

Peripheral Delivery of a ROCK Inhibitor Improves Learning and Working Memory

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Previously, utilizing a series of genome-wide association, brain imaging, and gene expression studies we implicated the *KIBRA* gene and the RhoA/ROCK pathway in hippocampal-mediated human memory. Here we show that peripheral administration of the ROCK inhibitor hydroxyfasudil improves spatial learning and working memory in the rodent model. This study supports the action of ROCK on learning and memory, suggests the potential value of ROCK inhibition for the promotion of cognition in humans, and highlights the powerful potential of unbiased genome-wide association studies to inform potential novel uses for existing pharmaceuticals.

Keywords: learning, memory, ROCK, fasudil, aging

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The molecular processes involved in learning and memory provide promising targets for putative memory-enhancing (i.e., nootropic) pharmaceuticals, which might be helpful in the treatment of Alzheimer's disease, other dementias, mild cognitive impairment, and other disorders associated with impairments in learning and memory. If safe and well tolerated, these medications could even have roles in the treatment of the nondisabling

learning and memory declines associated with healthy aging as well as in the enhancement of normal learning and memory.

To date, nootropic drug discovery efforts have focused on the enhancement of cholinergic, glutaminergic, and serotonergic neurotransmission and phosphodiesterase inhibition, and have had limited benefits (Sarter, 2006). Examples include the cholinesterase inhibitors and N-methyl-D-aspartate (NMDA) ago-

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nists currently approved for the treatment of Alzheimer's disease, α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptor agonists, and nicotinic α 7 receptor agonists.

Using a hypothesis-free 500K single nucleotide polymorphism (SNP) genome-wide association study, followed by functional brain imaging and gene expression studies, we recently associated the *KIBRA* gene with the variation in normal episodic memory performance in both young (median age of 22; ranging from 18 to 48 years) and late middle aged (median age of 55; ranging from 20 to 81 years) adults (Papassotiropoulos et al., 2006). Our initial genetic findings have been replicated by a different research team in a unique cohort of older individuals (mean age of 67) thereby further supporting *KIBRA*'s role in episodic memory as well as extending this relationship to the aged population (Schaper, Kolsch, Popp, Wagner, & Jessen, 2007). In addition, two other groups have published studies using independent cohorts that further support a genetic link between *KIBRA* and memory variation in healthy individuals (Almeida et al., 2008; Nacmias et al., 2008). The genetic link between *KIBRA* and human memory disorders has also been investigated. One group recently reported no effect on risk for development of mild cognitive impairment (Almeida et al., 2008), however, a manuscript published in 2007 as well as a recently published manuscript by members of our group support a link between *KIBRA* genetic variation and Alzheimer's disease in (Rodriguez-Rodriguez et al., in press) and (Corneveaux et al., in press). There has also been a single recent report suggesting no association between *KIBRA* and multiple-verbal memory tasks (Need et al., 2008).

Based on these findings and a pathway analysis approach, we hypothesized that *KIBRA* activity would be altered via the RhoA/ROCK/Rac pathway through the putative modulation of PKC- ζ (Van Kolen & Slegers, 2006). *KIBRA* is a demonstrated substrate for PKC- ζ (Buther, Plaas, Barnekow, & Kremerskothen, 2004) and has been shown to interact with Dendrin (Kremerskothen et al., 2003), a postsynaptic cytoskeleton modulatory molecule. Recently, *KIBRA* also has been shown to colocalize with both a postsynaptic marker protein (ProSAP2/Shank3) and in close contact with a presynaptic marker (bassoon) in primary rat hippocampal neurons (Johannsen, Duning, Pavenstadt, Kremerskothen, & Boeckers, 2008). In multiple-cell types the RhoA/ROCK/Rac pathway has been demonstrated to be upstream of PKC- ζ (Kampfer et al., 2001; Scott, Arioka, & Jacobs, 2007; Uberall et al., 1999; Van Kolen & Slegers, 2006). In addition, because the RhoA/ROCK/Rac pathway has been implicated in key neurobiological processes that underlie cognitive function, such as neurite outgrowth and growth cone modulation (Gopalakrishnan et al., 2008; Lingor et al., 2007; Loudon, Silver, Yee, & Gallo, 2006; Woo & Gomez, 2006), we postulated that an inhibitor of this pathway might be useful as a treatment for the enhancement of learning and memory.

Several existing compounds have the capability to modulate the RhoA/ROCK pathway. Although a recently developed inhibitor of ROCK, fasudil has been investigated in patients as a potential treatment for vasospasm and angina, fasudil or its active metabolite hydroxyfasudil has not been evaluated in laboratory animals or human subjects for effects on learning and memory (Hirooka & Shimokawa, 2005).

Method

Subjects and Treatment Procedures

Subjects were 27, 17-month-old Fischer-344 male rats (18-months old at the time of behavioral testing) born and raised at the aging colony of the National Institute on Aging at Harlan Laboratories (Indianapolis, IN). After arrival at Arizona State University, animals were pair housed with a same-age cage mate, had exposure to food and water ad lib, and were maintained on a 12-hr light-dark cycle. All procedures were approved by the local IACUC committee and adhered to NIH standards. The experimenters who performed the behavioral testing and brain dissections were blind to treatment group.

One daily injection of the assigned substrate began 4 days prior to behavioral testing and continued throughout testing. The half life of hydroxyfasudil in humans has been estimated at between 5 and 7 hr (Hinderling et al., 2007), therefore we administered the drug dose each morning prior to behavioral testing. The initial 4-day period before testing was incorporated to habituate the animals to daily drug or vehicle delivery. Injections were given subcutaneously into the scruff of the neck. There were three experimental groups: nine animals received saline vehicle ("aged vehicle"), nine animals received hydroxyfasudil (Sigma-Aldrich, St. Louis, MO) at a dose of 0.1875 mg ("aged low dose"), and nine animals received hydroxyfasudil at a dose of 0.3750 mg ("aged high dose").

Water Radial-Arm Maze

A schematic of the water radial-arm maze is shown in Supplementary Figure 1A. This win-shift radial-arm maze tests working and reference memory and utilizes water escape onto hidden platforms as the reinforcer (Bimonte & Denenberg, 1999, 2000; Bimonte, Granholm, Seo, & Isacson, 2002; Bimonte, Hyde, Hoplight, & Denenberg, 2000; Hyde, Hoplight, & Denenberg, 1998; Hyde, Sherman, & Denenberg, 2000). The eight-arm maze had submerged escape platforms placed in the ends of four of the arms. Each subject had different platform locations that remained fixed throughout the experiment. A subject was released from the start arm, which remained constant throughout testing and for all subjects, and had 3 min to locate a platform. Once a platform was found, the animal remained on it for 15 s, and was then returned to its heated home cage for 30 s until its next trial. During the intertrial interval, the just-chosen platform was removed from the maze. The animal was placed again into the start alley and allowed to locate another platform. The same sequence of events was repeated daily until all four platforms were located. Consequently, for each animal a daily session consisted of four trials per session, with the number of platformed arms reduced by one on each subsequent trial resulting in one more item of information needing to be remembered after every trial. Hence, the working memory system was increasingly taxed as trials progressed. Animals performed for four trials each day for 12 days.

Entry into an arm was counted when the tip of a rat's snout reached a mark delineated on the outside of the arm (11 cm into the arm). Errors were quantified for each daily session using the orthogonal measures of working and reference memory errors (Jarrard, Okaichi, Steward, & Goldschmidt, 1984), and blocked into the initial and latter test phases based on previous studies

using the water escape radial-arm maze (Bimonte & Denenberg, 1999, 2000; Bimonte et al., 2002, 2000; Bimonte, Nelson, & Granholm, 2003; Bimonte-Nelson, Francis, Umphlet, & Granholm, 2006; Bimonte-Nelson, Hunter, Nelson, & Granholm, 2003; Bimonte-Nelson, Singleton, Williams, & Granholm, 2004; Hyde et al., 1998, 2000). Day 1 was considered a training session because the animal had no previous experience in the maze. Days 2 through 12 were testing sessions. The 11 testing days were blocked into two phases: The initial phase consisting of testing Days 2 to 7, which is considered an acquisition measure as used in the learning index measure described below, and the latter phase consisting of Days 8 to 12, which is considered a more steady-state measure of memory after the rules of the task have been learned. Working memory correct errors were the number of first and repeat entries into any arm from which a platform had been removed during that session. Reference memory errors were the number of first entries into any arm that never contained a platform. Working memory incorrect errors were the number of repeat entries into an arm that never contained a platform in the past. A total error score was also obtained. The ability to handle an increasing working memory load was tested by evaluating performance as trials increased during the latter testing phase for the working memory correct and incorrect variables. We showed that young rats can learn to handle a high working memory load as testing days progress on this task, whereas experimentally unaltered aged rats cannot (Bimonte et al., 2003). Thus, to evaluate learning of the water radial-arm maze task at the highest memory load, we used a learning index. Total, working memory correct, and working memory incorrect errors on Trial 4 were used to determine the learning index for each day of testing following the first day. This measure is based on similar indexes of learning using a difference score, and corrects for potential differences in baseline variances across animals.

Learning index

$$= \frac{\text{errors made in the initial phase} - \text{errors made in the latter phase}}{\text{errors made in the initial phase}}$$

In addition to learning, we also measured memory competence by evaluating error scores on the latter testing phase via a repeated measures analysis of variance (ANOVA), with treatment as the

between-subjects factor and days and trials as the repeated measures. Each drug-treated group was compared to the saline group, set a priori.

Spatial Reference Memory Morris Maze

A schematic of the Morris water maze is shown in Supplementary Figure 1B. The Morris maze (Morris, 1984) consisted of a round tub (188 cm in diameter) filled with room temperature (19 to 22 °C) water made opaque with black nontoxic paint. A black platform (10 cm) was submerged just below the water surface. The rat was placed in the maze, facing the tub wall, from any of four locations (North, South, East, or West) and had 60 s to locate the hidden platform that remained in a fixed location throughout testing. After 15 s on the platform, the rat was removed from the maze and placed into its heated cage until the next trial. The intertrial interval was approximately 5 to 7 min. The rats were given five trials a day for 4 days, plus a probe trial on Trial 6, Day 5 in which the platform was removed to test platform spatial localization. A video camera suspended on the ceiling above the maze tracked the rat's path and a tracking system (Ethovision System, Noldus Instruments, Wageningen, The Netherlands) was used to analyze each rat's tracing. Performance was assessed by swim path distance to the platform. Distance scores were analyzed using repeated measures ANOVA with treatment as the between-subjects factor and days and trials as the repeated measures for the test trials.

Results and Discussion

Based on results from our prior whole-genome association study, we evaluated the effects of two doses of the ROCK inhibitor hydroxyfasudil on spatial learning and memory in aged rats. For the water radial-arm maze, the aged high dose group showed superior learning for all three measures evaluated (Figure 1a–c). For the measure of total errors (Figure 1a), the aged high dose group showed the best learning, with a significantly higher learning index compared to the aged vehicle group, $t(16) = 2.42$; $p = .028$. The aged high dose group also showed better learning of both orthogonal working memory measures at the most demanding memory load of the radial-arm maze, on Trial 4. Indeed, the aged high dose group had a higher learning index than the aged vehicle

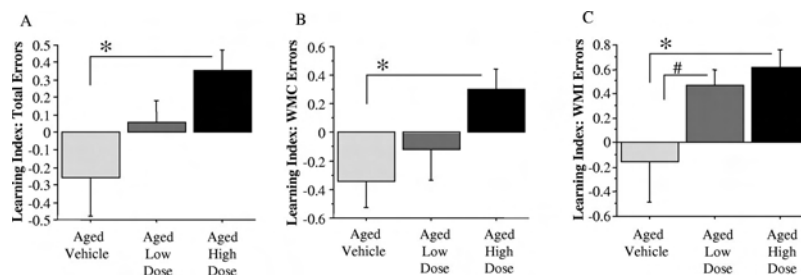


Figure 1. Hydroxyfasudil treatment is associated with a dose-related increase in learning proficiency in aged rats. Shown are the $M \pm SE$. Learning index scores for A) total errors, B) working memory correct (WMC) errors, and C) working memory incorrect (WMI) errors for each group for the trial with the highest memory demand. A higher learning index is indicative of better learning. Aged rats given high dose hydroxyfasudil showed better learning on all three measures, and linear trends showed that drug dose was correlated with a higher learning index for each of the three variables. * $p < .05$. # $p = .09$.

group for working memory correct, $t(16) = 2.70$; $p = .016$ (Figure 1b) and working memory incorrect, $t(16) = 2.19$; $p = .043$ (Figure 1c). The aged low dose group had only a marginally higher learning index for working memory incorrect errors compared to the aged vehicle group, $t(16) = 1.79$; $p = .09$ (Figure 1c). This marginal finding may be due to a slight under powering in the aged low dose animals. However, for every learning index measure, values for the aged low dose group were intermediate between the aged vehicle and aged high dose groups, thereby suggesting a dose-dependent effect on learning the working memory task. Regression analyses confirmed this relationship between treatment dose and performance, with significant linear trends across groups for the learning index for total, ANOVA for linear trend: $F(1, 25) = 7.24$; $p = .013$; working memory correct, ANOVA for linear trend: $F(1, 25) = 6.10$; $p = .021$; and working memory incorrect, ANOVA for linear trend: $F(1, 25) = 6.24$; $p = .019$ errors.

In addition to demonstrating better learning of the working memory task, the aged high dose group also outperformed the aged vehicle group on working memory on the latter phase of testing, with high dose hydroxyfasudil improving the ability to handle the maximally increased memory load, Drug Treatment \times Trial significant interaction for working memory incorrect: $F(3, 48) = 2.84$; $p = .048$ (Figure 2a); Drug Treatment \times Trial marginal interaction for working memory correct: $F(2, 32) = 3.14$; $p = .057$ (Figure 2b). Low-dose treatment did not significantly enhance working memory performance for any variable. Finally, neither dose of hydroxyfasudil was associated with significantly altered spatial reference memory performance on the water radial-arm maze or Morris water maze. Indeed, there were no drug treatment main effects or interactions for reference memory errors on the water radial-arm maze, nor for distance scores on the Morris maze.

The data presented here implicate ROCK activity in the processes of both learning and the ability to handle a high working memory load. In addition, there were no effects of hydroxyfasudil on reference memory. Thus, the effects of hydroxyfasudil were working memory specific, which may implicate effects in part, on the frontal cortex. Indeed, the frontal cortex is intimately involved with working memory in both humans and rodents (Miotto, Bullock, Polkey, & Morris, 1996; Poucet, 1990; Poucet & Herrmann, 1990). Although there has been some research evaluating the

effects of ROCK inhibition on variables such as pain perception and anxiety, and one study evaluating memory retention after hippocampal infusion of a ROCK inhibitor in the young rodent, aside from the current report there has been no study evaluating the effects of peripheral administration of a ROCK inhibitor on spatial cognition (Buyukafsar et al., 2006; Dash, Orsi, Moody, & Moore, 2004; Lamprecht, Farb, & LeDoux, 2002; Saitoh et al., 2006). Thus, the current study is the first to examine the effect of peripheral delivery of hydroxyfasudil on spatial cognition as well as the first to test the mnemonic effects of ROCK inhibition in an older population of animals.

These findings underscore the potential of the promises of genome-wide association studies. Our interest in ROCK as a potential target for cognitive enhancement arose from the elucidation of the genetic involvement of KIBRA in human episodic memory performance in our previously published association study (Papassotiropoulos et al., 2006). The evidence detailed in this manuscript illustrates that it is possible to move from a validated human genetic association to an informed pharmaceutical decision. In our approach we first searched for targets with existing pharmaceutical agents that were at an advanced clinical stage. The optimal study design might be envisioned to directly pharmaceutically target KIBRA, and the investigation of agents that may be capable of disrupting key KIBRA interactions are currently ongoing. In lieu of directly altering the activity of the genetically associated gene product, the approach used in the present study was to influence the biomolecules that interact with it, in turn, resulting in the functional consequences of cognitive change. This approach may be widely applicable to many of the associated genes currently being reported in the literature.

Our current working hypothesis is that PKC- ζ activity modulation alters learning and memory through KIBRA. It has been shown in astrocytoma cells that ROCK inhibition leads to a subsequent increase in Rac1 activity (Salhia et al., 2005). Rac1 activation has also been demonstrated to modulate the activity of PKC- ζ (Liu et al., 2006; Scott et al., 2007; Ueberall et al., 1999). In addition, pioneering work has shown that a unique form of PKC- ζ , termed PKM- ζ , is essential for long term potentiation and plays a significant role in the process of differing forms of memory as well (Ling et al., 2002; Pastalkova et al., 2006; Shema, Sacktor, & Dudai, 2007). Based on these reports, we hypothesized that inhi-

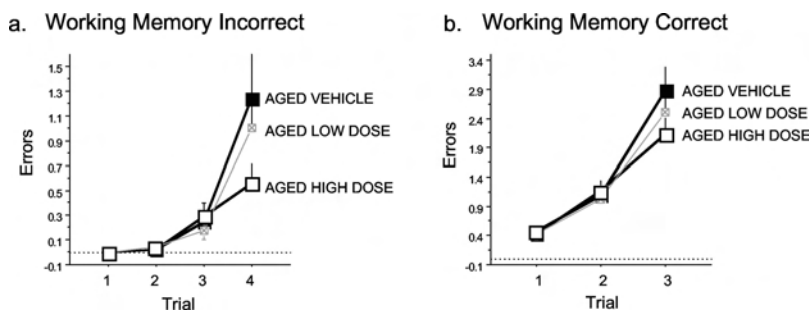


Figure 2. Hydroxyfasudil treatment improves working memory in aged rats. Shown are the $M \pm SE$ working memory incorrect errors across trials for the latter testing phase of the water-escape radial arm maze. Aged animals given high dose hydroxyfasudil treatment were better able to handle an increased memory load as compared to aged animals given vehicle. Drug treatment by trial interactions were $p = .048$ and $p = .057$, respectively.

bition of ROCK leading to activation of Rac1 and PKC- ζ would in turn phosphorylate KIBRA and alter its activity. However, as of yet the mechanism related to the nootropic benefits we observed with hydroxyfasudil are unknown. Fasudil, the parent compound, has been shown to exhibit direct neuroprotective effects against ischemia (Satoh, Toshima, Ikegaki, Iwasaki, & Asano, 2007; Yamashita et al., 2007) as well as influence vasodilation, which could then in turn influence cognitive function (Jiang et al., 2007; Nagata et al., 1993). Although further experiments are necessary to confirm the mechanism/s underlying hydroxyfasudil-induced functional improvements on learning and memory, the present results can be considered an initial step in that direction providing support for the hypothesis that ROCK inhibition initiates a cascade of events that can influence cognition.

Our findings suggest that peripheral administration of the ROCK inhibitor hydroxyfasudil improves spatial learning and memory, a finding that may have clinical relevance considering that the parent drug (Fasudil) was found to be safe and extremely well tolerated when used in the human clinic in multiple-dosage forms. The collected findings and the relative safety of fasudil support the potential of this ROCK inhibitor as a cognitive enhancer in humans that have age- or neurodegenerative-related memory dysfunction.

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