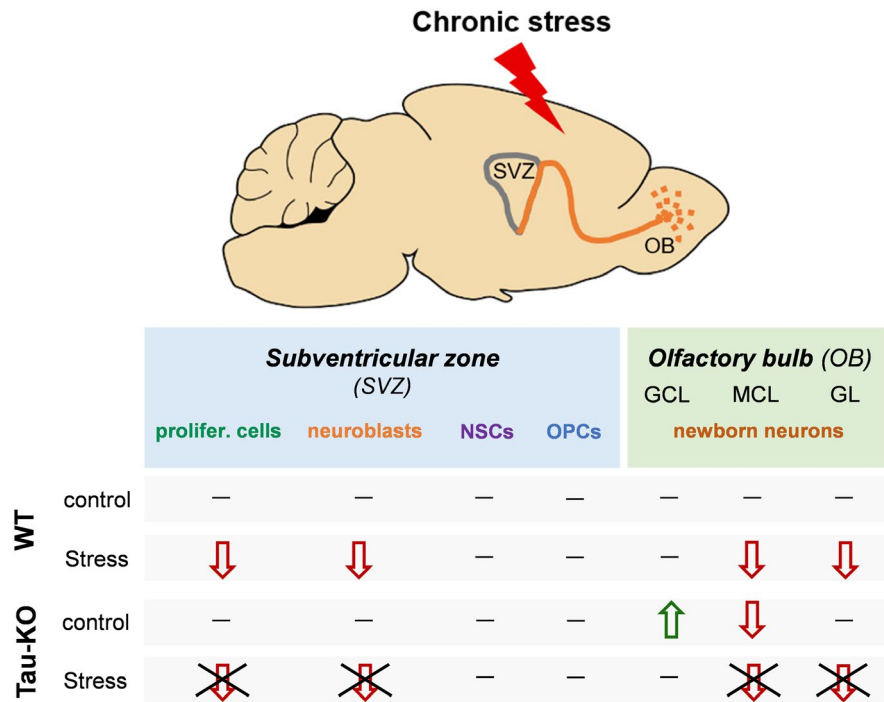
$P = .0026$ ). Further analysis revealed that stressed WT animals exhibited a reduced number of USVs when compared to WT controls ( $P = .035$ ) (Figure 4D). On the contrary, Tau-KO animals were not affected by stress as control and stressed Tau-KO animals showed similar levels of USVs ( $P = .327$ ). Cognitive performance was also monitored by Novel object recognition. We found an interaction between *Stress* and *Genotype* in the preference index (two-way ANOVA,  $F_{1,24} = 12.23$ ,  $P = .002$ ) (Figure 4E). Post hoc analysis revealed a significant reduction in the preference index in stressed WT animals when compared to control WTs ( $P = .002$ ) while no differences were found between stressed and control Tau-KO animals ( $P = .854$ ).

## 4 | DISCUSSION

Fifty-five years after the first report of neurogenesis in the adult brain,<sup>49</sup> the specific role and the mechanisms that regulate the generation of newborn neurons integrated into the adult brain are still to be fully understood. Although the extent and relevance of adult neurogenesis in humans are currently debated,<sup>15,16</sup> accumulating evidence supports the involvement of neurogenesis in brain morphofunctional response to different stimuli (eg exercise, environmental enrichment vs. sleep deprivation, environmental stress) and brain pathologies (depression, post-traumatic stress disorder,

Alzheimer's disease).<sup>28,50,51</sup> Furthermore, the possibility to induce neural precursors to generate new neurons is an attractive prospect for neuro-replacement therapy in different pathological conditions characterized by neuronal loss (eg. Alzheimer's disease, brain trauma). The role of SVZ neurogenesis in the adult brain function and pathology has been less investigated compared with hippocampal neurogenesis despite that the SVZ exhibits the highest number of proliferative cells in the adult brain.<sup>4,5</sup> Moreover, besides the OB (in rodents<sup>52,53</sup>; or the striatum (in humans<sup>54</sup>), other neocortical regions after stroke or trauma are shown to receive newly born cells/neurons generated in the SVZ.<sup>40,55,56</sup>

The current study focused on the analysis of neurogenesis in the SVZ-OB system of the adult rodent brain after prolonged exposure to environmental stress. Clinical and experimental evidence has long shown that exposure to stressful conditions is a strong precipitant of depressive pathology while a cardinal feature of the response to chronic stress is the atrophy of specific brain regions, as detected by both brain imaging and stereological techniques.<sup>57,58</sup> These plastic changes of the brain include dendritic atrophy and synaptic loss accompanied by the suppressed generation of newly born cells in specific areas of the adult brain.<sup>28,29,48,57,59</sup> Whereas the hippocampus has been the main focus of a plethora of clinical and experimental studies of depressed and/or stress-exposed human subjects and related animal models, clinical studies have also reported that adults with a history of early life stress or major depressive disorder present



**FIGURE 5** Summary of the impact of chronic stress on different cell populations in the subventricular zone - olfactory bulb neurogenic niche. Chronic stress suppresses proliferation, neuronal, but not oligodendrocytic, differentiation and maturation of newly born cells in the subventricular zone (SVZ) and olfactory bulb (OB) of the adult brain as assessed by decreased levels of proliferating cells and neuroblasts in SVZ and reduced newborn neurons in the mitral cell and glomerular cell layer (MCL and GL, respectively) of OB. However, the stress impact on the above cell populations was blocked in animals lacking Tau (Tau-KO). These findings suggest that Tau protein is essentially involved in the neurogenesis-suppressing role of chronic stress on the SVZ-OB neurogenic niche of the adult brain in line with previously reported reduction of the hippocampal neurogenesis by stress.<sup>22,33</sup> Interestingly, compared to wild type (WT), newborn neurons of Tau-KO animals seem to be accumulated in the first layer of the OB, the granular cell layer (GCL), indicating delayed migration of newborn neurons in the other OB cell layers and subsequently, decreased number of these cells in the mitral cell layer (MCL). GCL, granular cell layer; MCL, mitral cell layer; GL, glomerular cell layer; NSCs, neural stem cells; OPCs, oligodendrocytes progenitor cells; WT; wild-type, Tau-KO, Tau-knockout

reduced OB volume and odorant detection impairment<sup>60-63</sup> indicating the potential impact of chronic stress on the OB. In line with that clinical evidence, few experimental studies on rodents exposed to chronic stress and/or to high levels of stress hormones, glucocorticoids, have shown reduced SVZ neurogenesis and olfactory deficits along with depressive-like and anxiety symptoms.<sup>43,64,65</sup> Different stress paradigms, such as maternal separation, repeated exposure to forced swim stress and chronic administration of corticosterone resulted in reduced BrdU-labelled proliferating cells in the SVZ.<sup>43,64-66</sup> In line with these reports, we hereby demonstrate that 9-week exposure to a CUS protocol reduced both proliferation and neuronal differentiation of newly born cells in the SVZ, as assessed by the reduced number of BrdU-labelled cells as well as DCX/BrdU-labelled neuronal precursors and immature neurons in SVZ (Figure 5). On the other hand, exposure to short stress periods (e.g. 2-day repeated foot shock stress paradigm<sup>67</sup>) or a milder stress paradigm (e.g. chronic mild stress<sup>42</sup> or corticosterone<sup>64</sup>) do not seem to impact SVZ neurogenesis; nevertheless, the above stress paradigms reduced neurogenesis in the hippocampus indicating a differential vulnerability of proliferating cells to stress and stress hormones between the dentate gyrus (DG) and SVZ neurogenic niches (see also<sup>64</sup>). This is of great importance as different areas of the adult brain are shown to

exhibit different vulnerability to the detrimental effects of chronic stress on their plasticity and function (e.g. hippocampus vs. frontal cortex<sup>68</sup>) while other brain areas respond with opposite effects to chronic stress; for instance, stress causes atrophy to adult hippocampus whereas hypertrophy to amygdala and nucleus accumbens.<sup>48,69</sup>

Following a 4-week period of newly born cells survival and migration into the OB, we found that the 9-week CUS protocol also suppress the BrdU-labelled cell population in the MCL and GL of the OB; This was accompanied by a reduced number of NeuN/BrdU-labelled newborn neurons in OB of the adult brain (Figure 5). These findings indicate that chronic stress diminishes neuronal maturation as well as the survival rate of newly born neurons in the OB that may contribute to the previously described deficits of olfactory memory induced by prolonged stress and/or corticosterone exposure.<sup>43,64</sup> The current findings on both SVZ and OB brain areas suggest that 9 weeks of chronic stress exposure suppress proliferation, neuronal differentiation and maturation of newly born cells in the SVZ and OB, respectively (Figure 5). Together with previous studies showing that stress triggers neuronal atrophy and synaptic loss in pre-existing (older) hippocampal and cortical neurons,<sup>48,70</sup> as well as suppresses the genesis of newborn neurons in the hippocampal dentate gyrus,<sup>22,23,48,71</sup> the current study provides solid evidence about the participation of the

SVZ-OB neurogenic system in the network of brain areas damaged by chronic stress. Through an integrated manner, chronic stress damages various domains of behavioural performance such as different types of memory (i.e. associative, spatial and odour memories) and emotional status (e.g. anxiety levels, depressive behaviour).

Tau is an important protein involved in the regulation of cytoskeletal assembly and different cellular processes, such as axonal branching and transport, as well as in neuronal polarity, migration and differentiation.<sup>22,72-74</sup> Despite the compensatory mechanisms (e.g. increased expression of other cytoskeletal proteins) that have been suggested to attribute to the lack of gross behavioural and neurostructural abnormalities in animals that lack Tau protein (Tau-KO), the absence of Tau appears to cause a transient delay in the dendritic maturation of new-born neurons<sup>33</sup> as well as a delay in their migration.<sup>73,75</sup> Moreover, it was recently shown that 14-month-old Tau-KO mice exhibit increased BrdU-labelled proliferating cells in the SVZ.<sup>76</sup> In contrast, our analysis of BrdU-labelled proliferating cell in SVZ did not detect a significant difference between WT and Tau-KO animals; this difference may be attributed to the age difference between our (6-7-month-old mice) and Criado-Marrero study (14 months old).<sup>76</sup> However, our findings demonstrate a significant increase in the number of newborn neurons in the OB, a region that was not monitored in Criado-Marrero's study.<sup>76</sup> Moreover, this increase in the number of newborn neurons was detected in the first layer of the OB (granular cell layer; GCL) of Tau-KO animals accompanied by a tendency for a decrease in the next OB layers (mitral and glomerular cell layers—see Figure 3 and Suppl. Figure 3), indicative of a delay of neuronal migration in OB led by the absence of Tau. Although the precise mechanisms through which lack of Tau may induce neuronal migration deficits in the OB are still under investigation, inhibition of the Rho-ROCK signalling pathway by Tau absence in glioblastoma cells was recently shown to induce the remodelling of the actin cytoskeleton leading to delayed cell migration.<sup>77</sup> Alternatively, the interplay between Tau protein and the transduction of reelin, a protein that is crucial in neuronal migration and to the formation of synaptic connections in the brain, may have an equally important role here.<sup>78-80</sup> Moreover, the malfunction of Tau is causally related to cytoskeletal dysregulation, neuronal malfunction and atrophy under different pathological conditions including AD as well as stroke and brain trauma.<sup>48,70,81-83</sup> As SVZ neurogenesis is suggested to participate in the endogenous regenerative response of the brain to stroke or trauma,<sup>40,55,56</sup> future studies should clarify the potential role of Tau in the adult neurogenic process under these pathological conditions, too.

Recent animal studies from our team and others have proposed the involvement of Tau in the regulation of adult neurogenesis of the hippocampus after exposure to acute or prolonged stress.<sup>22,33</sup> Specifically, exposure to stress leads to Tau hyperphosphorylation and accumulation in newly born neurons of the adult brain.<sup>2</sup> It is known that dephosphorylated Tau binds more stable to microtubules while increased phosphorylation of Tau is shown to reduce its microtubule-binding capacity leading to microtubule instability.<sup>84</sup> Through a constant regulation of its phosphorylation-dephosphorylation equilibrium,

Tau protein is involved in many cellular functions as it regulates the cytoskeletal stability influencing morphogenesis of neurons.<sup>85</sup> Thus, stress-evoked alterations of the tight control of Tau phosphorylation could impair the complex and tight regulation of Tau, diminishing the cellular control over the cytoskeletal dynamics and network essential for proliferating cells and neuroblasts. Interestingly, animals lacking Tau were spared from the neurogenesis-damaging effects of chronic stress as, in contrast to WTs, stressed Tau-KO presented no reduction of proliferating cells and neuroblasts in the SVZ, followed by lack of decreased newborn neurons in the OB (Figure 5). Extending previous evidence about Tau-dependent suppression of the hippocampal neurogenesis under stress conditions,<sup>22,33</sup> the current study suggests the essential mediation of Tau in the stress-driven neurogenic deficits in the SVZ-OB system of the adult brain. Moreover, progenitor cells in the hippocampus and SVZ are also able to differentiate into non-neuronal cell types (e.g. astrocytes, oligodendrocytes); however, our knowledge about whether and how chronic stress impacts other types of newborn cells (non-neuronal ones) in the adult brain remains limited. Our findings suggest that the population of oligodendrocyte progenitor cells in the SVZ is not affected by stress indicating that the detrimental impact of chronic stress on the SVZ-OB niche is mainly neuronal with an essential mediating role for Tau protein. Indeed, the neuroprotective role of Tau reduction against chronic stress is extended beyond newly born cells (and neurogenesis) as it is also evident in old, pre-existing neurons of the hippocampus as well as of other brain areas (e.g. prefrontal cortex).<sup>48,70</sup> Moreover, emerging evidence from animal models of diverse brain pathologies (e.g. Alzheimer's disease, epilepsy, stroke, traumatic brain injury)<sup>81,83,86</sup> suggests Tau as a converging protein of neuronal damage between different insults and disorders highlighting its broad neuroplastic and neuropathological role.<sup>85</sup>

In summary, the current study provides novel insights about the involvement of Tau protein in the mechanisms that reduce cell proliferation, neuronal differentiation and migration within the SVZ-OB neurogenic niche under prolonged stressful conditions. Together with previous work suggesting the role of Tau in stress-evoked hippocampal plasticity changes,<sup>22,33,48</sup> these findings bring further information about the biological underpinnings of the stress-driven deficits on the adult brain circuits regulating mood and cognition. A better understanding of the mechanisms underlying the neuroplastic effect of chronic stress on the adult brain may help the development of targeted therapies for stress-related disorders, such as depression and Alzheimer's disease, which are characterized by deficits of neuronal plasticity.

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## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## AUTHOR CONTRIBUTIONS

CD was involved in all stages of the experimental procedures, data collection and analysis, interpretation and manuscript preparation. PP, CM, MM, and LGP have contributed to immunohistochemical staining and confocal microscope analysis and involved in the preparation of the manuscript. SV and JMB provide materials and critically reviewed the manuscript. LP and IS supervised these studies and were involved in interpretation and manuscript preparation.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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