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Sildenafil citrate attenuates a complex maze impairment induced by intracerebroventricular infusion of the NOS inhibitor N^{ω}-nitro-L-arginine methyl ester

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Abstract

In a previous study, our laboratory reported that sildenafil citrate, a cyclic nucleotide phosphodiesterase type 5 inhibitor, reversed a learning impairment in rats induced by systemic inhibition of nitric oxide synthase (60 mg/kg, i.p., N^{ω}-nitro-L-arginine methyl ester; L-NAME). To limit the peripheral effects of L-NAME and further localize the site of action of sildenafil, L-NAME (48 μg, i.c.v.) was infused bilaterally into the lateral cerebral ventricles 30 min prior to maze training. Saline or sildenafil citrate (1.5 or 3.0 mg/kg, i.p.) was administered systemically 15 min before training. Drug injections occurred 24 h after pretraining rats to avoid foot shock on a one-way active avoidance straight runway. Following drug treatment, the rats received 15 training trials on a 14-unit T-maze task that requires learning a complex sequence of turns to avoid mild foot shock. This complex maze paradigm is sensitive to aging and blockade of cholinergic, *N*-methyl-D-aspartate and nitric oxide signaling systems. Behavioral measures of performance included deviations from the correct pathway (errors), runtime from start to goal (latency), shock frequency and shock duration. Statistical analysis revealed that central infusion of L-NAME impaired maze performance and that sildenafil (3.0 mg/kg) significantly attenuated the impairment. These results suggest that sildenafil citrate may serve as a cognitive enhancer by modulating central nitric oxide/cGMP signal transduction following *N*-methyl-D-aspartate receptor activation. This pathway has been implicated in age-related cognitive decline and may be a useful target for pharmacological intervention of neurodegenerative disease.

Keywords: Phosphodiesterase inhibition; Nitric oxide; Cyclic GMP; Aging; Cognitive performance; Stone maze

1. Introduction

The development of improved drug treatments for Alzheimer's disease and other age-related neurodegenerative conditions remains a major challenge of current biomedical research. The two approved drug treatment strategies in clinical use for Alzheimer's disease afford only modest reductions in the progression of cognitive impairment during different stages of the disease. Cholinesterase inhibitors, such as tacrine (Cognex) and donepezil (Aricept), which increase cholinergic transmission by inhibiting enzymatic degradation, are used to treat early-to-moderate symptoms of the disease, while the more recently approved drug, memantine (Namenda), a moderate affinity uncompetitive antagonist of glutamatergic *N*-methyl-D-aspartate

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(NMDA) receptors, is used to treat 'moderately severe to severe' Alzheimer's disease in Europe and 'moderate-to-severe' cases in the United States. Although it has been suggested that memantine may only attenuate excessive activity that leads to excitotoxic cell damage without impairing normal synaptic transmission (Danysz and Parsons, 2003; Rogawski and Wenk, 2003), more recent preclinical evidence suggests that low doses of memantine insufficient to produce neuroprotection may impair memory formation in adult rats (Creeley et al., 2006, 2003, 2004). These findings are consistent with a large body of literature implicating *N*-methyl-D-aspartate receptor activation in synaptic plasticity and normal memory formation (Collingridge and Bliss, 1995; Martin et al., 2000).

The lack of alternative treatment options has stimulated novel approaches to drug discovery, several of which are based on the neuropathological features that define Alzheimer's disease, e.g., deposition of insoluble amyloid-β peptide, formation of neurofibrillary tangles and inflammatory responses leading to neuronal cell death (Vardy et al., 2006). Such drug discovery efforts are advantageous because they deal directly with the hallmark neurobiological consequences of the disorder. However, the major disadvantage is that no causal link between structural pathology and cognitive impairment has been established. Consequently, drug targets based on such changes may overshoot the critical signal transduction events that directly contribute to the cognitive decline and dementia.

An alternative approach is to start with events that contribute to the beginning stages of normal cognitive processing (e.g., learning and memory formation) and proceed systematically through the chain of signal transduction events that may become dysfunctional and eventually lead to the neuropathological endpoints. In this way, drug discovery may build upon prior knowledge that has obtained a certain level of justification and usefulness (Bartus, 2000; Bartus et al., 1982), or so-called proof of principle in current clinical practice.

Based on this strategy, evidence suggests that signal transduction mechanisms that closely follow neurotransmitter–receptor interactions may be useful targets in the search for alternative drug treatments. Phosphodiesterase (PDE) enzymes limit signal transduction by the second messengers, cyclic adenosine 3′-5′-monophosphate (cAMP) and/or cyclic guanosine 3′-5′-monophosphate (cGMP). Over fifty isozymes belonging to eleven different gene families have been cloned, characterized, and found to be expressed in specific cell types and tissues, particularly within the central nervous system (Menniti et al., 2006). Preclinical evidence suggests that PDE inhibitors targeting brain isozymes may enhance learning and memory formation in animals. Clinical trials for the treatment of Alzheimer's disease are being conducted with PDE type 4 (PDE4) inhibitors that increase cAMP signaling along a mitogen activated protein kinase (MAPK)-cAMP response element binding protein (CREB) pathway. Given that downstream targets, such as CREB, are ubiquitous and may influence diverse functions besides memory formation, targeting upstream components of the pathway that are brain region-specific may selectively enhance memory with fewer side effects (Barco et al., 2003). Although mixed preclinical results have been reported for first generation PDE4 inhibitors, such as rolipram, newer compounds may have less side effects and greater therapeutic value (Rose et al., 2005; Zhang et al., 2005a).

Preclinical studies using PDE type 5 (PDE5) inhibitors that increase cGMP signaling have also demonstrated improved learning and memory performance in rodents (Blokland et al., 2006; Devan et al., 2005; Prickaerts et al., 2004). For example, we have shown that the PDE5 inhibitor, sildenafil citrate (Viagra®), attenuates a complex maze learning impairment induced by systemic administration of the cholinergic muscarinic receptor antagonist, scopolamine (Devan et al., 2004) and the non-specific nitric oxide synthase inhibitor, N^{ω}-L-nitro-arginine methyl ester (L-NAME) (Devan et al., 2006).

Because systemic nitric oxide synthase inhibition is known to have several side effects including catalepsy (Araki et al., 2001; Del Bel et al., 1998, 2004, 2005), malaise (Prendergast et al., 1997), increased blood pressure (Weldon et al., 1995) and altered pain responsiveness (Khattab et al., 2004; Morgan et al., 1992), it is possible that the impairment resulting from systemically administered L-NAME may have been due to an indirect effect on learning performance. The fact that systemic L-NAME impaired performance on the initial trials of the 14-unit T-maze task, when the influence of learning was likely at a minimum, supports this possibility (Devan et al., 2006). However, the results of a simple one-way active avoidance control experiment conducted in the same study showed no L-NAME related impairment and thus indicated that L-NAME primarily impaired complex maze learning. To further investigate this issue in the present study, a small dose of L-NAME was infused directly into the brain to minimize its peripheral effects. In addition, the effectiveness of sildenafil in attenuating the learning impairment induced by central infusion of L-NAME was assessed.

2. Materials and methods

2.1. Subjects

A total of thirty-one 3-month-old virgin male Fischer-344 rats weighing ~250–300 g were shipped to the Gerontology Research Center from the National Institute on Aging colony of Harlan-Sprague–Dawley (Indianapolis, IN). The rats were housed in pairs in large, suspended plastic cages in a vivarium maintained at 21 °C and on a 12:12 h light:dark photocycle (lights on 07:00 h EST). Water was freely available via an automated water system, and food (NIH-07) was provided ad libitum. All rats were acclimatized to the vivarium for at least 1 week prior to the surgery. All procedures described below were approved by the National Institute on Aging Institutional Animal Care and Use Committee and were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

2.2. Surgery

Rats were anesthetized using the inhalation anesthetic isoflurane (2 1/2–5%) mixed with oxygen. The scalp was

shaved and cleaned with betadine followed by a 70% alcohol solution before being placed into a small animal stereotaxic instrument. A local anesthetic (1% xylocaine with epinephrine) was administered immediately prior to incising and retracting the scalp to expose the skull surface. Stereotaxic coordinates were determined using the atlas of (Paxinos and Watson, 1986) based on a flat skull position with bregma as the reference point. A hand-held Dremel tool was used to drill two holes (~1 mm diameter) through the skull surface. Two jeweler's screws were secured to the skull, and the dura was punctured with an aseptic needle before lowering bilateral guide cannula (22 gauge, C232G-2.4, Plastics One, Roanoke, VA) into the brain such that the cannula tips were positioned at the following stereotaxic coordinates relative to the bregma: −0.8 mm anterior–posterior; ±1.2 mm medial–lateral; −4.5 mm dorsal–ventral. Following stereotaxic positioning, the cannula assembly was fixed to the skull with cranioplastic cement, which was allowed to thoroughly dry before the wound was cleaned and closed with surgical clips. A topical antibiotic (Neosporin) was applied to the wound, and animals were allowed to recover from the anesthetic in a plastic holding cage placed on a warming pad maintained at 32 °C. Animals were injected with buprenorphrine, 0.03 mg/kg, intramuscular (i.m.) and monitored carefully during the 7–10 day recovery from the anesthetic and surgery.

2.3. Drug administration

Animals were randomly assigned to one of the following treatment conditions, each comprising an intracerebroventricular (i.c.v.) infusion of L-NAME or saline and an intraperitoneal (i.p.) injection of sildenafil or vehicle [30 µl dimethyl sulfoxide (DMSO), 90 µl Tween 80, 0.88 ml of saline], 30 and 15 min, respectively, before training in the 14-unit T-maze: (1) saline+vehicle (n=7), (2) L-NAME+vehicle (n=10), (3) L-NAME+sildenafil 1.5 mg/kg (n=7) and (4) L-NAME+sildenafil 3.0 mg/kg (n=7). Drugs were prepared fresh in aqueous solution each day. L-NAME (Sigma-RBI, MA), was dissolved in sterile physiological saline (NaCl 0.9%) at a concentration of 6 mg/ml. A total of 48 µg (24 µg/side) was infused bilaterally (rate: 1 µl/min for a total of 4 min) into the lateral ventricles using a CMA microinfusion pump with 50 µl Hamilton syringes connected to a double polyethylene tube assembly. Control animals received an equal volume of physiological saline. Injection cannulae were left in place for 1 min following infusions. Pure powder sildenafil (gratis from Pfizer, Inc.) was dissolved in DMSO and suspended in Tween 80 before being brought to volume in physiological saline. Systemic injection volume was ≤1 ml/kg body weight. Drug dosing and delivery was based on pilot work and the results reported in previous publications (Devan et al., 2004, 2006).

2.4. Behavioral apparatus and procedure

2.4.1. Pretraining on one-way active avoidance

As described in detail previously (Spangler et al., 1986), a straight runway 2 m long and constructed of clear plastic was used for pretraining in a one-way active avoidance. The runway had a grid floor comprised of stainless steel bars that were wired to receive scrambled shock (alternating current) from a Coulbourn Instruments (E13–08) grid floor shocker. Black plastic boxes with a guillotine door at the front and a movable rear wall served interchangeably as start and goal boxes. A hand-held switch was wired to a clock that automatically initiated a mild foot shock (0.8 mA; maximum duration 120 s, inter-shock interval 100 s) once 10 s had elapsed.

Prior to pretraining in the straight runway, the rats were moved to the testing room in their home cages and allowed to acclimatize for at least 30 min. A rat was then removed from the home cage, placed into one of the black boxes and in turn was placed into the start area over the grid floor. The guillotine door was opened, and the rat was pushed gently forward onto the grid floor using the movable back wall. Rats had 10 s to avoid scrambled foot shock by moving down the straight runway and entering a black box at the opposite end. After 10 s, the foot shock continued until either the rat entered the goal box, or 120 s had elapsed. The guillotine door was lowered after the rat entered the goal box. A 90 s inter-trial interval intervened between trials. Criterion for successful completion of straight runway pretraining was 13 out 15 successful avoidances, in 10 s or less per trial (maximum 30 trials). All rats that successfully met the criterion were assigned to one of the drug groups described above and trained in the 14-unit T-maze.

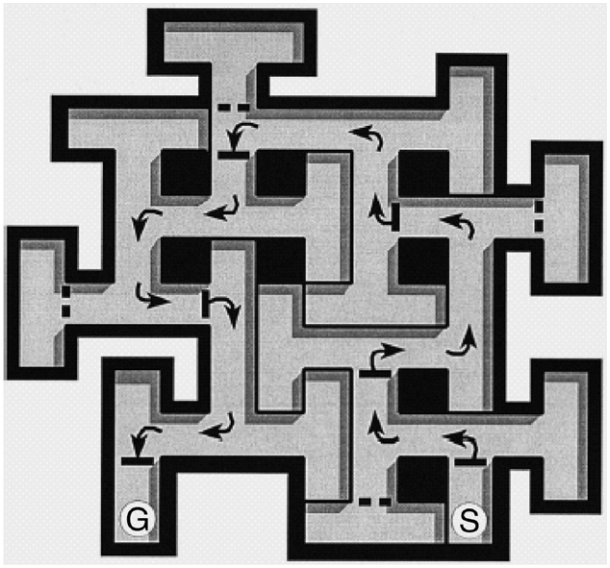

Fig. 1. Schematic diagram showing the configuration of the 14-unit T-maze. Arrows indicate the correct pathway. Errors are defined as any deviation from the correct pathway. S = start box, G = goal box, ——— = guillotine door, −−− = false guillotine door.

2.4.2. Complex maze training

Training was conducted in a clear plastic 14-unit T-maze that has also been described previously (Spangler et al., 1986; see Fig. 1). The maze was separated into five sections by guillotine doors that prevented animals from backtracking into previous sections of the maze. Non-functional guillotine doors were placed at the entry to each cul-de-sac of the maze to prevent the actual doors from being used as cues to the correct pathway. A

switchbox triggered a clock which, when timed out, initiated a second clock to record the duration of shock (maximum of 5 shocks per trial). Infrared photocells were positioned throughout the maze and were wired in series to a microprocessor that recorded movement through the maze, time elapsed from start to goal, and time between photocell interruptions.

Data collected from the photocells were analyzed by the microprocessor which calculated the number of errors (defined as any entrance into a maze section leading to a cul-de-sac) and runtime for each section of the maze. Data from the microprocessor were transferred to a personal computer for more detailed analysis as well as storage of raw data. The maze was surrounded by gray walls to reduce extra maze visual cues. Speakers were located under the maze and provided music to mask auditory cues. The maze could be hoisted by motor-driven pulleys in order to clean the grid floor and reduce the presence of odor cues.

Consistent with pretraining, rats were brought to the testing room in the home cage and allowed to acclimatize for at least 30 min before drug administration and acquisition training in the 14-unit T-maze. The rat was then taken from its home cage and placed into a black start box which was positioned at the start location of the maze. The rat was pushed gently into the first section of the maze, and the guillotine door was closed. A manual switch initiated a clock that controlled the shock contingency. The rat then had 10 s to pass through the first guillotine door and enter the next section. Failure to meet this criterion resulted in a scrambled foot shock (0.8 mA) that was terminated when the rat passed through the door or a maximum of 300 s elapsed, resulting in the termination of the trial (testing was discontinued after two terminated trials). The shock contingency was reset after the rat passed through each guillotine door, completing each of the five maze sections and entering the goal box. A 90 s inter-trial interval was used, during which time the box was placed in a holding area while the grid floor was cleaned with a 95% solution of ethanol solution. Each rat received a total of 15 massed trials (subsequently collapsed into five blocks of three trials for purposes of statistical analysis and data presentation).

2.5. Data analyses

Maze errors, runtime, shock frequency and shock duration were analyzed using one- or two-way mixed analysis of variance (ANOVA). Planned comparisons of overall mean errors and errors committed on the first trial and initial block of 3 trials were conducted a priori with the number of group comparisons restricted ($k-1$) to determine if the L-NAME group made more errors than controls and whether the sildenafil groups made fewer errors than the L-NAME group. Tukey post hoc tests were used with the other performance measures when appropriate.

3. Results

In a previous study from our laboratory (Devan et al., 2006), we demonstrated that systemic administration of L-NAME (60 mg/kg, i.p.) impaired performance on the 14-unit T-maze; however, the impairment was evident on the first trial block of training, suggesting that systemic administration may have produced non-specific effects on maze performance. Consequently, in the present study, it was important to determine whether limiting the dose and route of drug administration centrally (48 µg, i.c.v.) would spare performance on the initial trials of complex maze training. A one-way ANOVA on errors committed on the first trial or on the first block of 3 trials failed to show any significant differences among the groups tested [first trial: $F(3,27)=1.49$, $P>0.20$; first trial block: $F(3,27)=1.19$, $P>0.30$]. Hence, central administration in the present study was apparently effective in limiting the non-learning related performance effects of L-NAME.

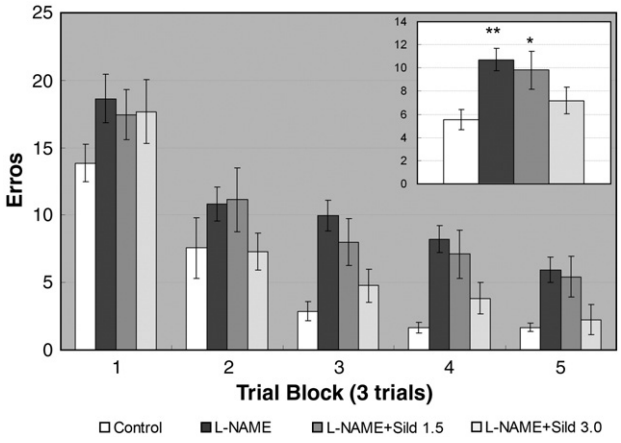

Fig. 2. Effects of central L-NAME and systemic sildenafil citrate on mean (±s.e.m.) number of errors during acquisition training in a 14-unit T-maze. The main graph shows data plotted in blocks of 3 trials and the bar graph (inset) shows data averaged across trial blocks. L-NAME and sildenafil were administered 30 and 15 min prior to maze training, respectively. Rats received central (i.c.v.) and systemic (i.p.) injections of either saline-vehicle ($n=7$), L-NAME (48 µg)+vehicle ($n=10$), L-NAME (48 µg)+sildenafil 1.5 mg/kg ($n=7$), or L-NAME (48 µg)+sildenafil 3.0 mg/kg ($n=7$). Symbols: **, significantly different from the saline-vehicle and L-NAME+sildenafil 3.0 groups; *, significantly different from the saline-vehicle group. See text for description of statistical results.

Assessment of learning performance was conducted by an overall analysis of error scores using 4×5 (Group×Trial block) mixed factorial ANOVA, with repeated measures on the second factor. The analysis revealed a significant main effect of Group [$F(3,27)=4.26$, $P<0.05$], Trial block [$F(4,108)=104.46$, $P<0.001$] but a non-significant Group×Trial block interaction [$F(12,108)=0.96$, $P>0.40$]. Planned comparisons of group marginal means revealed that the L-NAME group differed significantly from controls ($P<0.01$) and the 3.0 mg/kg sildenafil+L-NAME group differed from the L-NAME alone group ($P<0.05$).

Fig. 2 shows the mean errors for each group across blocks of 3 trials in the main graph and collapsed across all 15 trials in the inset graph. Central infusion of L-NAME clearly impaired overall performance while systemic administration of 3.0 mg/kg significantly reduced this effect. These results suggest that the 3.0, but not the 1.5, mg/kg dose of sildenafil, was effective in attenuating the learning impairment induced by central blockade of NOS with L-NAME.

Table 1
Effects of L-NAME and combined L-NAME + sildenafil treatments (expressed as the percent of change from control) on performance measures in the 14-unit T-maze task

Group (*n*)	Runtime	Shock duration	Shock frequency
L-NAME (10)	166.34 ± 41.22	394.62 ± 104.17	103.44 ± 18.20
L-NAME + sildenafil 1.5 (7)	113.83 ± 44.61	238.47 ± 92.89	86.93 ± 26.85
L-NAME + sildenafil 3.0 (7)	45.92 ± 14.15	97.98 ± 26.56	27.27 ± 20.72

Overall mean runtime, shock frequency and shock duration were averaged across the five trial blocks and are presented in Table 1 as percent of control values. One-way ANOVAs computed on the raw scores for each of these dependent measures revealed significant Group effects [runtime: $F(3,27)=4.54$, $P<0.05$; shock frequency: $F(3,27)=5.01$, $P<0.01$; shock duration: $F(3,27)=4.60$, $P=0.01$]. Tukey post hoc tests showed that only the L-NAME group differed significantly from the saline control group on each of the measures (Ps <0.05).

4. Discussion

The present results extend previous findings (Devan et al., 2006) by showing that (1) centrally administered L-NAME can induce a selective learning impairment as demonstrated by the lack of significant performance impairment on the early trials of complex maze acquisition and (2) that the PDE5 inhibitor, sildenafil citrate, can attenuate the overall impairment. Combined with previous findings showing that systemically administered L-NAME did not significantly impair performance of a simple one-way active avoidance response, it is likely that the performance enhancing effect of sildenafil is due in large part to specific facilitation of the central mechanisms involved in complex maze learning.

It is likely that central administration of L-NAME did not completely preclude peripheral or centrally-mediated effects of the drug on non-learning related components of maze performance. However, whereas previous systemic (i.p.) administration of 60 mg/kg L-NAME (Devan et al., 2006) is within the range that has been reported to produce cataleptic motor effects in rodents (e.g., 40–160 mg/kg) (Araki et al., 2001; Del Bel et al., 2004), central (i.c.v.) infusion of 48 μg (0.17 nmol) is ~1000 fold less than central doses (100–200 nmol) that produce similar motoric effects in rats (Del Bel et al., 2004). Further, central administration of L-NAME (150 μg, i.c.v.) did not alter pulmonary vascular tone in rats (Schwenke et al., 2006, 2005). While the effects of L-NAME on malaise have only been demonstrated systemically (Prendergast et al., 1997), both central (Kawabata et al., 1993) and peripheral (Guney et al., 1998; Khattab et al., 2004) effects of L-NAME on pain responsiveness have been reported; although a central effect on pain responsiveness cannot be completely ruled out in the present study, such an effect should have been observed or even pronounced at the beginning of training before any habituation or adaptation to shock could occur.

Previous studies of complex maze learning in rodents suggest an interaction between central cholinergic and *N*-methyl-D-aspartate systems (Ingram et al., 1996, 1994a,b; Meyer et al., 1998). In addition to the wealth of evidence supporting the cholinergic hypothesis of geriatric memory dysfunction (Bartus, 2000; Bartus et al., 1982), neurobiological research on the long-term potentiation of synaptic transmission suggests that the glutamatergic system may play an important role in synaptic plasticity as a coincidence detector mechanism for learning and memory (Bliss and Collingridge, 1993; Collingridge and Bliss, 1995; Martin et al., 2000). Other evidence indicates that the same excitatory amino acid system may be responsible for some of the clinical manifestations of Alzheimer's disease (Greenamyre and Young, 1989) which, as previously noted, is the current rationale for memantine treatment (Molinuevo et al., 2005). Interactions between acetylcholine and *N*-methyl-D-aspartate systems may coordinate states of learning new information with recall of previously acquired cognitive representations (Aigner, 1995; Hasselmo and Bower, 1993). Dysfunction among these systems may lead to aberrant synaptic plasticity (Hasselmo, 1997) that interferes with attention, learning and cognitive memory formation.

N-methyl-D-aspartate receptor activation leads to an influx of calcium and the activation of a calmodulin dependent form of nitric oxide synthase (Garthwaite, 1991; Garthwaite and Boulton, 1995). Evidence suggests that nitric oxide functions as a retrograde messenger in the brain, stimulating soluble guanylyl cyclase (sGC) to form the second messenger cGMP (Garthwaite, 1991; Hawkins et al., 1998). The three main classes of effector proteins are cGMP dependent protein kinases (PKG), cyclic nucleotide gated (CNG) ion channels and PDEs (Beavo and Brunton, 2002; Hofmann et al., 2000; Lohse et al., 1998). PKG regulates neural function by phosphorylating different proteins and influencing cAMP phosphodiesterases that regulate cAMP concentrations (Schmidt et al., 1993). CNG ion channels regulate calcium influx leading to depolarization of the presynaptic terminal and subsequent neurotransmitter release (Ahmad et al., 1994; Zufall et al., 1997). Finally, cGMP activates PDEs that degrade the second messengers thereby terminating their action (Bellamy and Garthwaite, 2001; Juilfs et al., 1999).

PDE5 inhibitors, such as sildenafil, provide a means to enhance the amplitude and duration of cGMP signal transduction that is strictly limited by cyclic nucleotide PDE degradation. Recently, it has been shown that cGMP directly activates PDE5, leading to the rapid decrease in cGMP and desensitization (Mullershausen et al., 2003). PKG increases the sensitivity of PDE5 for cGMP, producing multiple components of negative feedback (Koesling et al., 2005) that are reduced by PDE inhibition, extending cGMP signal transduction along the other parallel pathways.

Although there are many apparent advantages to targeting PDE5 and cGMP signal transduction, including links between Alzheimer's pathology and nitric oxide/cGMP signal transduction (Chalimoniuk and Strosznajder, 1998; de Vente, 2004; Paris et al., 1999; Puzzo et al., 2005) and *N*-methyl-D-aspartate receptor subunit expression (Hynd et al., 2004a,b), crosstalk with cAMP pathways (Schmidt et al., 1993) and reported neuroprotective effects of sildenafil (Zhang et al., 2005b, 2002),

other factors may limit the utility of this strategy for drug development. One major consideration involves localization of the isoenzyme which is most abundant in Purkinje cells of the cerebellum (Kotera et al., 2000, 1997; Shimizu-Albergine et al., 2003; van Staveren et al., 2003) although mRNA expression has been detected in all cortical layers, hippocampal subfields and in the caudate–putamen complex (van Staveren et al., 2003). Further, the concept of nitric oxide as a retrograde messenger has been questioned because cGMP immunoreactivity is primarily found in postsynaptic structures (Prickaerts et al., 2004; van Staveren et al., 2004). Finally, because phosphodiesterase inhibitors increase glutamatergic neurotransmission, it is possible that prolonged treatment could have deleterious effects via excitotoxicity, although studies on neuroprotection cited above would suggest otherwise.

In conclusion, further research should be conducted to determine the extent of the cognitive enhancing effects of PDE5 inhibitors, the precise mechanism(s) of action within different neural systems responsible for the behavioral–cognitive effects, and the potential role of these drugs in abating the neurodegeneration that accompanies aging. Given that multiple co-factors may contribute to age-related cognitive decline (McDonald, 2002), studies should assess the therapeutic potential of combining PDE5 inhibitors with other cognitive enhancing agents.

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