Neurobiology of Aging 34 (2013) 2431–2440

Contents lists available at SciVerse ScienceDirect

Neurobiology of Aging

journal homepage: www.elsevier.com/locate/neuaging

Soluble amyloid precursor protein-α rescues age-linked decline in neural progenitor cell proliferation

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ARTICLE INFO

Article history:
Received 15 October 2012
Received in revised form 7 March 2013
Accepted 14 April 2013
Available online 14 May 2013

Keywords:
Neurogenesis
Amyloid precursor protein
Adulthood
Aging
Cognition
Learning and memory
Alzheimer's disease

ABSTRACT

Neurogenesis is thought to play a role in cognitive function and hippocampal plasticity. Previous studies suggest that neurogenesis declines with aging. However, the onset and mechanism of declined neurogenesis are not fully elucidated. Here we show that the major decline in neurogenesis takes place during adulthood, before aging. Decline in neurogenesis takes place in the subgranular layer of the dentate gyrus and in the subventricular zone, and is primarily due to a reduced number of fast-proliferating neural progenitor cells. Importantly, this decline can be rescued by intraventricular injection of recombinant soluble amyloid precursor protein (sAPPα), which regulates neural progenitor cell proliferation in the adult brain. The counterpart, sAPPβ, a product of the amyloidogenic cleavage pathway of amyloid precursor protein, fails to exhibit a proliferative effect in vitro and in vivo, in equimolar concentrations to sAPPα. These observations suggest that adulthood is an appropriate time window for an intervention that upregulates neurogenesis, such as enhancement of sAPPα levels, for the prevention of declining brain plasticity and cognitive function.

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1. Introduction

Amyloid precursor protein (APP) can undergo metabolism via two distinct pathways leading to the production of a number of intra- and extracellular metabolites. The amyloidogenic pathway of APP cleavage, so termed for its involvement in the formation of beta amyloid, begins with cleavage by β-secretase, β-site APP cleaving enzyme 1, and releases soluble APPβ (sAPPβ) to the lumen (for review, see Vassar, 2012). In the non-amyloidogenic APP cleavage pathway, initial cleavage occurs via α-secretase enzymes, primarily composed of a disintegrin and matrix-metalloproteinase proteins, ADAMs, leading to the release of sAPPα (Asai et al., 2003; Buxbaum et al., 1998; Jorissen et al., 2010; Lammich et al., 1999). It has been previously reported that sAPPα has trophic properties in a number of different cell types including fibroblasts (Saitoh et al., 1989), thyroid epithelial cells (Pietrzik et al., 1998), embryonic stem cells (Ohsawa et al., 1999), and carcinoma cells (Ko et al., 2004). With respect to adult neural progenitor cells (NPCs), sAPPα has been shown to bind to and stimulate the proliferation of NPCs in vitro and in vivo (Caille et al., 2004; Demars et al., 2011).

In the aging brain, there is a marked decline in neurogenesis in the subventricular zone (SVZ) (Mirich et al., 2002; Shook et al., 2012) and subgranular layer (SGL) (Ben Abdallah et al., 2010; Bernal and Peterson, 2004; Bondolfi et al., 2004; Cameron and McKay, 1999; Encinas et al., 2011; Kronenberg et al., 2006; Kuhn et al., 1996; Miranda et al., 2012). This decline in neurogenesis has been shown to manifest in deficits in olfactory function and hippocampal-dependent learning and memory (Bizon et al., 2004; Dupret et al., 2008; Enwere et al., 2004). However, the cause of neurogenic decline in the aging brain remains somewhat controversial. Some studies argue that the proliferation of NPCs and their maturation declines with age (Heine et al., 2004; Kuhn et al., 1996; Morgenstern et al., 2008; Rao et al., 2005). Olariu and colleagues suggest that this decline is not because of alterations in the length of the cell cycle of NPCs in the SGL (Olariu et al., 2007). Encinas and colleagues suggest that neurogenic decline is because of the disappearance of neural stem cells (NSC) by their conversion into mature hippocampal astrocytes (Encinas et al., 2011). Bonaguidi et al. (2008) suggest that upregulation of bone morphogenetic protein signaling or downregulation of its antagonists might underlie age-linked reduced self-renewal of NSC leading to the decline in neurogenesis (Bonaguidi et al., 2008). Other groups have argued that the rate of proliferation does not decline with aging, but

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http://dx.doi.org/10.1016/j.neurobiolaging.2013.04.016

that cells might exhibit increased quiescence that might be attributed to a decline in the vascular niche (Hattiangady and Shetty, 2008). Although this debate is still ongoing, it is apparent that the neurogenic niche undergoes changes associated with aging that have the potential to underlie neurogenic decline (Ahlenius et al., 2009; Miranda et al., 2012; Villeda et al., 2011). For instance, it has been well established that the expression of many growth factors and their receptors that are integral to neurogenic processes peak during development, and decline in expression thereafter (for review, see Klempin and Kempermann, 2007). Neurogenesis in the adult central nervous system plays a role in hippocampus- and olfaction-dependent learning and memory (for review, see Bernal and Peterson, 2004; Kempermann and Gage, 2000; Lie et al., 2004). These findings raise the possibility that reduced neurogenesis might, at least in part, account for impaired learning and memory and cognitive deterioration in the elderly (Kempermann et al., 1998, 2002; Kuhn et al., 1996; Seki and Arai, 1995; Tropepe et al., 1997) and might enhance vulnerability to Alzheimer's disease (AD; for review, see Lazarov et al., 2010).

Aging remains the predominant risk factor for the development of the sporadic form of AD. Alterations in the cleavage pattern of APP are causative of familial forms of the disease (for review, see Selkoe, 2001). We have previously reported that deficits in proliferation and neurogenesis occur long before the appearance of pathological hallmarks or cognitive impairment in a mouse model of familial AD (Demars et al., 2010). Taken together with our observation that sAPPα is a proliferation factor of adult NPCs, this might suggest the hypothesis that increasing levels of sAPPα in the neurogenic niches would rescue neurogenic deficits.

Here we show that a dramatic decline in neurogenesis takes place at 7–9 months of age, compared with mice at 2 months of age, and only a minor further decrease takes place at 20 months. This suggests that the significant decline in neurogenesis takes place during adulthood, rather than during aging. This decline is characterized by a reduced numbers of fast proliferating NPCs (type II cells in the SGL and type C cells in the SVZ), but there is no decline in the number of NSC. Intracerebroventricular (ICV) injection of sAPPα ameliorates proliferation deficits in adult mice. In support of that, adding sAPPα to a neurosphere culture derived from 7–9-month-old or 20-month-old mice enhances NPC proliferation. Additionally, we show that at a similar concentration range, sAPPβ does not evoke the same proliferative effect as sAPPα. This study suggests that therapy aimed at rescuing decline in neurogenesis should be applied during adulthood rather than aging, and that enhancement of sAPPα levels might rescue this decline.

2. Methods

2.1. Animals

Wild type mice, 2 months, 7–9 months, and 20 months of age on a C57/Bl6XC3H background were maintained in our colony. Our colony is maintained via group housing (<5 mice per cage) in a barrier facility under a 14:10 light:dark cycle with free access to food and water. Mice were euthanized using isofluorane and cervical dislocation. Animal care and procedures were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.2. Recombinant sAPPα and sAPPβ

sAPPα (Sigma-Aldrich, St. Louis, MO, USA) was used at 10 nM concentrations unless otherwise indicated (dissolved in phosphate-buffered saline [PBS]). sAPPβ (Sigma-Aldrich) was also dissolved in PBS and was used in the indicated concentrations.

2.3. ICV injections

A PBS vehicle or recombinant sAPPα or sAPPβ at a concentration of 1 μM (1 μL per mouse; 0.25 μL/min) were stereotaxically injected into the left lateral ventricle of 7–9-month-old C57BL/6 mice using the following coordinates: anteroposterior, 0 mm; mediolateral, −0.8 mm; and dorsoventral, −2.0 mm from bregma. Mice were anesthetized using a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg). The heads of the mice were then shaved and wiped with 70% ethanol. Animals were placed into a stereotaxic frame and a centimeter incision was made in the midline of the scalp to reveal the bregma. The scalp was rinsed with 30% hydrogen peroxide and a small hole was drilled at the coordinate site measured from bregma according to the mouse atlas of Franklin and Paxinos (2008). Animals then received a unilateral injection of sAPP to the lateral ventricle. The sAPP was delivered through a 5-μL Hamilton syringe connected to a hydraulic injection system set to inject at a rate of 0.25 μL/min. The injection needle was then left in place for an additional minute to ensure distribution of the solution. The needle was slowly removed and the incision closed using EZ-clips from Stoelting. After 6 hours of recovery, mice were given a single intraperitoneal dose of 100 mg/kg BrdU solution. Twenty-four hours after the BrdU injection, mice were transcardially perfused and the brains processed for immunohistochemistry as described on subsequent pages in this article.

2.4. Neural progenitor cell culture

Mice were euthanized and their brains were removed and placed into sterile Dulbecco's modified Eagle's medium/F12 (Gibco, now part of Invitrogen Corporation, Carlsbad, CA, USA). A coronal slice (approximately 1 mm) was dissected starting 1–2 mm posterior to the olfactory bulb. The region occupying the lateral wall and anterior horn of the lateral ventricles was removed with the aid of a dissecting microscope and diced with a sterile scalpel. Neurosphere culture was prepared as previously described (Demars et al., 2011). Briefly, tissue pieces were collected in a mixture of Papain and DNase in Earl's balanced salt solution and incubated at 37 °C for 40 minutes. Then, tissue pieces were pelleted using centrifugation and dissociated to a single-cell suspension, and cells were plated in NPC media that included water, Dulbecco's modified Eagle's medium/F12, glucose (Sigma-Aldrich, St Louis, MO, USA), $NaHCO_3$ (Sigma-Aldrich), HEPES solution (N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)) (Sigma-Aldrich), L-glutamine (Invitrogen Corporation), penicillin/streptomycin (Invitrogen Corporation), putrescine (9.6 μg/mL; Sigma-Aldrich), apotransferrin (0.1 mg/mL; Sigma-Aldrich), insulin (0.025 mg/mL; Roche, Indianapolis, IN, USA), selenium (5.2 ng/mL; Sigma-Aldrich), progesterone (6.3 ng/mL; Sigma-Aldrich), bovine serum albumin (2 mg/mL; Sigma-Aldrich), heparin (4 μg/mL; Sigma-Aldrich), epidermal growth factor (EGF) (20 ng/mL; PeproTech Rocky Hill, NJ, USA), and basic fibroblast growth factor (bFGF) (10 ng/mL; Pepro-Tech), and passaged after 10 days.

2.5. Clonogenic assay

Briefly, neurospheres were singly dissociated using mechanical dissociation and plated at 1000 cells per well onto 96-well plates. Cells were then treated with the indicated molar concentration of GM6001 or GM6001 negative control (Millipore Corporation, Billerica, MA, USA), β-secretase inhibitor IV (EMD/Millipore), and the indicated molar concentrations of sAPP. Cells were treated every

day for 7 days. After 7 days in culture, neurospheres were counted using an inverted light microscope, and the average neurosphere diameter was calculated for each sphere by measuring 4 diameters for each to control for irregularly shaped neurospheres using a Zeiss AX10 microscope (Carl Zeiss Ltd, Hertfordshire, UK) and StereoInvestigator software (StereoInvestigator version 8, MBF Bioscience, Williston, VT, USA). After sphere size determination, cells were singly dissociated with a p200 pipette and counted with a hemocytometer. Each experiment was repeated 5 times using neurosphere cultures derived from 5 different animals.

2.6. Immunoprecipitation and Western blot analysis

The detection of sAPP in NPC culture was performed by conditioning media using 5×10^5 cells plated for 2 hours in fresh NPC media. Media was then spun (1000*g* for 10 minutes) to remove any cells and precleared with protein A agarose beads (Pierce, Thermo Scientific, Rockford, IL, USA). Next, media was incubated overnight with 22C11 antibodies against the N-terminus of APP. The next day, media was incubated for 30 minutes in protein A agarose beads, spun, and the pellet was resuspended in sample buffer. Samples were heated at 100 °C for 5 minutes and run on a 6% Tris-glycine gel. For the extraction of protein from the neurospheres used to condition media, a lysis buffer containing 150 mM NaCl, 50 mM Tris-Cl, 5 mM ethylenediaminetetraacetic acid (EDTA), 1% Triton X-100, 0.5% sodium deoxycholate, protease inhibitor cocktail (mammalian protease inhibitor cocktail; Sigma-Aldrich) and 250 μM phenylmethylsulfonyl fluoride (PMSF) was used. Quantification of protein was performed using the bicinchoninic acid (BCA) method (Pierce) and equal amounts of protein were subjected to direct immunoblot analysis. For quantification, $n \geq 3$ was used.

2.7. Brain tissue processing

For in vivo immunohistochemical staining, all mice were anesthetized using a mixture of ketamine (100 mg/kg) and xylazine (10 mg/kg) and transcardially perfused with 100 mL of ice-cold PBS. The brains were then removed and halved on the sagittal plane. The left half was immediately placed into 4% paraformaldehyde on ice.

2.8. Immunohistochemistry

Left hemibrains from PBS-perfused mice were postfixed in 4% paraformaldehyde for 3 days and stored in 30% sucrose at 4 °C. Hemibrains were sectioned sagittally at 50 μm by using a freezing stage microtome and placed into cryopreservent (47.6% PBS, 28.57% ethylene glycol, and 25% glycerin [vol/vol]). Sections were blocked by using a solution containing 0.25% (vol/vol) Triton X-100 (Sigma-Aldrich) and 5% (vol/vol) Normal Donkey Serum (Jackson ImmunoResearch Laboratories, Inc, West Grove, PA, USA) in Tris-buffered saline solution (TBS). The following antibodies were used: BrdU (1:400; Accurate Chemicals, Westbury, NY, USA), nestin (1:100; Millipore Corporation), doublecortin (DCX, 1:400; Santa Cruz Biotechnology, Inc, Santa Cruz, CA, USA) and glial fibrillary acidic protein (GFAP; 1:500; Millipore Corporation). Floating sections were incubated in primary antibodies for 72 hours at 4 °C before continuing with blocking, biotin conjugation (Jackson ImmunoResearch Laboratories, Inc), and secondary antibody incubation (cy2 Streptavidin, anti-mouse cy3, anti-goat cy5, and anti-rabbit cy5; Jackson ImmunoResearch Laboratories, Inc).

2.9. Stereological quantification

The number of positively stained cells in sagittal brain sections was quantified using design-based stereology (StereoInvestigator version 8, MBF Bioscience). For the analysis, every sixth section of brain tissue was quantified by applying the Nv × VRef method. The following parameters were used: for SVZ, sections were traced using a Zeiss AX10 microscope (Carl Zeiss Ltd, Hertfordshire, England) in low magnification (5×) and counting was performed at high magnification (63×), counting frame = 100 μm × 100 μm, grid size 100 μm × 100 μm, and all sections were counted using 12.5-μm top and bottom guard zones. For the dentate gyrus (DG), the counting frame was set equal to the grid size (100 μm × 100 μm) to count the entirety of the DG because of the relative paucity of cells. All other parameters remained the same.

3. Results

To examine the course of decline in neurogenesis with age we first assessed the extent of neurogenesis in the SGL and SVZ of 2- (young), 7–9- (adult), and 20- (aging) month-old mice ($n = 4$). For this purpose, we quantified the number of type B (nestin$^+$GFAP$^+$), total fast proliferating cells (BrdU$^+$), type C (BrdU$^+$nestin$^+$, BrdU$^+$DCX$^+$), and type A (BrdU$^-$DCX$^+$) cells. Unbiased stereology revealed a severe reduction in the number of total proliferating cells (Fig. 1A), and the number of type C and A cells by 7–9 months of age in the SVZ (Fig. 1B–D). Notably, the number of fast proliferating cells (BrdU$^+$) and NPCs (nestin$^+$BrdU$^+$) is significantly reduced by 7–9 months of age, and stays reduced without further significant reduction by 20 months of age (Fig. 1A and B). This might suggest that the reduced proliferation of NPCs in the SVZ by 7–9 months of age might play a major role in reduced extent of neurogenesis. The number of neuroblasts and immature neurons is significantly reduced by 7–9 months, and this number is further reduced by 20 months (Fig. 1C, D, and Kiii–v). The number of type B cells (GFAP$^+$nestin$^+$) was not significantly changed in any of the age groups (Fig. 1E), suggesting that the pool of NSC in the SVZ does not decline with age.

Examination of the cell population in the SGL of the different ages revealed similar observations. We quantified the number of type I (nestin$^+$GFAP$^+$), total fast proliferating cells (BrdU$^+$), type II (BrdU$^+$nestin$^+$, BrdU$^+$DCX$^+$), and type III (BrdU$^-$DCX$^+$) cells. The number of total proliferating cells (Fig. 1F), and the number of type II and III cells (Fig. 1G–I) was dramatically reduced by 7–9 months of age in the SGL. The number of type II and III cells remained reduced. The number of proliferating neuroblasts, BrdU$^+$DCX$^+$, was reduced severely by 7–9 months of age, with a further significant reduction in their number between middle and old age (Fig. 1H, Kiii and iv). The same is true for the total number of postmitotic immature neurons (BrdU$^-$DCX$^+$). A dramatic reduction in this population occurs between 2 and 7–9 months of age with a smaller but significant reduction taking place thereafter up to 20 months of age (Fig. 1I). Of note, the number of total proliferating BrdU$^+$ cells and of fast proliferating NPC type II (BrdU$^+$nestin$^+$, BrdU$^+$DCX$^+$) was dramatically reduced. Notably, there was no change in the number of NSC (Fig. 1J), suggesting that the pool of NSC in the SGL does not decline with age. We and others have shown that sAPPα is a proliferation factor of NPCs in the adult brain (Baratchi et al., 2012; Caille et al., 2004; Demars et al., 2011). Thus, in light of the dramatic reduction in proliferation of NPCs in the SVZ and the SGL of mice at the age of 7–9 months, we asked whether addition of sAPPα into the neurogenic areas can ameliorate deficits in proliferative cells in the brains of 7–9-month-old mice in vivo. To this end, we performed ICV injection of recombinant sAPPα or PBS (Supplementary fig. 1) in mice at 7–9 months of age followed 6 hours later with a single intraperitoneal dose of BrdU and sacrificed 24 hours later ($n = 4$). A single ICV injection of sAPPα in 7–9-month-old mice dramatically increased the number of proliferating (BrdU$^+$) cells in the SVZ (Fig. 2A, Ii and ii) and SGL (Fig. 2E, Iiii and iv). To assess the effect of ICV sAPPα injection on NPC proliferation, we quantified the

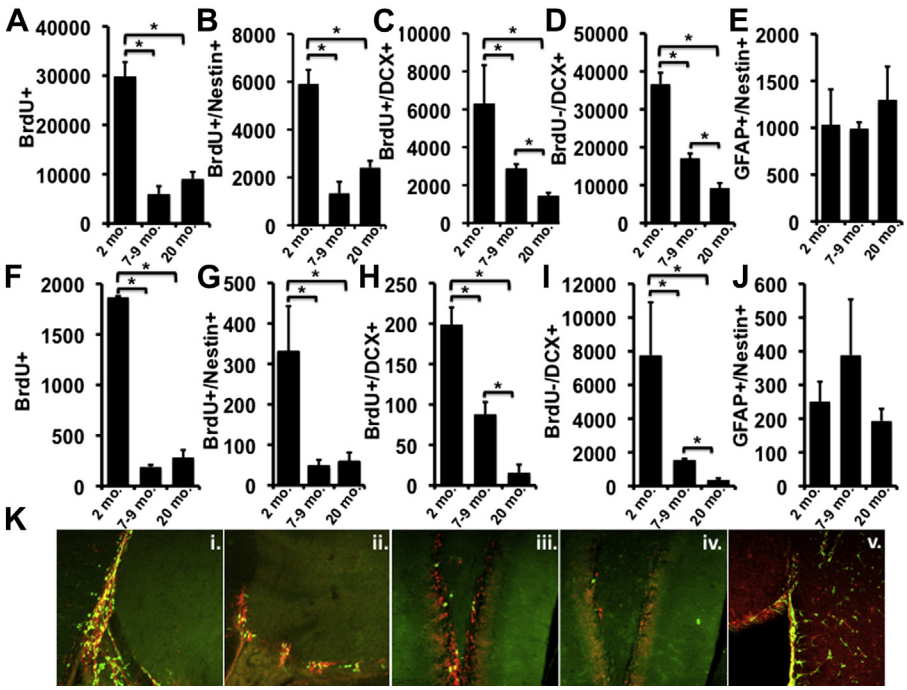

Fig. 1. Age-linked decline in proliferating neural progenitor cells and immature neurons in the subventricular zone (SVZ) and subgranular layer (SGL). Quantification of proliferating cells (BrdU$^+$ [A and F]), proliferating neural progenitor cells (NPCs) (BrdU$^+$/nestin$^+$ [B and G]), proliferating neuroblasts (BrdU$^+$DCX$^+$ [C and H]), immature neurons (BrdU$^-$/DCX$^+$ [D and I]), and neural stem cells (NSC) (GFAP$^+$/nestin$^+$ [E and J]) in the SVZ (A–E) and SGL (F–J) of 2-, 7–9, and 20-month-old mice. (K) Representative images of (i) 2-month-old SVZ (BrdU, green; DCX, red), (ii) 20-month-old SVZ, (iii) 2-month-old SGL, (iv) 20-month-old SGL, (v) representative image of GFAP (green) and nestin (red) staining. $n = 4$; * $p < 0.05$, analysis of variance with post hoc analysis. Abbreviations: BrdU, 5-bromo-2-deoxyuridine; DCX, doublecortin; GFAP, glial fibrillary acidic protein; NSC, neural stem cell.

number of cells colabeled with BrdU and nestin. We show that sAPPα injection rapidly and significantly increased the number of proliferating NPCs in the SVZ (Fig. 2B and Ivi) and SGL (Fig. 2F) of 7–9-month-old mice. Notably, in the SVZ, the number of proliferating neuroblasts was increased after sAPPα injection (Fig. 2C, Iv), suggesting that enhanced proliferation had a direct effect on the

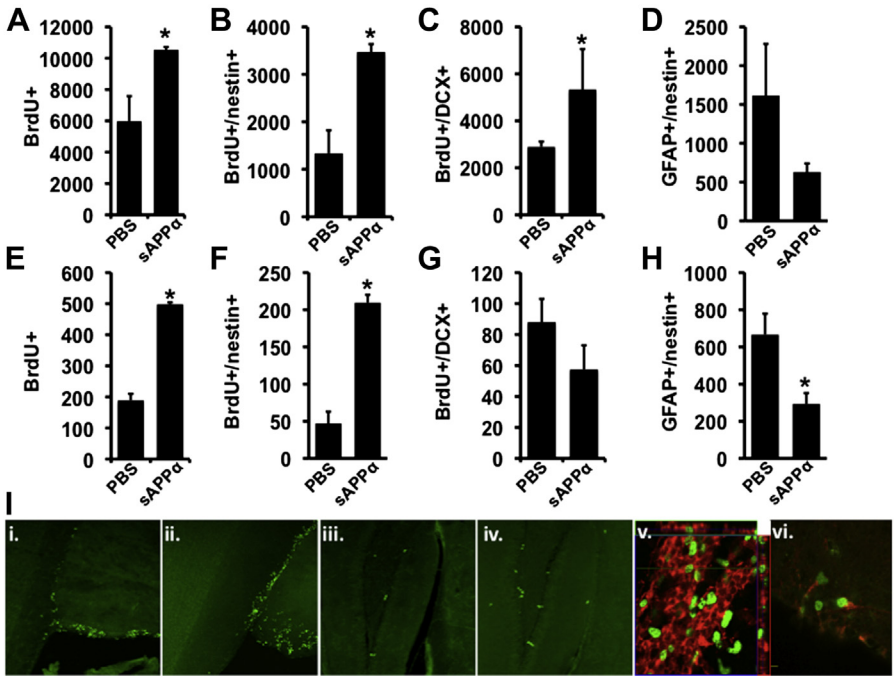

Fig. 2. Intracerebroventricular injection of soluble amyloid precursor protein (sAPP)-α ameliorates aging-linked proliferation deficits in the subgranular layer (SGL) and subventricular zone (SVZ). Quantification of proliferating cells (BrdU$^+$; SVZ [A] and SGL [E]), proliferating neural progenitor cells (NPCs) (BrdU$^+$/nestin$^+$; SVZ [B] and SGL [F]), neuroblasts (BrdU$^+$DCX$^+$; SVZ [C] and SGL [G]), and NSC (GFAP$^+$/nestin$^+$; SVZ [D] and SGL [H]) in 7–9-month-old mice injected intracerebroventricularly with either phosphate-buffered saline (PBS) or 1 μL of 1 μM sAPPα. (I) Representative images of (i) PBS-injected SVZ (BrdU, green), (ii) sAPPα-injected SVZ (BrdU, green), (iii) PBS-injected SGL (BrdU, green), (iv) sAPPα-injected SGL (BrdU, green), (v) high magnification of BrdU$^+$ (green)/DCX$^+$ (red) staining, and (vi) representative image of BrdU$^+$ (green)/nestin$^+$ (red) staining. $n = 4$; * $p < 0.05$, Student t test. Abbreviations: BrdU, 5-bromo-2-deoxyuridine; DCX, doublecortin; GFAP, glial fibrillary acidic protein; NSC, neural stem cell.

number of neuronally-committed NPCs. In the SGL, the number of neuroblasts appeared slightly reduced but this change was not statistically significant (Fig. 2G). Interestingly, there was a statistically insignificant reduction in the number of NSC ($GFAP^+/nestin^+$) in the SVZ (Fig. 2D). In the SGL, reduction in the number of NSC was significant (Fig. 2H). This might raise the possibility that sAPPα induces NSC asymmetric division resulting in more NPCs in vivo. Alternatively, sAPPα might facilitate their commitment to neuronal lineage. Taken together, these results suggest that sAPPα can enhance aging-linked reduced proliferation in the SVZ and SGL by stimulating NPC proliferation.

To further establish that the addition of sAPPα stimulates NPC proliferation, we isolated NPCs from the SVZ of mice at the ages of 2, 7–9, or 20 months, and treated the neurosphere cultures with either recombinant sAPPα or vehicle. Consistent with previous reports suggesting proliferation deficits could be masked by culture conditions (Ahlenius et al., 2009; Bouab et al., 2011), we saw no significant difference in neurosphere diameter or total number of NPCs in neurosphere cultures derived from mice that are 2, 7–9, or 20 months old (Fig. 3). However, we could stimulate proliferation with the addition of 10 nM sAPPα in neurospheres derived from 2-, 7–9, and even 20-month-old mice as indicated by increased neurosphere diameter (Fig. 3A and C), and increased total NPC numbers (Fig. 3B). This result suggests that NPCs derived from mice at various ages retain the ability to proliferate in response to sAPPα in vitro.

The cleavage of APP can produce either sAPPα or sAPPβ. Therefore, we sought to determine if NPCs respond differentially to the two sAPP species. We assayed sphere diameter of neurospheres derived from singly-dissociated NPCs treated with varying concentrations of either sAPPα or sAPPβ. Treatment with sAPPα increased neurosphere diameter at 10 nM concentrations with a trend toward significance at 1 nM. However, sAPPβ treatment failed to significantly increase proliferation at any of the concentrations tested (Fig. 4A; representative images in C). In our previous work, we showed that sAPPα is able to ameliorate deficits in NPC proliferation caused by matrix-metalloproteinase (MPP) inhibition that inhibits enzymes exhibiting α-secretase activity that yields sAPPα production (Demars et al., 2011). The use of the inhibitor is meant to inhibit endogenous production of sAPPα, thus preventing a masking of the proliferative effect exerted by recombinant sAPPα. Therefore, we sought to determine whether sAPPβ would enhance proliferation in the absence of endogenous production of sAPPα. As previously reported, 1 μM GM6001 MMP inhibitor treatment impairs proliferation, and sAPPα ameliorates these proliferation deficits (Fig. 4B; Demars et al., 2011). At 1 nM concentrations, sAPPβ enhances proliferation to the same extent as sAPPα. However, at concentrations greater than 10 nM, sAPPβ treatment does not rescue MMP inhibitor-induced proliferation deficits (Fig. 4B).

Treatment with MMP inhibitor likely exclusively reduces sAPPα. Thus, we next asked whether endogenous production of sAPPβ masks the proliferative effect of recombinant sAPPβ. For this purpose, we inhibited endogenous production of sAPPβ by treating NPC culture with β-secretase inhibitor IV. We showed that treatment of NPCs with β-secretase inhibitor reduces proliferation dramatically in assays of neurosphere diameter and total cell number (Fig. 5A). Though sAPPα ameliorates this deficit at 10-nM concentrations, 1 nM sAPPβ, the most effective dose in previous experiments, does not enhance proliferation after β-secretase inhibition (Fig. 5A and B). To further exclude the possibility that the presence of either endogenous sAPPα or sAPPβ mask the proliferative effect of recombinant sAPPβ, NPC cultures were treated with MMP and β-secretase inhibitor. Treatment of NPCs with GM6001 or β-secretase inhibitor IV reduced neurosphere diameter (Fig. 6A), and total cell number (Fig. 6B) to the same extent after 7 days in vitro. To assess the ability of sAPPα or sAPPβ to stimulate proliferation in conditions of minimal endogenous sAPP, we added exogenous recombinant sAPPα or sAPPβ to NPCs in dual secretase inhibition. sAPPα rescued proliferation deficits to control levels. Conversely, sAPPβ failed to ameliorate the proliferation deficits

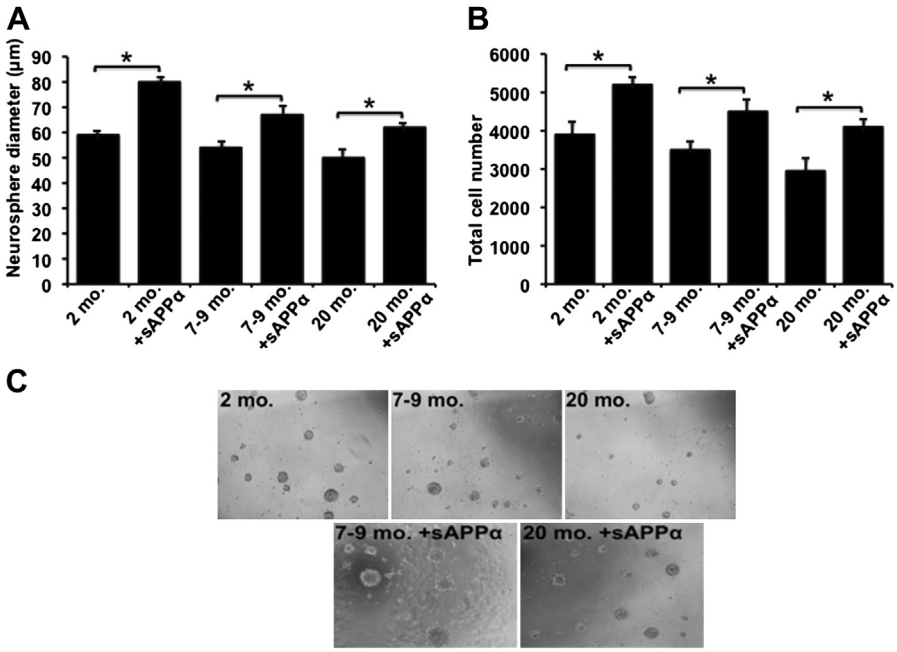

Fig. 3. Neural progenitor cells (NPCs) from aged mice retain the ability to proliferate in response to soluble amyloid precursor protein (sAPP). (A) Neurosphere diameter after 7 days in vitro. Singly-dissociated NPCs of 2-, 7–9, and 20-month-old mice were supplemented daily with 10 nM sAPPα or vehicle. (B) Total cell number of dissociated neurospheres quantified in (A). (C) Representative images of neurospheres in clonogenic analysis after 7 days in vitro with the indicated ages and treatments. $n = 5$; * $p < 0.05$, Student t test or analysis of variance with post hoc analysis.

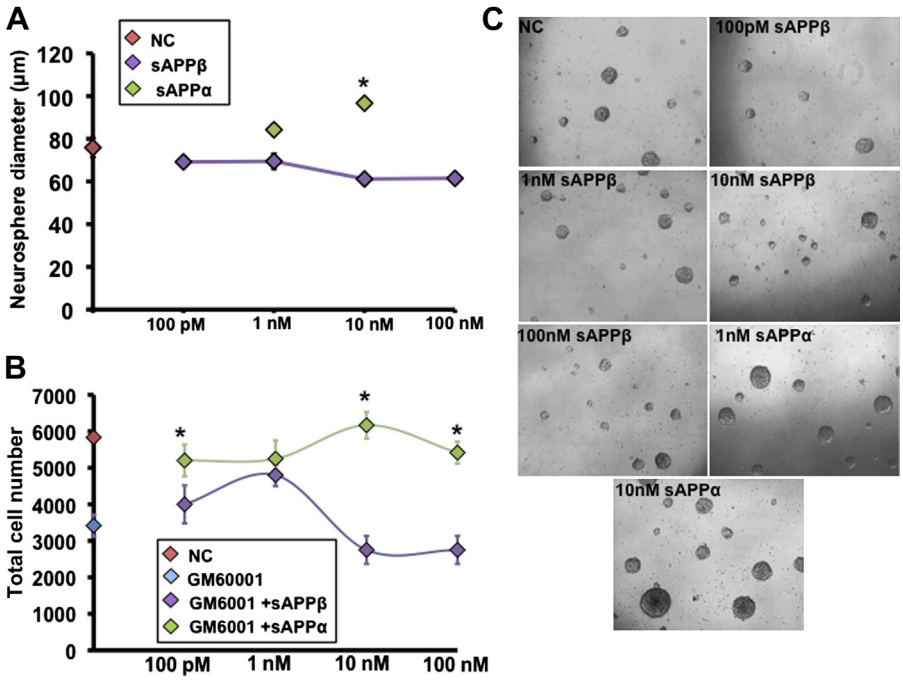

Fig. 4. Soluble amyloid precursor protein should be (sAPPα) and sAPPβ differentially affect proliferation of neural progenitor cells (NPCs) and proliferation rescue after matrix-metalloproteinase inhibition by treatment with GM6001. (A) Clonogenic assay after treatment with varying concentrations of sAPPα (green) or sAPPβ (purple) compared with phosphate-buffered saline-treated controls (NC; red). (B) Total cell number from dissociated neurospheres treated with GM6001 (blue), GM6001, and sAPPα (green) or GM6001 and sAPPβ (purple) compared with GM6001 negative control-treated controls (red). (C) Representative images of neurospheres from Fig. 4A, $n = 5$; * $p < 0.05$, analysis of variance with post-hoc analysis.

incurred from dual secretase inhibition (Fig. 6A and B). These results suggest that sAPPα is a more potent proliferation factor for NPCs than sAPPβ.

Finally, we sought to address whether, similar to sAPPα, sAPPβ would rescue age-dependent proliferation deficits of NPCs. Because age-dependent proliferation deficits are not apparent in vitro, we

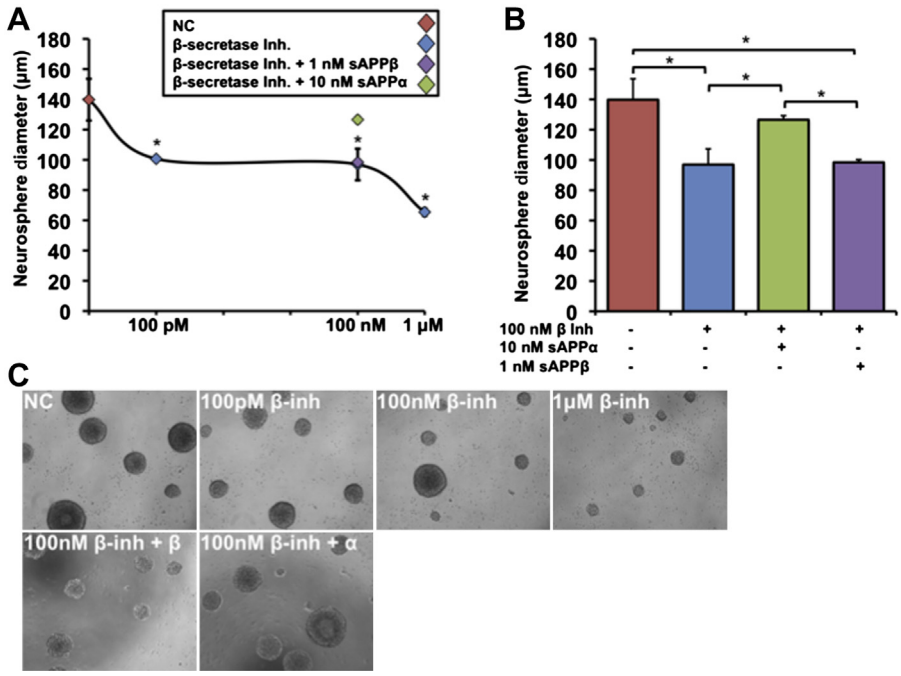

Fig. 5. β-secretase inhibitor (Inh)-induced proliferation deficits are ameliorated by soluble amyloid precursor protein should be (sAPPα) but not sAPPβ. (A) Neurosphere diameter in a clonogenic assay after 7-day in vitro treatment with β-secretase inhibitor IV (blue), β-secretase inhibitor IV and sAPPα (green), or β-secretase inhibitor IV and sAPPβ (purple), compared with DMSO-treated controls (NC) (red). (B) Neurosphere diameter in a cologenic assay after 7-day in vitro treatment with DMSO (NC) (red), 100 nM β-secretase inhibitor IV (blue), 100 nM β-secretase inhibitor IV and 10 nM sAPPα (green), and 100 nM β-secretase inhibitor IV and 1 nM sAPPβ (purple) Fig. 5B. (C) Representative images of neurospheres in clonogenic assay. $n = 5$; * $p < 0.05$, analysis of variance with post hoc analysis. Abbreviation: DMSO, dimethyl sulfoxide.

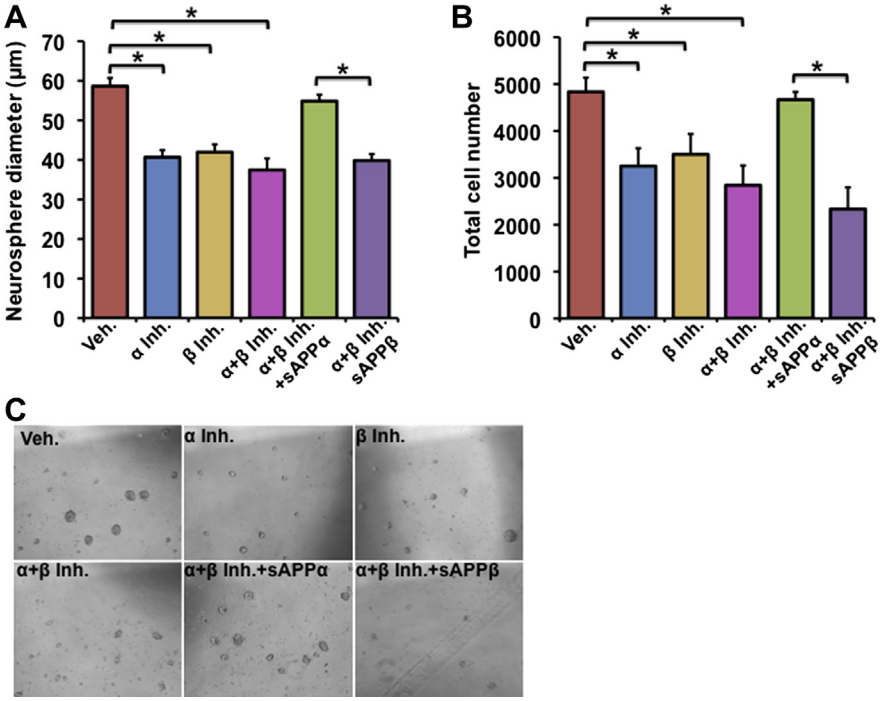

Fig. 6. Soluble amyloid precursor protein should be (sAPPα), but not sAPPβ, can rescue proliferation deficits incurred by dual secretase inhibition. (A) Neurosphere diameter in a clonogenic assay after 7 days in vitro; GM6001 (blue), β-secretase inhibitor IV (gold), dual secretase inhibition (light purple), dual secretase inhibition and sAPPα (green), or dual secretase and sAPPβ (dark purple) compared with DMSO (Veh)-treated (red) controls. (B) Total cell number of dissociated neurosphere from Fig. 6A. (C) Representative images of neurospheres after 7 days in vitro with the indicated treatments. $n = 5$; * $p < 0.05$, analysis of variance with post hoc analysis. Abbreviation: DMSO, dimethyl sulfoxide.

examined the effect of sAPPβ administration on proliferation in 7–9-month-old mice compared with the young mice in vivo ($n = 4$). We showed that ICV injection of sAPPβ led to a markedly reduced population of proliferating cells in the SVZ (Fig. 7A–C) and SGL (Fig. 7D–F), including a dramatically reduced population of $BrdU^+/DCX^+$ proliferating neuroblasts (Fig. 7B [SVZ] and E [SGL]). Interestingly, similar to the sAPPα-injected cohorts, sAPPβ-injected animals showed a decrease in $GFAP^+/nestin^+$ NSCs in the SGL (Fig. 7F) and a trend in the SVZ, albeit not significant (Fig. 7C). These results indicate that at equimolar concentrations, sAPPα and sAPPβ have significantly different effects on proliferating cells in the two neurogenic niches. Though sAPPα has the ability to ameliorate deficits in proliferation, sAPPβ reduces the number of proliferating cells in the already depleted brains of 7–9-month-old animals.

4. Discussion

This study reports several important observations. First, there is a dramatic decline in neurogenesis in adulthood, before aging. This is consistent with previous reports on neurogenesis in the aging brain (Bernal and Peterson, 2004; Cameron and McKay, 1999; Kronenberg et al., 2006). Second, the number of proliferating cells is dramatically reduced by 7 months of age in the SVZ and SGL, with no further significant decline. We further showed that the decline in proliferation is caused, in large part, by a reduction in the number of proliferating NPCs without any change in the number of NSCs in either region. The molecular mechanism underlying this decline in proliferation is not fully elucidated. It is possible that the level of essential proliferation factors declines with age. Notably, binding sites for sAPP are localized to rapidly proliferating C cells in the adult brain (Caille et al., 2004). However, our results cannot rule out the possibility that the age-linked decline in proliferating cells is because of increased quiescence of NSC and a reduced NPC pool. Because NSCs proliferate very slowly (Zheng et al., 2004), our paradigm of single-pulse BrdU injection likely will not capture this population. However, our results do indicate that the total number of NSCs is steady across all ages and suggest that a waning number of NSCs is not the cause of neurogenic decline.

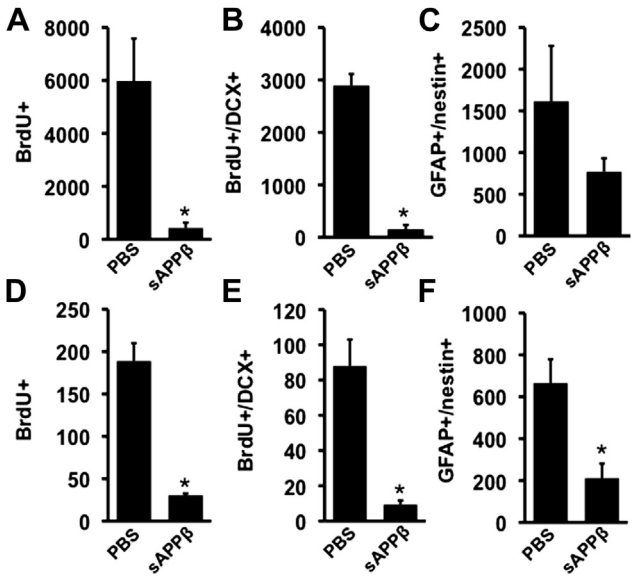

Fig. 7. Intracerebroventricular injection of soluble amyloid precursor protein (sAPP)-β exacerbates aging-linked deficits in proliferating cell numbers in the subventricular zone (SVZ) and subgranular layer (SGL). Quantification of proliferating cells ($BrdU^+$; [SVZ (A), SGL (D)]), proliferating neuroblasts ($BrdU^+DCX^+$ [SVZ (B), SGL (E)]), and NSC ($GFAP^+/nestin^+$ [SVZ (C), SGL (F)]) in 7–9-month-old mice injected intracerebroventricularly with either phosphate-buffered saline (PBS) or 1 μL of 1 μM sAPPα. $n = 4$; * $p < 0.05$, Student t test. Abbreviations: BrdU, 5-bromo-2-deoxyuridine; DCX, doublecortin; GFAP, glial fibrillary acidic protein; NSC, neural stem cell.

Third, we show that a single ICV injection of recombinant sAPPα is sufficient to significantly ameliorate age-linked deficits in the number of proliferative NPCs. It should be noted that though the final concentration of sAPP after injection is uncertain, it was designed to be diluted approximately 100-fold in the cerebrospinal fluid of the ventricular space. Considering the injected dose of 1 μM, this would result in a final concentration of approximately 10 nM, the most effective concentration of sAPPα in our culture conditions. The injection of sAPPα significantly increased the number of BrdU/nestin colabeled NPCs in the SGL and SVZ. A single injection of sAPPα into the lateral ventricle enhanced the number of neuroblasts in the SVZ but not in the SGL. One possibility is that because of the relatively low number of neuroblasts in the SGL compared with the SVZ, a single injection is not sufficient to evoke an increase in neuroblasts within 24 hours. It is reasonable to assume that some of the detected $BrdU^{+}DCX^{+}$ started to proliferate before the stimulation of recombinant sAPPα. Thus, more studies are warranted to determine whether injection of sAPPα leads to increased numbers of new neurons and to their survival and functional incorporation in the olfactory bulb and granular layer of the dentate gyrus.

Fourth, we showed that sAPPβ is a less potent proliferation factor than sAPPα in vitro and might only function to stimulate proliferation in a relatively small concentration range. Several of the mutations in APP, that are causative of familial Alzheimer's disease, lie in close proximity to the β-secretase cleavage site at the N-terminal portion of the beta amyloid region. These mutations cause a shift in the metabolism of APP toward the amyloidogenic pathway, increasing sAPPβ production at the expense of sAPPα (Thinakaran et al., 1996). Familial Alzheimer's disease-linked transgenic mice display impaired proliferation of NPCs before the onset of pathological hallmarks or the presentation of memory deficits. In vitro, NPCs derived from these mice have impaired proliferation, suggesting a potential intrinsic mechanism caused by the mutations (Demars et al., 2010). The differential activity between sAPPα and sAPPβ has been previously reported with respect to other trophic properties such as neuroprotection against glutamatergic or beta amyloid toxicity (Furukawa et al., 1996), promotion of axonal elongation and primary dendritic length (Chasseigneaux et al., 2011), hippocampal long-term potentiation (Taylor et al., 2008), and rescue of prenatal lethality in an APP/APLP2 knockout mice (Li et al., 2010; Weyer et al., 2011). However, the cause of this functional divergence between the two peptides has yet to be elucidated. The C-terminal portion of sAPPα alone is unable to stimulate proliferation in embryonic stem cells (Ohsawa et al., 1999). Whatever the cause of these differences, it is feasible that a shift in the homeostatic balance of APP processing favoring the amyloidogenic pathway would impair neurogenesis. Not only would this result in the production of the less trophic sAPPβ but it would also lead to increases in the transcriptionally active form of the APP intracellular domain, which has been shown to be preferentially produced by this pathway (Belyaev et al., 2010; Goodger et al., 2009), and to be a negative regulator of proliferation (Ghosal et al., 2010; Ma et al., 2008). Therefore, a shift in the balance of these cleavage pathways could underlie, at least in part, neurogenic deficits in aging and AD.

Finally, we showed that ICV injection of sAPPβ at equimolar concentrations to sAPPα does not enhance the number of proliferating cells in either the SVZ or SGL of the aging brain. Conversely, sAPPβ injection reduced the number of proliferating cells. There is evidence from peripheral neurons that during times of reduced growth factor support, sAPPβ is released and binds to death receptor 6 (DR6), inducing neurodegeneration (Nikolaev et al., 2009). The mechanism underlying this suppression of proliferation is not known. Future experiments will be aimed at unravelling the significance of sAPPβ-regulated decline in proliferation. It should be noted that the concentration of sAPPβ used was designed to be an equimolar dose to sAPPα injections. As we have shown in our in vitro assays, the sAPPs might have different optimal concentrations thus we might not have captured the optimal dose in our experiments. Alternatively, a difference in the half-life of sAPPα versus sAPPβ or their rate of metabolism might underlie this outcome.

In summary, this study shows that extent of neurogenesis declines well before aging, that the decline in neurogenesis as a function of age is largely because of a decline in the number of proliferating NPCs, and that this reduction can be reversed by a single-dose ICV injection of sAPPα, and the same dose of sAPPβ resulted in further impairment of proliferation. These findings highlight the differential activity of the sAPPs with respect to proliferation of NPCs. Together with previous studies, this work provides evidence that the regulation of APP processing plays a major role in the regulation of NPC proliferation in the adult brain. This paves the way for therapeutic intervention with an emphasis on maintaining a homeostatic balance in APP processing in AD and physiological aging. Though aging is the predominant risk factor for sporadic forms of AD, little is known about the expression of APP and APP metabolites during normal aging. One study of note did examine APP maturation and processing during cellular aging in a human lung fibroblast cell line and showed a decreasing metabolism with increasing cellular age including a decline in sAPPα (Kern et al., 2006). This group further showed that increasing membrane cholesterol levels correlated with increasing cellular age. Intriguingly, APP, β-site APP cleaving enzyme 1, and presenilin1 have been shown to associate increasingly with detergent-resistant, cholesterol-rich membranes or "lipid rafts" with increasing cellular age (Kang et al., 2006). Evidence suggests that amyloidogenic processing of APP is sequestered in these detergent-resistant membrane microdomains (Schneider et al., 2008; Simons et al., 1998). Thus, the possibility exists that in physiological aging and AD there is a shift in the metabolic pathway or cleavage pattern of APP favoring the amyloidogenic pathway over the seemingly more trophic nonamyloidogenic pathway.

Disclosure statement

The authors declare no competing financial interests.

Animal care and procedures were conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Acknowledgements

The work was supported by the NIA AG033570, NIA 1RC1AG036208-01 ARRA, The Brain Research Foundation, and The Alzheimer's Association Young Investigator Award (OL).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.neurobiolaging.2013.04.016.

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