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Research article

Chronic P7C3 treatment restores hippocampal neurogenesis

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HIGHLIGHTS

- Down syndrome (DS) patients are marked by diminished adult hippocampal neurogenesis.
- P7C3, a proneurogenic compound, was tested in the Ts65Dn mouse model of DS.
- Chronic P7C3 treatment had no effect on hippocampal neurogenesis in wild-type mice.
- Chronic P7C3 treatment increased hippocampal neurogenesis in Ts65Dn mice.

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ABSTRACT

Down syndrome (DS) is the most common genetic cause of intellectual disability and developmental delay. In addition to cognitive dysfunction, DS patients are marked by diminished neurogenesis, a neuropathological feature also found in the Ts65Dn mouse model of DS. Interestingly, manipulations that enhance neurogenesis – like environmental enrichment or pharmacological agents – improve cognition in Ts65Dn mice. P7C3 is a proneurogenic compound that enhances hippocampal neurogenesis, cell survival, and promotes cognition in aged animals. However, this compound has not been tested in the Ts65Dn mouse model of DS. We hypothesized that P7C3 treatment would reverse or ameliorate the neurogenic deficits in Ts65Dn mice. To test this, adult Ts65Dn and age-matched wild-type (WT) mice were administered vehicle or P7C3 twice daily for 3 months. After 3 months, brains were examined for indices of neurogenesis, including quantification of Ki67, DCX, activated caspase-3 (AC3), and surviving BrdU–immunoreactive(+) cells in the granule cell layer (GCL) of the hippocampal dentate gyrus. P7C3 had no effect on total Ki67+, DCX+, AC3+, or surviving BrdU+ cells in WT mice relative to vehicle. GCL volume was also not changed. In keeping with our hypothesis, however, P7C3-treated Ts65Dn mice had a significant increase in total Ki67+, DCX+, and surviving BrdU+ cells relative to vehicle. P7C3 treatment also decreased AC3+ cell number but had no effect on total GCL volume in Ts65Dn mice. Our findings show 3 months of P7C3 is sufficient to restore the neurogenic deficits observed in the Ts65Dn mouse model of DS.

1. Introduction

Down syndrome (DS) is the most common genetic cause of intellectual disability caused by the triplication of human chromosome 21 [11,17]. The mutation results in congenital neuropathologies including hypoplasia and reduced brain volumes [11,17,31,34] that contributes to diminished brain function in DS individuals [11,31]. Reduced hippocampal volume is evident during embryonic and early postnatal development [12,18,32], and persists into adulthood [34]. One key determinant of hippocampal volume is the lifelong process of neurogenesis in which dividing neural progenitor cells are generated in the granule cell layer (GCL) of the hippocampal dentate gyrus [22]. Both postnatal- and adult-generated progenitor cells ultimately differentiate into functional neurons important for many cognitive processes [22]. Decreased hippocampal volume and cognition in individuals with DS has been correlated with fewer proliferating cells and increased apoptotic cell death in the hippocampus [12,18,32], supporting a link between decreased brain size and impaired cognition with reduced neurogenesis.

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The reduced hippocampal neurogenesis that characterizes individuals with DS is also seen in trisomic mouse models of DS, providing additional evidence that reduced neurogenesis is a key, conserved neuropathological feature of DS [17]. The most studied trisomic DS mouse model is the Ts65Dn mouse, which contains a triplication of genes on mouse chromosome 16 that are orthologous for nearly half of the genes located on human chromosome 21 [11,17]. Ts65Dn mice recapitulate many of the neuropathologies seen in the human condition, including hypoplasia, decreased brain volume, and decreased cognitive function [11,17,23]. Notably, Ts65Dn mice demonstrate suboptimal hippocampal network architecture spurred by atypical synaptic connectivity [8,19,33], reduced glutamatergic input, and enhanced GABAergic inhibition [1,13,29]. Ts65Dn mice also display increased astrogliogenesis [7,27,28] and reduced neurogenesis [8,12], motivating interest in uncovering novel therapeutic strategies to target hippocampal neurogenesis as a means to improve cognitive function in individuals with DS.

Some of the neurogenic and cognitive deficits in Ts65Dn mice can be reversed with pharmacological intervention [2,6,13,14,19,33,37] or environment enrichment strategies [9,26]. These studies underscore the potential of harnessing neurogenesis to improve cognition in individuals with DS. However, the nonspecific nature and unknown molecular targets of these strategies precludes detailed mechanistic findings. More selective neurogenic-enhancing approaches are needed to test the hypothesis that reversal or amelioration of neurogenic deficits in Ts65Dn mice. One promising proneurogenic molecule is the aminopropyl carbazole P7C3 [30]. P7C3 was discovered for its ability to enhance cell survival and decrease apoptosis, thereby preventing the neurogenic and cognitive decline seen in aged rats [30] and rodent models of neurodegeneration [15,35], traumatic brain injury [4], and depression [38]. However, the therapeutic use of P7C3 as a proneurogenic and neuroprotective compound in restoring hippocampal neurogenesis in Ts65Dn mice has not been tested.

In this study we hypothesized that chronic P7C3 treatment would reverse or ameliorate the neurogenic deficits in Ts65Dn mice. WT and Ts65Dn mice were treated with Veh or P7C3 twice daily for 3 months and were examined for hippocampal neurogenesis. In keeping with our hypothesis, 3-month treatment with P7C3 rescued hippocampal neurogenesis in Ts65Dn mice, but had no effect in WT mice. Our findings show that chronic P7C3 treatment may be sufficient to restore the neurogenic deficits in the Ts65Dn mouse model of DS.

2. Methods

2.1. Animals

Age-matched male and female WT and Ts65Dn (B6EiC3Sn.BLiA-Ts(17^{16}) 65Dn/DnJ; stock 5252) mice were obtained from Jackson laboratory (Bar Harbor, ME) at 4–10 weeks old. Mice were group-housed, maintained on a 12:12 h light/dark cycle, and provided food and water ad libitum in accordance to NIH guidelines for the Care and Use of Laboratory Animals. Mice were monitored daily for general health and appearance throughout the study and cage changes were performed per routine scheduling. All experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center.

2.2. BrdU and P7C3 administration

All mice received 3 intraperitoneal (i.p.) injections of bromodeoxyuridine (BrdU; 150 mg/kg dissolved in 0.9% saline and 0.007 N NaOH; Boehringer Mannheim), one injection every 6 h to label dividing cells in S-phase. Two days following BrdU injections, mice were administered P7C3 (provided by Andrew Pieper, University of Iowa, Iowa City, IA) or vehicle as follows: P7C3 was dissolved in 2.5% dimethyl sulfoxide (DMSO) transferred to a glass vial containing 10% Cremaphor EL (Sigma, C5135, St. Louis, MO) and vortexed vigorously for 1 min. Sterile-filtered 5% dextrose (pH 7.0) was added to create a 1.25 mg/mL working solution of P7C3 and vortexted vigorously. P7C3 was prepared fresh daily and stored at 4 °C until use. Vehicle (Veh) consisted of 5% dextrose (pH 7.0) and 10% Cremaphor EL. Mice were administered 350 μL (437.5 μg) of P7C3 or Veh twice daily via i.p. injections between 8–9 am and 5:30–7 pm for 90 days.

2.3. Tissue preparation and immunohistochemistry (IHC)

One day following the final P7C3 treatment, all mice were anesthetized using chloral hydrate, intracardially perfused, and brains were removed and processed for IHC [24]. Tissues from all experimental groups were processed simultaneously.

2.3. Cell and volume quantification

Unbiased estimates for total DCX cell counts were obtained using stereological quantification on an Olympus BX51 System Microscope with a MicroFIRE A/R camera (Optronics, Goleta, California) and the Optical Fractionator Probe within the Stereo Investigator software (MBF Bioscience, MicroBrightField, Inc., Williston, Vermont) [24,25].

For Ki67, BrdU, and AC3 quantification, brightfield staining was visualized with an Olympus BX51 microscope using a 40*x*, 0.63 NA lens with continuous adjustment through the depth of the section [24,25].

Volume estimation of the granule cell layer (GCL) within the dentate gyrus was performed using the Cavalieri Probe within Stereo Investigator [24,25].

2.4. Statistical analyses and data presentation

Data are expressed as means ± s.e.m. from 6 mice (3 males and 3 females) per group. Statistics were performed with unpaired Student's *t*-test (GraphPad Prism 6.0). p values <0.05 were considered significant. Because WT and Ts65Dn mice were not littermates of each other, the main comparison in this study was within genotype, and not across genotype. Therefore, WT and Ts65Dn data were graphed separately. No main effects of sex were observed in WT or Ts65Dn mice, therefore, male and female mice were combined for final analysis.

3. Results

Adult WT and Ts65Dn mice were administered P7C3 twice-daily for 90 days to test the hypothesis that P7C3 would reverse deficits in hippocampal neurogenesis observed in adult Ts65Dn mice [8,11,12,18,23]. We chose the initial dose of the drug based in the initial weight of the animal. Similar to how most patients are treated with drugs in a clinical setting, this initial dose was kept fixed for the duration of the study regardless of whether the animal's weight changed during the study. During the 3-month treatment with P7C3 or Veh both WT and Ts65Dn mice steadily gained weight from 16–20 g at the beginning of the study to 25–31 g at completion (overall mean[g] ± s.e.m: WT/Veh: 26.7 ± 1.8; WT/P7C3: 24.2 ± 1.3; Ts65Dn/Veh: 26.6 ± 2.3; Ts65Dn/P7C3: 22.2 ± 1.5), resulting in a main effect of time (WT: $F_{(6182)} = 26.2$, $p < 0.0001$; Ts65Dn: $F_{(6168)} = 37.5$, $p < 0.0001$). In both WT and Ts65Dn mice, there was also a significant main effect of drug (WT: $F_{(1,182)} = 16.6$, $p < 0.0001$; Ts65Dn: $F_{(1,168)} = 48.1$,

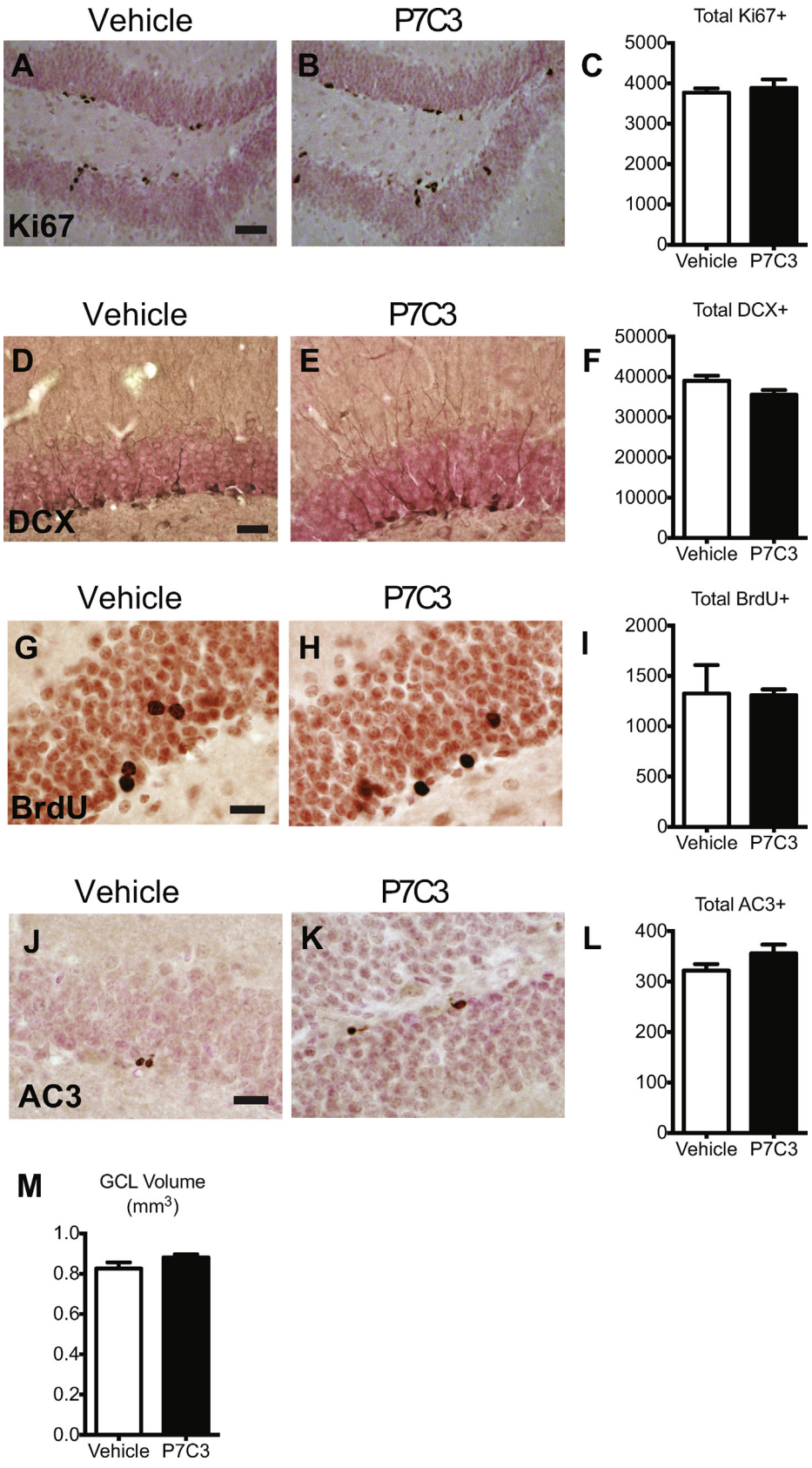

Fig. 1. Representative images of the dentate gyrus in WT mice stained for Ki67 (A and B), DCX (D and E), BrdU (G and H), and AC3 (J and K) (brown) and counterstained with fast red (pink). Scale bar = 25 μm. Total number of Ki67+ (C), DCX+ (F), BrdU+ (I), and AC3+ (L) cells in the GCL are not changed in WT mice. Total GCL volume is also not changed in P7C3-treated WT mice (M). (C,F,I,L,M) Data analyzed by unpaired Student's *t*-test, $N = 6$/group. Data represent means ± s.e.m.

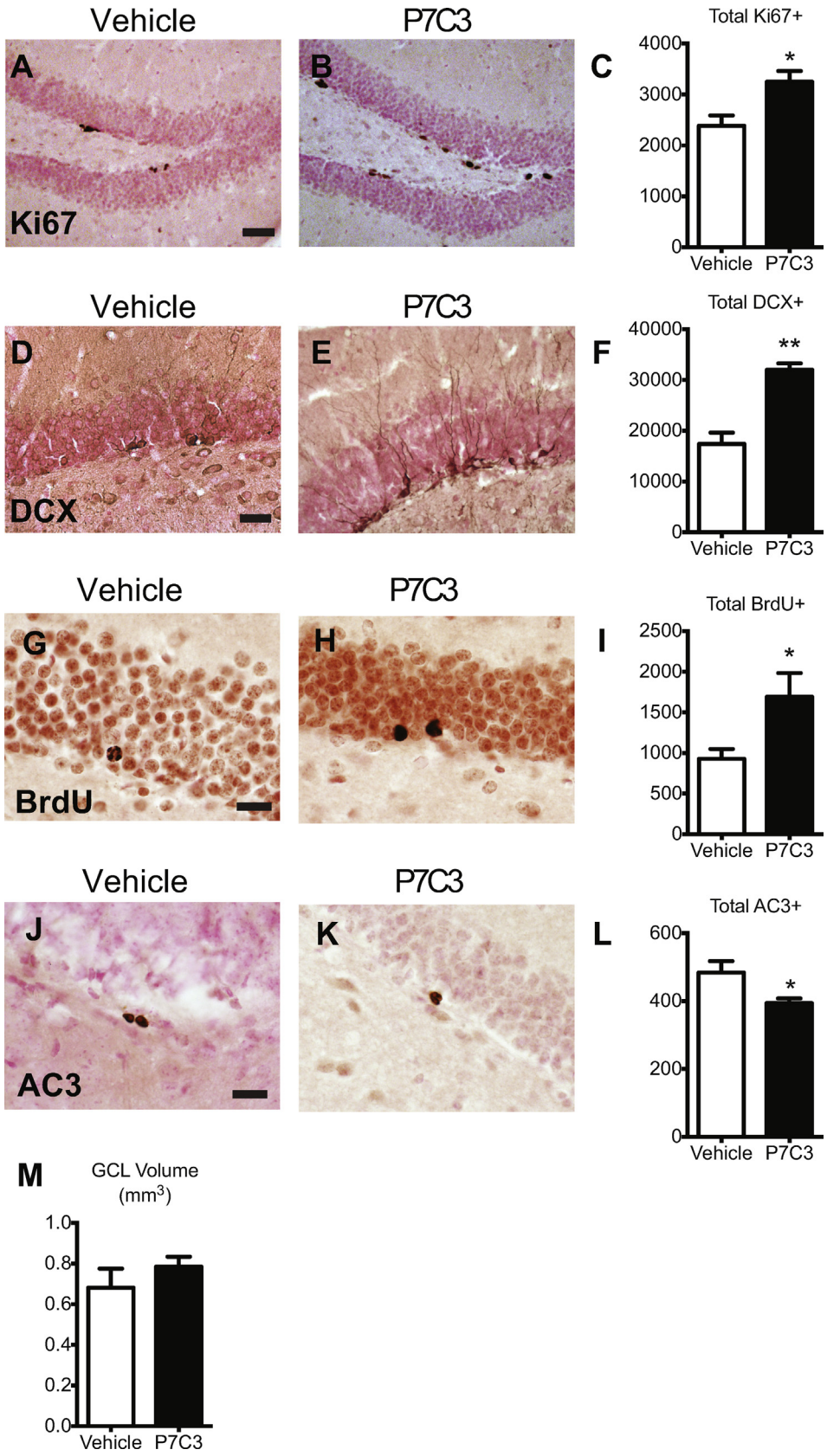

Fig. 2. Representative images of the dentate gyrus in Ts65Dn mice stained for Ki67 (A and B), DCX (D and E), BrdU (G and H), and AC3 (J and K) (brown) and counterstained with fast red (pink). Scale bar = 25 μm. Total number of Ki67+ (C) DCX+ (F), and BrdU+ (I) cells increased in P7C3-treated Ts65Dn mice. Total number of AC3+ cells decreased in P7C3-treated Ts65Dn mice (L). Total GCL volume is not changed in P7C3-treated Ts65Dn mice (M). (C,F,I,L,M) Data analyzed by unpaired Student's t-test. $N = 6$/group. Data represent means ± s.e.m.

$p < 0.0001$), with P7C3-treated mice weighing significantly less than their respective Veh-treated controls. Subsequent posthoc analysis revealed that P7C3-treated Ts65Dn mice weighed significantly less than Veh-treated Ts65Dn mice only during the last 20 days before kill, and not prior to that. No interaction existed between time and drug in either WT or Ts65Dn mice (WT: $F_{(6,182)} = 0.78$, $p > 0.05$; Ts65Dn: $F_{(6,168)} = 1.45$, $p > 0.05$). The mechanism underlying the slight decrease in body weight during the last 20 days in P7C3-treated mice relative to their Veh-treated counterparts is unknown, but may be due to the extended period of drug administration used in the present study, as shorter P7C3 treatment periods did not trigger significant weight loss or slower weight gain in mice or rats [30,38]. Despite the slightly reduced weight of P7C3-treated mice compared to Veh-treated mice during the last 20 days, all mice remained healthy appearing throughout the study.

All mice were administered 350 μL of a 1.25 mg/mL P7C3 working solution or Veh twice each day. This resulted in a daily P7C3 dose of 22–27 mg/kg at the beginning, which, due to weight gain, steadily decreased to 14–17 mg/kg prior to kill. In both WT and Ts65Dn mice, there was a significant main effect of time (WT: $F_{(6,182)} = 29.6$, $p < 0.0001$; Ts65Dn: $F_{(6,168)} = 55.0$, $p < 0.0001$) and drug (WT: $F_{(1,182)} = 13.7$, $p < 0.001$; Ts65Dn: $F_{(1,168)} = 37.3$, $p < 0.001$), but no significant interaction (WT: $F_{(6,182)} = 0.1$, $p > 0.05$, Ts65Dn: $F_{(6,168)} = 0.06$, $p > 0.05$). Despite the range of P7C3 dose administered, the dose was maintained well above 10 mg/kg, a dose shown to effectively reverse the neurogenic and cognitive decline in aged rats [30] and in rodent models of neurodegeneration [15,35] and traumatic brain injury [4].

Mice were killed one day following the final P7C3 dose and adult hippocampal neurogenesis was assessed by quantifying key markers of neurogenesis (Figs. 1A–K and 2A–K): Ki67+ cell number was used as an endogenous marker for proliferating cells (Figs. 1A–C and 2A–C) [21]; DCX+ cell number was used to label neuroblasts/immature neurons (Figs. 1D–F and 2D–F) [5]; BrdU+ cell number (with BrdU given prior to P7C3/saline injections and mice killed 90 days later) was used as an index of adult-generated cell survival (Figs. 1G–I and 2G–I) [40]; and AC3+ cell number was used as an indicator of apoptosis (Figs. 1J–L and 2J–L).

When P7C3 was administered twice daily for 90 days to WT mice, there was no significant change in total Ki67+ (Fig. 1C), DCX+ (Fig. 1F), surviving BrdU+ (Fig. 1I), or AC3+ (Fig. 1L) cell number relative to Veh-treated WT mice ($p > 0.05$). Ki67+, DCX+, BrdU+, and AC3+ cells were also analyzed along the rostro-caudal extent of the dentate gyrus based on literature showing that the dentate gyrus differs in cellular composition and function along this axis [16,20]. Analysis of these markers across the longitudinal axis in WT mice revealed a main effect of Bregma ($p < 0.0001$) but not drug ($p > 0.05$), and no significant interaction (all $p > 0.05$; data not shown). These negative findings are consistent with previously published literature that P7C3 does not significantly affect neurogenesis in young, healthy WT mice [30].

In contrast to WT mice, stereological quantification revealed a substantial increase in the number of Ki67+ (Fig. 2C, $p < 0.05$), DCX+ (Fig. 2F, $p < 0.001$), and surviving BrdU+ (Fig. 2I, $p < 0.05$) cells in P7C3-treated Ts65Dn mice relative to Veh-treated Ts65Dn mice. P7C3 also decrease apoptosis in Ts65Dn mice (Fig. 2L, $p < 0.05$). When these markers were analyzed along the rostro-caudal extent of the dentate gyrus, there was a main effect of Bregma ($p < 0.0001$) and drug ($p < 0.0001$) such that P7C3-treated Ts65Dn mice had more immunoreactive cells along the rosto-caudal extent of the hippocampus – particularly from −2.8 to −3.4 mm Bregma – relative to Veh-treated Ts65Dn mice (data not shown). No significant interaction existed between Bregma distance and drug (all $p > 0.05$; data not shown). These results suggest that chronic P7C3 treatment increases Ki67+, DCX+, and surviving BrdU+ cell number and decreases apoptosis exclusively in Ts65Dn mice, but not in WT mice.

Because these data show that P7C3 increased adult hippocampal neurogenesis solely in Ts65Dn mice, we examined whether P7C3 led to an increase in GCL volume in Ts65Dn mice. There was no significant change in total GCL volume between Veh- or P7C3-treated WT (Fig. 1M, $p > 0.05$) or between Veh- or P7C3-treated Ts65Dn mice (Fig. 2M, $p > 0.05$). When examined across the rostrocaudal axis of the hippocampus in WT mice, there was a significant main effect of Bregma ($F_{(9,100)} = 120.6$, $p < 0.0001$), but not of drug ($F_{(1,100)} = 0.07$, $p > 0.05$), nor an interaction ($F_{(9,100)} = 0.43$, $p > 0.05$). In Ts65Dn mice, however, there was a significant main effect of both Bregma ($F_{(9,100)} = 69.9$, $p < 0.0001$) and drug ($F_{(1,100)} = 8.2$, $p < 0.001$), but no interaction ($F_{(9,100)} = 0.95$, $p > 0.05$).

4. Discussion

Restoring hippocampal neurogenesis to healthy levels by pharmacological [2,6,13,14,19,33,37] or physiological [9,26] means has shown promise to improve cognition in DS mouse models. We hypothesized that treatment with a neurogenesis-enhancing molecule, P7C3, would restore neurogenesis in Ts65Dn mice. Our findings indicate that chronic P7C3 treatment yielded genotype-dependent effects on hippocampal neurogenesis. We show that twice-daily P7C3 treatment for 3 months in Ts65Dn mice increased neurogenesis and cell survival relative to Veh-treated Ts65Dn mice, but had no effect in WT mice.

Although the molecular targets for P7C3 remain unknown, the proneurogenic actions of P7C3 are proposed to be due to reduced apoptosis of newborn neurons via an oxidative stress and mitochondria-dependent mechanism [30,39]. Our BrdU and AC3 data support the possibility that the P7C3-induced increase in neurogenesis in Ts65Dn mice may be linked to increased cell survival. Whether or not the P7C3-induced increase in cell survival is mitochondria-dependent [30,39], earlier studies in which Ts65Dn mice were treated with neuroprotective peptides demonstrate increased neuronal survival and mitochondrial function [6,36].

Several hypotheses may explain the disparity in P7C3's neurogenic effects in WT and Ts65Dn mice. First, P7C3 appears to be most effective in enhancing neurogenesis in instances where neurogenesis levels are inherently below normal, such as in aged rats or in Npas3 deficient mice [30]. One interpretation is that there may be a ceiling effect for P7C3-induced neurogenesis in which mice with healthy levels of neurogenesis may require a higher concentration [38] or longer dosing duration to observe an increase in neurogenesis. However, both our work (15–25 mg/kg, 90 days) and prior work (20 mg/kg, 12 days) highlight the inefficacy of twice-daily injections of P7C3 to increase neurogenesis or hippocampal-dependent function. An alternative concept is that proneurogenic molecules with greater potency and efficacy, such as P7C3 derivatives [15,35], may be required to stimulate neurogenesis in WT mice.

Regardless of the underlying cause for the genotype-dependent neurogenic outcomes following P7C3 treatment, P7C3 and derivatives of this compound may represent a new class of therapeutic drugs to selectively target hippocampal physiological regulatory mechanisms seen only in pathological conditions. While other physiological and pharmacological manipulations enhance hippocampal neurogenesis in both Ts65Dn and WT mice, here we show only Ts65Dn mice are responsive to P7C3 treatment. For example, chronic lithium [13] or fluoxetine [3,10,19,33] increases neurogenesis in both Ts65Dn and WT mice. Likewise, environmental enrichment and exercise also increase neurogenesis in both Ts65Dn and WT mice [9,26]. The genotype-specificity of P7C3 shown here may have beneficial clinical implications, as P7C3 may only induce alterations in the context of abnormally low levels of neurogen-

esis. Limiting alterations to unique physiological contexts could prevent unwanted side effects, such as uncontrolled proliferation. This genotype-specificity, therefore, encourages additional studies to understand the dynamic process of neurogenesis in Ts65Dn mice and to uncover the molecular targets of P7C3 [39].

Authors' contributions

SEL designed and carried out the neurogenesis studies, analyzed and interpreted results, and prepared and edited the manuscript. TCJ administered P7C3 injections. PDR designed and carried out the neurogenesis studies, analyzed and interpreted results. AJE and CMP conceived and participated in the design of the study, interpreted results, and wrote and edited the manuscript. All authors read and approved the final manuscript.

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