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Research Report

Selective septohippocampal – but not forebrain amygdalar – cholinergic dysfunction in diencephalic amnesia

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ABSTRACT

A rodent model of diencephalic amnesia, pyriithiamine-induced thiamine deficiency (PTD), was used to investigate diencephalic–limbic interactions. In-vivo acetylcholine (ACh) efflux, a marker of memory-related activation, was measured in the hippocampus and the amygdala of PTD-treated and pair-fed (PF) control rats while they were tested on a spontaneous alternation task. During behavioral testing, all animals displayed increases in ACh efflux in both the hippocampus and amygdala. However, during spontaneous alternation testing ACh efflux in the hippocampus and the alternation scores were higher in PF rats relative to PTD-treated rats. In contrast, ACh efflux in the amygdala was not suppressed in PTD treated rats, relative to PF rats, prior to or during behavioral testing. In addition, unbiased stereological estimates of the number of choline acetyltransferase (ChAT) immunopositive neurons in the medial septal/diagonal band (MS/DB) and nucleus basalis of Meynert (NBM) also reveal a selective cholinergic dysfunction: In PTD-treated rats a significant loss of ChAT-immunopositive cells was found only in the MS/DB, but not in the NBM. Significantly, these results demonstrate that thiamine deficiency causes selective cholinergic dysfunction in the septo-hippocampal pathway.

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Diencephalic lesions are seen in a range of disorders: malnourishment, ischemic infarcts, viral infections, tumors and traumatic damage. Damage to certain nuclei and fiber systems within the diencephalon interrupts the flow of information between key memory structures and thus causes severe and long-lasting amnesia (Langlais and Savage, 1995; Mair, 1994; Reed et al., 2003). Although there is evidence that lesions to particular diencephalic nuclei result in memory impairment in their own right, there is also evidence that damage to diencephalic nuclei can disrupt memory circuits leading to dysfunction in other regions of the brain (Bentivoglio

et al., 1997; Mair, 1994; Reed et al., 2003; Savage et al., 2003; Vann and Aggleton, 2003).

In cases of diencephalic amnesia associated with thiamine deficiency (i.e., Wernicke–Korsakoff Syndrome [WKS]) there is neuronal loss and lesions in multiple limbic thalamic nuclei, mammillary bodies (Langlais and Savage, 1995; Langlais et al., 1996; Mair et al., 1988; Mair, 1994), as well as degeneration in key fiber tracts connecting limbic structures (fornix and mammillothalamic tract; Langlais and Zhang, 1993; Langlais and Zhang, 1997). The damage to both key diencephalic nuclei and fiber tracts likely results in a “disconnection syndrome”

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within the limbic system (see Jenkins et al., 2002; Markowitsch and Pritzel, 1985; Warrington and Weiskrantz, 1982).

At one point, WKS patients were labeled as “temporal lobe-related amnesiacs” based on their memory performance (Cohen and Squire, 1980). This clinical population was critical for the development of the memory system classifications (i.e., declarative vs. nondeclarative memory (Squire, 1982)). However, it is clear from neuroimaging studies that the most consistent neuropathology of WKS is damage to the cortex and the diencephalon—particularly the thalamus and mammillary bodies (Jacobson and Lishman, 1997; Kopelman, 1995). One of the best predictors of memory loss in WKS is pathology of the anterior thalamus (Harding et al., 2000). In contrast, the hippocampus is damaged in less than 10% of WKS cases. Although the two types of amnesia differ in structural correlates, the *functional distinction* between temporal lobe amnesia and diencephalic amnesia has been questioned, given the behavioral similarities and the neural connections between the diencephalon and the hippocampus (Aggleton and Brown, 1999; Squire, 1982).

Using a rodent model of WKS, pyriethamine-induced thiamine deficiency (PTD), we have demonstrated that the hippocampus is *functionally impaired*: When PTD-rats are performing a spontaneous alternation task (Savage et al., 2003) or learning a nonmatching-to-position task (Roland and Savage, 2007) the rise in hippocampal acetylcholine (ACh) efflux is blunted relative to control rats. This impairment in hippocampal ACh efflux is only evident during behavioral testing as baseline levels of hippocampal ACh are normal in the PTD model. Given the known importance of ascending cholinergic systems in a range of behaviors and neural physiology within the limbic system, understanding the role of cholinergic dysfunction in diencephalic amnesia will both further our understanding of the complex role of ACh in learning and memory and aid in the development of pharmacotherapies.

The impairment of functional ACh hippocampal release during behavioral testing in the PTD model could be driven by a number of neuroanatomical changes associated with thiamine deficiency and diencephalic amnesia. We have previously documented a loss of choline acetyltransferase (ChAT) immunopositive neurons in the medial septal/diagonal band (MS/DB) in the PTD model using a profile counting method (Pitkin and Savage, 2001; Pitkin and Savage, 2004). Furthermore, Nakagawasai and colleagues (Nakagawasai et al., 2000; Nakagawasai et al., 2004; Nakagawasai, 2005) have demonstrated a decrease in the intensity of ChAT positive fibers in the hippocampus, cortex and thalamus after thiamine deficiency. However, this decrease in ChAT immunofluorescence is not observed in the amygdala. Pires and colleagues have also demonstrated that after mild thiamine deficiency (Pires et al., 2001) or PTD treatment (Pires et al., 2005) stimulated ACh release is decreased in the cortex. Furthermore, these authors found that acetylcholinesterase (AChE) activity was decreased in both the hippocampus and cortex after PTD treatment (Pires et al., 2005). In addition, compounds that enhance ACh levels decrease mortality and sickness behaviors (attacking, antinociception, altered startle response) as well as improve learning in thiamine-deficient mice (Nakagawasai et al., 2000) and spontaneous alternation in PTD-treated rats [unpublished data].

Reviews of the pathology produced by the PTD treatment have consistently demonstrated that although mild to moderate cell loss occurs outside the diencephalon, the anterior and midline thalamic damage are critical and responsible for the majority of the loss of learning and memory function that occurs after thiamine deficiency (Langlais et al., 1996; Mair, 1994). These changes, along with the white matter loss that occurs in key fiber tracts from the diencephalon after PTD treatment (Langlais and Zhang, 1997), suggest that thiamine deficiency likely causes system level dysfunction. There is neurobiological evidence that even discrete diencephalic damage alters the activation of other limbic regions—in particular the hippocampus (see Jenkins et al., 2002; Reed et al., 2003; Savage et al., 2003). Thus, damage to the diencephalon produced by thiamine deficiency could produce dysfunction in other limbic regions.

In the current experiment we examined whether thiamine deficiency, which causes diencephalic pathology, alters both hippocampal and amygdalar ACh efflux when rats are actively exploring a maze. Measurement of ACh efflux in the brains of rats during learning appears to be a useful marker of activation of a given neural system—particularly the hippocampus and amygdala (Chang and Gold, 2004; Gold, 2003; McIntyre et al., 2002; McIntyre et al., 2003a; McIntyre et al., 2003b). We chose a task, spontaneous alternation, which is sensitive to diencephalic damage (Langlais and Savage, 1995) and to changes in release of ACh in both the hippocampus and amygdala (Chang and Gold, 2004; McIntyre et al., 2002; McIntyre et al., 2003a). In addition, to understand the neuroanatomical correlates of this dysfunction we used unbiased stereological techniques to estimate ChAT cell populations in the MS/DB (that projects to the hippocampus) as well as the nucleus basalis of Meynert (NBM; that projects to the amygdala). Given our behavioral data (Savage and Langlais, 1995), that suggests intact amygdala functioning in the PTD model, we expect that during behavioral testing there will be a blunted rise in hippocampal – but not amygdalar – ACh efflux in the PTD treated rats. Furthermore, we expect PTD-treated rats to display a loss of ChAT immunopositive cells in the MS/DB (Pitkin and Savage, 2001; Pitkin and Savage, 2004). However, no one has examined cell loss in the NBM of this model. Our goal was to determine the selectivity of cholinergic dysfunction by measuring both ACh efflux during behavioral testing in two structures and ChAT immunopositive cell loss in the regions that project to these structures. The data from this study which combines behavioral, neurochemical and histological measures will give a more complete picture of the role of cholinergic abnormalities to the learning and memory problems associated with the thiamine deficiency form of diencephalic amnesia.

1. Results

1.1. Statistical analyses

The mean percent alternation and total number of arm entries during spontaneous alternation, as well as ChAT immunopositive cell counts (MS/DB, NBM) were analyzed with a 1-factor (Group=PF vs. PTD) ANOVA. The neurochemical microdialysis/HPLC data were analyzed with a one-between (Group), two-

within factors repeated measures (phase, sample) AVOVA. Simple regression was used to correlate ACh efflux in the hippocampus and amygdala with alternation scores.

1.2. Histology

Figs. 1A, B displays the PTD neuropathology compared to a PF brain. Figs. 1C, D displays representative cannula placement in the hippocampus and amygdala. Two PTD and one PF subjects had misplaced amygdalar cannulae and data from those subjects was discarded.

1.3. Stereological estimates of ChAT positive cells

In the MS/DB region, the PTD rats ($n=7$) had significantly fewer ChAT positive cells than the PF rats ($n=7$; $F[1,12]=9.29$, $p<0.01$). The PTD group displayed about a 20% loss of ChAT positive cells in the MS/DB region (see Table 1). However, in the NBM region, the loss of ChAT positive cells (-5%) in the PTD model was nonsignificant ($F[1,12]=1.42$, $p>0.2$). Fig. 2 displays the regions sampled and the localization of the ChAT immunopositive staining.

1.4. Spontaneous alternation

As shown in Fig. 3A, PTD-treated rats ($n=8$) had a significant decrease in spontaneous alternation rates relative to PF ($n=8$) control rats ($F[1, 14]=8.56$, $p<0.01$). However, the PTD rats were more active on the maze than the PF rats (see Fig. 3B; $F[1, 14]=5.10$, $p<0.05$). Thus, we ran a second analysis including alteration rates for only the first 22 trials. Equating number

Table 1 – Estimated total numbers of ChAT immunopositive cells (Mean \pm SEM) in the medial-septum-diagonal band (MS/DB) and the nucleus basalis (NB) of PF and PTD treated male rats

Group	MS/DB	NB	Total
PF ($n=7$)	10187.14 \pm 488.44 ($s=4.71$)	6848.57 \pm 340.75 ($s=5.00$)	17035.71 \pm 510.08
PTD ($n=7$)	8173.57 \pm 445.01* ($s=4.71$)	6334.86 \pm 262.64 ($s=5.14$)	14508.43 \pm 444.85*

n=number of subjects in the Group; *s*=mean number of brain sections from the region from each subject; *=significant difference ($p<0.01$).

of arms entered across PF and PTD-treated rats on the maze still resulted in impaired alternation rates in the PTD-treated rats relative to the PF rats ($F[1, 14]=8.27$, $p<0.02$).

1.5. Acetylcholine efflux

Hippocampal and amygdalar ACh levels increased during behavioral testing relative to baseline levels in the holding cage (both F 's[1, 14] >164.00 , p 's <0.0001 ; see Figs. 4A, B). Basal amounts of ACh in the hippocampus (58.73 ± 15.5 fM) and amygdala (34.43 ± 10.2 fM) were not different as a function of Group (both F 's[1, 14] <1.5). However, there was a significant Group (PF vs. PTD) \times Phase (Baseline, Maze) interaction ($F[1, 14]=13.78$, $p<0.01$) for the hippocampal ACh samples. There was no significant main effect of Sample time ($p>0.10$); however, the Group \times Phase \times Sample interaction approached significance ($F[3, 42]=2.46$, $p<0.08$). Fig. 4A

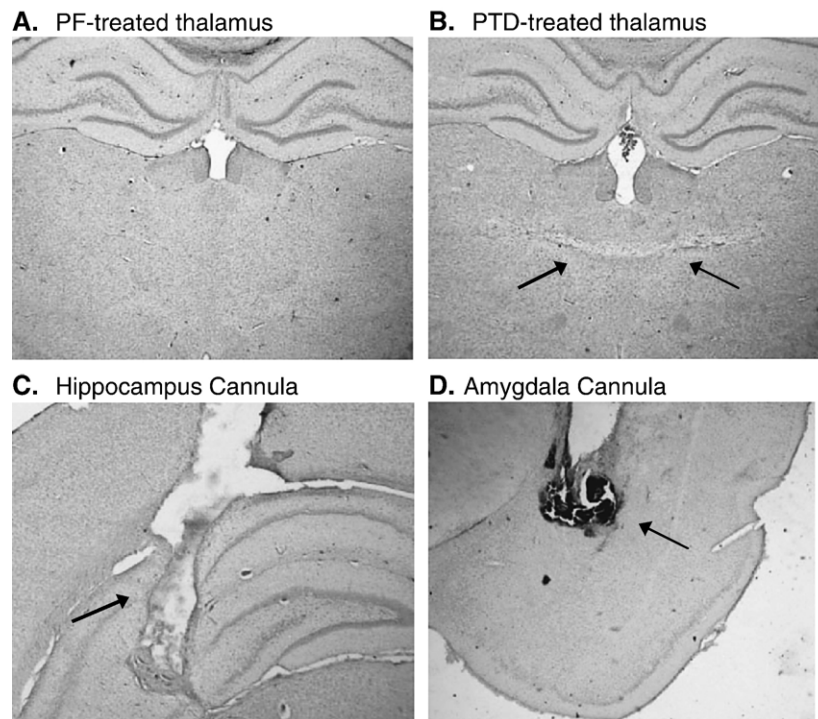


Fig. 1 – Photomicrographs illustrating a midline thalamic lesion produced by PTD treatment (2B), relative to a PF control (2A). The second row of photomicrographs are examples of acceptable probe placement in the hippocampus (C) and amygdala (D).

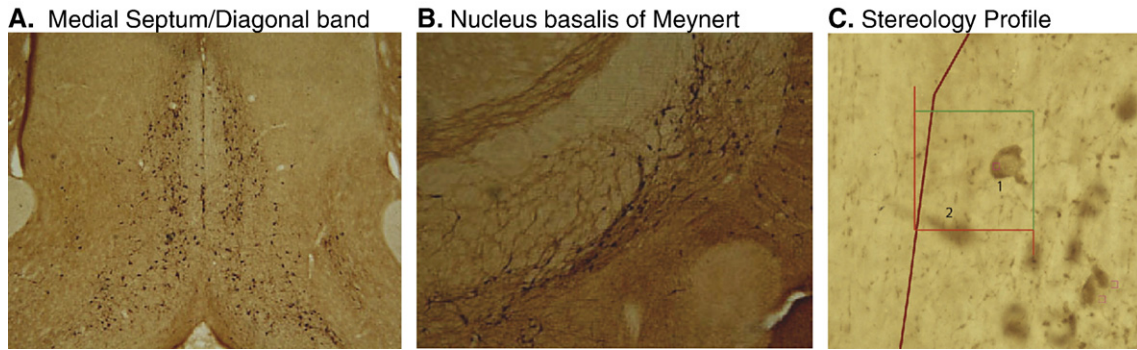


Fig. 2 – Sections of the medial septum/diagonal band (A) and nucleus basalis of Meynert (B) stained for choline acetyltransferase (ChAT) immunoreactivity as a marker for cholinergic neurons. The third panel is a high magnification (40 \times) demonstrating the disector counting rule (cell 1 counted, cell 2 was not) within the counting frame (50 μm \times 50 μm).

shows that PTD-treated rats had a significantly lower level of hippocampal ACh release (expressed as percent increase above baseline values) during behavioral testing, relative to PF rats ($F[1,14]=13.18$, $p<0.01$). Furthermore, there was a significant correlation between hippocampal ACh efflux and alternation rate ($r=0.50$, $p<0.05$).

In contrast, the Group \times Phase interaction did not reach significance for the amygdala ACh samples ($F[1,14]=3.17$, $p=0.09$). The rise in amygdala ACh efflux during behavioral testing in the PTD group was slightly higher than the amygdala ACh rise in the PF groups. The variable of Sample time was not significant ($p=0.08$) and it did not interact with any variables (all p 's >0.2). Thus, in contrast to hippocampal functioning, amygdala ACh functioning was not suppressed in the PTD model. In

addition, there was no relationship between amygdala ACh efflux and alternation score ($r=-.31$, $p>0.2$) (Fig. 5).

2. Discussion

This study provides evidence that the cholinergic dysfunction in the PTD model is selective to the septo-hippocampal axis. The data replicate that PTD rats have impaired hippocampal ACh release that is evident only under conditions of spatial exploration or place learning (Roland and Savage, 2007; Savage et al., 2003). In addition, the unbiased stereological estimate of cell counts also replicates the profile counting method of assessment of loss of ChAT immunopositive cells in the MS/DB of PTD treated rats (Pitkin and Savage, 2001; Pitkin and Savage, 2004). The novel findings from this study are (1) Amygdala functioning is intact in the PTD-model: The behavioral relevant increase in amygdala ACh efflux was not suppressed. (2) The number of ChAT positive neurons in the NBM region, which projects to the amygdala, was not significantly altered in PTD rats. These results together suggest that the forebrain cholinergic innervation of limbic structures likely modulate the activation of these structures during environmental-behavioral demand and the degree of activation precipitates behavioral adaptation.

Although there have been previous data that suggests normal amygdala functioning in the PTD model, until the microdialysis data from the present study there has been no direct evidence for that claim. In a previous study we modified the reward contingencies on a matching-to-position task such that reward expectancies, rather than spatial location, could be used to solve the task. This slight manipulation completely eliminated the learning impairment in PTD treated rats (Savage and Langlais, 1995). Modifying a task such that reward expectancies can be used to solve the task requires the amygdala (Blundell et al., 2001; Savage, 2001). Second, the intensity of ChAT positive fibers in the amygdala remains unchanged after thiamine deficiency in mice (Nakagawasai et al., 2000). Our ChAT immunocytochemistry data provide evidence that the loss (hippocampus) or sparing (amygdala) of ChAT-positive fibers reported in the Nakagawasai paper is due to a decrease of cells staining positive for ChAT in the MS/DB,

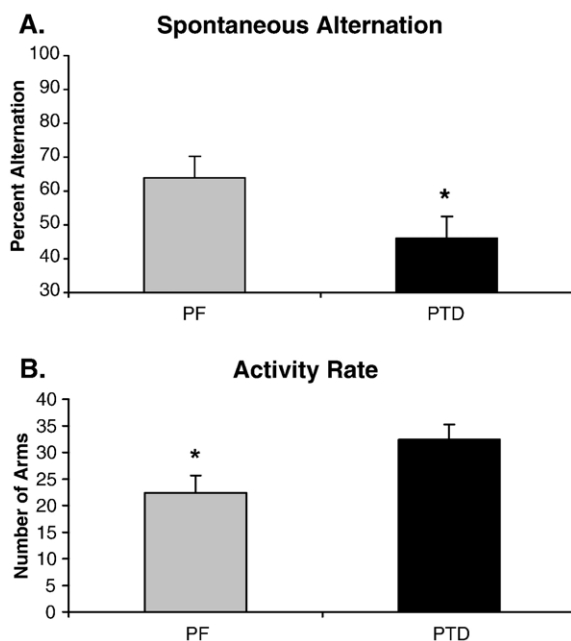


Fig. 3 – Behavioral data (Mean \pm SEM) from a single session of spontaneous alternation testing for PTD and PF rats. Panel A shows the significant difference between PTD and PF rats in alternation behavior. Panel B shows that the PTD rats transversed more arms during the session.

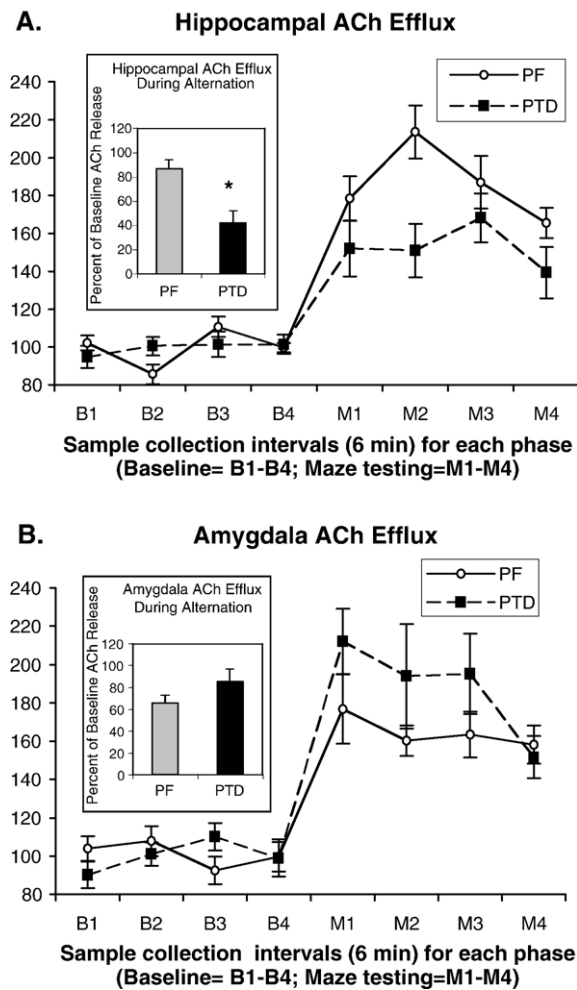


Fig. 4 – Profiles of ACh efflux (Mean \pm SEM) from the hippocampus (A) and the amygdala (B) during 6-min sample bins prior to and during spontaneous alternation. The PF rats' hippocampal ACh efflux increased to 80% above baseline values, whereas the PTD rats displayed a significantly blunted ACh efflux of only 40% above baseline values. In the amygdala, the PF rats had about 65% increase in ACh efflux above baseline and the PTD rats displayed a greater 84% ACh efflux, but this change was not significant. Insert graphs are the mean increase of ACh efflux across samples obtained from the Group \times Phase interactions.

but not the NBM. We do not know if the reduction in the number of neurons staining positive for ChAT in the PTD model represents specific or general cell death in the MS/DB region. Analysis of total (cresyl violet) or other cell population (such as GABA) would shed light on this question.

It was interesting that there was a trend ($p=0.09$) for the ACh efflux in the amygdala to be higher in the PTD-treated rats than that observed in the PF controls. This suggests that there is some effort towards a compensatory response in PTD-treated rats. As stated above, PTD rats do show a compensatory response on a matching-to-position task if they can use reward information (amygdala-dependent process) rather than spatial location (hippocampal-dependent process) to

solve the task (Savage and Langlais, 1995). ACh efflux in the hippocampus, amygdala and dorsal striatum has provided a neurochemical marker of differential activation during maze exploration and learning (Chang and Gold, 2003; Gold, 2004; McIntyre et al., 2003a). Although a previous study found a correlation between alternation performance and amygdala ACh efflux in normal rats (McIntyre et al., 2003a), we did not observe such an effect in our study that included the behaviorally impaired PTD rats. Increases in amygdalar ACh levels are unique in that they can be achieved under either complementary or compensatory reactions (McIntyre et al., 2003a; McIntyre et al., 2003b). However, our data suggest that when the hippocampus is dysfunctional, activation of the amygdala does not lead to behavioral recovery on a spatial exploration task without reward in the PTD model. Thus, given the demands of the task, level of training and integrity of the brain, amygdalar ACh response can vary to correlate with behavior or not.

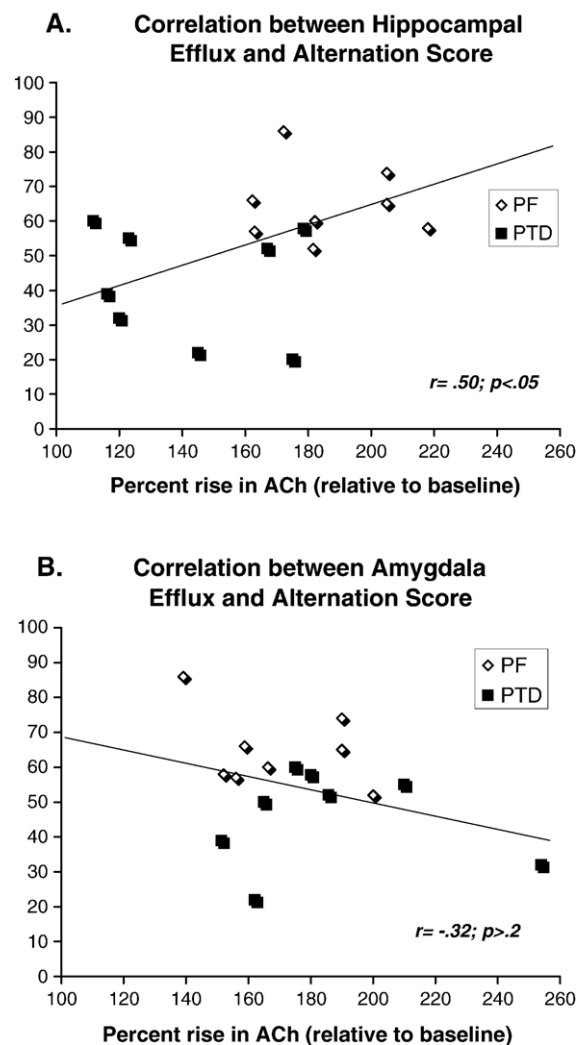


Fig. 5 – Correlative relationship between spontaneous alternation rate and ACh efflux in the hippocampus (top) and amygdala (bottom). Trend lines on each graph represents the overall combined regression analysis for the PTD and PF groups.

This example exemplifies that the role of ACh in learning and memory is not a simple story (Gold, 2004; Hasselmo, 2006; McKinney and Jacksonville, 2005; Parent and Baxter, 2004). A current theory is that the ACh level sets the appropriate physiological tone in the hippocampus (and potentially other structures) for learning and later memory as well as related neural adaptation (Elvander et al., 2004; Gold, 2004; Hasselmo, 2006). Thus, ACh tone across structures modulates adaptation of behavioral strategies (Gold, 2003; Gold, 2004).

The ascending cholinergic pathways of the rat brain are important for providing tonic increases in excitability in cortical and subcortical structures—primarily those that are part of the limbic system (Kirk and Mackay, 2003; Kolmac and Mitrofanis, 1999; Sotty et al., 2003). Specifically, the basal forebrain complex (MS/DB, NBM, substantia innominata) provides cholinergic input to a number of limbic structures. As noted in the introduction, the MS/DB provides the major cholinergic input to the hippocampus and the NBM provides the major cholinergic input into the amygdala as well as the cortex (Wainer and Mesulam, 1990). Interestingly, these subpopulations of cholinergic neurons respond differently to some neural toxic events and appear to have differential vulnerability to oxidative stress (McKinney and Jacksonville, 2005). The cholinergic neurons in the NBM that project to the amygdala are not damaged by the immunotoxin IgG-saporin, presumably because they do not express the p75 receptor (Berger-Sweeney et al., 1994; Heckers et al., 1994). Our results suggest that these neurons are also less sensitive to the neural insults caused by thiamine deficiency. Our population estimates of ChAT immunopositive cells in the NBM, and the MS/DB, are similar to other published results using stereological estimation techniques (Miettinen et al., 2002; Yoder and Pang, 2005). It should be pointed out that significantly lower number of ChAT immunopositive cells does not necessarily indicate cell death, although it is commonly used as a measure of cholinergic cell loss (McKinney and Jacksonville, 2005; Yoder and Pang, 2005). The exact mechanisms of cholinergic neural degeneration remain controversial (McKinney and Jacksonville, 2005).

Multiple mechanisms of cell death, as a result thiamine deficiency, have been proposed for the diencephalic damage. The data suggest that after thiamine deficiency there is mitochondrial dysfunction that leads to impairments in cerebral energy metabolism, lactic acidosis, glutamate receptor-mediated excitotoxicity and oxidative stress (Todd and Butterworth, 1999). Thus, the two main theories to explain neuronal death in the PTD model are the excitotoxicity hypothesis and the oxidative stress hypothesis, but these mechanisms are not mutually exclusive (Desjardins and Butterworth, 2005). Currently, we do not know the mechanisms of ChAT-immunopositive cell loss in the PTD model. However, degeneration of basal forebrain cholinergic neurons also involves mitochondrial abnormalities and oxidative stress (McKinney and Jacksonville, 2005).

Another question that remains to be answered is: Why is the cholinergic activation of the hippocampus selectively impaired during behavioral testing in the PTD model? However, to answer that question we must also understand the molecular mechanisms responsible for the cholinergic modulation of hippocampal activity that regulates behavior. Our

data suggests that baseline cholinergic tone in the hippocampus and amygdala is normal in the PTD model. However, when environmental or behavioral demands are placed on the hippocampal system there is not enough of an increase in ACh efflux to produce successful behavioral change on spatial tasks. This lack of adaptation in the hippocampal cholinergic response may be due to the loss of MS/DB cells in the PTD model. There is evidence that both cholinergic and GABAergic cells in the MS/DB modulate neural activation of the hippocampus (Alreja et al., 2000; Yoder and Pang, 2005; Wu et al., 2003). Cholinergic activation in the hippocampus triggers rhythmic activity that can persist for hours and such rhythmic activity is related to both learning and neural plasticity (Colgin et al., 2003). Furthermore, our recent data demonstrated that when the hippocampal ACh levels are increased in the hippocampus by direct application of physostigmine, the behavioral impairment in spontaneous alternation in PTD rats is eliminated (unpublished data). Limited clinical trials have also revealed that anticholinesterase inhibitors improve cognitive functioning in patients with WKS (Angunawela and Barker, 2001; Cochrane et al., 2005). Thus, although cholinergic dysfunction may be an overlooked feature in thiamine deficiency-induced amnesia, increasing ACh levels does lead to significant behavioral recovery in the human disorder of WKS and the PTD model.

In the PTD model there is impairment on “hippocampal dependent” tasks—with the most common behavioral measures being assessment of spatial abilities (Langlais and Savage, 1995; Mair et al., 1988; Savage and Langlais, 1995). There are two disease features of thiamine deficiency-induced amnesia that could result in this selective hippocampal dysfunction: (1) As the present study suggests, the loss of ChAT positive neurons in the MS/DB produces an inadequate activation of the hippocampus during behavioral testing; (2) Lesions of discrete diencephalic regions (anterior thalamus, mammillary bodies) can lead to memory impairment as well as changes in hippocampal physiological activity (Jenkins et al., 2002; Reed et al., 2003; Vann and Aggleton, 2004; Vertes et al., 2004; Warburton et al., 2001). Additional research is needed comparing the two factors before we can identify the primary mechanisms for ACh dysfunction in diencephalic amnesia.

In summary, a picture is emerging that suggests there are significant cholinergic abnormalities in thiamine-deficiency related amnesia. However, these abnormalities appear selective to the septohippocampal axis. The present findings further demonstrate the complexity of the role of ACh in learning and memory—particularly across different memory-related structures. Brain integrity interacts with task demands to determine whether compensatory mechanisms or strategies will be successful in behavioral adaptation.

3. Experimental procedures

3.1. Subjects

Twenty-two young male Sprague–Dawley rats (3–4 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-h/12-h light–

dark cycle (onset at 7:00 am). All rats were fasted the night before behavioral testing.

3.2. PTD treatment

Animals were first randomly assigned to one of the following treatments: (i) pair-ed control (PF, $n=11$), or (ii) pyriethamine-induced thiamine deficiency (PTD, $n=11$) 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 pyriethamine hydrobromide (Sigma, St. Louis, MO). On days 14–16 of treatment, animals display signs of local tonic movement of the front and hind limbs, and generalized convulsions (seizures). Within 4 h after observing the onset of seizure, PTD-treated animals were given an injection of thiamine (100 mg/kg, i.p.) every 8 h 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 (0.4 mg/kg i.p.). After treatment all subjects were placed on regular chow and allowed to regain the weight lost during treatment.

3.3. Surgery

Sixteen days after recovering from PTD or PF treatments stereotaxic surgery (David Kopf Instruments, USA) was performed on animals anesthetized with an i.p. injection (.1 ml/kg) of a Ketamine (83 mg/kg)/Xylazine (17 mg/kg) mixture. Each subject was implanted with two plastic guide cannulae (CMA/12 mm, Carnegie Medicine Associates, Chelmsford, MA). One cannula was lowered into the left hippocampus (5.0 mm posterior to bregma, 5.0 mm lateral to the midline, and 4.2 mm DV) and another guide cannula (CMA/12) was lowered into the lateral amygdala (3.0 mm AP, 4.8 mm ML, 6.8 mm DV) according to the atlas of Paxinos and Watson (Paxinos and Watson, 1986). Cannula placement and cannulae size were based on a previous study assessing ACh efflux in the amygdala and hippocampus (McIntyre et al., 2003a). Two days after surgery animals were handled daily (5 min/day) for 5 days prior to behavioral testing.

3.4. Microdialysis/HPLC

One week following surgery two microdialysis probes (CMA/12) were inserted into the guide cannulae (hippocampus—3 mm; amygdala—2 mm). The probes were connected to plastic tubing and driven by a microinfusion system (CMA/100 pump). The dialysis probes were perfused continuously at a rate of 2.0 μ l/min with artificial CSF (in mM: 128 NaCl, 2.5 KCl, 1.3 CaCl₂, 2.1 MgCl₂, NaH₂PO₄, 1.3 NaHPO₄, and 1.0 glucose, brought to a pH of 7.4), which contained the acetylcholinesterase inhibitor neostigmine (500 nM). Prior to maze testing, the microdialysis probes were inserted into the hippocampal and amygdalar cannulae and the animal was placed into the holding cage (41 cm \times 30 cm \times 35 cm) located in the testing room. After 60 min of stabilization, dialysis samples (sample volume 12 μ l) were collected every 6 min for a period of 24 min in the holding cage to determine basal levels of ACh in awake

rats. During this initial baseline phase the animal was free to move about the holding cage. After initial exploration, most animals sat in a corner and occasionally groomed. After 4 baseline samples were collected, the rat was gently picked up and placed on the center of the maze. The plus-maze used for behavioral testing was made of wood with clear Plexiglas sidewalls (12 cm high) and a painted black floor with the four arms of equal distance (55 cm) was used for training. It was elevated 80 cm from the floor. The rat was allowed to transverse the maze freely for 24 min. The number and sequence of arms entered were recorded to determine alternation scores (see Chang and Gold, 2004; Savage et al., 2003).

An alternation was defined as the choice of an arm to the left or right of the rat when it reached the center area, not the arm straight ahead or a re-enter in to the same arm. The percent alternation score is equal to the ratio of: (actual alternations/possible alterations) \times 100. The maze testing room contained various extramaze cues (posters, doors, tables, etc). Upon completion of 24 min of maze testing, rats were transferred back to the holding cage for an additional 24 min. One PTD and 2 PF rats had microdialysis collection problems during behavioral testing and their data were discarded.

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 acetylcholinesterase and choline oxidase, and a peroxidase wired working electrode. The detection limit of this system is 10 fmol. ACh peaks were quantified by comparison to peak heights of standard solutions and corrected for in-vitro recovery of the probe.

3.5. Tissue fixation and histological analysis

At the conclusion of behavioral testing, animals were anesthetized with 0.5 mg/kg, i.p. injection of Sleep-away (26% sodium pentobarbital in 7.8% isopropyl alcohol and 20.7% propylene glycol solution; Fort Dodge, Iowa) and perfused transcardially using 0.9% saline solution and 4% phosphate-buffered paraformaldehyde. Their brains were removed, post fixed in a 10% formalin solution for at least 72 h and then transferred to a 30% sucrose solution. Coronal sections from the brains were cut (60 μ m thick) on a vibratome from the level of the anterior commissure to the level of the posterior pontine tegmentum, sections were collected into wells in sequential order and every fifth section was stained with cresyl violet for assessment of probe location.

3.6. Immunocytochemical (ICC) analysis

Every 6th section (with a random start point within the first six sections) throughout the extent of the MS/DB and the NBM were systematically collected and were used for immunocytochemical analysis of choline acetyltransferase (ChAT) to assess the integrity of the basal forebrain cholinergic system. For this assay, 0.1 M phosphate buffer (PB) of pH 7.3 was used for all washes, and all steps until the chromogenic reaction were carried out at 4 $^{\circ}$ C. Sections were first placed in PB

overnight; the next day, sections were washed in PB three times for 10 min each. A quench of 30% H₂O₂ in 60 ml of PB was then undertaken for 20 min. Three 5-min PB washes were performed, after which the sections were incubated in the primary antibody (goat anti-ChAT, 1:100; Chemicon, CA) for approximately 24 h. Next, sections were washed in PB three times for 5 min each, then incubated in the secondary antibody (biotinylated rabbit anti-goat, 1:100; Vector Laboratories, CA) for 1 h, followed by three more 5-min PB washes. Sections were then incubated in avidin–biotin complex (Vector) at 4 °C for 1 h followed again by three 5-min PB washes. The sections were placed in the chromogenic (DAB fast tab; Sigma-Aldrich, IL) reaction for approximately 1 min. The reaction was stopped with three 5-min PB washes, after which the sections were mounted on gelatinized slides, air-dried overnight, dehydrated through ascending alcohols, and cover-slipped. To prevent variability in staining due to ICC procedures, the brains of 6–8 animals, equated across experimental groups, were processed at the same time using the same reagents and temperature conditions. In each ICC run, control for specificity was conducted using 4 sections (2 MS/DB, 2 NMB) from a randomly selected PF and PTD rat, using the above procedures with the exception that the primary antibody was replaced by normal goat serum. Under those conditions no immunostaining was observed.

One of the PF subject's tissue was in poor condition and would not withstand immunocytochemistry procedure. Thus, the brains from seven subjects each from the PF and PTD groups were used to estimate ChAT cell populations.

3.7. Stereology

Unbiased stereology was used to estimate ChAT immunopositive cell counts in the MS/DB and NMB. Parameters for sampling were based on previously published studies assessing the number of ChAT positive neuron in the basal forebrain (Miettinen et al., 2002; Yoder and Pang, 2005). A low power image was captured with a digital camera (DVC-1310; DVC Company, Austin Texas) with a 5X lens on a Zeiss microscope (Axioscope 2-plus) with an attached 3-axis motorized stage. A contour was drawn around the region of interest using StereoInvestigator software (MicroBrightField; Williston, VT). These cholinergic nuclei are well defined and have clear boundaries particularly when stained with a ChAT antibody (see Figs. 2A, B). Anatomical boundaries used to define the basal forebrain were the corpus callosum on the dorsal aspect and the walls of the lateral ventricles. Sections began with the appearance of the rostral genu of the corpus callosum crossing hemispheres and ended caudally with the last section containing the full anterior commissure (approximately IA 10.6–8.74 mm [Paxinos and Watson, 1986]). For the NMB the anatomical landmarks were the appearance of the fornix and the anterior thalamus. The boundaries were set by the internal capsule, optic tract and globus pallidus (approximately IA 7.7–5.86 mm [Paxinos and Watson, 1986]). ChAT positive neurons must have an immunosignal in the soma to be counted. The neurons were counted with a 40× objective using optical fractionator approach (see Fig. 2C). The fractionator sampling consisted of a section sampling fraction (ssf=1/6), an area sampling fraction representing ratio between counting frame

size and grid size (asf=50 μm×50 μm/100 μm×100 μm) and a height sampling fraction (hsf=20 μm/60 μm). The equation for determining cell estimates was: $N = \sum Q^- \times 1/ssf \times 1/asf \times 1/hsf$, where Q^- is the number of neurons actually counted in the specimens, whereas N represents the total cell estimation.

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