

Review Article

Cholesterol and Copper Affect Learning and Memory in the Rabbit

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Received 27 June 2013; Accepted 31 July 2013

Academic Editor: Rosanna Squitti

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A rabbit model of Alzheimer's disease based on feeding a cholesterol diet for eight weeks shows sixteen hallmarks of the disease including beta amyloid accumulation and learning and memory changes. Although we have shown that feeding 2% cholesterol and adding copper to the drinking water can retard learning, other studies have shown that feeding dietary cholesterol before learning can improve acquisition and feeding cholesterol after learning can degrade long-term memory. We explore the development of this model, the issues surrounding the role of copper, and the particular contributions of the late D. Larry Sparks.

1. Introduction

In 2001, we were looking for nontransgenic animal models of Alzheimer's disease (AD) in which we could study the effects of potential treatments on AD deficits in learning and memory. A review of the literature revealed very few options other than aged animals that would take many months or even years to reach a point at which they could be studied [1–4]. One exception was a cholesterol-fed rabbit model of AD that Sparks and colleagues showed had several hallmarks of Alzheimer's pathology, particularly beta amyloid accumulation, that developed in as little as 8 weeks of being fed a 2% cholesterol diet [5–8]. Surprisingly, given the well-characterized rabbit eyeblink conditioning preparation first published by Gormezano and colleagues in the 1960s [9–13], there were no studies in the literature examining learning and memory in these cholesterol-fed rabbits. We contacted Larry Sparks to ask why no one had published learning and memory studies with this model and the answer was as clear and emphatic as only Larry Sparks could make it: he had tried to convince researchers for years to do the experiments but no one seemed to be interested.

One possible reason for this apparent lack of interest in studying learning and memory in a rabbit model of AD was the fact that standard rabbit eyeblink conditioning experiments in which a tone preceded and overlapped with

a puff of air to the eye was mediated in large part by the cerebellum [14, 15], and the cerebellum is the last and least affected brain structure in patients with AD [16]. However, this mediation of learning by the cerebellum is only true for the most basic of classical conditioning paradigms known as delay conditioning in which the stimuli overlap [17]. If there is a substantial trace between the two stimuli and the tone and air puff do not overlap, there is good evidence that the hippocampus and prefrontal cortex are engaged and become critical to successful learning and memory [18–30]. The hippocampus and cortex are among the areas that are the first and most profoundly affected structures in patients with AD [16, 31].

2. The Effects of Cholesterol on Learning

In collaboration with Sparks, we sought to assess the effects of a cholesterol diet on trace conditioning of the rabbit nictitating membrane response [32]. The results of these first experiments with the nictitating membrane response (NMR) were to start us on a ten-year odyssey that still continues to challenge us and has been deeply affected by the untimely death of our colleague Larry Sparks.

In order to study NMR conditioning in cholesterol-fed rabbits, we instituted a standard set of procedures that began

with rabbits being fed 2% cholesterol or standard Purina rabbit chow (0% cholesterol) for eight weeks and then presented the rabbits with pairings of a brief tone (100 ms, 82 dB, 1 kHz) as the conditioned stimulus (CS) followed by an eyeblink-eliciting air puff (100 ms, 4 psi) or periorbital electrical pulse (100 ms, 2.0 mA, 60 Hz) as the unconditioned stimulus (US). In some experiments, half of the rabbits received explicitly unpaired presentations of the CS and US to assess nonassociative contributors to responding [9, 13, 33–36]. Importantly, the interval between the CS and US for paired rabbits was more than 500 ms creating a significant trace which previous studies by a number of groups have shown made classical conditioning dependent on the hippocampus [18–20, 37–40] and prefrontal cortex [23, 25, 27, 29, 41–45] in addition to the cerebellum. In each of our trace conditioning experiments, acquisition of a conditioned response was a function of the trace interval and usually took many days of training to reach asymptote, and this asymptote tended to be lower than that seen using delay conditioning [17]. Importantly, subsequent delay conditioning and sensory thresholds were always the same for cholesterol-fed rabbits and normal chow controls [32, 46]. Cholesterol-fed unpaired control subjects showed low levels of responding that were consistent with previous observations in rabbits fed normal chow [9, 33, 47]. In all of these experiments, the cholesterol diet continued throughout the course of the behavioral manipulations.

With the previously well-documented accumulation of intracellular beta amyloid induced by feeding 2% cholesterol for 8 weeks reported by Sparks and his colleagues [5, 6, 8], the first experiments we conducted were surprising because of the expectation that we would see a beta amyloid-induced deficit in learning when, in fact, we saw a facilitation of NMR conditioning [32]. This is a finding we have seen in many of our subsequent experiments [48–50]. The facilitated conditioning was indexed by higher levels of responding to the CS [32, 48] and heightened responsivity to the US measured after conditioning, and known as a conditioning-specific reflex modification [32, 48–50]. When the levels of beta amyloid accumulation in our cholesterol-fed rabbits were examined by the Sparks laboratory, the immunoreactivity was relatively light although significantly higher than in the rabbits fed normal chow. At that point, we discussed the results with Sparks and it became clear that there was an as yet untold part of the story involving the drinking water given to rabbits.

3. The Contents of Tap Water

The effects of drinking water on beta amyloid accumulation have been discussed at length in a number of articles by Sparks and colleagues and will only be summarized here [51–53]. The original finding that the contents of tap water might be important to the level of beta amyloid immunoreactivity began with the observation by Sparks that after moving his laboratory from Kentucky to Arizona, cholesterol-fed rabbits showed significantly less intense beta amyloid immunoreactivity. Upon investigation, and ruling out other potential causes, it came to light that the rabbits in the Arizona facility were being given distilled water to

drink and, when they were returned to tap water, the beta amyloid immunoreactivity became more intense [51]. An analysis of trace metals in Morgantown tap water by an independent laboratory showed virtually no detectable levels of copper [32]. In contrast, rabbits that had been switched to and maintained on tap water in Arizona showed high levels of beta amyloid immunoreactivity and high levels of copper in the tap water [32, 51]. Subsequent manipulation of copper in distilled drinking water showed that the level of beta amyloid immunoreactivity was, indeed, a function of copper in the drinking water [46, 51–54]. In his own inimitable style, Sparks initially broached the entire subject of the role of drinking water in beta amyloid accumulation when he began a conversation about our initial light beta amyloid staining results with “And now for the rest of the story. . .”

4. The Effects of Copper on Learning

We next conducted a seminal experiment [46] in which we added 0.12 parts per million (ppm) copper as copper sulfate to distilled drinking water and found that the levels of beta amyloid in cholesterol-fed rabbits had increased over previous levels to the point of generating extracellular plaques and, importantly, these rabbits showed a deficit in trace conditioning relative to controls. The photo montage in Figure 1 shows evidence of extracellular plaque-like structures as well as dense intracellular immunoreactivity to the beta amyloid antibody 10D5 shown in detail at the bottom left of Figure 1. Figure 1 also shows a thioflavin-S stained neuron in detail (top left) in a rabbit fed cholesterol and given copper in its drinking water. Figure 2 shows that the level of trace conditioning acquired by rabbits given cholesterol and copper was significantly lower than rabbits fed cholesterol and given distilled water and rabbits fed normal chow and given copper in their drinking water. Although there is a suggestion that the cholesterol-fed animals drinking distilled water might have had higher terminal levels of responding than rabbits fed normal chow, the differences were not significant. As noted above, all rabbits were able to acquire a simple delay conditioning task in which the tone and air puff overlapped and all showed very similar auditory thresholds indicating that the cholesterol and copper did not affect sensory processing or simple delay conditioning [46]. The essential aspects of these initial findings of increased beta amyloid and lower levels of trace eyeblink conditioning were subsequently replicated by another rabbit conditioning laboratory [55, 56]. The beta amyloid accumulation resulting from rabbits being fed cholesterol has also been independently confirmed by the Ghribi group who have gone on to study some of the underlying molecular mechanisms [57–60].

To further explore the role of copper and tap water, we conducted a simple experiment in which we fed rabbits 2% cholesterol and provided them with Morgantown tap water which had been supplemented with 0.12 ppm copper. These rabbits were compared to rabbits that were fed normal chow and given tap water supplemented with 0.12 ppm copper. The data in Figure 3 show both the level of responding during trace conditioning and the number of beta amyloid

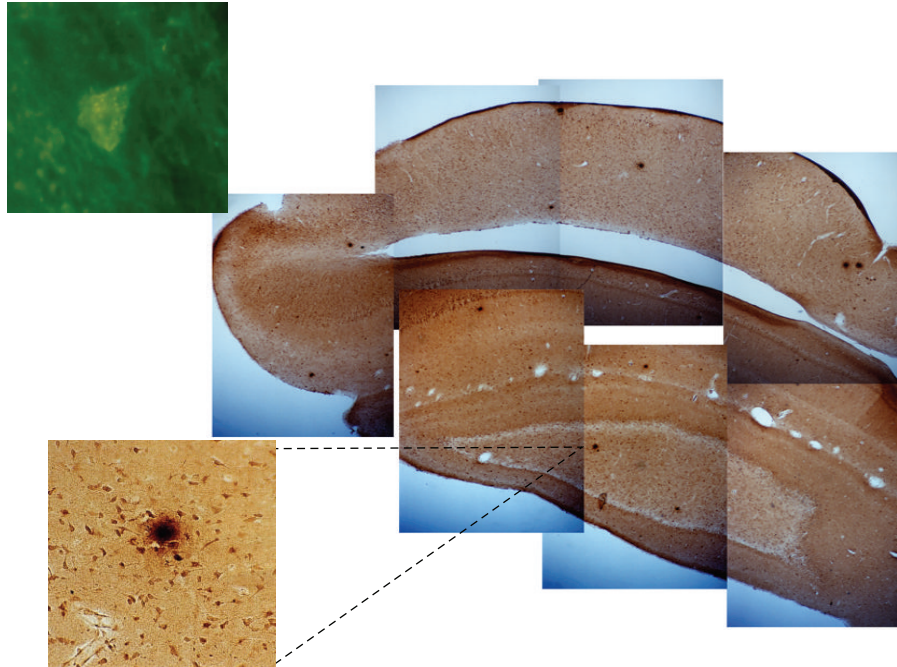


FIGURE 1: Photo montage of temporal lobe and hippocampus for a cholesterol-fed rabbit given distilled water supplemented with 0.12 parts per million copper as copper sulfate (right). Note the numerous dark spots that appear to be plaque-like structures that are shown in detail at bottom left. The top left image shows a thioflavin-S stained neuron from a cholesterol-fed rabbit given distilled water supplemented with 0.12 parts per million copper.

immune-positive neurons in the cortex and hippocampus of the two groups. Figure 3 shows and statistical analysis confirmed that the level of trace conditioning acquired by rabbits given cholesterol and copper in tap water was significantly lower than rabbits fed chow and given tap water with copper ($P < .005$). Once again all rabbits were able to acquire delay conditioning and showed identical auditory thresholds indicating that the cholesterol and copper did not affect sensory processing or simple response acquisition. The inset of Figure 3 shows that the number of beta amyloid immune-positive cells was significantly higher in the cortex and hippocampus of rabbits fed cholesterol and given copper in tap water than those fed chow. These data help confirm the original findings shown in Figure 2 and suggest that there may be more to the effects of water than first thought. For example, a subsequent analysis of the tap water supplemented with 0.12 ppm copper stored in standard carboys for four-five weeks yielded a large number of components similar to our original analysis of the tap water in Morgantown [32] but, surprisingly, the level of copper was only 0.085 ppm. The level of copper in tap water supplemented with 0.12 ppm copper and stored for only two weeks was 0.104 ppm. Clearly, storage in polypropylene carboys caused significant changes in copper concentration as a function of time and, as a result, we instituted weekly preparation of fresh copper-supplemented water that was administered to rabbits in glass bottles.

5. Cholesterol, Copper, and Beta Amyloid

At this point, we began a series of parametric experiments in which we manipulated the concentration [48] and duration

[50] of cholesterol and routinely included copper in the distilled drinking water. We continued to see the facilitating effects of cholesterol on NMR conditioning [48, 50] but, surprisingly, we did not see the debilitating effects on NMR conditioning with the addition of copper to the cholesterol. Importantly, although we continued to see higher levels of beta amyloid immunoreactivity with copper added to the drinking water of cholesterol-fed rabbits compared to those on distilled water, there was no evidence of extracellular beta amyloid plaques. This was even true when we doubled the copper concentration in the drinking water to 0.24 ppm although, in that case, the cortical levels of beta amyloid immunoreactivity were significantly higher in chow-fed rabbits given 0.24 ppm copper compared to those given distilled water suggesting that copper by itself was having an effect on beta amyloid accumulation [61].

During this second phase of behavioral experiments, a heightened sensitivity by veterinary staff to the hepatotoxic effects of the cholesterol diet [53, 62–64] meant that animals were being given supplementary feeding or withdrawn from studies earlier and more often than had occurred in our original studies. It is possible that the beta amyloid load was not as severe and the consequent extracellular plaque formations were no longer being detected because of this earlier withdrawal from the studies. In a separate development, the Sparks laboratory began to notice a decrease in the intensity of beta amyloid staining with the 10D5 antibody and the problem became worse with succeeding batches of antibody. Nevertheless, in an unpublished study, we continue to find beta amyloid immunoreactivity with a commercial human beta amyloid enzyme linked-immunosorbent assay

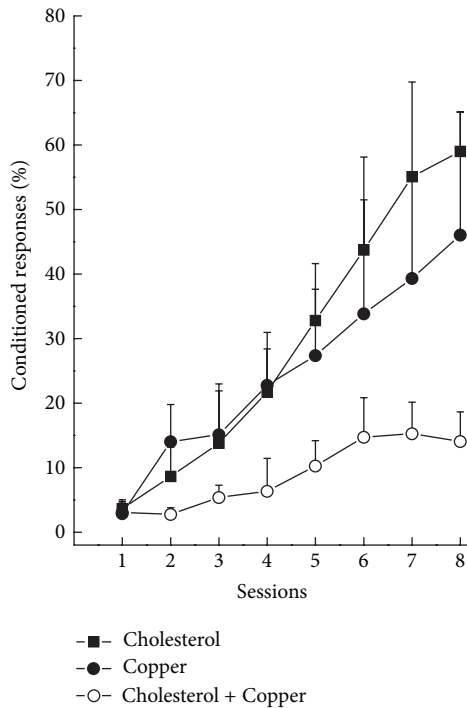


FIGURE 2: Mean percent (\pm SEM) conditioned responses to a tone conditioned stimulus as a function of eight days of pairings (sessions) of the tone and air puff to the eye for rabbits fed 2% cholesterol in their rabbit chow (Cholesterol), fed normal chow and given 0.12 parts per million copper as copper sulfate in their distilled drinking water (Copper), or fed 2% cholesterol and given 0.12 parts per million copper in their distilled drinking water (Cholesterol + Copper). The data show lower levels of trace conditioning of the nictitating membrane response in rabbits fed cholesterol and given copper in their drinking water. The data are modified from Sparks and Schreurs [46].

and immunofluorescent labeling based on the 6E10 beta amyloid antibody. Moreover, other groups have also reported significant elevations in beta amyloid as a result of feeding rabbits cholesterol [55, 58, 65, 66].

6. Imaging the Effects of Cholesterol and Copper

At the conclusion of many of our behavioral experiments, we began structural MRI imaging of the rabbits' brains to explore the effects of cholesterol and copper on rabbit ventricular volume [61, 67] and cerebrovascular diameter [67]—indices that have been noted to change in patients with AD [68–71]. The four panels of Figure 4 show structural MRI scans of rabbits that received normal chow and distilled water (a), normal chow and 0.12 ppm copper added to the distilled water (b), 2% cholesterol and distilled water (c), and 2% cholesterol and copper (d), with insets that show the area of the third ventricle. The data in Figure 4 illustrate clearly that a cholesterol diet significantly increased the area of the third ventricle and consequently, when the entire rabbit brain was analyzed, the volume of the third ventricle was

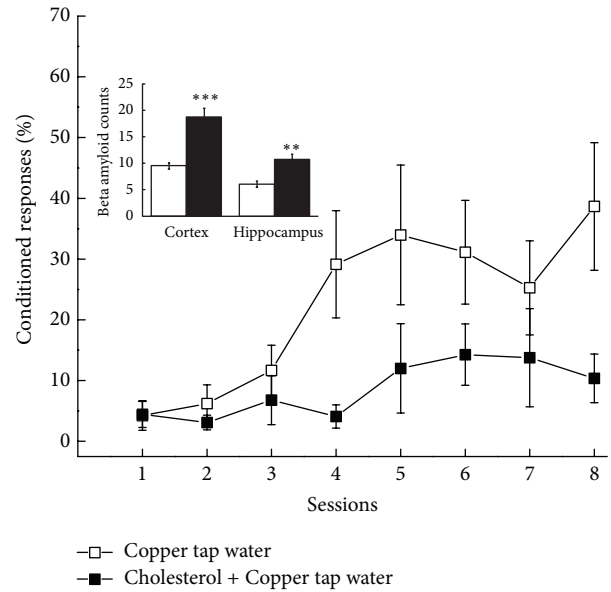


FIGURE 3: Mean percent (\pm SEM) conditioned responses to a tone conditioned stimulus as a function of eight days of pairings (Sessions) of the tone and air puff to the eye for rabbits fed normal rabbit chow and given 0.12 parts per million copper as copper sulfate in their tap water (Copper Tap Water) or fed 2% cholesterol and given 0.12 parts per million copper in their tap water (Cholesterol + Copper Tap Water). The inset shows the mean (\pm SEM) number of counts of beta amyloid immunoreactive cells in the cortex and hippocampus. The data show lower levels of trace conditioning of the nictitating membrane response and number of beta amyloid positive cells in rabbits fed cholesterol and given copper in their tap water.

found to be higher for the cholesterol-fed rabbits than the normal chow-fed controls [72]. This was true regardless of whether the rabbits were given copper in the drinking water or whether the concentration of that copper was 0.12 ppm [72] or 0.24 ppm [61]. In all of these experiments, the levels of beta amyloid immunoreactivity to the 10D5 antibody was always higher in cholesterol-fed rabbits than normal chow controls, and the addition of copper tended to increase the intensity of that immunoreactivity even further although this copper-induced increase was not always significantly higher [48–50, 61]. Figure 5 shows the blood vessels in a rabbit brain that were visualized by time-of-flight angiography during our MRI studies and they include the common carotid arteries, the basilar artery, the internal carotids, and the posterior communicating arteries [67]. Figure 6 shows that the basilar, internal carotid, and posterior communicating arteries were all narrowed by a 2% cholesterol diet compared to normal chow controls and that the addition of 0.12 ppm copper to the drinking water did not significantly increase the narrowing [72]. There were no significant differences in diameter of the common carotid arteries.

7. The Effects of Cholesterol on Other Forms of Learning

As important as the behavioral effects of cholesterol were with the rabbit NMR, there were two broader questions. First,

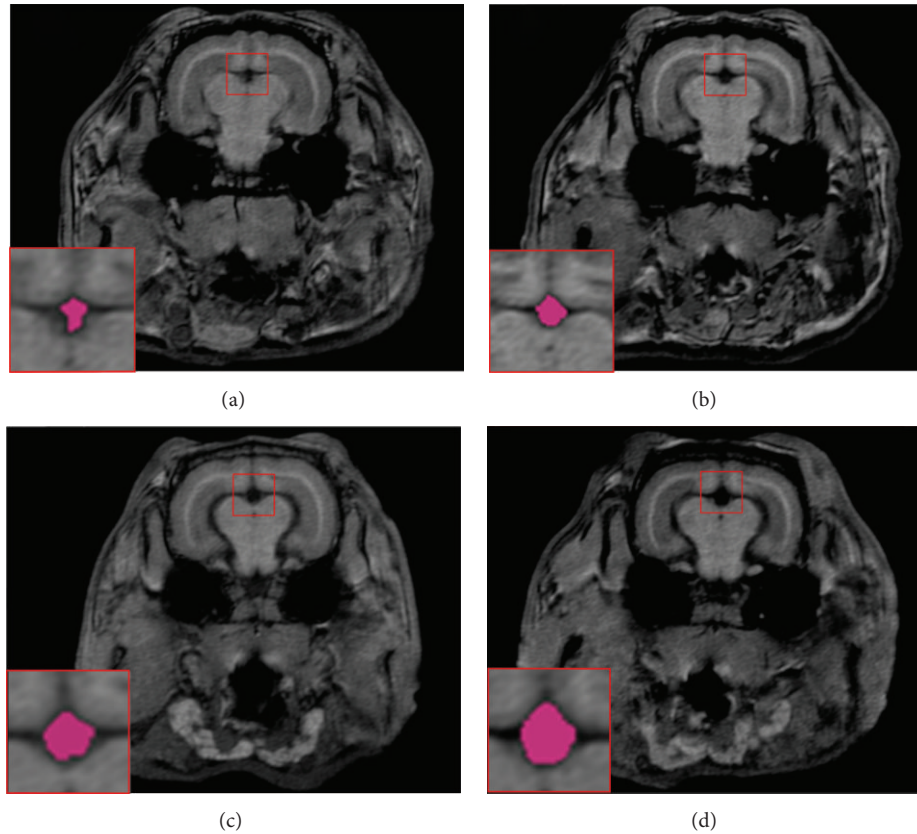


FIGURE 4: Structural MRI scan of a rabbit that received normal chow and distilled drinking water (a), normal chow and 0.12 ppm copper added to the distilled drinking water (b), 2% cholesterol and distilled drinking water (c), or 2% cholesterol and copper added to the distilled drinking water (d). The inset shows the area around the third ventricle in each rabbit and the red shading illustrates the significantly larger area of the third ventricle for cholesterol-fed rabbits ((c) and (d)) compared to the chow-fed control rabbits ((a) and (b)). The data show significant increases in the area of the third ventricle as a function of being fed cholesterol. Data are modified from [72].

would the effects of cholesterol on acquisition of the rabbit NMR hold true for other forms of learning, and second, what were the effects of cholesterol on memory? Although the majority of the research into learning in rabbits is based on changes in skeletal responses, particularly the closure of the upper eyelid and the sweep of the nictitating membrane [35], a significant body of research has examined an autonomic response—deceleration of heart rate [73–80]. The adult rabbit typically shows an unconditioned increase in heart rate (HR) to electrical stimulation and an unconditioned decrease in HR to tones. The unconditioned HR acceleration to electrodermal stimulation is an acute response to a stressful stimulus and has been used as a measure of the animal's “defense reaction” [80]. The unconditioned deceleration in HR to a tone is an orienting response that can be habituated by tone-alone presentations. Heart rate classical conditioning occurs when rabbits that receive pairings of tone and shock show a conditioned deceleration in HR to the tone relative to rabbits that show little or no change in HR when they receive explicitly unpaired tone and shock presentations [73, 81, 82]. This type of autonomic conditioning usually occurs within a relatively few pairings that consist of tones that are separated from shock by a second or more [83]. Using a trace conditioning paradigm, we found that cholesterol facilitated

rabbit HR conditioning and that the unconditioned HR response to shock was also modified by conditioning [49]. The significant facilitation of HR conditioning suggests that the effects of cholesterol on learning were not specific to one form of conditioning involving a skeletal response but to an autonomic response as well. Importantly, a major anatomical locus for rabbit HR conditioning is the amygdala [75, 84–86] and we found that, in addition to the hippocampus and cortex, the level of beta amyloid staining in the amygdala was higher in cholesterol-fed rabbits than in controls [49].

Our findings of cholesterol-induced facilitated learning are consistent with experiments in a number of other animal models that have reported that modifying dietary cholesterol can improve learning [87]. For example, increasing cholesterol in mutant mice in which hippocampally dependent spatial learning is normally impaired improves performance in the Morris water maze [88, 89]. Feeding cholesterol to young, normal rats also improves performance in the Morris water maze [90, 91]. Feeding cholesterol to rats that are either deficient in cholesterol or have cholesterol synthesis blocked reverses problems with learning in the water maze and acquisition of eyeblink conditioning [92–95]. These animal data are also consistent with some human literature showing that higher cognitive functioning is correlated with high

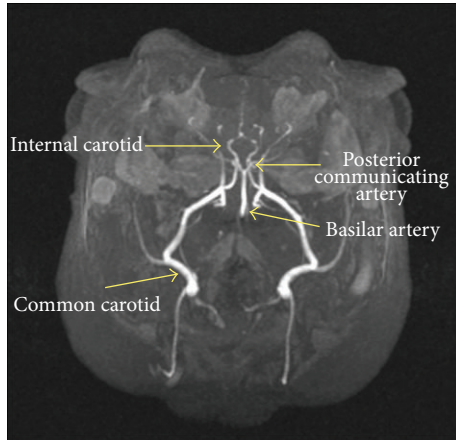


FIGURE 5: Blood vessels in the rabbit brain that were visualized by time-of-flight magnetic resonance angiography during our MRI studies and analysis of vessel diameters focused on the left and right common carotid arteries, the basilar artery, the left and right internal carotids arteries, and the left and right posterior communicating arteries.

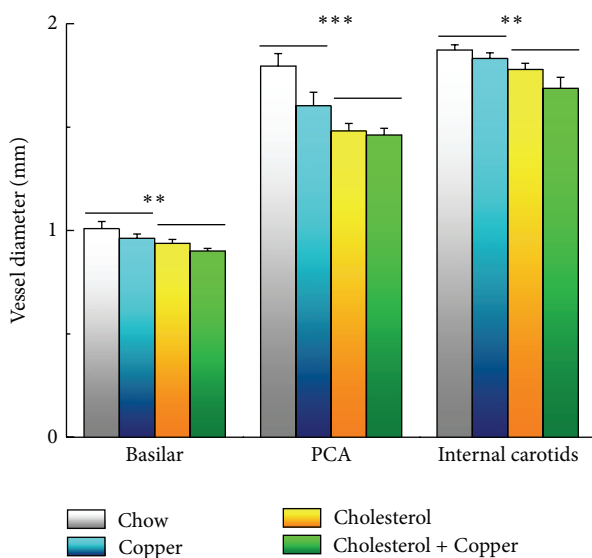


FIGURE 6: Mean (\pm SEM) vessel diameter of the left and right internal carotid arteries, basilar artery, and left and right posterior communicating arteries (PCA) for rabbits fed normal chow and given distilled drinking water (Chow), 2% cholesterol in their rabbit chow and given distilled drinking water (Cholesterol), fed normal chow and given 0.12 parts per million copper as copper sulfate in their distilled drinking water (Copper), or fed 2% cholesterol and given 0.12 parts per million copper in their distilled drinking water (Cholesterol + Copper). Data are modified from [72].

cholesterol [96, 97] and that cholesterol may protect against cognitive decline especially in the elderly [97–100].

8. The Effects of Cholesterol on Memory

The majority of research with humans suggests strongly that cholesterol is detrimental to memory. A significant number of

studies show that elevated serum cholesterol is a risk factor for mild cognitive impairment [101–105] and dementia [106, 107] and that cholesterol levels are correlated with measures of intelligence [103, 108–111] except in the very elderly [99, 112]. Low HDL cholesterol has been correlated with deficits and declines in memory in midlife [113]. A study of cholesterol synthesis showed the level of the cholesterol precursors lanosterol and lathosterol are correlated with low memory performance as subjects age [114]. It is to this second question—the effects of cholesterol on memory—that we next turned our attention.

In an experiment by Darwish and colleagues, we trained rabbits to asymptotic levels of NMR tracing conditioning and then instituted an 8-week diet of 2% cholesterol or normal chow before assessing the memory of trace conditioning by presenting the tone alone over a period of days during extinction [115]. Rabbits fed normal chow showed response levels of about 60% at the beginning of extinction which was very consistent with a previous assessment of long-term memory for trace conditioning of the rabbit NMR after 8 weeks [116]. In contrast, cholesterol-fed rabbits showed significantly lower response levels of only 30%—a level that was not significantly higher than cholesterol-fed unpaired control rabbits [115]. We were able to replicate this finding with different concentrations of cholesterol that all showed lower levels of responding during extinction than a normal chow control group [117]. Importantly, neither of these experiments involved the addition of copper to the distilled drinking water suggesting that cholesterol by itself can degrade a previously acquired memory.

9. The Effects of Cholesterol and Copper on Both Learning and Memory

In a recent study, we combined our behavioral procedures and cholesterol feeding regimens into a single paradigm. We used discrimination reversal conditioning to assess the effects of a cholesterol diet and copper in the water on the memory of a previously acquired association and the ability of the rabbits to acquire a new and opposite association. In brief, rabbits were trained to discriminate between two tones of different frequency (1 kHz and 8 kHz), then placed on an 8-week cholesterol diet with or without 0.12 ppm copper in their drinking water, then tested for their memory of the original discrimination, and subsequently trained to reverse that discrimination [118–122]. The data showed that cholesterol and distilled water degraded the ability of rabbits to remember the original discrimination but facilitated their ability to learn the reversal of that discrimination. Interestingly, the addition of copper to the water of cholesterol-fed rabbits had the opposite effect on both phenomena—the rabbits were able to recall the original discrimination but were less able to learn the reversal of that discrimination. Importantly, a rabbit's ability to successfully reverse a discrimination is dependent on an intact, functioning hippocampus which allows the rabbit to inhibit responding to a previously paired stimulus [118, 120]. We found that cholesterol-fed rabbits were better able to inhibit responding than cholesterol-fed rabbits given copper

and have shown elsewhere that the membrane excitability of hippocampal neurons is increased by cholesterol feeding that is decreased by the addition of copper to the drinking water [123]. Membrane excitability has been shown to increase as a function of learning [124–128]. Taken together, these data show that cholesterol by itself and cholesterol supplemented by copper have opposite effects on behavior as well as on one of the underlying neural mechanisms associated with learning and memory.

10. Effect of Cholesterol and Copper on Beta Amyloid Accumulation and Learning and Memory in This Model

It is clear that feeding rabbits cholesterol increases the level of beta amyloid in the brain at the same time that it has significant systemic effects particularly in the liver and vasculature [87, 129–133]. Our research shows that against a backdrop of increased beta amyloid immunoreactivity in the cortex and hippocampus, there are replicable effects of feeding cholesterol on learning and memory. Given the essential role of the cortex and hippocampus in the acquisition and recall of trace conditioning in both eyelid and heart conditioning [26, 39, 55, 134–138], it is tempting to draw causal inferences from the accumulation of beta amyloid in those structures and the observed changes in learning and memory particularly given our findings of cholesterol-induced changes in the membrane properties of hippocampal neurons [123]. In fact, this recapitulates the inferences the field continues to make concerning beta amyloid and Alzheimer's disease. However, the recent very public failures of clinical trials designed to mitigate the effects of beta amyloid have given some researchers grounds to revisit other potential mechanisms that may play a part in the development of Alzheimer's disease including the role of vascular factors, inflammation, oxidative stress, and the role of trace metals including copper, to name a few. For example, there is strong evidence that human cognitive impairment is correlated with the extent of cholesterol-induced atherosclerosis both in peripheral arterial disease [139] and in carotid atherosclerosis [140]. There is also a growing awareness of the effects of inflammation on cognitive decline [141–144]. Similarly, there are a significant number of peripheral effects of cholesterol in the rabbit that may have effects on learning and memory including atherosclerosis [145–147], inflammation [5, 133, 148, 149], oxidative stress [150–152], and copper [153–155]. With the compromise of the rabbit's blood brain barrier that occurs with cholesterol feeding [5, 58, 156–158], these peripheral effects may very well also become central effects. Finally, beta amyloid is present in the brain from birth to death and, in normal concentrations, is critical for cell function, synaptic plasticity, and memory [159]. In other words, there are a number of complex effects of cholesterol, copper, and beta amyloid and it is probably a combination or interaction of several of these effects that can best explain their influence on learning and memory.

11. Summary

From our first published rabbit NMR study [32], the majority of experiments have shown that cholesterol facilitates learning and the addition of copper, which increases the level of beta amyloid immunoreactivity, reverses this facilitation, and, in at least two cases, makes it significantly worse than controls [46]. More recently, we have found that cholesterol degrades long-term memory both of simple acquisition as well as discrimination learning, and, in the latter case, copper returns responding to control levels. On the other hand, as noted above, the addition of copper to cholesterol tends to exacerbate the level of beta amyloid immunoreactivity but only slightly increases other indices of pathology including increases in ventricular volume and cerebrovascular diameter beyond those induced by cholesterol alone [61, 67]. Taken together, these results have raised a number of important questions including the nature of the effects of cholesterol on learning and memory, the potential mechanisms of these effects, and the role copper may have in modifying the effects of cholesterol and in elevating beta amyloid.

Acknowledgments

This paper is dedicated to the memory of D. Larry Sparks—friend and colleague. The authors also wish to acknowledge the important contributions of past and present members of the Sparks and Schreurs laboratories: Roger Bell, Lauren Burhans, Deya Darwish, Stephen Deci, Jimena Gonzales-Joekes, Susan Lemieux, Sherry Johnson-Traver, Jeff Lochhead, Sylwia Mrowka, Carrie Smith-Bell, Goran Stankovic, and Chuck Ziolkowski. Preparation of the paper and many of the experiments described herein was supported by National Institutes of Health/National Institute on Aging Grant AG023211 to Bernard G. Schreurs and D. Larry Sparks. The contents of this paper are solely the responsibility of the author and do not necessarily represent the official views of the National Institute on Aging.

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