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Allopregnanolone Increases the Number of Dopaminergic Neurons in Substantia Nigra of a Triple Transgenic Mouse Model of Alzheimer's Disease

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Abstract

More than a third of Alzheimer's disease (AD) patients show nigrostriatal pathway disturbances, resulting in akinesia (inability to initiate movement) and bradykinesia (slowness of movement). The high prevalence of this dysfunction of dopaminergic neuron in the nigrostriatal pathway in AD suggests that the risk factors for AD appear also significant risk factors for substantia nigra pars compacta (SNpc) lesions. Previously, we have demonstrated that allopregnanolone (APα) promotes neurogenesis and improves the cognitive function in a triple transgenic mouse model of AD (3xTgAD). In this study, we sought to exam 1) the SNpc lesions in 3xTgAD mice and 2) the impact of APα on promoting the regeneration of new dopaminergic neurons in SNpc of the 3xTgAD mice. The number of Nissl-stained total neurons, tyrosine hydroxylase (TH) positive neurons, and BrdU/TH double positive newly formed neurons were analyzed with unbiased stereology. In the SNpc of 3xTgAD mice, TH positive neurons was 47 ± 18 % ($p = 0.007$), total neurons was 62 ± 11.6 % ($p = 0.016$), of those in the SNpc of non-Tg mice, respectively. APα treatment increased the TH positive neurons in the SNpc of 3xTgAD mice to 93.2 ± 18.5 % ($p = 0.021$ vs. 3xTgAD vehicle) and the total neurons to 84.9 ± 6.6 ($p = 0.046$ vs. 3xTgAD vehicle) of non-Tg mice. These findings indicate that there is a loss of neurons, specifically the TH positive neurons in SNpc of 3xTgAD mice, and that APα reverses the lesion in SNpc of 3xTgAD by increasing the formation of new TH neurons.

Keywords

Allopregnanolone; neurogenesis; tyrosine hydroxylase (TH); substantia nigra; dopaminergic neurons

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INTRODUCTION

More than a third of Alzheimer's disease (AD) patients have motor disturbances including akinesia (inability to initiate movement) and bradykinasia (slowness of movement) [1]. Neuropathological reports have shown that, in addition to intraneuronal and extraneuronal amyloid beta (Aß) deposits and the intraneuronal hyperphosphorylated tau and neurofibrillary tangles in the hippocampus, lesions are present in the nigrostriatal pathway and in the locus coeruleus in the brains of AD subjects. These lesions are evidenced by the loss of tyrosine hydroxylase (TH, a rate limiting enzyme for the synthesis if catecholamines, including dopamine) expressing neurons [2-11]. The loss of TH neurons in nigrostriatal pathway and locus coerulous are common pathological features of AD [12-14] and suggests that AD risk factors are significant risk factors for lesions in nigrostriatal pathway [13,15].

This hypothesis is further supported by experimental investigations in subjects carrying mutations in familial AD genes. For example, individuals with an early onset presenilin 1 mutation (V272A) experience a subcortical dementia at 26-36 years of age with age of death between 36-46 years [2]. The pathology of these individuals showed, besides the classical lesions of Alzheimer disease, Lewy bodies in both the cortex and substantia nigra [2]. Perez and colleagues reported an elevated expression of Aβ plagues and a reduction in the dopamine metabolite DOPAC in striatum in APP-swe/PS1DeltaE9 transgenic mice in comparison with age-matched wild-type controls [16]. O'Neil and colleagues measured the catecholaminergic neuronal loss in brains of the transgenic (APPswe/PS1DeltaE9) female mice and reported a significant decrease in TH expressing neurons in the locus coeruleus with a trend to decrease in the substantia nigra [17]. These studies, from both human and animal model, further support the concept that the mutations in genes associated with human familial AD contribute to dysfunctions of the nigrostriatal pathway in the disease.

The triple transgenic mouse model of AD (3xTgAD) carries mutations in two human familial AD genes (APP_{Swe}, $PS1_{M146V}$) and one frontal temporal dementia-linked tau mutation (tau_{P301L}), and manifests age-dependent neuropathology of both Aβ plaques, ptau neurofibrillary tangles [18-20] and insoluble pSer129 a-synuclein [21]. Recently, it has been demonstrated by long-term two-photon confocal *in vivo* imaging stereology that 3xTgAD mice specifically exhibit an early loss of somatosensory cortex layer III neurons at 4 months of age [21], this is consistent with our fluorescence active cell sorting data that loss of total hippocampal cells occur in 3xTgAD mice at 3 months of age [20]. At 3 months old, no apparent AD pathology was observed [21, 20], there is already a significant deficits in neural progenitor cell proliferation at both hippocampal dentate gyrus and cerebral subventricular zone [20]. Obviously, the early neurogenic deficits contribute to the cellular basis for early neuron loss in 3xTgAD mice.

The neurosteroid allopregnanolone (APα, 3α-hydroxy-5α-pregnan-20-one) is a reduced metabolite of progesterone and is generated *de novo* in the CNS [22]. Several reports have demonstrated that administering relatively large doses of progesterone during the first few hours to days after injury significantly limits central nervous system damage, reduces loss of neural tissue, and improves functional recovery [23-25], and allopregnanolone was found to be a more efficacious than progesterone [25]. In our study, we demonstrated that both progesterone and allopregnanolone promoted neural progenitor proliferation *in vitro*, although allopregnanolone had greater efficacy at the same concentration [26-27]. These data suggest that for neuroprotection and also for neurogenesis, the progesterone metabolite, APα is the primary effective agent. Recently, we further demonstrated that *in vivo*, APα reversed the neurogenic deficits in hippocampal dentate gyrus and cerebral subventricular zone, and restored the cognitive function in 3, 6 and 9 month old male 3xTgAD mice [19-20,28-30].

Collectively, these findings led us to determine firstly the neuronal lesions in the SNpc of 3xTgAD mice and secondly the efficacy of APα to induce a regenerative response to increase and to restore the number of TH expressing cells to normal. To address these issues, we conducted unbiased quantitative stereology to quantify the number of total neurons, TH positive neurons and the number of newly formed TH positive neurons in the SNpc of three month old male 3xTgAD mice 24 days after APα treatment.

MATERIALS AND METHODS

Experimental Designs and Tissue Preparation

Experiments were performed using 3-month-old male 3xTgAD, an age neurogenic deficits have been observed [19-20,28-30], and non-Tg mice which were obtained from Dr. Frank LaFerla (Univ of California-Irvine). All mice were kept under controlled temperature, humidity, a 12h light/12h dark cycle with continuous access to food and water. All procedures were in accordance with the Guide for the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (National Academy Press, Washington, DC, 1996). Mice (6 per group) received a single subcutaneous injection of APα (10 mg/kg) or vehicle (PBS containing 0.002% of alcohol). One hour after APα injection, mice were intraperitoneally injected with 5-bromo-2'-deoxyuridine (BrdU) at a concentration of 100mg/kg. Mice were sacrificed and sampled 24 days after injection. Brains were removed and dissected into two hemispheres, one of which was fixed immediately in cold 4% paraformaldehyde overnight at 4°C and cryoprotected with 30% sucrose for 24 h at 4°C. The tissue containing the midbrain was embedded in optimum cutting temperature (OCT) compound (Tissue Tek). Frozen serial coronal midbrain sections (40 μm) were collected utilizing a Leica cryostat, preserved in 0.01 M PBS, and processed for immunohistochemistry followed by unbiased stereological analysis.

Immunofluorescence for TH and BrdU

The TH and BrdU positive neurons were identified using double immunofluorescence labeling. For those labeled with TH-IR alone, free-floating sections were incubated with rabbit anti-TH polyclonal antibody (1: 500 dilution, Pel-FreeZ® Analysis Certificate, Rogers, Arkansas), followed by incubation with Texas red conjugated goat anti-rabbit IgG secondary antibody (1:5000, Vector Laboratories, Burlingame, CA). For those double labeled with TH- and BrdU-IR, the TH-IR was visualized with an anti-TH antibody (1:1000, ab112 from abcam) and an FITC conjugated goat-anti rabbit IgC (1:10,000, FI 1000, Vector). After washing three times (5 min each) with PBST, the sections were fixed with 4% paraformaldehyde in PBS for 10 min. The sections were then denatured using 50% formamide/2xSSC (NaCl 17.53g, sodium citrate 8.82g, pH 7.0) for 90 min at 65°C, 2N HCl solution for 30 min at 37°C, and then neutralized with sodium borate buffer (0.1M, pH 8.5) for 2×5 min. After extensive washes with 0.01M PBST (pH 7.4), the sections were incubated in blocking solution containing 0.1% Triton X-100 and 4% normal horse serum for 90 min. Subsequently, the sections were placed in an incubating solutions containing 3% normal horse serum with mouse anti-BrdU antibody (1:300, NB500, NOVUS), for 24 h at 4°C with gentle agitation, followed by Texas red-conjugated horse anti-mouse IgG secondary antibody (1:5000 dilution, Vector Laboratories, Burlingame, CA). All the sections were washed 3×5 min with PBS, rinsed with water, and mounted onto gelatinized slides using mounting medium (VECTASHIELD®, Vector Laboratories, Burlingame, CA). Immunoreactive controls were carried out by stepwise omission of antibodies or by replacement with normal serum. In addition, a set of free floating sections were mounted onto gelatin-coated slides, and stained with 0.5 % Cresyl Violet, dehydrated and cover-slipped as described by McCormack *et al.* [31].

Unbiased Quantitative Stereology of TH or TH/BrdU Double Labeled Neurons

The numbers of total Nissl-stained neurons and TH-immunopositive neurons in the SNpc were quantified, double blinded to the mouse codes, using optical fractionator methodology in the stereology module of SlideBook 5.0 (Innovative Intelligence, Inc, Denver, CO) in a Zeiss fluorescence microscope, equipped with a motorized stage driven in the X, Y and Z planes and coupled to a scientific grade CCD camera (Hamamatsu, Japan) as described previously [20]. One of every six serial sections of the mid-brain was counted to cover the entire mouse SNpc as defined in Franklin and Paxinos mouse brain atlas [32]. Standard neuroanatomical boundaries were used to define the nuclei that comprise the DA cell complex in the mouse according to different cell size, morphology, packing density and proximity to fiber bundles. The dorsal border of the SNpc was separated from the overlying VTA by the medial lemniscus at the level of the interpeduncular nucleus [33]. For each section, the SNpc was masked and optical fractionator frames were randomly assigned to the masked area. In each frame area, 20 images were taken (one per μm from top to the bottom) to cover the 20-μm thick Z optical sections under Z axis control, using a 63 ×/1.4 NA plan-apochromat oil objective. Only well focused TH, BrdU/TH double and Nissl positive neurons which fall inside the optical fractionator frames were counted (as demonstrated in Fig. (1B)). The total positive cell number was calculated by the reference factors; one over six of the sections analyzed; one of 16 of the 50μm × 50μm counting frame each of 200 μm × 200 μm area; and the tissue shrink factor.

Statistical Analysis

The data were expressed as the mean ± standard deviation (SD). Statistically significant differences were determined by a two-way ANOVA followed by a post-hoc Neuman-Keuls analysis. In all analyses, differences were considered significant at probability (p) values less than 0.05.

RESULTS

3xTgAD Mice have Fewer Nissl-Stained and TH-Expressing Neurons in SNpc Compared to Non-Tg and APα Reverses the Decline

Representative photomicrographs are shown in Fig. (1A) to demonstrate the TH immunoreactive neurons in SNpc. The image of Fig. (1B) presents the Nissl-stained neurons in SNpc and also demonstrates how a positive neuron was counted according to the optical fractionator criteria. The number of Nissl-stained neurons and TH positive neurons was determined from series of coronal sections. The data was presented as average ± SEM in Fig. (1C). There were 13840 ± 3342 Nissl-stained neurons and 10390 ± 3007 TH positive neurons in SNpc of non-Tg, and 8608 ± 1603 Nissl-stained neurons and 4848 ± 1860 TH positive neurons in SNpc of 3xTgAD, demonstrating a significant decline of both total neurons (37.8 %, $p = 0.016$) and TH positive neurons (53.4 %, $p = 0.007$) in SNpc of 3xTgAD mice. These data suggest that the mutations in the genes which are responsible for the early onset of AD also play a role in the reduction of both total and TH positive neurons in SNpc. In 3xTgAD that received APα, the number of Nissl-stained neurons was increased to 11744 ± 916 and TH-positive neurons to 9687 ± 2034 in SNpc, both are significantly higher than that in vehicle treated 3xTgAD mice ($p = 0.046$ for Nissl-stained neurons and $p = 0.021$ for TH positive neurons, Fig. (1C)). No significant difference was observed between the APα treated 3xTgAD and non-Tg background mice for both Nissl-stained neurons and TH-positive neurons in the SNpc. These data demonstrate that APα treatment specifically reversed the deficits of both of the total neurons and TH positive neurons in SNpc of 3xTg AD mice to generate a magnitude of total neurons and TH positive neurons comparable to that of normal non-Tg mice.

APα Increases the Number of Double Labeled BrdU/TH Cells in the SNpc of the 3xTg AD Mice

To determine whether the APα-induced restoration of total and TH positive neurons in SNpc of 3xTgAD is contributed by APα-induced formation of new TH neurons, the number of double labeled BrdU/TH neurons was assessed. In the representative images in Fig. (2A), several well differentiated TH-positive neurons (TH alone, green), several newly formed cell (BrdU positive, red) and a BrdU/TH double positive cell (indicated by an white arrow), are observed. The results of the assessment of BrdU/TH double positive cells in SNpc are presented in Fig. (2B). In SNpc of non-Tg that received vehicle, there was a small number of BrdU/TH double positive neurons (51 ± 33), suggesting that new TH neurons may be routinely produced under physiological conditions. The number of BrdU/TH double positive cells was significantly lower (15 ± 15) in the SNpc of 3xTg that received vehicle than that in the non-Tg receiving APα (78 ± 48, P = 0.012). Although it was not significantly lower than that in the non-Tg that received vehicle due to the high intragroup variation, the average level was 3 times lower. In SNpc of 3xTgAD mice that received APα a significant increase in the number of double labeled BrdU/TH positive cells (99 ± 48, p=0.008 vs. 3xTgAD received vehicle) was observed.

DISCUSSION

Using immunohistochemistry combined with unbiased stereology, we demonstrate a reduced number of total neurons and TH positive neurons in the SNpc of 3xTgAD mice compared to non-Tg mice. This reduction of TH neurons in 3xTgAD mice may be due to the decline of the newly formed TH positive neurons. To the best of our knowledge, this is the first demonstration that the TH positive neurons and total neurons are reduced in the midbrain substantia nigra of 3 month old male 3xTgAD mice. These findings are in agreement with the discovery of TH positive cell loss in locus coeruleus of aged (16-23 months) female APPSwe/PS1deltaE9 mice [17] and the loss of total neurons in cortex layer III and hippocampus of 3-4 month old 3xTgAD mice [20,34]. More interestingly, APα, a neurogenic steroid, reversed these deficits in the SNpc of 3xTgAD. Our data support the hypothesis that genetic risk factors found in familial AD, i.e., mutations in APP, PS1 and tau phosphorylation genes, also play a role in SNpc neuropathology and atrophy. Our data further suggest that APα may prove useful as a therapeutic agent for AD and also for nigrostriatal pathway related disease, such as PD.

The Loss of Neurons in 3xTgAD Mice

Aggregation of Aβ, hyperphosphorylated tau and α-synuclein are pathological hallmarks of several neurological disorders, including AD and PD. In brain of 3xTgAD mice age-dependent neuropathology of β-Amyloid plaques, neurofibrillary tangles [35] and insoluble pSer129 a-synuclein [21] have been observed. Our previous study reported early (at 3 months old) neurogenic deficits in 3xTgAD dentate gyrus and subventricular zone [20,36]. The low neurogenic ability leads to a lower total cell number in the hippocampus of 3xTgAD male mice compared to the age and gender matched non-Tg mice [20]. Similarly, Bittner and colleagues reported that 3xTgAD mice exhibit an early loss of neurons in layer III of somatosensory cortex at 4 months of age by two-photon *in vivo* imaging stereology [34]. The authors further clarified that the significant neuron loss occurred specifically in 3xTg-AD mice, whereas neuron loss was not observed in non-Tg control mice at this age. Consistent with these studies, the current report indicated that the total neurons in SNpc of 3xTgAD mice are 38% less than that of the non-Tg mice. This data indicate that the atrophy regions in brain of 3xTgAD mice are not only in cortex and hippocampus, but also in SNpc. The early deficits in the generation of new cells in 3xTgAD may limit the replacement of damaged neurons with new cells, and also causes a deterioration of the microenvironment. A

degenerative milieu will further accelerate the degeneration of old neurons and inhibit the recovery of damaged neurons and the survival of newly formed neurons [37] and eventually result in a decrease of total neurons in specific brain areas.

Mutations in Genes Associated with Human Familial AD Contribute to Atrophy in SNpc and Loss of TH^+ Neurons

The present study, using double immunofluorescence combined with unbiased stereology, demonstrated that that there were ~10,000 neurons in the SNpc on one side of the brain in Non-Tg mice. This number is consistent with previous reports of approximately 21,000 neurons in SNpcs on both sides of the brain in the C57BL/6 strain [38]. However, in the SNpc of 3xTgAD mice, the number of TH neurons was 53 % less than that in the SNpc of non-Tg mice.

Besides the occurrence of plaques and tangles and hippocampal atrophy, atrophy in brain nuclei containing TH expressing neurons is also a neuropathological feature of late-onset AD [39-41]. For example, in a comparison of results from a number of groups by Zarow *et al.*, [41], there are consistent high neuron loss, 52 - 76 %, in Locus coerulous, and a variable neuron loss, 4 – 50 %, in SNpc in post-mortem brain of late onset AD subjects. The current study indicated a 53 % loss of TH neurons and a 38 % loss of total neurons in SNpc of 3xTgAD mice, in agreement with those early studies from AD subjects [41].

Accumulated evidence also suggests that dysfunctions in the nigrostriatal pathway and TH expressing neurons occur in early onset AD. For example, AD subjects with an early onset presenilin 1 mutation (V272A) [2] or mutation (S170F) [42] showed Aβ-plaques, a classical lesions of AD, and Lewy bodies, a common pathological marker in PD and are composed of alpha-synuclein, in both the cortex and substantia nigra. Supportive evidence also from the transgenic APP/PS1 mouse model of AD, in which hyperaccumulated Aβ-42 residues lead to the early appearance of amyloid plaque formation when compared to mice with only the single transgene APP [16-17]. In the APP/PS1 double mutant mice there was a significant 24% reduction in TH-positive neurons in the locus coeruleus in comparison to their background controls [17]. Interestingly, the loss of TH expressing neurons was not observed in transgenic mouse model with APP23 [43] nor PADPP [44]. These findings suggest that the loss of TH positive neurons may be a result of the double APP/PS1 mutations, rather than a single APP mutation. Only a trend that, not statistically significant, was observed in the reduced number of TH expressing neurons in SNpc [17].

It has been proposed that tau protein abnormalities play a more important role in the loss of neurons in AD, and that deposition of amyloid plaques does not correlate well with neuron loss [45-47]. Neurofibrillary tangle formation is composed of hyperphosphorylated microtubule-associated protein tau that appears to accumulate within vulnerable neurons and may eventually kill the cell, leaving behind only a ghost tangle and no neuron [48-50]. The 3xTgAD mice carry, in addition to two mutations in human familial AD genes (APP_{Swe}, $PS1_{M146V}$), one frontal temporal dementialinked tau mutation (tau_{P301L}) and mimic multiple aspects of AD neuropathology in relevant brain regions [18,35]. The reduction of TH-immunoreactive neurons in SNpc of 3xTgAD male mice at 3 months old, extends our previous report and supports the hypothesis that early neurogenic deficits lead to the reduction of total neuron numbers in multiple brain regions of AD subjects [37] including SNpc. SN lesions are frequently present in AD and include pigmented neuronal cell loss, gliosis, Lewy bodies, α-synuclein-stained structures, and hyperphosphorylated tau accumulation in neurofibrillary tangles as well as neuritis [12-14], suggesting that AD is a significant risk factor for SN lesions [13,15].

APα Reverses the Loss of the TH$^+$ Neuron in 3xTgAD Mice

The synthesis of the neurosteroids, progesterone, and its metabolite APα in brain was first described by Baulieu and is now well established [51-52]. It has been consistently reported in our own studies as well as by others that APα is a neurogenic agent which can reverse neurogenic deficits in transgenic mouse model of AD [20,26-27,53-55]. In this study, we further extend this discovery by demonstrating that APα also reversed the deficit in TH expressing neurons in SNpc of 3xTgAD mice.

APα is a lipophilic molecule (logP = 3.97 with a low molecular weight 318.5 Da). These two features facilitate APα penetration of the blood brain barrier. APα-induced neurogenesis is dose-dependent and the concentrations required to induce neurogenesis *in vitro* are comparable to those found in both rat and human brain [27,53,56-57]. The most effective dose *in vitro* also has neurogenic effects *in vivo* that are accompanied with a reversal of the cognitive deficits in 3xTgAD mice [20].

Previously, we have demonstrated that the basal level of APα in brain of 3xTgAD mice was significantly lower than that in non-Tg mice [20], suggesting that these mutations in AD genes may somehow either impairs APα synthesis or accelerats APα metabolism in 3xTgAD mice brain. Interestingly, 3xTgAD mice exhibited a consistently lower level of APα in cortex for both the vehicle and APα-treated mice relative to non-Tg. Given that both non-Tg and 3xTgAD mice received the same doses of APα or vehicle, the lower levels of APα in the 3xTgAD support accelerated APα metabolism in 3xTgAD mice, which could be mediated by increased expression of 17β-hydroxysteroid dehydrogenase (17βHSD, aka ERAB, ScHAD, or ABAD) [58-59]. Indeed, the lower levels of APα in serum and CSF of Parkinson's patients [60-61] and the reduction of expression of genes which encode enzymes responsible for APα synthesis in post-mortem brain samples of PD subjects [61] provide experimental evidence to support this hypothesis. These studies further suggest a lower level of APα in the brain may trigger the progress of AD or PD and supported by the observation that, in post-mortem AD brain, there is a significant decrease in the temporal cortex in APα levels [56-57] and that APα levels in the temporal cortex are inversely correlated with the neuropathological disease stage (Braak & Braak grade) [56- 57].

In the 3xTgAD mouse cortex, a 10-mg/kg dose of APα resulted in a cortical level of 21.92 ± 8.57 ng/g APα/wet tissue 24 h post-injection [20], which is comparable to the concentration reported for pregnant day-19 rats (15.9 ± 2.5 ng/g) [62] and mice (20.9 ± 2.6 ng/g) [63], a time that embryonic brain is developed the most; for example, hippocampus is formed at this time. Our data indicated that a single injection of APα followed by analysis of outcome either 24 hours later in the case of proliferation or 7-29 days later in the case of behavior and BrdU positive cell survival [20,27] regenerated new neurons in hippocampus and improved learning and memory in 3xTgAD mice. In agreement with our study, either as a single injection or repeatedly, APα delays demyelination and enhances survival of Niemann-Pick C mice [64]. In addition, these data may suggest that to promoting NPC to enter the mitotic cycle (set the ball rolling) needs only one shot. While multiple re-entry may need repeated stimulation but in an optimum frequency[65].

Our previous studies indicated biphasic dose-dependent efficacy of APα on neurogenesis [20,27]. At 100, 250, and 500 nM concentrations, APα significantly increased BrdU incorporation (lower concentrations were not statistically different from control). At 1000 nM, a reversal of the dose–response was first apparent, with higher doses shifting the response to significant repression of proliferation at 100–1000μM. Recently, we titrated the optimal regimen for therapeutic efficacy of APα treatment *in vivo* [65]. Both APα treatment regimens of a single exposure of 1/month and of repeatedly 1/week/6 months APα treatment significantly increased the survival of cells that were generated at the first exposure to APα.

The 1/week/6 months APα treatment regimen had greater regenerative efficacy. However, the 3/week/3 months regimen significantly reduced regenerative efficacy [65], this may be due to the accumulation of APα in brain into level high enough to enter the second phase of dose response of APα on neurogenesis.

Earlier *in vitro* investigations indicated that APα-induced proliferation was mediated via $GABA_A$ receptor-activated voltage gated L-type Ca^{2+} channels leading to a rapid rise in intracellular calcium in neural progenitors [26-27]. In neural progenitor cells, the high intracellular chloride content leads to an efflux of chloride through the $GABA_A$ channels upon opening, which leads to depolarization of the membrane and influx of Ca^{2+} through voltage dependent L-type Ca^{2+} channels and activation of the CREB transcription factor [19,26,37,66-67]. Through this pathway, APα stimulation of $GABA_A$-mediated excitation and CREB signaling can activate a key pathway in adult neurogenesis, to promote the proliferation, survival, and differentiation of neural progenitor cells.

In addition, our study found a surprisingly low number of newly formed dopaminergic neurons (BrdU/TH double positive) in the SNpc of 3xTg vehicle treated mice (reduced up to ~71% in comparison with non-Tg), indicating impaired neurogenesis of TH expressing neurons in SNpc of 3xTgAD mice. Several studies support this hypothesis [68-69] and suggest neurogenesis may exist in SN, either at a very low level under physiological conditions or in a elevated level in pathological conditions, although these conclusions are somewhat controversial [70]. Furthermore, we found that the TH/BrdU double positive neurons also frequently occurred in and near the subventricular zone (SVZ, data not shown), suggesting a possibility that new SNpc TH^+ cells might be originally generated in SVZ and then migrate from the SVZ to the SN as reported by others [71-72].

In summary, results of the current analysis show a loss of total neurons and TH expressing neurons in SNpc of 3 month-old male 3xTgAD mice and that APα treatment restored the number of neurons to control levels and increased the number of newly formed TH neurons. Because loss of hippocampal [20] and TH expressing neurons [73] are early pathological events in Alzheimer's disease (AD), this study supports use of the neurogenic agent APα as a potential therapeutic modality for treating AD, AD with extrapyramidal symptoms, as well as Parkinson's disease.

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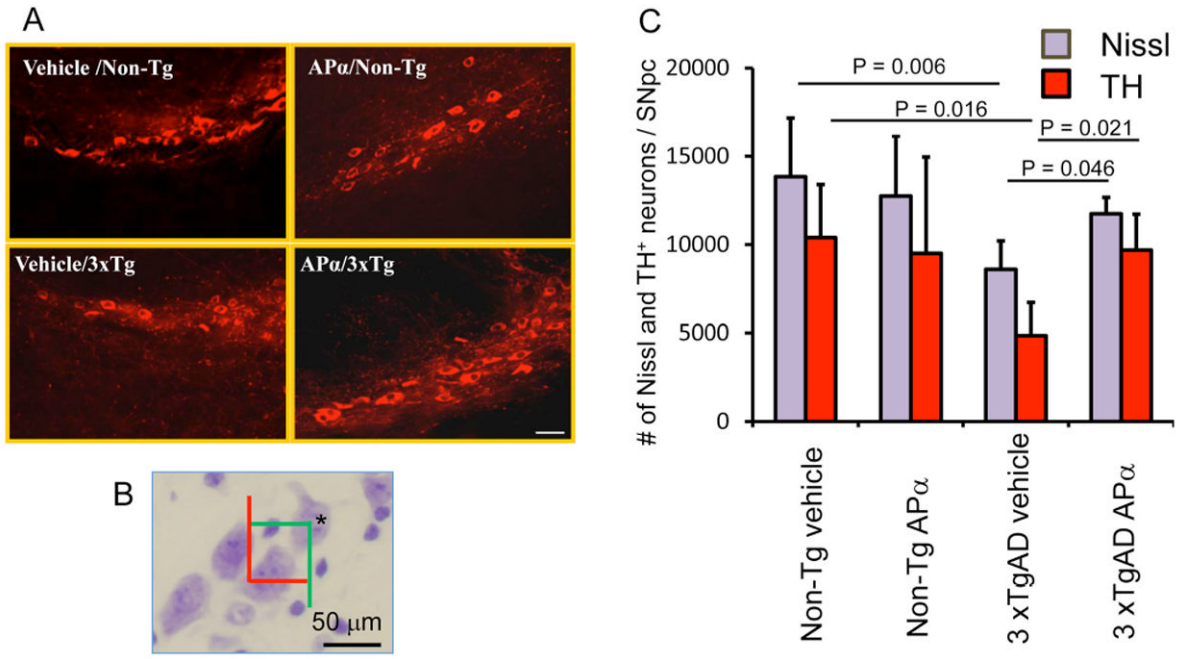

Fig. (1).

Allopregnanolone reverses the decline of total neurons and tyrosine hydroxylase expressing neurons in substantia nigra of 3 month old male 3xTgAD mice. Representative immunofluorescent images of TH positive cells are presented in A. The representative Nissl-stained neurons and the optical fractionator counting frame are presented in B. The countable Nissl-stained neuron is marked with *. Scale bars = 50μm. The results of unbiased stereological counting of Nissl-stained neurons and TH neurons in SNpc are presented as mean ± SD and the statistical significance between groups is indicated. The ANOVA analysis indicated $F(3,20) = 3.33$; $p = 0.04$.

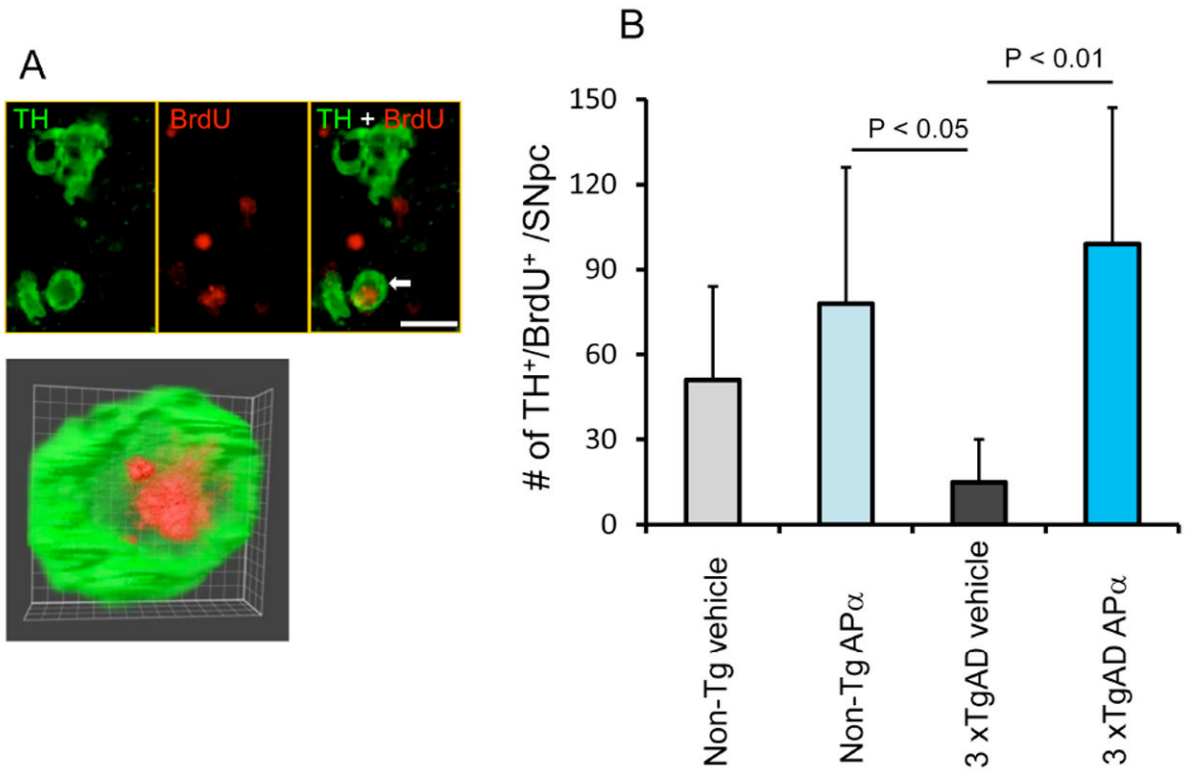

Fig. (2).

(A) Allopregananolone increases the newly formed (BrdU positive) TH expressing cells in SNpc of 3xTgAD mice. The representative BrdU (red) and TH (green) double positive (indicated by an arrow) immunofluorescent images are presented in A. The 3D volume-view of z-series images of the double immune-stained newly formed TH neuron in A demonstrates a co-localization of BrdU incorporation (nuclear) and TH (cytoplasm) IR in the same cell. Scale bar = 50μm. The number of BrdU/TH double positive cells are presented as mean ± SD and the statistical significance between groups are indicated. **(B)** The ANOVA analysis indicated $F(3,20) = 3.20$; $p = 0.026$.