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Allopregnanolone as regenerative therapeutic for Alzheimer's disease: Translational development and clinical promise

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Abstract

Herein, we review a translational development plan to advance allopregnanolone to the clinic as a regenerative therapeutic for neurodegenerative diseases, in particular Alzheimer's. Allopregnanolone, an endogenous neurosteroid that declines with age and neurodegenerative disease, was exogenously administered and assessed for safety and efficacy to promote neuroregeneration, cognitive function and reduction of Alzheimer's pathology. Allopregnanolone-induced neurogenesis correlated with restoration of learning and memory function in a mouse model of Alzheimer's disease and was comparably efficacious in aged normal mice. Critical to success was a dosing and treatment regimen that was consistent with the temporal requirements of systems biology of regeneration in brain. A treatment regimen that adhered to regenerative requirements of brain was also efficacious in reducing Alzheimer's pathology. With an optimized dosing and treatment regimen, chronic allopregnanolone administration promoted neurogenesis, oligodendrogenesis, reduced neuroinflammation and beta-amyloid burden while increasing markers of white matter generation and cholesterol homeostasis. Allopregnanolone meets three of the four drug-like physicochemical properties described by Lipinski's rule that predict the success rate of drugs in development for clinical trials. Pharmacokinetic and pharmacodynamic outcomes, securing GMP material, development of clinically translatable formulations and acquiring regulatory approval are discussed. Investigation of allopregnanolone as a regenerative therapeutic has provided key insights into mechanistic targets for neurogenesis and disease modification, dosing requirements, optimal treatment regimen, route of administration and the appropriate formulation necessary to advance to proof of concept clinical studies to determine efficacy of allopregnanolone as a regenerative and disease modifying therapeutic for Alzheimer's disease.

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Disclosure statement

Patents pending on allopregnanolone as a therapeutic for mild cognitive impairment and Alzheimer's disease.

Keywords

Neurogenesis; Neurosteroid; Regenerative medicine; Treatment regimen; Pharmacokinetics; Pharmacodynamics

1. Introduction

Neurogenic mechanisms in brain are novel therapeutic targets to sustain neurological function and to prevent, delay or treat neurodegenerative diseases such as Alzheimer's and Parkinson's (Brinton, 2013). More than a decade of research has amassed since adult mammalian neurogenesis was detected in human brain (Eriksson et al., 1998) and has been confirmed and extensively studied in preclinical animal models. The seminal study by Eriksson et al. demonstrated unambiguously that the cell-birth marker BrdU indeed labeled adult human neurogenesis. It was recently demonstrated that a significant amount of neurogenesis occurs in the healthy aging human brain measured by a radiometric carbon dating method to birthdate neuronal and nonneuronal genomic DNA during the human lifespan (Spalding et al., 2013). The two most prominent adult neurogenic niches are the hippocampal dentate gyrus subgranular zone (SGZ) (Altman and Das, 1965; Cameron et al., 1993) and subventricular zone (SVZ) of the lateral ventricles (Altman, 1969; Luskin, 1993) reviewed by Liu and Brinton (2010). Although the evidence is less robust, data exist to support additional neurogenic niches in mammals including the cortical layers (evidence for interneurons generated after neocortical development), hypothalamus (Kokoeva et al., 2007), and cerebellum (Lee et al., 2005; Ponti et al., 2008).

Newly born dentate gyrus granule cells of the hippocampal neurogenic niche localize to the granule cell layer in the dentate gyrus and integrate into the adjacent molecular cell layer. As the new neural cells mature, dendrites sprout into the molecular cell layer to receive glutamatergic afferents from the perforant pathway of the entorhinal cortex and their axonal projections form mossy fiber synapses in the CA3 subfield to strengthen neural circuitry and function of the hippocampus. In less-described cortical neurogenesis, newly born interneurons, most destined to be GABAergic, are dispersed throughout the relatively voluminous cortical space. In the neocortex and dentate gyrus, adult neurogenic rates of replacement are similar despite differences in cell type, function, and relative numeric ratios to surrounding mature cells (Cameron and Dayer, 2008). Regenerative potential of these brain regions is possible throughout the lifespan with a neurogenic decline in aged brain (Cameron and McKay, 1999; Kuhn et al., 1996). In the SGZ, immature granule cells reach their highest level within weeks following birth in rodents; early postnatal rodents are roughly equivalent to birth maturity in humans.

Newborn granule cells are present in advanced aged mammalian brains and animal models demonstrate that the new granule cells possess intrinsic neuronal properties required for integration into the neural network. While the significance of functional neurogenesis in human AD remains unknown, new evidence demonstrates that a significant amount of neurogenesis occurs in the healthy aging human brain. Knoth et al. examined a large number of post-mortem brains that covered the lifespan range of 0–100 years. It was reported

that human neurogenesis declines with age and doublecortin-positive cells decreased about tenfold from childhood to old age (Knoth et al., 2010) and the numbers roughly parallel the rate of neurogenesis in aging rodent models (Fig. 1). Recently, Spalding et al. used a ^{14}C labeling method that relied on a serendipitous spike in atmospheric ^{14}C levels resulting from nuclear bomb fallout (Spalding et al., 2013). The results of these studies revealed that the age of the ^{14}C -labeled DNA within adult hippocampal neurons indicate that the neurons were born during adulthood. Spalding et al. determined that each year approximately 1.75 percent of the neurons turnover within the self-renewing fraction with only a modest decline during aging. A best-fit scenario model predicted that approximately 35 percent of the hippocampal cells were cycling corresponding to slightly less than the proportion that constitute the entire dentate gyrus region. The authors estimated that the hippocampal dentate gyrus of human brain produces around 700 new neurons per day. At this rate, enough neurons could be replaced in the hippocampus to regenerate the entire hippocampal neurogenic region over the lifespan suggesting that the potential amount of neurogenesis is very significant. Interestingly, the rate of neurogenesis in adult humans was similar by comparison to the rate determined in adult rodents but the rate did not decline as steeply as is known in rodents suggesting that humans may rely on neurogenesis more during the aging process. Compared to developmental periods there are relatively few new granule neurons generated in the advanced aged rodent hippocampus yet the cells are functionally capable of survival and retain normal granule cell morphology and spine density (Morgenstern et al., 2008). Adult born neurons may also be distinctly different and possess higher plasticity and functionality than aged mature neurons. Compared to the perinatal period of rapid brain development, adult neurogenic granule cells mature at a slower rate which could be attributed to less than optimal levels of neurosteroids and other neurotrophic factors. Newly born adult rodent model neurons that survive possess appropriate synaptic density and can form afferent glutamatergic connections (Morgenstern et al., 2008). Newborn dentate granule cells have been shown to functionally integrate into the neural circuitry (van Praag et al., 2002) and to influence hippocampal-dependent processes including spatial pattern recognition (Clelland et al., 2009).

Glutamatergic and cholinergic synaptic inputs penetrate the SGZ of the adult dentate gyrus, yet the adult dentate gyrus SGZ retains an embryonic-like state with nearly exclusive GABA_{A} chloride channel depolarizing inputs influencing the local micro-environment of progenitors in the neurogenic niche (Tozuka et al., 2005). Mature neurons typically respond to GABA as an inhibitory neurotransmitter. In neural progenitor cells, the chloride ion gradient is reversed and GABA is excitatory to efflux of chloride. Depolarization of the progenitor cell activates molecular events that regulate cell proliferation. Adult-generated granule cells initially receive GABAergic input from local interneurons, isolated from extrinsic excitatory input (Overstreet Wadiche et al., 2005). GABA_{A} excitation initiates a cascade of events leading to calcium influx in adult neuroprogenitor cells subsequently inducing the accumulation of a neurogenic transcription factor, NeuroD (Tozuka et al., 2005). Upon GABA exposure, Type-2a cells display inward currents in SGZ (Tozuka et al., 2005). Depolarization events promote subsequent transcription factor-activated processes, mitosis, and granule cell maturation in the weeks following cell division (Overstreet-Wadiche et al., 2006).

2. Allopregnanolone (Allo) and neurogenesis

2.1. Allopregnanolone in brain development, aging and Alzheimer's disease (AD)

Humans are naturally exposed to Allo and other neurosteroids throughout life. During the third trimester of human pregnancy endogenous levels of Allo in humans can naturally reach 150–200 nmol/L for both mother and fetus (Fig. 1) (Luisi et al., 2000). The placenta is both a barrier and a major source of Allo and other neurosteroids during gestation. During embryonic development, neuroepithelial cells proliferate in response to neurogenic cues, often from contact with the vascular niche, to form the embryonic brain (Shen et al., 2004). Neuroepithelial cells then develop the ventricular zone in the embryonic brain and give rise to mitotic radial glial cells that can differentiate into either glial cells or neurons (Noctor et al., 2001). Asymmetric radial glial cell expansion of the cortical layers could be the factor that allowed humans to possess remarkably evolved cortical layers required for human cognitive function (Kriegstein et al., 2006). In uteroplacental deficiency or very premature infancy slower brain development can result because of early withdrawal of the critical neurosteroid milieu. The preterm brain is also protected by *de novo* synthesis of neurosteroids in rat and sheep fetal stress models such as growth restriction or induced-hypoxia increased Allo levels for neuroprotective benefits (Hirst et al., 2009; Trotter et al., 1999). Neural progenitors have been isolated *in vitro* from post-mortem 11-week post-natal and adult human brain demonstrating that these progenitors are present in adults (Palmer et al., 2001). The long-term consequences of early neurosteroid deprivation remain to be studied in brain development (Saria et al., 2010) and could be associated with increased risk for cognitive impairment.

At low concentrations, neurosteroids bind to GABA_AR at sites that differ from GABA, benzodiazepines, ethanol, and barbiturate binding sites and can act as positive or negative modulators of GABA_AR function (Gee et al., 1987, 1988). Allopregnanolone (Allo; 3 α -hydroxy-5 α -pregnan-20-one) (also known as AP, AP α , or THP) is a potent positive allosteric activator of the GABA_AR channels whereby at nanomolar concentrations enhances the action of GABA at GABA_AR and at higher concentrations directly activates GABA_AR. Allo binds to two transmembrane sites of the hetero-pentameric GABA_AR (Hosie et al., 2006). GABA and neurosteroid transmembrane binding sites each occur twice per channel complex. GABA_AR binding sites are conserved in all receptor complex subtypes with the general subunit stoichiometry 2 α :2 β :1 γ . The γ subunit in a subset of extrasynaptic GABA_AR channel complexes can be replaced by the neurosteroid-sensitive δ subunit (Meldrum and Rogawski, 2007). The $\alpha\beta\gamma$ and $\alpha\beta\delta$ receptor composites are pharmacologically distinct. GABA plays a key role in generation of the spontaneous network activity of immature dentate granule cells (Owens and Kriegstein, 2002; Sipila et al., 2004). In neuroprogenitor cells, GABAergic depolarization of the uniquely reversed membrane potential underlies the trophic actions of Allo. Adult dentate granule cells, devoid of the $\alpha 1$ subunit, are also subjected to tonic GABAergic signaling *via* δ subunit containing GABA_ARs mediated by surrounding synaptic boutons of local interneurons (Overstreet Wadiche et al., 2005). By electron microscopy, membrane bound GABA_ARs have been observed on BrdU co-labeled adult hippocampal SGZ progenitor cells (Mayo et al., 2005). GABAergic stimulation of neural progenitor cells elicits an efflux of chloride and

a concomitant influx of calcium that contributes to the induction of cell signaling events leading to gene transcription of mitotic genes and down-regulation of anti-mitotic genes (Wang et al., 2005). GABAergic signaling likewise controls proliferation of adult progenitor cells within the SVZ neurogenic niche (Liu et al., 2005).

Allo and other trophic factors were diminished in both blood and brain of AD patients compared to age-matched controls (Fig. 2) (Marx et al., 2006; Naylor et al., 2010; Weill-Engerer et al., 2002). An early indicator of AD is the loss of episodic and semantic memory (Perry et al., 2000). AD diagnostic imaging studies using volumetric MRI revealed a decreased hippocampal volume due to atrophy of gray matter, *i.e.* neurodegeneration in amnesic subtype mild cognitive impairment in the progression to AD (Whitwell et al., 2007). Neuropathological hallmarks of AD include extracellular and intracellular deposition of β -amyloid (A β) protein, neurofibrillary tangles and neurodegeneration. Several studies have reported decreased neurogenesis in AD mouse models (Lazarov and Marr, 2010).

Despite substantial evidence in AD animal models that demonstrate reduced neurogenesis with degree of AD pathology, data in human AD brain have yet to definitively demonstrate an AD-related decline in neural proliferation. In a cohort of senile patients, doublecortin, a marker for new neurons was increased and the investigators claimed that neurogenesis was increased as a compensatory mechanism of neurodegeneration (Jin et al., 2004). These unexpected findings have been disputed and other studies have demonstrated that in presenile human AD brain, most cell proliferation could be attributed to glial and vasculature-associated changes and found no evidence for altered neurogenesis in the dentate gyrus (Boekhoorn et al., 2006). Another explanation suggests that in AD brains a subpopulation of dysfunctional neurons undergo aberrant entry into the cell cycle that could lead to false positives in searches for neurogenic cell proliferation in AD brains. The aberrant cells replicate their genomes but fail to divide leading to apoptotic mechanisms instead of neurogenesis (Busser et al., 1998; Herrup, 2010). Recently, it was demonstrated that in humans, hippocampal neurogenesis occurs throughout adulthood and only modestly tapers with advanced age (Spalding et al., 2013). In future studies, the method of carbon dating used by Frisén and colleagues to retrospectively birthdate neuronal nuclei could also be used to determine the level of neurogenesis in AD victims. Thus the potential for regenerative therapeutics in AD remains an active area of investigation.

2.2. Neurosteroidogenesis

Neuroactive steroids are endogenous molecules that are present in the central nervous system and the entire peripheral system as the gonads and adrenal glands also produce neurosteroids. In the central and peripheral nervous systems, neurosteroid synthesis occurs in myelinating glial cells, astrocytes, and several neuronal cell types. Cholesterol, transported from ApoE containing LDL particles, is supplied to these cell types and presented to the mitochondria (Fig. 2). The 18 kDa outer mitochondrial membrane translocator protein (TSPO) in steroidogenic cells, controls the uptake of cholesterol and thus the synthesis of neuroactive steroids (Rupprecht et al., 2010). Cholesterol binding protein TSPO associates with other proteins that include the 30 kDa steroidogenic acute regulatory protein (StAR), 32 kDa voltage-dependent anion channel protein (VDAC),

and 30 kDa adenine nucleotide transporter protein (ANT) to form a macromolecular signaling complex to transport cholesterol through the mitochondrial inner membrane to the mitochondrial matrix. TSPO regulates cholesterol compartmentalization in the mitochondria and in addition to cholesterol trafficking, could be important for mitochondrial biogenesis and thus mitochondrial function.

Activated by several different neurosteroids, cAMP dependent mechanisms then modulate protein kinase C epsilon (PKC ϵ) to increase TSPO expression (Batarseh and Papadopoulos, 2010). Calcium ion induced nuclear translocation of ERK requires protein kinase A (PKA) activation. ERK-PKA crosstalk was found to be important for calcium-stimulated CREB-dependent transcription (Impey et al., 1998). Calcium entry also stimulates calmodulin dependent protein kinases II/IV to activate CREB genes important for neurogenesis and learning and memory. Activated PKC ϵ translocates to the outer mitochondrial membrane, to interact with the TSPO-associated proteins VDAC and ANT.

Within the mitochondrial matrix, transported cholesterol is converted into the neurosteroid pregnenolone by the cytochrome P450 side-chain cleavage (CYP450 $_{scc}$) enzyme (Liu et al., 2006) (Fig. 2). Pregnenolone diffuses through the mitochondrial membrane whereby its conversion to progesterone is facilitated by the 3 β -hydroxysteroid dehydrogenase (3 β -HSD) enzyme in the cytosol. Progesterone is converted by enzymatic reactions to its metabolites including Allo in the brain and periphery. Allo is synthesized in the brain from progesterone by the sequential enzymatic steps of 5 α -reductase (5 α -R) type-I and 3 α -hydroxysteroid dehydrogenase (3 α -HSD) (Mellon, 2007; Mellon et al., 2001). The rate-limiting step in neurosteroidogenesis is the unidirectional reduction of progesterone to 5 α -dihydroprogesterone (5 α -DHP) by 5 α -R. Subsequently, 3 α -HSD catalyzes conversion of 5 α -DHP into Allo. Both 5 α -R and 3 α -HSD enzymes are responsible for Allo synthesis and have been found to functionally express in pluripotent progenitor cells (Melcangi et al., 1996). This neuroactive steroid synthesis and degradation system is also importantly influenced by so-called endocrine disrupting chemicals that mimic endogenous neurosteroids such as phytochemicals and synthetic pollutants (Melcangi and Panzica, 2009). Amyloid-beta binding alcohol dehydrogenase or ABAD (aka ERAB; 17 β HSD; SCHAD) is an enzyme that associates with mitochondria and has many enzymatic functions including retro-conversion of Allo to 5 α -DHP (Yang et al., 2005). Intracellular oxidation of Allo *via* ABAD appears to maintain the normal function of GABAergic neurons (He et al., 2005). ABAD was abnormally overexpressed in activated astrocytes and enzymatic levels were high in AD brains (He et al., 2005). It is plausible that elevated expression of ABAD leads to the observed decrease in cortical Allo observed in AD. Our previous data show that neurosteroids can lower ABAD load (Chen et al., 2011). Hippocampus and cortex regions display specific expression patterns of progesterone converting enzymes important for their unique demand for neurosteroids (Mellon, 2007; Mellon et al., 2001).

The GABAergic system and its interrelation with the stress response controls the neurogenic niches in brain and presents unique molecular targets for neurosteroids and their rationale-based chemical structure derivatives (Kaminski et al., 2004). Stress has been shown to be an important determinant of neurogenic status. High levels of glucocorticoids, key mediators of the stress response, were correlated with a decline in granule cell proliferation and thus

neurogenic potential (Cameron and McKay, 2001). In addition, chronic stress was shown to cause memory impairment in rodents (de Quervain et al., 1998) and humans (de Quervain et al., 2000) and genetic variations in the glucocorticoid system associate with AD risk (de Quervain et al., 2004). Chronically elevated levels of Allo that occur under severe chronic stress conditions are associated with down-regulation and malfunction of GABA_AR and can cause memory impairment. Risk exposure assessments of chronic and acute exposure to neurosteroids can be estimated based on our current knowledge of the neuroendocrine system in normal and disease states.

3. Regenerative neurosteroid: allopregnanolone (Allo)

3.1. Allo efficacy in promoting neurogenesis

Our studies have demonstrated a correlation between Allo-induced neural progenitor cell survival and improved memory function in the triple transgenic mouse model of AD (3xTgAD) (Brinton, 2013; Chen et al., 2011; Irwin et al., 2011; Singh et al., 2011; Wang et al., 2010). The 3xTgAD mouse was developed by overexpression of Swedish mutant APP, mutant P301L Tau in the homozygous mutant of presenilin 1 (M146V) knock-in mouse and age-dependently develop cortical and hippocampal tangle-like pathology, intraneuronal A β deposition, extraneuronal A β deposition, and neurological deficits (Oddo et al., 2003a, 2003b). Consistent with the human AD brain neurosteroid profile, basal concentration of Allo in blood plasma (0.47 ± 0.88 ng/ml) was significantly lower ($p < 0.05$) than in cortex (10.36 ± 1.43 ng/g) in young adult male non-transgenic (nonTg) mice, indicating a higher brain accumulation, attributed to locally synthesized Allo in specific brain regions as required for synaptic function. 3xTgAD mice exhibited a lower basal level of Allo in the cerebral cortex (3xTgAD, 6.49 ± 2.02 ng/g *versus* nonTg, 10.36 ± 1.43 ng/g), suggesting either impairment of upstream Allo enzymatic production (Fig. 1) or accelerated Allo metabolism in 3xTgAD mice brain (Wang et al., 2010). Within the SGZ and SVZ in male and female 3xTgAD mice a decline in neurogenesis correlated with age related AD-like pathology progression (Brinton and Wang, 2006; Rodriguez et al., 2008, 2009; Wang et al., 2010). Our studies demonstrated that Allo promoted neurogenesis in the hippocampal SGZ to reverse learning and memory deficits. Neural progenitor cell proliferation and subsequent cell survival were determined by analysis of BrdU incorporation into the mitotic cells labeled shortly after exposure to acute Allo. Triple-immunolabeling coronal sections verified the phenotype of *in vivo* newly formed BrdU-positive cells in mouse hippocampus that were stereologically analyzed and derived from 3-month-old 3xTgAD mice treated with a single subcutaneous injection of 10 mg/kg Allo (Wang et al., 2010). Confocal microscopy identified BrdU-positive cells colocalized with DCX labeled young immature neurons alone or together with NeuN a marker for mature neurons. Neural progenitor cell survival was further confirmed by immunolabeling coronal sections derived from Allo (subcutaneous, 10 mg/kg) treated 3xTgAD mouse dentate gyrus 22 days post-Allo treatment and post-behavioral analyses. Analyses of Allo-treated mouse brains revealed BrdU-positive cells located deep within the granular cell layer, indicating the migration of newly formed cells from the SGZ to the granule cell layer (Wang et al., 2010). These *in vivo* data indicated that newly formed cells, generated following Allo treatment, expressed a neuronal phenotype.

3.2. Allo efficacy in promoting learning and memory

Previously we assessed associative learning and memory function using the hippocampal-dependent trace eye-blink conditioning paradigm. In 3xTgAD mice, 3-months of age, the basal level of BrdU-positive cells in the SGZ of 3xTgAD mice was significantly lower relative to non-transgenic (nonTg) mice, despite the lack of evident AD pathology (Wang et al., 2010). Allo significantly increased, in a dose-dependent manner, BrdU-positive cells in the SGZ of 3xTgAD mice and restored SGZ proliferation to normal magnitude of nonTg mice (Wang et al., 2010). Coinciding with deficits in adult neural progenitor proliferation, 3xTgAD mice exhibited deficits in learning and memory. Allo reversed the cognitive deficits to restore learning and memory performance to the level of normal nonTg mice (Wang et al., 2010). In 3-month-old 3xTgAD mice, Allo increased proliferation and promoted the survival of newly born hippocampal dentate granule cells (Wang et al., 2010). Neural progenitor cell numbers significantly correlated with Allo-induced memory performance. Deficits in neurogenesis were detected in this genetic model of AD prior to immunodetectable A β . Comparable to the 3-month mice, Allo increased proliferative activity, promoted the survival of newly born hippocampal dentate granule cells, and restored cognitive function in 6- and 9-month-old 3xTgAD mice while having no significant impact on their age-matched normal nonTg counterparts (Singh et al., 2011). BrdU-positive neural progenitor cell survival was assessed after 3 weeks, following a single exposure to Allo (10 mg/kg). During the 3-week period after neural stimulation by a single dose of Allo, trace conditioning, a hippocampal-dependent associative learning and memory task, was performed. The 3-week period allowed granule cells sufficient time to proliferate, migrate to the border between the hilus and the granule cell layer and incorporate into the existing neural network. Thus we found that Allo significantly increased survival of BrdU-positive cells and recovered hippocampal-dependent cognition in 6-, and 9-month-old 3xTgAD mice, in the presence of intraneuronal A β . It is important to state that Allo was ineffective after development of extraneuronal A β plaques that appear in 12-month-old 3xTgAD mice. Remarkably, cognition was restored to maximum by the first day of trace eyeblink conditioning, only 1 week following a single exposure to Allo in pre-plaque mice. Hippocampal-dependent associative learning accomplished by repeated trials of an auditory tone followed by a mildly aversive shock stimulus, was sustained throughout behavioral training. Allo efficacy promoted learning and memory function in 3xTgAD mice to the extent that 100% increase in magnitude was observed in learning at the start of training compared to the age-matched vehicle-treated group. The remarkable increase in associative learning was comparable to the maximal normal nonTg mouse performance. In nonTg at 12-months of age, we observed an upward trend toward Allo-enhanced learning that did not reach significance. Single dose Allo-treatment to 15-month-old nonTg mice revealed a therapeutic benefit (Singh et al., 2011). A decline in Allo has been measured in blood samples of 40-year-old healthy males and may suggest that a decline in Allo precedes many age-related diseases (Genazzani et al., 1998). The therapeutic benefit found in aged nonTg mice suggests that at least in males, Allo therapy could reverse an age- and gender-related neurosteroid decline and thus maintain or improve cognitive abilities. Our preclinical findings in the AD mouse model provided evidence that Allo-promoted survival of newly generated neural cells and paralleled restoration of cognitive performance in the pre-plaque phase of AD pathology and in late-stage normal aging.

3.3. Allo efficacy in preventing the development of and reducing severity of Alzheimer's pathology

Increasing evidence indicates that altered cholesterol metabolism is linked to AD pathology (Brinton, 2013; Mellon et al., 2008; Schumacher et al., 2004). In addition to the mechanism of action whereby Allo induces neurogenesis through excitation of GABA_AR chloride channels in neural progenitor cells (Brinton, 2013), Allo regulates cholesterol homeostasis *via* mechanism that influences the Liver-X-receptor (LXR) and its associated pregnane-X-receptor (PXR) system (Chen et al., 2011). LXR is a nuclear hormone receptor abundant in the brain and acts as a molecular sensor of cholesterol levels and initiates cholesterol clearance (Jakobsson et al., 2012; Whitney et al., 2002). Loss of either LXR α or LXR β subtype expression exacerbated AD-related pathology in APP/PS1 double transgenic mice (Zelcer et al., 2007). Loss of LXR has been shown to repress cortical neurogenesis particularly during late-embryonic stage development of layer II/III (Fan et al., 2008). LXR activation increases cholesterol efflux through increased ABCA1 and ApoE expression, and prevents overactivation of γ -secretase and overproduction of A β (Jiang et al., 2008; Shenoy et al., 2004; Whitney et al., 2002). LXR activation improved cognitive function in multiple mouse models of amyloidogenesis (Donkin et al., 2010; Leduc et al., 2010a; Schultz et al., 2000; Whitney et al., 2002; Xiong et al., 2008; Yang et al., 2006). LXRs are recruited to ABCA1 gene promoter regions and ApoE expression to decrease A β plaque formation and increase A β clearance (Koldamova et al., 2010) through phagocytosis by microglia (Terwel et al., 2011). Both fibrillar A β (Koenigsnecht-Talboo and Landreth, 2005) and soluble A β (Mandrekar et al., 2009) are cleared by microglia. Further, LXRs are recruited to ABCG1 promoter in a ligand-dependent manner to alter epigenetic histone methylation allowing for a relaxed chromatin structure accessible to further gene expression and cholesterol efflux (Jakobsson et al., 2012). LXRs reduce neuroinflammation by inhibition of several inflammatory genes that were shown to include Ccl2, Ccl5, Cxcl10, Ifit1, Il1b, Lcn2, Ptgs2, Slc7a11, TNfrsf5, and Traf1 (Zelcer et al., 2007). Inflammatory cytokines reach high levels in AD and when suppressed by LXR activation, enhance phagocytic activity of microglia and thus A β clearance. LXR ligands activate pregnane-X-receptor (PXR) (Riddell et al., 2007). Results from our analyses indicated that in parallel with an Allo-induced increase in LXR expression in the pre-pathology condition, Allo also increased PXR expression in the pre-pathology 3xTgAD mouse brain (Chen et al., 2011). PXR activation induces cytochrome P450 3A (CYP3A) enzymes including CYP3A4 and CYP3A13 and this leads to cholesterol hydroxylation and activation of organic anion transporters for cholesterol extrusion (Sun et al., 2003). In addition to increased LXR and PXR expression, Allo treatment initiated in pre-A β pathology 3-month-old 3xTgAD mice treated once per week for six months displayed increased expression of 3-hydroxy-3-methyl-glutaryl-CoA-reductase (HMG-CoA-R) (Chen et al., 2011).

The increase in HMG-CoA-R is at first paradoxical as it is the rate-limiting enzyme in cholesterol synthesis. HMG-CoA reductase is also required for production of oxysterols that activate LXR and PXR-mediated gene transcription for cholesterol and lipid transport proteins (Leduc et al., 2010b). Thus, the Allo-induced increase in brain LXR and PXR leads to increased cholesterol efflux, thereby reducing γ -secretase activation by cholesterol-laden lipid rafts. Allo stimulated cholesterol efflux is a plausible mechanism for the observed

reduction of 27 kDa and 56 kDa intraneuronal A β oligomers after 6 months of once per week treatment (Chen et al., 2011).

In vivo, brain cholesterol homeostasis and intraneuronal A β are tightly coupled to Allo efficacy (Chen et al., 2011). Deposition of A β in the extracellular compartment disconnected this coupled pathway and led to a loss of Allo efficacy in advanced stages of AD-like pathology in the 3xTgAD model. Allo significantly reduced A β generation in hippocampus, cortex, and amygdala, which was paralleled by decreased mitochondrial A β -binding-alcohol-dehydrogenase (ABAD) and reduced microglia activation assessed as reduced expression of Iba-1 (Chen et al., 2011). A reduction in ABAD is important for decreased mitochondrial dysfunction and simultaneously for decreasing the amount of Allo that is enzymatically back-converted to its precursor steroid 5 α DHP. In organotypic slice cultures of rat cerebellum (Schumacher et al., 2004) Allo increased myelin basic protein and delayed demyelination in Niemann-Pick C mice (Mellon et al., 2008). Allo may stimulate oligodendrocyte progenitor cells in addition to neural progenitor cells and as a metabolite of progesterone, could be responsible for observed oligodendrogenesis with progesterone treatment (Schumacher et al., 2012). In oligodendrocytes, the myelin marker CNPase was increased by once per week Allo, indicating myelinating capabilities in this mouse model (Chen et al., 2011). In summary, in the 3xTgAD mouse model Allo promoted neural proliferation while simultaneously reducing AD pathology.

4. The optimal allopregnanolone treatment regimen

4.1. Allo *in vivo* once per week promotes neurogenesis and reduces AD pathology

To further advance efforts to assess the preclinical efficacy of Allo for AD, our group designed long-term treatment studies. The studies were designed to test Allo using the same age of enrollment (3xTgAD male 3-months of age; prior to overt intraneuronal A β), efficacious dose of 10 mg/kg *via* subcutaneous route of administration, matching our previous studies. In addition to neurogenic efficacy, the long-term studies were extended to determine the disease modifying effects afforded by the therapeutic regimen. Once per month, once per week, and every other day treatment regimens were tested (Chen et al., 2011). Based on measured endpoints of A β oligomers by immunostain and immunoblot approaches, the every other day treatment regimen was maximally efficacious but failed to increase neurogenesis. The once-per-month Allo treatment was efficacious for proliferation of neural progenitor cells but not for decreased A β pathology. Once per month treatment regimen (Chen et al., 2011) was similar to the single exposure treatment paradigm that improved learning and memory performance after a single exposure (Singh et al., 2011). Analyses to determine the optimal treatment paradigm indicated that Allo administered once per week for six months was maximally efficacious for both neurogenic and anti-amyloidogenic endpoints. In parallel to the 3-month-old mice administered once per week Allo, we simultaneously began treatment of a 6-month-old male 3xTgAD group. When Allo was administered beginning at 6-months of age, the age at which intraneuronal plaques are apparent in this mouse model, the appearance of A β pathology paralleled cessation of Allo efficacy. Once intraneuronal A β is extracellularly localized, the efficacy of Allo is largely diminished. This suggested to us that intraneuronal A β accumulation is a determining

factor that focuses the window of Allo therapeutic efficacy on the early stages of AD. We hypothesize that an intermittent Allo regimen will help maintain the normal rate of neurogenesis and that early clinical intervention will lead to greatest therapeutic benefit.

4.2. Chronic continuous unrelenting exposure to Allo opposes therapeutic efficacy

Bengtsson et al. investigated the effect of chronically elevated Allo levels, mimicking physiological chronic stress levels, on cognition and brain tissue A β levels in female and male transgenic APPSwePSEN1 E9 mice (see Table 1) (Bengtsson et al., 2012). When the neurosteroid Allo was administered to young adult mice, neurotransmission and intracellular A β release were adversely inhibited, possibly by GABAergic mechanisms, thereby leading to increased A β accumulation and decrements in learning and memory function. The findings point to Allo's biphasic effects on disease states. The results in a rapid AD progression model should not be generalized to conclude that Allo is deleterious to AD progression. More likely, the chronic continuous exposure that mimics stress levels of Allo was a treatment paradigm chosen to demonstrate that dysregulation of the mechanisms controlled by neurosteroids will further exacerbate genetically programmed AD progression and negatively impact behavior as was measured by the forced swim test. Allo was administered by continuous release at the rate of: 4.7 nmol/h = 0.0015 mg/h \times 24 = 0.036 mg/d and another group of mice were given a double Allo level of 9.3 nmol/h = 0.003 mg/h \times 24 = 0.072 mg/d which yields 2.4 mg/kg/day (Table 1). On a single day of the continuous release regimen, Allo at the rate of 4.7 nmol/h amounts to approximately 0.036 mg/d, assuming 30 g mouse yields 1.2 mg/kg/day. This amount of Allo is approximately 8-fold less (0.036 mg vs 0.3 mg) than our single subcutaneous dose studies (Table 1) (Chen et al., 2011; Singh et al., 2011; Wang et al., 2010) wherein the mice were subcutaneously treated with a single dose: 10 mg/kg = 0.3 mg/30 g mouse, single bolus by subcutaneous injection, not daily. However, the continuous release treatment paradigm was much different pharmacokinetically and pharmacodynamically than the "treat and excrete" approach wherein the neurobiological system is stimulated and then allowed to return to baseline over the period of 1 week or longer duration. Allo plasma concentration after 24 h measured: 5.4 nmol/L, 3xTgAD vehicle and 12.6 nmol/L, 3xTgAD Allo 10 mg/kg. In nonTg mice, Allo plasma levels were 1.48 nmol/L, nonTg vehicle and 9.5 nmol/L, nonTg Allo 10 mg/kg. Allo cerebral cortex concentration after 24 h measured: 6 ng/g, 3xTgAD vehicle and 15 ng/g, 3xTgAD Allo 10 mg/kg. NonTg cerebral cortex levels measured 10 ng/g, nonTg vehicle and 22 ng/g, nonTg Allo 10 mg/kg (Wang et al., 2010). Although the levels of Allo were higher in the single pulse dose experiments, the neurobiological system was able to respond in a proactive manner to induce neurogenesis and learning and memory improvements and return to homeostasis. Comparison of intermittent bolus dosing and continuous release treatment regimens further demonstrate the importance of precise neurosteroid treatment paradigms.

Human blood levels of Allo are highest in the third trimester of pregnancy at levels up to 157 nmol/l (50 ng/ml) or higher under certain pregnancy conditions. High Allo during pregnancy is associated with drowsiness although high Allo is not associated with any severe adverse effects for either mother or fetus (Luisi et al., 2000). Post-mortem human brain Allo levels from women revealed levels in the range of 14–21 ng/g (Bixo et al., 1997). In comparison,

Allo levels in human reached 150 nmol/L, 10 min after intravenous administration 0.09 mg/kg compared to baseline levels in the range of 0.4–2.4 nmol/L (van Broekhoven et al., 2007). In rodents, many studies demonstrate that stress elevates Allo in plasma and brain: Allo increased in acute stress swimming in ambient temperature water (Purdy et al., 1991); Allo increased inhalation of CO₂ (Barbaccia et al., 1994, 1997); and Allo increased handling in naïve rats or foot shock in handling habituated rats (Barbaccia et al., 1997). Chronic stress decreases the baseline Allo (Serra et al., 2000) interestingly such that the ability to respond to acute stress (to elevate Allo) is maintained in chronic stressed animals (Serra et al., 2007). Stress activates the HPA axis leading to an increase in cortisol levels. Allo decreases corticotropin-releasing hormone and adrenocorticotropic hormone expression (Patchev and Almeida, 1996; Patchev et al., 1994) whereas administration of exogenous corticotropin-releasing hormone increases Allo levels (Genazzani et al., 1998). These studies strengthen the point that the dosing regimen is not trivial but rather a critical parameter of successful therapeutic intervention design. The treatment intervention point is just as critical as dosing regimen, a hard lesson learned from other once-promising AD therapeutic efforts that failed in clinical trials (Mullard, 2012). Many of the learning and memory reports associated with Allo were reported in animals and humans and it is important to understand that these studies were either acute measures of memory performance for example, participants were asked to recall a list of words within minutes of a mildly sedative Allo dose (Kask et al., 2008) or chronic treatment paradigms that mimic sink-or-swim high stress conditions in rodents (Turkmen et al., 2006). When high Allo doses or chronic continuous treatment regimens are employed, Allo acts like the benzodiazepines to impair learning and memory. Allo administered twice daily at high doses to male rats for several consecutive days decreased performance on the Morris water maze, escape latency, pathlength, and thigmotaxis and pretreatment induced a partial tolerance against acute Allo effects (Turkmen et al., 2006).

Bengtsson et al. extended their investigations of the effect of chronically elevated Allo levels (3-months elevated followed by 1-month withdrawal), on cognition and brain tissue A β levels in female and male transgenic APP_{Swe}PSEN1 E9 (Bengtsson et al., 2012). The follow-up study included both APP_{Swe}PSEN1 E9 (predominately elevated A β 1–42 levels; plaques as early as 6-months) and A β PPSweArc (predominately elevated A β 1–40 levels; slower plaque development beginning at 9-months) mouse models of Alzheimer's disease with a 1-month treatment followed by 1-month washout (Bengtsson et al., 2013) (Table 1). In young AD mice, the neurosteroid Allo adversely inhibits neurotransmission and intracellular A β release leading to increased A β accumulation and decrements in learning and memory function. While expected cognitive deficits were observed in both AD mouse models compared to their non-transgenic littermates, Allo treatment effects were not observed in non-transgenic mice. The effect on the GABAergic system due Allo held chronically at a stress level followed by the washout period leading to changes in various GABA receptor subunit combinations could cause deleterious withdrawal effects as well as A β processing changes that could far outweigh the direct effect of chronic elevation of Allo. The important findings point to Allo's biphasic effects on disease states. Under some but not all conditions in these chronic Allo studies, the treatment paradigm further exacerbated genetically programmed AD progression and negatively impacted behavior measured by

the MWM swim test. From the chronic continuous exposure studies it was difficult to conclude or generalize that prior stress levels of Allo impair memory. The treatment paradigm (1-month treatment followed by one month wash-out) was such that the direct effect of Allo remained unclear. The paradigm primarily looked at long-term effects of a continuous unrelenting Allo episode in an amyloidogenic model. It could be speculated that both amyloidogenesis was exacerbated and neurogenesis and neuronal differentiation and integration to be specific were chronically inhibited that amounted to long lasting deleterious effects. In translation to humans, the chronic treatment paradigm and in young mice with very early stage AD-like pathology would not be an ideal therapeutic approach. Data varied between mouse genders within each model, brain regions, and behavior tests highlighting the importance of each variable as they relate to neurosteroid efficacy (Bengtsson et al., 2012, 2013). The long-term consequences of a previous stressful period where Allo levels were constantly elevated and then suddenly relieved in two mouse models of genetic AD could relate to a subset of stressed or depressed human cases that progress to AD either sporadically or more relevantly to familial AD.

5. Requirements for translation of preclinical allopregnanolone outcomes into clinical Alzheimer's disease therapy

5.1. Allopregnanolone and Lipinski's rule of 5

Lipinski's rule of five is a mnemonic developed by Christopher A. Lipinski and colleagues for predicting success of a drug by oral route of administration (Lipinski et al., 2001). They found that four criteria with values close to multiples of five were predictive of oral availability. While the rules are based on requirements for oral administration of a drug, they are widely applied as an initial indicator for successful drug development. The rule of five predicts that poor absorption or permeation are likely if: (1) there are more than five hydrogen donors (Allo has one); (2) the molecular weight is more than 500 Da (the molecular weight of Allo is 318.49 Da); (3) the Log *P* value is just over five (Allo is 5.042); and (4) there are more than 10 hydrogen acceptors (Allo has two). Allo meets three of the four Lipinski rules whereas it does not meet the requirement for a Log *P* value less than five, although it should be mentioned that some estimates report Allo's Log *P* to be just below 5. The Log *P* value is an indicator of lipophilicity. Molecules with a high octanol to water partition constant are preferentially distributed to hydrophobic compartments such as lipid bilayers of cells, whereas hydrophilic molecules (low octanol to water partition coefficients) are preferentially distributed in hydrophilic compartments such as blood serum. The chemical properties of Allo are not optimal for oral dosage and would require advanced formulation techniques. However, Allo availability by parenteral and other routes with proper formulations are readily delivered from the peripheral system, penetrating through the blood brain barrier to reach the central nervous system (Table 1). A Log *P* value of slightly greater than five indicates that Allo preferentially distributes to lipophilic compartments, a typical feature of steroid molecules.

5.2. Allopregnanolone formulation

Determining the best formulation and route of delivery is a formidable challenge for Allo and other neurosteroids. The chemical structures of most neurosteroids are not well suited for oral delivery because of poor solubility properties in aqueous environments. Neurosteroids precipitate readily from aqueous solution and thus display poor bioavailability followed by first-pass metabolism in the digestive tract and liver. Formulation of delivery vehicle for Allo is critical, as the Allo formulation will determine its bioavailability, pharmacokinetic and pharmacodynamic properties. For example, a sterile Allo suspension dosage form could be administered subcutaneously or intramuscularly whereas only soluble, particle-free sterile-filtered products would be acceptable for intravenous administration. Simple rapid-release formulations may be fine-tuned by changing the complexation agent ratio or adding viscosity agents to improve the desired pharmacokinetic profile. The complexation ratio has limitations based on solubility properties of combination of the excipients and the neurosteroid. Solubility is a major concern for most hydrophobic neurosteroids and efforts have been made to synthesize water-soluble analogs of neurosteroids, including Allo and progesterone with the goal to maintain structure-activity relationships (MacNevin et al., 2009). Excipients with prior regulatory approval are most desirable for initial formulations since they are more likely to gain regulatory acceptance in new formulations (Stella and He, 2008) whereas new excipients are required to undergo their own extensive quality and safety assessments. Use of existing excipients will, however, often limit patent strategy.

Complexing agents are substances that allow for polar-based solvents, ideally water, to complex with otherwise water-insoluble neurosteroids to enhance solubility and bioavailability. The most common complexing agents for Allo are cyclodextrins that ideally form 1:1 ratios with neurosteroids in aqueous solution (Fig. 3) (Stella and He, 2008). Clinical and translational scientists should note that cyclodextrins when delivered *in vivo* could form inclusion complexes with other small molecules including other drugs that may be present in the circulation or endogenous molecules including peripheral endogenous steroids and cholesterol. It is generally thought that cyclodextrins with high molecular weight cyclic saccharide structures do not penetrate the blood brain barrier when administered in reasonable quantities sufficient for drug delivery to CNS.

Other formulations that are potentially viable options for neurosteroids include injectable suspension depots with standard dispersed systems, oral emulsions, or advanced nano-encapsulation technologies. Devices such as controlled release pumps theoretically could be used to cyclically deliver one or more neurosteroids to the brain although this approach remains to be investigated.

Stability of the neurosteroid formulation must be tested and often the formulation must be further developed to avoid typical stability issues such as precipitation, freeze-thaw, maintaining potency, pH fluctuation, or packaging issues. When a final Allo drug product is prepared for the regulatory approval inspection process, it is critical to consider packaging (*e.g.* volume, shape, cap closure system, label, materials, interaction of package with product under various conditions including time and temperature) with scale-up capability. For example, the best solvents for neurosteroids may also interact with the chemical components

of typical choices for packaging such as synthetic plastics (as containers or coatings), rubber compounds, or in some cases even glass to fracture, leach or adsorb thus interfering with the intended drug product. The drug container will undergo a rigorous quality assessment by regulators. Changing any variable of the formulation requires another costly round of all-encompassing quality assessment and could require further safety and efficacy tests.

5.3. Allopregnanolone route of administration, pharmacokinetics and pharmacodynamics

From our studies (Table 1), subcutaneous and intravenous soluble cyclodextrin-based Allo and subcutaneous suspension Allo depots induced rapid neurogenic responses. Multiple formulations of Allo: (1) promoted neurogenesis with no serious adverse effects following both acute and chronic exposure at optimal intervals; (2) reduced burden of AD pathology; (3) exhibited efficacy in both male and female 3xTgAD mice; (4) reversed learning and memory deficits assessed by hippocampal dependent behavioral assays relevant to cognition in humans with AD (Wang et al., 2010; Singh et al., 2011; Chen et al., 2011; Brinton, 2013). Rapid exposure to a sub-sedative dose of Allo is sufficient to induce the Allo mechanism of neurogenic action, achievable *via* rapidly bioavailable intravenous or subcutaneous routes solubilized in a cyclodextrin formulation of 2-hydroxypropyl β -cyclodextrin (H β CD; Trappsol[®]) or sulfobutyl ether β -cyclodextrin (SBE β CD; Captisol¹) a non-nephrotoxic derivative (Stella and He, 2008). Based on neurogenic regenerative efficacy determined in rodents we hypothesize that Allo will likewise induce neurogenesis in humans at risk for or affected by AD (Brinton, 2013).

Initial pharmacokinetic modeling of IV administration of Allo predicted that an intravenous infusion rate over 5 min would induce a sub-sedative peak of Allo in brain while extending the duration (area under the curve) of exposure to more closely simulate conditions generated by subcutaneous bioavailability of cyclodextrin-solubilized Allo exposure. We chose absolute exposure doses in the mouse to correspond, factoring for allometric species conversion, to the expected intravenous dose range per average 70 kg human in order for results to best translate our preclinical results to hypothetically safe conscious-sedative clinical doses (Reagan-Shaw et al., 2008). Mice samples collected 5 min, 15 min, 30 min, 4 h, 24 h post infusion start time were used to determine Allo levels in brain and plasma to provide data for PK modeling and indication of cell cycle activation and differentiation marked by pCREB (neurogenic cell signaling) and NeuroD (developing neural cell) (Table 1). Both intravenous and subcutaneous cyclodextrin-formulated Allo had similar dose-dependent sedation profiles and elimination occurred prior to 4 h. Our preclinical data indicate that the regenerative effect of Allo occurred at sub-sedative doses given in a regimen consistent with the time course for regeneration – *i.e.* maximum exposure of once per week – not daily as is typical for most therapeutics (Brinton, 2013). Allo efficacy *via* rapidly bioavailable routes is the result of rapid calcium signaling to activate CREB regulated genes to ultimately stimulate endogenous neurogenesis and subsequent neural cell survival to enhance memory function. Phospho-CREB upregulation (Irwin et al., 2013) was induced by a rise in Allo concentration in blood and brain following IV dose of Allo 1.5 mg/kg. Pharmacokinetic analyses (Table 1) indicated that Allo doses that were mild-sedative (less than 10 min ataxia) or sub-sedative (no change in motor activity) were sufficient to activate neurogenic mechanisms by multiple routes and formulations including

IV (1.5 mg/kg) and SC (10 mg/kg suspension). PK parameters of Allo in mouse plasma and brain cortex were determined by LC-MS/MS after mild-sedative IV (Allo 1.5 mg/kg) or sub-sedative administration in aged adult male nonTg mice. The C_{\max} reached in plasma was 215 ng/ml (675 nmol/l) with a corresponding T_{\max} of 5 min. Brain exposure to Allo was reached a C_{\max} of 639 ng/g with a corresponding T_{\max} of 5 min in mice IV 1.5 mg/kg. During the initial phase of distribution the plasma half-life $t_{1/2,1}$ was 12.6 min followed by $t_{1/2,z}$ of 5.1 h for the elimination phase. The Allo pharmacokinetics in mouse models were comparable to humans, with distribution phase ranging in humans between $t_{1/2,1}$ of 18.9 ± 5.3 min and 43.9 ± 7.3 min followed by elimination phase half-life $t_{1/2,z}$ of 4.4 ± 1.7 h treated with IV Allo cumulative dosing over 90 min (van Broekhoven et al., 2007; Timby et al., 2006). Thirty minutes after 1.5 mg/kg IV administration in the mouse, the plasma Allo concentration was 51 ng/ml (160 nmol/l) and 192 ng/g in brain cortex, comparable by 30 min post-treatment with our previously tested subcutaneous formulation (Allo 10 mg/kg s.c.) that reached 34 ng/ml (107 nmol/l) in plasma and 159 ng/g in brain cortex within 30 min. Although the peak exposure briefly spiked with IV, the mouse brain and blood exposure levels of IV (1.5 mg/kg) and suspension SC (10 mg/kg) and were nearly equal at 30 min and neurogenic effect was comparable thereby bridging IV and subcutaneous formulation and Allo exposure.

In a previous clinical study, Allo volume of distribution, elimination half-life, and the area under the curve (AUC) adjusted for body weight did not differ between men and women (van Broekhoven et al., 2007). Following dosing of Allo 0.09 mg/kg (three cumulative doses of 0.015, 0.03, 0.045 mg/kg within 1 h) fully bioavailable intravenous administration, maximum blood levels of 48 ng/ml (150 nmol/L) in men and 32 ng/ml (100 nmol/L) in women were achieved (Table 1) (van Broekhoven et al., 2007). Mean levels of endogenous plasma Allo ranged from 0.13 to 0.76 ng/ml (0.4–2.4 nmol/L) at baseline. Clinically, a dose range of 0.5–6 mg is equivalent to 0.007–0.086 mg/kg IV for a 70 kg (154 lb) adult. Within the sub-sedative to mild-sedative dose range, Allo in cyclodextrin-based formulations approximates pharmacokinetically with previously published human experiences with albumin-based formulation summarized in Table 1. Previous intravenous studies found minimal risk of adverse effects other than mild self-reported sedation in a subset of participants. The clinical plan and safety surveillance should be discussed in the US, at pre-investigational new drug application (pre-IND) meetings (21 CFR 312.82), end-of-phase 1 meetings (21 CFR 312.82), end-of-phase 2 and pre-phase 3 meetings (21 CFR 312.47), pre-new drug application license application meetings (21 CFR 312.47) with the United States Food and Drug Administration (FDA), or equivalent regulatory agencies in other countries.

Selection of starting dose is a critical first step in translating drug discovery to early clinical development. Clinical subcutaneous dose range estimates can be determined in part by computer simulations to predict pharmacokinetic and pharmacodynamic profiles using data from animal studies and known cases of exposure to the neurosteroid regarded as previous human experience. Such was the case with clinical intravenous Allo from Bäckström's group (0.03–0.09 Allo mg/kg body weight) (Kask et al., 2008, 2009; Timby et al., 2006; van Broekhoven et al., 2007) (Table 1) to serve as a reference point for future clinical pharmacokinetic studies. *In silico* pharmacokinetic models can be validated and used

to predict the plasma and brain concentrations of Allo resulting from low doses with no predicted pharmacodynamic response up to higher doses with defined maximal tolerated side effects in the proposed study population. Computer simulations are used to predict the total neurosteroid blood and brain compartment exposure and pharmacodynamic responses such as changes in saccadic eye movement as indicator of sedation to more accurately estimate the safe dosage range.

Our preclinical studies with Allo predict that the optimal Allo treatment regimen will be a once per week parenteral, intravenous or subcutaneous administration (Fig. 4; Table 1). Again referring to clinical studies by Bäckström's group at Umeå University, Sweden, Allo is safe with short-lived, mild self-reported drowsiness. Following dosing of Allo 0.09 mg/kg (three cumulative doses of 0.015, 0.03, 0.045 mg/kg within 1 h) fully bioavailable intravenous administration, van Broekhoven et al. measured maximum blood levels of 150 nmol/L (48 ng/ml) in men and 100 nmol/L (32 ng/ml) in women (Table 1) (van Broekhoven et al., 2007). Mean blood levels of Allo were found to be higher in men compared to women at baseline (men, 2.4 nmol/L or 0.76 ng/ml *versus* women, 0.4 nmol/L or 0.13 ng/ml) and after each of three doses of intravenous Allo indicating possible sex differences in absorption and metabolism. However, other pharmacokinetic parameters when adjusted for body weight did not differ between the young healthy men and women in the clinical study (van Broekhoven et al., 2007).

Allo pharmacokinetics and pharmacodynamics have not been studied in AD trials on men and post-menopausal women age approximately 60–80 years old. Possible differences in metabolism and clearance of Allo, unique to AD subjects, will require careful monitoring. However, clinical studies of intravenous Allo in young adult males and females have proven useful to understand the pharmacokinetics and pharmacodynamics including sedation measured by saccadic eye movement and memory tests as well as blood level differences in metabolism (Table 1). Clinically, Allo has only been studied intravenously but has not been studied by alternative routes of administration such as subcutaneously or intramuscularly in rapid soluble release or slow depot form in depot forms that would have very different pharmacodynamics and pharmacokinetic profiles that may be required to achieve the desired pharmacokinetic profile for efficacy (Fig. 4).

5.4. Safety requirements to conduct AD clinical trials

Toxicology studies are required by regulatory agencies prior to acute and chronic clinical trials with the Allo drug product. The required final Good Laboratory Practices (GLP) studies conducted using drug product generated under Good Manufacturing Practices (cGMP) will include acute and chronic studies in rodents and non-rodent species to meet or exceed the duration of the clinical trial (Steinmetz and Spack, 2009). Preclinical safety studies should seek regulatory oversight by the FDA. Useful guidance from the FDA or other regulatory agencies typically follows the International Conference on Harmonization guidelines. Meeting with the appropriate regulatory agency is crucial prior to developing an IND application. Both IND approval in the US, or its equivalent in other countries, and a complete review by the Institutional Review Board or Ethics Committee are necessary to move a preclinical development program to early clinical trial stages. In some cases where

existing human exposure data is available, the neurosteroid, as is the case with Allo, may be considered safe at blood concentrations at or below levels that have been reported to occur endogenously (Luisi et al., 2000) thus guiding the toxicology study recommendations.

Several clinical studies have detected vascular edema with clinical dosing of anti-amyloid therapies (Salloway et al., 2009; Sperling et al., 2011, 2012) and have alarmed regulatory agencies to screen new drug candidates in pre-clinical stages. If the drug product affects beta-amyloid mechanisms for example, nonGLP preclinical studies may be required to include assessment of associated risk for cerebral angiopathies or microhemorrhages in an appropriate animal model. Microhemorrhage risk assessment studies require advanced age animals, 20 months old in AD mice for example, and present real challenges because of the age related attrition rates observed in AD mouse models (Pfeifer et al., 2002; Racke et al., 2005). A positive control must be included in the study to demonstrate accelerated microhemorrhage development (Demattos et al., 2012; Racke et al., 2005). These toxicology studies are important to demonstrate minimal additional risk of the drug product and therapeutic regimen in a late stage AD model and need not be aimed solely at finding an efficacious outcome on reduction of AD pathology.

5.5. Chemistry, manufacturing, and controls (CMC) and patent considerations

To develop Allo or other neurosteroids as a potential therapeutic, there are several critical manufacturing issues to carefully consider. Neurosteroids are often only available commercially in small quantities and most are manufactured for nonclinical research use only. To overcome this technical barrier, one must obtain a source of active ingredient in sufficient quantity and most importantly, manufactured under current Good Manufacturing Practice guidelines (cGMP) to conduct required preclinical toxicology and clinical trial studies. In addition to the active pharmaceutical ingredient (Allo) a cGMP batch of inactive ingredients (excipients) must be obtained. The choice of excipients is not a moot point with regards to neurosteroid formulation (refer back to Figs. 3 and 4). The precise formulation must be decided upon including concentration of stock solution and final drug product. The combination of Allo plus excipient in a drug formulation may or may not be proprietary. It is important to understand the scope of formulation patents and method of use patents at this step. Once decisions regarding dose, formulation, and treatment regimen have been carefully planned, the next step is to manufacture the cGMP drug product. The cGMP Allo product will then undergo a series of analytic tests. In addition, the proper storage conditions must be in place to maintain cGMP status of the product components and the final product. The final drug product is prepared in batches and the container is selected to minimize risk of contamination, decomposition by drug or vehicle over time and must remain sterile. Sterility testing and stability testing of the stock concentration and final drug product are required to conduct further studies. As part of an IND (investigational new drug) application, a well-defined plan for manufacturing, delivery and regulatory monitoring must be approved by national regulatory agencies, FDA for the US and the European Medicines Agency (EMA) for Europe.

5.6. Alzheimer's therapeutic landscape and global unmet medical need

Currently approved AD drugs offer only transient benefits for symptoms of AD. There are no treatments developed to delay or reverse the progression of AD. The FDA and EMA have not approved any new drugs for Alzheimer's disease in the past decade. Of the five FDA-approved AD drugs, four are cholinesterase inhibitors and one is an *N*-methyl-D-aspartate receptor antagonist. It should be noted that none of the approved AD drugs target the brain's regenerative system. In several animal models, the regenerative system does improve cognitive function and is thus a viable candidate mechanism for AD therapeutic development. Other plausible mechanistic AD targets may not target the disease soon enough as recent clinical trials have demonstrated. Phase 3 clinical trials have failed to therapeutically modify AD *via* the inhibition of β -amyloid cascade targets (Mullard, 2012; Karran et al., 2011). Passive immunotherapy Anti-A β antibodies, delivered as human recombinant antibodies have had many challenges to overcome and thus far have resulted in lack of efficacy in human trials (Mullard, 2012). Preclinical studies in AD mouse models have demonstrated efficacy of anti-A β antibodies. However, A β 42 immunization clinical trials for example have not shown appreciable clinical efficacy. Several studies have shown that anti-A β antibodies increase the risk for micro-hemorrhages or cerebral vascular risks in animal models and humans (Pfeifer et al., 2002; Racke et al., 2005; Salloway et al., 2009; Sperling et al., 2011, 2012). Treatment approaches to AD must be reevaluated to target the endogenous regenerative system before rather than after the neurobiological system is unresponsive to therapy. The amyloid hypothesis has been tried at the highest level and failed to demonstrate appreciable efficacy. Lessons can be learned from the costly failed AD trials (Brinton, 2013; Mullard, 2012). From a scientific standpoint, it is ineffective to evaluate therapeutic efficacy in victims that have reached the later stages of AD, when irreversible damage (beta-amyloid plaques, tauopathic tangles, and neurodegeneration) has occurred. To modify disease progression, innovative therapies such as targeting neuroregeneration should be applied early to the appropriately responsive cohorts to intervene with cognitive impairment and AD progression.

Expansion and aging of the global population is projected to increase the number of persons affected by Alzheimer's worldwide. Current estimates of dementia indicate that there were 36 million people living with dementia worldwide in 2010, increasing to 66 million by 2030 and 115 million by 2050 (World Health Organization and Alzheimer's Disease International, 2012). According to estimates from the World Health Organization Dementia report there were 7.7 million new cases of dementia in the year 2010, or one new case every 4 s which is three times as many as HIV/AIDS (2.6 million per year). Based on assumptions that that incidence will increase in parallel with prevalence, as global aging is driving both incidence and prevalence, the incidence is projected to increase to 24.6 million new cases annually by 2050. Based on the projected incidence over this time frame, in less than 40 years there will be 646 million new cases in addition to the current 36 million cases of Alzheimer's disease. Summing both the current and projected number of persons living with predicts that within the next four decades 682 million people will live with dementia. To put these numbers into perspective, within 40 years the number of 682 million persons living with dementia will exceed the current population of the US and Canada (542 million) combined or nearly equal the population of Europe (738 million) (Alzheimer's Disease

International, 2012). In the United States an epidemic of Alzheimer's disease exists where 5 million persons with Alzheimer's currently live with the disease two-thirds of whom are women (Alzheimer's Association, 2013; Brinton, 2008, 2009; Mosconi et al., 2007, 2009). Projections of incidence and prevalence will not change unless there is a cure or a treatment that delays the onset or progression of the disease.

6. Concluding remarks

Preclinical and translational research evidence support therapeutic development of allopregnanolone for neurodegenerative diseases including Alzheimer's. The dose and treatment regimen of Allo must be tailored to a route of administration and pharmacokinetic profile required for neurogenesis and AD pathology reduction. Allo induces both rapid neural progenitor cell cycle gene expression (Wang et al., 2005) and induces key regulators of cholesterol homeostasis (Chen et al., 2011) while reducing toxic amyloid-beta and neuroinflammation. In a pre-clinical mouse model, intermittent treatment regimens begun prior to and during the early stages of AD pathology significantly increased the regenerative response in brain while also reducing burden of pathology in an AD mouse model and remarkably improved cognitive function measured by associative learning and memory tasks (Singh et al., 2011; Wang et al., 2010). In addition to the dosing frequency, intervention with disease progression at early stages with neurosteroid supplementation is critical. The magnitude of AD pathology and remaining neurogenic capacity at initiation of Allo treatment may influence the efficacy endpoints of the clinical trial.

Translational efforts should focus on both the pharmacodynamic effectiveness and minimized toxicological risks in both rodent and non-rodent species to predict the clinical response to the intended disease population. A clinical plan and safety surveillance program should be discussed with regulatory agencies as early as possible in the process of neurosteroid therapeutic development. Regulatory agencies on many levels offer valuable recommendations toward successful therapeutic development programs. Therapeutic development lessons can be learned from previous successful and unsuccessful drug development efforts. Several recent Alzheimer's clinical trials have provided critical insights into the important parameters required to minimize failure (Mullard, 2012). A remaining translational and clinical development challenge is to establish neuroimaging indicators of neurogenesis and oligodendrogenesis comparable to those developed to monitor disease progression or rate of decline (*e.g.* white matter hyperintensity, cerebral blood flow, glucose metabolism, and amyloid-related imaging abnormalities (Salloway et al., 2009; Sperling et al., 2011, 2012). In conclusion, translational research required for development of Allo as a regenerative therapeutic has provided key insights into mechanistic targets for both neurogenesis and disease modification, dosing requirements, optimal treatment regimen, route of administration and the appropriate formulation necessary to advance to proof of concept clinical studies to determine efficacy of Allo as a regenerative and disease modifying therapeutic for Alzheimer's disease.

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Abbreviations:

ANT	adenine nucleotide transporter protein
Allo	allopregnanolone
AD	Alzheimer's disease
ABAD	amyloid-beta binding alcohol dehydrogenase
AUC	area under the curve
Aβ	β -amyloid
CMC	chemistry, manufacturing, and controls
CYP3A	cytochrome P450 3A
CYP450scc	cytochrome P450 side-chain cleavage
EMA	European Medicines Agency
FDA	United States Food and Drug Administration
GLP	Good Laboratory Practices
cGMP	Good Manufacturing Practices
IM	intramuscular
IN	intranasal
IV	intravenous
IND	investigational new drug
HβCD	2-hydroxypropyl β -cyclodextrin
HMG-CoA-R	3-hydroxy-3-methyl-glutaryl-CoA-reductase
3β-HSD	3 β -hydroxysteroid dehydrogenase
5α-R	5 α -reductase
3α-HSD	3 α -hydroxysteroid dehydrogenase
5α-DHP	5 α -dihydroprogesterone
LXR	liver-X-receptor
MED	minimal effective dose

MTD	maximum tolerated dose
nonTg	non-transgenic
PXR	pregnane-X-receptor
PKA	protein kinase A
PKCϵ	protein kinase C epsilon
StAR	steroidogenic acute regulatory protein
SBEβCD	sulfobutyl ether β -cyclodextrin
SGZ	subgranular zone
SVZ	subventricular zone
SC	subcutaneous
TD	transdermal
TSPO	translocator protein
3xTgAD	triple transgenic mouse model of AD
VDAC	voltage-dependent anion channel protein.

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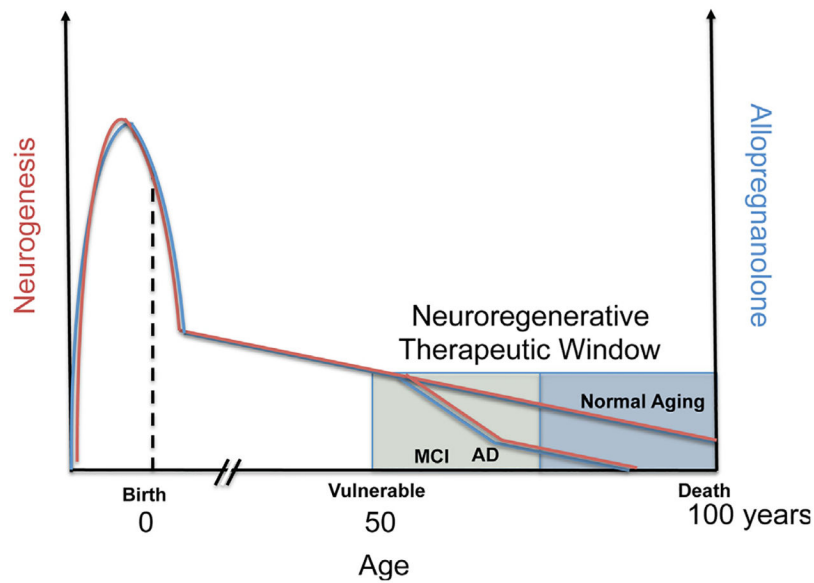


Fig. 1.

Optimal allopregnanolone (Allo) therapeutic regimen applied to therapeutic window to protect from Alzheimer's disease (AD). Endogenous Allo (blue colored line) is produced both locally within the brain and from peripheral sources, including maternal circulation pre-natal, and post-natal and adult from the gonads and adrenal cortex in both male and female. In this generalized diagram, Allo and neurogenesis (red colored line) correlate throughout the life span and in disease. Most notable is the elevated level of Allo during fetal brain development that peaks and then sharply declines before birth (Hirst et al., 2009; Nguyen et al., 2003; Westcott et al., 2008; Luisi et al., 2000). Not depicted are the cyclic fluctuations in progesterone and closely follows progesterone levels in vivo. Also not depicted are the fluctuations in Allo during puberty. In adults, levels of neurosteroids including Allo gradually decline with advanced age and can be associated with chronological age or more specifically by what is often called endocrine age which considers the unique or categorical endocrine system status. In the presymptomatic and mild cognitive impairment (MCI) stages before AD, circulating blood and brain cortex Allo levels sharply decline correlating with onset of AD (Marx et al., 2006; Naylor et al., 2010). In most animal models of AD, the decline in neurogenesis correlates with temporal progression of AD pathology (Lazarov and Marr, 2010). Human neurogenesis declines with age and doublecortin-positive cells decreased approximately ten-fold from 0 to 100 years of age (Knoth et al., 2010) and human data roughly parallel the rate of neurogenesis in aging rodent models. Another method in which post-mortem brain genomic DNA samples containing unique radiometric signatures due to a spike in atmospheric ^{14}C levels from global nuclear bomb fallout were used to determine neuronal and nonneuronal cell birthdates (Spalding et al., 2013). The results of these studies revealed that the age of the ^{14}C -labeled DNA within adult hippocampal neurons were incorporated during adulthood. Each year approximately 1.75 percent of neurons turned over within the self-renewing fraction with only a modest decline during aging. A best-fit scenario model predicted that approximately 35 percent of the hippocampal cells were cycling corresponding to slightly less than the proportion that constitute the entire dentate gyrus region. Spalding and colleagues estimated that the hippocampal dentate

gyrus of human brain produces around 700 new neurons per day. At this rate, enough neurons could be regenerated in the hippocampus to replace the dentate gyrus region over the human lifespan suggesting that the potential amount of neurogenesis is very substantial. Interestingly, the rate of neurogenesis in adult humans was similar by comparison to the rate determined in adult rodents but the rate did not decline as steeply as is known in rodents suggesting that humans may rely on neurogenesis more during the aging process. Chronic stress is associated with depressed responsiveness to constant levels of Allo and in correlation, chronic stress inhibits neurogenesis and exacerbates AD and loss of memory (not depicted) (Bengtsson et al., 2012, 2012). Further, neurodegeneration, a hallmark of AD negatively correlates with levels of Allo (also not depicted). The graphical representation suggests a prominent role of Allo in the neuroendocrine axis, its role both in neurogenesis and AD progression.

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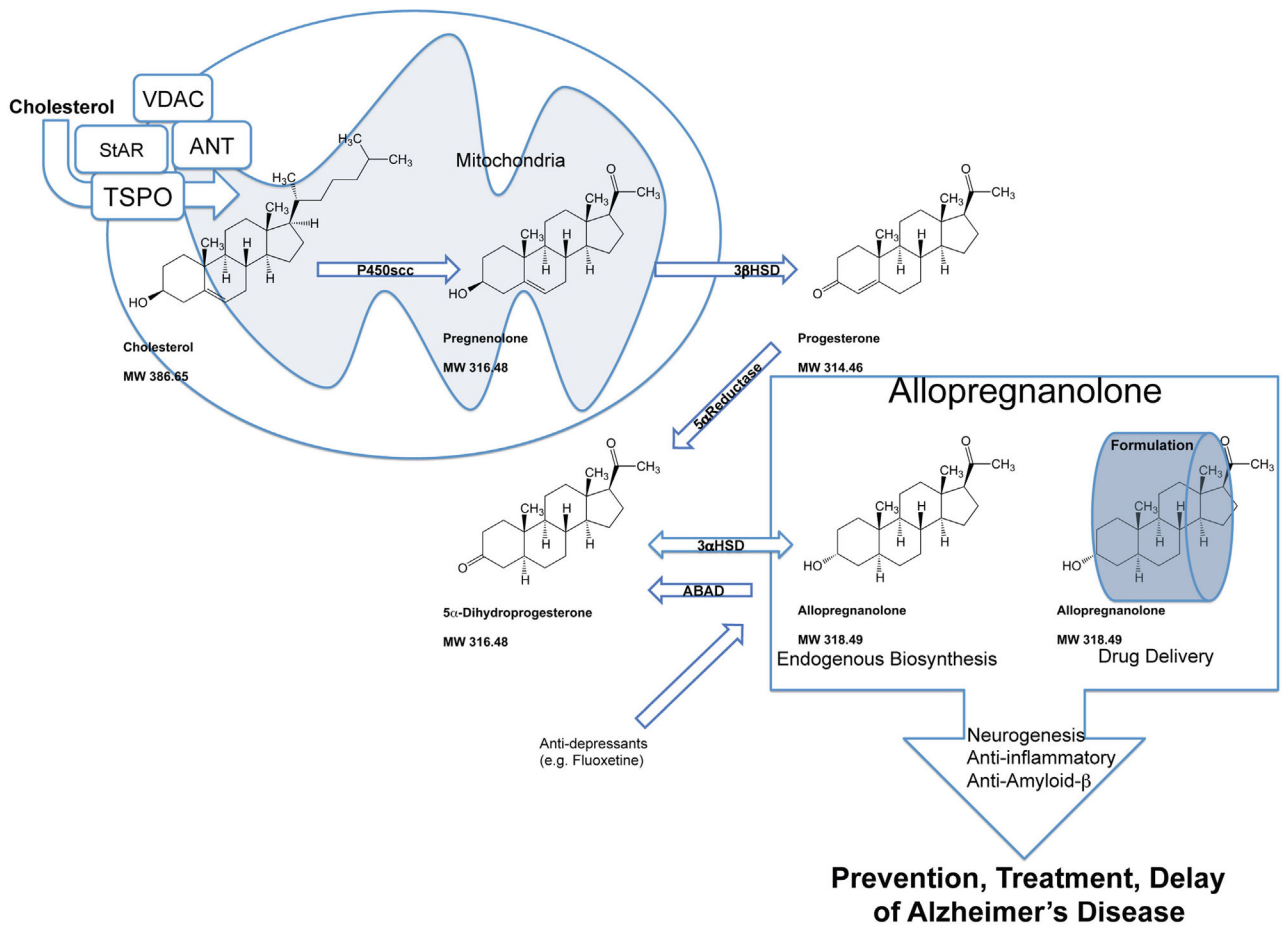


Fig. 2. Neurosteroid biosynthesis is a multi-step enzymatic pathway. Cholesterol homeostasis, recruitment to the mitochondrial compartment, enzymatic reduction and transport to neural and glial cells or their precursor cells require regulation of multiple mechanisms critical to Alzheimer’s disease treatment. Allo mechanisms of action promote neurogenesis, oligodendrogenesis, and on a systems-level inhibit excess inflammation and β -amyloidogenesis. In the central and peripheral nervous systems, neurosteroid synthesis occurs in myelinating glial cells, astrocytes, and several neuronal cell types including neural progenitors. Cholesterol is supplied to these cell types and presented to the mitochondria. The mitochondrial membrane translocator protein (TSPO) controls the uptake of cholesterol and the synthesis of neuroactive steroids (Rupprecht et al., 2010). TSPO-associated proteins form a cholesterol transport pore in the mitochondrial inner membrane that include the steroidogenic acute regulatory protein (StAR), voltage-dependent anion channel protein (VDAC), and adenine nucleotide transporter protein (ANT). The mitochondrial pore transports cholesterol to the mitochondrial matrix to be converted into pregnenolone by the cytochrome P450 side-chain cleavage (CYP450scc) enzyme (Liu et al., 2006). Pregnenolone diffuses to the cytosol and then is converted to progesterone by the 3 β -hydroxysteroid dehydrogenase (3 β -HSD) enzyme. Peripherally derived progesterone and Allo cross the blood-brain barrier to also contribute to the neurosteroid concentration. Allo is synthesized from progesterone in two enzymatic steps by 5 α -reductase (5 α -R) type-I and

3 α -hydroxysteroid dehydrogenase (3 α -HSD) in the brain *de novo* (Mellon, 2007; Mellon et al., 2001). The rate-limiting step in neurosteroidogenesis is the reduction of progesterone to 5 α -dihydroprogesterone (5 α -DHP) by 5 α -R. Subsequently, 3 α -HSD catalyzes conversion of 5 α -DHP into Allo. Amyloid beta-binding alcohol dehydrogenase or (also known as ABAD; SCHAD; 17 β HSD10) is an enzyme that associates with mitochondria and facilitates back conversion of Allo to 5 α -DHP (He et al., 2005; Yang et al., 2005). Additionally, anti-depressants such as fluoxetine are pro-neurogenic and have been shown to increase Allo production in the brain (Malberg et al., 2000; Uzunova et al., 2004, 2006). Endogenous Allo or optimal therapeutic dose of Allo with proper drug delivery strategy promote neurogenesis, oligodendrogenesis, and on a systems-level inhibit excess inflammation and β -amyloidogenesis.

Neurosteroid Solubility and *In Vivo* Dissociation Rate
 Complexation Ratio
 Cyclodextrin : Neurosteroid

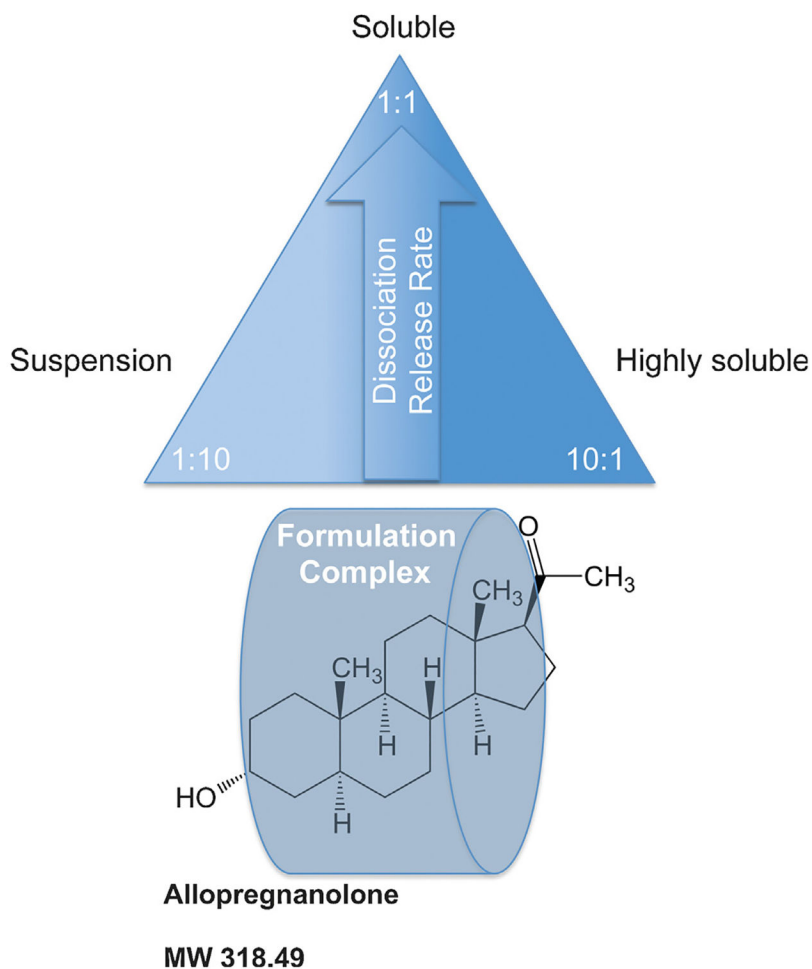


Fig. 3. The neurosteroid release rate pyramid describes the relationship between release rate of active neurosteroid and aqueous cyclodextrin vehicle. Molar complexation ratios greatly alter the release rate or dissolution profile of neurosteroids including allopregnanolone (Allo) as illustrated by the release rate pyramid. Typical cyclodextrin carrier formulations utilize the cyclodextrin such as hydroxypropyl beta-cyclodextrin, at 5–30% in aqueous solution such as water or normal saline with a maximal achievable cyclodextrin concentration between 45–60% weight to volume. Generally, a 1:1 stoichiometry inclusion complex of neurosteroid and a cyclodextrin exists at the apex of the pyramid hypothetically set at a threshold dose that requires maximal delivery rate of the neurosteroid. For Allo, the molar complexation ratio (suitable cyclodextrin:Allo) that is optimal for release rate is closer to 1:2. The same Allo dose would exhibit a slower release rate with a cyclodextrin:Allo of 1:10 complexation ratio since most of the Allo would be suspended in a depot formulation and less accessible for release and absorption into the biological system. Likewise, a

complexation ratio that possesses cyclodextrin:Allo of 10:1 at the same dose of Allo would be readily soluble in an aqueous environment similar to the 1:10 suspension formulation that sits on the opposite side of the solubility spectrum. The 10:1 formulation would also be less accessible for absorption compared to the 1:1 formulation as the Allo is held within the overly abundant empty cyclodextrin molecules governed by the binding and dissociation rate. We hypothesize that the 10:1 formulation displays a re-uptake phenomenon whereby the neurosteroid is relatively less likely to be released into the biological system and therefore slower to release and interact with the receptor targets. A desired pharmacokinetic profile can be titrated by understanding the complexation ratio and release rate to optimize a measurable biological effect such as neurogenesis, anxiolytic activity, or sedation. For Allo, ataxia or sedation can be used as a measurable biomarker of target engagement and can establish the maximally tolerated dose in relation to formulation and delivery route. The lowest possible dose to experience sedation for example at 1:1 formulation would elicit little or no effect with 1:10 or 10:1 formulation complexes.

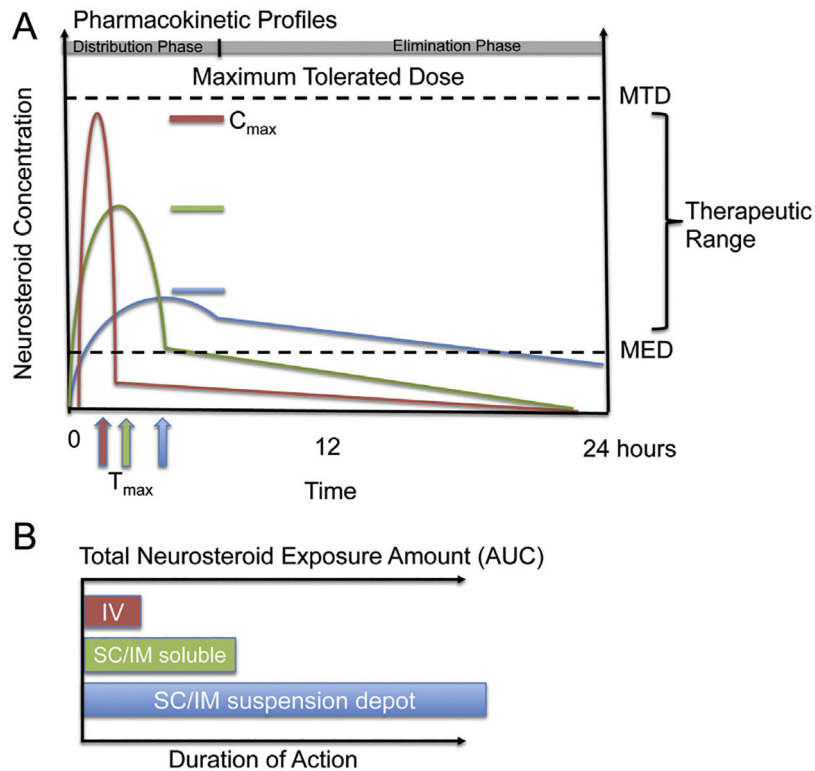


Fig. 4.

Neurosteroid pharmacokinetic profiles with consideration of formulation and route of administration. A. Pharmacokinetic profiles in blood plasma are generalized within the therapeutic range between the maximum tolerated dose (MTD) and the minimal effective dose (MED) to highlight the importance of formulation release rates and maximum exposure levels following a single equal neurosteroid bolus infusion dose. Neurosteroids readily cross the blood–brain barrier after absorption into the blood stream. The profiles: (1) Intravenous (IV) soluble dose (red colored line) – rapid release rate to reach T_{max} ; relatively highest concentration C_{max} , rapid elimination rate; optimal for rapid cell signaling and non-genomic mechanisms within minutes and a 5–30 min distribution phase and rapid clearance. (2) Subcutaneous (SC)/intramuscular (IM) soluble dose (green colored line) – medium release; medium elimination; optimal for rapid cell signaling and non-genomic mechanisms; activates some slow mechanisms in distribution phase of 10–60 min range. (3) SC/IM suspension dose (blue colored line) – slow release; slow elimination; less optimal for rapid cell signaling, optimal for mechanisms that require slow or prolonged activation. (B) Intravenous (IV) soluble dose (red colored bar) is plotted on dual axes as the systemic amount of neurosteroid exposure or area under the curve (AUC) required to activate therapeutically relevant mechanisms and the duration of action. For example, a short burst of the neurosteroid Allo reaches MTD defined by level of conscious-sedation with relatively low exposure AUC *via* a bolus IV infusion of fully soluble neurosteroid. SC/IM soluble dose (green colored bar) depicts the AUC of neurosteroid that could be required for longer duration of action compared to IV. With soluble SC/IM injection, relatively less total AUC is required to activate the biological system compared to a hypothetical SC/IM suspension dose (blue bar) with relatively low solubility and slow release rate and minimal peak level

at a given dose. Depending on the desired biological effect, each route has advantages and disadvantages that should be considered when developing a therapeutic strategy.

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Table 1

Allopregnanone (Allo) pre-clinical research translation in AD mouse models and existing clinical exposure data in healthy subjects. Allo blood plasma and brain concentrations and reported safety and efficacy by multiple routes of administration. Intravenous (IV) dose studies determine the pharmacokinetic properties. Assumed to be 100% bioavailable, the IV route of administration is the most quantitative, and useful for gauging alternative routes of administration. Our pre-clinical Allo dosing studies with subcutaneous (SC), transdermal (TD), intranasal (IN), and IV routes of administration that resulted in beneficial effects that decreased hallmarks of AD pathology and improved cognitive performance were tabulated alongside with other pre-clinical studies that tested long-term chronic continuous exposure to SC Allo that resulted in deleterious effects on AD pathology and long-term memory impairment. Human IV dosing is included to compare the mouse pharmacokinetic parameters to clinical Allo pharmacokinetics demonstrates the safety of Allo when blood levels are below the maximum tolerated dose indicated by sedation level. When bioavailability and species-specific allometric conversions are taken into account the pre-clinical studies that demonstrate neurogenerative efficacy in an AD mouse model are within the safe range of dosage for humans. The AD mouse models were administered both chronic and acute dosing regimens. Allo treatment induced a significant increase in neurogenesis, with the latter regimen yielding the greater increase in neurogenesis. However, the 3/week/3 months (12/month) treatment induced a significant decrease in neurogenesis. Brain sections from 3xTgAD mice treated with Allo or vehicle were immunostained. Aβ immunoreactivity was detected and indicated that the 1/week/6 months (4/month) Allo treatment significantly decreased Aβ immunoreactivity. Efficacy of Aβ reduction in 4-month was comparable to the 12/month Allo treatment whereas the 1/month Allo treatment was ineffective at reducing Aβ immunoreactivity (Chen et al., 2011). Allo tested to mimic chronic stress levels associated with AD. In a series of studies, continuous subcutaneous osmotic pump infusion of Allo proved to be detrimental and decreased learning and memory performance while simultaneously exacerbating AD pathology markers (Bengtsson et al., 2012, 2013). Although not measured, neurogenesis possibly was inhibited in the continuous Allo exposure studies. From these tabulated studies we hypothesized that the dose and frequency of Allo are critical components of efficacy at neurogenic and pathological endpoints.

Purpose	Species/Strain	Route	ALLO mg/kg	Vehicle	Frequency of exposure	Endpoint	Blood [ALLO]	Brain [ALLO]	Results	Safety	Reference
Pre-clinical	Mouse (male) 3xTgAD: APP ^{Swe} × PS1 M146V × Tau P301L	SC	10	PBS/5% EtOH	Single dose	24h	4 ng/ml (12.5 nmol/L) C _{24h}	15 ng/g C _{24h}	Increased neurogenesis	No adverse effects	Wang et al. (2010)
		SC	10	PBS/5% EtOH	Single dose	24 h time course	34ng/ml (107nmol/L) C _{max} ; 0.5h T _{max}	159ng/g C _{0.5 h}	Increased neurogenesis	No adverse effects	Irwin et al. (2013)
		IV	1.5	6% HβCD	Single dose	24 h time course	215 ng/ml (675 nmol/L) C _{max} ; 0.08 h T _{max}	639 ng/g C _{max}	Increased CREB signaling and neurodifferentiation markers	No adverse effects other than acute mild sedation	Irwin et al. (2013)

Purpose	Species/Strain	Route	ALLO mg/kg	Vehicle	Frequency of exposure	Endpoint	Blood [ALLO]	Brain [ALLO]	Results	Safety	Reference
		TD	10-50	Various soluble formulations	Single dose	4h; 24h	44 ng/ml (138nmol/L) C _{4h}	-	Increased neurogenesis	No adverse effects	Irwin et al. (2013)
		IN	3-10				-	-			
		SC	10	PBS/5% EtOH	Single dose	3 wk	-	-	Increased neurogenesis and cell survival; improved hippocampal-dependent learning and memory	No adverse effects	Singh et al. (2011)
		SC	10	PBS/5% EtOH	Three/wk	3 mo	-	-	No effect on neurogenesis; decreased β-amyloid	No adverse effects	Chen et al. (2011)
		SC	10	20% HβCD	Once/wk	6 mo	-	-	Increased neurogenesis and cell survival; acute hyperactivity followed by brief sedation	No serious adverse effects	Chen et al. (2011)
Allo pre-clinical efficacy and regimen development in mouse model of Alzheimer's disease	Mouse APPSwe × PS1 E9 (male and female, n = 6-11)	SC	1.2-2.4 mg/kg/day osmotic pump	60% SBEβCD	Continuous release	3 mo	1.3-2.2 ng/ml (4-7 nmol/L) C _{max} wk 4	2.5-4.8 ng/g C _{max} wk 4	Chronic treatment for three months increased soluble Aβ ₁₋₄₂ ; impaired cognitive learning assessed one month after treatment to observe long-term effects	Accelerated Alzheimer's pathology and memory impairment	Bengtsson et al. (2012)
	Mouse APPSwe × PS1 E9 (male and female)	SC	2.4-4.8 mg/kg/day osmotic pump	60% SBEβCD	Continuous release	1 mo	-	-	Brief but chronic Allo for one month increased soluble Aβ ₁₋₄₀ and Aβ ₁₋₄₂ but was not sufficient to impair cognitive learning	Accelerated Alzheimer's pathology	Bengtsson et al. (2013)
	Mouse APPSweArc (male and female)	SC	2.4mg/k g/day osmotic pump	60% SBEβCD	Continuous release	1 mo	-	-	Brief but chronic treatment for one month did not affect soluble Aβ ₁₋₄₀ and Aβ ₁₋₄₂ but impaired cognitive learning	Memory impairment	Bengtsson et al. (2013)
Clinical	Human(healthy male, n = 9) Human (healthy female on oral	IV	0.09	Albumin solution	Acute 1 h cumulative doses	During 1 h cumulative dosing	48 ng/ml (150nmol/L) C _{max}	-	Male - decreased saccadic eye movement; decrease in contentedness	No adverse effects other than self	van Broekhoven et al. (2007)

Purpose	Species/Strain	Route	ALLO mg/kg	Vehicle	Frequency of exposure	Endpoint	Blood [ALLO]	Brain [ALLO]	Results	Safety	Reference
	contraceptive, <i>n</i> = 9)						32 ng/ml (100nmol/L) <i>C</i> _{max}	-	Female – decreased saccadic eye movement; increase in contentedness	reported sedation	
Clinical trial	Human (healthy female, <i>n</i> = 10)	IV	0.09	Albumin solution	Acute 1 h cumulative doses	During 1 h cumulative dosing	22 ng/ml (70nmol/L) <i>C</i> _{max}	-	Decreased saccadic eye movement	No adverse effects other than fatigue, mild nausea	Timby et al. (2006)
Clinical trial	Human (healthy female, <i>n</i> = 28 crossover)	IV	0.07	Albumin solution	Single dose	10–55 min post-dosing	22–45 ng/ml (70–140nmol/L) <i>C</i> _{max}	-	Acutely impaired free verbal recall episodic memory due to mild sedation with high variability; no acute effect on semantic or working memory	No adverse effects other than self reported sedation	Kask et al. (2008)
Clinical trial	Human (healthy female, <i>n</i> = 12; PMDD, <i>n</i> = 16)	IV	0.05	Albumin solution	Single dose	0–20 min post-dosing	16–22 ng/ml (50–70 nmol/L) <i>C</i> _{max}	-	No effect on startle response or prepulse inhibition to startle	No adverse effects other than self reported sedation	Kask et al. (2009)