Treatment Strategies Targeting Excess Hippocampal Activity Benefit Aged Rats with Cognitive Impairment

Ming Teng Koh, Ph.D.[1], **Rebecca P. Haberman, Ph.D.**[1], **Stacey Foti, Ph.D.**[2], **Thomas J. McCown, Ph.D.**[2,3], and **Michela Gallagher, Ph.D.**[1]

[1] Department of Psychological and Brain Sciences, Johns Hopkins University

[2] Gene Therapy Center, University of North Carolina at Chapel Hill

[3] Department of Psychiatry, University of North Carolina at Chapel Hill

Abstract

Excess neural activity in the CA3 region of the hippocampus has been linked to memory impairment in aged rats. We tested whether interventions aimed at reducing this excess activity would improve memory performance. Aged (24–28 mo-old) male Long-Evans rats were characterized in a spatial memory task known to depend on the functional integrity of the hippocampus, such that aged rats with identified memory impairment were used in a series of experiments. Over-expression of the inhibitory neuropeptide Y 13–36 in the CA3 via adeno-associated viral transduction was found to improve hippocampal-dependent long-term memory in aged rats that had been characterized with impairment. Subsequent experiments with two commonly used antiepileptic agents, sodium valproate and levetiracetam, similarly produced dose-dependent memory improvement in such aged rats. Improved spatial memory with low doses of these agents was observed in both appetitve and aversive spatial tasks. The benefits of these different modalities of treatment are consistent with the concept that excess activity in the CA3 region of the hippocampus is a dysfunctional condition that may play a key role underlying age-related impairment in hippocampal-dependent memory processes. Because increased hippocampal activation occurs in age-related memory impairment in humans as observed in functional neuroimaging, the current findings also suggest that low doses of certain antiepileptic drugs in

Correspondence: Dr. MT Koh, Department of Psychological and Brain Sciences, Johns Hopkins University, Ames Hall, 3400, North Charles Street, Baltimore, MD 21218 USA. Tel: +1 410 516 7724; Fax: +1 410 516 0494; mtkoh@jhu.edu.

cognitively impaired elderly humans may have therapeutic potential and point to novel targets for this indication.

Keywords

neurocognitive aging; neural hyperactivity; water maze; radial arm maze; mild cognitive impairment; NPY

INTRODUCTION

Aging is often associated with a decline in memory functions that depend on the hippocampus. Recent research on neurocognitive aging has focused on localized increased neural activity that may drive dysfunction underlying memory impairment in the hippocampal network (Wilson *et al*, 2006). Excess neural activity in the CA3 region of the hippocampus occurs in aged rats with memory impairment when such neurons are unable to encode new information in a manner usually observed in young rats (Wilson *et al*, 2003; Wilson *et al*, 2005). The current research was designed to test the functional significance of such excess activity on behavioral performance in hippocampal-dependent spatial memory tasks.

Specialized functions of the subregions of the hippocampus have recently elucidated a critical role for the CA3/dentate gyrus in the encoding of information. When young rats first explore a familiar arena and then are placed in a novel environment, CA3 cells with location-specific encoding (e.g., place fields) rapidly form different patterns of spatial encoding for the two environments (Leutgeb *et al*, 2004; Leutgeb *et al*, 2007). In aged rats, on the other hand, hippocampal CA3 neurons can have very similar spatial representations across environments (Wilson *et al*, 2003; Wilson *et al*, 2005). Hence, rather than creating a new representation for a novel environment, aged rats tend to retrieve a previously stored representation, a process known as 'pattern completion', distinguishing it from 'pattern separation'. Furthermore, the extent of this failure to encode new information predicts the degree of spatial learning impairment (Wilson *et al*, 2003). This positive correlation between behavior and physiology supports the idea that the encoding failure of hippocampal CA3 neurons contributes to impaired performance in settings that depend on spatial memory. The concurrent localization of excess activity in the CA3 population of pyramidal neurons also suggests a dysfunctional role for such elevated firing rates which may shift the computational properties of this network (Wilson *et al*, 2006).

We tested whether treatments aimed at reducing excess neuronal activity in aged rats would improve memory performance. Boosting neuropeptide Y (NPY) neurotransmission, especially through Y2 receptors, has been shown to effectively attenuate neuronal excitability in seizure models (Foti *et al*, 2007; Morris *et al*, 2007), indicating a potential application to reduce elevated activity associated with neurocognitive aging. Hence, we first examined whether increasing NPY Y2 receptor activation via adeno-associated virus (AAV) delivered into the CA3 of the hippocampus would improve memory in cognitively impaired aged rats. We then tested two different pharmacological agents that are widely used antiepileptic agents, sodium valproate (VPA) and levetiracetam (LEV). In each case,

improved behavioral performance was produced by these treatments in aged rats with identified cognitive impairment, suggesting that excess neural activity in the CA3 region contributes to behavioral memory deficits. A test of LEV in young adult rats showed no such benefit, further supporting efficacy in the condition of memory impairments in aging.

METHODS

Subjects

Aged, male Long-Evans rats were obtained at 8–9 mo of age from Charles River Laboratories (Raleigh, NC) and housed in a vivarium at Johns Hopkins University until 24–26 mo of age. Young rats obtained from the same source were housed in the same vivarium and tested at 6 mo of age. All rats were individually housed at 25°C and maintained on a 12 hr light/dark cycle. Food and water were provided ad libitum unless noted otherwise. The rats were examined for health and pathogen-free status throughout the experiments, as well as necropsies at the time of sacrifice. All procedures in the current investigations were approved by the Institutional Animal Care and Use Committee in accordance with the National Institutes of Health directive.

Background behavioral characterization

All rats were screened in a standardized assessment of spatial cognition prior to the studies with experimental treatments. That background assessment used a well-established Morris water maze protocol as described in detail elsewhere (Gallagher *et al*, 1993). Briefly, the rats were trained for eight days (three trials per day) to locate a camouflaged escape platform that remained at the same location throughout training in a water maze. Every sixth trial consisted of a probe trial (free swim with no escape platform) that served to assess the development of a spatially localized search for the escape platform. During these probe trials, a learning index was generated from the proximity of the rat to the escape platform and was used to define impairment in the aged rats. The learning index is the sum of weighted proximity scores obtained during probe trials, with low scores reflecting a search near the escape platform and high scores reflecting searches farther away from the platform (Gallagher *et al*, 1993). Cue training (visible escape platform) occurred on the last day of training to test for sensorimotor and motivational factors independent of spatial learning. Aged rats with impaired spatial memory performance (i.e., those with learning index scores outside the young "normative" range) but successful cued training performance were used for the studies as described below.

Surgery and treatments

In the NPY experiment, memory-impaired aged rats were assigned to either NPY or control treatment (n = 6 per group), with group assignment matched for learning index scores in the background characterization. The rats were injected with either an AAV vector that expresses and constitutively secretes NPY 13–36 (targeting Y2 receptors) or control substances (AAV-GFP or saline) bilaterally into the CA3 area of the hippocampus (2 μl per side). That surgery, performed under isoflurane anesthesia, occurred two weeks prior to further behavioral assessment. Stereotaxic coordinates were 3.6 mm posterior to bregma, 3.0 mm lateral to midline, and 3.8 mm ventral to the skull surface. AAV-NPY 13–36 vector

contained a chicken beta-actin (CBA) promoter driving expression of a fragment of the NPY gene encoding amino acids 13–36 fused to the fibronectin signal sequence (FIB-NPY 13–36). The addition of the fibronectin signal sequence results in constitutive, unregulated secretion of NPY 13–36 protein from the transduced cells. While the FIB-NPY 13–36 mRNA is readily detected, the secreted protein degrades rapidly making immunohistochemical detection difficult (Haberman *et al*, 2003). The AAV-GFP control vector contained the CBA promoter driving enhanced green fluorescent protein. AAV virus was made using standard procedures (Foti *et al*, 2007). All virus titers were approximately 1 $\times 10^{12}$ particles per ml.

In an initial VPA experiment, memory-impaired aged rats were implanted subcutaneously in the intrascapular region with osmotic mini-pumps (ALZET, Durect Corporation, Cupertino, CA) with VPA (50 or 100 mg/kg/day; Sigma-Aldrich, St. Louis, MO) or saline vehicle starting two weeks prior to the intervention experiments. The VPA treatment at 100 mg/kg/day via osmotic mini-pump was determined to produce a mean total VPA concentration of 10 μg/ml (SEM = 1.28; n = 20) in the plasma of aged rats, a level that agrees with those reported by others when similar dosing with VPA was also chronically administered via osmotic mini-pumps (Stout *et al*, 2001). Other behavioral experiments used acute administration of VPA or LEV (Sigma-Aldrich, St. Louis, MO or Tecoland Corporation, Edison, NJ) given by systemic injection 30–40 min prior to test sessions.

Behavioral assessment in the water maze

Rats were trained and tested in a novel water maze environment to assess the effect of the treatments. The water maze used here was housed in a different building and was surrounded by curtains with a novel set of patterns relative to the maze used for initial assessment of cognitive status. The training protocol used in the NPY experiment and in an assessment of LEV consisted of 6 trials per day for 2 days to locate a submerged escape platform. On each trial, a rat was released in the maze from one of four equally spaced starting positions around the perimeter of the pool. The starting position varied from trial to trial. If the rat did not locate the escape platform within 60 s on any trial, the experimenter guided and placed the rat on the platform, where it remained for 20 s. The rat was then removed from the platform and placed in a holding cage for another 40 s before the next trial. Approximately 24 hr after the last training trial, a probe test in the absence of the escape platform was given to assess spatial memory.

The training protocol for the VPA experiment in the water maze was based on a modified water maze task that has been shown to be hippocampal-dependent (de Hoz *et al*, 2005; Steele & Morris, 1999). Unlike the traditional water maze protocol (such as that used in the background assessment and the NPY and LEV experiments) wherein the escape platform location remained constant throughout training, the escape platform location in this version of the task varied from day to day. During each day's training session, rats were given six trials to locate the submerged escape platform. At the end of the training session, the rats were returned to their home cages. After a 6-hr delay, the rats were returned to the water maze for a retention trial with the submerged platform located in the same position as in the prior session of training trials. Each rat was given 60 s to locate the platform. Rats were pre-

trained on this protocol for two days, and then trained and tested in this manner for three consecutive days. Performance on these three days was averaged for analysis using the escape latency and path to locate the platform as measures of retention; spatial bias in a free swim was not used because the repeated training protocol encourages rats to search for a new location when escape is not readily achieved.

Behavioral assessment in the radial arm maze

A radial arm maze task was used to assess effects of acute drug treatment with LEV, VPA, and the combination of those drugs. This protocol allowed within-subject assessment across drugs at different doses and in combinations. The radial maze consisted of eight arms projecting from each side of an octagonal center platform, with a food well located at the distal end of each arm. Plexiglas blocks could be positioned to prevent entry into any arm. Extra-maze cues were provided in the room surrounding the maze and illumination was provided by an overhead light.

Pre-training, as described in detail in Chappell et al. (1998), consisted of habituation, standard win-shift training, and win-shift training with delays interposed between information and memory test phases. Drug treatments began two days after the completion of pre-training. Three arms were blocked at the beginning of each trial (information phase). The identity and configuration of the blocked arms were varied across trials. Food-deprived rats were allowed to retrieve food reward (Kellogg's Froot Loops cereal) from the three unblocked arms. The rat was then removed from the maze for 60 min (retention interval), during which time the barriers on the blocked arms were removed allowing access to all eight arms. Rats were then placed back onto the center platform and allowed to retrieve the remaining food rewards (memory test phase). An error consisted of returning to an arm (all four paws on the arm) from which food had already been obtained. Memory-impaired aged rats (n = 10) were first tested with a series of LEV doses in ascending/descending order; each dose was thus tested twice, with one washout day in between each determination. At the completion of LEV assessment, the same rats (n = 9, because one rat died) were tested with a series of VPA doses in the same fashion as that for LEV assessment with performance at each dose tested twice. About a week after the completion of the individual drug assessments, the rats were given combination drug dosing in which ineffective doses of LEV and VPA in monotherapy were administered in combination (n = 8, one rat had to be dropped due to health deterioration). The number of errors made in the retention phase after the one hour delay was used to assess memory performance.

To determine whether treatment efficacy is age specific, young rats (n = 8) were tested with varying doses of LEV in the same fashion as that in the aged rats, except that a 5- hr retention interval was used to approximate the number of errors committed by memory-impaired aged rats under drug free condition as described above.

In situ hybridization

At the end of the NPY experiment, the brains of the rats were harvested following perfusion. A riboprobe template targeting the FIB NPY 13–36 sequence was generated by PCR from rodent whole hippocampus RNA and modified to contain SP6 and T7 RNA polymerase

sites. ^{35}S-UTP labeled riboprobe was generated using the Maxiscript kit (Ambion). Brain sections (40 μm) from across the entire extent of the hippocampus were hybridized overnight at 60°C in buffer containing the radiolabeled riboprobe. Hybridized sections were then extensively washed and mounted onto slides.

RESULTS

Background assessment of cognitive status

Rats were tested for cognitive status prior to the intervention experiments using a protocol developed in this study population. Figure 1 shows the mean learning index of memory-impaired aged rats (n = 67) subsequently used in the current experiments, and for comparison, the learning index of young rats (n = 122) that were tested concurrently with these aged rats during background characterization. Higher learning index scores signify worse performance by reflecting search at a greater distance from the escape location during memory probe tests. As only aged rats that performed beyond the normative range of young adult rats in this study population were selected for the subsequent experiments, it is not surprising that the performance of these aged rats was significantly impaired relative to the young rats, $t(187) = 13.97$, $p = .001$.

Spatial memory under AAV-NPY treatment

Rats were assigned to treatment groups based on the background characterization [mean learning indexes were 273 (SEM = 14.5) and 279 (SEM = 14.7) for experimental and control groups, respectively]. Two weeks after NPY or control injection, aged rats were trained and tested in a novel water maze environment. Figure 2A shows the average escape latencies over four blocks of training trials (3 trials per block). An analysis of variance (ANOVA) with repeated measures showed significant improvement over the course of training as indicated by decreased escape latencies in a main effect of Trial Block, $F(3, 30) = 3.24$, $p = .036$. Rats in the NPY group also demonstrated significantly better performance than the age/impairment-matched control group as indicated by a significant Group effect, $F(1, 10) = 6.28$, $p = .031$. Even though the Group × Trial Block interaction was not significant, $F(3, 30) = 1.12$, $p = .358$, the overall better performance of the NPY Group was not evident on the first training trial, $t(10) = 0.537$, $p = .603$. The group difference in escape latencies was also not due to swim speed performance because there was no significant difference on that measure during training trials for rats in the two groups (NPY = 17.1 cm/s, SEM = 1.37; control = 15.6 cm/s, SEM = 2.48).

A probe test was subsequently given 24 hr after training in which the escape platform was removed. As expected for aged rats characterized with impairment, the control group showed no evidence of memory, searching a similar amount of time in all quadrants of the maze (Figure 2B). In contrast, the NPY group exhibited retention for the escape platform location as indicated by a strong spatial bias for the target quadrant. An ANOVA with repeated measures showed a marginally significant Group × Quadrant interaction overall, $F(3, 30) = 2.91$, $p = .051$. Further analysis confirmed that rats in the NPY group demonstrated a spatial bias for the target quadrant, $t(5) = 2.86$, $p = .035$ (compared to the average search in the other quadrants). In contrast, that analysis showed no significant

spatial bias in the performance of rats in the control group, t < 1. Finally, rats in the NPY group spent significantly more time in the target quadrant compared to those in the control group, t(10) = 2.33, p = .042.

Photomicrographs of hippocampal sections of aged rats in the NPY group confirmed that FIB-NPY 13–36 mRNA was present in the area of AAV injection (Figure 3), while control brains demonstrated absence of specific probe hybridization.

VPA treatment on memory performance in the water maze task

The effect of chronic treatment with the antiepileptic drug, VPA, was assessed in separate groups of aged rats implanted with osmotic mini-pumps as described in methods (50 mg/kg, n = 9; 100 mg/kg, n = 13; vehicle treatment, n = 17). Group assignment was matched for learning index scores [291 (SEM = 10.8), 290 (SEM = 5.4), and 297 (SEM = 7.7) for VPA 50 mg/kg, VPA 100 mg/kg, and vehicle groups, respectively]. In this study, rats were trained to new spatial locations in each day's training session and a retention test occurred at a 6 hr delay. Figure 4 shows the path length (A) and escape latency (B) data for rats that received treatment of vehicle (VEH), VPA at 50 mg/kg/day (VPA 50), or VPA at 100 mg/kg/day (VPA 100) averaged over three new escape locations. During training, rats in all three groups improved within the day's session, attaining similar proficiency in performance by the last training trial. For path length, an ANOVA with repeated measures showed a significant main effect of Trial, F(5, 180) = 19.94, p = .001. There was also a main effect of Group, F(2, 36) = 4.02, p = .027, reflecting somewhat longer paths in rats that received VPA at 50 mg/kg relative to the groups that received vehicle or VPA at 100 mg/kg. The three groups however were not different from each other at the end of training, p > .304 (Trial 6). Similar results were obtained in the analysis of escape latency. Rats took progressively less time to escape over trials, F(5, 180) = 6.27, p = .001, with a trend for group differences, F(2, 36) = 2.81, p = .073, but no performance difference among the groups at the end of training sessions, p > .661.

The rats were given a retention trial after a 6-hr delay to assess memory of the prior session's learning. Better retention was evident in rats treated with VPA at 100 mg/kg/day for both escape latency and path. A one-way ANOVA indicated a significant group effect, F(2, 36) = 4.258, p = .022 for path length, and F(2, 36) = 7.606, p = .002 for escape latency. Further group comparisons confirmed that rats in the VPA 100 group had significantly shorter path lengths and escape latencies than those in the VEH and VPA 50 groups (all p values < .05). The VEH and VPA 50 groups were not different from each other on either measure, p values > .927. Taken together, these results indicate that chronic VPA treatment dose-dependently improves retention of new spatial information in aged rats previously characterized with hippocampal-dependent cognitive impairment.

VPA, LEV, and combination therapy on the radial arm maze task

To further assess the effects of antiepileptic drugs in aged impaired rats, we used a radial arm maze task that allowed a within-subject design to compare two different compounds, VPA and LEV. This task also extended the behavioral assessment to spatial memory using appetitive motivation. An independent set of aged rats (n = 10), which were characterized

with impairment in the background water maze assessment (mean learning index = 266, SEM = 7.1) served as subjects. Figure 5 shows the memory performance of those rats treated with varying doses of VPA by subcutaneous (s.c.) injection and LEV by intraperitoneal (i.p.) injection as indexed by the number of errors made during post-delay memory tests. Differences in memory performance were evident under VPA treatment as a function of dose, $F(4, 32) = 2.87$, $p = .039$. The VPA doses at 100 and 200 mg/kg significantly reduced errors relative to vehicle, $t(8) = 6.54$, $p = .001$ and $t(8) = 2.39$, $p = .044$, respectively. LEV also appeared to decrease errors in the post-delay tests. An overall analysis of LEV administration in the aged rats revealed differences in performance as a function of dose, $F(5, 45) = 2.38$, $p = .053$, with rats making fewer errors on 5 and 10 mg/kg of LEV compared to saline, $t(9) = 3.53$, $p = .006$ and $t(9) = 2.25$, $p = .051$, respectively. These results therefore confirm the initial findings in the water maze with VPA, extend the improvement seen with that treatment to an appetitive spatial memory task, and suggest that another antiepileptic drug, LEV, has similar benefit in a low dose range.

We also tested combinations of LEV and VPA at sub-threshold doses (when administered individually) to assess the possibility that these drugs might improve memory when given in combination. Prior to those drug tests, we first re-established baseline performance with a combination of i.p and s.c. saline injections. Figure 6A shows the results of combination doses and the comparison vehicle treatment. Compared to vehicle, the combination of VPA 50 mg/kg and LEV 2.5 mg/kg, as well as the combination of VPA 50 mg/kg and LEV 1.25 mg/kg, were effective at significantly reducing memory errors, $t(7) = 2.49$, $p = .042$, and $t(7) = 2.68$, $p = .032$, respectively. The combination of VPA 25 mg/kg and LEV 2.5 mg/kg, however, did not differ from vehicle. We plotted those data in an isobologram. As indicated in the isobologram (Figure 6B), the dose combination of VPA 50 mg/kg and LEV 1.25 mg/kg falls below the line/isobole of additivity, suggesting super-additive or synergistic interaction between VPA and LEV in that combination of doses.

LEV treatment on memory performance in the water maze task

In a final experiment, acute treatment with LEV at 10 mg/kg was given to impaired aged rats (n = 6; mean learning index = 280, SEM = 6.6) during water maze training using a within-subject design to compare drug performance with saline. Hence upon completion of assessing the first treatment condition (drug or vehicle, counterbalanced), the rats received the other treatment starting the next day in the same maze but surrounded by a new set of curtains with a novel configuration/color of cues, and a new escape platform location. Figure 7A shows performance during training trials. An ANOVA with repeated measures revealed that the rats improved during training as their escape latencies significantly decreased over trials, $F(3, 15) = 5.58$, $p = .009$. Although performance appears better under drug than saline at the end of training, no statistical difference was revealed involving treatment; that is, no significant Treatment × Trial Block interaction ($p > .25$) or main treatment effect ($p > .60$).

A memory probe test was given 24 hr after the end of training (data in Figure 7B). An ANOVA with repeated measures showed a strong trend for a Treatment × Quadrant interaction, $F(3, 15) = 3.14$, $p = .057$ in which LEV treatment during training augmented subsequent retention for the escape platform location. A spatially localized search was

evident in time spent in the target quadrant, which was significantly greater when rats had been treated with LEV compared with saline, $t(5) = 2.80$, $p = .038$. Overall then, these results indicate that LEV treatment at 10 mg/kg benefits long-term spatial memory in aged rats that are otherwise impaired.

LEV treatment on memory performance in young rats

Based on our hypothesis of targeting excess hippocampal activity, we expected that these treatments might only benefit aged rats but not young rats. In a study run in parallel with the aged rats, injection of AAV-NPY into the CA3 of young rats (6-mo old) produced no improvement in the water maze performance beyond that of control rats (data not shown). That study however was limited by strong memory performance in the young control rats, thus possibly precluding the detection of improvement in the experimental treatment group. In order to address that limitation, we ran a separate study with a new batch of young rats using the radial arm maze and imposing a lengthy delay between information and test phase (5-hr delay) to attain a number of errors similar to those of aged rats at the shorter delay. Prior to treatment, these young rats had received the same background water maze assessment (mean learning index = 191, SEM = 10.1) as the aged rats. They were then tested on the radial arm maze with a series of LEV doses in the same fashion as that in the aged rat study. Figure 8 shows the memory performance of young rats treated with varying doses of LEV as indexed by the number of errors made during post-delay memory tests. No significant differences in performance were found as a function of dose, $F(5, 35) = 0.46$, $p = .806$. The data indicate that this pharmacological treatment aimed at reducing excess hippocampal activity only benefits memory performance in aging.

DISCUSSION

The studies reported here were designed to test the hypothesis that excess neural activity in the hippocampus of aged rats contributes to memory impairment. We first over-expressed an inhibitory neuropeptide (NPY 13–36) targeting the CA3 region of the hippocampus of aged rats using methods shown to attenuate aberrant excitability in limbic seizure models. We also tested two widely used antiepileptic agents administered systemically. In each case, aged rats with established memory impairment exhibited improved behavioral performance in spatial memory tasks. The converging evidence for benefits of these treatments indicates that excess excitation in the hippocampus may play a key role underlying age-related impairment in hippocampal-dependent memory processes as proposed for this model (Wilson *et al*, 2006).

Aged rats in the current research came from a study population with well-characterized individual differences in memory impairment. Importantly, prior studies have shown a high degree of test-retest reliability for the presence and severity of those individual differences in behavioral assessments; aged rats that fall outside the range of normative young performance in the standardized assessment exhibit a similar behavioral profile in subsequent tests of hippocampal-dependent memory (Colombo *et al*, 1997; Robitsek *et al*, 2008). Such evidence increases confidence that the assignment of subjects based on the standardized background assessment ensured a valid test of treatment efficacy in

experiments using between group comparisons. In addition, the data obtained in the experiments using within-subject designs support the reliability of individual differences because impairment was routinely evident under vehicle treatment in those studies, as discussed further below.

The hypothesis examined here is also grounded in much background research in this model demonstrating that behavioral status in the aged rat study population is strongly correlated with neurobiology in the underlying circuitry of the hippocampal formation. While aged rats in this study population maintain similar numbers of neurons and largely preserved synaptic connections in the medial temporal lobe system (Geinisman *et al*, 2004; Rapp & Gallagher, 1996; Rapp *et al*, 2002; Smith *et al*, 2000), functional alterations are closely tied to age-related cognitive decline. The striking increase in neural activity in the CA3 region led to a proposal for the basis of cognitive impairment, which links that excess activity to loss of encoding that normally depends on the CA3/dentate gyrus subdivisions of the hippocampal network (Wilson *et al*, 2006). Because the majority of synaptic input to the CA3 pyramidal neurons comes from the excitatory recurrent CA3 collaterals, elevated activity would be expected to promote pattern completion, a computational function attributed to the CA3 autoassociative network that favors retrieval of previously stored representations (Guzowski *et al*, 2004; Treves and Rolls, 1994). In addition, the joint occurrence of elevated activity and reduced plasticity is well established in studies of neural networks, including the hippocampus; synaptic plasticity most readily occurs in an optimal range of activity, and thresholds for synaptic modification can rise in conditions of excess activity (see Abraham *et al*, 2001 for example, and Abraham, 2008 for recent review). Recording data from hippocampal neurons in this study population are in agreement with this concept linking excess activity to a failure to encode new information in the CA3 network (Wilson *et al*, 2005). By that view, experimental treatments that lower activity would be expected to lower the threshold for synaptic modification to allow the encoding of new information.

As an initial test of this idea, we locally expressed an inhibitory neuropeptide, NPY13–36, which has been shown elsewhere to be effective in modulating neuronal excitability under both normal and epileptic conditions (Foti *et al*, 2007; McQuiston & Colmers, 1996; Vezzani *et al*, 1999). NPY13–36 preferentially activates the Y2 receptor subtype which plays a predominant role in mediating the NPY-induced inhibition of glutamate release in the CA1 and CA3 of the hippocampus (El Bahh *et al*, 2005; Silva *et al*, 2005). We engaged the inhibitory property of Y2 receptor activation by constitutively expressing NPY 13–36 via an AAV vector delivered into the CA3; this resulted in significantly improved hippocampal-dependent memory in the aged rats. In situ histochemistry for the vector-produced mRNA confirmed that expression of NPY13–36 was restricted to the hippocampus and centered primarily on the CA3, likely providing only local control of inhibition in that area. Because no elevated activity is observed in the CA1 subregion of the hippocampus in our aging model (Wilson *et al*, 2005), and CA3 connections (Shaffer collaterals) onto CA1 neurons have reduced efficacy in the aged brain (Lister and Barnes, 2009), boosting inhibition in the adjacent CA1 area would not be expected to have beneficial effects on network function. These data therefore support the view that controlling excess neural activity in the CA3 of aged rats improves hippocampal network function and hippocampal-

dependent memory performance, and serves as a proof of principal for further exploration into more clinically accessible methods of modulating excess neural activity.

Subsequent tests centered on the same rationale of targeting excess excitation by administering two widely used antiepileptic agents, VPA and LEV, which each showed the ability to improve spatial memory in aged rats. In the first of those studies, VPA was assessed in a spatial memory task when aged memory-impaired rats learned new escape platform locations in each day's session in the water maze. Rats implanted with osmotic mini-pumps to administer 100 mg/kg had substantial savings after a 6 hr delay, escaping with similar proficiency relative to the end of the prior session's training trials. Those rats differed from the control vehicle group and a group receiving a lower dose of VPA, which exhibited little savings for prior training after the delay. In a subsequent study using a within-subject design, VPA doses of 100 and 200 mg/kg significantly improved memory performance on the radial maze after a 1-hr delay. The improvement obtained with effective doses of VPA reduced errors to levels typically seen in young rats tested on this task (Chappell *et al*, 1998). Together those experiments demonstrate a benefit from VPA treatment across spatial memory tasks with different motivational and performance demands. It is important to note that blood levels of VPA in rats at the doses effective for neurocognitive aging (10 μg/ml; see methods for details) are well below those needed for antiepileptic efficacy to control seizure activity (225 μg/ml and above; Tulloch *et al*, 1982). Indeed, high dosing might be less effective for cognitive improvement in aging by producing more pronounced and widespread effects on brain function.

Similar to VPA, LEV was also found to improve memory performance on the radial maze at doses substantially lower than those used in seizure models. Significant improvement was observed at 5 and 10 mg/kg but not at a higher dose of 20 mg/kg. Typical antiepileptic doses of LEV in rodent seizure models are in a range of 50–150 mg/kg (e.g., Ji-qun *et al*, 2005; Stratton *et al*, 2003). Additionally, the two antiepileptic compounds VPA and LEV exhibited benefit in combination at doses that were otherwise ineffective. Comparing the dose response for the individual drugs with the combinations provided evidence for drug synergy rather than merely additive effects. As an extension of the data obtained in the radial maze, improved retention over a 24 hr delay was confirmed with LEV at 10 mg/kg in an independent set of memory-impaired aged rats trained in a novel water maze environment. Finally, as expected under the view that benefit is due to dampening excess activity, we found no improvement in memory performance when young adult rats were given LEV.

Although the route by which VPA and LEV exert antiepileptic efficacy is not known with certainty, and those mechanisms may not directly apply to the doses used in the current studies, these two compounds share little apparent mechanistic overlap. The efficacy of VPA as an antiepileptic potentially involves a variety of mechanisms, including increased gamma-amino butyric acid (GABA)-ergic transmission, increased uptake of excitatory amino acids, and blockade of voltage-gated sodium channels (Perucca, 2002). At the low doses found to be effective in the current research, the most likely mechanisms involve control of excitatory transmission by reducing extracellular accumulation of glutamate and increased GABAergic transmission. An up-regulation of hippocampal glutamate transport occurs in rats treated with VPA (Hassel *et al*, 2001). We confirmed this finding at doses

used in our aged rats and further observed that low doses of VPA administered systemically increase the hippocampal expression of GLT1b, an isoform of the glutamate transporter (M. Gallagher, unpublished data). In contrast to the localization of GLT1a in astrocytes, GLT1b is localized to neurons in association with the post-synaptic density (González-González *et al*, 2008). Thus, VPA may permit the regulation of excitatory transmission at synapses that drive neuron activity in addition to boosting inhibition. LEV is thought to mediate its effects via a synaptic vesicle protein Sv2a, which renders primed synaptic vesicles competent for activity-dependent (calcium-mediated) release (Chang and Sudhof, 2009). At concentrations of up to 10 μM, LEV does not demonstrate binding affinity for a variety of known receptors, such as those associated with benzodiazepines, GABA, glycine, and NMDA (N-methyl-D-aspartate). *In vitro* and *in vivo* recordings of epileptiform activity from the hippocampus have shown that LEV inhibits burst firing. In vivo data also show that LEV opposes a pilocarpine-induced increase of the orthodromically-activated population spike in the CA3 region of hippocampus (Klitgaard *et al*, 2003). Synergistic activity has also been noted for use of VPA and LEV in combination in studies of seizure models (Kaminski *et al*, 2009).

Current data suggest that modifying excitatory-inhibitory balance through different routes can ameliorate behavioral memory impairment in aging. The benefit observed with these different treatments suggests that neural activity and encoding properties in the hippocampal network are ameliorated by targeting excess activity. Studies to directly monitor such effects, using electrophysiological recordings in awake behaving rats, are beyond of scope of the current investigation but will be informative as to whether such restoration is a final common pathway for behaviorally effective treatments in this model.

The functional significance of aberrant activity in the hippocampus may not be limited to animal models of aging but may also have direct implications for memory decline experienced during aging in humans. A number of investigations using functional neuroimaging in human aging have reported increased activation in the medial temporal lobe during memory encoding. This finding has now been observed in patients with mild cognitive impairment (MCI; Celone *et al*, 2006; Dickerson *et al*, 2004; Dickerson *et al*, 2005; Hämäläinen *et al*, 2007), in older individuals with genetic or familial risk for developing Alzheimer's disease (Bassett *et al*, 2006; Bookheimer *et al*, 2000), and in aged subjects exhibiting poor performance in memory but not sufficiently impaired to meet a diagnosis of MCI (Miller *et al*, 2008a). The original view of such increased activation was that it served a beneficial function by recruiting additional resources to provide "compensation" for a failing neural network. A possible detrimental role of excess activation in humans, however, has been suggested by findings in follow up with MCI patients (Dickerson *et al*, 2004; Miller *et al*, 2008b). Specifically, Miller and colleagues (2008b) found that higher activation in the hippocampus, but not in signals imaged elsewhere in the brain, predicted greater subsequent cognitive decline 4–8 yrs after initial evaluation. Consequently, those authors suggested that elevated hippocampal activation might serve as a therapeutic target for early intervention in cognitive decline. An assessment of whether reduction in elevated hippocampal activation in humans leads to worse memory performance, as suggested by a compensation view, or improves performance, consistent with the animal data obtained here, would provide a needed experimental test of the

alternative interpretations now existing in the clinical literature. If the condition in humans parallels the excess activity observed in studies of aged animals with memory impairment, such a test would be feasible using FDA approved compounds (VPA and LEV) that are effective in aged animals, albeit at lower doses than those used for other clinical indications.

Not much more than a decade ago it was widely believed that loss of neurons in the brain was the cause of memory impairment associated with aging. Now it is generally accepted that memory decline is predominantly a functional disorder in aged brains that are largely structurally intact. This view gives hope to the prospect for corrective therapies that will aid in the restoration of function in the aged brain. Many recent approaches to this problem have focused on boosting mechanisms underlying neural plasticity in young adults (Josselyn & Nguyen, 2005; Rose *et al*, 2005 for reviews). The current research supports a novel approach based on a disorder specific to the condition of neurocogntive impairment in the aged brain. As such, the findings may point to novel targets for therapies in this indication.

Acknowledgments

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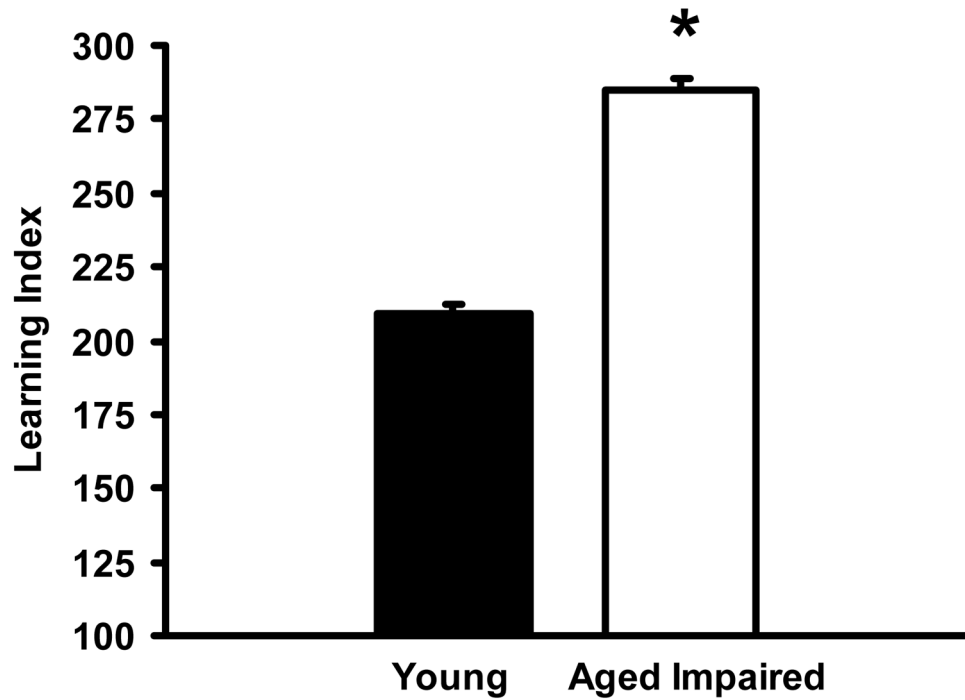
Figure 1.
Background behavioral characterization. The cognitive status of aged and young rats was assessed in a standardized water maze task before intervention experiments. Higher learning index scores signify worse performance by reflecting search at a greater distance from the escape location during memory probe tests. Aged rats that performed beyond the normative range of young adult rats in this study population were selected for the present studies. * p = .001.
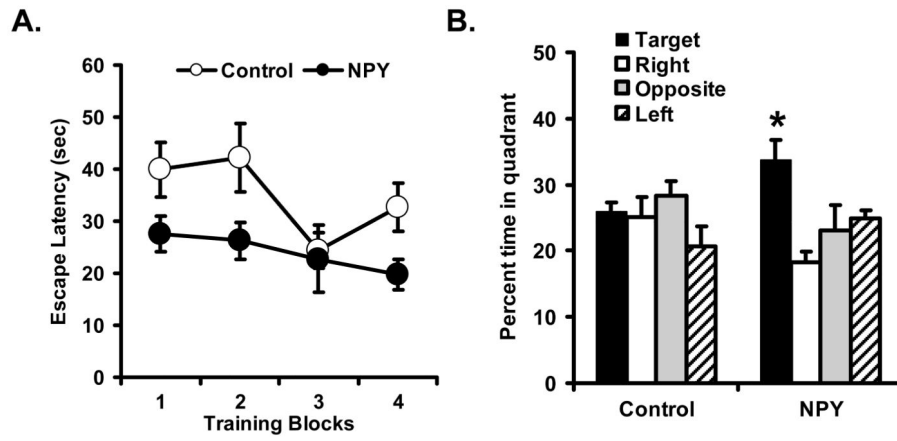
Figure 2.
Behavioral assessment of NPY treatment. Memory-impaired aged rats that received AAV-NPY 13–36 injections into the CA3 of the hippocampus two weeks prior performed better than control rats during water maze training (A), and showed stronger memory retention for the escape platform location in a probe trial 24 hr after training (B). See text for details of data analysis.
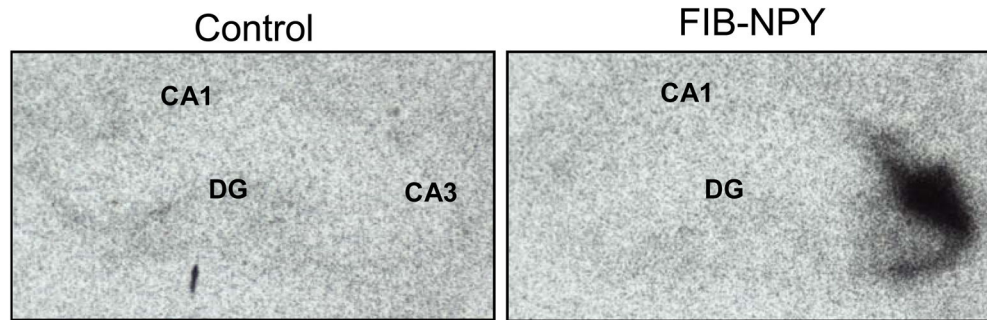
Figure 3.
AAV-NPY transduction in the hippocampus. Photomicrographs of coronal hippocampal sections of aged rats showing FIB-NPY 13–36 mRNA (right panel) was present in the NPY group, and the absence of specific probe hybridization in the brain of a control rat (left panel). In testing for the FIB-NPY in situ probe, we also generated and hybridized a sense control probe. This control probe showed very little signal as expected (not shown). DG = dentate gyrus.
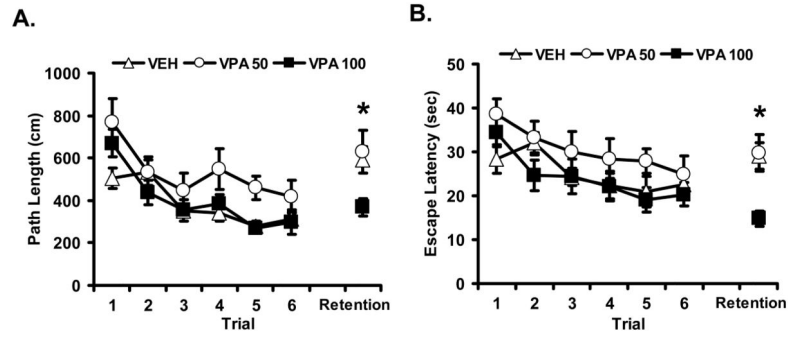
Figure 4.
VPA assessment in the water maze. Memory-impaired aged rats that received chronic treatment with VPA or saline performed at a comparable level at the end of the water maze training phase (Trial 6), but those treated with VPA at 100 mg/kg/day (VPA 100) showed less forgetting as measured by path length (A) and escape latency (B) after a 6-hr delay (Retention Trial) compared to their counterparts treated with saline (VEH) or VPA at 50 mg/kg/day (VPA 50). See text for details of data analysis.
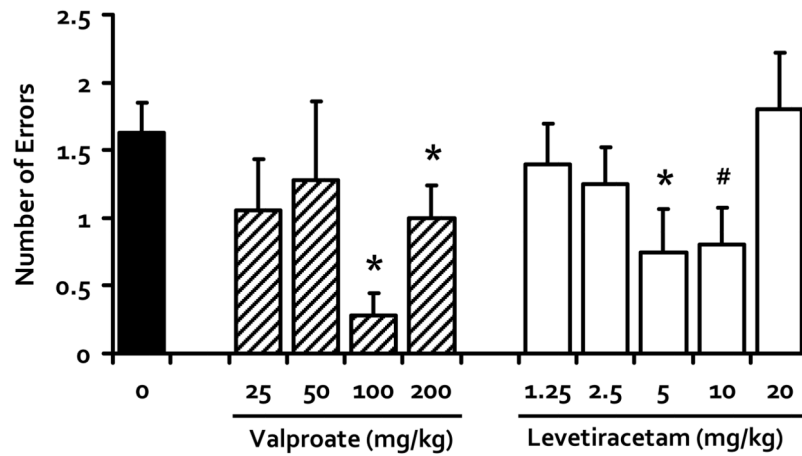
Figure 5.
VPA and LEV assessment in the radial maze. Efficacious doses of VPA and LEV to improve memory performance of aged rats with cognitive deficit were determined using a within-subject design in an eight-arm radial maze. Over the course of the study, the same rats were tested repeatedly with vehicle saline (0 mg/kg), VPA, and LEV. Post-delay memory errors were significantly lower than vehicle baseline when rats were treated with VPA at 100 and 200 mg/kg, as well as with LEV at 5 and 10 mg/kg. * $p < .050$ and # $p = .051$ compared to vehicle treatment (0 mg/kg).
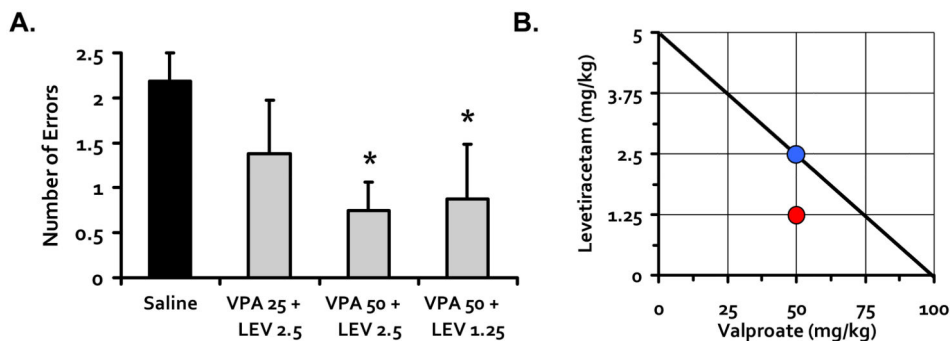
Figure 6.
Combination VPA/LEV assessment in the radial maze. Combination therapy consisting of VPA and LEV was assessed in the radial arm maze after each drug was tested individually over a range of doses. (A) Baseline performance was established with combination saline injections, and sub-threshold doses of each drug (VPA at 25 or 50 mg/kg, and LEV at 1.25 or 2.5 mg/kg) was administered in combination. To facilitate interpretation, effective combinations were then plotted on an isobologram (B). The diagonal straight line is the line of additivity, anchored on each axis by the lowest effective doses of VPA and LEV when assessed individually. Efficacious combinations at lower sub-threshold doses that fall on the line is interpreted accordingly as a simple additive effect, as is the case for the treatment of VPA 50 mg/kg combined with LEV 2.5 mg/kg. The dose combination of VPA 50 mg/kg and LEV 1.25 mg/kg falls below the line of additivity, suggesting super-additive or synergistic interaction between the two drugs.
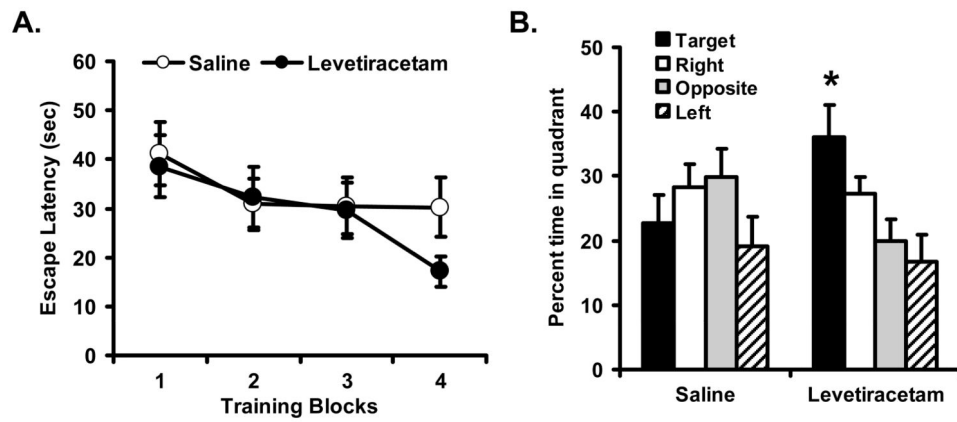
Figure 7.
LEV assessment in the water maze. Memory-impaired aged rats were injected with LEV at 10 mg/kg or saline vehicle before training in the water maze (A). Better memory retention was observed under LEV than saline treatment 24-hr later in a probe test (B). * $p < .050$ compared to percent time spent in target quadrant under saline treatment.
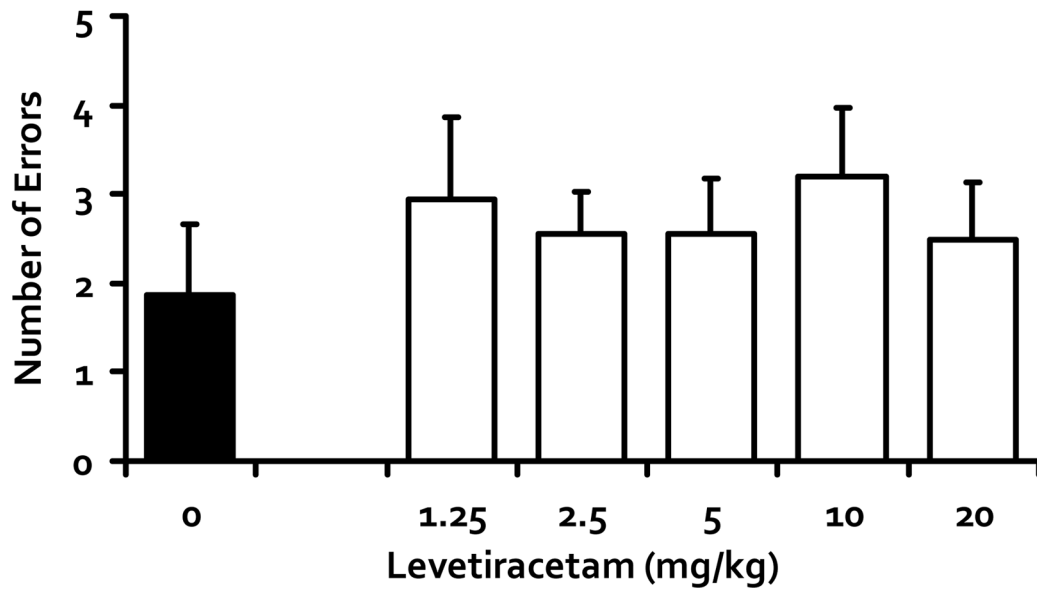
Figure 8.
LEV treatment in young rats. Young rats were treated with LEV at varying doses before testing in the radial arm maze using a 5-hr retention delay. No differences in memory performance were found as a function of dose.