Nitric Oxide Mimetic Molecules as Therapeutic Agents in Alzheimer's Disease

Gregory R.J. Thatcher*, Brian M. Bennett[1] and James N. Reynolds[1]

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Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, 833 S. Wood St. Chicago, IL 60612-7231, USA, [1]Dept. of Pharmacology & Toxicology, Queen's University, Kingston, ON, Canada

Abstract: Nitric oxide is multifunctional messenger molecule in the brain, playing important roles including in learning and memory and in regulating the expression of trophic factors that may be reduced with aging. Small molecules that mimic the biological activity of NO, NO mimetics, will bypass cholinergic receptor activation and are anticipated to provide multiple pathways of treating and circumventing dementia in Alzheimer's disease. Activation of soluble guanylyl cyclase and cGMP formation in the brain represents one element of effective neuroprotective pathways mediated by NO. Substantial evidence suggests that NO mimetics may display cGMP-dependent and cGMP-independent activity and may operate *via* multiple biochemical signaling pathways, both to ensure the survival of neurons subjected to stress and also to provide cognition-enabling pathways to circumvent dementia. GT 1061 is an NO mimetic compound currently in clinical trials for Alzheimer's. A survey of current research indicates that NO mimetics will provide a combined neuroprotective and cognition-enabling approach to anti-neurodegenerative therapy.

Keywords: Cognition, dementia, nitric oxide, cGMP, nitrate, Alzheimer's, neurodegeneration, BDNF

INTRODUCTION

Alzheimer's disease (AD) is the most common cause of dementia in older individuals. AD is a neurodegenerative disorder characterized by a progressive and global deterioration in mental function, most notably in cognitive performance. Progressive impairment of memory and visual and spatial cognition are accompanied by changes in affective behaviour, including depression and aggression, leading to disintegration of intellectual skills, personality, and the ability to function in everyday life. Mild cognitive impairment (MCI) is often apparent as a prelude to AD; an estimated 50-80% of individuals with MCI will progress to develop AD [1, 2]. AD is characterized by disruption of both excitatory amino acid and cholinergic neurotransmission most notably in temporal lobe structures and regions of the cerebral cortex. In particular, loss of cholinergic neurons, and subsequent deficits in cholinergic neurotransmission in the hippocampus and cerebral cortex, is strongly correlated with clinical signs of cognitive impairment and dementia in AD patients [3, 4]. Currently the only FDA-approved therapeutic agents for treatment of mild-to-moderate AD are the acetylchol-inesterase inhibitors (ACIs), predominantly donepezil (Aricept). There has been criticism of ACI therapy including reference to modest efficacy and lack of efficacy in segments of the patient population [5]. Based upon the cholinergic hypothesis of neuronal dysfunction, it might be suggested that ACI therapy is inherently flawed, since ACI therapy attempts to maintain the residual function of an apparatus that is progressively degrading. A preferred therapeutic would be a neuroprotective agent that circumvents the damaged apparatus, supplementing cholinergic function down stream of acetylcholine (ACh). Small molecules that mimic the biological activity of nitric oxide, termed NO mimetics, represent such a therapeutic strategy. The NO mimetic, GT 1061, a novel nitrate ester, is currently in clinical trials for AD.

*Address correspondence to this author at the Department of Medicinal Chemistry and Pharmacognosy, College of Pharmacy, University of Illinois at Chicago, 833 S. Wood St., Chicago, IL 60612-7231, USA; Tel: (312) 355-5282; Fax: (312) 996-7107; E-mail: thatcher@uic.edu

NO SIGNALING IN THE CNS

NO signaling is essential for normal physiological function in the CNS, including learning and memory, and is compromised in many disease states including neurodegenerative disorders, where reduced intracellular NO levels may result from upstream blockade, as in cases where cholinergic neurons are damaged and acetylcholine is depleted. The enzyme soluble guanylyl cyclase (sGC) has often been referred to as the "NO receptor", because of its central role in binding NO and relaying the NO signal [6]. The soluble isoforms of sGC are activated by NO, which is the product of the enzyme action of NO synthase (NOS) on L-arginine, leading to the formation and elevation of intracellular levels of the second messenger molecule, cGMP. In many CNS regions, NOS activation and elevation of tissue cGMP levels follows as a consequence of activation of both the N-methyl-D-aspartate (NMDA) subtype of excitatory amino acid receptors and cholinergic muscarinic receptor subtypes [7-9]. The NO/sGC/cGMP signal transduction system is considered to be important for modulating synaptic transmission and plasticity in brain regions such as the hippocampus and cerebral cortex, which are critical for learning and memory [10-13]. In the CNS, NO can serve as a retrograde synaptic messenger, as an intracellular messenger, and as a lateral diffusible

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messenger in NO plays a critical role in signal transduction cascades that are compromised in AD and thereby contribute to the symptoms of cognitive impairment and dementia that characterize AD. There is evidence that NO may positively impact learning, memory and cognition through cGMP-dependent and independent pathways [14]. NO mimetics are thus proposed to bypass cholinergic receptor activation and are anticipated to provide multiple pathways of treating and circumventing dementia.

NO AND NEUROPROTECTION

Models of glutamate-induced excitotoxic neurodegeneration have frequently implicated a major role for the elevation of postsynaptic NO levels as causative in neuronal damage primarily *via* generation of free radicals and the oxidizing cytotoxin, peroxynitrite [15]. One of the initiating events in excitotoxic, neuronal cell death is excessive release of the excitatory amino acid glutamate. Prolonged or overactivation of the N-methyl-D-aspartate (NMDA) subtype of ionotropic glutamate receptors has long been associated with ischemic brain injury [16]. Prolonged NMDA receptor activation allows the excessive influx of calcium into the postsynaptic neuron, which initiates multiple processes that contribute to cellular injury and death, including the activation of proteases, and inhibition of mitochondrial respiration leading to failure of cellular energy stores and apoptosis. The increase in intracellular calcium also results in activation of a number of calcium/calmodulin-dependent enzymes, including constitutive nitric oxide synthase (NOS). Excessive production of NO, *via* excitotoxic activation of NMDA receptors, may lead to generation of cytotoxic peroxynitrite, which would be a contributing factor in ischemic injury and cell death in part due to inhibition of mitochondrial energy production [17, 18]. Support for a neurotoxic role for NO includes observations of neuroprotection in NOS knockout mice and on treatment with NOS inhibitors [19-22], however, several studies also clearly demonstrate the anti-neurodegenerative properties of NO and of NO/cGMP signaling [23-25]. The hypothesis of induction or activation of NOS as a central, universal, causal factor in neuronal damage is not tenable. Modifications of this paradigm propose a threshold NO concentration above which neurotoxicity is observed, or suggest a neuroprotective role for nNOS but a neurodestructive role for iNOS. In the long run, these are likely also to prove too simplistic.

Lipton's seminal work on the interaction of NO with NMDA receptors demonstrated that some NO donors and nitrovasodilators are neuroprotective in models of NMDA receptor-mediated excitotoxic neuronal injury, and provided a role for NO as a neuroprotective agent in inhibiting NMDA receptor-mediated excitotoxicity [17]. Putative mechanisms of regulation of NMDA receptor activity by endogenous NO and exogenous NO donors include modification of the thiol-disulfide redox regulatory site and modification of other receptor cysteine residues, involving conformational changes to the NMDA receptor induced by reversible protein thiol *S*-nitrosation [17, 26]. This direct modification and inhibition of the NMDA receptor provides one cGMP-independent neuroprotection pathway for NO.

The biological actions of NO can be categorized as either cGMP dependent or independent, and amongst the cGMP independent properties are protein nitrosation, protein nitration, and antioxidant action. There is good evidence that NO can act as a potent chain-breaking antioxidant and that certain organic nitrates may manifest antioxidant activity [27, 28]. An NO donor was shown to be neuroprotective against an oxidative stress-induced neuronal cell injury in the substantia nigra [29]. Thus, the neuroprotective effects of NO and NO mimetics include: the action of NO as an antioxidant; NO-mediated inhibition of caspases; NO-mediated modulation of NMDA receptor activity; and cGMP-dependent pathways, such as those that inhibit apoptosis [30-32]. Therefore, sGC activation and cGMP formation in the brain represents one element of an effective NO mimetic neuroprotective strategy.

NO IN LEARNING AND MEMORY

Recent studies show that NO/sGC/cGMP signaling is important in multiple forms of synaptic plasticity, and several reports have provided experimental evidence suggesting that the sGC/cGMP signal transduction system is important for acquisition of new learning and memory. Passive avoidance learning in the rat is associated with an increase in the level of cGMP in the hippocampus, and administration of the membrane permeant cGMP analog, 8-bromo cGMP, enhances memory performance [33]. Conversely, in the same paradigm, inhibition of either sGC activity or cGMP-dependent protein kinase (PKG) immediately post-training blocks memory formation [34]. Selective inhibition of nNOS with 7-nitroindazole impairs object recognition memory in rats, whereas treatment with zaprinast, a selective cGMP phosphodiesterase inhibitor, both facilitates object recognition and reverses the memory deficit induced by 7-nitroindazole [35]. Post-training infusion of 8-bromo-cGMP bilaterally into the hippocampus improves object recognition memory, whereas 8-bromo-cAMP is ineffective [36].

The animal studies that implicate the NO/sGC/cGMP signal transduction system in learning and memory are supported by numerous *in vitro* studies showing that long-term potentiation (LTP) in the hippocampus can be blocked by inhibition of sGC [12, 13, 37-40], and that NO and cGMP can induce long-lasting enhancement of presynaptic neurotransmitter release [37, 41]. Furthermore, the close temporal relationship between activation of the NO/sGC/cGMP signal transduction cascade and improvements in learning and memory suggest a mechanistic link between the two phenomena [40]. Activation of sGC leading to cGMP accumulation will activate PKG that in turn initiates protein phosphorylation cascades leading to activation of transcription regulating factors such as cAMP response element binding protein (CREB), a critical event in both LTP and the establishment of long-term memory [13, 42, 43].

Acetylcholine plays a critical role in modulating synaptic function in the cerebral cortex and hippocampus. The pro-cognitive actions of ACh in these brain regions are mediated *via* activation of muscarinic receptors, which induce primarily excitatory effects involving multiple different ionic conductances [44, 45]. In the hippocampus, cholinergic muscarinic receptor activation leads to increased tissue levels of cGMP (4). Importantly, the inhibition of the slow after-hyperpolarizing current (a calcium-activated potassium conductance that underlies spike-frequency adaptation) induced by muscarinic receptor activation in hippocampal CA1 pyramidal neurons can be blocked by inhibitors of sGC and PKG [45]. Therefore, the neuromodulatory effects of ACh in the brain must, at least in part ,involve NO/sGC/cGMP signaling.

Behavioural studies have demonstrated that NMDA receptors also play an important role in both spatial working memory and long-term memory processes. Blockade of NMDA receptors, or inhibition of NOS activity, impairs performance in the Y-maze test, a model of spatial working memory [46]. The impairment induced by NMDA receptor blockade could be reversed by intracerebroventricular administration of the nitrosothiol *S*-nitroso-*N*-acetylpenicillamine (which acts in part as NO donor), L-arginine (the substrate for NOS), or dibutyrl-cGMP [46]. From these studies it can be proposed that both ACh and glutamate activate receptor systems coupled to NO/sGC/cGMP signal transduction and that this biochemical pathway is important for synaptic plasticity and the formation of memory.

NO-stimulated sGC activity is severely decreased in the cerebral cortex of patients with AD, and aberrant signaling by NO has been reported to occur in the brain in AD [13]. These findings lead to the prediction that sGC activation and cGMP formation in the brain may be an effective strategy for mitigating the cognitive dysfunction that occurs as a consequence of cholinergic deficits in the CNS. Therefore, in contrast to the cholinesterase inhibitors that attempt to salvage the functionality of a degenerating cholinergic system, NO mimetics are postulated to bypass this system, to modulate the normal function of signaling pathways downstream from cholinergic receptor activation.

NO, MAPK SIGNALING AND CREB ACTIVATION

The NO/cGMP signal transduction system is linked to several signaling pathways in the brain that have been associated with neuroprotection. NO possesses neuroprotective properties related to activation of sGC and the production of cGMP, since cGMP has been found to protect neurons against excitotoxic injury [47], and to promote neuronal survival and inhibit apoptotic cell death in a number of neuronal cell types [25]. Furthermore, cyclic nucleotides (cGMP and cAMP) attenuate lipid peroxidation-mediated neuronal injury [48], and cGMP decreases both resting intracellular Ca^{2+} levels and the elevations in intracellular Ca^{2+} concentrations that follow exposure to glutamate [23]. Elevating cellular levels of cGMP depresses excitatory synaptic transmission in the hippocampus, possibly *via* a direct, PKG-independent interaction between cGMP and the α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) subtype of excitatory amino acid receptors [49]. Soluble β-amyloid precursor protein (APP) has neuroprotective properties that have been attributed to selective elevation of intracellular cGMP levels and activation of PKG [50]. Conversely, elevation of cGMP leads to inhibiton of the proinflammatory action of β-amyloid peptide (Aβ) itself, on microglia [51]. Microglial activation leading to release of proinflammatory cytokines and neurotoxic factors is strongly implicated in the pathogenesis of neurodegenerative disorders [52]. In microglial cell culture, inhibitors of cytokine release, including NO donors and cGMP analogs, operate *via* cGMP/PKG signaling and the mitogen-activated protein kinase (MAPK) cascade[53].

A key role for NO and the importance of MAPK signaling cascades is also emphasized by studies on neuroprotection resulting from preconditioning. In cell culture experiments, activation of nNOS activation triggers NO/cGMP/PKG signaling which in turn mediates activation of signaling cascades *via* ERK1/2 (extracellular signal-regulated kinases) and c-Jun, leading to upregulation and activation of proteins including brain derived neurotrophic factor (BDNF), thioredoxin and superoxide dismutase, as well as the Bcl-2 anti-apoptotic factor [54, 55]. In these experiments, the authors discounted a role for protein S-nitrosation, however, in other work, neuronal preconditioning was reported to be mediated by NOS activation, and replicated by NO donors, *via* NMDA-dependent, Ras-dependent, but cGMP-independent pathways [56]. Again in the cGMP-independent pathway, activation of ERK1/2 by phosphorylation was observed to be essential for neuroprotection.

NO/cGMP signaling is closely linked with behavioural responses, learning, and memory [37, 57-61]. Animal behavioural studies have shown that NO is involved in both short and long term learning and memory [43, 62, 63]. LTP, widely held to be centrally important to learning and memory, has early and late phases that require NO/cGMP signaling and CREB phosphorylation. Recent results suggest that NO/cGMP/PKG signaling provides a parallel pathway to PKA-signaling in both phases of LTP, with PKG and PKA pathways performing complementary roles [13]. NO/cGMP and PKG contribute to CREB phosphorylation, in part mediated by the ERK cascade, but NO *via* cGMP-dependent or independent mechanisms may also mediate CREB phosphorylation *via* PKC and the CaMK cascades [13, 43, 64]. Interestingly, experiments with YC-1 an agent that augments sGC activation, produced an enhancement of LTP in rat hippocampus and amygdala *via* an NO/cGMP/PKG/ERK pathway culminating in CREB phosphorylation [42].

The critical involvement of the ERK cascade in mediating hippocampus-dependent long term memory and amygdala/hippocampus-dependent fear conditioning has only been known for a few years, but has led to considerable research demonstrating the importance of this signal cascade in several brain regions [65-69]. ERK1/2 are members of the MAPK super-family. ERK was shown to be activated in the rat hippocampal CA1 region following NMDA receptor stimulation [70], but has now been shown to be activated by a number of stimuli in the hippocampus, cerebral cortex, and amygdala [71-74]. The membrane-associated G-protein, Ras can activate the ERK pathway *via* the kinase Raf-1, which is a MAPKKK (MEK kinase) [75]. Stimulation of several neuroreceptors, including NMDA, serotonin, muscarinic and nicotinic acetylcholine receptors can lead to ERK activation *via* the protein kinases, PKA or PKC. CREB can be activated and phosphorylated *via* CaMK IV, PKA, and RSK2, the last mediating the activation of CREB by ERK [76].

CREB activation, which can be elicited by NO [13, 43, 77], is a focus of investigations into the cellular mechanisms underlying cognition and depression [66, 67, 78-83], but understanding of the detailed upstream pathways is incomplete, owing to the number and complexity of signaling cascades and substantial cross-talk (Fig. **1**). CaMK IV may drive fast-onset CREB-activation [84], whereas the PKA and ERK pathways may activate CREB in a slower manner. The combination of multiple signaling pathways may be advantageous for the precise control of gene expression and the integration of multiple converging signals for optimal activation of CREB [84, 85].

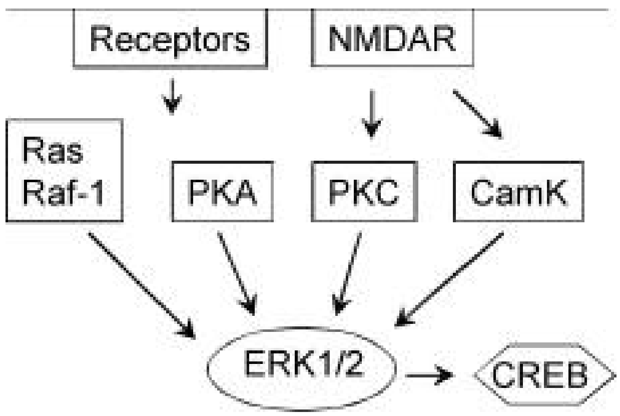

Fig. (1). The ERK/MAPK cascade in neurons may be triggered by a number of pathways and initiated by a number of receptors including the NMDA receptor (NMDAR) in the hippocampus. Various mechanisms are available to NO in eliciting ERK activation including cGMP-dependent pathways *via* NO/sGC/PKG signaling and cGMP-independent pathways. Mechanisms of NO action *via* PKC, CamK and Ras have been proposed, although feedback between cGMP and cAMP pathways requires that NO/cGMP will also influence PKA signaling.

One factor that differentiates NO from other messenger molecules is transmembrane diffusion, allowing retrograde signal transduction stimulating cGMP in the presynaptic terminus, and paragrade signaling as a lateral diffusible messenger sensitizing adjacent postsynaptic neurons. The exact role of cGMP signaling in learning, memory, and affective behaviour is not completely defined. However, there are a number of important ideas that are emerging: the importance of linking presynaptic and postsynaptic activity in a pathway specific manner; the observation that cGMP signaling in LTP may be brief and phasic; and the importance of cGMP signaling in the early consolidation phase of learning and memory. Clearly, NO mimetics that manifest cGMP-dependent and independent activities may operate *via* multiple biochemical pathways, to ensure the survival of neurons subjected to ischemic injury, Ca^{2+} overload, or oxidative stress, and also to provide cognition-enabling pathways to circumvent dementia. Emerging research would support activation of ERK and CREB as important in both neuroprotective and cognition-enhancing pathways.

THE EXTENDED CHOLINERGIC HYPOTHESIS

Despite criticisms of ACI therapy and the cholinergic hypothesis, it is notable that the very recent AD2000 clinical trial designed in part to correct putative bias in previous trials (although not achieving all of the intended endpoints), clearly replicated the reported significant efficacy of donepezil treatment in mild-to-moderate AD [86, 87]. Moreover, there are numerous reports that ACIs may be disease modifying in addition to providing symptomatic relief [88, 89]. The simple cholinergic hypothesis posited that cognition deficits resulted from loss of ACh-containing neurons early in AD, reflected by reduced ACh-transferase (ChAT) activity and choline uptake [90-93]. Thus, in animal models, both use of the muscarinic ACh receptor antagonist, scopalamine, and lesioning of cholinergic neurons resulted in cognition deficits as demonstrated by impaired ability to perform learning and spatial memory tasks, such as the Morris water maze task (MWT). Moreover these functional deficits are alleviated by administration of an ACI [94-104]. The observations that degradation of cholinergic neurons and ChAT activity is often not a feature of early AD seems contradictory to the hypothesis [105-107], but recent findings suggest that the cholinergic hypotheses should be extended not discarded, for example, the cortical and hippocampal cholinergic synaptic systems [108, 109], and trophic factors [110] can either reduce or accelerate pathogenesis and progression of AD by effects on APP levels, metabolism and processing [111, 112], and therefore cerebrocortical plaques and degeneration of afferents must be linked in a multifactorial progression.

ACI therapeutics represent symptomatic early treatments for AD if their function is simply to augment diminishing levels of ACh produced by degenerating neurons, but there are lessons to be learned: firstly, ACIs utilize mechanisms in addition to simple AChE inhibition, mediated through targets such as butyrylcholinesterase[89]; secondly there is evidence that ACIs and ACh-mimetics positively impact non-cognitive behavioural dysfunction in AD, which is a major symptom of AD linked to cognitive decline [113, 114]; and thirdly, the elevation of ACh in early AD may be beneficial through the interplay of the cholinergic system with noradrenergic and serotonergic systems, APP and trophic factors in AD pathogenesis.

RELEVANCE TO THE AMYLOID CASCADE

It has long been proposed that the neurodegeneration in AD may be caused by the deposition of Aβ in plaques found in brain tissue [115, 116], although an objection to this hypothesis rests in the fact that the number and localization of amyloid deposits in the brain do not correlate well with the degree of cognitive impairment [117], an observation recapitulated in transgenic mouse models of AD. Recent cognition studies on hAPP transgenic mice have shown that cognition deficits precede amyloid deposition and correlate with small soluble forms of Aβ [118-120]. supporting a role in memory failure in AD for small, soluble oligomers of Aβ [121]. Inhibition of amyloid deposition in AD remains a major drug target, including approaches directed at reducing aggregation and increasing clearance of $A\beta_{1\text{-}42}$ [122]. Blocking Aβ production has targeted BACE [123], γ-secretase inhibitors [124], modulating APP synthesis, [125] and the upregulation of α-secretase [126]. However, the role of APP, $A\beta_{1\text{-}40}$, and the secretases in normal physiological function [127-130] presents a problem in providing effective and safe approaches to AD therapy. As we have described above, APP has neuroprotective properties that may be mediated by cGMP/PKG signaling [50].

It has been commented that the pattern and progression of memory impairment and cognitive decline seen in AD, in particular the early loss of short term and recent memory, is not typical of that following neuronal cell death [131, 132]; instead, loss of synapses leading to degradation of synaptic plasticity [133, 134], counterbalanced by synaptic scaling, is proposed as a causal mechanism [135], prevalent in MCI and early stage AD. Memory formation through synaptic scaling is strengthened by increased cholinergic activity, and this may occur in early progression of AD, since ChAT levels increase whereas AChE levels are generally normal in MCI and early AD [107, 136, 137]. However, Aβ may elevate AChE levels in early stage AD through binding to α7-nicotinic ACh receptors (nAChRs) [138, 139], and there is evidence for upregulation of the nAChR in AD from animal models and clinical data [140, 141]. The proposal of elevation of ChAT, AChE, and nAChRs in early AD, associated with Aβ toxicity, is compatible with the observed efficacy of ACIs in early AD, where AChE inhibition supports synaptic scaling [135]. However, after early stage AD, the degeneration and loss of cholinergic neurons greatly reduces the potential efficacy of the ACI therapeutic approach.

The nAChR may act as a receptor for Aβ (1-42), eliciting responses including deranged ERK signaling, and consequent downregulation of ERK2 and disruption of downstream events including CREB phosphorylation [142]. The interplay of Aβ with the nAChR couples the amyloid cascade to the cholinergic hypothesis in the hippocampus in pathways that impact synaptic plasticity and memory and operate *via* the MAPK cascade and CREB phosphorylation. Interestingly, compromised CREB signaling has been reported in AD and in cell culture in response to Aβ [143, 144].

AMYLOID CASCADE, CHOLINERGIC HYPOTHESIS AND TROPHIC FACTORS

A transgenic mouse deficient in nerve growth factor (NGF) has been shown to exhibit more typical AD neuropathologies than the "amyloid cascade models" (*viz* the mutant hAPP and presenelin transgenics), including: amyloid plaques, hyperphosphorylated tau, neurofibrillary tangles in cortical and hippocampal regions, and marked cholinergic neuron degeneration [110, 145]. The APP and presenelin animal models of AD have often been criticized because of the uncertain causal connection between observed abnormal protein deposits on the one hand and cognition deficits and degeneration of the cholinergic neurons on the other [146]. Depletion of NGF in rats leads to deterioration of cholinergic CNS basal forebrain neurons and synapses, decreased ChAT levels, and elevated APP [101, 108]. Conversely, mice which exhibit deficits in both basal forebrain cholinergic neurons and hippocampal terminal cholinergic axonal fields have impaired retrograde transport of NGF from hippocampus to the basal forebrain. Individuals with AD show signs of impaired NGF transport with NGF levels in the basal forebrain decreased compared with age-matched controls [147]. Interrelationships between APP and NGF have been described [148-150]. Links between the cholinergic system and APP are seen in the high affinity binding of $A\beta_{1-42}$ to α7-nicotinic ACh receptors, leading to decreased Ca^{2+} influx [151].

Cholinergic neurons and synapses associated with cholinergic nerve terminals rely on the trophic action of NGF for their function [152-155]. A pathological cascade in AD memory impairment is suggested linking aberrant APP/Aβ processing, cholinergic neuronal dysfunction, and trophic factor loss in target regions, leading to degeneration of cholinergic nerve terminal function in the hippocampus and cerebral cortex, and thence both decreased NGF release/uptake and degradation of cholinergic neurons [156]. LTP is seen as central to synaptic plasticity and learning in the hippocampal formation [151]. Significant release of both NGF and brain-derived neurotrophic factor (BDNF) after LTP induction in the hippocampus has been reported [157-159]. Enhanced LTP and afferent synaptic strength *via* cholinergic and other transmitter systems in the hippocampus has been shown to enhance memory function, whereas impairment of these transmitter systems reduces LTP and memory function in hippocampal-dependent tasks [160]. Taken together, these observations support the concept that perturbation of the homeostasis between hippocampal, neurotrophic factors, APP and ACh-mediated activity will progressively lead to memory impairment *via* imbalances in neurotransmission, synaptic damage, neuronal dysfunction, and consequently neuronal cell loss [156]. This concept holds the corollary that inhibition of amyloid deposition alone will not be a successful therapeutic strategy without maintenance of cholinergic and synaptic function.

BDNF, NO AND OXIDATIVE STRESS

Neurotransmitters and neurotrophic factors are fundamental to regulation of synaptic plasticity and neuronal adaptation, for example in response to aging. In particular, BDNF activates genes that regulate neurogenesis, neuronal plasticity and survival, preventing cell death caused by stress, ischemia and trauma. The action of BDNF at synapses is to enhance LTP *via* the MAPK cascade and activation of the transcription factor CREB, which regulates genes controlling LTP and memory formation [161-163]. BDNF itself is a gene product of CREB, as are other proteins important to cell survival and synaptic function, including nNOS, anti-apoptotic Bcl-2, and glutamate receptor subunits [152, 164-167]. Aged rats show decreased levels of both BDNF and CREB in cortical and hippocampal formations, leading to age-related susceptibility to neurodegenerative factors [168-170].

Excitatory amino acid receptor activation increases CREB activity and upregulation of BDNF [171]. The interaction of 5HT receptors with BDNF has been proposed to have an important role in age-related changes in neuronal plasticity and neurodegeneration. 5-HT is important in regulation of neurite growth, synaptogenesis and cell survival, with specific receptors ($5HT_{2A}$, $5HT_{2C}$) mediating memory formation [172]. 5HT and BDNF activate genes that serve complementary functions in neuronal plasticity and survival, with 5HT signal transduction utilizing PKA and PKC signaling cascades. Stimulation of 5HT receptors and activation of CREB induces transcription of the BDNF gene, providing a mechanism of interaction with the cholinergic system in facilitating learning and memory [173]. Reduced levels of 5HT and degradation of serotoninergic neurons accompany cognitive decline in AD and major depression is a symptom of AD [174]. It is possible that 5HT and BDNF work cooperatively to suppress age-related oxidative stress, and thus that reduced levels of either will lead to increased susceptibility to oxidative stress and neuronal degeneration [175, 176].

In cell culture, Aβ induces oxidative stress, triggering upregulation of BDNF as a stress response [177, 178], and the neuroprotective actions of BDNF are argued to be mediated by NO [179]. BDNF and NO share neurological actions, including promoting neuronal survival and enhancing synaptic plasticity [180-184]. Furthermore, the interplay of BDNF with NO is central to the activity of BDNF since NO provides a positive feedback loop that regulates neurogenesis: NO regulates BDNF production; and, BDNF induces nNOS expression [166]. BDNF has been shown to induce the upregulation of nNOS in newly generated neuronal cells and mature cerebrocortical neurons [166, 185]. Elevation of BDNF and NO plays an important role in attenuating neurodegeneration resulting from stress including oxidative stress, but it has also been proposed recently, in light of studies linking neurogenesis to learning and memory [186], that upregulation of BDNF and NO signaling plays an important role in enhancing learning and memory in response to environment [166]. Therefore, elevation of NO levels in the aging brain is likely to be essential to both synaptic and neuronal survival and function, including learning and memory, that may be compromised in response to oxidative stress.

NITRATES AS NO MIMETIC THERAPEUTICS IN AD

Classical nitrates have been in clinical use since the introduction of nitroglycerin (GTN) for treatment of angina pectoris in the 1870's, and continue to make headlines as novel therapeutic agents, most recently in the halting by the FDA of a successful 2004 Phase III clinical trial for prophylactic treatment of heart failure in African-Americans with isosorbide dinitrate. Although the most studied, GTN is somewhat atypical in being much more potent hypotensive agent than most other classical nitrate vasodilators [187-189]. GTN shows serendipitous venodilator selectivity and displays NO mimetic cardiovascular activity, with a clinical safety record proven over 130 years. However, GTN is contraindicated for CNS indications, such as cerebral ischemia, because of peripheral hypotension and tolerance [190, 191]. GTN and other nitrates are often described as NO donors, requiring bioactivation to NO, but no protein capable of bioactivation of GTN to NO is known [192], and at pharmacological concentrations, NO release in target tissues can be at a level too low to measure [193].

The biological and medicinal chemistry of nitrates has recently been thoroughly reviewed [189]. The biological activity of nitrates is NO mimetic: nitrates may exploit bioactivation pathways for selectivity, but importantly in contrast to true NO donors such as nitrosothiols, nitrates at therapeutic doses, will not release large, potentially harmful fluxes of NO. With over a century of human clinical experience, nitrates represent ideal NO mimetic therapeutics. Hybrid nitrates, which consist of a classical nitrate grafted onto a primary drug pharmacophore, have received much attention recently. These molecules have yielded exciting data, but in most cases it is difficult to dissect and attribute the observed activity to the pharmacophore versus the nitrate moiety. Of relevance to this present paper, in animal studies on hybrid nitrates, both microglial activation and accompanying amyloid clearance [194, 195], and inhibition of neurotoxin induced microglial activation by a hybrid nitrate have been reported [196]. Hybrid nitrate therapeutics, in particular the so called NO-NSAIDs (NO donor non-steroidal anti-inflammatory drugs), have received regulatory approval and completed human clinical trials [197].

Novel nitrates in which the neuromodulatory activity and systemic hypotensive effects can be dissociated represent good candidates for NO mimetic therapeutic agents in AD, because the nitrate functional group is inherently lipophilic supporting satisfactory CNS bioavailability. The novel nitrate, GT 715, represents a prototype for one such approach [198, 199]. GTN and GT715 were shown to exert differential effects on cardiovascular function, with GT715, being a weaker vasodilator with minimal effects on mean arterial pressure in the whole animal compared to GTN [190]. GT715 was both more potent and more effective as an activator of sGC in the brain, and more effective in elevating cGMP levels in hippocampal brain slices, compared to GTN, whereas GTN produced a much greater accumulation of cGMP in vascular tissue [200]. GT 715 supported the postulate that neuromodulatory and hypotensive effects of nitrates can be dissociated. Demonstration of the neuromodulatory effects of GT 715 was observed in a variety of models of neuroprotection: (a) in the middle cerebral artery occlusion (MCAO) rat model of focal ischemic stroke [201], (b) in the 6-hydroxydopamine-lesion rat model of Parkinson's Disease, and (c) in the malonate-lesion rat model of excitotoxic neurodegeneration [202].

Intrastriatal injection of malonate, a mitochondrial succinate dehydrogenase inhibitor, into the brain of rats produces energy depletion, secondary excitotoxicity, and free radical production that ultimately leads to neuronal cell death [203]. In this model of neuronal injury [204] GT 715 was observed to significantly decrease the brain injury induced by the malonate neurotoxin at both the behavioural and neurochemical levels. Preservation of GABA levels in the striatum after malonate injection, measured as an index of the neuronal cell population, and a markedly decreased response to apomorphine in ipsolateral turning indicated that neuronal injury was significantly inhibited by GT 715 administration, and that normal function within the neostriatum was maintained [202]. These results reinforce our previous unpublished observations in a Parkinson's animal model and reported observations on the neuroprotective activity of GT 715 in the rat transient MCAO model of ischemic stroke wherein s.c. delivery of drug 2h or 4h after ischemia produced a significant reduction in infarct volume (58% reduction in total and 72%reduction in cortical infarct volumes at 4h) [190].

The novel nitrate GT 715 represents a prototype for neuromodulatory novel nitrates that have been studied in a number of behavioral tests used to demonstrate memory improvement and the reversal of cognition deficits in rat models of dementia. In AD, loss of cholinergic neurons and subsequent deficits in cholinergic neurotransmission in the hippocampus and cerebral cortex, are strongly correlated with clinical signs of cognitive impairment and dementia. Central cholinergic muscarinic receptor blockade produces profound cognitive impairments in human and animal subjects, thus the use of cholinergic muscarinic antagonists, such as scopolamine, in animal models to mimic the cognitive impairment observed in AD is well established, and has proven to be a useful model system for understanding and developing treatment strategies for neurodegenerative diseases in humans [205]. Data have been published for the novel nitrates, GT 715 and GT 061, demonstrating the reversal of cognitive deficits produced by scopolamine in rats tested in the MWT [200, 202]. All members of this class tested to date in behavioural models of dementia show cognition enhancing activity, and various members manifest additional biological activity, including anticonvulsant and analgesic activity, and AMPA receptor modulation [206]. GT 1061 is a salt form of GT 061, which was selected as a drug candidate based upon CNS activity ancillary to ability to reverse cognition deficits in a variety of models.

GT 1061 has been studied in a variety of experimental paradigms where cognition deficits are induced, including: (a) injection of the muscarinic receptor antagonist scopolamine [200]; (b) administration of the cholinergic neurotoxin 192 IgG-saporin *via* intracerebroventricular infusion and other routes [98]; and (c), chronic, daily, bilateral, intracerebroventricular infusion of β-amyloid peptide (Aβ1-40) [207]. The behavioural models used to measure reduction of cognition deficits have included the MWT (both fixed and moving platform versions), step-through passive avoidance test (STPA), contextual memory after STPA, and visual delayed matching to sample (DMTS), in all of which tacrine and donepezil ACIs were used for validation and comparison.

That dysfunction in the rat hippocampus causes spatial learning deficits in tasks such as the MWT is well established [208, 209], however, the role of the hippocampus in visual memory and recognition has not been proven until recently [210]. The observation of similarities between cognitive processing in the rat and man is important, since visual association tests are reported to reliably detect a substantial proportion of AD patients up to a year before diagnosis [211], and further to distinguish against non-Alzheimer’s dementia (including vascular dementia, frontotemporal dementia, subcortical dementia [211]. A reliable rat model of visual recognition memory deficit has been reported [210]. In this model a lesion induced by intracerebroventricular infusion of 192 IgG-saporin induced a deficit in visual recognition memory that was completely reversed in a dose dependent manner by oral administration of GT 1061, which proved superior to donepezil: moreover, drug administration was observed to elevate levels of phosphorylated ERK in the hippocampus [212].

SUMMARY

AD is a multifactorial disease, with contributions from synaptic, dendritic and neuronal damage and dysfunction, and the formation of abnormal protein aggregates throughout the brain. The interlinked, contributing factors to AD are complex and regulate the timeline of disease progression *via* processes including inflammation, oxidative stress, apoptosis, and aberrant kinase signalling. NO mimetics are likely to impact multiple factors responsible for synaptic and neuronal dysfunction in AD. The combination of neuroprotection with cognition enhancement demonstrated by novel nitrates represents exciting potential for new approaches to AD therapy.

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