

Therapeutic Potential of Neurogenesis for Prevention and Recovery from Alzheimer's Disease: Allopregnanolone as a Proof of Concept Neurogenic Agent

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Abstract: 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 holds 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. Basic science analyses and human trials indicate that constituents of microenvironments within the brain determine the neurogenic potential, phenotypic differentiation of neural stem cells and magnitude of the neural stem cell pool. Multiple analyses have documented that dentate neurogenesis is regulated by multiple growth factors which are abundant during development and which dramatically decline with age. While the cause(s) of age-associated decline in neurogenesis remains to be fully determined, loss in growth factors, FGF-2, IGF-1 and VEGF, in the microenvironment of the subgranular zone (SGZ) are prime contributors to the reduced neurogenic potential. The decline in dentate neurogenesis can be observed as early as middle age. In the aged and AD brain, both the pool of neural stem cells and their proliferative potential are markedly diminished. In parallel, the level of potential regenerative factors is diminished in the brains of Alzheimer's patients compared to age-matched controls. Our efforts have been directed towards discovery and development of small, blood brain barrier penetrant molecules to promote endogenous proliferation of neural stem cells within the brain. These endeavors have led to the discovery that the neurosteroid allopregnanolone (AP) is a potent and highly efficacious proliferative agent *in vitro* and *in vivo* of both rodent and human neural stem cells. Results of our *in vitro* studies coupled with our more recent analyses in the triple transgenic mouse model of AD suggest that AP 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. A brief overview of issues impacting the therapeutic potential of neurogenesis and the factors used to promote neurogenesis in the aging and degenerating brain is presented. Also included is a review of our current research into the neurogenic potential of the small molecule, blood brain barrier penetrating, neurosteroid allopregnanolone (AP).

Keywords: Alzheimer's disease, allopregnanolone, neurogenesis, hippocampus, cell cycle, therapeutics, neural stem cells.

PREVENTION OF NEURAL DECLINE AND RESTORATION OF FUNCTION FOLLOWING NEURODEGENERATIVE DISEASE: THE ROLE OF NEURAL STEM CELL GENERATION

Regeneration of lost neurons and neural circuitry to restore cognitive function in victims of Alzheimer's disease (AD) remains a major challenge not yet addressed by current therapeutic interventions. Strategies that halt 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 to restore neural populations lost to the disease and regeneration of neural circuits necessary for cerebral function.

While the potential of regeneration of brain through 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 faces the challenge of distributing cells throughout the brain as AD is characterized by a diffuse pattern of degeneration. Moreover, because AD disease progression is not a uniform process, the microenvironments of degenerating versus degenerated neural circuits present challenges for integration of neural progenitors and their phenotypic differentiation. An alternative strategy to exogenous delivery of neural stem cells is to promote endogenous proliferation of neural stem cells within the brain. Although in low abundance, neural stem cells and progenitors could be induced to proliferate. One of the challenges 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 indwelling catheters in the brain. In contrast, small molecules that pene-

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trate the blood brain barrier and which induce a controlled targeted proliferation of neural stem or progenitor cells are promising therapeutic strategies [1].

The following review provides a brief summary of issues impacting the therapeutic potential of neurogenesis and the factors used to promote neurogenesis in the aging and degenerating brain. Also presented is a review of our current research into the neurogenic potential of the small molecule, blood brain barrier penetrating, neurosteroid allopregnanolone (AP).

NEURAL STEM CELL GENERATION: THERAPEUTIC OPPORTUNITY IN THE AGED AND ALZHEIMER'S BRAIN

In the developing brain, most stem cells and microenvironments are spatially shifting and are temporally transient as the cellular and molecular programs of neurogenesis and morphogenesis are "assembled and disassembled".[2] In contrast to the undulating milieu of stem cell zones in the embryonic nervous system, the adult brain restricts neural stem cells and their proliferation to select microenvironments. These specialized domains, the subventricular zone (SVZ) of the lateral ventricle and the dentate gyrus subgranular zone (SGZ) of the hippocampus, retain developmental potential throughout the life span. [2]

Within the dentate gyrus of the rat, a remarkable degree of neurogenesis occurs on a daily basis, with 9,400 dividing cells proliferating with a cell cycle time of 25 hours.[3] This degree of proliferation would generate 9,000 new cells each day, or more than 250,000 per month.[3] Within 5–12 days of BrdU injection, a substantial pool of immature granule neurons, 50% of all BrdU-labeled cells in the dentate gyrus, express neuron-specific markers. The number of new granule neurons generated each month is 6% of the total size of the granule cell population and 30–60% of the size of the afferent and efferent populations.[3] While the death rate of newly generated neurons is significant, still 3% granular cells are replaced every month which corresponds to ~0.1 % per day in young adult mice and rats.[3, 4]

Importantly, neurogenesis in the dentate gyrus is not a phenomenon limited to the rodent brain. In the human brain, neurogenesis was first detected in victims of squamous cell carcinomas who were injected with BrdU to assess proliferative activity of the tumor cells.[5] Following succumbing to the cancer, the brains of these individuals were analyzed for evidence of mitosis. Results of this analysis demonstrated the generation of new neurons from dividing progenitors in the dentate gyrus of the human hippocampus.[5] These findings in the human brain were consistent with basic science analyses conducted decades earlier by Altman and colleagues who found evidence for neurogenesis in the rodent hippocampus and brain.[6-8] Subsequent to Altman's findings, Nottebohm's laboratory discovered gonadal steroid-induced neurogenesis in the song bird. The new neurons generated in the bird brain are required for the generation of song and undergo a cycle of generation and degeneration through the reproductive / song season.[9-11] Parallel to the analyzes in human brain, Gould and colleagues confirmed the earlier findings of Altman in the rodent brain and further

found that that neurogenesis within the dentate gyrus was required for hippocampal learning.[3, 12]

While the human and rodent dentate gyrus both exhibit neurogenesis, there are differences in survival of the newly generated neurons. In the human brain, newly generated neurons in the adult human dentate gyrus have been detected up to 781 days post BrdU injection[5] whereas the detection maximum is 112 days post-BrdU injection in the adult mouse olfactory body.[13] In the rat, many newly generated neural progenitor cells in the dentate gyrus die between the first and second week[14] and those in the olfactory body die between 2 and 6 weeks after they are born.[15] It appears that the rodent brain has an accelerated generation and demise of neural progenitors whereas the human has a slower generation rate but an increased survival rate.

In the adult endogenous stem cells are activated in response to various injuries, but their capacity to migrate and to undergo either neurogenesis or gliogenesis differ according to the lesion-type and the germinative zone from which they arise.[16] The SGZ and SVZ differ dramatically in the migratory potential of the newly generated cells with the SVZ winning the migratory distance race. Neural progenitors generated by the SGZ are short distance runners and remain in the dentate gyrus whereas progenitors from the SVZ are long-distance runners and can migrate, under conditions of injury, to regions beyond their normal olfactory bulb destination.[16]

Although production of new neurons from proliferating stem/progenitor cells in the subgranular zone (SGZ) of the dentate gyrus (DG) is maintained throughout life in multiple species including humans [17, 18], the magnitude of the neurogenesis declines with age. Age-associated decline in neurogenic potential in the dentate gyrus (DG) has been observed as early as middle age.[19, 20] In the aged and AD brain, both the pool of neural stem cells and their proliferative potential are markedly diminished.[19, 21] The decline in neurogenesis has been proposed to contribute to age-related learning and memory impairments.[22] [23-25] In parallel, the level of potential regenerative factors such as AP is diminished in the brains of Alzheimer's patients compared to age-matched controls.[26]

GROWTH FACTORS AND NEUROGENESIS

Basic science analyses and human trials indicate that constituents of microenvironments within the brain determine the neurogenic potential, phenotypic differentiation of neural stem cells and magnitude of the neural stem cell pool.[2, 27-32] Multiple analyses have documented that dentate neurogenesis is regulated by FGF-2, IGF-1, and VEGF.[11, 32-38] For example, FGF-2 enhanced dentate neurogenesis in both neonatal and adult brain[36, 37, 39, 40] [32, 41-43] and intracerebroventricular (ICV) infusions of FGF-2 upregulated dentate neurogenesis in the aged brain.[19, 27, 32, 35, 44] Likewise, IGF-1 increased dentate neurogenesis in the adult and aged brain following ICV administration of IGF-1.[25, 33, 45] VEGF can promote dentate neurogenesis in both the intact and the injured adult brain following ICV administration [46-49]. VEGF neurogenesis may be mediated by a chemoattractant function specifically targeting FGF2 stimulated neural progenitors.[38]

While mechanism of age-associated decline in neurogenesis remains to be fully determined, loss in growth factors, FGF-2, IGF-1 and VEGF, in the microenvironment of the subgranular zone (SGZ) is a prime contributor to the reduced neurogenic potential of SGZ.[50] Recent studies demonstrated that multiple stem/progenitor cell proliferation factors, FGF, IGF, VEGF, exhibit early decline during the course of aging in the hippocampus. [51] [37] The average concentration of growth factors, FGF-2, IGF-1, and VEGF, each showed a >50-60% decline compared to hippocampal levels of young rat hippocampi.[37] These results suggest that the dramatic decline in dentate neurogenesis observed as early as middle age [19, 20] could be linked to reduced concentrations of FGF-2, IGF-1, and VEGF in the hippocampus, as each of these factors can individually influence the proliferation of stem/progenitor cells in the SGZ of the DG. In the aged and AD brain, both the pool of neural stem cells and their proliferative potential are markedly diminished.[19, 21] In parallel, the level of potential regenerative factors is diminished in the brains of Alzheimer's patients compared to age-matched controls.[26]

GENERATION OF PROGESTERONE DERIVED NEUROSTEROIDS IN BRAIN: ENDOGENOUS NEUROGENIC SYNTHESIS

The case for progesterone metabolism in brain to neuroactive metabolites, first identified by Baulieu, is now well established by many laboratories and documented in multiple species including rodent and human brain.[52-55] Neurosteroids are synthesized in the central and peripheral nervous system, particularly in myelinating glial cells, but also in astrocytes and several neuron types. A region specific expression pattern of P₄ converting enzymes in brain is evident in both hippocampus and cortex.[56] Specifically, both 5 α -reductase and 3 β -hydroxysteroid dehydrogenase are expressed in hippocampus of both the rodent and human brain. [54, 55] Remarkably, the enzymes, 5 α -reductase and 3 β -hydroxysteroid dehydrogenase, required to convert P₄ to its 3 β metabolites, are present and functional in pluripotential progenitors.[57] With the exception of progesterone, very little is known about the metabolism of most progestogens.

ALLOPREGNANOLONE AS A SMALL MOLECULE, BLOOD BRAIN BARRIER PENETRATING NEUROGENIC AGENT

Our own work in this area over the past two decades has demonstrated that the neurosteroid allopregnanolone (AP ; AKA 3 α -hydroxy-5 α -pregnan-20-one, 3 β -hydroxy, 5 α -tetrahydroprogesterone,) is a potent and stereoisomer specific allosteric modulator of the GABA chloride channel complex to increase conductance through the channel which can be protective against seizure activity.[58-60] This finding is particularly important to our current understanding of AP mechanism of neurogenesis [61]. Our investigation into the neurogenic potential of AP began after we discovered that AP induced neurite regression of hippocampal neurons in culture.[60] As we pursued the significance of this rather dramatic effect, we discovered that cultures treated with AP contained a great many cells that appeared as mitotic doublets. This finding led to us to conduct an extensive charac-

terization of AP -induced proliferation of hippocampal progenitor cells *in vitro*. Results of these analyses indicated that AP is a potent, stereoisomer specific promoter of neurogenesis of both rat hippocampal neural progenitor cells and human cortical neural stem cells [61].

AP induced neurogenesis ranged from 20-30 % in the rodent neural progenitor cells to 37-49% in the human neural stem cells. The efficacy of AP as a neurogenic factor is comparable to that induced by bFGF + heparin from our own study and also in agreement with previously published results [62] after 3 days treatment and a 25 % increase of BrdU incorporation in 3 month old rat brain [44]. In immature rat cerebral granular cells AP induced ~ 20% increase in thymidine incorporation [63] and a 20-30% increase of PSA-NCAM positive progenitor proliferation derived from rat brain [64]. Together, these data indicate that AP can promote neurogenesis of neural stem cells derived from multiple sites within the rodent brain and from the cerebral cortex of human brain [1, 61].

Our analyses demonstrating that AP increased BrdU incorporation are consistent with gene array and real time RT-PCR data [61]. AP increased expression of genes that promote transition through the cell cycle and proliferation, such as cyclins and CDKs including CDC2, cyclin B and PCNA. Correspondingly, AP 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 [61]. In our study, AP not only regulated the expression of cell cycle proteins and DNA amplification but also drove a complete mitosis of the rodent neural progenitor cells. This conclusion is supported by data showing that AP increased the MuLV-GFP positive cell number as the GFP signal can only be observed in cells which traverse a complete cell cycle [65-67]. Consistent with this finding AP increases total cell number [61].

AP -induced neurogenesis was a dose dependent process with concentrations within the low to mid 10⁻⁹ to 10⁻⁷ M range promoting proliferation while concentrations in excess of 10⁻⁶ M significantly inhibiting neurogenesis [61]. The biphasic dose response profile of AP -induced neurogenesis could account for the disparity between our *in vitro* data and reports of AP -induced decrease in neurogenesis *in vivo* [68]. In these studies AP inhibited neurogenesis of rat subventricular zone (SVZ) cells following intracerebral ventricular (ICV) injection of 7.8 mM AP .[68, 69] Considering that the ICV injected concentration is diluted into the cerebrospinal fluid, the volume of CSF in a 300 g rat is ~ 580 μ l[70], the final concentration would be more than 50 μ M micromolar. The inhibition of neurogenesis at micromolar concentrations of AP in this study is consistent with our dose response data which demonstrates inhibition of neural progenitor cell proliferation at micromolar concentrations and promotion of neurogenesis at nanomolar concentrations. Alternatively, AP may differentially regulate neurogenesis, promoting proliferation in the SGZ while inhibiting it in the SVZ. An interesting, but not yet fully understood finding came from studies in mouse models of Niemann-Pick dis-

ease. Griffin and Mellon found that the neurodegenerative disease, Niemann-Pick type C, involves disrupted neurosteroidogenesis and that early administration of AP substantially delays progression and severity of the disease in a transgenic NP mouse model.[71] The level of potential regenerative factors such as AP is diminished in the brains of Alzheimer's patients compared to age-matched controls.[26] Consistent with the human data, our own analyses have revealed a lower rate of neurogenesis in the SGZ of the triple transgenic mouse model of AD even prior to expression of AD pathology which was restored to nontransgenic levels following a single exposure to AP (unpublished observations).

NEUROGENESIS, CELL CYCLE CONTROL AND ALZHEIMER'S DISEASE

In parallel to the decline in both the pool of neural stem cells and their proliferative potential [19, 21], the level of potential regenerative factors such as AP is diminished in the brains of Alzheimer's patients compared to age-matched controls.[26] Multiple basic and clinical studies indicate that constituents of microenvironments within the brain determine the neurogenic potential, phenotypic differentiation of neural stem cells and magnitude of the neural stem cell pool.[2, 27-31]

A simplistic replacement of growth factor strategy is complicated by the biology of ectopic mitosis that occurs during the degenerative course of Alzheimer's disease. In Alzheimer's disease, cell cycle gene expression is upregulated [75] and evidence suggests a dysregulation of mitotic signaling [76]. Herrup and colleagues have found that ectopic cell cycle protein expression predicts the site of neuronal cell death in AD brain which led these investigators to propose that dysregulation of various elements of the cell cycle contributes to regionally specific neuronal death in AD [77]. They further found that DNA replication precedes neuronal death in AD brain [78]. Most disturbing for strategies targeting neurogenesis in the AD brain, Herrup and colleagues found that cell cycle events precede neuronal death at *all* stages of AD, from MCI to late stage AD [76]. These findings suggest that promoting entry into the cell cycle could potentially be a double-edged sword with benefit in healthy brains while potentially exacerbating ectopic mitosis in brains destined to develop AD or with existing AD. We are currently addressing this issue by investigating the impact of AP on cell cycle gene expression and neurogenesis at different stages of pathology progression in the triple transgenic AD mouse model.

THERAPEUTIC POTENTIAL OF AP TO PROMOTE NEUROGENESIS

In the aged and AD brain, both the pool of neural stem cells and their proliferative potential are markedly diminished [21]. In parallel, potential regenerative factors, such as AP, are diminished in the brains of Alzheimer's patients compared to age-matched controls [26]. While *de novo* synthesis of AP in brain is possible, therapeutic agents that augment endogenous stores of growth factors have the potential of promoting regenerative neurogenesis. One important caveat for optimal therapeutic results is critical. Multiple

analyses have demonstrated that, with respect to growth factors, more is definitely not better [33, 42, 61]. Growth factor responses are tightly regulated and often can exhibit an inverted-V shaped dose response profile. It is not uncommon to observe that low doses of neurogenic factor induce proliferation whereas high doses do not and may even induce adverse off target side effects [21].

Pharmacokinetic properties are crucial when considering brain targeted therapeutics. Large molecular weight growth factors, such as FGF and neurotrophins, which do not readily pass the blood brain barrier can induce untoward side effects in humans [21]. In contrast, AP with a steroidal chemical structure, 3-hydroxy-5-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 [59, 60].

A critical issue for therapeutic translation is safety. Substantial toxicity and pharmacokinetic analyses were performed in animals and Phase I safety analyses were conducted in humans as part of the translational development of AP as an antiepileptic / antianxiety therapeutic by CoCensys, [72]. Results of these analyses indicated no toxicology issues in healthy human volunteers [72] and therapeutic benefit without adverse events in children with refractory infantile spasms [73]. The major side effect of AP is drowsiness which occurs in a dose dependent fashion [72]. The therapeutic development driven analyses of safety are consistent with the safety profile of AP in all humans as levels of AP reach 100 mg/24 h during pregnancy in humans, which while associated with drowsiness, *is not associated with adverse effects* for either mother or fetus [74].

The outcomes of *in vitro* analyses of AP regulation of both rat and human neural progenitors coupled with our more recent analyses in the triple transgenic mouse model of AD suggest that AP is a promising therapeutic strategy for promoting neurogenesis in the aged brain. Moreover, AP may prove efficacious in restoring neuronal populations in brains recovering from neurodegenerative disease or injury [1]. Studies are currently underway to determine the neurogenic potential of AP in rodent models of aging and Alzheimer's disease.

REMAINING CHALLENGES AND THERAPEUTIC OPPORTUNITIES

The greatest therapeutic potential for neurogenic factors such as AP is likely to be in the healthy aged population where replacement with a relatively safe neurogenic molecule could contribute to sustaining cognitive and neurological function while also preventing or delaying neurodegenerative disease such as Alzheimer's. A major unmet challenge is the development of biomarkers to monitor therapeutic neurogenic efficacy. It remains to be determined whether promoting neurogenesis in persons diagnosed with MCI could delay progression of cognitive decline. Treatment of AD with neurogenic factors does not address the disease process and could even exacerbate the disease as discussed above. However, once therapeutics to halt AD are developed, small molecule, blood brain barrier penetrant neurogenic therapeutics such as AP could act as critical factors to promote repopulation of neural circuits for restoration of function.

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