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Cellular mechanisms for altered learning in aging

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Abstract

Getting gray hair is part of the natural progression of aging. People expect it and they can change their hair color, if they choose. People also expect increases in memory lapses and learning difficulties as they get older. However, unlike hair color, there is no magic cure or option to fix learning and memory difficulties, because the cellular mechanisms of learning and aging in all the different types of neurons throughout the brain have yet to be discovered. This review describes our efforts to identify a cellular biomarker in hippocampal pyramidal neurons that has been demonstrated to reliably change with learning and with aging – the postburst afterhyperpolarization. We propose that this biomarker, which plays a critical role in regulating neuronal excitability, can be used as a benchmark for future studies in order to understand and identify the cellular mechanisms of learning and aging in the hippocampus, as well as in other cortical regions.

Keywords

calcium channels; eyeblink conditioning; Henry Gustav Molaison; hippocampus; postburst afterhyperpolarization

The idiom ‘you can’t teach an old dog new tricks’ is mainly used to describe habitual behavior of an individual that is difficult to correct. However, a liberal interpretation of the phrase implies that an old dog cannot learn something new. While it is generally accepted that memory lapses increase and that learning new things becomes more difficult with normal aging, some healthy aging subjects can successfully learn and retain new ‘tricks’. The focus of our research is to identify the cellular mechanisms that are engaged for successful learning and to elaborate on these findings to identify the potential cellular stumbling blocks that prevent successful learning with normal aging. In this review, we will discuss one such mechanism that we have found to be consistently altered by learning and in normal aging – the postburst afterhyperpolarization (AHP).

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Before delving into the cellular mechanisms, the eyeblink conditioning (EBC) behavioral learning task, which our laboratory has successfully used to study these questions, will be described and elaborated upon.

Eyeblink conditioning

A Pavlovian conditioning task, EBC, is a simple behavioral assay that was first designed and tested on humans [1]. In most cases, the subject is presented with a tone (a neutral, conditioned stimulus) that is repeatedly paired with an airpuff to the eye (an aversive, unconditioned stimulus), which evokes the reflexive, eyelid closure (an unconditioned response); similar to Pavlov's training protocol to evoke salivation from his dogs to the ringing of a bell. After repeated pairings, the subject will learn that the tone is a predictor of the airpuff and avoid the noxious airpuff to the eye by closing the eyelid after the tone presentation but prior to the airpuff, producing a conditioned response.

There are two main forms of EBC: delay and trace. In delay EBC, the subject is presented with a conditioned stimulus that stays on and coterminates with the unconditioned stimulus, and, thus, there is no temporal gap between the conditioned and unconditioned stimuli (Figure 1A). In trace EBC, there is a temporal, stimulus-free gap between the offset of the conditioned stimulus and the onset of the unconditioned stimulus (Figure 1B). The temporal gap between the conditioned and unconditioned stimuli makes trace EBC much more difficult to learn than the delay EBC task [2]. Cumulative work by many laboratories suggest that neural connections in the cerebellum are essential for delay EBC, whereas the neural interconnections in the forebrain regions (especially the hippocampus) are required for successfully learning trace EBC [3,4]. Thus, these two similar, yet distinct, EBC paradigms can be used to examine the cellular alterations in the specific brain region(s) that are engaged and essential for learning each variant of the task.

Behavioral research with EBC in animals has been especially important in identifying potential cellular mechanism(s) of learning and memory, since the behavioral findings with animals are similar to those observed with humans. As a case in point, aging subjects – including humans – have difficulty learning the EBC response compared with young adults [2,5–11]. In animal studies, nearly half of the aging subjects tested on trace EBC failed to meet the learning criteria [2,10], and those that do learn require many more trials than young animals in order to do so [2,12]. More importantly, this age-related impairment on learning the EBC task has been used successfully in preclinical trials with aging rabbits to test potential therapeutic compounds that have been designed to slow cognitive decline in Alzheimer's disease patients [13]. Therefore, systematic examination of the cellular alterations during learning and with normal aging in animals may have a direct functional relevance to the neural alterations in our own brains when we learn something new, and may also provide information as to why it becomes progressively more difficult to learn new things as we age.

But where (in what brain region) should we look for the learning-related alterations?

The hippocampus, a brain structure within the medial temporal lobe, has garnered much scientific attention with the landmark publication by Scoville and Milner that described the memory deficits of ten patients who had portions of their medial temporal lobe removed bilaterally for various reasons [14]; the most famous of them being Henry Gustav Molaison who passed away in December 2008. Specifically, the patients with bilateral removal of a majority of the hippocampus and the surrounding tissue (including the uncus and amygdala) had profound anterograde amnesia (i.e., they could not form new long-term memories of events and facts that are part of their daily lives [declarative memory]). However, motor skill learning (such as mirrortracing) was intact [15]. Notably, patients with amnesia [16]

and animals with hippocampal lesions [17,18] are impaired in learning trace EBC, while learning delay EBC has been found to be intact [17,19]. Thus, the work in our laboratory has focused on systematically identifying the cellular mechanisms in the principle output neurons of the hippocampus (CA1 and CA3 pyramidal neurons) that underlie learning trace EBC.

Cellular mechanism of learning: the postburst afterhyperpolarization

In the brain, the flow of information from one neuron to another in neural circuits is mainly achieved by action potentials that release neurotransmitters in the synapses, which neurons use to communicate with each other. In essence, the action potential is the building block of the neuronal language, and the frequency and the number of action potentials are used by neurons to communicate with one another. To learn and create memories, the neurons have to be able to generate action potentials and relay the critical information during learning and retrieval of memories in a meaningful way. Thus, neurons have to have a cellular mechanism to act as a ‘gatekeeper’ that also controls the frequency and number of action potentials.

In hippocampal and cortical pyramidal neurons, the postburst AHP is such a neuronal mechanism that is generated after a burst of action potentials to prevent a neuron from firing repeatedly to a sustained depolarizing input [20,21]. For example, Gant and Thibault recently demonstrated in CA1 pyramidal neurons that the postburst AHP significantly dampens the probability of action potential generations to a subsequent synaptic stimulation, which was sufficient to induce an action potential prior to the AHP [22]. In essence, the postburst AHP is a cellular brake to prevent a neuron from being overexcited. The postburst AHP amplitude is correlated with the number and frequency of action potentials evoked during the burst: a higher frequency and number of action potentials yields a larger AHP [23,24]. In hippocampal pyramidal neurons, the postburst AHP has two segments (medium and slow) that are mainly mediated by calcium-dependent potassium currents and modulated by numerous neurotransmitters and neuropeptides [21,25,26]. This review refers to both the medium and the slow periods of the AHP, unless specified.

So why is the postburst AHP an important cellular mechanism for learning and for the learning impairments observed with aging?

First, many studies have demonstrated that the postburst AHP is significantly altered in cortical and hippocampal pyramidal neurons from animals that successfully learned an associative task [21]. Originally described in CA1 hippocampal pyramidal neurons after rabbits successfully learned delay EBC [23,27], the learning-related postburst AHP reductions have been reported in rats and rabbits after trace EBC [12,28–32], trace fear [33], spatial water maze [34] and olfactory discrimination [35–37] tasks. This learning-related modulation of the postburst AHP has been recently extended to prefrontal cortical pyramidal neurons after fear conditioning in rats [38]. Thus, the modulation of the postburst AHP in pyramidal neurons appears to be an important and generalized cellular substrate of learning.

Second, the learning-related postburst AHP reduction in hippocampal pyramidal neurons following trace EBC is transient and learning specific [29,30]. The hippocampus is one of several key medial temporal lobe regions that is important for learning new declarative memories, but it is not the site of permanent memory storage. For example, Henry Gustav Mollaison was severely impaired in learning new declarative memories, but his memories for events prior to his surgery were relatively intact [14,15]. Similarly, Richard Thompson and colleagues demonstrated that the hippocampus is essential for learning trace EBC, but not the memory of the learned CS–US association recalled 1 month after successful learning [17]. Thus, the hippocampal neurons must be engaged for learning, but are not part of the

essential neural circuitry for recall of the learned event. This is effectively demonstrated in two studies that found learning-related postburst AHP reductions in CA1 and CA3 pyramidal neurons are transient (lasting up to 1 week) after trace EBC (Figure 2) [29,30]. More importantly, the postburst AHPs measured in the same cohort of animals 2 weeks after learning the trace EBC task were no different from pseudoconditioned and behaviorally naive animals, while the behavioral response to the learned CS–US association (or memory) was intact [29,30]. Thus, the postburst AHP had to be reduced for successful learning to occur, but reverted back to the original state once consolidation of the learned memory had occurred – presumably in the neocortical regions.

Third, the postburst AHP is significantly enlarged in CA1 pyramidal neurons from aged animals. Originally reported by Landfield and Pitler [39], we and others have demonstrated that the enlarged postburst AHP may be due to enhanced calcium influx into the neurons via the voltage-dependent L-type calcium channels and release of calcium from internal calcium stores (such as the endoplasmic reticulum) [21,40–44]. Although the postburst AHP is much larger in CA1 neurons from aged animals, it is significantly reduced in CA1 neurons from aged animals that learned the trace EBC task compared with those measured in neurons from aged animals that were trained but failed to learn [12]. Furthermore, the postburst AHP measured from CA1 neurons from aged animals that learned trace EBC was nearly identical to that measured in CA1 neurons from young adult animals that also learned the trace EBC task [12], indicating that the cellular mechanism for modulating the postburst AHP with learning may be unchanged with normal aging.

Our working hypothesis is that the enlarged postburst AHP in neurons from aged animals presents a higher threshold for neurons to fire action potentials in a meaningful way while learning a task (i.e., the large postburst AHP in aged neurons will prevent them from firing repeatedly during a prolonged depolarization, and thus reduces the excitability of these neurons in the neural circuit). This in turn would prevent the neural network from working in a proper manner to encode the associations. One way in which we tested this hypothesis was via pharmacological testing of potential therapeutics for Alzheimer's disease [13]. For example, we found that 3 weeks of treatment with a cholinesterase inhibitor, metrifonate, prior to the start of trace EBC in aged rabbits alleviated the learning deficit in these aged animals (Figure 3) [45,46]. In order to identify the potential source of this behavioral rejuvenation in the aged rabbits, we carried out another set of experiments that examined the firing property of CA1 neurons of the animals treated with metrifonate. We found that metrifonate pretreatment restored the excitability of CA1 neurons to a 'young-like' state (Figure 3) [47]. This may have been a critical component that allowed the neural circuitry to function in a proper state for learning. This hypothesis has garnered support from a study that took advantage of the transient nature of the learning-related AHP reduction [48] (i.e., the learning-related postburst AHP reduction observed in hippocampal pyramidal neurons returns to baseline levels [nearly identical to those of naive animals] within 2 weeks after learning has occurred) [29,30]. In this study, young adult and aged rats were trained to find a hidden platform on a spatial water maze task. Approximately 2 weeks after the training, the postburst AHP was measured in CA1 neurons from aged animals that learned, aged animals that failed to learn, and young adult animals that learned the task. The results demonstrated that the aged animals that succeeded in learning the spatial water maze task had postburst AHPs that were similar to those of young adult animals; and both groups had AHPs that were significantly smaller than the aged animals that failed to learn [48]. Taken together, these data suggest that the postburst AHP and the excitability level of hippocampal pyramidal neurons from aged subjects must be in a 'young-like' state for successful learning, and if not, then it can potentially be restored to a 'young-like' state with pharmacological interventions.

Additional support for our postburst AHP hypothesis comes from measuring the firing behavior of hippocampal pyramidal neurons *in vivo* while animals are learning the EBC task. The hippocampal neuronal activity *in vivo* has been demonstrated to be highly correlated with behavioral acquisition of delay [49] and trace [50,51] EBC in rabbits. More importantly, if the postburst AHP of pyramidal neurons is a key regulator of neuronal firing and network activity *in vivo*, then neuronal activity of these hippocampal neurons should increase during the learning process (i.e., reduction of the postburst AHP should allow the neurons to fire more readily and more often). This is indeed the case. The baseline activity of hippocampal pyramidal neurons from young animals, measured *in vivo*, are significantly elevated after learning trace EBC [52]. Furthermore, aged animals that succeeded in learning trace EBC demonstrated significantly increased basal firing rates in a manner similar to that observed in young adult animals that learned the task [52]. Aged animals that failed to learn trace EBC had significantly lower basal firing rates compared with both the young adult and aged animals that succeeded in learning [52]. Notably, an L-type calcium channel antagonist, nimodipine, has been demonstrated to reduce the postburst AHP of CA1 pyramidal neurons *in vitro* [53], increase the firing rate of hippocampal pyramidal neurons *in vivo* [54] and ameliorate the learning deficit on trace EBC in aged animals (Figure 3) [55]. Thus, the postburst AHP is a key regulator of neuronal activity that has a direct impact on network activity during learning.

Conclusion

The cumulative experimental evidence, thus far, points to the postburst AHP as a key regulator of neuronal excitability and, by extension, of successful learning. It is clear that a transient reduction of the postburst AHP must occur in hippocampal pyramidal neurons for successful learning and that the increase in the postburst AHP in these neurons with age is a significant factor in age-related learning difficulties. Thus, the challenge is: first, to identify the cellular signaling mechanism cascade that is activated during learning; and second, to identify the cellular mechanisms that contribute to the age-related postburst AHP enlargement, as well as how to restore the postburst AHP to a 'young-like' state.

Future perspective

The cellular signaling cascade that is engaged for the learning-related postburst AHP reduction is still not understood. We have started to systematically search for proteins within the cell that are recruited for maintaining the learning-related postburst AHP reduction, and have found that PKA is one such protein [32]. Activity of PKA has been linked to numerous cellular signaling cascades; including alterations in gene transcription, long-term modification of synaptic proteins and reductions in the postburst AHP [25,32,56]. However, the interaction of PKA and its effectors that ultimately leads to the learning-related postburst AHP reduction remains to be determined. Furthermore, the link between PKA activity and the learning-related postburst AHP reduction in hippocampal pyramidal neurons have been examined after the subject has met the learning criteria. It remains to be seen whether PKA and/or other kinases (such as PKC and calcium/calmodulin-dependent protein kinase II) are involved in mediating the cellular signaling cascade(s) in earlier phases that lead to learning.

The molecular identity of the slow AHP channel has yet to be discovered. While the apaminsensitive SK2 channels have been identified as a candidate for the medium AHP in pyramidal neurons [21,26], the molecular identity of the slow AHP channel has remained elusive. Considering the importance of the slow AHP in modulating the neuronal excitability of pyramidal neurons, this calcium-dependent potassium channel is an important target for potential therapeutics. Once the identity of the slow AHP channel is known, it will be possible to directly determine if the expression level of these channels is a source of the

learning- and aging-related postburst AHP alterations. If the expression level of the slow AHP channel is increased with age, then specific pharmacological therapeutics that are designed to block these channels would be beneficial for ameliorating learning difficulties in the aging population.

In the absence of the molecular identity of the slow AHP channel, studies in our laboratory are being conducted to systematically examine the potential alteration of calcium levels in regulating the postburst AHP with learning and aging, considering that the slow and medium AHP channels rely on calcium in the cytosol of neurons for activation. At present, we are using molecular (western blotting and immunohistochemistry) and calcium imaging (including the new innovative multiphoton laser scanning microscopy system) techniques to examine alterations in protein levels that regulate calcium entry into the cytosol (such as the L-type calcium channels), which may have a direct impact on the resting calcium level dynamics with learning and with normal aging. If these calcium handling proteins are altered to enlarge the postburst AHP in normal aging, then genetic manipulations to silence these proteins (via virally delivered siRNAs) will be used to further validate these proteins as targets for future therapeutics to alleviate the learning deficits associated with normal aging.

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Executive Summary

Introduction

- Increases in memory lapses and in learning difficulties are expected as part of the normal aging process.
- In order to prevent or alleviate these symptoms of aging, it is essential to identify and understand the cellular mechanism(s) of learning.
- This review is mainly focused on our experimental findings that have yielded a biomarker of learning and aging – the postburst afterhyperpolarization (AHP). We propose that the postburst AHP can be used to identify the cellular signaling events that are engaged for learning and that may be altered with aging, which leads to learning and memory difficulties.

Eyeblink conditioning

- Eyeblink conditioning (EBC) is a simple, yet powerful, behavioral assay to examine the function of various brain regions.
- There are two main versions of EBC: delay and trace.
- The proper function of the cerebellum and its associated circuitry has been shown to be essential for learning of the delay EBC task.
- The proper function of the forebrain (especially the hippocampus) and its associated circuitry has been demonstrated to be essential for learning the trace EBC task.
- Learning the trace EBC task has been shown to be difficult in aging subjects, including humans.

Postburst AHP

- The postburst AHP is a cellular mechanism for controlling the frequency and number of action potentials; thus, it is a key regulator of neuronal activity in the neural network.
- The postburst AHP is significantly reduced in a transient manner (up to 1 week) in hippocampal pyramidal neurons after a subject has successfully learned a hippocampus-dependent task.
- The postburst AHP is significantly larger in CA1 pyramidal neurons from aged animals compared with young adults.
- Pharmacological compounds that reduce the postburst AHP also ameliorate the learning deficits observed in aged animals.

Future perspective

- We propose that a critical factor in successfully encoding declarative memories depends on maintaining the intrinsic excitability of hippocampal pyramidal neurons with normal aging by having the postburst AHP of these neurons in a ‘young-like’ state.
- Experiments are currently underway to examine the cellular signaling mechanism(s) that are recruited for successful learning and that are altered with normal aging in the hippocampal pyramidal neurons.

- Future studies are necessary to identify the cellular mechanisms that are altered with successful learning and normal aging in other neurons throughout the brain. These future studies will be greatly aided by using the experimental findings in hippocampal pyramidal neurons with the use of the postburst AHP as a biomarker of learning and aging.

Figure 1. Integrated electromyography recordings of eyeblink responses during delay and trace eyeblink conditioning from a young adult rat

In the delay EBC paradigm, the onset of the CS precedes and often coterminates with the US. **(A)** Delay EBC, the CS turns off simultaneously with the onset of the US; there is no temporal gap between the CS and the US. **(B)** Trace EBC paradigm, the CS and the US are separated by a temporal gap. Note that in both cases the eyelid closure (as indicated by the increase in the EMG signal) starts well after the onset of the tone CS. In trace EBC, the eyelid is closed during the trace interval in anticipation of the airpuff.

CS: Conditioned stimulus; EBC: Eyeblink conditioning; EMG: Electromyography; US: Unconditioned stimulus.

Figure 2. Postburst afterhyperpolarization was transiently reduced in hippocampal pyramidal neurons while performance remained persistent after learning trace eyeblink conditioning in young adult rabbits

(A) Postburst AHP in CA1 neurons from a N rabbit and from trace-conditioned rabbits studied 24 h after initial learning (trace 24 h) or 24 h after receiving an additional training session given 14 days after initial learning (R). The AHPs were evoked with a 100 ms minimal depolarizing current injection (~0.6 nA, solid black line) that reliably elicited a burst of four action potentials. Action potentials are truncated for visualization of the AHP.

(B) Learning-related reductions of the AHP amplitude were transient, lasting approximately 1 week in slices prepared at various times after learning (1 h [0 days], 1, 3, 5, 7 or 14 days). Such changes were not observed in N, P or S control rabbits. Numbers in parentheses indicate the ratio of individual cells with reduced AHPs to number of cells studied in that group. The pseudoconditioned animals receive the CS and US in an unpaired manner, so there is no CS-US association learned in this group. Pseudoconditioned group is important to assess potential alterations in neuronal properties due to exposure of the animals to the CS and US (i.e., training-related, and not learning-related, neuronal alterations). S rabbits were

defined as rabbits that did not reach criterion within 15 training sessions, and that exhibited less than 30% conditioned responses on the last training session. R rabbits received an additional 80 trial training session on day 14 after initial learning.

*Data significantly different from all three control groups: $p < 0.001$.

AHP: Afterhyperpolarization; CS: Conditioned stimulus; N: Naive; P: Pseudoconditioned;

R: Retention; S: Slow-learning; US: Unconditioned stimulus.

Adapted with permission from [29].

Figure 3. Pharmacological compounds that reverse the learning deficit in aged rabbits also reduce the postburst afterhyperpolarization in hippocampal pyramidal neurons

(A) Treatment with nimodipine, an L-type calcium channel blocker, ameliorated the learning impairment in aged rabbits, and allowed the aged rabbits to learn the trace eyeblink conditioning task at a similar rate to young adult animals. (B) Nimodipine also reduced the postburst afterhyperpolarization in CA1 neurons from aged animals to a level found comparable as that observed in CA1 neurons from young adults (dark colour represents aCSF; light colour represents 10 mM nimodipine). (C) Treatment with metrifonate, a cholinesterase inhibitor, also ameliorated the impairment in learning the trace eyeblink conditioning task observed in the aging population. (D) Furthermore, the excitability of CA1 neurons from aged animals was significantly increased with subchronic treatment with metrifonate compared with those aged animals treated with vehicle.

* $p < 0.001$ as compared with aCSF; † $p < 0.01$ as compared with vehicle.

aCSF: Artificial cerebrospinal fluid; nim: Nimodipine; veh: Vehicle.

Adapted with permission from [46,47,53,55].