Eyeblink Conditioning As Models of Declarative and Non-declarative Memories

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Abstract

Learning and memory are the crucial cognitive functions, which equip animals for the ability to preserve our experiences of both knowledge and motor skills that we have learned. Eyeblink conditioning is a basis form of associative learning that conditions the eyeblink reflex to a neutral stimulus that predicts an aversive stimulus. With small manipulations, eyeblink conditioning procedures have been used as learning models of both declarative and non-declarative memories. The procedures can then be used to examine the basic mechanisms of memory and the pathophysiology of memory afflicted diseases, and to test new or alternative treatments of memory disorders. This paper introduced the methods of eyeblink conditioning as well as the memory circuitry responsible for mediating the memory, and the clinical applications of the procedures were also reviewed.

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Memory is one of the mental faculties generated by the brain and it acts as the glue that binds our mental life. Some memory afflicted diseases such as Alzheimer's disease have devastating effects on patients especially when the diseases progress to advanced states that the patients start to forget their names or their close relative's names, or to lose their way home, basically they are losing themselves. Moreover, when we talk about memory, we always have to talk about learning as well, because they are connected processes. Kandel et al. (2000) stated that "learning is the process by which we acquire knowledge about the world, whereas memory is the process by which that knowledge is encoded, stored and later retrieved."[1] Moreover, not only are learning and memory important for our normal functions, but they can also lead to abnormal behaviors such as post-traumatic stress disorders. Therefore, the study of learning and memory is very important for the understanding of the behaviors of human being. In this area of study, there are many learning paradigms that have been developed such as fear conditioning, maze learning, vestibulo-ocular reflex. Each has been the major learning paradigm and has great benefit in its own arena. However, one of the learning paradigms that has an extensive understanding in both neurobiological and behavioral science and has a promising potential for clinical applications is eyeblink conditioning.[2]

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Memory system

Before we delve into the details, the system of memory will be introduced briefly. The memory can be classified into two major forms: declarative (explicit) memory and non-declarative (implicit) memory. The declarative (explicit) memory is the memory that you can use to describe what and where something is or how and when it happened, and it requires conscious awareness when it is recalled; for example memories about personal experiences, knowledge, fact, or directions. More importantly, this kind of memory depends on the integrity of the hippocampus during the acquisition period. On the other hand, the non-declarative (implicit) memory does not require conscious awareness to recall. Usually, the implicit memory is recalled or is expressed in terms of performance; for example memories of how to perform some activities e.g. driving a car, playing tennis. This kind of memory does not require an intact hippocampus. The non-declarative memory is a collective term of many memory abilities, and it is comprised of skills and habits, the phenomenon of priming, nonassociative forms of memory like sensitization and habituation, and simple forms of classical conditioning including emotional conditioning (e.g. fear conditioning) and somatic conditioning (e.g. delay eyeblink conditioning). Each kind of memories requires diverse brain areas during the memory processes.[1]

Eyeblink conditioning paradigm

Eyeblink conditioning learning paradigm initially has performed in rabbits,[3] and then progressed to include rats[4] and mice.[5] Eyeblink conditioning has also been done in human for a long time and has become more active nowadays.[2,6] Eyeblink conditioning is a form of Pavlovian conditioning in which the training trial consists of a paired presentation of two stimuli – a neutral conditioned stimulus (CS) and an eyeblink-eliciting unconditioned stimulus (US) where the CS precedes the US. During the learning trials, animals or subjects learn about the relationship between

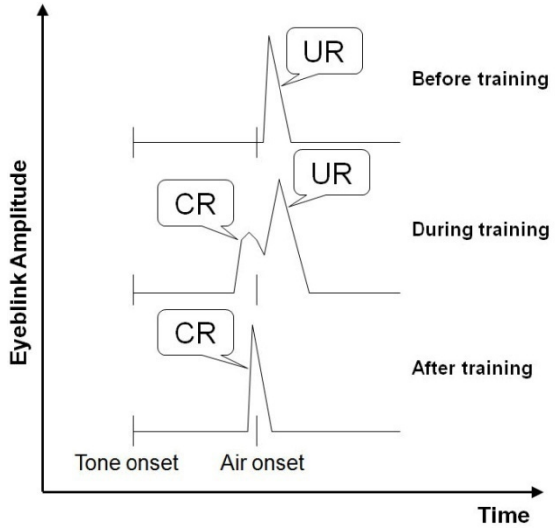

Figure 1 Eyeblink response: comparing eyeblink amplitude before, during and after training.

two stimuli. The conditioned stimulus (CS) typically is a neutral stimulus, which elicits no responses. The eyeblink-eliciting unconditioned stimulus (US), on the other hand, is a strong and significant stimulus that produces a reflexive response.

In eyeblink conditioning paradigm,[7] the CS is a tone or light whereas the US is an airpuff pumped directly into the eyes or a periorbital electrical shock. The eyeblink conditioning paradigm varies into many procedures depending on the durations of and the timing relationship between the two stimuli. Although each procedure has different neural circuitry responsible for learning and memory processes, the logic behind the learning is the same. When the paired presentation of stimuli is initially given to animals, the animals do not respond to the CS, but instead respond to the US as a reflex by blinking the eyelid and moving the nictitating membrane backward. The nictitating membrane is a protective membrane covering both eyes of rabbits. The movement of the nictitating membrane and the eyelid, or the electromyogram (EMG) recorded from musculus levator labii superior (MLLS) and musculus orbicularis oculi (MOO) are measured and recorded. The elicited response to an US is called an unconditioned response (UR). With the continuous training over time, the animals learn to respond to the CS, producing the same movement of the eyelid and the nictitating membrane after the onset of the CS, before the onset of the US, to protect the eye from receiving an airpuff or an electrical shock. This later response is called a conditioned response (CR) because it is elicited by the CS, see Figure 1. The CR shares many characteristics with the UR but it is typically smaller than the UR, has a faster onset, and occurs after the CS presentation before the US onset. The peak of the CR usually falls around the onset of the US presumably in order to maximize the eye protection (8). In general, during the training period, the animals learn the temporal relationship between the CS and the US, and use the CS to predict the onset of the US, and then accordingly produce well-time adaptive conditioned responses (CR) to the CS.

As mentioned before, the variants of the paradigm depend on the durations of and the relationships between the stimuli. The two procedures that have been employed in many experiments are delay and trace eyeblink conditioning procedures. The major difference between the two procedures is the stimulus free gap between the CS and the US. This gap surprisingly affects not only the way that the brain learns these two tasks, but also the contribution of each brain area during the memory processes.

Delay *vs* trace eyeblink conditioning procedures

Procedure

In both procedures, the CS precedes the US; however, as mentioned, the major difference between the delay and trace eyeblink conditioning procedures is the silent gap between the CS and the US. The essential components of delay and trace eyeblink conditioning procedures are shown in Figure 2. The example of delay eyeblink conditioning procedure is 250-ms standard delay conditioning which is used in mouse and rabbit experiments.[9,10] In this procedure, the CS lasts for 350 ms from t = 0 ms to t = 350 ms, and the US onset is at t = 250 ms with a duration of 100 ms. Both stimuli are overlapped for 100 ms and co-terminated at the end of the trial. The term 250-ms signifies the delay between the CS onset and the US onset; this delay is also called "inter-stimulus interval". Thus, in this procedure, the relationship between the two stimuli is apparent because they are physically associated through the overlapping period of both stimuli. Trace eyeblink conditioning procedure, on the other hand, has a silent gap between two stimuli, resulting in the involvement of the higher brain areas that are required during the trace eyeblink conditioning training, not during the delay conditioning training. Those brain areas are such as the hippocampus and the forebrain. The lengths of the gap that determine the higher brain involvement are also critical and vary among different mammalian species. That critical length is 250 ms in mice[11] and rats,[12] over 300 ms in rabbits[13] and 1000 ms in humans.[14,15] The example of trace eyeblink conditioning procedure used in rabbit experiments is called "500-ms

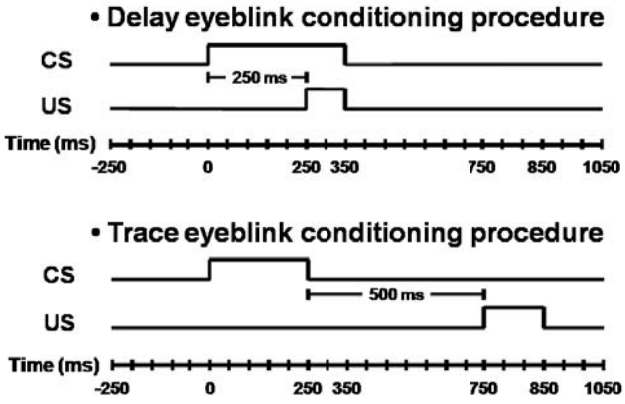

Figure 2 Delay and trace eyeblink conditioning procedures in rabbits.

trace conditioning", which is derived from the 500-ms gap interval.[16] The procedure starts with a 250-ms of CS, then followed by a 500-ms of a stimulus free gap, and ends with a 100 ms of US. To learn the trace eyeblink conditioning procedure, the brains have to overcome the gap in order to make the association between both stimuli.

Delay eyeblink conditioning as a model of non-declarative memory

In delay conditioning in which there is no stimulus-free interval between the stimuli, the brain areas that are necessary for the acquisition and retention of the memory are confined only to the brain stem nuclei and the cerebellum.[17] Decerebrated animals that were transected above the red nucleus still showed significant levels of learning in the delay conditioning training.[18] Along with the results of other studies that lesioned other nuclei in the brain stem and the cerebellum, the evidence revealed that the brain stem and the cerebellar circuit are necessary and sufficient to acquire and retain the temporal relationship of two stimuli that are temporally overlapped, and to use the association for the expression of the learned adaptive behavioral response.[17,19] Higher brain areas such as the prefrontal cortex, the motor cortex, and the hippocampus showed activity-related changes during delay eyeblink conditioning training although they did not play essential roles during the acquisition and retention of the memory. The lesion made in the hippocampus did not impair the acquisition of the delay eyeblink conditioning memory,[20] causing the delay eyeblink conditioning memory a hippocampal independent form of memory. Moreover, the availability of conscious awareness, which depends on the higher brain functions, of the CS-US contingency did not affect the ability of subjects to learn delay eyeblink conditioning.[14] These data suggest that delay eyeblink conditioning can be considered as a learning model of non-declarative memory.[21] However, the higher brain areas might help in the facilitation of the memory acquisition.[22] For the cerebellum, it is widely accepted that the deep cerebellar interpositus nucleus is essential for the acquisition and retention of delay eyeblink conditioning and the cerebellar cortex plays a facilitory role in acquiring the normal CR in terms of the rate of acquisition and the timing of appropriately timed response.[10,19,23]

Trace eyeblink conditioning as a model of declarative memory

Trace eyeblink conditioning, on the other hand, requires higher brain areas during the learning and memory processes. The presence of the gap in trace eyeblink conditioning is surprisingly very significant, and determines which brain areas are necessary in the acquisition, retention, and consolidation processes of the memory. One of the essential areas involved in the acquisition and short-term retention of the memory is the hippocampus, leading to the consideration that the trace eyeblink conditioning memory is a hippocampus-dependent form of memory.[24-26] Moreover, conscious awareness plays a significant role in the acquisition of the memory. Clark and Squire showed that subjects who were aware of the contingency of CS and US learned better in trace eyeblink conditioning. This procedure thus is considered an experimentally controllable model of declarative memory in which the declarative knowledge of the stimulus contingencies are acquired and consolidated over time.[14,21] Other areas such as the prefrontal cortex, the anterior cingulated gyrus, and the splenial cortex showed the involvement in trace eyeblink conditioning as well. It has been proposed that the forebrain circuitry plays a major role in bridging the temporal gap in trace conditioning through pontine-cerebellar nuclear connections.[22,27] This connection can then bypass the cerebellar cortex as it appears that the cerebellar cortex play much less role in trace than delay eyeblink conditioning.[8]

The detailed circuitry responsible for trace eyeblink conditioning has been investigated systematically and extensively in the recent years. The cerebellar circuitry seems to be involved in trace eyeblink conditioning as it is in delay eyeblink conditioning. The deep cerebellar interpositus nuclei have been shown to be crucial for the acquisition, and the short- and long-term retentions of the trace conditioning memory.[16,28] The cerebellar cortex, on the other hand, seems to play differential roles between delay and trace eyeblink conditioning.[28] Reports from mouse and rat studies demonstrated that functional disruptions of the cerebellar cortex impaired delay, but not trace, eyeblink conditioning.[9,29-32] The effect of cerebellar cortex lesions on human patients in eyeblink conditioning training pointed toward the same conclusions as those found in animal studies. In patients who had cerebellar cortex lesions, delay eyeblink conditioning was more strongly impacted than was trace eyeblink conditioning training.[6] The patterns of activation in the cerebellum were investigated using a metabolic mapping technique.[33] The authors demonstrated that there was more widespread activation in the cerebellar cortex in the delay eyeblink conditioning group than in the trace conditioning or the unpaired groups, and different regions of the deep cerebellar interpositus nuclei were activated in each training procedure. The anterior interpositus nucleus was activated more in the delay conditioning group than in the unpaired group whereas the posterior interpositus nucleus was activated more in the trace conditioning groups than in the delay conditioning and the unpaired groups. The evidence indicating less involvement of the cerebellar cortex in trace than in delay conditioning suggests that the cerebellar cortex plays a less significant role in trace eyeblink conditioning than in delay conditioning. Moreover, it seems that different areas in interpositus nuclei are recruited during each paradigm, suggesting a complicated interaction within the cerebellar structures and also between the cerebellum and other brain areas.[27] Woodruff-Pak and Disterhoft (2008) concluded that temporal gaps in trace eyeblink conditioning training were responsible for forebrain dependency and for the activation of different cerebellar circuits during the memorization of conditioned responses.[8]

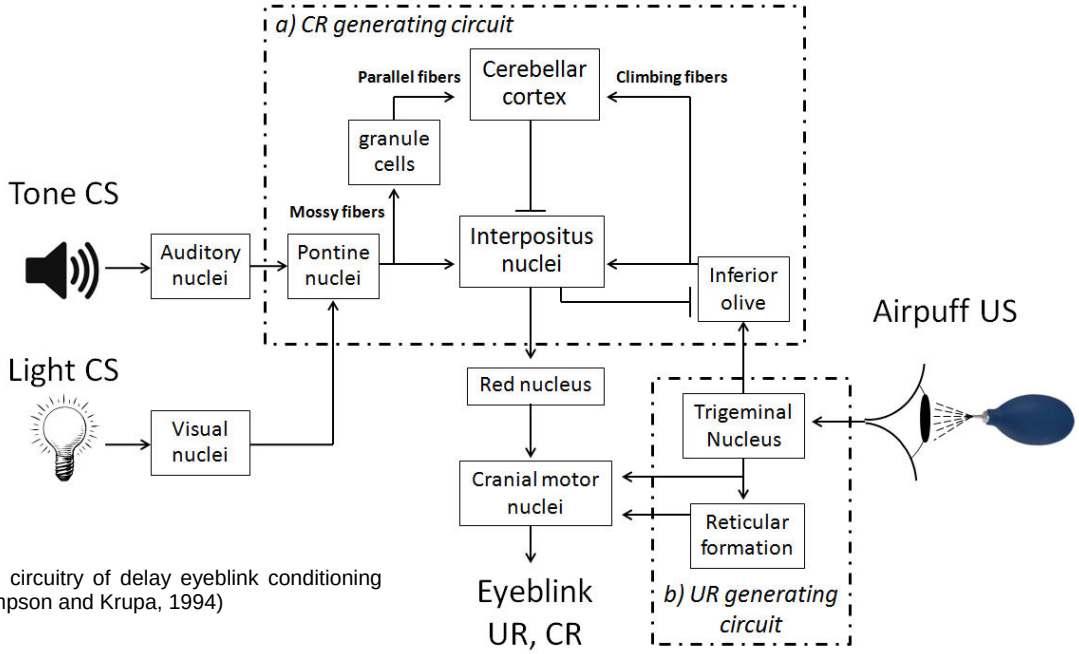

Figure 3 Essential circuitry of delay eyeblink conditioning (Modified from Thompson and Krupa, 1994)

Circuitry of delay eyeblink conditioning

The circuitry of the eyeblink conditioning procedure has been investigated extensively for several decades. Because of the well-organized neuronal arrangement in the cerebellum, most of the circuitry essential for delay eyeblink conditioning has been determined.[19] Figure 3 shows the essential circuitry of delay eyeblink conditioning, which localizes with in the brain stem and the cerebellum.

US pathway

The US pathway begins at the trigeminal sensory neurons receiving the sensory signals from the eye that is trained by either an airpuff or a periorbital shock as a US, and the unconditioned response (UR) is produced through the reflex pathway as shown in Figure 3b. The UR occurs after the onset of US, and it is not adaptive, but rather reflexive in response to the US. The sensory neurons convey the signals via a direct projection to cranial motor nuclei which are the accessory abducens and abducens nuclei, and the facial nucleus,[34,35] and an indirect projection via the brain stem reticular formation to the facial nucleus.[36,37] These cranial motor nuclei are on the same side of the trained eye. In the adaptive pathway, which is involved in the production of the conditioned response (CR) as shown in Figure 3a, the trigeminal sensory neurons ipsilateral to the trained eye send the efferent axons across the midline and form synapses with the inferior olivary neurons particularly in the dorsal accessory olive. The inferior olivary neurons then convey the airpuff signals through the climbing fibers. The climbing fibers cross the midline again and form excitatory synapses onto the proximal dendrites of the cerebellar Purkinje cells ipsilateral to the trained eye.[19] Each Purkinje cell forms synapses with only a single climbing fiber, producing the most powerful excitatory synapse in the nervous system.[38] The action potentials from the climbing fibers produce the complex spikes in the Purkinje cells.

CS pathway

The most common sensory modality for being used as a CS in eyeblink conditioning study has been sound.[22] However, other sensory modalities such as light and somatosensory stimulation can also act as a CS. In general, the CS signals are conveyed through the pontine nuclei to be later processed in the cerebellar circuit.[27] The pontine nuclei receive signals from many sensory cortices including auditory, visual, somatosensory cortices, as well as from prefrontal areas.[39-43] Although showing some overlap, the terminations of these projections are localized in specific regions of the pons, and appropriate lesions of the pontine nuclei could selectively impair the CR performance elicited by a particular type of CS.[19] For example, the inactivation of the medial pontine nuclei severely impaired the retention of CR performance elicited by the light CS, but not the tone CS, whereas the inactivation of the lateral pontine nuclei had a greater effect on the tone CS elicited CR performance.[19,44,45] In case of a tone CS, the signal is initially processed by the auditory nuclei in the brain stem. The auditory nuclei on the same side of the trained eye send the efferent axons across the midline and the axons form excitatory synapses with the lateral pontine nuclei contralateral to the trained eye.[19] On the other hand, the visual nuclei – the lateral geniculate nucleus (LGN) and nucleus of the optic tract – contralateral to the trained eye receives the visual CS input from the side of the trained eye. The efferent fibers from the visual nuclei then project ipsilaterally to the medial pontine nuclei contralateral to the trained eye.[44] Recently, the activation of somatosensory modality has been employed as a CS by the use of the mystacial vibrissae stimulation.[46,47] The vibrissae stimulation CS signals reach the cerebellum also via the pontine nuclei contralateral to the trained eye.[22,27,48] However, the detailed circuitry carrying this CS signals is still needed some more investigation. After receiving the CS signals,

the pontine nuclei, send the mossy fibers across the midline into the cerebellum ipsilateral to the trained eye. The mossy fibers end in contact with the dendrites of the granule cells whose axons – parallel fibers – form the excitatory synapses with the distal dendrites of Purkinje cells. The stimulation of the parallel fibers produces the simple spikes in the Purkinje cells. Both pathways – climbing and mossy fibers – also send collateral branches to form the excitatory synapses with the deep cerebellar interpositus nuclei.

Mechanisms of memory storage in the cerebellum

In the quest for the mechanisms underlying learning and memory, two major processes which are long-term potentiation (LTP) and long-term depression (LTD) are believed to play major roles in the information storage in learning and memory. LTP is a phenomenon defined as a persistent enhancement in synaptic strength (amplitude EPSP) following high-frequency stimulation of the presynaptic afferent (repeated learning) and it was discovered in the rabbit hippocampus by Terje Lomo in 1996.[49] LTD, on the other hand, is a long-lasting decrease of synaptic efficacy following low-frequency stimulation of the presynaptic afferent.[50]

In the cerebellum, after receiving both CS and US signals, Purkinje cells in the HVI cerebellar cortex ipsilateral to the trained eye send inhibitory axons to the deep cerebellar interpositus nuclei. This inhibitory axons are the only output signal of the cerebellar cortex. Earlier, many investigations focusing on the synaptic responses at the Purkinje cell-climbing fiber synapses and at the Purkinje cell-parallel fiber synapses were performed by Rawson and Tilokskulchai.[51-53] In 1982, Ito discovered the long-term depression (LTD) at the parallel fiber-Purkinje cell synapses in the cerebellar cortex.[54] Building on the prior model of Marr (1969) and Albus (1971), this mechanism has been hypothesized by Ito (1984) to be the main learning mechanism of the cerebellum although there are other mechanisms at other synaptic sites that might play a role in the cerebellar learning as well.[55-57] The theory states that when the parallel fibers are stimulated and followed by the activation of the climbing fibers in the appropriate time period for an extended time during the training, the co-activation of these two Purkinje cell inputs induces a long-term input-specific depression of synaptic strength at the relevant parallel fiber-Purkinje cell synapses.[58,59] With this depression, the Purkinje cells decrease the response to the CS signals, resulting in the reduction of the inhibitory efferent signals onto the interpositus nuclei. The reduction of the inhibitory signals allows the interpositus neurons to be more excitable and to increase its firing rates in response to the CS signals coming from the mossy fiber collaterals. The increased output signals of the interpositus neurons, in turn, activate neurons in the Red nucleus. The Red nucleus then relays the signals to motor nuclei of the accessory sixth and seventh nerves responsible for the movement of the nictitating membrane and the external eyelid closure, respectively, generating the conditioned response (CR).[17,19] Through the learning process, the inhibitory signals onto the interpositus neurons are reduced at the appropriate time when the interpositus neurons are stimulated by the excitatory CS signals via mossy fibers from the pontine nuclei, resulting in the increase of firing rates of the interpositus neurons after the CS onset, but before the US onset. This well-time adaptive output of the interpositus nucleus stimulates the cranial motor nuclei via the Red nucleus, generating the appropriate CRs.[17,19,27] The neuronal activities at different areas in the circuit during both resting and learning states are demonstrated in Figure 4. The increased activities of the interpositus neurons correspond with the behavior of eyeblink conditioned responses; therefore, the activity of the interpositus neurons was considered as the model of the eyeblink CR.[23] The CR is an adaptive response because animals learn to adapt and to produce the response earlier that the US onset in order to prevent the eye from the noxious quality of the US, and the peak of the CR usually coincides with the US onset in order to fully protect the eye from the airpuff US. The influence of LTD in eyeblink conditioning learning was tested by mGluR1 mutant mice that showed the deficit in LTD. The LTD deficit mutant mice demonstrated the impairment in the eyeblink conditioning learning.[5]

As the LTD mechanism works at the parallel fiber-Purkinje cell synapses by producing the long-lasting depression of the synaptic activities, long-term potentiation (LTP), on the other hand, is a mechanism that causes an increase in synaptic activities at mossy fiber-interpositus neuron synapses (60), and the increase in synaptic activity

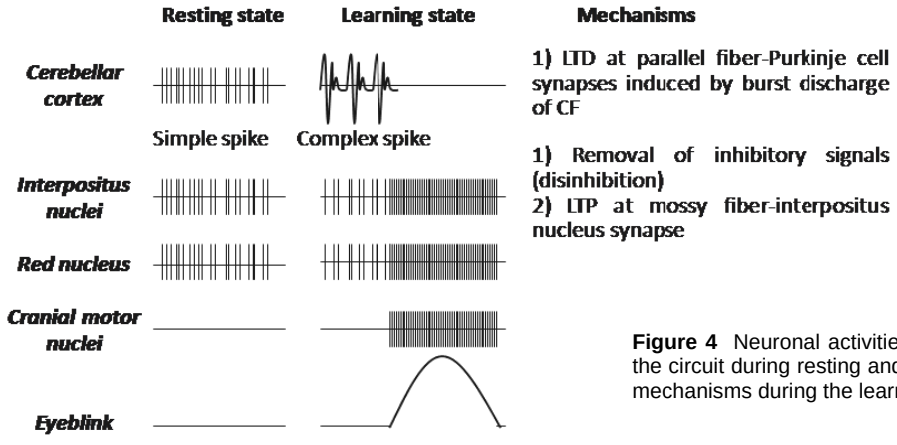

Figure 4 Neuronal activities at different synaptic sites in the circuit during resting and learning states. The possible mechanisms during the learning state are included.

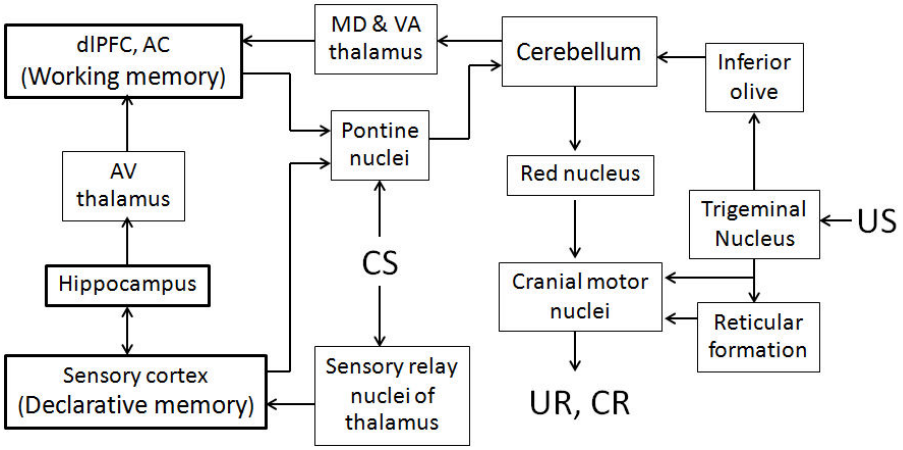

Figure 5 The circuitry of trace eyeblink conditioning. AV thalamus, anteroventral thalamus; MD thalamus, dorsomedial thalamus; VA thalamus, ventral anterior thalamus; dlPFC, dorsolateral prefrontal cortex; AC: anterior cingulate gyrus. (Modified from Weiss & Disterhoft, 2011)

due to the LTP is important of the learning of eyeblink conditioning.[61] Anatomical evidence looking for the changes of ultrastructures in the synapses has shown that there was an significant increase in the length of excitatory synapses at the interpositus neurons, supporting the influence of LTP at the interpositus nucleus synapses after eyeblink conditioning training.[62] Therefore, when the LTD reduces the parallel fiber-Purkinje cell synaptic activities, removing the interpositus neurons from inhibitory efferent signals of the cerebellar cortex, the LTP, on the other hand, enhances the synaptic activities of the interpositus neurons, allowing the neurons to be efficiently stimulated by the mossy fibers. The efferent signals of the interpositus neurons then transmit to the Red nucleus and then to the cranial motor nuclei, producing the conditioned response.[19] The cerebellar circuitry that is required for the generation of conditioned responses is shown in Figure 3a. The interpositus nuclei have been shown to be critical for the acquisition, short- and long-term retention of both delay and trace eyeblink conditioning.[16,28] Moreover, several lines of evidence supported the role of the interpositus nuclei as a short- and long-term storage site of both delay and trace eyeblink conditioning memory.[16,19,62-65]

Circuitry of trace eyeblink conditioning

In delay eyeblink conditioning, only the cerebellum and the brain stem are essential and are required for the acquisition and retention of the memory. In trace eyeblink conditioning as a model of declarative memory, the brain stem and the cerebellum are still required for the CR generation, especially for the cerebellum because it is the site where the basic association of CS and US is formed and permanently stored.[16,65] However, many other higher brain areas are critically involved in the memory processes of trace conditioning. The higher brain areas such as the hippocampus, the prefrontal cortex, the anterior cingulate gyrus, the thalamus, the striatum and the sensory cortex showed the involvement in eyeblink conditioning and played different roles at each stage of the memory processes.[19,22] Clark et al. (2011) hypothesized that it may be the declarative knowledge of the stimulus contingencies that is stored and is consolidated in the higher brain areas during the memory processes.[21] Nonetheless, the true mechanisms of the higher brain involvement in trace eyeblink conditioning are the challenging tasks. Weiss and Disterhoft (1996, 2011) proposed the interaction of the higher brain areas with the cerebellum as shown in Figure 5, and the actions of the forebrain and the hippocampus are to potentiate the effect of the CS signals at the pontine nuclei through the cortico-pontine projections. The result of the potentiation will effectively expand the duration of the CS signals such that the CS signals overlap with the US signals even though physically there are no overlap of the CS and the US due to the stimulus free gap of trace eyeblink conditioning.[27,66] The involvement of some major brain areas during the trace eyeblink conditioning is reviewed here.

Hippocampus

For many years, the hippocampus has been the center of intensive studies for learning and memory. Its anatomical connections are very intricate and complex, and it plays a significant role in the acquisition of declarative memory. Although the contribution of the hippocampus in eyeblink conditioning has been intensively investigated, it is a challenging task to define the true nature of hippocampal-cerebellar communications since there is no direct connection between these two areas.[16] The hippocampus receives inputs from and sends outputs to a wide range of neocortical areas such as the posterior parietal cortex (area 7), the inferior temporal association area (area 20, 37), and the retrosplenial cortex and subcortical areas such as the basal forebrain and hypothalamic nuclei. The neocortical signals reach the hippocampus through the entorhinal cortex, whereas the subcortical signals mainly reach the hippocampus through the fimbria-fornix pathway.[67,68] However, it appears that there is no direct connection between the hippocampus and the cerebellum. Weiss and Disterhoft (1996, 2011) proposed that these two regions could be functionally connected via the frontal cortex and the pons. The possible indirect connections consist of the pathway from the hippocampus to the retrosplenial cortex which conveys through the entorhinal cortex and the pathway from the hippocampus to the mamillary nucleus and thalamic nuclei which passes through the fimbria-fornix pathways. Both pathways eventually connect with the cerebellum through the pontine nuclei, which relay and send the signals via mossy fibers into the cerebellar

circuit.[27,66]

The influence of the hippocampus on eyeblink conditioning has been demonstrated in many studies. Hippocampal lesions prevented the acquisition of trace eyeblink conditioned response,[12] and abolished a recently learned trace conditioned responses.[13,28,69] However, this effect of the hippocampal lesions was limited only to the recently acquired trace eyeblink conditioned responses.[24-26] The electrophysiology studies showed an increase in the firing rate of CA1 of the hippocampus during the acquisition of eyeblink conditioning both in delay eyeblink procedure[70] and in trace eyeblink procedure.[71,72] This conditioned hippocampal pyramidal neuron activity was then abolished or prevented by lesioning the deep cerebellar interpositus nucleus.[73,74] These bidirectional lesion effects of the hippocampus and the cerebellum are evidence that these two regions interact during the learning process of eyeblink conditioning, and this interaction is especially required for mediating trace eyeblink conditioning. The increase of hippocampal activities has very specific characteristics. The activities increase very early during the training in the US period. The increased activities then shift forward into the CS period about the time point when conditioned responses appear. The increased activities eventually decline with the continued training. Generally, the increase of the activities in a given hippocampal neuron represents only some limited time period of conditioned responses during the trial. The summation of such increase models the learned conditioned responses, suggesting the distributive behavioral representation over space and time in the hippocampus.[19] Although the hippocampus shows synaptic changes during the acquisition of the memory, it is not critical for the acquisition of delay eyeblink conditioning. The hippocampus, instead, seems to play a modulatory role because disruption of the septo-hippocampal system retarded the acquisition of delay eyeblink conditioning,[75] but lesions of the hippocampus itself did not affect the acquisition of the delay eyeblink conditioning.[20] When the interstimulus interval (ISI) between the CS onset and the US onset is extended for 1400 ms to match in difficulty to a short trace procedure, the task becomes a very-long-delay eyeblink conditioning. Hippocampal lesions in rats impair the acquisition of the memory, but the animals can learn significant amount of memory with extensive training.[76] It indicates that the hippocampus is important, but not essential, in acquiring delay conditioning, especially when the ISI is very long. In trace eyeblink conditioning if the trace interval exceeds some species-specific length (300 ms in rabbit), the hippocampus becomes essential in acquisition and short-term retention of trace eyeblink conditioning.[24] When the critical trace time interval in trace conditioning is exceeded this critical length, the cerebellum alone can no longer form an association between the CS and the US. Therefore, the hippocampus may be recruited in order to help in forming the association between two stimuli.[32] These behavioral data concur with the previous physiological data, emphasizing the involvement of the hippocampus in both delay and trace eyeblink conditioning.

Prefrontal cortex

In primates, the dorsolateral prefrontal cortex (dlPFC) is a plausible area to mediate the trace interval during trace eyeblink conditioning because of its involvement in working memory processes.[22] Studies of dlPFC areas showed neurons that had sustained activity during the delay period in delayed matching to sample tasks.[77-79] In anatomical studies, dlPFC was discovered to receive input from the cerebellum via the mediodorsal (MD) thalamus, and lesions of the MD thalamus impaired trace eyeblink conditioning learning, but not delay conditioning learning.[80] The rabbit homologue of the primate dlPFC was identified by injection of HRP or WGA-HRP into the MD thalamus, and it was shown to be located at the midline regions of the prefrontal cortex (prelimbic area).[81] The medial prefrontal cortex (mPFC) was shown to be involved in trace eyeblink conditioning. Lesions of mPFC lesions retarded trace, but not delay, eyeblink conditioning learning in rabbits.[82] When the mPFC lesion was made one day after the learning, the animals exhibited a slight decline in performance compared to the severely impaired performance in the hippocampal lesion group and the cerebellar lesion group. When the lesions were made one month after the learning, only the mPFC and cerebellar lesion groups showed a deficit in retention whereas the hippocampal lesion group showed no deficit.[25] This result concurs with the study by Oswald et al. (2008) in which the mPFC lesion made one week after learning produced deficits in the expression of trace eyeblink conditioning.[83] The study of the single-unit activity of neurons in the mPFC during eyeblink conditinonig showed different subpopulations of neurons exhibiting both mono and biphasic responses to the simuli.[84] These studies demonstrated that trace eyeblink conditioning essentially required the hippocampus and to a lesser degree the mPFC during the acquisition phase. During the consolidation period, the memory was reorganized and the mPFC critical for the long-term retention of the memory, suggesting the critical involvement of the mPFC in trace eyeblink conditioning.[21]

Caudal anterior cingulate gyrus (cAC)

Studies have shown that the cAC to be involved in trace eyeblink conditioned learning. Lesions in the cAC prevented the acquisition of a trace conditioned response.[85,86] The in-vivo electrophsiological study recorded from this area showed learning-related changes in neuronal activity very early prior to the exhibition of a conditioned response (during the first 10 CS-US pairings), even earlier than the onset of neuronal changes during the acquisition of trace conditioning in other regions such as the hippocampus.[87] The CS elicited an increase in the neuronal firing rate throughout the paired training sessions, with the greatest increase during the first two training sessions, whereas the CS-elicited response declined rapidly to baseline levels in the unpaired training sessions. The US-elicited response in the paired training trials was also greater than the response in the unpaired trials. With these characteristics, Weiss et al. (2006) proposed an attentional role for cAC during conditioning learning.[22] This notion

was supported by the later study on the anatomical connections of cAC with neuronal tract tracing techniques.[43] The cAC was shown to be anatomically connected with the basal forebrain cholinergic system: the horizontal limb of the diagonal band of Broca (HDB) and the nucleus basalis magnocellularis (NBM). The basal forebrain cholinergic system is involved in the control of attention and also eyeblink conditioning learning.[88,89] Moreover, the cAC was demonstrated to have projections to lateral pontine nuclei,[43] providing possible direct communications with the cerebellar circuit via the pontine nuclei.

Sensory cortex

The ideas that primary sensory cortex takes part in the storage of long-term memory have been around for a long time.[22] In eyeblink conditioning, the multiple unit recording from the somatosensory cortex showed the increase in firing rates corresponding to the time-course of the conditioned response during delay eyeblink conditioning training with tone CS and airpuff US.[90] Disterhoft and colleagues have investigated the involvement of sensory cortex in eyeblink conditioning by using the stimulation of the mystacial vibrissae. Because of the well defined somatotopic patterns in the sensory cortex of the neurons sensitive to whisker stimulations in rabbits, the changes of activities in the sensory cortex during the eyeblink conditioning training were assessed conveniently.[46,47] Tactile information from each whisker travels to cortex in a one-to-one orientation from the trigeminal nerve to medullary barrelets, to thalamic barreloids, and eventually to somatosensory barrel cortex.[91,92] The involvement of the sensory cortex in trace eyeblink conditioning has been investigated by Galvez et al. (2006). The expansion of sensory cortex after trace eyeblink conditioning were demonstrated by cytochrome-oxidase-staining.[47] The sensory cortex is critical for the acquisition and the retention of trace eyeblink conditioning. Galvez et al. (2007) found that pretraining lesions of the sensory cortex sensitive to whisker stimulation prevented the acquisition of the trace eyeblink conditioning memory whereas the lesions made after 10 days of acquisition period significantly reduced the expression of previously learned conditioned responses, suggesting that sensory cortex was the site for long-term storage of whisker trace eyeblink conditioning.[93] Weiss et al. (2011) proposed that sensory cortex is the site that represent the declarative memory about the behavioral significance of the CS.[27]

Advantages and Applications

Eyeblink conditioning procedures play a significant role in learning and memory research because of its advantages in many aspects. Some of the important aspects are that the procedures have the excellent controlled environment, and provide a wealth of data available for the comprehension of learning and memory mechanisms.[65,66] For example, UR offers an independent measure of performance which can be compare with the effects of other parameters acting on CR, and the behavioral CR is robust, reliable, and discrete, and the exact amplitude-time course of the response is measurable.[17] Moreover, it is powerful and useful because of its simplicity, reliability and repeatability, especially in rabbits, as their learning develops very uniformly and depends directly on the amount of training trials the animal receives. Owing to the extensive knowledge of the neurobiology and psychobehavior of eyeblink conditioning especially the delay procedure, the procedures can be used to thoroughly investigate the mechanisms of memory formation during all periods – acquisition, consolidation, and retention – and the mechanisms of memory extinction (forgetting). Trace eyeblink conditioning as a model of declarative memory is a powerful tool for studying the interaction of multiple brain memory system, the involvement of higher cognitive functions e.g. conscious awareness, or system consolidation – a characteristic of declarative memory.[21] With only small adjustments in the configuration of the CS and the US, eyeblink conditioning procedures can be used to examine the mechanisms of both declarative and non-declarative memories and the interaction between both kinds of the memories as well. Nowadays with modern molecular techniques, mouse eyeblink conditioning increasingly becomes popular and important because of its subcellular specificity that can target small populations of neurons or only some molecules involved in memory mechanisms as demonstrated in the studies mentioned earlier. Therefore, molecular techniques significantly promote in-depth examination of memory mechanisms. Moreover, many genetically engineered mice were linked with many diseases. These mice were used as the models in order to examine the pathophysiology of the diseases that affect the learning and memory capability of both declarative and non-declarative memories such as Alzheimer's disease.[94-96]

Not only has eyeblink conditioning been employed in animal studies, but also used in human studies as well. The history of human eyeblink conditioning has begun over hundred years ago.[2] Recently, human eyeblink conditioning has been under active investigations for both basic mechanisms of learning and the use of this learning paradigm in memory afflicted disorders,[15,97-99] and the evidence from the human studies corresponds with the data from animal studies that has been review previously. For example, by using the fMRI, Cheng et al. (2008) found that the strong and comparable activities in the cerebellum of subjects during both delay and trace eyeblink conditioning training, whereas the greater activities in the hippocampus during trace than during delay eyeblink conditioning training.[98] Therefore, human eyeblink conditioning would be a potential tool for investigating the memory disorders in the future. For example, the deficit in the expression of type-I metabotropic glutamate receptors (mGlu I) in the cerebellar Purkinje cell has been found in multiple sclerosis patients.[100] Delay eyeblink conditioning was used for the investigation of the cerebellar impairment in multiple sclerosis patients.[101] Also the impairments in eyeblink conditioning learning were also being examined in many diseases e.g. schizophrenia, attention deficit hyperactive disorder.[102-104] Furthermore, not only can the eyeblink

conditioning procedures help to enhance our understanding of the diseases, but they could also be applied to assess the potential of new treatments. For example, Alzheimer's patients showed the deficit in declarative memory due to the impairment in the hippocampus during the early stage of the disease.[105] A new approach for the treatment of Alzheimer's disease conducted by Wang et al. (2010) was tested. In this experiment, the benefit of allopregnanolone, a metabolite of progesterone, was examined in 3xTgAD mice, an animal model of Alzheimer's disease, and it was found that allopregnanolone induced the survival of neural progenitor cells in the hippocampus, and increased memory performances of the mice in trace eyeblink conditioning procedures.[106]

Conclusion

Eyeblink conditioning paradigm can be learning models of both declarative and non-declarative memories. Delay conditioning procedure as the model of non-declarative memory depends only on the cerebellum and the brain stem nuclei during the acquisition and the retention periods. Trace conditioning procedure, on the other hand, as the model of declarative memory requires the cerebellum, the brain stem nuclei, and other higher brain areas during the memory processes. The cerebellum is always required for both delay and trace eyeblink conditioning because it is the location where the basic association of CS and US is formed and stored. Higher brain areas such as the hippocampus showed activity related changes during delay eyeblink conditioning, but they are not necessary for the acquisition and the retention of the memory. In turn, higher brain areas play very significant and differential roles during each stage of the memory processes in trace eyeblink conditioning. Although, eyeblink conditioning paradigm has been studies exhaustively and extensively, there are still lots to be learned from this potential learning paradigm such as the basic knowledge of memory mechanisms. By utilizing the eyeblink conditioning procedures, animal models and patients were investigated for the pathophysiology of the memory afflicted diseases and the new therapeutic approaches can be tested. For example, some alternative substances such as some potential herbal medicines can be tested for their therapeutic effects. Moreover, the advancement in molecular techniques and the advent of new techniques such as optogenetics will drastically increase our understanding of the memory processes and might be able to shed some light to the cure of the memory disorders such as Alzheimer's disease.

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Conflict of Interest

None to declare.

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