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Lactate as a Memory-Enhancing Metabolite Across the Lifespan in Male Fischer 344 Rats

A Capstone Project Submitted in Partial Fulfillment of the
Requirements of the Renée Crown University Honors Program at
Syracuse University

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And Renée Crown University Honors
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Abstract

Aging is often associated with cognitive decline, including problems with working memory and difficulties forming new memories. These deficits can be directly linked to the hippocampus, an area of the temporal lobe of the brain that is engaged during spatial working memory. Age-related declines may be influenced by changes in important modulatory pathways that impact hippocampal function, including regulation of the metabolite lactate. When astrocytic stores of glycogen are hydrolyzed, lactate is released into the extracellular space where it can be taken up by neurons and used as fuel during moments of activation. We previously found that the extracellular concentration of lactate rises in the hippocampus of young male rats while they performed a spatial working memory task (Newman et al., 2011). Infusions of lactate into the hippocampus of young adult males improved their memory performance on this same task (Newman et al., 2011). Recent data has shown that lactate does not rise as robustly in the hippocampus of old rats during spatial working memory when compared to their young counterparts. To investigate whether age-related memory deficits were a result of reversible shifts in metabolic regulation, young (3 months) and old (24 months) male Fischer 344 rats were given infusions of lactate or a vehicle control directly into the hippocampus immediately before a spatial working memory task. Old rats that received infusions of lactate showed robust improvement on this task compared to saline controls or young rats with the same treatment. These results indicate that age-related shifts in memory may be due to changes in metabolic regulation and may be reversed with the application of specific useful metabolites.

Executive Summary

Aging can be defined as the changes in an organism's physiology associated with the passage of time. Elderly individuals frequently experience memory deficits that can drastically influence their quality of life. These deficits often manifest as difficulty forming new memories and problems with working memory. It is unclear whether these memory deficits are a result of permanent changes to the brain tissue itself, to changes in the systems that modulate the brain's function, or both. An important modulator of memory shown to change across the lifespan is metabolism. The human brain is an extraordinarily hungry organ that relies on metabolic fuel delivered by the body. Although the brain is only 5% of the bodies' total weight, it consumes approximately 20% of the bodies' total energy requirements. Activation of specialized areas of the brain result in localized increases in metabolic demand.

The brain consists of different cell types that take on different functional roles. Neurons are cells that send electrical and chemical signals creating intricate circuits, integrating important messages and allowing for cognition. Astrocytes are a variety of glial cell that surround the brain vasculature and neurons and help to maintain the environment of the central nervous system. Astrocytes play a role in mediating how metabolites are removed from the blood vessels and distributed to the neurons. Unlike neurons, astrocytes store glycogen, a large branched molecule constructed with small glycosyl units that can be broken down and used for energy in the form of lactate. The Astrocyte-Neuron Lactate Shuttle Hypothesis is a theory that describes the metabolic dynamics between different cell types in the brain during moments of activation. In moments of high metabolic demand, it is theorized that astrocytes release lactate to be used as metabolic fuel by the highly activated neural circuitry. An important example of this

phenomenon is the metabolic activation of the hippocampus, an area of the temporal lobe associated with the formation of new memories, during tasks that rely on spatial working memory. Previous research has shown that extracellular lactate concentrations increase in the hippocampus in young rats when they perform tasks of this kind. Moreover, infusions of lactate directly into the hippocampus immediately prior to a working memory tasks improve performance of these tasks in young rats. These studies provide evidence that lactate is a powerful modulator of hippocampal-sensitive memory. Changes in the availability or use of this metabolite across the lifespan could explain the memory deficits experienced by elderly populations.

To investigate whether lactate retained its memory enhancing effects across the lifespan old (24 months of age) and young (3 months of age) male Fischer 344 rats were given infusions of lactate (100 nmol/ 0.5 μ l) directly into the hippocampus immediately prior to a spatial working memory task, spontaneous alternation. Guide cannula were surgically implanted into the dorsal hippocampus to allow for infusions directly into this brain region. Each rat received either an infusion of lactate or saline vehicle immediately prior to spontaneous alternation testing. Spontaneous alternation is a hippocampus sensitive task that exploits the rat's tendency to explore novelty to measure spatial working memory. Old rats that did not undergo surgery or infusions displayed age-related memory deficits when compared to their young counterparts. Young animals showed mild improvement with lactate infusion compared to saline infusion, but the enhancement was not statistically significant. Importantly, the old animals displayed a robust improvement with the infusion of lactate, raising memory scores to levels comparable to those of young rats. These impressive improvements were directly related to an increased percent of

correct choices on the task and not other behavioral changes such as increased overall movement or increased number of possible correct choices.

These results support the claim metabolic modulation influences age-related memory deficits in the hippocampus and that these deficits are indeed reversible. Thus, the findings suggest that aged rats can learn and remember new information but need a boost, such as an increase in metabolic support, to engage memory mechanisms as is done endogenously in young rats. However, the underlying cause of these age related changes in metabolic modulation remains unclear. This shift may be influenced by changes in how the astrocytes are activated to produce lactate for use by neurons. One possibility is decreased adrenergic signaling associated with the aging process, potentially leading to a decreased breakdown of glycogen and subsequent decline in available lactate during moments of high metabolic demand.

Scientific inquiry into the mechanics of memory and how it changes across the lifespan are extremely relevant in the twenty-first century. Advances in technology, agriculture, and medicine have improved the general quality of life in many areas of the world and extended average life expectancy dramatically. This demographic development has resulted in a large and ever expanding population of elderly people, resulting in a large population suffering from age-related mild cognitive decline and other more severe dementias. The impending social and economic consequences of this transition to an older population continues to motivate the scientific community to investigate the underlying mechanisms of memory and their shifts across the lifespan.

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Introduction

Due to advances in technology and medicine, people are living longer than ever before. This distinct demographic shift has increased the proportion of the population reaching old age. The quality of life for elderly individuals is often degraded by the development of age-related memory deficits and more severe dementias. Age-related memory deficits associated with the natural aging process may be directly linked to shifts in brain metabolism occurring across an organism's lifetime. Previous research focused on the metabolic demands of learning and memory has highlighted several pathways that may contribute to age-associated cognitive decline. This experiment was designed and performed to investigate how the application of the metabolite lactate influences working memory across the lifespan in a rodent model.

Epinephrine's Influence on Glucose, Glycogen, and Memory

The adrenergic signaling molecule epinephrine represents an important modulator of both metabolism and memory. The release of epinephrine is a result of sympathetic activation and is often correlated with stressful or arousing situations. Increased epinephrine release has been shown to enhance memory in rats (Gold and van Buskirk 1975 and 1976). This phenomenon was first shown in rodent models tested on an inhibitory avoidance learning paradigm. In this task, rats escape a well-lit chamber and enter a dark chamber where they receive a foot shock at variable intensities. At a later time, the same rats are placed into the apparatus and their latency to cross into the dark chamber represent retention or memory of the shock. Researchers found that application of a high foot shock showed good retention while mild shock produced poor

retention. Rats exposed to mild shock but also given an injection of epinephrine at the time of training showed good retention, suggesting that the increased release of epinephrine associated with increased arousal enhances memory (Gold and van Buskirk 1975). These early studies also suggest that the influence of epinephrine on memory is time- and dose-dependent, illustrated by an inverted-U dose-response curve.

There are several features of this pathway that have focused scientists' attention to the importance of glycogen breakdown and glucose mobilization in association with memory retention. Once epinephrine is released from the adrenal medulla into circulation it does not readily enter the brain from blood (Axelrod 1959); therefore a peripheral action must be responsible for changes in the central nervous system during arousal. Epinephrine released during arousal activates adrenergic receptors in the liver and in the muscles, triggering the breakdown of glycogen and a release of glucose into the surrounding tissue or the blood stream. Glucose, the major source of metabolic energy in the body, is a robust memory-enhancing agent. The body closely regulates the concentration of glucose circulating in the blood and available to cells in the brain and periphery. This increased circulating blood glucose supplies the brain with energy and enhances memory retention (Gold 2014).

Epinephrine acts to breakdown peripheral stores of glycogen to increase the circulating levels of glucose during moments of arousal. Glycogen is a large, branched molecule composed of glucose residues connected by easily broken α -1, 4-glycosidic linkages (Berg 2002). Instead of turning glucose directly to fatty acids, the production of glycogen has several advantages. Glycogen, particular that stored in liver, is broken down as a metabolic buffer in between influxes of glucose during mealtimes, acting to maintain metabolite levels high enough to

maintain proper function. Unlike fatty acid metabolism, glycogen can be broken down without the presence of oxygen, acting to support anaerobic activity (Berg 2002). Glycogen granules are stored within the cytoplasm of cells and can range in size from 10 to 40 nm (Berg 2002). The largest stores of glycogen in the body are found in the liver and in skeletal muscle. Liver glycogen is broken down after activation by epinephrine and releases glucose into the bloodstream while skeletal muscle glycogen is broken down and used to power contractions.

There are several enzymatic mechanisms responsible for the accumulation and breakdown of glycogen. Two key enzymes include glycogen synthase and glycogen phosphorylase. Glycogen Synthase creates the chains of glycogen by adding the activated form of glucose (UDP-glucose) to an already existing glycogen chain. The chain of glycosyl units originates from a primer, specifically the dimeric protein glycogenin. Without glycogenin the chain has no starting point and glycogen synthesis cannot occur (Berg 2002). Glycogen phosphorylase performs the opposite reaction termed glycogenolysis that releases single glucose-1-phosphate molecules from the chain. This free molecule is converted to glucose-6-phosphate and may have several different fates, including being turned into lactate through glycolysis (Newman 2011). These enzymes are regulated by both allosteric feedback mechanisms and by hormonal regulation (Berg 2002).

Once adrenergic activation has caused the peripheral breakdown of glycogen, the resulting glucose is released into the circulating blood it becomes readily available to the brain for consumption. This physiological fact has lead researchers to believe that glucose is the molecule acting on the brain to improve cognition (Korol & Gold 1998; Gold 2014; Gold and Korol, 2014). Glucose, like epinephrine, has been shown to enhance cognition in a dose and

time dependent manner, illustrated by dose response curves (Gold, 2014; Gold and Korol, 2014). Blocking epinephrine's function with adrenergic receptor antagonists blocks the effects of epinephrine on memory but does not block the memory-enhancing effects of glucose, supporting the claim that glucose acts downstream from adrenergic activation to enhance neuronal function (Hall and Gold, 1992).

Glycogen in the Brain

The arrival of glucose to the cells of the nervous system is limited by peripheral mechanisms and blood flow. As discussed above, circulating levels of glucose are regulated by endocrine mechanisms including the release of epinephrine. If glucose becomes unavailable, the tissue must switch metabolic strategies to maintain proper cellular function. One way the body maintains function under hypoglycemic conditions is by storing energy rich glucose in the form of glycogen for later use. Glycogen is stored in a variety of different tissues throughout the body, including but not limited to hepatic tissue, muscles and the brain. When glucose is delivered to an area of tissue it is rapidly stored as glycogen. Glycogen can be broken down in the future, ensuring that the basal metabolic rate of the cells is maintained in the absence of glucose. In the brain, the storage and breakdown of glycogen is isolated to a type of glial cell termed the astrocyte. The breakdown of glycogen and release of lactate from these astrocytes (Newman et al., 2011) influence neuronal function including learning and memory.

Astrocytes support the brain's vast network of neurons by creating and maintaining stores of glycogen. Astrocytes are expressed in all brain regions and frequently outnumber neurons. Long thought to be the "glue" holding the neurons together, astrocytes actually command a degree of control over the neurons and the flow of nutrients entering the brain from the blood.

Fibrous and protoplasmic, astrocytes extend their processes to make connections with neurons and blood vessels (Abbott et al., 2006). Astrocytes release signaling molecules such as glutamate, D-serine, and ATP that can change the local environment to influence neuronal activity and vascular tone (Haydon 2006). Astrocytes also contain many different metabotropic receptors reacting to an array of different neurotransmitters and hormones (norepinephrine, acetylcholine, GABA, estrogen, etc.). Activation of these receptors results in an increased internal calcium concentration and alterations in the astrocytes actions on the surrounding environment (Haydon 2006). These features of astrocytes allow them to mediate the metabolites within neuronal tissues between vascular elements, neurons, and the astrocytes themselves.

The most important feature of astrocytes for the purpose of this investigation is their ability to synthesize, store, and breakdown glycogen. Most of our knowledge of glycogen comes from studies performed on peripheral tissues, however, glycogen plays an important role in the central nervous system. Glycogen seems to be isolated to astrocytes in brains of healthy adults, supporting the idea that these cells function as modulators of metabolism in nervous tissue (Cataldo and Broadwell, 1986). Immunolabeling with GFAP and NeuN to visualize astrocytes and neurons, respectively, shows colocalization of glycogen with GFAP, supporting the claim that glycogen is isolated to astrocytes (Newman et al 2011). Glycogen phosphorylase is found only within astrocytes, although glycogen synthase is found in both neurons and astrocytes (Pellegeri 1996). The brain contains relatively low concentrations (6-12 μmol) of glycogen compared to other regions of the body such as the liver (100-500 μmol) or skeletal muscle (300-350 μmol), implying that these stores are for local consumption during moments of high metabolic demand (Chryssanthopoulos et al., 2004). Levels of brain glycogen change very little

with starvation compared to the liver. Large changes in intracellular glucose concentration are not physiologically possible because of enormous osmotic pressure changes that would lead cells to burst. The accumulation of glycogen allows for the intracellular accumulation of metabolic resources without this dangerous change in osmotic pressure.

Under the appropriate conditions glycogen is transformed into lactate, a carbohydrate that can be metabolized by neurons in need of immediate energy. The availability of lactate may be important for proper cellular function during periods of intense activation or arousal. Brain has a high resting metabolic rate that is increased during local brain activation (McKenna et al 2006). When an organism experiences a strenuous task, such as learning, brain tissue rapidly consumes the local supplies of glucose and must produce another metabolic fuel (Brown and Ransom 2007). Activity-dependent hypoglycemic conditions lead to the breakdown of glycogen into lactate through glycogenolysis. In this model, lactate protects against hypoglycemic injury, allowing neurons to continue to function during intense activation.

Astrocytes can create and export lactate into the surrounding parenchyma through specific monocarboxylate transporters (MCT) 1 and MCT4 (Newman et al 2011). Astrocytes release lactate into the extracellular fluid where neurons have access to this molecule and retain the capability to function at full capacity (Brown and Ransom 2007, Magistretti 2006). Neurons express MCT2 that take up lactate from the surrounding extracellular fluid. Neurons also contain the enzyme lactate dehydrogenase, which converts lactate to pyruvate in preparation for oxidative phosphorylation (Brown and Ransom 2007). Low brain glycogen concentrations imply they can only provide support a short period of support in the absence of glucose or other energy substrates, being completely consumed in a few minutes under aglycemic conditions

(unlike liver glycogen supplies that would support euglycemia for up to 24 h). Glycogen provides sufficient energy during hypoglycemia and increases past baseline level afterwards (Criego 2005). Inhibition of glycogen degradation quickly ends basic neuronal functions under high stimulation or aglycemia and increases glycogen accumulation once returned to normoglycaemic conditions (Brown 2004). These data indicate that the small astrocytic stores of glycogen have major implications for brain function, including support of the mechanisms important for learning and memory. Shifts in this metabolic pathway across the lifespan may influence the cognitive changes experienced with age (Gold and Korol, 2014).

Lactate and memory

Previous experiments performed by Lori Newman and other members of the Gold and Korol labs have highlighted several important trends during spatial working memory tasks in rats. In these experiments, young male rats were tested on a hippocampus sensitive task (spontaneous alternation) in which rats were placed on a plus maze surrounded by extra maze cues. The spontaneous alternation task makes use of the rats' natural tendency to seek novelty. Cannula were implanted directly into the hippocampus and biosensor probes were used to measure single second recordings of extracellular glucose and lactate concentrations. These experiments showed a decrease in glucose concentrations at the start of learning with a slow but steady increase returning to baseline levels before the end of training. In contrast, there was a robust and almost immediate increase of lactate concentrations that persisted to the end of training (Newman et al., 2011). In a similar experiment 1, 4-dideoxy-1, 4-imino-D-arabinitol (DAB) was injected into the hippocampus to pharmacologically inhibit the production of lactate from glial glycogen stores. These animals displayed impaired performance on spatial working

memory tasks (Newman et al., 2011). The results of this experiment indicate that without the production of lactate in astrocytes and release into the extracellular fluid, the hippocampal neurons did not have access to normal metabolic resources provided by astrocytes during periods of extended activity, limiting the rats' learning and memory capabilities.

The metabolites glucose and lactate act to support the actions of the brain, and therefore replenishment of these compounds should act to enhance cellular function and enhance learning. Injections of either lactate or glucose directly into the hippocampus both acted to enhance young male rats' performance on a hippocampus-sensitive spatial working memory task. Injections of either of these metabolites also reversed the memory impairments caused by pharmacologically blocking glycogenolysis with DAB (Newman, et al., 2011). These results support previous claims that glucose is an important metabolic resource for the brain and a potent enhancer of memory, but also bring lactate into the spotlight as an important modulator of brain state.

Age-Related Changes

In terms of glucose regulation, there seems to be an uncoupling of epinephrine and glucose release with age (Gold and Korol 2014). There is a larger release of epinephrine into blood of old rats when performing a swim task and during inhibitory avoidance training when compared to young rats (Mabry et al. 1995a, b). Following the model described previously, it would be safe to assume that this increased release of epinephrine should be correlated with increased circulating glucose levels in old animals. However, blood glucose levels were significantly lower in the old animals than the young animals when compared to baseline (Mabry et al., 1995a). Injections of epinephrine enhanced memory of an inhibitory avoidance task in young rats, but failed to significantly enhance memory in old rats (Morris et al., 2010). Despite

this uncoupling, glucose remains a robust enhancer of memory, significantly enhancing memory in both old and young rats (McNay and Gold 2001; Salinas and Gold, 2005; Morris et al., 2010; cf. Gold and Korol, 2014), improving memory in aged rats to scores comparable to young rats. The effectiveness of glucose as a memory-enhancing substance has also been tested on human subjects. The data collected from these experiments that elderly participants showed cognitive enhancement after consuming glucose (Hall 1989; Manning et al., 1993, 1998; cf. Korol and Manning, 2001; Korol, 2002; Gold and Korol, 2014).

Epinephrine-initiated glucose release, however, is only one mechanism supporting the metabolic state of an actively learning brain. The shift from glucose to lactate metabolism may also change across an organism's lifespan, thereby influencing cognitive decline. Recently collected data from the Gold and Korol labs show that 2-year-old rats displayed a reduced release of lactate in the hippocampus compared to young rats aged 3 months during training on a hippocampus-sensitive place learning task. These data suggest that a lack of immediate metabolic support may influence memory impairments in these aged animals (Newman et al., unpublished 2016).

These results raise several questions about mechanistic changes associated with age and how biochemical shifts might be influencing cognition. Just as glucose acts as an enhancer of memory across lifespan regardless of the changing effectiveness of epinephrine, lactate may maintain its status as a memory-enhancing substance irrespective of changes in upstream signaling cascades. The memory-enhancing effects of lactate have been shown in young rats, but it was unclear how lactate might influence learning in an old brain. This experiment was designed to investigate whether or not the mechanism by which lactate enhances memory is

retained across the lifespan. If the results depict difference across age groups, the differential use of lactate across the lifespan may influence some of the shifts in learning experienced by the elderly population.

Outline of the Experiment

The purpose of this experiment is to investigate further how changes in brain metabolism, specifically lactate, influence spatial learning memory across the lifespan in rats. First, we assessed both young and old rats on a spatial memory task, spontaneous alternation. Three-month-old and 24-month-old Fischer-344 (F344) rats were tested on a spontaneous alternation task for 20 minutes. On the basis of previous work, we hypothesized that old rats will alternate significantly less than young rats due to age-related memory impairments. Next, we infused lactate into the hippocampus of young and old rats before spontaneous alternation training to assess the memory-enhancing effects of this metabolites across the lifespan. Old and young F344 rats underwent surgery for the bilateral implantation of guide cannula into both the left and right dorsal hippocampus. On the day of testing, rats received an injection of lactate or saline into the hippocampus 15 minutes before spontaneous alternation testing. During all phases of the experiment the rats were euthanized immediately following behavior and samples were collected for the analysis of glycogen content. Behavior on the maze was analyzed to determine if lactate enhances working memory. We hypothesized that these infusions will enhance learning in both age groups. Enhancement with lactate infusions in the old age group imply that age-related deficits in spatial working memory are influenced by shifts in metabolic modulation and are reversible.

Methods

Subjects and Experimental Design

These experiments were conducted using male F344 rats supplied by the National Institute on Aging (NIA) colony managed by Charles River. All of the procedures performed during these experiments were approved by the Syracuse University Animal Care and Use Committee (IACUC), accredited by the Association for Assessment and Accreditation of Animal Care (AAALAC). The two experimental age groups included 3-month-old rats (young) and 24-month-old (old) rats. The 24-month-old group was examined daily for age-related health issues that might confound their performance in the study. These rats were housed individually and had free access to food and water at all times. Animal housing was kept under a 12:12 hour light:dark cycle and animals were behaviorally tested during the light portion of this cycle.

Experiment 1 compared performance of young and old animals on the spontaneous alternation task. The rats used for this portion of the experiment did not undergo the surgical procedures described below. Behavioral data and tissue samples were collected from this group. For Experiment 2, rats underwent surgery to implant bilateral guide cannula into the dorsal hippocampus. Young and old rats were randomly selected to receive either a 0.5 μ l injection of 100 nmol sodium L-lactate in a saline vehicle or a saline control immediately before spontaneous alternation training. All experimental procedures and analyses were performed by the author of this document.

Bilateral Cannula Implantation Surgery

In experiment 2, all rats underwent stereotaxic surgery to implant bilateral cannula (6 mm) into the dorsal hippocampus (coordinates: anterior posterior +3.8 mm; medial-lateral 2.5

mm, and ventral 1.5 mm from dura). Rats were anesthetized using isoflurane and the area of the incision was shaved and prepared with betadine. Each rat received a subcutaneous injection of the non-steroidal anti-inflammatory pain-relieving drug flunixin (1 mg/kg) and an intramuscular injection of the antibiotic penicillin (0.3 mg/kg) to avoid infection. After surgery, rats received saline (10 ml injected subcutaneously) to avoid dehydration. Rats were given ibuprofen (47 mg in 500 ml of drinking water) as a postoperative analgesic. To insure their health, all rats were allowed one week to recover and were checked daily.

Infusions

Before spontaneous alternation training, rats received either a 0.5 μ l infusion of L-lactate (100 nmol in 0.9% saline, pH 7.2) or saline (0.9%, pH 7.2). The injections were made with 7 mm injection needles attached to a CMA injection pump running at a flow rate of 0.25 μ l/min for 2 min, ending 5 min before spontaneous alternation training.

Training Procedure

As a measure of spatial working memory, all experimental groups underwent spontaneous alternation training. This is a useful task when examining metabolism in vivo because the task does not require a food reward or food restriction prior to training. Spontaneous alternation takes advantage of the rat's natural tendency to be motivated by novelty. The testing room is decorated with extra-maze cues on the walls surrounding the maze. The apparatus for this task is a four arm, plus-shaped maze made of black Plexiglas. Each arm was designated either A, B, C, or D. After receiving an injection of lactate or vehicle control animals were placed in the training room for 15 minutes in order to acclimate to their surroundings. The rat was then placed on the maze and allowed to move freely from arm to arm for a 20-minute

period. During this session arm entries were recorded by the investigator. Scores on this task were determined by calculating each subject's percent alternation. An alternation was defined as each time the rat entered all four arms in five consecutive arm choices. The measure of percent alternation was determined by dividing the number of alternations performed by the animals by the number of possible alternation times 100. This creates a score out of 100%; with this measure, chance performance results in an alternation score of 44%. Rats which made fewer than 10 arm choices during the 20 minute training period were excluded from the study.

Post-training Procedure

After spontaneous alternation training was completed, the subjects were immediately overdosed using an intraperitoneal injection (1 ml) of sodium pentobarbital. For this experiment several different methods of post mortem tissue collection were used. For Experiment 1, which tested rats without surgery or brain infusions, tissue samples were removed and flash frozen on dry ice. Portions of the brain (frontal cortex, cerebellum, brainstem, striatum, and hippocampus) and the periphery (leg muscle, diaphragm, and liver) were collected and stored at -80° C. These tissue samples will be analyzed for glycogen content using a colorimetric assay in the near future. This analysis will be important for determining if there are age-related changes in the accumulation and breakdown of this energy reservoir.

Rats with surgically implanted cannula and pre-training infusions from Experiment 2 were deeply anesthetized with sodium pentobarbital and were then perfused immediately after training. The vasculature was flushed with saline then tissue was fixed using 4% paraformaldehyde. The brains were removed and stored for two days in paraformaldehyde then stored in glycerol until sectioning. These tissue samples were sectioned and nissl stained to

confirm the correct placement of the cannula tip within the dorsal region of the hippocampus. If the cannula was not in the correct region of the brain that animal was excluded from the final analyses. Sections were collected and stored in cryoprotectant at -20°C in the hopes of performing immunohistochemistry in the near future. This immunohistochemistry will be performed to assess the abundance and distribution of specific adrenergic receptors to determine if age-related changes in metabolic modulation are linked to altered adrenergic signaling.

Data Analysis and Statistics

Statistical analyses were performed using SPSS software. The behavioral data collected during spontaneous alternation training was analyzed using T tests to assess difference between old and young groups in experiment 1 and differences between treatment groups (saline control or lactate) within the age group in experiment 2. Within the age groups, the scores of unoperated animals from experiment 1 were compared to the cannulated animals in experiment 2 using a t-test as well. These tests were run with an alpha level of 0.05.

Results

Experiment 1: Age-related deficit on spontaneous alternation task

Previous studies claim that there is an age-related decline in spatial working memory. Before examining the influence of lactate on learning, it was important to determine the extent of age-related deficits on the spontaneous alternation task in rats that had not received surgical cannula implantation and did not receive infusions of lactate or vehicle control. Young rats (N=8) averaged a percent alternation score of $60.8 \pm 1.6\%$. Old animals (N=8) averaged a percent alternation score of $52 \pm 0.9\%$ (see Figure 1). Young animals scored significantly higher than old animals ($p < 0.05$) on this task. This difference supports the claim that the 24 month old animals experience spatial working memory deficits compared to the 3 month old animals within the parameters of this task.

Experiment 2: Lactate enhances spontaneous alternation scores in old rats

Young rats that received the vehicle control (N=4) infusion of 0.9% saline showed a significantly reduced percent alternation score ($p < 0.05$) compared to the intact controls with an average percent alternation score of $45.0 \pm 1.9\%$ (see Figure 2). Compared to saline controls, young rats that received infusions of lactate (N=5) showed slightly higher percent alternation scores with an average percent alternation score of $51 \pm 1.3\%$, although this difference was not statistically significant ($p > 0.05$).

Similar to the young rats, old rats (N=5) that received the vehicle control infusion of 0.9% saline showed a significantly reduced percent alternation score ($p < 0.05$) compared to the unimplanted rats (Experiment 1) with an average percent alternation score of $34.3 \pm 3.9\%$ (see

Figure 3). Compared to these saline controls, old rats that received infusions of lactate (N=4) showed an enhanced percent alternation scores with an average percent alternation score of $54.7 \pm 2.6\%$ (see Figure 4). Old animals that received infusions of lactate had statistically significantly higher percent alternation scores when compared to animals that received infusions of saline ($p < 0.05$) (see Figure 3). Unlike the young experimental group, the percent alternation scores of old animals that received lactate (54.7%) exceeded the average scores of the intact controls (52%), although this was not a significant difference ($p > 0.05$).

Differences in number of arm entries between age groups

The number of arm entries made by rats from both age groups provides further evidence that infusion of lactate acts directly on working memory during this task. Young animals who received infusions of lactate showed a slight increase in the number of arms entered during their time on the maze (42.2 ± 1.1 arm entries) compared to the vehicle group (34.5 ± 2.8), but this difference was not significant ($p > 0.05$) (see Figure 5). Old animals did not display a change in the number of arm entries made between experimental groups: the lactate group entered an average of 17.3 ± 2.4 arms and the saline group entered an average of 17.2 ± 1.6 arms (see Figure 5). Compared to the young cohort, the old animals made fewer overall arm choices regardless of their treatment group. Old rats that received lactate performed at an average higher percent alternation score than age-matched saline controls or the young rats without changing the average number of arms entered.

These results indicate that both young and old rats show enhancement on a spatial working memory task with the direct infusion of lactate into the hippocampus. In the near future additional animals will be added to all experimental groups in an effort to strengthen the effects

already seen with such small sample sizes. These rats were perfused with 4% paraformaldehyde immediately post training and will be sectioned in the near future. This measure is to ensure that the infusions of lactate were indeed deposited directly into the hippocampus. Therefore, the results in Figures 2 and 3 remain preliminary until histological confirmations of placements are complete.

Discussion

Data collected from these experiments and many others seem to indicate shifts in metabolite availability in the hippocampus across the lifespan. Data collected during experiment 1 indicate that old rats' percent alternation scores were significantly lower than their younger counterparts, supporting the claim that old animals experience spatial working memory deficits. When interpreting these data it is important to note that the percent alternation scores recorded during this experiment differ slightly from those recorded in other similar experiments (Newman et al., 2011). This difference may be due to the use of a different strain of rat in this experiment: this procedure used F344 rats while previous work used Sprague -Dawley rats (Newman et al. 2011). This trend of low scores may be specific to the F344 strain of rats. In the near future, the tissue samples collected from experiment 1 will be analyzed for glycogen content. Variable levels of this energy storage molecule immediately after training across age groups may indicate age related shifts in the dynamics of lactate release. If consistent with previous findings, the old animals are expected to have higher levels compared to the young, indicating a mechanistic difference with glycogen breakdown between experimental groups.

When infusions of lactate or saline were provided before spontaneous alternation training, young rats showed insignificant enhancement of spatial working memory with lactate compared to saline controls. Although the groups were not statistically significant from one another, the direction of this trend supports previous findings made by our lab showing that young rat's spatial working memory is enhanced by the addition of lactate (Newman et al. 2011). More young animals may be added to this experiment in the near future to determine the

reproducibility of these data. In contrast, the old rats showed significant enhancement of spatial working memory when infused with lactate prior to spontaneous alternation. This robust enhancement indicates that age-related deficits in spatial working memory are fully reversible with the application of the metabolite lactate.

Analysis of the number of arm entries made by each group supports our claim that lactate is enhancing memory and not causing additional behavioral changes which might be interpreted incorrectly within the confines of this task. There was no change in the number of arm choices between the rats which received lactate and the rats which received the saline control. This indicates that the enhancement was not due to overall increased activity, but instead an overall increase in the amount of correct arm choices. Regardless of their treatment old rats made a very limited number of arm choices compared to the young, supporting the claim that their spatial working memory was enhanced with the application of lactate. Several old animals were excluded from the final evaluation because they made fewer than 10 arm choices during the twenty-minute testing period. This lack of activity in the old animals is most likely due to age related lethargy seen in animals of this age. Animals were regularly checked for health related problems that may have limited their motility on the maze apparatus and no animals showed signs of illness or injury that would hinder their physical performance of the task.

The results of these experiments support the claim that changes in metabolic modulation are responsible for age-related memory deficits. Previous experiments have shown that activation of the adrenergic signaling system modulates both metabolism and memory. Epinephrine is produced by the adrenal medulla and when this gland is removed surgically by adrenalectomy rats experience learning deficits on the inhibitory avoidance task (Borrell, 1983).

These deficits are attenuated by the subcutaneous administration of epinephrine or norepinephrine (Borrell, 1983). Peripheral epinephrine enhances memory in young rats (Gold and van Buskirk 1975, 1976) by eliciting the release of glucose from the liver, thereby increasing circulating glucose available to the brain (Gold 2014). In contrast, aged animals display a larger release of epinephrine during learning or arousal compared to young animals, but show a significantly smaller increase in plasma glucose (Mabry et al, 1995). This shift in plasma glucose may have important implications for the amount of glucose reaching the brain, potentially changing the strategy used by cells like astrocytes to keep the brain metabolically stable. In this way, age-related memory deficits can be linked to changes in adrenergic regulation of metabolic reserves.

Dysregulation of peripheral epinephrine in old animals may influence metabolic mechanisms specific to the nervous system. Early studies have shown that circulating levels of epinephrine influence the levels of norepinephrine found within the brain (Gold and van Buskirk, 1978). After the application of peripheral epinephrine, norepinephrine release increases through the brain, including the hippocampus (van Buskirk and Gold, 1978a, b; Miyashita and Williams, 2004). Blocking β -adrenergic receptors in the periphery or directly in the amygdala using the antagonist propranolol inhibits the memory enhancing effects of epinephrine (Gold and van Buskirk, 1978; Liang et al, 1985). These results imply that peripheral epinephrine's modulatory influence on learning is in part due to its influence on norepinephrine production and signaling in the brain.

Noradrenergic receptors are expressed on both neurons and astrocytes (Hertz et al, 2010). Activation of these receptors has been shown to modulate glucose uptake, oxidative metabolism,

glutamate uptake and hydrolysis and most importantly for this study the creation and breakdown of glycogen (Dienel, 2015). Activation of each specific receptor type (α_1 , α_2 , β_1 , and β_2) has been shown to initiate different intracellular cascades. Activation of α_1 and α_2 adrenergic receptors results in the accumulation of glycogen while activation of β_1 , and β_2 adrenergic receptors results in the breakdown of glycogen stores (O'Donnelle, 2012). By having the potential to increase or decrease glycogen stores within astrocytes depending on the variety of receptor on the surface of the astrocyte, norepinephrine has the ability to modulate long term metabolic dynamic in the brain. Norepinephrine-producing neurons projecting from the locus coeruleus spread throughout the nervous system influencing many different cell types, potentially influencing glycogen metabolism in various areas of the brain (O'Donnell et al 2012). A disruption at any point in this signaling pathway may lead to the accumulation of glycogen and lack of behavior induced release described in our preliminary work. Shifts in this pathway in addition to the inability of epinephrine to induce glucose release from the liver may combine to produce learning deficits in aged organisms.

At this point it is unclear how metabolic modulation is changing with age, but the dynamics of glycogen accumulation and breakdown may be important in this pathway. Previously collected data indicate that old animals retain more glycogen in the hippocampus than do young animals after memory testing (Newman, unpublished). Old animals also release less lactate in the hippocampus during the hippocampus-sensitive spontaneous alternation task when compared to young rats (Newman, unpublished). Although lactate retains its memory enhancing effects, old animals may lack the capability to break down the glycogen during moments of high energy demand. This indicates a disconnection between accumulation of glycogen and its timely

breakdown. This shift could be linked to dysfunction with the noradrenergic signaling cascade leading to the breakdown of glycogen within the brain.

The data collected from this experiment pose several new questions that should be explored with further research. This study focused on hippocampal sensitive spatial working memory but there are other learning strategies that are heavily influenced by other areas of the brain, including the striatum. Learning strategy determines the metabolic response of the brain area most strongly activated. Shifts in learning strategy across the lifespan may influence each brain areas response to activation (Gold, 2014). This experiment was also performed on only male rats. Estrogens have been shown to influence learning, memory and bioenergetics in the brain. It is important to determine if the same results appear in female subjects or if circulating estrogens change the metabolic strategy of the brain or how these strategies shift with age. As mentioned above, noradrenergic signaling also plays a role in the dynamics of glycogen in the brain. Future work will investigate the role the noradrenergic signaling system plays in determining age-related memory deficits. Future investigations will help the scientific community to define how memory is metabolically modulated and how this changes across the lifespan. Research focused on the shifts in brain bioenergetics associated with age is extremely relevant in an ever-expanding aging society and has the potential to define many of the mechanisms underlying both healthy and pathological aging.

Figures

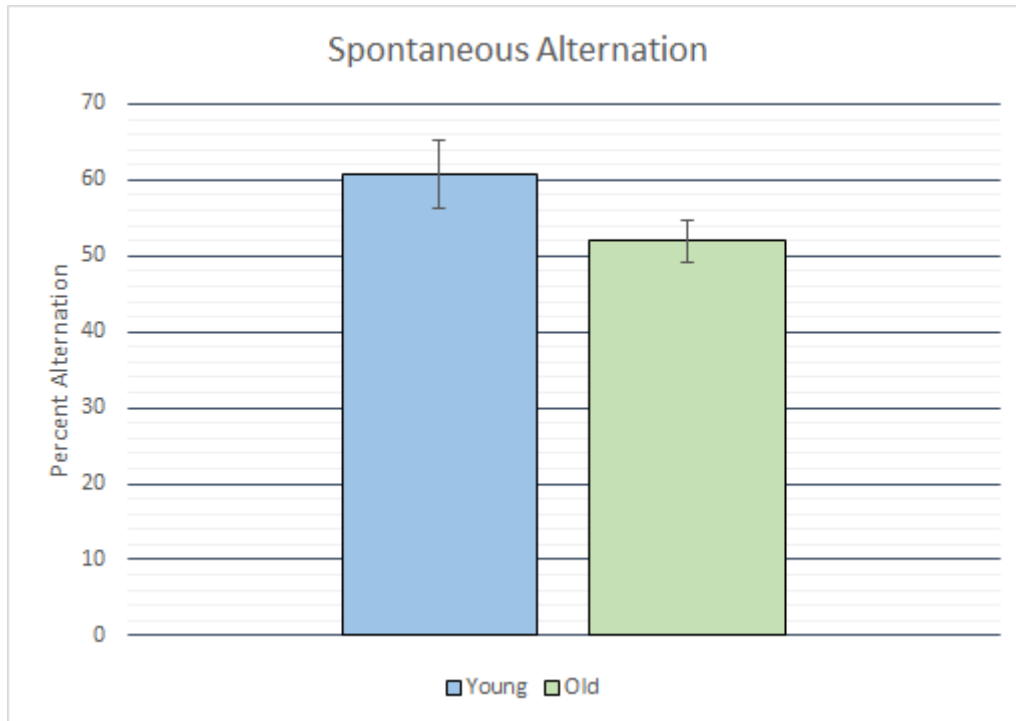


Figure 1: Age related deficit on spontaneous alternation task. Young animals (N=8) mean percent alternation (60.78% with a standard deviation of 4.56%) was statistically significantly higher ($p=0.0333$ at a significance level of 0.05) compared to old animals (N=8) mean percent alternation (51.96% with a standard deviation of 2.68%) on this task.

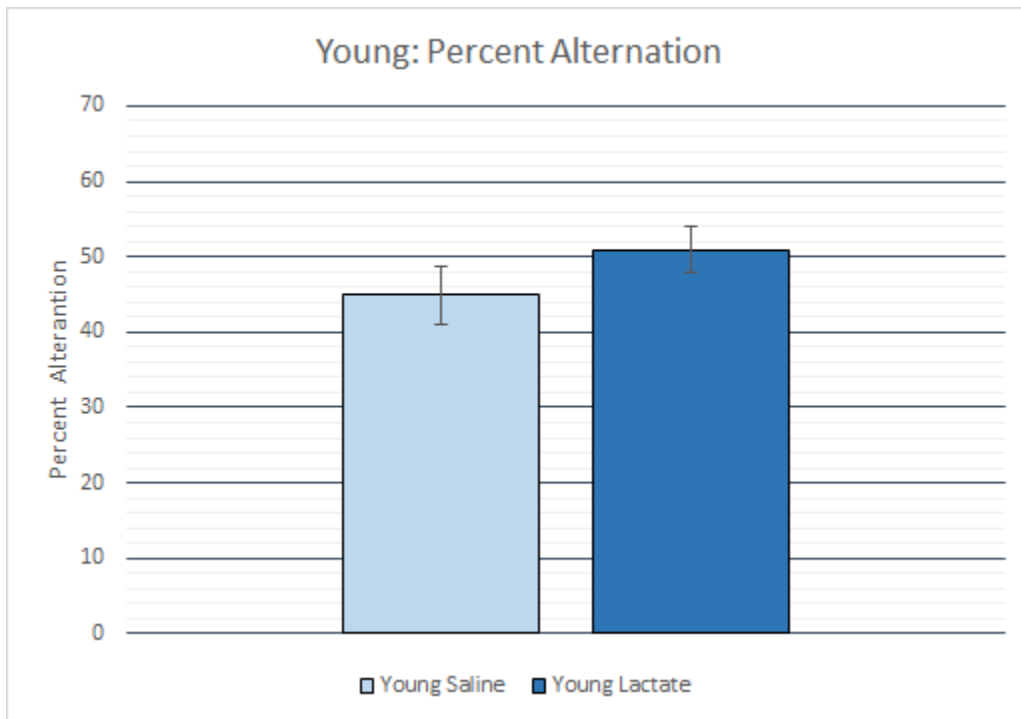


Figure 2: Lactate fails to enhance spontaneous alternation scores in young rats. Although these groups were not statistically different from one another ($p=0.25$ with a significance level of 0.05), young rats that received lactate ($N=5$) had a higher mean percent alternation score (50.98% with a standard deviation of 2.96%) compared you young rats who received saline ($N=4$, mean of 44.975% with a standard deviation of 3.9%).

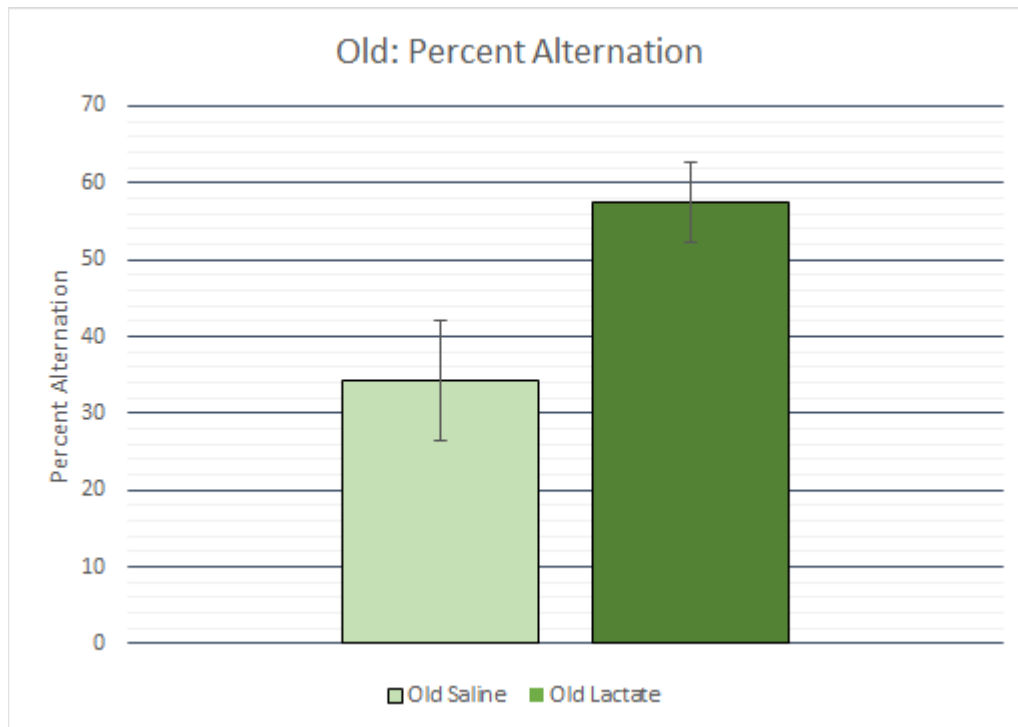


Figure 3: Lactate enhances spontaneous alternation scores in old rats. Old rats that received lactate (N=5) had a higher mean percent alternation score (57.45% with a standard deviation of 7.76%) compared you young rats who received saline (N=4, mean of 34.275% with a standard deviation of 5.24%). This difference was statistically significant ($p=0.048$ with a significance level of 0.05)

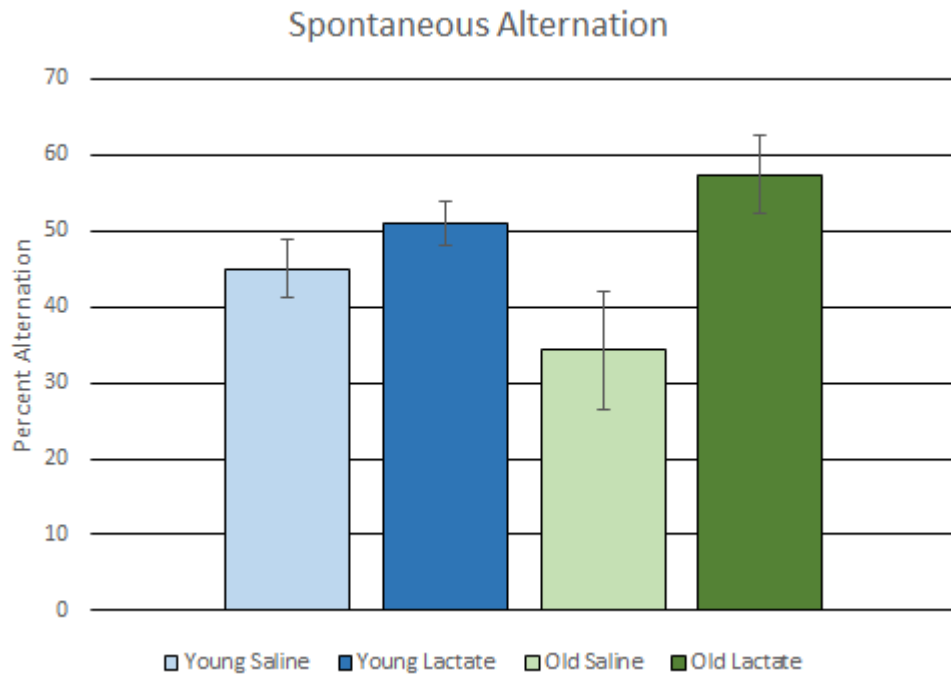


Figure 4: Old rats show a robust increase in percent alternation score with lactate compared to young rats. Young animals that received infusions of lactate performed approximately 6.0% higher on the spontaneous alternation task compared to age matched saline controls. Old rats that received infusions of lactate performed approximately 23.2% higher on the spontaneous alternation task compared to age matched saline controls.

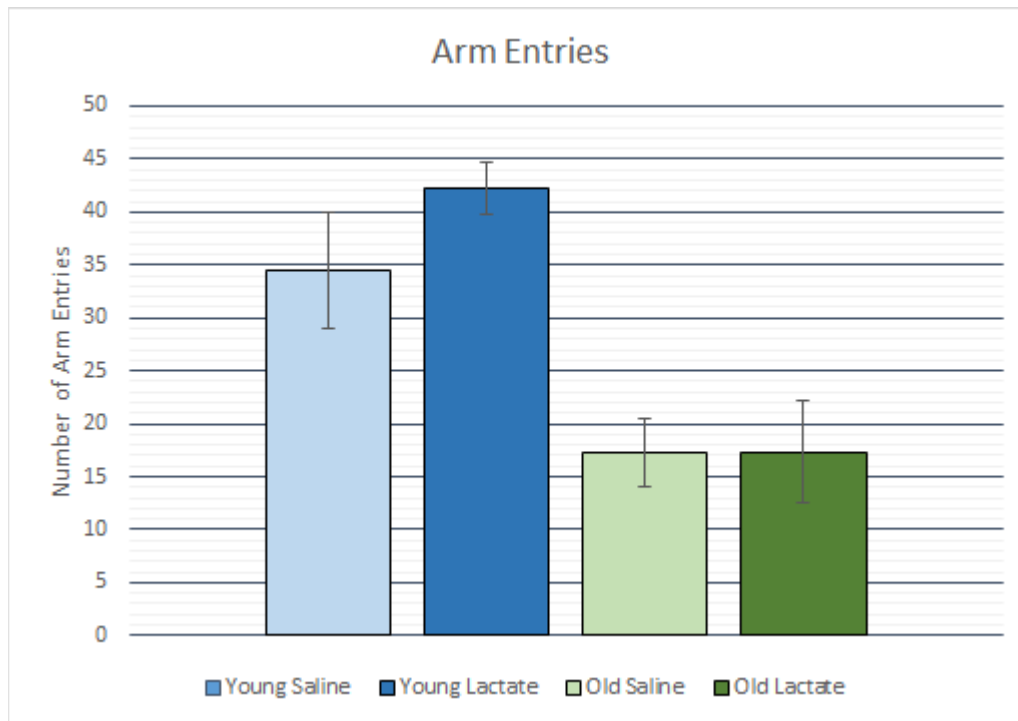


Figure 5: Differences in number of arm entries between age groups. Although young animals that received lactate made slightly more arm choices (mean of 42.2 arm choices) compared to those that received saline (mean of 34.5 arm choices), there was no significant difference between these two groups. Old animals made approximately the same number of arm choices regardless of their treatment with saline or lactate.

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