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Increasing Hippocampal Acetylcholine Levels Enhances Behavioral Performance in an Animal Model of Diencephalic Amnesia

Jessica J. Roland^a, Katherine Mark^a, Ryan P. Vetreno^a, and Lisa M. Savage^{a,*}

^aBehavioral Neuroscience Program, Department of Psychology, Binghamton University-SUNY, Binghamton NY, 13902

Abstract

Pyrithiamine-induced thiamine deficiency (PTD) was used to produce a rodent model of Wernicke-Korsakoff syndrome that results in acute neurological disturbances, thalamic lesions, and learning and memory impairments. There is also cholinergic septo-hippocampal dysfunction in the PTD model. Systemic (Experiment 1) and intrahippocampal (Experiment 2) injections of the acetylcholinesterase inhibitor physostigmine were administered to determine if increasing acetylcholine levels would eliminate the behavioral impairment produced by PTD. Prior to spontaneous alternation testing, rats received injections of either physostigmine (systemic = 0.075 mg/kg; intrahippocampal = 20, 40 ng/μl) or saline. In Experiment 2, intrahippocampal injections of physostigmine significantly enhanced alternation rates in the PTD-treated rats. In addition, although intrahippocampal infusions of 40 ng of physostigmine increased the available amount of ACh in both Pair-fed (PF) and PTD rats, it did so to a greater extent in PF rats. The increase in ACh levels induced by the direct hippocampal application of physostigmine in the PTD model likely increased activation of the extended limbic system, which was dysfunctional, and therefore led to recovery of function on the spontaneous alternation task. In contrast, the lack of behavioral improvement by intrahippocampal physostigmine infusion in the PF rats, despite a greater rise in hippocampal ACh levels, supports the theory that there is an optimal range of cholinergic tone for optimal behavioral and hippocampal function.

Keywords

Hippocampus; diencephalons; microdialysis; acetylcholine; spontaneous alternation; rat

Introduction

Although cholinergic abnormalities are seen in a range of neurological disorders affecting cognition, the role of acetylcholine (ACh) in learning and memory still perplexes us (Gold 2004; Parent and Baxter 2004). Pharmacological augmentation of septohippocampal cholinergic activity enhances learning and memory performance in cognitively impaired animals (Frick et al. 1996; Markowska et al. 1995; Sabolek et al. 2004a) and reverse drug-induced amnesia (Degroot and Parent 2000; 2001; Flood et al. 1998). Cholinergic enhancing

*Corresponding Author, Phone: (607) 777 - 4383, FAX: (607) 777 - 4890, E-Mail: lsavage@binghamton.edu.

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drugs also prolong and optimize hippocampal rhythms (Colgin et al. 2003) as well as facilitate neurogenesis (Mohapel et al. 2005). However, selective lesions of the cholinergic neurons of the medial septal/diagonal band region (MS/DB) produced by the immunotoxin 192 IgG-Saporin produce contradictory results: In some cases behavioral impairment is observed (Chang and Gold 2004; Lehmann et al. 2003) and in other cases no impairment is observed (Pang and Nocera 1999; Vuckovich et al. 2004). Factors such as regional specificity, degree of cell loss, timing of behavioral training and nature of the task all appear to be important for understanding the role of ACh in cognitive impairment. A study readdressing the role of ACh in learning and memory stresses a more plastic role of ACh in neural activation. Rather than being the sole or key neuromodulator of learning and memory, ACh sets the appropriate dynamics for neural activity and for the occurrence of learning/memory within the extended hippocampal system (Gold 2004; Hasselmo and McGaughy 2004).

Although Wernicke-Korsakoff syndrome (WKS) was key in the development of the distinction between declarative (hippocampal-dependent) and non-declarative (nonhippocampal-dependent) memory systems, only a very limited number of such patients actually display gross anatomical hippocampal damage (Caulo et al. 2005; Victor et al. 1971). The key neuroanatomical damage in this disorder is primarily localized to the diencephalon, in particular the anterior and midline thalamus as well as the mammillary bodies (Harding et al. 2000; Kopelman 1995). This neuropathological profile is supported by thiamine deficiency in an animal model (Langlais et al. 1996; Mair 1994). However, human imaging studies of WKS patients (Caulo et al. 2005) and studies using animal models of WKS (Pires et al. 2005; Roland and Savage 2007; Savage et al. 2003) show that the hippocampus is not fully activated when challenged by behavioral demands. Therefore, although gross anatomical abnormalities in the hippocampus may not be common in WKS or the pyridoxamine-induced thiamine deficiency (PTD) rodent model of the disorder, it appears that functioning of the hippocampus is altered—particularly when environmental demands require hippocampal processing.

Furthermore, in the PTD model numerous cholinergic abnormalities have been documented in the septohippocampal pathway. A loss of neurons (Pitkin and Savage 2001; 2004; Savage et al. 2007) and fibers (Nakagawasai et al. 2000) staining positive for choline acetyltransferase (ChAT) in the MS/DB region and hippocampus have been observed. There is also a loss of functional efflux of ACh in the hippocampus during behavioral challenge (Roland and Savage 2007; Savage et al. 2003).

Thus, the septohippocampal system is dysfunctional in PTD animals. There is some limited evidence that this may also be the case in alcoholic-induced WKS in humans (Arendt 1994; Cullen et al. 1997). Two case study reports have found that five WKS patients demonstrated cognitive improvement when given anticholinesterase drugs (Angunawela and Barker 2001; Cochrane et al. 2005). The animal model data (loss of cholinergic neurons in the MS/DB, loss of infiltration of ChAT positive fibers into the hippocampus, decreased hippocampal ACh efflux during function testing) suggests such drugs would improve cognitive function by increasing available ACh levels within the hippocampus. The goals of the current studies were to replicate the systemic effects observed in case reports of five WKS patients (Angunawela and Barker 2001; Cochrane et al. 2005) in the PTD rat model and demonstrate that the cognitive enhancing effects of acetylcholinesterase (AChE) inhibitors in WKS and the PTD model are directly due to enhancing ACh levels in the hippocampus. Understanding the direct mechanism of action of drugs that improve cognitive functioning in WKS will provide the pathway for therapeutic development of cognitive enhancers.

Experiment 1

The cause of WKS is prolonged thiamine (vitamin B₁) deficiency. WKS is being diagnosed in countries all over the world and it is found most often in alcoholics; however, other groups are also at risk, such as those with eating disorders, persistent emesis, systemic disease, starvation, patients on long-term IV feeding, and those on dialysis (Harper 2006). The animal model of WKS, PTD, has provided a wealth of data regarding the brain and behavioral dysfunction that accompanies thiamine deficiency (Langlais et al. 1996; Mair 1994). However, despite knowing the etiology of WKS little progress has been made in terms of long-term treatment. The current treatment protocol for WKS consists of thiamine repletion, stabilization of electrolytes and nutritional modifications. All of which are only successful during the acute phase. Success of this treatment is highly variable and although many of the patients recover full ocular function and have improvement in their ataxia, only 20% of patients recover completely from the learning and memory impairments (Victor and Martin 1994).

Drugs that inhibit the breakdown of ACh (i.e. AChE inhibitors) have been shown to enhance learning/memory performance in humans with Alzheimer's disease (Braidia and Sala 2001; Darreh-Shori et al. 2006; Knapp et al. 1994; Sano et al. 1993) and in animal models of memory disorders (Barnes et al. 2000; Chang and Gold 2004; Dong et al. 2005; Mulder et al. 2005; Nakagawasai et al. 2000). Given the data suggesting cholinergic dysfunction in the animal models of WKS (Nakagawasai et al. 2000; Pires et al. 2005; Roland and Savage 2007; Savage et al. 2003) and case study reports showing improvement of cognitive function in WKS patients receiving drug treatment (Angunawela and Barker 2001; Cochrane et al. 2005), we assessed whether an i.p. injection of physostigmine would enhance spontaneous alternation performance in PTD-treated rats.

Subjects

A total of 32 male Sprague-Dawley rats (Harlan Co.) weighing 300–350 g were used. Prior to surgery and testing, subjects were housed two per cage on a 12-hr/12-hr light/dark cycle (light on at 7:00 am) with *ad libitum* access to Purina rat chow and water. Food was withheld for 12 hr prior to behavioral testing.

PTD treatment

Animals were first randomly assigned to one of the following treatments: (i) pair-fed control (PF, n = 16), or (ii) pyrithiamine-induced thiamine deficiency (PTD, n = 16) groups. Subjects in the PTD group were free-fed a thiamine-deficient chow (Teklad Diets, Madison, WI) and given daily injections (0.25 mg/kg, i.p.) of pyrithiamine HBr (Sigma, St. Louis, MO). On days 14–16 of treatment, animals display signs of local tonic-clonic movement of the front and hind limbs, and generalized convulsions (seizures). Within 4 hr after observing the onset of seizure, PTD-treated animals were given an injection of thiamine (100 mg/kg, i.p.) every 8 hr until the seizure activity disappeared and the rats regained upright posture. The PF animals were fed an amount of thiamine-deficient chow equivalent to the average amount consumed by the PTD groups on the previous day of treatment, and were given daily injections of thiamine HCl (0.4 mg/kg, i.p.). After treatment all subjects were placed on regular chow and allowed to regain the weight lost during treatment.

Pharmacological testing

The design of the experiment was a complete between-subjects model: Subjects in both the PTD and PF groups were further randomly subdivided into saline (PF, n = 8; PTD, n = 8) or physostigmine (PF, n = 8; PTD, n = 8) treatment groups. Thirty min prior to behavioral testing the animals received either an i.p. injection of physostigmine (0.075 mg/kg; Sigma, St. Louis, MO) or saline solution of the equivalent volume.

Behavioral testing

Thirty min after drug/saline injection, rats were placed on the center of the plus-maze with clear Plexiglas sidewalls (12 cm high) and a black floor with the four arms of equal distance (55 cm) situated 80 cm above the floor. Rats were allowed to transverse the maze freely and the number and sequence of arms entered were recorded to determine alternation scores. The percent alteration score is equal to the ratio of: (actual alternations/possible alterations) $\times$ 100. With the exception of the first arm selected, every time the animal chooses an arm there is the possibility of making an alternation. Therefore, possible alternations are defined as the total number of arms entered minus one. The maze testing room contained various extra-maze cues (posters, doors, tables, etc.).

Histology

Once the behavioral testing was completed, animals were anesthetized with Nembutal (~2.0 mg/kg, i.p.) and decapitated. Their brains were removed, post fixed in a 10% formalin solution for at least 72 hr, and then transferred to a 30% sucrose solution. The brains were blocked, first cutting 1 mm anterior to the optic chiasm and then 1 mm posterior to the pituitary, after which they were frozen and cut in 40 μ m sections. The brain was sliced and every 5th section was slide mounted until the posterior commissure and the end of the mammillary bodies were reached. The mounted sections were stained with cresyl violet stain and were evaluated for diencephalic damage (see Figure 1AB).

Results

Figure 2A displays a significant difference in the percent alternation between PF- and PTD-treated animals under saline and a lack of difference when groups were administered physostigmine. A two-factor ANOVA revealed a significant effect of Treatment (PF vs. PTD: $F [1, 28] = 9.96, p < .01$) and a significant effect of Drug (Saline vs. Physostigmine: $F [1, 28] = 9.71, p < .01$). The Treatment $\times$ Drug interaction failed to reach significance ($F [1, 28] = 2.32, p > .13$): Both PF and PTD rats injected with physostigmine did display some improvement over those injected with saline. However, planned contrasts revealed that main effect of Drug was primarily due to the dramatic enhancement of alternation rates (from 48.35% to 67.44%) of PTD rats in the physostigmine condition, relative to the saline condition ($F [1, 14] = 8.90, p < .01$). Although there was also a rise in alternation rate in the PF rats injected with physostigmine compared to those given saline (67.6% vs. 74.15%), it was not significant ($F [1, 14] = 1.60, p > .2$). The main effect of Group was driven by the saline condition in which PF rats had a higher rate of alternation than the PTD rats ($F [1, 14] = 12.77, p < .01$). In contrast, following an i.p. injection of physostigmine, the alternation levels of the two groups were not significantly different ($F [1, 14] = 1.16, p > .2$).

Figure 2B shows no significant difference in the average number of arms entered by PF- and PTD-treated animals, regardless of drug condition (all F 's $[1, 28] < 1$).

Discussion

The results of Experiment 1 demonstrated that cholinesterase inhibitors can enhance behavioral performance in the PTD model and that this increase in performance was not due to an increase in locomotor activity level. This effect could be due to the fact that the PTD-treated rats have an altered cholinergic state. Recently a number of studies have demonstrated that there are abnormalities in the forebrain cholinergic system of PTD-treated rats. Nakagawasai and colleagues (Nakagawasai 2005; Nakagawasai et al. 2000; 2004) demonstrated a decrease in the intensity of ChAT positive fibers in the hippocampus, cortex and thalamus following thiamine deficiency. Pires and colleagues have found that AChE activity was decreased in the hippocampus and cortex after PTD treatment (Pires et al. 2005). Furthermore, we have

demonstrated that there is a selective loss of MS/DB cholinergic neurons after PTD treatment (Pitkin and Savage 2001; 2004; Savage et al. 2007) and these abnormalities likely play a role in the functionally blunted hippocampal ACh release during behavioral testing (Savage et al. 2003).

The i.p. dose of physostigmine chosen was based on previous studies assessing physostigmine's ameliorative effects on drug-induced (Stone et al. 1991; Dennes and Barnes 1993) or lesion-induced (Arendt et al. 1990; Beninger et al. 1995; Dokla and Thal 1988; Murray and Fibiger 1985; 1986) behavioral impairments. The dose chosen completely eliminated the behavioral impairment produced by the PTD treatment. Studies using tasks similar to ours, such as maze or operant alternation (Ordry et al. 1988; Shannon et al. 1990) or nonmatching-to-position (Dunnett and Martel 1990; Herremans et al. 1995) have demonstrated that young control rats do not show a dose-dependent enhancement in alternation behavior. Rather, there is no significant change across doses or high doses of physostigmine produce slight impairment.

In summary, the data from Experiment 1 are in line with the WKS case study reports involving the use of anticholinesterases to enhance cognitive ability (Angunawela and Barker 2001; Cochrane et al. 2005). To provide further support that altered cholinergic function in the septohippocampal pathway plays a significant role in the amnesic syndrome in WKS and PTD, we conducted Experiment 2 that directly administered physostigmine to the hippocampus. If directly increasing ACh tone within the hippocampus reduces the behavioral impairment in PTD-treated rats, it will provide significant support for the role of cholinergic dysfunction in diencephalic amnesia produced by thiamine deficiency.

Experiment 2

We hypothesized that the enhancing effects of physostigmine from the systemic administration in Experiment 1 in the PTD-treated rats were due to increased levels of ACh in the hippocampus as a result of reduced degradation of ACh within the hippocampus. Increased ACh in the hippocampus can lead to enhanced theta rhythm within the hippocampus (Buzsaki 2002; Yoder and Pang 2005), which in turn enhances function throughout the Papez circuit (Vertes et al. 2001). Thus, Experiment 2 tested the hypothesis that intrahippocampal infusions of physostigmine would recover impaired alternation performance in PTD-treated rats. In addition, hippocampal ACh levels were measured via *in vivo* microdialysis during pre-drug baseline, drug administration and behavioral testing. This allowed for further assessment of differential ACh sensitivity in PF- and PTD-treated rats under behavioral and pharmacological challenges.

Thiamine is a cofactor for the production of the enzyme pyruvate dehydrogenase (PDH) (Hoyumpa 1980). Furthermore, PDH is essential for the biosynthesis of ACh because it converts pyruvate to acetyl-CoA during the process of glycolysis and acetyl-CoA is the rate-limited step in the production of ACh (Todd and Butterworth 1999). During the late acute neurotoxic phase of PTD treatment there is decreased ACh synthesis and decreased levels of ACh in the brain—including the hippocampus (Barclay et al. 1978; Vorhees et al. 1978). Furthermore, injections of physostigmine during PTD treatment reduce symptomatology and prolong survival (Cheney et al. 1969). These results suggest that decreased ACh levels may be critical in the pathogenesis of thiamine deficiency. However, the prolonged role of ACh dysfunction after acute thiamine deficiency has not been fully established.

The doses of physostigmine chosen for intrahippocampal administration were adopted from a study that demonstrated that spontaneous alternation performance was enhanced by intrahippocampal administration of physostigmine in rats with cholinergic MS/DB lesions (Chang and Gold 2004). We expected to observe a dose-dependent enhancement of spontaneous alternation performance only in the PTD-treated rats.

Subjects

Forty-eight male Sprague-Dawley rats (2–3 months; 250–300 g) were used as subjects in this study. They were housed one per cage with unlimited access to water and Purina rodent chow in a colony room with a 12-hr/12-hr light/dark cycle (onset at 7:00 am). All rats were fasted the night before behavioral testing.

Note: An additional 8 young male Sprague-Dawley rats (2–3 months; 250–300 g) were used to assess the effects of repeated spontaneous alternation testing.

Treatment

Animals were randomly assigned to either PF (n = 24) or PTD (n = 24) groups. The same treatment regime used in Experiment 1 was used in Experiment 2.

Surgery

Two-three weeks after recovering from PTD or PF treatments stereotaxic surgery (David Kopf Instruments, USA) was performed on animals anesthetized with a Ketamine (8.25 mL)/Xylazine (1.75 mL) mixture (50 mg/kg, i.p.). Each subject was implanted with two plastic guide cannulae: a microdialysis cannula (CMA/12 mm; Carnegie Medicine Associates, Chelmsford, MA) and a typical drug cannula (28 g; Plastic One, Roanoke, VA). In a single surgery session, the cannulae were bilaterally implanted into the hippocampus (5.0 mm posterior to bregma, 5.0 mm lateral to the midline, and 4.2 mm DV) according to the atlas of Paxinos and Watson (1986; see Figures 1C & 1D for acceptable probe placement). The location (left/right) of the microdialysis/drug cannulae was counterbalanced across subjects. Four days after surgery animals were handled daily (5 min/day) for 5 days prior to behavioral testing.

Drug Infusion, Microdialysis, Behavioral testing and HPLC

Nine days following surgery, prior to maze testing, the microdialysis probe (CMA/12, 3 mm) was inserted into the hippocampus and the animal was placed into the holding cage (41 cm × 30 cm × 35 cm) located in the testing room. The probe was connected to plastic tubing and driven by a microinfusion system (CMA/100 pump). The dialysis probe was perfused continuously at a rate of 2.0 µL/min with artificial CSF (in mM: 128 NaCl, 2.5 KCl, 1.3 CaCl₂, 2.1 MgCl₂, 0.9 NaH₂PO₄, 2.0 NaHPO₄, and 1.0 glucose, brought to a pH of 7.4), which contained the AChE inhibitor neostigmine (500 nM). After 60 min of stabilization, dialysis samples (sample volume 12 µL) were collected every 6 min for a period of 18 min in the holding cage to determine basal levels of ACh in awake rats. This is the common procedure for obtaining baseline levels of neurotransmitter prior to drug or behavioral manipulation (Chang and Gold 2004; Fadel et al. 1999; Moor et al. 1998a; 1998b). During the initial baseline phase the animal was free to move about the holding cage. After the 18 min baseline phase, the rat was gently picked up and the microdialysis probe and the stilet from the drug cannula were both removed. Both injection cannulae were inserted and 0.5 µL/per side of either saline or physostigmine (20 or 40 ng/µL) was delivered slowly over a 4-min period. After the infusion, both the microdialysis probe and drug stilet were reinserted. Infusion, rather than reverse-microdialysis, was used to achieve maximal bilateral diffusion in the hippocampus. Each subject received only one drug dose and saline infusion. We counterbalanced within groups which compound (saline or physostigmine) was administered on session 1 or session 2. The two testing sessions were separated by 24 hr. We ran an additional group of normal rats to assess changes in behavior as a function of repeated testing. Neither the rate of alternation (Day 1: 64.8%; Day 2: 69.7%; $F(1, 7) = 1.21$, $p > .3$) nor the number of arms entered (Day 1: 35.38; Day 2: 32.25; $F(1, 7) < 1$, $p > .9$) was altered across the two successive sessions of spontaneous alternation testing. The time between sessions (24 hr) is well past the half-life of physostigmine in the brain (Somani et al. 1991).

After the infusion, the rat was then placed back into the holding cage for 14 more min. This total 18-min microdialysis period was referred to as the drug infusion phase. Following the drug infusion phase, the rat was gently placed on the center of the maze (same maze set-up used in Experiment 1). The rat was allowed to traverse the maze freely for 18 min (maze phase), while *in vivo* microdialysis samples continued to be collected. As in Experiment 1, the number and sequence of arms entered were recorded to determine percent alternation scores. After maze testing, post baseline microdialysis samples were collected for an additional 18 min. Each phase (baseline, drug, maze, post baseline) produced 3 dialysate samples that were frozen until the time of assay (total samples per rat = 12).

Thawed dialysate samples were assayed for ACh using HPLC with electrochemical detection (Bio Analytic Systems, West Lafayette, IN). The system included an ion-exchange microbore analytical column, a microbore ACh/Ch immobilized enzyme reactor containing AChE and choline oxidase, and a peroxidase wired working electrode. The detection limit of this system is 5 femto-moles (fmol). ACh peaks were quantified by comparison to peak heights of standard solutions (100 and 20 nM) and corrected for *in vitro* recovery of the probe.

Histology

After the completion of testing, animals were deeply anesthetized with a pentobarbital cocktail (Sleepaway, Fort Dodge, Iowa: 26% sodium pentobarbital in 7.8% isopropyl alcohol and 20.7% propylene glycol solution; 0.2 mg/kg, i.p.) and decapitated. As in Experiment 1, cresyl violet stained sections were evaluated for presence of diencephalic lesions and cannulae location (see Figure 1CD). Histological analysis revealed that eight rats had incorrect cannula placement and those rats were removed from the study. The final number of rats was 20 PF and 20 PTD.

Results

An initial analyses of order effects revealed that counterbalancing the drug condition across sessions did not influence alternation rates, number of arms entered or ACh efflux in either group (all p 's > .12).

Behavioral Performance—Figure 3A depicts the spontaneous alternation data for both PF and PTD groups. An ANOVA revealed that there was a significant Group (PF vs. PTD) $\times$ Dose (saline, Low = 20 ng, High = 40 ng, physostigmine) interaction ($F [2, 54] = 5.60, p < .01$). Follow-up analyses revealed that in the PF group, physostigmine did not enhance alternation behavior: Rather, there was a trend for the high dose of physostigmine to impair alternation behavior ($p = .078$). In contrast, physostigmine enhanced alternation scores of the PTD rats ($F [2, 27] = 5.28, p < .01$). This was particularly evident at the high dose where the increase in alternation rate was about 20% in the PTD rats ($p < .01$).

There was a significant Group difference: PF rats had higher alternation scores than PTD-treated rat, when subjects were administered saline ($F [1, 18] = 24.89, p < .0001$). This was not seen when the both groups of rats were administered the high dose of physostigmine ($p > .3$). Thus, the high dose of physostigmine recovered the behavior of PTD rats to that observed in PF rats without drug.

In Figure 3B we see that there are no differences in activity (number of arms entered) as a function of Group, Drug, or the interaction between those variables (all p 's > .14).

Hippocampal ACh Levels—Basal amounts of ACh (PF = 55.13 ± 6.9 fmol; PTD = 58.61 ± 9.2 fmol) in the hippocampus were not different as a function of Group ($F [1, 38] < 1$). As shown in Figure 4, intrahippocampal infusions of physostigmine dose-dependently increased

hippocampal ACh levels in both groups, but to a greater extent in the PF group. This is supported by the statistical analysis conducted with a mixed-design repeated measures ANOVA (**Group** = PF vs. PTD; **Dose** = Saline, Low, High; **Phase** = Baseline, Infusion, Maze, Post Baseline; **Sample Time** = First, Second, Third). The analyses revealed significant main effects for Dose ($F [2, 54] = 4.49, p < .05$) and Phase ($F [3, 162] = 43.08, p < .0001$), as well as significant interactions for Dose $\times$ Phase ($F [6, 162] = 6.33, p < .0001$), Group $\times$ Dose $\times$ Time ($F [4, 108] = 3.17, p < .05$) and Group $\times$ Dose $\times$ Phase $\times$ Time ($F [12, 324] = 2.07, p < .05$).

Follow-up analyses revealed that, although there was a significant effect of dose on hippocampal ACh levels in both PF and PTD groups (both F 's $> 3.22, p < .05$); when administered either dose of physostigmine, the PF group had a higher rise in hippocampal ACh levels than PTD rats for the infusion and maze testing phases (both F 's $> 3.93, p < .05$). For the high dose of physostigmine, the greatest difference between the groups was the last sample during the infusion phase ($F [1, 18] = 11.64, p < .01$). During the maze testing phase, the greatest differences were observed during the last two sample time periods, in which the PF rats had higher ACh levels relative to baseline than PTD rats (both F 's $> 4.50, p < .05$). There were no significant Group differences in the post baseline ACh levels (all p 's $< .18$). Thus, the microdialysis/HPLC data demonstrated that although the PF rats exhibited increased levels ACh in the hippocampus after infusions of physostigmine, there was no parallel increase in spontaneous alternation performance. This is in contrast to PTD-treated rats, which displayed both an increase in available ACh and greater behavioral change after the 40 ng hippocampal infusion of physostigmine.

Discussion

The key points of Experiment 2 are that an intrahippocampal infusion of physostigmine dose-dependently enhanced alternation behavior in PTD-treated rats, while resulting in a non-significant trend toward decreased alternation behavior in control PF rats. Furthermore, although intrahippocampal infusion of physostigmine prevented the breakdown of ACh, therefore enhancing hippocampal ACh availability and thus ACh levels in both groups, it did so to a greater degree in PF animals. However, an increase above normal hippocampal ACh levels did not map onto greater behavioral success in PF subjects. Our experiment also shows that although there was an increase in ACh levels in the PF rats after physostigmine was delivered directly to the hippocampus, there was no enhancement of alternation rates. Actually, there was a non-significant trend of decline in PF rat's alternation rates when 40 ng of physostigmine was directly administered into the hippocampus. Physostigmine could potentially be increasing ACh beyond functional levels.

Studies aimed at increasing the cholinergic levels in the septohippocampal pathway have found that, in normal animals, high doses of cholinergic drugs also produce amnesic effects (Bunce et al. 2004; Elvander et al. 2004). Such data suggest that there is likely an inverted U-shaped dose response effect with enhanced cholinergic activity (Lalonde 2002). In fact, recent data suggests there is an optimal range of cholinergic tone within the hippocampus for successful encoding and recall of spatial information (Hasselmo 2006).

However, in the PTD rats, increases in hippocampal ACh levels by physostigmine enhanced behavior performance on the spontaneous alternation task. These results resemble previous studies in that drugs that increase cholinergic levels facilitate behavioral performance in some rodent models of cognitive impairment (Barnes et al. 2000; Chang and Gold 2004; Dong et al. 2005; Mulder et al. 2005; Nakagawasai et al. 2000). Furthermore, physostigmine has been shown to reverse the memory impairing effects of septal muscimol (GABA_A agonist) (Degroot and Parent 2001).

The mechanisms by which cholinergic drugs may enhance or impair performance may be a factor of the integrity of the septo-hippocampal pathway (Bunce et al 2004). In addition to its behavioral effects, physostigmine and other cholinomimetics facilitate theta rhythm within the hippocampus (Lawson and Bland 1993; Olpe et al. 1987; Rowntree and Bland 1986; Yoder and Pang 2005) and i.c.v. physostigmine administration significantly increases hippocampal ACh levels (Hallak and Giacobini 1987).

In a slice preparation application, physostigmine and the cholinergic agonist carbachol both induced rhythmic activity that resembled long-term potentiation in the hippocampus that persisted for hours (Colgin et al. 2003). The proposed functional significance of these prolonged rhythms produced by intense cholinergic activity is to synchronize brain and behavioral function (Colgin et al. 2003). Thus, application of cholinomimetics directly to the hippocampus of awake animals would also likely change the activity within limbic circuits for extended periods of time. In the PTD rat, the significant rise in hippocampal ACh that occurred after the infusion of 40 ng of physostigmine may have boosted the rhythmic activity in the animals to a more functional level. In contrast, this same amount of drug in the PF control animals could have led to an over excitation of the system resulting in a disorganization of rhythm through out the limbic system and compromised behavioral success.

Physostigmine may also be increasing behavioral performance in brain-altered subjects through ACh-GABAergic feedback mechanisms. When ACh activates pyramidal neurons, the hippocampal GABAergic interneurons are also activated and send feedback to the medial septal region (Wu et al. 2000). Several studies have also demonstrated the presence of presynaptic muscarinic autoreceptors on medial septal projection cholinergic neurons, which help regulate ACh production through negative feedback (Levey et al. 1995; Van der Zee and Luiten 1994; Vilaro et al. 1992).

In the hippocampus such muscarinic autoreceptors that mediate feedback inhibition for ACh release (Starke, Gothert and Kilbinger, 1989). Recent evidence has determined that M2 and M4 receptors are the major cholinergic autoreceptors in the hippocampus. Specifically, the M2 receptor plays a key role in regulating pharmacologically and physiologically evoked ACh release in this region (Tzavara et al 2003). The excessive increases in ACh levels in PF rats following the hippocampal infusion of physostigmine could impede further ACh release by over activation of such receptors. This receptor driven feed back mechanism has been shown to desensitized in rats with lesions of the fimbria-fornix (Cassel et al, 1995; Levey et al, 1995). Thus, in rats with damage to the septo-hippocampal pathway increases in hippocampal ACh levels, via direct physostigmine, are less likely to impact autoregulation of ACh release. Given that PTD rats have a reduction in medialisepal/diagonal band cholinergic neurons (Savage et al, 2007) and degeneration of the fornix (Langlais & Zhang, 1997), such a compensatory mechanism could explain why PTD rats have a more effective behavioral response to intrahippocampal infusion of physostigmine. However, our in-vivo microdialysis paradigm cannot confirm this hypothesis. The data do suggest that whether physostigmine enhances or impairs performance is a function of the integrity of the septal-hippocampal circuit.

Previous studies using the in-vivo microdialysis technique have shown that increased and decreased ACh levels can correlate with increased and decreased memory performance, respectively (Ragozzino et al., 1994; 1996). In non-drug manipulated PF and PTD rats we have shown a correlation between hippocampal ACh efflux and percent alternation score (Savage et al. 2007). However, there was no correlation between ACh levels and behavior in the current study, given that physostigmine produced a greater rise in hippocampal ACh levels in PF rats relative to PTD rats, but there was a trend for impairment in PF rats and enhanced performance in PTD rats. The trend towards behavioral dysfunction when ACh levels are very high could be a function of altered septohippocampal feedback mechanisms. This data suggest that there

is not a simple one-to-one correlation between hippocampal ACh levels and behavior (see also Parent & Baxter, 2004).

It should be noted that other brain pathology exists in the PTD model and likely contributes to the behavioral impairment. Previous studies have shown that the level of learning and memory impairment in the PTD rat is closely related to the amount of damage in diencephalic-related structures. In particular two key nuclei within the limbic thalamus: The internal medullary lamina (IML) (Langlais and Savage 1995; Mair et al. 1991) and the anteroventral (AV) thalamic nucleus. The AV is connected to the hippocampus through the fornix and also receives input from the mammillary bodies (Aggleton and Brown 1999). The “limbic thalamus” is believed to play an integrative role in the production of numerous complex functions--including learning and memory. Furthermore, these medial thalamic nuclei have been partially segregated into neural systems with proposed distinctive functional roles in learning and memory (Bentivoglio et al. 1997; Mitchell and Dalrymple-Alford 2005; Smith et al. 2002; Van der Welf et al. 2002). The severity of diencephalic amnesia is likely to be related to the extent of damage to multiple sites across these systems. However, enhancing activity in the hippocampus, by drug manipulations such as increasing ACh levels, enhances activity in a number of brain circuits (Vertes et al. 2001). The recovery of function observed in the present study may reflect enhanced activity throughout limbic circuits.

These data therefore provide additional evidence for cholinergic dysfunction in PTD rats. Furthermore, increasing the available amount of ACh in hippocampus, by preventing its breakdown via physostigmine, eliminates the behavioral impairment observed after PTD treatment. However, in the normal rats excessive activation of cholinergic activity can become dysfunctional. Thus, high ACh levels within the hippocampus do not always predict high levels of behavioral success. A number of studies have concluded that there is a limited range of ACh levels for optimal cognitive and hippocampal function (Bunce et al. 2004; Elvander et al. 2004).

General Discussion

The current results add several new findings to the existing literature. Both Experiment 1 and Experiment 2 demonstrate a statistically significant effect of physostigmine on increasing the rate of spontaneous alternation in the animal model of WKS. This increase in spontaneous alternation rate was directly due to an increase in the number of alternations made and not simply due to the increase in the activity of the physostigmine-treated PTD rats. More importantly, we observed complete behavioral recovery after administration of physostigmine: PTD-treated rats injected with physostigmine reach alternation levels similar to the PF animals in an un-drugged state. The fact that these results are reproducible despite different routes of the administration (systemic [Exp 1] vs. site-specific [Exp 2]) can be attributed to the physical properties of the physostigmine. It is a lipid soluble carbamate, a property that allows it to easily cross the blood brain barrier and mediate ACh levels in structures such as the hippocampus.

The increase in alternation performance seen in PTD-treated rats after either systemic or intrahippocampal injections of physostigmine could be a combination of two events: (1) an increase in the excitability (2) and a decrease in the inhibition of the hippocampus and the surrounding circuitry. The hippocampus receives the large majority of ACh input from the cholinergic projection neurons located in the MS/DB; however, there is a small population of cholinergic neurons within the hippocampus (Frotscher et al. 1986). Acetylcholine plays an excitatory role in the hippocampus: Beyond exciting hippocampal pyramidal neurons, pharmacological activation of hippocampal muscarinic ACh receptors also directly excites GABAergic interneurons (Widmer et al. 2006; Wu et al. 2003b).

In the PTD model there is a decrease in the cholinergic fibers that infiltrate that hippocampus (Nakagawasai, 2000) and a loss of cholinergic cells in the MS/DB that project to the hippocampus (Pitkin and Savage 2001; 2004; Savage et al. 2007). It is likely that these cholinergic alterations in the MS/DB region are partially responsible for the down regulation of ACh release in the hippocampus reported in PTD-treated rats. However, the septohippocampal axis consists of not only cholinergic projections, but also GABAergic and glutamate projections between the MS/DB and the hippocampus (Colom et al. 2005; Sotty et al. 2003). Currently, we know very little about the loss of the GABA or glutamate MS/DB neurons in disorders affecting learning and memory. However, electrophysiological evidence suggests that cholinergic, GABAergic, and glutamatergic projection neurons from the MS/DB may each uniquely contribute to hippocampal rhythmicity and therefore behavior (Sotty et al. 2003).

Whether cholinergic drugs produce their effects on cholinergic or noncholinergic neurons has been questioned (Wu et al. 2000). Although cholinomimetics facilitate theta rhythm (Lawson and Bland 1993; Olpe et al. 1987; Yoder and Pang 2005) and can enhance learning and memory in brain-altered subjects (Arendt 1994; Beninger et al. 1995; Dennes and Barnes 1993; Dokla and Thal 1988; Givens and Olton 1990; Murray and Fibiger 1985; 1986; Stone et al. 1991), these effects may not be a direct result of cholinergic activation, but may be caused by the disinhibitory mechanisms due to increased impulse flow in the septohippocampal GABAergic pathway (Wu et al. 2000). Thus, the interactions between ACh, GABA, and glutamate are critical for normal hippocampal and extended limbic system functioning. How cholinomimetic drugs change extended hippocampal neural activity and behavioral outputs in the PTD model, WKS and other disorders of memory still requires further investigation.

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Figure 1.

Cresyl violet stained sections demonstrating the thalamus of a PF-rat (A) for comparison to a PTD-induced lesion of the thalamus (B). The arrows on panel B point to the medline thalamic lesion caused by thiamine deficiency. Cresyl violet stained sections showing acceptable hippocampal placement of the microdialysis probe (C) and the drug probe (D). The arrows in panel C point to the top and bottom of where the microdialysis probe was located. The arrow in panel D points to where in the tissue the injection needle would have extended (0.75 mm beyond the drug cannula).

A.

B.

Figure 2.

Experiment 1: Behavioral data (Mean $\pm$ SEM) from spontaneous alternation testing for PF and PTD rats after systemic administration of saline or 0.075 mg/kg of physostigmine. Panel A displays that there are group differences under the saline condition, but not when subjects are administered physostigmine. In addition, physostigmine significantly improved alternation in the PTD group. Panel B shows that activity did not change as a function of drug.

A.

Experiment 2: Intrahippocampal Infusion

B.

Figure 3.

Experiment 2: Behavioral data (Mean $\pm$ SEM) from spontaneous alternation testing for PF and PTD rats after intrahippocampal infusions of saline or physostigmine (20 ng, 40 ng). Panel A shows the significant difference between PTD and PF rats in alternation behavior after saline was infused. Intra-hippocampal infusion of physostigmine only enhanced alternation rates in the PTD group. Panel B shows that activity did not change as a function of drug.

A.

B.

Figure 4.

Experiment 2: Profiles of ACh efflux (Mean percent rise above baseline $\pm$ SEM) from the hippocampus of PF (Panel A) and PTD (Panel B) rats as a function of drug dose during the phases of baseline, drug infusion, behavioral testing, and post-baseline.