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Chronic aspartame affects T-maze performance, brain cholinergic receptors and Na^+,K^+-ATPase in rats

Brandon Christian, Kenneth McConnaughey, Elena Bethea, Scott Brantley, Amy Coffey, Leigha Hammond, Shelly Harrell, Kasee Metcalf, Danielle Muehlenbein, Willie Spruill, Leslie Brinson, Mona McConnaughey*

Department of Pharmacology, Brody School of Medicine, East Carolina University, Greenville, NC 27858, USA
North Carolina School of Science and Mathematics, Durham, NC 27811, USA

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Abstract

This study demonstrated that chronic aspartame consumption in rats can lead to altered T-maze performance and increased muscarinic cholinergic receptor densities in certain brain regions. Control and treated rats were trained in a T-maze to a particular side and then periodically tested to see how well they retained the learned response. Rats that had received aspartame (250 mg/kg/day) in the drinking water for 3 or 4 months showed a significant increase in time to reach the reward in the T-maze, suggesting a possible effect on memory due to the artificial sweetener. Using [^{3}H]quinuclidinyl benzilate (QNB) (1 nM) to label muscarinic cholinergic receptors and atropine (10^{-6} M) to determine nonspecific binding in whole-brain preparations, aspartame-treated rats showed a 31% increase in receptor numbers when compared to controls. In aspartame-treated rats, there was a significant increase in muscarinic receptor densities in the frontal cortex, midcortex, posterior cortex, hippocampus, hypothalamus and cerebellum of 80%, 60%, 61%, 65%, 66% and 60%, respectively. The midbrain was the only area where preparations from aspartame-treated rats showed a significant increase in Na^+,K^+-ATPase activity. It can be concluded from these data that long-term consumption of aspartame can affect T-maze performance in rats and alter receptor densities or enzymes in brain.

Keywords: Aspartame; Cholinergic receptors; Chronic; T-maze; Memory; ATPase

1. Introduction

Anecdotal reports on the toxic effects of aspartame (NutraSweet) are numerous, and various issues continue to be raised today, more than 20 years after aspartame approval by the FDA. Concern relating to possible adverse effects has been raised due to the metabolic components, phenylalanine, aspartic acid, diketopiperazine (DKP) and methanol as well as the compound itself. (Trocho et al., 1998) There are many accounts of situations in which aspartame is believed to have caused negative effects on specific human functions. These include brain tumors, memory loss, seizures, headaches, confusion, personality disorders, visual difficulty and dizziness. (Tollefson and Barnard, 1992) There is very little scientific evidence in the literature to prove an aspartame connection in these instances. Shortly after aspartame was marketed, the FDA began to receive an increased number of reports concerning adverse reactions related to aspartame (Garriga and Metcalfe, 1988). However, conclusive evidence was not found (Aspartame, 1985; Butchko and Stargel, 2001; Butchko et al., 2002; Stegink, 1987; Stegink et al., 1981; Yost, 1989).

Numerous short-term studies have been conducted and none of these have suggested any relationship between aspartame consumption and memory loss (Moser, 1994). Very few long-term studies have been done. Most short-term studies consisted of either giving one large dose of aspartame or treating for a short time (a few days or weeks) and then assessing aspartame's effects on learning or memory. Whether done in either humans or animals, these studies have shown no adverse effects of aspartame on memory (Lapierre et al., 1990; Mullenix et al., 1991; Saravis et al.,

* Corresponding author. Tel.: +1-252-744-3301; fax: +1-252-744-3203.
E-mail address: mcconnaugheym@mail.ecu.edu (M. McConnaughey).

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1990; Shaywitz et al., 1994; Spiers et al., 1998; Stokes et al., 1994; Tilson et al., 1991; Wolraich et al., 1994). In a longer study, Holder (1989) showed that 50 days of NutraSweet had no effect on reflex or spatial memory development. Another study (Leon et al., 1989) showed no persistent changes in vital signs, body weight or standard laboratory tests in subjects receiving aspartame for 24 weeks; however, extensive memory testing was not done. A few chronic studies have implicated aspartame consumption in learning or memory. Potts et al. (1980) showed that administration of aspartame as 9% of the diet for 13 weeks altered learning behavior in male rats. Using a much lower daily dose of aspartame, Dow-Edwards et al. (1989) treated pregnant guinea pigs throughout gestation and demonstrated the aspartame-treated pups showed a disruption of odor-associative learning.

Various neurochemical effects due to aspartame consumption have been reported (Coulombe and Sharma, 1986; Goerss et al., 2000; Pan-Hou et al., 1990). Neuropeptide Y concentrations have been shown to be lower in arcuate nucleus in rats treated with aspartame for 14 weeks (Beck et al., 2002). Certain brain amino acid levels have been shown to be increased after aspartame consumption (Dailey et al., 1991; Diomede et al., 1991; Yokogoshi et al., 1984). Neurochemical changes following high-dose aspartame with dietary carbohydrates have also been reported (Wurtman, 1983). Taken collectively, these studies suggest that aspartame might affect brain neurotransmitters and receptors, and these effects may become more prominent with long-term consumption.

Numerous studies have implicated muscarinic cholinergic receptors in learning and memory (Bartus et al., 1982; Granon et al., 1995; Kadar et al., 1990; Mezey et al., 1999; Rose et al., 1980; Russell, 1996; Uchida et al., 1991; Van der Zee and Luiten, 1999; Vogt et al., 1991;) In the rabbit, elevated muscarinic binding has been shown in the anterodorsal nucleus early in the learning process, and this increase was maintained throughout subsequent training (Vogt et al., 1991). The density of muscarinic receptors in the CNS has been correlated with cognitive performance in aging Wistar rats (Kadar et al., 1990). Two or more muscarinic receptor states have been suggested to be associated with age-related memory deficits in laboratory animals (Lippa et al., 1985). Muscarinic receptor binding has been shown to be altered in forebrain and midbrain regions of chicks during passive avoidance learning (Longstaff and Rose, 1981). It has been suggested that nicotinic transmission may be important in delayed response tasks, while the muscarinic system may be involved in general working memory processes (Granon et al., 1995). These studies lead us to hypothesize that if memory impairment were seen with chronic aspartame consumption in the rat, then we might see an alteration in brain muscarinic cholinergic receptor densities.

Ionic involvement has been suggested to be involved in memory formation and the Na^+,K^+-ATPase enzyme is crucial for maintaining ionic gradients in neurons and tissues (Conrad and Roy, 1993; Ng et al., 1992). Na^+,K^+-ATPase activity has been found to change in young chicks after taste stimulation using a chemical aversant (Hajek et al., 1994). Bourre et al. (1989) have found that a diet rich in sunflower oil can affect Na^+,K^+-ATPase activity in rat brain cells and alter learning tasks measured with the shuttle box test. Because these studies suggest that Na^+,K^+-ATPase activity could potentially be involved with memory, we also wanted to investigate the possibility that chronic aspartame treatment might affect the levels of this enzyme in the brain.

The specific aim of this study was to determine if long-term aspartame administration (4 months) would lead to memory loss using rats trained in a T-maze and if so, to explore a possible biochemical explanation by measuring muscarinic cholinergic receptor densities and Na^+,K^+-ATPase activity in nine brain areas. We chose the aspartame dose of approximately 250 mg/kg/day because this dose is consistent with other values in the literature and could be easily within the limits of human consumption after species factor correction. Dose comparisons between humans and rats have usually been corrected by a factor of 5 since rats metabolize aspartame faster than humans (Fernstrom, 1989); however, a factor of 60 has also been suggested as a better value to use (Wurtman and Meher, 1987). The everyday consumption of NutraSweet by people is increasing and it is important to know if this substance has long-term adverse effects under certain conditions. Such studies are necessary to prove or disprove existing fears concerning aspartame.

2. Methods

2.1. T-maze

The T-maze was brown and had a start arm and a left and right arm (80×7×30 cm, Fig. 1). A dark screen covered the top of the entire maze. At the extremity of each arm, there was an opening to a 12×15-cm room. In the middle of the right room, a 1-g piece of chocolate was placed as the reward. Latency to find the reward was recorded as the seconds from the time the animals entered the maze until they found the chocolate.

2.2. Animals

Male Sprague–Dawley rats (225 g) were housed two per cage with unlimited access to laboratory chow. Control rats received regular tap water and treated rats received aspartame in the drinking water (250 mg/kg/day). Body weight as well as food and water consumption was recorded throughout the 4 months. Drinking solutions of aspartame were prepared to provide the appropriate dose of aspartame in the expected volume consumed. The

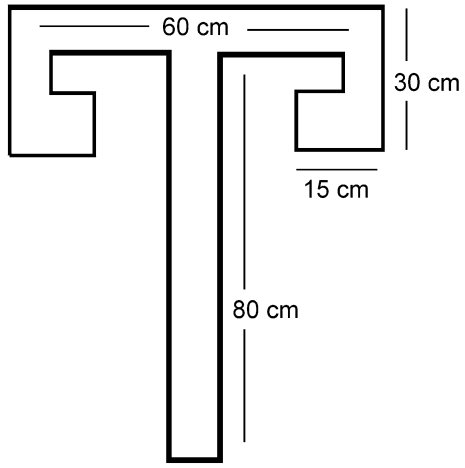

Fig. 1. T-maze dimensions are depicted as described in Methods. Reward was always placed on the right side at the end of the maze.

gain in body weight and the amount of water consumed during the 4 months of treatment were not affected by aspartame. Rats were trained three times/day in the T-maze for 2 weeks. At the end of this time all animals would consistently find the reward (piece of chocolate) at the end of the maze within 12 s. The animals were then periodically tested in the same T-maze at the same time each day (4:00 p.m.) for the next 4 months and the seconds to reach the reward recorded. At the end of the 4 months, the animals were anesthetized with pentobarbital (60 mg/kg), sacrificed by decapitation and brains quickly removed and frozen at −70 °C until time of assay. The experimental protocol was approved by the East Carolina University Institutional Review Committee for the Use of Human or Animal Subjects.

2.3. Membrane preparations

For whole-brain preparations, the frozen brains were thawed and homogenized for 15 s with a Brinkmann Polytron PT-10 in 10 ml of ice-cold homogenization buffer (50 mM Tris base, 150 mM sucrose, 5 mM $MgCl_2$, pH 7.4 with HCl). The homogenate was then centrifuged at 500×*g*, the pellet discarded, and the supernatant centrifuged at 10,000×*g* for 20 min. The pellet was resuspended in cold homogenization buffer to a concentration of 8–10 mg/ml.

For individual brain areas, the brains were thawed and the nine areas dissected. These sections were then homogenized in 3–5 ml of ice-cold buffer (50 mM Tris base, 150 mM sucrose, 5 mM $MgCl_2$, pH 7.4 with HCl) for 10 s with a Brinkmann Polytron PT-10. The homogenate was centrifuged for 15 min at 10,000×*g*. The pellet was resuspended in 1.5–2 ml of ice-cold homogenization buffer and immediately assayed. Excess membrane preparations were frozen at −70 °C and were stable up to 4 months when stored in this manner. Protein was determined by the method of Lowry et al. (1951).

2.4. Radioligand binding assay

Maximal binding capacity (B_{max}) was determined by the use of [^{3}H]quinuclidinyl benzilate (QNB, Perkin-Elmer) to label the receptors. Briefly, 1 nM [^{3}H]QNB was incubated with 40–50 μg membrane protein in 200 μl total volume (buffer: 50 mM Tris, 5 mM $MgCl_2$, pH 7.4) for 30 min at 27 °C. At the end of the incubation period the tubes were placed on ice for 10 min, rapidly filtered through Whatman GF/C glass fiber filters and washed with 12 ml of ice-cold incubation buffer. Nonspecific binding was determined in the presence of 10^{-6} M atropine. Radioactivity remaining on the filters was quantified using a Beckman scintillation counter.

2.5. Na^+,K^+-ATPase assay

Na^+,K^+-ATPase activity was measured at 37 °C by monitoring the release of inorganic phosphorus from 3 mM Tris ATP (Blumenthal et al., 1982; McConnaughey et al., 1979) Total Na^+,K^+-ATPase activity was unmasked in membrane preparations by pretreating the membranes with sodium dodecyl sulfate (SDS) (Besch et al., 1976) Briefly, freshly thawed preparations (approximately 1 mg/ml) were diluted 1:2 in 30 mM imidazole–HCl buffer (pH 7.1) containing 3.8 mM SDS. After preincubation for 20 min at room temperature, 20 μl of the diluted suspension was added to previously prepared reaction tubes containing 1 ml incubation medium (50 mM histidine, 3 mM $MgCl_2$, 100 mM NaCl, 10 mM KCl, pH 7.4). Na^+,K^+-ATPase activity was defined by the activity inhibited by 8 mM ouabain.

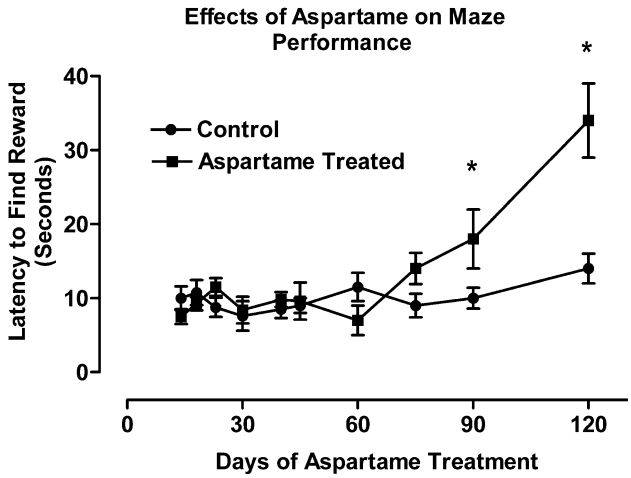

Fig. 2. Aspartame effects on latency to find reward in the T-maze. Seconds were measured from time of T-maze entry to when the reward (chocolate) was found for control and aspartame-treated rats. At 90 days of aspartame treatment and at 120 days of treatment the treated animals took significantly longer to find the reward than the controls. *P<.05 (n=12).

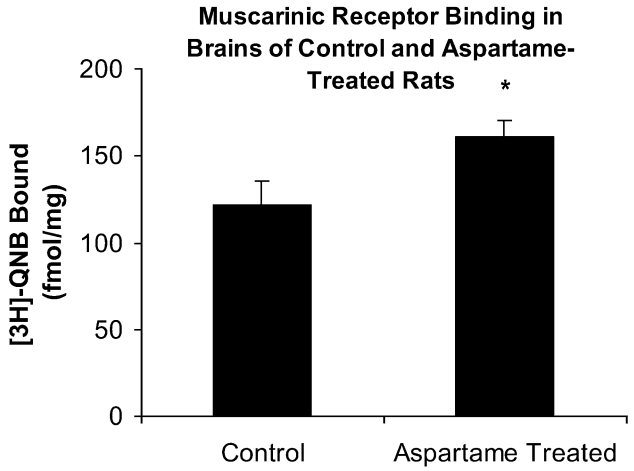

Fig. 3. [^{3}H]QNB binding for control and aspartame-treated rats (4 months) was performed as described in Methods. Brain tissue from aspartame-treated rats had significantly more apparent muscarinic cholinergic receptors when compared to controls. *P<.05 (n=6).

2.6. Statistics

Data are expressed as the mean±S.E.M. All values were compared with a Student's t test. The level of statistical significance for these experiments was P<.05.

3. Results

The results of this study demonstrated that rats consuming aspartame in the drinking water for 3–4 months took longer to find the reward in a T-maze (Fig. 2). After 90 days of treatment, rats that had received aspartame showed a significant increase (P<.05) in time to reach the reward, with controls taking 10±1.4 s and aspartame-treated rats taking 18±4 s. After 120 days of treatment, control rats took 14±2 s to reach the reward and aspartame-treated rats took 34±5 s (P<.05). The aspartame-treated animals did not show any differences in the amount of food or water consumed when compared to controls, and at the end of the 4 months both groups had gained a similar amount of weight (data not shown). Aspartame-treated rats learned at the same rate as control rats initially when maze training took place during the first 2 weeks.

Table 1
Na^+,K^+-ATPase in control and aspartame-treated rats (μmol Pi/mg protein/h)

Tissue	Control	Aspartame-treated
Frontal cortex	3.86±0.05	3.98±0.40
Midcortex	3.74±0.05	3.88±0.09
Posterior cortex	3.41±0.06	3.47±0.06
Hypothalamus	4.55±0.08	4.67±0.09
Hippocampus	2.99±0.08	2.83±0.09
Pons	2.59±0.02	2.45±0.07
Medulla	2.53±0.08	2.41±0.10
Cerebellum	3.42±0.16	3.04±0.19
Midbrain	3.58±0.05	4.39±0.04*

Na^+,K^+-ATPase activities were assessed as described in Methods. The midbrain area was the only one that showed a difference between control and aspartame-treated animals.

* Significantly different from control (P<.05). Determinations were on four to five separate pooled preparations each containing two to six brain areas each.

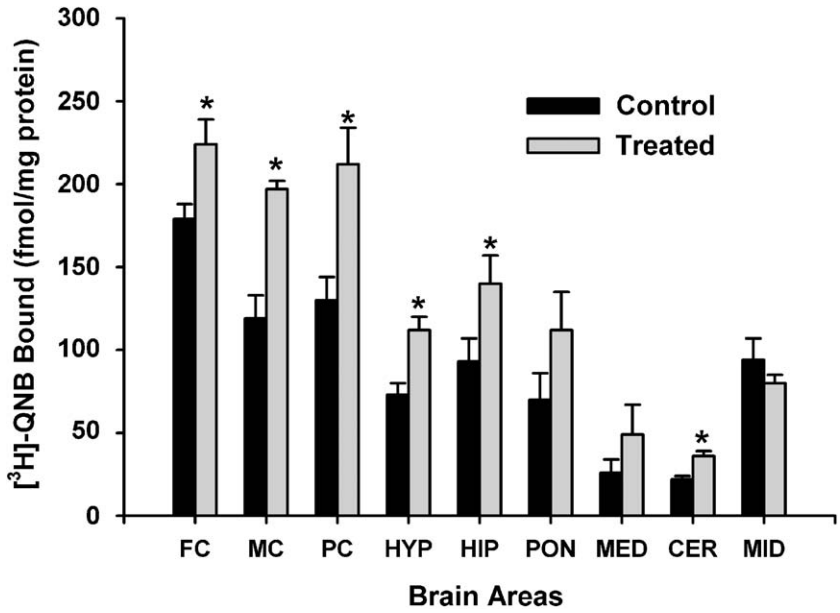

Fig. 4. Aspartame effects on muscarinic receptor binding in various brain regions. [^{3}H]QNB binding for control and aspartame-treated rats (4 months) was performed as described in Methods. Areas included frontal cortex (FC), midcortex (MC), posterior cortex (PC), hypothalamus (HYP), hippocampus (HIP), pons (PON), medulla (MED), cerebellum (CER) and midbrain (MID). FC, MC, PC, HYP, HIP and CER tissue from aspartame-treated rats had significantly more apparent muscarinic cholinergic receptors when compared to controls. *P<.05 (determinations were on three to five separate pooled preparations each containing two to six brain areas each).

As shown in Fig. 3, muscarinic cholinergic receptor densities were found to be significantly higher ($P<.05$) in whole-brain preparations from aspartame-treated rats (161±16 fmol/mg protein) when compared to controls (122±8 fmol/mg protein). When particular brain areas were investigated, it was found that apparent muscarinic receptor numbers were significantly higher ($P<.05$) in all three areas of the cortex as well as the hypothalamus, hippocampus and cerebellum (Fig. 4). No significant differences were observed in the pons, medulla or midbrain. Affinities of the muscarinic receptor for the agonist methacholine were not different between control and treated animals (data not shown).

Na^+,K^+-ATPase activities were similar in all areas of brain tested (Table 1) with the exception of the midbrain where the activities were significantly increased in the aspartame-treated animals ($P<.05$).

4. Discussion

This study produced the novel findings that chronic aspartame consumption lengthened the time it took rats to find the reward in a T-maze and increased muscarinic receptor numbers in specific brain areas. We postulate this first finding to represent impaired long-term memory retention. This effect was seen only after prolonged aspartame administration supporting short-term studies finding no effects. The impairment was seen only after 90 days of aspartame consumption and increased with longer exposure to up to the 120-day conclusion of the study. At this final endpoint, not only did the aspartame-treated rats take longer to find the reward, but two of the treated rats even went to the wrong side of the T-maze, totally forgetting where the reward was. These results indicate the aspartame-treated animals did not retain the learned behavior as well as the control rats. Other explanations for these results might include a decrease in smell to locate the reward or a decreased desire for the chocolate reward; however, once the rats did locate the reward, they devoured it immediately. Other physiological markers including weight gain and water and food consumption appeared stable throughout the study, making it less likely that an impaired sensory or metabolic effect of the chemical could be the cause of the impaired maze performance. Aspartame did not affect learning early in the course of the experiment when the animals were being trained in the maze. We found the aspartame-treated rats learned at the same rate as the control rats.

This study did not address which stage or stages of memory could possibly be affected by aspartame. The rats seemed more vulnerable to forgetting the learned T-maze task after 4 months, and it is certainly possible that various memory stages including short-term, long-term, semantic, recognition, implicit or memory consolidation could be affected (Brunelli et al., 1997, Murre et al., 2001). If long-term memory or memory recall involves synthesis of proteins and gene expression, then it is certainly possible that chronic exposure to high amounts of aspartame could affect these processes. Since hormonal as well as neural influences can regulate memory consolidation (McGaugh, 2000), then long-term exposure to aspartame may also play a part in impairing this consolidation. Three distinct stages of memory were recently described by Walker et al. (2003) involving initial, sleep dependent and recall phases. The recall phase allows a previously stabilized memory to be modified, and it is certainly possible that chronic aspartame could influence this phase.

We hypothesized that if long-term aspartame consumption appeared to affect memory retention in the rats, then brain muscarinic cholinergic receptor densities might also be altered by the chronic aspartame. The second major finding of this study demonstrated that after 4 months of aspartame treatment, muscarinic receptor densities were increased in numerous brain areas. If we relate these increases to decreased memory retention, then our data are contradictory to the results of others who show a correlation between muscarinic blockers or a decreased number of brain muscarinic receptors and impaired memory (Granon et al., 1995; Okuma et al., 2000; Power et al., 2000; Uchida et al., 1991). Considerable evidence supports an increase in cholinergic receptor binding being associated with learning and memory (Gill and Gallagher, 1998; Loullis et al., 1983; Vogt et al., 1991); however, other studies have suggested a decrease in muscarinic receptors may be involved with improved memory. Anagnostaras et al. (2003) showed that M_1-deficient mutant mice showed enhanced memory for tasks that involve matching-to-sample problems. Lerer et al. (1984) showed that diisopropyl fluorophosphate administration caused a decreased number of muscarinic receptors and that this was associated with enhanced performance on memory tasks. These studies are consistent with the idea that if muscarinic receptors are down-regulated, then certain memory functions may be enhanced. Our results indicate that an increase in muscarinic receptors may be related to memory-retention problems and that chronic consumption of aspartame may be partially responsible.

Our study investigated total number of muscarinic receptors but did not evaluate specific receptor subtypes. It is possible that aspartame may selectively affect both numbers and affinities of muscarinic receptor subtypes in the different brain regions. Various studies have implicated muscarinic subtypes to be involved in memory formation (Ortega et al., 1996; Patterson et al., 1990). By decreasing M_2 receptors with antisense oligonucleotides, Galli et al. (2000) showed that scopolamine-induced memory impairment in the Morris water maze was reversed; thus learning and memory improved. These authors postulated that there might be an increase in acetylcholine to compensate for the decrease in receptors and that this increase could possibly be related to the improved memory. The up-regulation of muscarinic receptors that we observed after 4 months of

aspartame consumption could be related to a compensatory decrease in acetylcholine levels or be due to other compensatory mechanisms such as sprouting.

Although Na^+,K^+-ATPase has not attracted as much attention dealing with memory and learning as muscarinic receptors, this enzyme has been implicated in memory function (Brunelli et al., 1997; Klink and Alonso, 1997; dos Reis et al., 2002; Nakazato et al., 2002). It is interesting that the only area of brain where we showed Na^+,K^+-ATPase activity to be altered was the midbrain area. This may be an effect unrelated to memory retention, but may be specific for chronic aspartame consumption.

It is certainly possible that the increases we observed in muscarinic receptor densities are unrelated to the memory deficits observed after 4 months of aspartame consumption. In addition, aspartame may be producing nonspecific increases in cholinergic receptor densities since these increases are similar in brain areas known to involve memory formation such as the hippocampus, as well as areas not associated with memory formation such as the hypothalamus. Our data support the idea that the inability to remember where the reward is in the T-maze could be related to an increased density of brain muscarinic receptors; however, it is certainly possible that other receptors, enzymes or transmitters are altered with long-term aspartame treatment and contribute to this decreased maze performance. Conflicting data exist concerning aspartame's effects on various receptors and transmitters. Pan-Hou et al. (1990) demonstrated that aspartame caused a significant change in affinity of L-[^{3}H]glutamate binding, whereas Reilly et al. (1989) found no changes in receptor binding for six amine neurotransmitter receptors after 30 days of aspartame treatment. Others have reported various neurochemical alterations due to aspartame consumption (Beck et al., 2002; Fernstrom et al., 1986; Goerss et al., 2000; Melchior et al., 1991). These data taken collectively suggest that the possibility is there for other receptors or transmitters to be altered by chronic aspartame treatment in addition to the increased density of muscarinic receptors that we have shown.

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