Interleukin 6 (IL-6) reduces allopregnanolone synthesis in the brain and contributes to age-related cognitive decline in mice

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Abstract

Cognitive decline with age is a harmful process that can reduce quality of life. Multiple factors have been established to contribute to cognitive decline, but the overall etiology remains unknown. Here, we hypothesized that cognitive dysfunction is mediated, in part, by increased levels of inflammatory cytokines that alter Allopregnanolone levels, an important neurosteroid in the brain. We assessed the levels and regulation of allopregnanolone and the effects of allopregnanolone supplementation on cognitive function in 4 month old and 24 month old male C57BL/6 mice. With age, the expression of enzymes involved in the allopregnanolone synthetic pathway were decreased and corticosterone synthesis increased. Supplementation of allopregnanolone improved cognitive function. Interestingly, IL-6 infusion in young animals significantly reduced the production of allopregnanolone compared to controls. Of note, inhibition of IL-6 with its natural inhibitor, soluble membrane glycoprotein gp130 (sgp130), significantly improved spatial memory in aged mice. These findings were supported by in vitro experiments in primary murine astrocyte cultures, indicating that IL-6 decreases production of allopregnanolone and increases corticosterone levels. Our results indicate that age-related increases in IL-6 levels reduce progesterone substrate availability, resulting in a decline in allopregnanolone levels and an increase in corticosterone. Furthermore, our results indicate that allopregnanolone is a critical link between inflammatory cytokines and the age-related decline in cognitive function.

Key Words: aging, Alzheimer’s disease, inflammation, steroid hormones, enzyme regulation, interleukin 6 (IL-6), allopregnanolone (AlloP), progesterone, neurosteroid, cognitive function
**Introduction**

Previous studies have clearly indicated that aging is the primary risk factor for cognitive decline and neurodegenerative disease [1-3]. Several factors have been implicated in cognitive decline with age, including a decrease in circulating growth factors and neurosteroids, and/or an increase in inflammatory cytokines [4]. The age-related changes in the levels of neurosteroids are of particular interest since they are produced both in the periphery and the brain, have a complex biochemistry and physiology and affect multiple pathways involved in cognitive function. Progesterone is the precursor for the vast majority of these compounds and enzymatic conversion to Allopregnanolone, androgens and corticosterone (CORT) have effects on neurogenesis, synaptic density and learning and memory [5]. Allopregnanolone (AlloP) has been shown to be reduced with age and in several neurodegenerative disease models, such as AD and multiple sclerosis (MS) [6-8]. Restoring the levels of AlloP in these disease models increases neurogenesis, reduces inflammation, and improves cognition [9]. Nevertheless, a rigorous analysis of the age-related decline in AlloP and the cellular mechanisms that contribute to the decline in AlloP has not been undertaken.

Previous studies indicate that interleukin 6 (IL-6), an inflammatory cytokine, increases with age, and, in the adrenal cortex, upregulates the expression of two (2) enzymes, 11β-hydroxylase and 21-hydroxylase, which convert PROG to CORT [10]. These enzymes, as well as others that lead to AlloP synthesis, are found in the endoplasmic reticulum, and expressed throughout various cell types in the brain [11]. Since CORT and AlloP share the same precursor, increased synthesis of CORT has the potential to reduce precursor availability for synthesis of other neurosteroids and effectively decrease AlloP levels. Whether similar changes in AlloP and CORT occur in brain and contribute to the age-related decline in cognitive function is unknown.

The purpose of the present study was threefold: a) to establish whether neurosteroid levels in brain decrease with age, b) to determine whether AlloP replacement improves cognitive function in older animals and c) to assess whether inflammatory cytokines that have been shown to alter steroidogenic enzyme activity in the periphery have a similar effect in the CNS and establish their mechanisms of action. In this study we investigated the effects of AlloP on cognitive function in aged (24 mo) mice as well as the impact of IL-6 on AlloP synthesis. Using liquid chromatography/mass spectroscopy (LC/MS) to quantify the levels of AlloP in the cerebral cortex of male mice, we observed a significant decline with age. Interestingly, administration of a single subcutaneous (SC) injection of AlloP improved learning and memory in 24 mo aged mice to a level comparable to that in young. We also report significant differences in the levels of other steroidogenic enzymes with age in the mouse cerebral cortex. Our data show that the expression and
activity of PROG metabolizing enzymes change with age, likely contributing to the reduced production of AlloP.

Methods

Animals: 3, 12 and 24 mo old male C57BL/6N mice were obtained from the National Institute on Aging (NIA) Aged rodent colony. C57BL/6J mice were bred in house to generate pups for culture experiments. Animals were housed in the pathogen-free rodent barrier facility at OUHSC as described previously [12, 13]. All animals were free of helicobacter and parvovirus, and kept on a 12-hour light/12-hour dark cycle at 21 °C. All mice were group housed except for those undergoing ICV infusion surgeries. Mice undergoing surgery were singly housed from the day of surgery until day 14, at which time the mice were euthanized. The mice had access to standard irradiated bacteria-free rodent chow (5053 Pico Lab; Purina Mills, Richmond, IN, USA) and reverse osmosis water ad libitum. All procedures were approved by and followed the guidelines of the Institutional Animal Care and Use Committee of OUHSC.

Drug Treatments & Antibodies: Allopregnanolone (AlloP, 3α-hydroxy-5α-pregnan-20-one) was purchased from Steraloids, Inc. (Newport, Rhode Island, USA). ALLOP was reconstituted in 22.5% (W/V) 2-hydroxypropyl-β-cyclodextrin, purchased from Sigma (St. Louis, MO). Sgp130 was purchased from R&D systems (Cat # 468-MG) and reconstituted in vehicle (0.1% BSA in saline) as described previously [14]. For ICV injections of IL-6 and sgp130, 1.4 ug was dissolved in 100 uL of vehicle to deliver 100 ng per day via a 14 day osmotic pump, at a pump rate of 0.25 uL per hour (Alzet, mini-osmotic pump, model 1002). 0.1% BSA in saline was used as the vehicle control for both the sgp130 and IL6 ICV injection studies. IL-6 was purchased from Invitrogen and reconstituted in the vehicle (0.1% BSA in saline). Primary antibodies used for immunoblot analysis included NeuN, Calnexin (Millipore), Cox 4 (Invitrogen), as well as p-STAT3 and STAT3 (Cell Signaling). Secondary Antibodies used included Goat anti mouse IRDye 800 and IRDye 680 (LI-COR), as well as Goat anti rabbit IRDye 800 and IRDye 680 (LI-COR) were used as secondary antibodies.

LC/MS analysis of steroids: Progesterone (PRG), Allopregnanolone (AlloP) and 5α-dihydroprogesterone (5α-DHP) were analyzed by Liquid Chromatography/Mass Spectrometry (LC/MS) at the Wayne State University Lipidomics core facility. Cerebral cortices from 3, 12, and 24 month old mice were dissected from the brain and flash frozen under liquid nitrogen prior to processing. C18 extraction columns (30 mg sorbent, 1 ml; Strata-X; Phenomenex, Torrance, CA, USA) were used to isolate steroidal hormones as described earlier for eicosanoids [15, 16]. Deuterium-labeled internal standards (progesterone-d9, allopregnanolone-d9, and dihydroprogesterone-d9) were added to the samples prior to analysis via high performance liquid chromatography (HPLC). HPLC was conducted using a Prominence XR system.
(Shimadzu) with a Luna C18 (3μ, 2.1x150 mm) column. The mobile phase gradients were: A; water-acetonitrile-formic acid (10:90:0.1 v/v) and B: water-acetonitrile-formic acid (5:95:0.1 v/v). Following HPLC elution, mass spectrometry was performed using an ESIQTRAP5500 mass analyzer (SCIEX) in the positive ion mode. Unique molecular ions for each steroid analyzed were detected by Multiple Reaction Monitoring (MRM). Analyst 1.6.2 software (SCIEX) was used to collect the data, and each MRM transition chromatogram was quantified by MultiQuant software (SCIEX). Each analyte was quantified following normalization to the internal standards in each chromatogram. Steroid concentrations are expressed as pg/mg tissue weight.

**Tissue preparation and solid phase extraction (SPE) of steroids for ELISAs**

Steroids were extracted from brain tissue homogenates as described previously [17]. Briefly, each tissue sample was weighed and homogenized in ice cold ddH2O (3X sample volume). Methanol was then added at a volume of 4X the homogenate volume (approx. 400 uL) and sonicated at room temperature. The homogenates were then shaken at 1000 rpm for 1 hour at room temperature, and then stored overnight at 4°C. The following day, the homogenates were centrifuged at 3000 x g for 10 minutes at 4°C. The supernatant was collected and diluted with 9 mls of ddH2O prior to SPE extraction.

Bond Elut C18 columns (part no: 12102052) were purchased from Agilent Technologies and used for extraction of steroids. Three (3) mls of HPLC-grade EtOH was added to each column. Two (2) minutes later the columns were then rinsed with 5 mls of ddH20 (x2). The samples were then added to the columns at a volume of 5 mls (x2) (10 mLs total sample). The columns were again rinsed twice with 5 mls of ddH20, and steroids were extracted with the addition of 2 mls 90% MeOH. The column eluates were then dried under nitrogen at 45°C and stored at -20°C until further use. Prior to ELISA, the samples were reconstituted in sample buffer (provided in both AlloP and CORT ELISA kits.)

**ELISAs:** The following enzyme immunoassays were used to quantify steroids and proteins in both cortical tissue samples as well as tissue culture lysates. Allopregnanolone and CORT (Arbor Assays), Mouse IL-6 (Abcam), Mouse 21-Hydroxylase (Aviva Systems Biology), and 5-α-Reductase (LifeSpan BioSciences). Plates were read at 450 nm wavelength using a SpectraMax M2 plate reader (Molecular Biosystems). Sample concentrations were determined using a standard curve of the standards provided in each kit. Steroid concentrations were quantified as pg/mg tissue weight, and protein levels were quantified as pg/mg total protein.

**RAWM spatial memory assessment:** To assess the effects of AlloP on spatial memory in aged mice, ALLOP was subcutaneously injected into aged mice at 10 mg/Kg body weight in a volume of 100 uL. 2-hydroxypropyl-β-cyclodextrin was used as the vehicle for both young and aged control groups and given
at a volume of 100 uL. Both AlloP and vehicle injections were administered 10 days prior to behavioral testing. The radial arm water maze (RAWM) was used to measure spatial learning and memory. The RAWM consists of an 8-arm maze in which the mouse swims from a starting arm to the platform in the target arm to escape the water as previously described [13]. Briefly, each mouse underwent 8 (60 sec) trials per day over 3 consecutive days. Movement of mice in the maze was detected by a video tracking system located above the maze and parameters were measured using Ethovision software version 10 (Noldus Information Technology Inc., Leesburg, VA, USA) as described previously [18]. Performance was measured by Velocity, Latency (time taken to reach the target platform), and Errors (entries into incorrect arms). Data were analyzed using JMP statistical software.

Spatial memory assessment using PhenoTyper with Cognition Wall: Spatial memory was assessed following vehicle (0.1% BSA in saline) or Sgp130 ICV infusion using the PhenoTyper cognition wall (Model 3000, Noldus Information Technology, Netherlands). Sgp130 was delivered icv at 100 ng/day over 14 days. The mice began behavioral testing on day 10 post ICV infusion surgery, and completed testing on day 14, after which the mice were euthanized. To familiarize the mice with the precision rodent pellets used in the PhenoTyper, each mouse was singly housed for 4 days prior to testing with access to traditional rodent chow and precision rodent pellets ad libitum. The PhenoTyper home cage testing apparatus was used to assess initial discrimination learning and reversal learning as described previously[12]. During initial discrimination, the mouse must enter the left of 3 entrances 5 times (FR5) to receive a food pellet. During the reversal period, the mouse must enter the right entrance 5 times to receive a food pellet. Activity of the mouse was recorded using Ethovision software version 11 (Noldus).

Tissue Preparation: After completion of the experiments, mice were euthanized by cervical dislocation and both hemispheres of the brain were dissected, frozen in liquid nitrogen, and later used for mRNA expression analysis, protein quantification, or steroid extraction.

Microsome Isolation: Microsomes containing the endoplasmic reticulum were isolated from a separate cohort of animals as described previously [19]. 3, 12, and 24 month old mice were euthanized and the cerebral cortex was dissected and homogenized in sucrose buffer (250 mM sucrose, 5 mM HEPES-KOH, pH 7.4, 1 mM EGTA, 1 mM DTT and protease inhibitors). Tissues were homogenized on ice using a dounce homogenizer. To remove the nuclear fraction, the homogenates were centrifuged at 500 x g for 15 minutes. The supernatant was then collected and centrifuged at 8000 x g for 15 minutes to remove the mitochondrial fraction. Finally, the supernatant containing the microsomal fraction was transferred to a new tube and diluted with 125 mM sucrose, followed by centrifugation at 21000 x g for 90 minutes. The resulting pellet containing the microsomes was then resuspended in storage buffer (250 mM sucrose, 5 mM HEPES-KOH, pH 7.4, 1 mM EGTA, protease inhibitors, and 30% glycerol) and kept at -80 °C until future use.
**Progesterone Metabolism Assays:** Microsomes were rinsed in sodium phosphate buffer (0.01 mol/L, pH 7.0) containing protease inhibitors. For all incubations, 150 μg of protein was suspended in 1 mL of sodium phosphate buffer containing NADPH (10^{-3} mol/L) and 0.1 mCi/mL 4-C\textsuperscript{14}-progesterone (10 nM), as described previously [20]. Following analysis of the Time Course and Dose response curves, we determined the optimum incubation conditions to be 60 minutes using 10 nM 4-C\textsuperscript{14}-progesterone. The reaction was incubated at 37 °C in a shaking water bath. After 60 minutes, the reaction was stopped by placing the samples on ice and adding 1 ml of ice cold Methylacetate.

**Extraction of progesterone metabolites:** Each sample was centrifuged at 1000 x g for 10 minutes at 4 °C. The upper phase containing the steroid metabolites was transferred into a new tube. 700 uL of Methylacetate was then added to the remaining lower fractions and resuspended, followed by centrifugation at 1000 x g for 10 minutes. The upper phase was again transferred to a separate tube and rinsed 3 more times. The collected upper phases containing the steroid fractions were combined and evaporated under a gentle stream of N\textsubscript{2} gas and stored at -20 °C until future use.

**Thin Layer Chromatography (TLC):** Steroid fractions were dissolved in methanol and resolved using TLC plates (TLC Silica gel 60 F\textsubscript{254} glass plates 20 x 20 cm, EMD Millipore) [20]. To visually confirm the location of the individual metabolites, a solution of 9 reference standards [Progesterone (Q2600), 5α-Dihydroprogesterone (P2750), 20α-Dihydroprogesterone (Q3600), 17-Hydroxyprogesterone (Q3360), Deoxycort (Q3460), CORT (Q1550), Epiallopregnanolone (P3830), Allopregnanolone (P3800), and Testosterone (A6950), (Steraloids)] (10 ug each) dissolved in methanol were pipetted onto the individual plates at a volume of 10 μL. Each sample was run on an individual TLC plate containing the reference standards. Each plate was run two-dimensionally using methylacetate-ethylendicholoride (65:35) for one direction and Hexanol-hexane (75:25) for the other direction. The plates were dried overnight, and then stained with an aqueous solution of 3% cupric acetate, 8% phosphoric acid to visualize the location of reference standards. The plates were then dried for 30 minutes at 110 °C. Individual spots (Figure 4B) containing each 14C labeled metabolite were identified based on their migration on the plate, scraped from the plate, and transferred to scintillation vials. Each spot was quantified by scintillation counting and specific activity calculated as pmols/mg/hour.

**Gene expression:** Total RNA was isolated from hippocampus and cortex using the Qiagen RNeasy kit, and reverse transcribed using a High Capacity RNA-to-cDNA kit (Applied Biosystems). Taqman primers were purchased from ThermoFisher. Gene expression of steroidogenic enzymes (5α-Reductase type 1) [Mm00614213], 21-Hydroxylase [Mm00487230], 17α-Hydroxylase [Mm00484040]), and IL-6 (Mm00446190) were quantified using the Quantstudio 12K Flex System (Applied Biosystems, Life technologies, Waltham, MA, USA) using Taqman universal master mix reagents (ThermoFisher). All data
were normalized to the housekeeping genes, HPRT [Mm03024075_m1] and GAPDH [Mm99999915_g1], and presented as % mRNA expression relative to young control groups.

**Western Blotting:** Tissues and cell culture lysates were homogenized in lysis buffer (specify components of lysis buffer) containing protease inhibitors, followed by sonication, and centrifugation at 15000g. The supernatants containing the soluble proteins were quantified by the Bio-Rad DC (detergent compatible) protein assay and denatured with 1% SDS. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. Immunoblots were probed with the appropriate primary antibodies, rinsed, and then probed with fluorescent secondary antibodies (IRDye 800CW or IRDye 600CW) (LI-COR Biosciences) as described previously [21]. An Odyssey infrared imaging system (LI-COR Biosciences) was used to image and quantify all protein bands.

**Cell cultures:** Primary mouse cortical neurons and astrocytes were used in the in vitro experiments. Cortical neurons were isolated from E18-E20 C57BL/6J mouse pups according to approved Institutional Animal Care and Use Committee guidelines. Cortical astrocytes were isolated from post-natal day 2-4 (PN2-4) C57BL/6J mouse pups. Astrocytes and neurons were isolated as described [13, 21]. Briefly, following papain enzymatic digestion and trituration, cortical cells were resuspended in growth media (Neurobasal medium containing 2% NuSerum (BD Biosciences), 2% B27 (Life Technologies), penicillin/streptomycin (10 μg/ml), and L-glutamine (29.2 μg/ml)). Neurons were seeded on 50 mg/ml poly-D-lysine-coated 6-well plates at a density of 2 x 10^5 cells/well and half of the media was replaced with fresh media every 2-3 days. After 72 h of incubation, the neuronal cultures were treated with 5-fluor-2’-deoxyuridine (1.5 μg/ml) and uridine (3.5 μg/ml) for inhibition of astrocyte proliferation. For astrocyte cultures, cells were seeded on 50 mg/ml poly-D-lysine-coated 10 cm^2 dishes at a density of 1.5x10^6 cells/dish. After 7 days in vitro (DIV7), cells were split with 0.25% Trypsin-EDTA and seeded on 50 mg/ml poly-D-lysine-coated 6-well plates at a density of 2 x 10^5 cells/well. For IL-6 experiments, drug administration began on day 10 for neurons and day 3 for astrocytes. Astrocyte and neuron cultures were treated with vehicle (0.1% BSA in saline) or 100 ng/mL IL6 (per well in a 6 well plate) for 24 hours, after which the lysates were harvested for mRNA and protein analyses. For cell culture AlloP and CORT analyses, the cells were treated with vehicle or 100 ng/mL IL6 for 24 hours, followed by the addition of 10 nM progesterone for one hour. Cell culture media was then harvested for neurosteroid analysis. The cells were maintained at 37 °C with 5% CO2 in a tissue culture incubator.

**Statistics:** Systat (version 11, Systat Software, San Jose CA), JMP statistical software package (JMP Pro Version 14.1, SAS Institute), and Graphpad Prism were used for data analysis and graphing. LC/MS and gene expression data were quantified using a One-way or Two-Way ANOVA, as appropriate. Post-hoc comparisons were made using the Holm-Sidak method. Data collected for analysis of cognitive function
were analyzed using treatment group, task (initial discrimination or reversal), hour and phase (day or night) as independent variables. RAWM data were also analyzed with treatment group, task, trial, and day as independent variables. Significant differences in main effects or their interactions were analyzed using the Tukey HSD for pairwise comparisons. All data are represented as the mean+/- SEM.

Results

Allopregnanolone levels are altered with age

AlloP is synthesized from progesterone via 2 enzymatic steps. It can be synthesized locally in the brain or can easily cross the blood brain barrier after production by peripheral endocrine organs. To quantify the levels of AlloP and its upstream metabolites with age, we performed LC/MS analysis of cortical brain tissue from young (3 mo), middle-aged (12 mo) and old (24 mo) male mice. Interestingly, progesterone levels were not significantly different with age (Fig. 1A). In contrast, the levels of 5-alpha-dihyproprogesterone (p = 0.009) and AlloP (p = 0.001) decreased at both middle-age and old age (Fig. 1B, C).

Allopregnanolone improves working memory in 24 mo mice

In addition to the reduction of AlloP in neurodegenerative models, previous studies have also shown that restoration of AlloP in young AD mice improves learning and memory. To determine if restoring AlloP in aged mice could improve cognition, we assessed spatial memory following AlloP administration. A single subcutaneous injection (sc) of AlloP (10 mg/Kg) or vehicle (β-Cyclodextrin) was administered to young and aged mice and, after 10 days, spatial memory was assessed using the Radial arm water maze (RAWM) (Fig 1D). As expected, young mice made fewer entries into incorrect arms than aged control mice (p < 0.05, Fig 1G). Interestingly, AlloP treated aged mice also performed better than control aged mice, and showed similar performance to young mice. Both young and AlloP-treated aged mice had a shorter latency to platform compared to aged control mice (p < 0.05, Fig. 1F). These differences were not due to differences in swimming velocity. Thus a single injection of AlloP is sufficient to improve spatial learning in advanced age mice.

Neurosteroid synthesis is altered with age

The production of AlloP is dependent on the expression and activity of multiple steroidogenic enzymes (Fig. 2). Considering that progesterone can be metabolized into several other neurosteroids, it is possible that precursors are shunted to alternative pathways leading to the reduction in AlloP. We first quantified the expression of the initiating enzymes that metabolize progesterone. Interestingly, the mRNA expression of 5α-Reductase, which initiates the production of AlloP, or 17-hydroxy-progesterone did not change with
age (Fig. 3A, B). However, the expression of 21-Hydroxylase, which leads to the synthesis of CORT was
significantly upregulated at both middle (p = 0.003) and old age (p = 0.042) compared to young. (Fig. 3C).
To further investigate the hypothesis that increased 21-hydroxylase contributed to the reduction in AlloP
synthesis with age, we next quantified the protein levels of progesterone metabolizing enzymes. Similar to
the mRNA expression levels, protein levels of 5-alpha Reductase and 17α-Hydroxylase were not changed
with age (Fig 3D, E), while those of 21-Hydroxylase were significantly upregulated with age (Fig 3F).

To assess the activity of progesterone metabolizing enzymes with age, microsomes, containing the
endoplasmic reticulum, were isolated from the mouse brain cortex via subcellular fractionation, using a
sucrose gradient. Validation of an enriched microsomal fraction was determined by the absence of the
mitochondrial marker, cox 4, and nuclear marker, NeuN, from the microsomal fraction (Fig. 4A). We
assessed progesterone metabolite formation at 60 minutes after addition of 10nM 14C Progesterone (Fig.
4C, D). The assay revealed that progesterone metabolism into both Testosterone, and its precursor 17-
hydroxyprogesterone declined with age (Fig. 4 F, I) (p = 0.002), as well as AlloP and its precursor 5a-DHP
(Fig. 4 E, H) (p = 0.042). In contrast, the metabolism of progesterone into CORT, and its precursor
DeoxyCort, significantly increased with age (Fig 4 G, J) (p = 0.014).

IL-6 increases in the brain with age and modulates neurosteroid synthesis and working memory

Inflammation is known to increase throughout the body with age, and IL-6 has been implicated in the
regulation of steroidogenic enzyme expression in the HPA axis [10, 22]. To determine if IL-6 had a role in
the age related changes in enzyme activity and expression, we quantified mRNA expression and protein
levels of IL-6 in the cortex of young, middle-aged and old mice. In accordance with previous literature,
mRNA expression and protein levels of IL-6 increased with age (p = 0.001 and 0.017 respectively) (Fig
5A, B) [2]. To determine if IL-6 administration to young mice could suppress AlloP production, we
administered IL-6 directly into the lateral ventricle via ICV infusion for 14 days. At the end of the 14 day
period, the mice were euthanized and the cerebral cortices were isolated for analysis (Fig 5C). The levels
of AlloP were significantly reduced in cerebral cortex of IL-6 treated mice (p = .002) (Fig 5 E), while those
of CORT were increased (p = 0.034) (Fig 5F), suggesting that IL-6 regulates AlloP and CORT synthesis in
the brain.

To further investigate the effects of IL-6 on steroidogenic enzyme expression and working memory in old
mice, we administered the IL-6 inhibitor, Sgp130, locally to the brain. Sgp130 was administered to the
lateral ventricle of young and aged mice via icv infusion using an osmotic pump. After 14 days the mice
were assessed for spatial memory. Using the cognition wall as previously described [12] (Fig. 6A), we
assessed the independent learning index (Fig. 6B), which represents the average learning index per group
across each hour of the initial and reversal periods. We also assessed the cumulative learning index (6C), which represents the average learning index per hour accumulated over consecutive hours in the initial and reversal periods. Although there were strong trends for aged saline groups performing poorly, and aged sgp130 groups performing better during the initial discrimination period (Fig. 6D), the results were not significant. However, during the reversal period, aged control mice performed significantly worse than both young mice treated with sgp130 and young mice treated with saline. Aged mice treated with sgp130 for 14 days had significantly improved working memory compared to aged matched controls (p < 0.003) (Fig. 6E). Additionally, area under the curve (AUC) analyses of the cumulative learning index revealed a significant difference between young (102.6 ±1.126) and aged (76.6 ±2.78) control mice (p < 0.0001) as well as between aged control (76.6 ±2.78) and aged mice treated with sgp130 (100 ± 2.32) (p < 0.0001) when assessed by two-way ANOVA. Young mice treated with sgp130 showed no differences compared to young control mice. These results suggest that inhibiting IL-6 signaling in aged animals could be a therapeutic target for treating or preventing cognitive decline.

Aged mice treated with Sgp130 had significantly reduced IL-6 mRNA expression compared to control aged mice (Fig. 6F), while young mice were unaffected by Sgp130 treatment. This is in accordance with our previous data showing lower levels of IL-6 in young mice, potentially making them less likely to respond to IL-6 inhibition. Similar to our previous results, 5α-Reductase was not significantly different across age, however there was a trend for an increase in 5α-Reductase in response to Sgp130 treatment in aged mice (Fig. 6G). As we observed in our earlier LC/MS data, AlloP was reduced with age (p=0.043). Although there was a trend for an increase in the levels of AlloP in sgp130 treated mice, the results were not significantly different (Fig. 6H). The levels of CORT increased with age, but statistical analysis indicated no significant differences between groups (Fig. 6I).

**IL-6 regulates enzyme activity and controls ALLOP synthesis specifically in astrocytes**

To assess cell-specific effects of IL-6 on steroidogenic enzyme expression and neurosteroid synthesis, we performed *in vitro* studies using primary neuronal cultures from ED19-20 pups, and primary astrocyte cultures from PND3 pups. Astrocytes treated with IL-6 had significantly lower mRNA expression and protein levels of 5α-Reductase (Fig. 7A and B) (p < 0.001) and higher mRNA expression of 21-Hydroxylase (Fig. 7D) (p = 0.001), and a trend for increased 21-Hydroxylase protein levels. (Fig. 7E). Due to the differences in enzyme expression, we next quantified the production of both AlloP and CORT in primary astrocytes. 24 hours following IL-6 or vehicle treatment, 10 nM of progesterone was added as an initiating substrate. After 4 hours, the cell culture media was harvested and steroids were extracted. IL-6 significantly reduced the production of AlloP (p = 0.015) and partially increased the production of CORT although the later effect was not significant (Fig. 7C and F). In contrast, primary neuron cultures had no reduction in
mRNA expression or protein levels of 5α-Reductase following IL-6 treatment (Fig. 7G and H). Nevertheless, IL-6 did increase the mRNA expression of 21-hydroxylase in primary neurons (p = 0.003), similar to that in astrocytes (Fig 7J). Conversely, IL-6 treatment reduced the protein levels of 21-Hydroxylase (p = 0.02) (Fig. 7K). When the production of AlloP and CORT in neuronal cultures was quantified, IL-6 had no effect on the production of AlloP (Fig. 7I). Interestingly, in contrast to the increased expression of 21-Hydroxylase, IL-6 significantly reduced the production of CORT in primary neurons (p = 0.012) (Fig. 7L). These data suggest that IL-6 may play unique roles in different cell types in the brain.

Discussion

Previous studies have shown that AlloP declines in neurodegenerative disease models [6, 23]. However, there have been no studies beyond the age of 14 months [23]. Considering that male C57BL/6 mice have an average lifespan of 28 months, 14 months is widely considered to be middle-aged [24, 25]. We sought to determine the levels of AlloP in 24 month old mice which is more indicative of physiological aging and is consistent with the decline in cognitive function that is generally observed beginning at 21 months of age [26-28]. To our knowledge, we are the first to demonstrate that AlloP declines in 24 month old mice, and more importantly, that replacement of AlloP can improve learning and memory in advanced age.

The mechanism by which AlloP declines with age has not been well studied. Like all steroid hormones, the synthesis of AlloP is dependent on the expression and activity of multiple steroidogenic enzymes in the brain [29, 30]. Using microsomes isolated from the cerebral cortex, we found that PRG metabolism into both AlloP and Testosterone were reduced with age, while PRG metabolism into CORT was elevated. Thus, the elevation of CORT synthesis with age could be a contributing factor to the reduction in AlloP due to limitations of precursor availability.

The expression patterns of a variety of steroidogenic enzymes have been established in cell types throughout the brain, however the regulation of their expression is poorly understood [11, 31]. In the adrenal glands of the HPA axis, IL-6 has been shown to increase the expression of both 11β-hydroxylase and 21-Hydroxylase, leading to the increased production of CORT [10]. IL-6 is one of several cytokines known to be elevated in the brain with age and contributes to the age-associated increase in inflammation [32, 33]. IL-6 can act upon cells via classical or trans-signaling [34]. Classical signaling involves IL-6 binding to its membrane bound receptor, which then dimerizes with the membrane bound gp130 receptor to elicit downstream signaling. Trans-signaling occurs when IL-6 binds to the soluble IL-6 receptor, followed by dimerization with membrane bound gp130. Several studies have implicated IL-6 trans-signaling as the major contributor to chronic inflammation with age as well as in neurodegenerative disease models [2, 34, 35].
The inhibition of trans-signaling is dependent upon the levels of the soluble gp130 (sgp130), which can bind to the soluble IL-6 receptor and prevent its signaling. Previous studies have shown that when sgp130 was administered to mice following lipopolysaccharide (LPS) treatment, there was a significant reduction in inflammation in the brain as well as reduced sickness behavior [14]. We reasoned that the age-related increase in IL-6 contributes to altered neurosteroid levels and reduced production of AlloP since a) Both IL-6 mRNA and protein levels increased in the cerebral cortex with age and b) ICV administration of IL-6 to young mice significantly reduced the levels of AlloP specifically in the cerebral cortex of male mice. Importantly, we also demonstrated that inhibition of IL-6 signaling via sgp130 ICV infusion significantly improved spatial memory in aged mice and increased the levels of AlloP in the brain, although the latter effect failed to reach statistical significance.

Further studies are needed to determine if the increased AlloP synthesis following IL-6 inhibition is part of the mechanism responsible for cognitive improvement. Previous studies indicate that both synapse number and function are reduced with age, and contribute to cognitive impairments [36]. To determine if IL-6 inhibition affected synaptic abundance, we quantified the levels of the presynaptic marker, synaptophysin, and the post synaptic marker, post synaptic density protein 95 (PSD-95) in the hippocampus via western blot. Using this approach we observed a trend for a decrease with age, but the results were not significant, nor were there differences in the sgp130 treated groups. In addition, we quantified the astrocyte marker, GFAP, and microglial marker, IBA-1 in the hippocampus to investigate whether IL-6 influenced the age-related increases in reactive astrocytes or microglia [37]. Although there were trending increases in these markers with age, the results were not significant and IL-6 inhibition did not alter GFAP or IBA-1 levels.

IL-6 is known to contribute to a variety of inflammatory pathways and likely has several downstream effects that may affect cognitive function directly, in addition to inhibiting AlloP synthesis [35]. For example, chronic elevation of IL-6 in the brain has been shown to induce apoptosis, increase blood brain barrier (BBB) permeability, and induce neuronal excitotoxicity [38-40]. All of these processes are associated with neurodegenerative diseases. Therefore, inhibition of IL-6 trans-signaling likely has multiple beneficial effects in the brain.

Our studies show that IL-6 administration resulted in a reduction in both 5a-Reductase expression, protein levels, and AlloP production specifically in astrocytes, rather than neurons. In contrast, IL-6 reduced 21-hydroxylase protein levels and reduced the production of CORT in neurons. Thus, the effect of IL-6 on steroidogenic enzymes is likely cell type specific and interactions between multiple cell types likely have a key role in the cognitive actions of IL-6[5]. Although the use of primary cultures is an important and useful tool in assessing cell type specific functions, it cannot recapitulate all of the complex changes that are evident in aged animals. Nevertheless, attempts were made to isolate astrocytes from the brains of aged
mice, but, based on the viability of these cells, we were not convinced that the response of these cells would provide useful information. Future studies using acutely isolated astrocytes from aged mice would be useful to determine if IL-6 specifically regulates AlloP synthesis in astrocytes, as we observed in our primary culture studies of embryonic mouse astrocytes.

Although the majority of cell types in the brain contain all the enzymes necessary to metabolize PRG into various neurosteroids, the relative levels of these enzymes likely differ [41]. For instance, glial cells are thought to secrete more AlloP [42] than other cell types in the brain, thus the impact of IL-6 on AlloP production that we observe in vitro could also be occurring locally in the brain. Previous studies have shown that AlloP can increase hippocampal neurogenesis, therefore its reduction with age is likely a contributing factor to the age-related decline in hippocampal neurogenesis.

Multiple factors are known to contribute to cognitive decline with age. These can include elevated inflammation, DNA damage, oxidative stress and cellular senescence [3, 4, 43]. It is possible that the reduction in AlloP with age is dependent on the elevation of IL-6 and is a major factor contributing to cognitive decline. Future studies are needed to determine the mechanism by which IL-6 downregulates 5α-Reductase expression in the brain, as well as its effects on specific cell types throughout the brain.

Data Availability Statement

All data reported in this study is located within the manuscript.

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Fig. 1. Allopregnanolone decreases in the brain with age, and its restoration improves working memory. A-C: LC/MS quantification of progesterone (A), 5α-Dihydroprogesterone (B), and allopregnanolone (C) in the cerebral cortex from young (3 m), middle (12 m), and old mice (24 m). \( n = 6 \) per group; one-way ANOVA. D: Illustration of the RAWM. Orange boxes indicate incorrect arms, and the yellow box indicates the target arm containing the escape platform. E-G: Performance in the RAWM in young (3 m), old (24 m), and old mice treated with Allopregnanolone (AlloP). E: Average velocity of the mice in each group while in the RAWM. F: Average latency from the starting arm to the target platform. G: Average entries into incorrect arms. E-G: Data represent the average of 8 trials per day over 3 days. \( n = 9 \) per group; Two-Way ANOVA and Tukey HSD pairwise comparisons. AlloP, allopregnanolone. * \( p < 0.05 \). Statistics were adjusted for multiple comparisons. Only significant differences are depicted in the figure. Data are presented as mean+/- SEM.
Fig. 2. Biosynthetic pathways leading to neurosteroid synthesis. A: Illustration of steroidogenic enzymes produced in the mitochondria and endoplasmic reticulum (ER) leading to the synthesis of allopregnanolone, testosterone, and corticosterone. B: Table outlining the steroidogenic enzymes associated with the respective numbers in A. Cholesterol is the precursor for all neurosteroid synthesis. Cholesterol is first metabolized by P450Scc (1) housed in the mitochondria to form Pregnenolone. After exiting the mitochondria, pregnenolone can then be metabolized by 3β-Hydroxysteroid dehydrogenase (2) located in the ER, to form progesterone. Progesterone can then be metabolized by several different enzymes to form unique steroid metabolites. Allopregnanolone is formed via 5α-Reductase (3) and 3α-Hydroxysteroid Dehydrogenase (4), respectively. Testosterone can be formed via P450c17 (3b) and 17β-Hydroxysteroid Dehydrogenase (4b), respectively. Corticosterone can also be formed from 21-Hydroxylase (3c) and 11β-Hydroxylase (4c), respectively.
Fig. 3. The mRNA expression and protein levels of steroidogenic enzymes are altered with age. A-C: % mRNA expression of 5α-Reductase, 17α-Hydroxysteroid Dehydrogenase, and 21-Hydroxylase in the cortex of young (3 m), middle (12 m), and old (24 m) mice. (n = 8 per group; one-way ANOVA). D-F: Protein levels (pg/mg protein) of 5α-Reductase, 17α-Hydroxysteroid Dehydrogenase, and 21-Hydroxylase in the cortex of young (3 m), middle (12 m), and old (24 m) mice. (n = 8 per group; one-way ANOVA). Statistics were adjusted for multiple comparisons. Only significant differences are depicted in the figure. Data are presented as mean±/− SEM.
Fig. 4. Progesterone metabolism via steroidogenic enzymes are altered with age in the cerebral cortex. A: Representative western blot showing the separation of subcellular fractions. Calnexin is a marker for the microsomal fraction, Cox4 marks the mitochondrial fraction, and NeuN marks the nuclear fraction. (n = 3, with 3 replicate experiments). B: Representative image of the TLC plates used to separate the individual steroid metabolites. C: Dose response curve of increasing amounts 14C-Progesterone to 14C-metabolites in microsomes. (n = 3). Data represent the average total 14C-Metabolite pmols formed per mg of protein per hour. D: Time Course of 14C-Metabolite formation in microsomes following the addition of 14C-Progesterone. (n = 3). E-J: Metabolite formation of (E) 5α-Dihydroprogesterone (5αDHP), (F) 17α-Hydroxyprogesterone (17αOHP), (G) Deoxycorticosterone (DOC), (H) Allopregnanolone (AlloP), (I) Testosterone (Test), and (J) Corticosterone (CORT) in microsomes isolated from the cerebral cortex of young (3m), middle (12 m), and old (24 m) mice. Progesterone metabolite formation is shown as the pmols of each 14C-Metabolite formed per mg of microsomal protein per hour. (n = 4 per group; one-way ANOVA). (* p < 0.05.) Statistics were adjusted for multiple comparisons. Only significant differences are depicted in the figure. Data are presented as mean +/- SEM.
Fig. 5. IL-6 increases in the brain with age and its administration to young mice reduces AlloP synthesis and increases CORT synthesis. A and B: Cerebral cortex tissues from young (3m), middle (12 m), and old (24 m) mice. A: % mRNA expression of IL-6 across age relative to young. (n = 8 per group; one-way ANOVA). B: Levels of IL-6 (pg/mg protein) in the cortex across age. (n = 6 per group; one-way ANOVA). C: Experimental design of Saline or IL-6 infusions to the mouse brain. Saline or IL6 was loaded into an osmotic pump that was coupled to a cannula and inserted into the lateral ventricle of the brain in young (4 m) mice. The osmotic pump delivered saline or IL-6 (100 ng/day) continuously for 14 days. D: Levels of IL-6 (pg/mg protein) in the cortex following Saline or IL6 ICV infusion. E-F: Allopregnanolone and Corticosterone levels in the cortex of young mice following 2 week ICV infusions of either saline or IL-6 (100 ng/day) to the lateral ventricle of the brain. (n = 8 per group, unpaired students t-test, * p < 0.05). Statistics were adjusted for multiple comparisons. Only significant differences are depicted in the figure. Data are presented as mean+/- SEM.
Fig. 6. The effects of IL6 inhibition on neurosteroid synthesis and working memory. A: Illustration of the Phenotyper home cage system, containing the cognition wall, used to assess working memory. On days 1-2, the initial learning period, the mouse must learn to enter the left most entry of the cognition wall at least 5 times to receive a food pellet. On days 3-4, the reversal period, the mouse learns to enter the right most entry at least 5 times to receive a food pellet. B-C: Working memory assessment in young and old mice following a 2 week ICV infusion of either saline or Sgp130 (IL6 inhibitor) to the lateral ventricle of the brain. Learning indices are calculated by (correct entries – incorrect entries)/total entries made per hour. B: The independent index represents the average learning index per group across each hour of the initial learning and reversal periods. C: The cumulative index represents the average learning index per group accumulated over consecutive hours in both the initial learning period and the reversal period. D: Average learning indices per group during the initial discrimination period. E: Average learning indices per group during the reversal period (n = 5-8 per group, Two-Way ANOVA, Tukey HSD pairwise comparisons * p < 0.05). F-I: Cerebral cortex tissues from young and old mice treated with saline or, the IL-6 inhibitor, Sgp130. F: % mRNA expression of IL6 across groups relative to young. G: % mRNA expression of 5α-Reductase across groups relative to young. H-I: Allopregnanolone and Corticosterone levels in the cortex following saline or Sgp130 treatment. F-I: (n = 5-8 per group, Two-Way ANOVA, * p < 0.05). Statistics were adjusted for multiple comparisons. Only significant differences are depicted in the figure. Data are presented as mean+/- SEM.
**Fig. 7.** IL6 reduces the synthesis of allopregnanolone specifically in astrocytes and reduces the synthesis of corticosterone specifically in neurons. Primary astrocyte and neuronal cultures were treated with or without IL-6 (100 ng/mL) for 24 hours and used for the following experiments. Cell culture lysates were used for %mRNA expression (relative to vehicle control) and protein levels of 5α-Reductase and 21-Hydroxylase. Cell culture media was used for allopregnanolone and corticosterone quantification. A and G: 5α-Reductase mRNA expression in astrocytes and neurons. B and H: 5α-Reductase protein levels in astrocytes and neurons. C and I: Allopregnanolone in cell culture media from astrocytes and neurons. D and J: 21-Hydroxylase mRNA expression in astrocytes and neurons. E and K: 21-Hydroxylase protein levels in astrocytes and neurons. F and L: Corticosterone in cell culture media from astrocytes and neurons. (n = 4 replicate experiments; students unpaired t-test; Bonferroni method for multiple comparisons, * p < 0.05). Data are presented as mean±/ SEM.