Preclinical Analyses of the Therapeutic Potential of Allopregnanolone to Promote Neurogenesis *In Vitro* and *In Vivo* in Transgenic Mouse Model of Alzheimer's Disease

Roberta Diaz Brinton and Jun Ming Wang[1,*]

[1]*Department of Molecular Pharmacology and Toxicology and Program in Neuroscience, Pharmaceutical Science Center, 1985 Zonal Avenue, University of Southern California, Los Angeles, CA 90033, USA*

Abstract: Herein, we present data to support a preclinical proof of concept for the therapeutic potential of allopregnanolone to promote neurogenesis. Our recent work has demonstrated that the neuroactive progesterone metabolite, allopregnanolone (3α-hydroxy-5α-pregnan-20-one), (APα) induced, in a dose dependent manner, a significant increase in proliferation of neuroprogenitor cells (NPCs) derived from the rat hippocampus and human neural stem cells (hNSM) derived from the cerebral cortex [1]. Proliferative efficacy was determined by incorporation of BrdU and ^{3}H-thymidine, FACS analysis of MuLV-GFP-labeled mitotic NPCs and quantification of total cell number. Allopregnanolone–induced proliferation was isomer and steroid specific, in that the stereoisomer 3β-hydroxy-5β-pregnan-20-one and related steroids did not increase ^{3}H-thymidine uptake. Immunofluorescent analyses for the NPC markers, nestin and Tuj1, indicated that newly formed cells were of neuronal lineage. Furthermore, microarray analysis of cell cycle genes and real time RT-PCR and western blot validation revealed that allopregnanolone increased the expression of genes which promote mitosis and inhibited the expression of genes that repress cell proliferation. Allopregnanolone-induced proliferation was antagonized by the voltage gated L-type calcium channel blocker nifedipine consistent with the finding that allopregnanolone induces a rapid increase in intracellular calcium in hippocampal neurons via a GABA type A receptor activated L-type calcium channel. Preliminary *in vivo* data indicate that APα for 24 hrs significantly increased neurogenesis in dentate gyrus, as determined by unbiased stereological analysis of BrdU positive cells, of 3-month-old male triple transgenic Alzheimer's disease mice. The *in vitro* and *in vivo* neurogenic properties of APα coupled with a low molecular weight, easy penetration of the blood brain barrier and lack of toxicity, are key elements required for developing APα as a neurogenic / regenerative therapeutic for restoration of neurons in victims of Alzheimer's disease.

Keywords: Allopregnanolone, neurogenesis, hippocampus, cell cycle genes, L-type calcium channel, therapeutics.

BACKGROUND: CHALLENGE OF REGENERATIVE THERAPEUTICS FOR ALZHEIMER'S DISEASE

A major challenge not yet addressed by current therapeutic interventions for Alzheimer's disease (AD) is the regeneration of lost neurons and neural circuitry to restore cognitive function. Therapies that lead to cessation of the degenerative process still leave the brain riddled with deteriorated neural circuits and reduced neuron number. The discovery of neurogenesis in the adult brain and the regenerative potential of neural stem cells hold the promise for restoration of neural populations and regeneration of neural circuits necessary for cerebral function.

While the regenerative potential of neural stem cells is great, so too is the challenge of delivering neural stem cells to the brain. Direct delivery of neural stem cells to the brain, with all the difficulties of neurosurgery in the elderly, also faces the challenge of distributing cells throughout the brain as AD is characterized by a diffuse pattern of degeneration. An alternative strategy to cell therapy is to promote endogenous proliferation of neural stem cells within the brain, which although in low abundance, can still be induced to proliferate. The challenge of this approach is the delivery of growth factors to the brain for promotion of neurogenesis. Large molecular weight growth factors do not readily cross the blood brain barrier and thus require direct infusion into the brain *via* acute or chronic catheterization of the brain. Our efforts have focused on translating a serendipitous discovery of allopregnanolone-induced mitosis of hippocampal neurons *in vitro* into a low molecular weight neurogenic therapeutic for promotion of neurogenesis in aging and to restore neuron populations lost to neurodegenerative diseases such as Alzheimer's.

ALLOPREGNANOLONE NEUROSTEROID AND ALLOSTERIC MODULATOR OF THE GABA CHLORIDE CHANNEL: SELECTIVE TARGET FOR NEUROGENESIS

The neurosteroid, allopregnanolone (APα, 3α-hydroxy-5α-pregnan-20-one), is a reduced metabolite of progesterone (P_4) and is generated *de novo* in the central nervous system (CNS) (for review, see [2-4]. Developmentally, P_4 and APα are synthesized in the CNS throughout the embryonic period in the pluripotential progenitor cells [5, 6] and reach their highest concentration in late gestation [7, 8]. A region spe-

*Address correspondence to this author at the Department of Molecular Pharmacology and Toxicology, Pharmaceutical Sciences Center, University of Southern California, 1985 Zonal Avenue, Los Angeles, CA 90089-9121, USA; Tel: 323-442-1430; Fax: 323-442-1489;
E-mail: rbrinton@hsc.usc.edu

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cific expression pattern of progesterone converting enzymes, P450scc, 5α reductase and 3α hydroxysteroid dehydrogenase, in brain is evident in both hippocampus and cortex [4, 9-11]. In the peripheral and central nervous system both APα and progesterone can promote oligodendrocyte proliferation and myelination [5, 12, 13]. Remarkably, the enzymes, 5α-reductase and 3α-hydroxysteroid dehydrogenase, required to convert progesterone to its 3αmetabolites, are present and functional in pluripotential progenitors [6, 14]. In mice, the concentration of APα in brain tissue was 1.5 to 2.5 times higher than vehicle control within 8 min of peripheral injections and diminished within 20 min [15].

In the aged and Alzheimer's disease (AD) brain, both the pool of neural stem cells and their proliferative potential are markedly diminished [16, 17]. In parallel, APα content, along with a host of other factors, is diminished in the brains of AD patients compared to age-matched controls [18].

In mature neurons, APα is well known as an allosteric modulator of the gamma-aminobutyric acid type A receptor ($GABA_AR$)/chloride channel to increase chloride influx thereby hyperpolarizing the neuronal membrane potential and decreasing neuron excitability [7, 19-21]. These properties led to the pursuit of APα and derivatives as an antiepileptic and antianxiety therapeutic drug [22, 23]. In marked contrast to differentiated neurons, the flux of chloride in developing neurons, *is opposite* to that of mature neurons [24, 25]. In immature neurons, the high intracellular chloride content leads to an *efflux* of chloride through the $GABA_AR$, depolarization of the membrane and opening of L-type voltage gated Ca^{2+} channels (VGLCC) [26-29]. Thus, the $GABA_AR$-mediated depolarization could act as a trigger for spontaneous, activity-independent $[Ca^{2+}]_i$ rise in early precursor cells or subventricular zone radial precursor cells, thereby influencing developmental events, such as neurogenesis and synaptogenesis [30-32]. Results of our analyses indicate that APα induces calcium influx into hippocampal progenitor cells *in vitro* that is blocked by an L-type calcium channel inhibitor [28].

ALLOPREGNANOLONE AS A NEUROGENIC FACTOR

Previous work from our laboratory demonstrated that exposure of embryonic day 18 (E18) rat hippocampal neurons to APα induced regression of neurite outgrowth within 1 hour [19]. Subsequent microscopic morphological observation indicated that within 24 hrs, APα significantly increased the number of cells exhibiting morphological features of mitotic events (unpublished observations). These findings led us to hypothesize that APα promoted neurite regression as a prelude to hippocampal progenitor cell proliferation to thereby act as a neurogenic agent. Therefore, we undertook a series of cellular, morphological, biochemical, and genomic analyses to determine the neurogenic potential of APα in cultured rat neural progenitor cells (rNPCs), human neural stem cells (hNSC) and in the triple transgenic mouse model of Alzheimer's disease.

Results of these analyses demonstrated that APα specifically increased proliferation of rat hippocampal NPCs, increased the formation of neurospheres of neural stem cells from both embryonic and adult rodent brain, and human cerebral cortical NSCs in a dose dependent fashion (see Figs. **1**-**2**) [1]. In parallel, APα significantly increased expression of genes that promote progression through the cell cycle while inhibiting expression of genes involved in cell cycle repression. Immunocytochemical labeling for NPC markers indicated that the newly formed cells are of neuronal lineage (Table **1**). Further, we have determined that the mechanism for APα−induced neurogenesis requires activation of voltage gated L-type calcium channels (VGLCC).

POTENCY AND EFFICACY OF APα-INDUCED NEUROGENESIS

APα-induced neurogenesis was a dose dependent process with concentrations within the 10^{-9} to mid 10^{-7} M range promoting proliferation while concentrations in excess of 10^{-6} M significantly inhibiting neurogenesis [1]. The biphasic effect of APα on neurogenesis is supported by a recent study showing that nanomolar levels of APα increase while micromolar levels of APα inhibit the proliferation of polysialylated form of the neural cell adhesion molecule (PSA-NCAM) positive neural progenitors [5]. At high concentrations (i.e. in micromolar), APα can be converted by 20α hydroxysteroid dehydrogenase to allopregnanediol (5α-pregnan-3α, 20α-diol) [33] and hence may increase the local concentration of allopregnanediol that inhibited DNA replication of rNPC, thereby inducing a biphasic dose response.

APα was a more potent neurogenic factor for hNSCs with a minimally effective dose of 1 nM whereas in the rNPCs the minimally effective dose was greater than 10 nM [1] (see Fig. **2**). The concentrations of APα required to induce neurogenesis *in vitro* are comparable to those found in both rat and human brain. APα levels are 12 ng/g (~ 38 nM) in the pregnant maternal rat brain and 19 ng/g, (~ 60 nM) within the embryonic rat brain [7, 34]. In the human premenopausal female, APα levels in serum are 4 nM in the middle of menstrual cycle [17, 35] and are 160 nM during the third trimester in healthy pregnant women [36]. Because a similar concentration has been detected in the umbilical cord, it is suggested to be an indicator of fetal levels of APα [36].

In contrast to fetal development, an age associated decrease in serum APα was observed in men more than 40 yr of age but remarkably not in women [16]. Interestingly, a significant decrease (about 3 fold) in APα levels was observed in patients with Alzheimer's disease compared to the age matched control group [18, 37]. In parallel, in the aged brain, both the pool of NSC and their proliferative potential are markedly diminished [38-40].

APα induced neurogenesis ranged from 20-30 % in the rodent NPCs to 37-49% in the human neural stem cells. The efficacy of APα as a neurogenic factor was comparable to that induced by bFGF + heparin from our own study and also in agreement with previously published results. For example, bFGF induced a 0.4 fold increase in cultured rat brain derived progenitor cells [41] after 3 days treatment and a 25 % increase of BrdU incorporation in 3 month old rat brain [39]. Additional support comes from the recent studies that APα induces about a 20% increase in thymidine incorporation in immature rat cerebral granular cells [42] and a 20-30% increase of PSA-NCAM positive progenitor proliferation de-

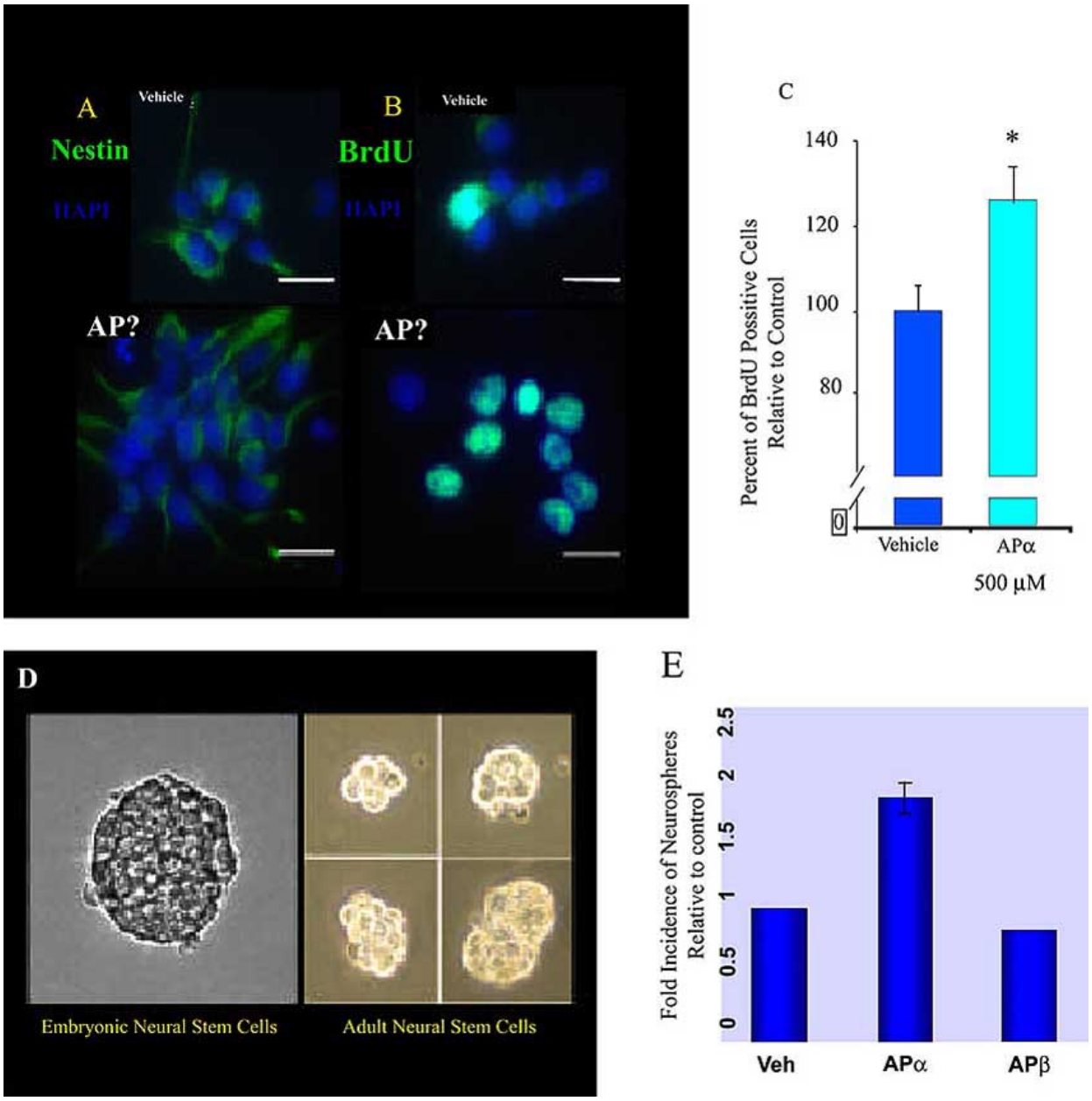

Fig. (1). Allopregnanolone (APα) promotes proliferation of hippocampal derived progenitors and neurosphere expansion of both embryonic and adult derived neural stem cells. Panels A and B. Nestin immunoreactivity and BrdU incorporation was visualized by specific antibody immunofluorescence. Photomicrographic images are representative of control (upper panel) and 500 nM APα treated (lower panel) rat E18 hippocampal cultures after 1 DIV APα exposure. Bars = 20 _m. Panel C. Bar graph depicts quantitation of BrdU positive cells from 10 randomly selected fields per slide. Total number of cells analyzed / slide = 800-1000. Three slides / condition were analyzed for a total of 8964 cells contributing to the analysis. Data are from 3 independent experiments and the results are plotted as mean ± SEM of BrdU positive cells in percent of vehicle group (set as 100%). * $p < 0.05$ vs. vehicle. Panel D. Neural stem cells from late stage rat embryos and 2 month old mouse brain were successfully cultured *in vitro* and formed aggregate neurospheres. Panel D (left) shows an embryonic neurosphere following 4 days *in vitro*. In the right panel of D is an image of adult neural stem cells derived from the dentate gyrus of 10-week old C57bl/6 mouse. Panel E. APα promotes embryonic neural stem cell proliferation. The neurogenic effect of APα was evident as an increase in dissociated single cell growth, neurosphere expansion and original neurosphere formation. Results of neurosphere expansion assay in the absence and presence of growth factors (GF= EGF 20ng/ml and FGF 10ng/ml) indicate that APα was neurogenic in the absence of growth factors and markedly potentiated the neurogenic efficacy of the growth factors. APβ diminished neurosphere expansion in the presence and absence of growth factors. Panel C. APα stimulates quiescent neural stem cells to proliferate and form neurospheres. Neural stem cells in the adult brain are maintained at G_0 phase to preserve genome integrity. We sought to determine whether APα would increase the pool of quiescent stem cells to enter the cell cycle. Neural stem cells were harvested from the periventricular region of 18.5 embryonic rat brains. Dissociated cells were seeded at a density of 200 cells/well. As shown in Panel C, exposure to APα increased the incidence of neurosphere formation by nearly two fold.

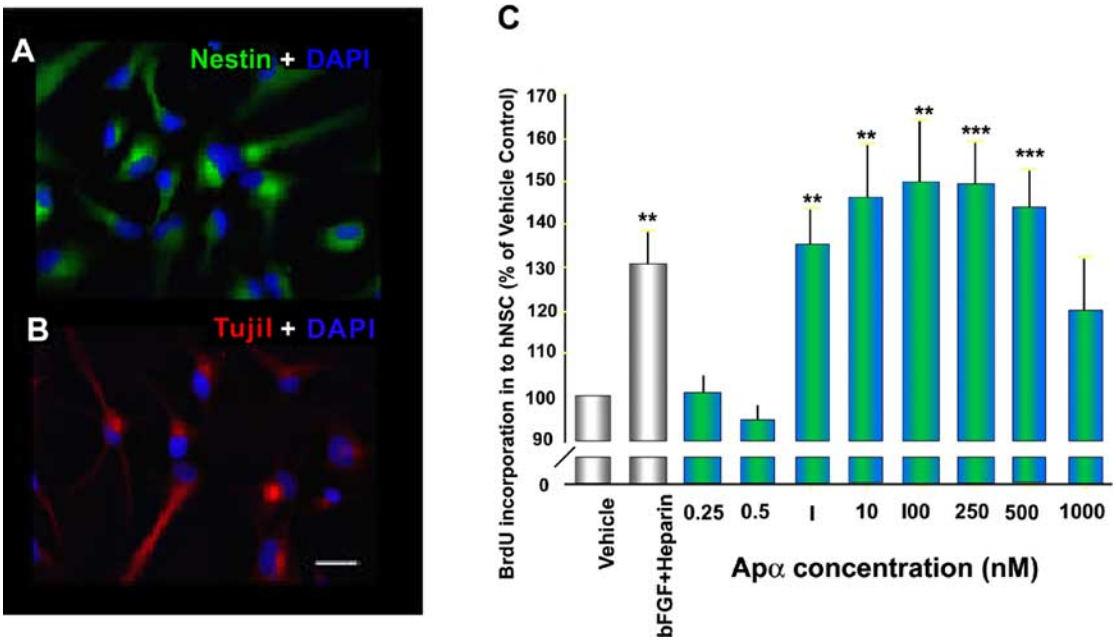

Fig. (2). Allopregnanolone (APα) increases BrdU incorporation in human neural stem cells from the cerebral cortex in a dose dependent manner. Panel A shows a representative cluster of nestin positive neural stem cells while Panel B shows Tuj1 positive human neural stem cells. Cell nuclei were counterstained with DAPI. Scale bar = 15μm. Panel C shows the dose response profile of APa-induced BrdU incorporation in human neural stem cells. BrdU incorporation in human cerebral cortical stem cells was measured by a chemiluminescence BrdU ELISA. Data are from at least three independent assays conducted in octuplets. The positive control, bFGF (20 ng/ml) plus heparin (5 μM/ml) induced a 30 % increase in BrdU incorporation vs. control. APα induced a dose dependent biphasic increase in BrdU incorporation that was biphasic. The minimally effective concentration was 1 nM with the maximally effective effect (50 % increases) occuring at 100 and 250 nM. At 1000 nM a reversal of the increase in BrdU incorporation was apparent. Results were plotted as % of vehicle control (mean ± SEM). ** $P < 0.01$ vs. vehicle, *** $P < 0.001$ vs control.
Human cortical neural stem cells were provided by Dr. Clive Svendsen at the University of Wisconsin

Table 1. Allopregnanolone Promotes Expression of Genes that Promote Progression through the Cell Cycle and Inhibits Genes that Inhibit Progression

Gene Category	Direction of change APα vs Control	
Cyclins	$P < 0.05$	increased
CDK	$P < 0.05$	increased
PCN	P <	increased
CDK inhib	P <	decreased
Ubiquitin relatives	P <	decreased
Actin GAPD	P >	no change

Cultures of hippocampal neurons were treated with vehicle or 500 nM APα for 24h and followed by extraction of total RNA. **A.** Gene array of cell cycle related genes expression**.** Non-radioactive probes were prepared and hybridized to 96 genes miniarray. Two housekeeping genes (β-actin and GAPDH) were used as internal controls. Two blanks and pUC18 from bacterial plasmid were used as negative controls. Data were represented as fold of change of mRNA expression in APα treated neurons vs. control (mean ± SEM) as determined by optical density. Results of these analyses in cultured hippocampal neurons indicate that APα induced a marked increase in genes that promote progression through the cell cycle while inhibiting genes associated with exiting the cell cycle. APα induced an 8 fold increase in cyclin E which promotes progression from G1 to S phase and a > 2 fold increase in two well defined cell proliferation markers, cell dividing control protein 2 (CDC2, also known as CDK1) and proliferating cell nuclear antigen (PCNA). In parallel to an increase in cell cycle progression genes, APα inhibited the expression of CDK 4&6 inhibitors and the ubiquitin, cullin3, which are associated with exiting the cell cycle. Two housekeeping genes, actin and GAPDH, were unchanged. Data were from three separate experiments and multivariant ANOVA statistical analysis indicated the increase and decrease were statistically significant as presented in the table 3. **B**. Validation of the gene array results by real time RT-PCR. APα–induced a significant increase in mRNA expression of cyclin A2, cyclin B1, cyclin E, CDC2 and PCNA mRNA, whereas APα-induced a significant reduction in mRNA for cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4) and ubiquitin-activating enzyme E1. These Q-PCR data are entirely consistent with the gene array results. Data are depicted as fold change of mRNA expression in APα treated neurons vs. vehicle control (mean ± SEM) from three separate experiments. Controls were set to zero. * = $P < 0.05$.

rived from rat brain [5]. Together, these data indicate that APα can promote neurogenesis of neural stem cells derived from multiple sites within the rodent brain and from the cerebral cortex of human brain.

GENETIC AND PROLIFERATIVE PROPERTIES OF APα-INDUCED NEUROGENESIS:

Our gene array and real time RT-PCR data are consistent with a neurogenic effect of APα. Genes that promote transition through the cell cycle and proliferation, such as cyclins and CDKs including CDC2, cyclin B and PNCA, were upregulated by APα. Correspondingly, APα down regulated the expression of genes involved in inhibition and degradation of CDKs and cyclins, such as CDK4 and CDK6 inhibitor P16, P18, cullin 3 and ubiquitin-activating enzyme E1(Ube1x), enzymes that are required for ubiquitination of mitotic cyclins and promote exit from the cell cycle [43, 44]. In our study, APα not only regulated the expression of cell cycle proteins and DNA amplification but also drove a complete mitosis of the rNPCs. This conclusion is supported by the data showing that APα increased the MuLV-GFP positive cell number, because GFP signal can only be observed in the cells which tranversed a complete cell cycle [45-47]. Moreover, the APα-induced increase in total cell number further supports this conclusion.

MECHANISM OF APα-INDUCED NEUROGENESIS

It is well known that APα acts as an allosteric modulator of the $GABA_AR$ to increase chloride influx thereby hyperpolarizing the neuronal membrane potential and decreasing neuron excitability [48-50]. In marked contrast to this action in mature neurons, activation of $GABA_AR$ by GABA or APα in immature neurons, leads to an *efflux* of chloride. The high intracellular chloride content in embryonic cells, reverses the concentration gradient for chloride whereby the efflux of chloride leads to depolarization of the membrane and opening of VGLCC [25, 28, 51, 52]. Blockade of APα-induced neurogenesis by an inhibitor of VGLCC is consistent with our finding of an APα-induced rise in intracellular calcium via activation of VGLCC [15].

Increases in intracellular calcium can activate calcium dependent mechanisms of mitosis in early precursor cells and human NSCs to promote neurogenesis [30, 32, 53]. Our analyses indicate that APα induced a rapid and developmentally regulated influx of calcium via $GABA_AR$ activation of VGLCC [15] in cultured hippocampal neurons that may evoke neurogenesis. Thus, we propose that the $GABA_AR$ activated VGLCC and subsequent calcium influx plays a key role in the APα stimulated neurogenesis in both rat neural progenitors and human neural stem cells. A schematic description of our working model is shown in Fig. **3**.

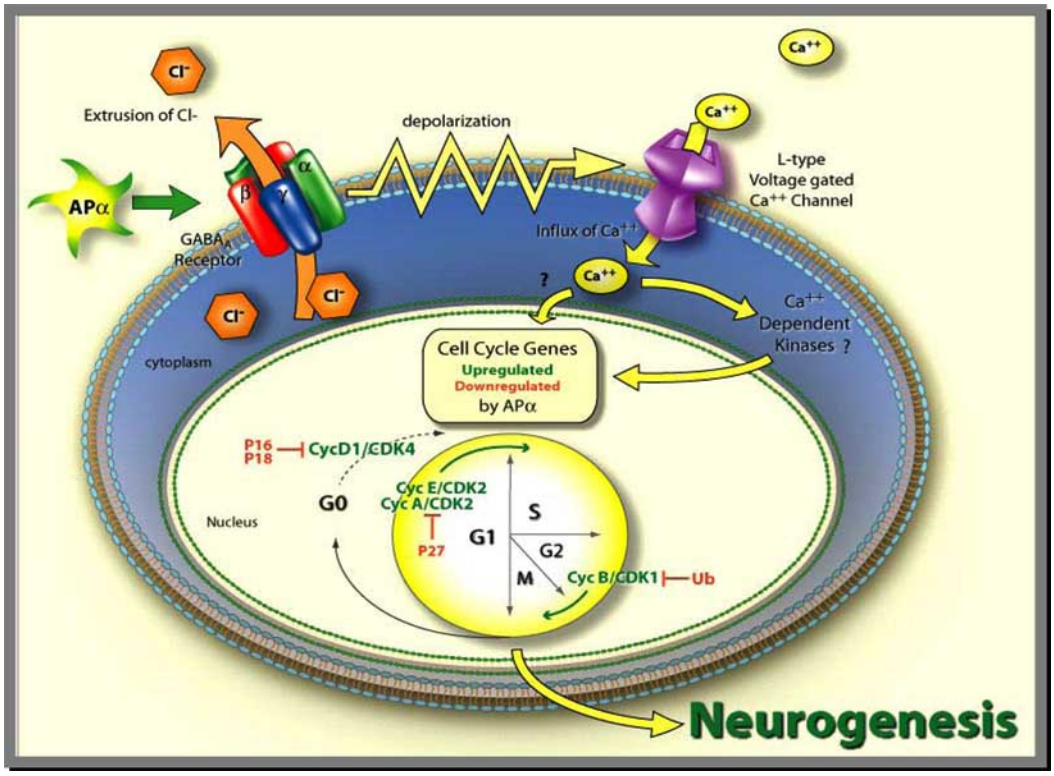

Fig. (3). Working model of Allopregnanolone (APα)- induced neurogenesis. APα activates the $GABA_A$ receptor to trigger the efflux of Cl^- from neural progenitor and neural stem cells. Efflux of Cl^- from the intracellular compartment leads to membrane depolarization and activation of the voltage dependent L-type Ca^{2+} channel. The $[Ca^{2+}]_i$ rise activates Ca^{2+} dependent kinases that ultimately lead to regulation of gene expression and protein synthesis of cell cycle proteins. APα upregulates the expression of cell cycle genes that promote mitosis while simultaneously down regulating genes that repress cell division. The mechanism of APα-induced neurogenesis capitalizes on the developmentally regulated direction of Cl^- flux to induce neurogenesis in those cells that are phenotypically competent to divide while not activating those mechanisms in mature neurons.

THERAPEUTIC POTENTIAL OF APα TO PROMOTE NEUROGENESIS

In the aged and AD brain, both the pool of neural stem cells and their proliferative potential are markedly diminished [54]. In parallel, the level of potential regenerative factors such as APα is diminished in the brains of Alzheimer's patients compared to age-matched controls [18]. While *de novo* synthesis of APα in brain is possible, peripheral delivery of therapeutic agents to promote neurogenesis will have the greatest potential utility. Pharmacokinetic properties are crucial when considering brain targeted therapeutics. Unlike large molecular weight growth factors, such as FGF and neurotrophins, which do not readily pass the blood brain barrier and induce untoward side effects in humans [54], APα with a steroidal chemical structure, 3α-hydroxy-5α-pregnan-20-one, and low molecular weight of 318.49, easily penetrates the blood brain barrier to induce CNS effects including anxiolytic and sedative hypnotic properties [19, 48].

In the course of developing APα as an antiepileptic / antianxiety therapeutic by CoCensys, substantial toxicity and pharmacokinetic analyses were conducted in animals and Phase I safety analyses were conducted in humans [23]. Results of these CoCensys sponsored studies indicated no toxicology issues in healthy human volunteers [23] and therapeutic benefit without adverse events in children with refractory infantile spasms [22]. The principle side effect of APα is drowsiness which occurs in a dose dependent fashion [23]. During pregnancy in humans, levels of APα reach 100 mg/24 h, which while associated with drowsiness, ***is not associated with adverse effects*** for either mother or fetus [55].

Results of our substantial *in vitro* studies coupled with our more recent analyses in the triple transgenic mouse model of AD suggest that APα is a promising strategy for promoting neurogenesis in the aged brain and potentially for restoration of neuronal populations in brains recovering from neurodegenerative disease or injury. Studies are currently underway to determine the neurogenic potential of APα in rodent models of aging and Alzheimer's disease.

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