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## Sensory system contributions to the development of trace and delay eyeblink conditioning

Mary Goldsberry-Troyer  
*University of Iowa*

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SENSORY SYSTEM CONTRIBUTIONS TO THE DEVELOPMENT OF TRACE AND  
DELAY EYEBLINK CONDITIONING

by

Mary Goldsberry-Troyer

A thesis submitted in partial fulfillment  
of the requirements for the Doctor of Philosophy degree  
in Psychology  
in the Graduate College of  
The University of Iowa

May 2016

Thesis Supervisor: Professor John H. Freeman

Graduate College  
The University of Iowa  
Iowa City, Iowa

CERTIFICATE OF APPROVAL

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PH.D. THESIS

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This is to certify that the Ph.D. thesis of

Mary Goldsberry-Troyer

has been approved by the Examining Committee for  
the thesis requirement for the Doctor of Philosophy degree  
in Psychology at the May 2016 graduation.

Thesis Committee:

---

John H. Freeman, Thesis Supervisor

---

Mark Blumberg

---

Alan K. Johnson

---

Amy Poremba

---

Jason Radley

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## ABSTRACT

Research concerning the development of learning and memory suggests that there are multiple memory systems. These systems differ in complexity, underlying neural substrates, and consequently, their developmental emergence. Pavlovian conditioning, and specifically eyeblink conditioning (EBC), allows researchers to investigate both simple and complex forms of learning and memory early in development. Delay EBC, which is considered a relatively simple form of learning, involves the association of a conditioned stimulus (CS) with an unconditioned stimulus (US). Research from our laboratory suggests that the emergence of delay EBC is dependent on the development of sensory input to the pontine nucleus. Trace EBC, a more complex form of learning, involves the association of a CS with a US over a stimulus-free trace interval. Due to its relatively late emergence, the developmental time course of trace EBC has been traditionally regarded as independent of sensory system development. Rather, it is the involvement of late-developing structures such as the hippocampus which is considered the principle limiting factor in the emergence of trace EBC.

The current collection of studies investigates the developmental emergence of delay and trace conditioning. We found that both delay and trace conditioning are facilitated by using an early-developing somatosensory CS. This suggests that the sensory system development plays a role in even late-developing trace EBC. Moreover, hippocampal CA1 neuronal activity shows increased responsiveness in even very young animals when trained with an early-developing somatosensory CS compared to those trained with a tone CS. Combined, these data suggest that both hippocampal and sensory system development may play key roles in the developmental emergence of learning.

## PUBLIC ABSTRACT

Research concerning the development of learning and memory suggests that there are multiple memory systems. These systems differ in complexity, underlying neural substrates, and consequently, their developmental emergence. Simpler forms of learning emerge early in development and are believed to be heavily dependent on sensory system development. More complex forms of learning depend on the circuitry involved in simple forms and additionally require the involvement of late-developing structures such as the hippocampus. Due to their late development, the emergence of complex forms of learning is generally believed to be independent of sensory system development. Indeed, it is this late development of the hippocampus which is currently believed to be the principle limiting factor in the development of complex forms of learning.

The current collection of studies investigates the developmental emergence of both simple and complex forms of learning. We found that using an earlier-developing sensory modality facilitates both simple and complex forms of learning. This would suggest that sensory system development is continuing to play a crucial role, even in late-developing complex forms of learning. Moreover, when we examined the activity of hippocampal neurons during complex learning tasks we found that they were more responsive when using an early-developing sensory modality. This would suggest that hippocampal function is more mature than originally believed. Therefore, both sensory system and hippocampal development are important contributing factors to the developmental emergence of simple and complex forms of learning.

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## INTRODUCTION

Eyeblink conditioning (EBC) is a form of Pavlovian conditioning in which an animal is presented with a conditioned stimulus (CS) that does not elicit a blink followed by an unconditioned stimulus (US) that does elicit a blink. Generally, the CS is a tone or a light and the US is either a puff of air (in rabbits and humans) or a periorbital shock (in rats and rabbits). After repeated pairing of the CS and US, the animal will come to show a conditioned response (CR) to the CS. That is, it will blink to the CS before the US onset, therefore demonstrating an association between the CS and the US.

Generally, when animals are trained in EBC they are trained in what is referred to as “delay” conditioning. In delay conditioning the US onset can either occur at CS offset or immediately before CS offset. In the latter case, the two stimuli co-terminate, thus overlap during the US interval. Alternatively, trace conditioning includes a stimulus-free “trace” interval between CS offset and US onset. Although delay and trace EBC both rely on the cerebellum and related circuitry, trace conditioning additionally requires forebrain structures such as the hippocampus and anterior cingulate cortex (Moyer, Deyo, & Disterhoft, 1990; Weible, McEchron, & Disterhoft, 2000; Weiss, Bouwmeester, Power, & Disterhoft, 1999).

The EBC paradigm is ideal for developmental studies of learning. Because the CR is relatively simple (a blink), late-developing response systems can be completely bypassed. Moreover, there is a vast amount of research concerning the neurobiological circuitry underlying adult EBC, therefore allowing developmental studies to build off of findings from adult animals. Finally, because the acquisition of EBC generally requires several trials, it is an ideal paradigm for electrophysiology studies.

## **Neurobiology of Delay Eyeblink Conditioning in Adults**

The neural circuitry underlying delay EBC in adults is very well established. EBC models propose that the cerebellum is responsible for associating the CS with the US during conditioning (Thompson, 1986). Specifically, the interpositus nucleus (IPN) of the cerebellum receives CS input from the pontine nucleus via mossy fibers and US input from the inferior olive via climbing fibers (Gould, Sears, & Steinmetz, 1993). Because the IPN is the site of CS-US association, lesions and reversible inactivations completely abolish the acquisition of the CR (Lavond, Hembree, & Thompson, 1985). Moreover, when later tested for retention, animals show no evidence of savings, therefore suggesting that the IPN must be online during training in order for conditioning to occur (Krupa & Thompson, 1997).

Stimulation studies further support the role of the IPN as the site of association formation. Acquisition of delay EBC occurs when an inferior olive stimulation US is paired with a tone CS, when a pontine stimulation CS is paired with an air puff US, and even when pontine and inferior olive stimulation serve as CS and US, respectively (Jirenhed, Bengtsson, & Hesslow, 2007; Mauk, Steinmetz, & Thompson, 1986; Steinmetz, Lavond, & Thompson, 1989; Steinmetz, Rosen, Chapman, Lavond, & Thompson, 1986). Moreover, stimulation of the mossy fibers and climbing fibers that project to the cerebellum are sufficient to produce learning (Lavond, Steinmetz, Yokaitis, & Thompson, 1987; Thompson, 1986).

The cerebellar cortex is also involved in delay EBC. However, unlike in the case of the IPN, its role is not entirely clear. Aspiration of the cerebellar cortex results in heavily impaired acquisition and retention of conditioning. However, after extensive training animals are eventually able to show poorly timed short-latency CRs (Lavond & Steinmetz, 1989; McCormick & Thompson, 1984). It is therefore currently hypothesized that the cerebellar cortex

may contribute to CR timing through its inhibitory projection to the IPN (Green & Steinmetz, 2005).

The output of the CR depends on projections from the superior cerebellar peduncle to the red nucleus which sends its output to brain stem motor neurons (McCormick, Guyer, & Thompson, 1982; Rosenfield & Moore, 1983). Reversible inactivation of the red nucleus or superior cerebellar peduncle by muscimol during acquisition is able to temporarily prevent conditioned responding. However, when tested drug-free, animals show high levels of responding, therefore indicating that although the superior cerebellar peduncle and red nucleus are responsible for the expression of the CR, they are not involved in CS-US association formation (Krupa, Thompson, & Thompson, 1993; Krupa & Thompson, 1995).

Prior to arriving at the level of the cerebellum, sensory information from the CS and the US must pass through a number of nuclei. Sensory information concerning the US is received by the trigeminal nucleus, which sends projections to the inferior olive. Lesions of the inferior olive following acquisition of EBC result in an extinction-like decrease in conditioned responding (McCormick, Steinmetz, & Thompson, 1985). However, stimulation of the inferior olive as a US supports conditioned responding (Mauk et al., 1986). US information is then sent along excitatory climbing fibers from the inferior olive to the Purkinje cells of the cerebellar cortex and the deep cerebellar nuclei where it is associated with CS information (Thompson, 1988). Finally, in order to prevent redundant plasticity, the cerebellum has an inhibitory projection to the inferior olive (Lang, 2003). Early in training inferior olive neurons have high rates of responding to the US, however as training continues there is a decrease in US-related inferior olive activity which is not observed in unpaired training (Sears & Steinmetz, 1991). This negative feedback loop is hypothesized to prevent redundant plasticity by maintaining climbing fiber activity in a

state of equilibrium between CS-US pairings (Bengtsson & Hesslow, 2006; Kenyon, Medina, & Mauk, 1998; Thompson, Thompson, Kim, Krupa, & Shinkman, 1998).

Adult studies of EBC have used auditory, visual, and somatosensory stimuli. The majority of research, however, has focused on the auditory pathway. Although there is a direct projection from the cochlear nucleus to the pontine nucleus (Steinmetz et al., 1987), recent work suggests that tone CS information passes through a number of nuclei before reaching the pontine nucleus (Nowak, Kehoe, Macrae, & Gormezano, 1999). Specifically, tone input is sent from the cochlear nucleus to the inferior colliculus (Freeman, Halverson, & Hubbard, 2007) which in turn sends projections to the auditory thalamus (Campolattaro, Halverson, & Freeman, 2007). CS input from the auditory thalamus is then relayed to the lateral pontine nucleus which sends sensory information along mossy fiber projections to the cerebellum (Steinmetz & Sengelaub, 1992). Inactivation studies of the pontine nucleus with cooling, lidocaine, or tetrodotoxin blocks conditioned, but not unconditioned responding, regardless of CS sensory modality (Bao, Chen, & Thompson, 2000; Clark, Gohl, & Lavond, 1997; Knowlton & Thompson, 1988). Conversely, stimulation of the pontine nucleus results in robust levels of conditioned responding (Nowak et al., 1999). Therefore, the pontine nucleus is considered to be the area responsible for CS input to the cerebellum.

### **Neurobiology of Trace Eyeblink Conditioning in Adults**

Whereas the neural circuitry underlying delay EBC in adult animals is very well understood, the circuitry involved in trace EBC has remained somewhat elusive.

As previously mentioned, in delay EBC the cerebellum and related circuitry are sufficient to form the association between the CS and the US (Mauk et al., 1986). However, if the two stimuli are separated by a sufficiently long trace interval, 250 ms in rats and 500 ms in rabbits,

additional structures, such as the hippocampus and anterior cingulate cortex, are required for task acquisition (Kalmbach, Ohyama, Kreider, Riusech, & Mauk, 2009; Kronforst-Collins & Disterhoft, 1998; Weiss, Knuttinen, et al., 1999; Weiss, Kronforst-Collins, & Disterhoft, 1996).

Although both the hippocampus and the anterior cingulate cortex have been demonstrated to be involved in trace EBC, the majority of studies have focused on the role of the hippocampus. Lesions of the hippocampus that occur either prior to or within one week of training result in an inability to learn trace EBC (Solomon, Vander Schaaf, Thompson, & Weisz, 1986; Takehara, Kawahara, & Kirino, 2003). However, lesions that occur one week or more after training have no effect on retrieval. The hypothesized time-dependent role for the hippocampus is further supported by *in vitro* electrophysiology. Pyramidal cell activity in slices of well-trained animals shows increased excitability up to three but not seven days following training (Moyer, Thompson, & Disterhoft, 1996). These data suggest that the hippocampus most likely plays a role in the acquisition and consolidation but not the retrieval of trace EBC.

This hypothesis concerning a time-dependent role for the hippocampus is further supported by *in vivo* recording studies. Neuronal recordings indicate that hippocampal CA1 pyramidal cells show learning-related increases in responsiveness to trial stimuli during acquisition (Green, Arenos, & Dillon, 2006; McEchron & Disterhoft, 1997; Solomon, Vander Schaaf, et al., 1986). These increases in excitation begin even prior to the animal behaviorally demonstrating conditioning acquisition and continue throughout learning. However, once the animal has reached asymptotic levels of responding, pyramidal cell activity begins to decline (McEchron & Disterhoft, 1997). This activity pattern lends further support to the above-mentioned hypothesis that the hippocampus is involved in EBC acquisition and consolidation.



Like the hippocampus, the anterior cingulate cortex is hypothesized to play a time-dependent role in trace EBC. As in the case of the hippocampus, lesions that are made prior to training result in impaired acquisition (Kronforst-Collins & Disterhoft, 1998). However unlike the hippocampus, lesions that are made weeks after the animal has reached asymptotic levels of performance result in impaired retrieval. Interestingly, this impairment is only temporary and animals are able to reacquire the task with continued training (Solomon, Vander Schaaf, et al., 1986; Takehara et al., 2003), therefore demonstrating that although the anterior cingulate is necessary for the initial acquisition, it is not necessary in reacquisition.

Neuronal recordings of anterior cingulate cortex neurons during the acquisition and retrieval of trace EBC support the findings from the above-mentioned lesion studies. Early in training both paired and unpaired animals show an immediate increase in anterior cingulate cortex activity in response to the CS, possibly indicating a role for the anterior cingulate cortex in attentional processes. However, as training continues neuronal excitation to the CS drops off in unpaired training and shifts to the later part of the CS period and trace interval in paired training. Trace interval excitability is then maintained for the remainder of training (Takehara-Nishiuchi & McNaughton, 2008; Weible, Weiss, & Disterhoft, 2003). Importantly, the maintenance of neuronal excitability across the trace interval could play a key role in trace conditioning. The anterior cingulate cortex sends direct and indirect projections to the pontine nucleus and hippocampus (Weible, Weiss, & Disterhoft, 2007). Therefore, maintaining neuronal excitability across the trace interval could result in prolonged activity in the pontine nucleus and hippocampus, therefore bridging the trace interval.

## **Development of Delay Eyeblink Conditioning and Underlying Neurobiology**

Developmental studies of EBC using an auditory or visual CS suggest that delay EBC develops between P17 and P24 (Ivkovich, Paczkowski, & Stanton, 2000; Stanton, Freeman, & Skelton, 1992). Although CS pathway development is thought to be the principal limiting factor in the development of delay eyeblink condition, there is evidence that both the cerebellum and US pathway are also developing in young animals.

Adult research on the US pathway highlights the importance of the inhibitory projection from the IPN to the inferior olive (Lang, 2003). However, developmental work concerning the inferior olive suggests that this pathway is late to develop in young animals. Recording studies of the inferior olive show a developmental difference in neuronal firing with the older animals showing less activity than younger animals (Nicholson & Freeman, 2000). Moreover, quantitative electron microscopy indicates a significant age-related change in the number of inhibitory synapses at the inferior olive. In P24-25 pups 50% of all synapses were inhibitory whereas in P17-18 pups only 35% were (Nicholson & Freeman, 2003).

Research concerning CS-US pathway integration in the cerebellum indicates that both the IPN and the cerebellar cortex may also be undergoing developmental changes. Neuronal recording studies of the IPN show age- and learning-related changes in both the percentage of units and the magnitude of responding (Freeman & Nicholson, 2000). Simple spike activity of the cerebellar cortex also showed developmental changes. Although both P17-18 and P24-25 pups showed learning-related changes in neuronal activity, the percentage of units showing these changes was higher in older animals (Nicholson & Freeman, 2004).

## **CS Pathway Development and Delay Eyeblink Conditioning**

As previously mentioned, the pontine nucleus provides the cerebellum with CS-related input. Neuronal recordings from the pontine nucleus during delay EBC show an age-related change in neuronal activity, suggesting weakened sensory input in younger animals (Freeman & Muckler, 2003). This weakened sensory input results in decreased input to the cerebellum downstream, which could account for developmental differences in conditioned responding. This hypothesis is further supported by stimulation studies. When pontine stimulation is used as a CS, thus bypassing late-developing sensory systems, pups as young as P12-13 show nearly adult-like levels of conditioned responding (Campolattaro & Freeman, 2008). Therefore, although the neural mechanisms involved in the CS-US association are sufficiently developed to support learning by P12-13, auditory and visual sensory system development may be limiting EBC development.

In rat pups, the auditory and visual systems develop relatively late, with the meatal canal of the ears and the eyes not opening until P12 and P14, respectively. Moreover, the development of learning in a given modality follows the developmental emergence of sensory input from that modality (Hyson & Rudy, 1984). Therefore, although the meatal canal of the ears opens at approximately P12, the neural pathways allowing tone information to be used in learning may not yet be fully developed. Indeed, literature concerning the developmental emergence of fear conditioning shows that auditory system development is far more complex than meatal canal opening. In fact, the auditory system has low levels of functioning even prior to meatal canal opening. At as early as P10, pups are able to respond to a tone. However, conditioning to that same tone does not emerge until nearly 5 days later, at P15 (Hyson & Rudy, 1984).

If late-developing sensory input to the pontine nucleus is limiting the ontogenetic emergence of EBC, using an earlier developing sensory system should result in higher levels of conditioned responding. The somatosensory system is the earliest developing sensory system (Alberts, 1984). Behavioral studies indicate that pups as young as embryonic day (E) 16 show evoked responses to tactile stimulation of the forepaw palmar surface (Narayanan, Fox, & Hamburger, 1971). Moreover, there is evidence that the somatosensory system is sufficiently developed by P0 to support low levels of conditioned fear (Bachevalier & Blozovski, 1980; Caldwell & Werboff, 1962).

### **Somatosensory CS pathway**

Somatosensory information from the vibrating grid floor CS is received by mechanoreceptors located in the palmar surface of the forelimbs and hindlimbs (Cohen, 1958). From there, sensory information is carried up the axon to the cell body located in the dorsal root ganglia of the spinal cord and then to the dorsal column nuclei of the medulla, known as the gracile and cuneate nuclei. Neurons that receive afferents from the hindlimbs project to the gracile nucleus whereas afferents to the cuneate nucleus carry information pertaining to the forelimbs (Rosen & Sjolund, 1973). At the dorsal column nuclei these neurons synapse onto secondary neurons which decussate and form the medial lemniscus, projecting to the ventral posterior lateral nucleus of the thalamus, the pontine nucleus, the inferior cerebellar peduncle, and the cerebellar cortex (Kosinski, Lee, & Mihailoff, 1988; Massopust, Hauge, Ferneding, Doubek, & Taylor, 1985; Rinvik & Walberg, 1975). Despite the fact that there are direct projections from the dorsal column nuclei to the inferior cerebellar peduncle and cerebellar cortex, lesion studies suggest that these routes are not the principle pathway for the vibration CS. Lesions of the medial cerebellar peduncle, which receives input from the pontine nucleus, completely abolish

conditioned responding to a vibration CS (Lewis, Lo Turco, & Solomon, 1987; Solomon, Lewis, LoTurco, Steinmetz, & Thompson, 1986). Therefore, the proposed CS pathway for vibratory stimuli is from the dorsal column nuclei to the pontine nucleus.

### **Development of Trace Eyeblink Conditioning and Underlying Neurobiology**

As in adults, trace EBC in young animals depends on both delay conditioning circuitry and additional forebrain structures, such as the hippocampus and anterior cingulate cortex (Ivkovich & Stanton, 2001). Auditory and visual trace EBC emerge ontogenetically between P19 and P30 (Ivkovich et al., 2000). This ontogenetic change closely matches the developmental timeline observed in another Pavlovian conditioning paradigm, trace fear conditioning (Barnet & Hunt, 2005; Moye & Rudy, 1987a). Because of the parallel developmental emergence of trace conditioning, the development of forebrain systems, such as the hippocampus, has been considered to be a major limiting factor in both trace fear and EBC development (Ivkovich et al., 2000; Moye & Rudy, 1987a, 1987b). Indeed, recently published work on hippocampal place cell development showed that although pups as young as P16 had place cells, stability of place fields continued to develop until at least P30 (Langston et al., 2010; Wills, Cacucci, Burgess, & O'Keefe, 2010). Although these papers suggest that hippocampal development is still ongoing at this point in development, they did not address the role of hippocampal development in learning and memory. One possible way to examine the role of hippocampal development would be to record neuronal activity from the hippocampus while pups are trained in a hippocampus-dependent task, such as trace EBC.

## **Trace Eyeblink Conditioning with a Somatosensory CS**

Research concerning the development of delay EBC suggests that its ontogeny is dependent on sensory input to the pontine nucleus. However, as mentioned above, due to the parallel emergence of trace fear conditioning and trace EBC, the developmental time course of trace conditioning has traditionally been believed to be dependent on hippocampal development (Ivkovich & Stanton, 2001; Moye & Rudy, 1987b). An alternative hypothesis, however, could be that it is actually the development of sensory input to the hippocampus, which is limiting the acquisition of trace conditioning. One possible way to test this hypothesis would be to train animals in trace conditioning with an early-developing sensory modality. If the original hypothesis is correct, then pups trained in somatosensory trace EBC should show identical levels of learning and neuronal responsiveness to those trained in auditory trace conditioning. However, if trace EBC is facilitated by using an earlier-developing sensory system, it would imply that auditory input to the hippocampus (via the perirhinal/entorhinal cortex) is not fully mature. Moreover, if it is indeed sensory input to the hippocampus (and not an alternative structure, such as the anterior cingulate) which is responsible for facilitated trace eyeblink acquisition with a somatosensory CS, then we would expect neuronal activity in the hippocampus to be greater when pups are trained with a vibration CS.

### **Overview of dissertation experiments**

The goal of the current set of studies is to determine how sensory system development affects both delay and trace EBC. Previous work from our laboratory suggests that sensory input to the pontine nucleus is the principle limiting factor in the development of delay EBC (Campolattaro & Freeman, 2008; Freeman & Rabinak, 2004; Freeman, Rabinak, & Campolattaro, 2005; Freeman & Muckler, 2003). The goal of Experiment 1 (Goldsberry, Elkin, & Freeman, 2014) is

to employ an early-developing sensory modality (vibration) as the CS, therefore bypassing late-developing sensory input to the pontine nucleus. If sensory system development is indeed a principle rate-limiting factor in the development of delay EBC we would expect vibration-trained pups to perform similarly to those trained with a pontine stimulation CS.

Although trace EBC is believed to be primarily limited by hippocampal development (Ivkovich et al., 2000; Ivkovich & Stanton, 2001; Moye & Rudy, 1987a, 1987b; Stanton, Ivkovich Claflin, & Herbert, 2009), sensory system development could play a role in its ontogenetic emergence. The goal of Experiment 2 is to test the hypothesis that sensory system development may be contributing to the developmental emergence of trace EBC. If the development of trace EBC is limited by sensory system development we would expect pups trained with a vibration CS to demonstrate learning at an earlier age than those trained with a tone CS. However, if sensory system development does not play a role in the ontogenetic emergence of trace EBC we would anticipate that tone- and vibration-trained pups would perform similarly.

The goal of Experiment 3 (Goldsberry, Kim, & Freeman, 2015) is to determine whether hippocampal neuronal activity is related to the developmental emergence of auditory trace EBC. Numerous studies in adult animals have demonstrated that hippocampal neuronal activity corresponds to trace EBC acquisition (Green & Arenos, 2007; McEchron & Disterhoft, 1999; McEchron, Tseng, & Disterhoft, 2003; McEchron, Weible, & Disterhoft, 2001; Solomon, Vander Schaaf, et al., 1986; Weible, O'Reilly, Weiss, & Disterhoft, 2006; Weiss et al., 1996). However, there have not been any attempts to characterize how developmental changes in learning are related to hippocampal neuronal activity. If the hippocampus or upstream pathways are indeed contributing to the developmental emergence of trace EBC, then we would expect to

see age- and learning- related increases in hippocampal neuronal activity. However, a lack of age- and learning-related changes in hippocampal activity would suggest that development critical to learning is actually occurring downstream of the hippocampus.

Experiment 2 revealed that training with an early-developing sensory modality facilitates the developmental emergence of trace EBC. Therefore, the goal of Experiment 4 is to determine whether the age- and learning-related changes in hippocampal activity observed in Experiment 3 are due to delayed hippocampal maturation (Goldsberry et al., 2015; Ivkovich et al., 2000; Ivkovich & Stanton, 2001) or limited input to the hippocampus from developing upstream sensory (auditory) pathways. If hippocampal neuronal activity is limited uniquely by the maturation of the hippocampus, then we would anticipate that CS modality would not influence hippocampal activity. This would imply that sensory system modulation of trace EBC is occurring downstream from the hippocampus. However, if using an early-developing CS modality increases age- and learning-related activity, then this would suggest that the development of sensory input upstream of the hippocampus may play a role in the ontogenetic emergence of hippocampal neuronal activity during trace EBC.



## **CHAPTER 1: SENSORY SYSTEM DEVELOPMENT INFLUENCES THE ONTOGENY OF DELAY EYEBLINK CONDITIONING**

Eyeblink conditioning (EBC), a type of Pavlovian conditioning, is a particularly useful paradigm for studying the development of learning and memory. Because the unconditioned response (UR) is relatively simple (i.e., eyelid closure), even very young animals are able to perform the response as well as adults. This limits the influence of response system development and allows for easier across-age comparisons. Furthermore, the vast amount of research concerning both the behavior and neural circuitry involved in adult EBC makes it an ideal paradigm for studying the ontogeny of EBC in young animals (Stanton et al., 1992).

The majority of previous research using EBC in adult animals has used a tone or light as the conditioned stimulus (CS). Consequently, developmental work concerning EBC has also used auditory and visual CSs. Developmental studies using an auditory or visual CS suggest that delay EBC develops between P17 and P24 (Paczkowski, Ivkovich, & Stanton, 1999; Stanton et al., 1992). Pups trained on P17 have low levels of conditioned responding to auditory or visual CSs. However, by P24-26 pups demonstrate high levels of conditioned responding to both auditory and visual CSs.

The developmental trajectory of EBC is related to sensory neural pathway development and the development of learning-related plasticity in the cerebellum (Freeman, 2010). At very young ages, sensory pathway development is a potential contributor to the ontogeny of learning (Alberts, 1984; Gottlieb, 1971). Although infant rats are able to respond to some auditory stimuli by P9, the ear canal does not fully open until approximately P13, and adult levels of auditory discrimination are not reached until approximately P16 (Crowley & Hepp-Reymond, 1966). Likewise, the rat visual system is considered late-developing. Pups do not open their eyes until

approximately P14 (Gramsbergen, Schwartz, & Precht, 1970). Because auditory and visual sensory system development is necessary to perceive a tone or light CS, the earliest conceivable age at which EBC could be observed is P13 and P14 for auditory and visual stimuli, respectively. However, auditory and visual conditioning emerge nearly a week after ear canal and eye opening occurs. Literature concerning the development of another Pavlovian paradigm, fear conditioning, demonstrates that the development of learning in a given modality follows the developmental emergence of sensory input from that modality. Specifically, although pups may be able to respond to a given sensory stimulus, conditioning with that same stimulus does not emerge until nearly five days later (Moye & Rudy, 1985; Rudy, 1993; Rudy & Hyson, 1984).

Previous work from our laboratory concerning the development of CS inputs to the cerebellum supports the hypothesis that sensory pathway development is a primary rate-limiting factor in the ontogeny of EBC (Freeman, 2010). The most proximal part of the CS pathway in EBC is the pontine nuclei and their mossy fiber projections to the cerebellum (Halverson & Freeman, 2010a; Steinmetz et al., 1987). Neuronal activity in the pontine nuclei during auditory conditioning shows developmental changes in the amplitude of responding and learning-related activity between the ages of P17-18 and P24-25 (Freeman & Muckler, 2003). These data suggest that pontine activity may play a role in the development of EBC by affecting CS input to the cerebellum. If pontine input to the cerebellum in younger animals is weak, cerebellar neurons will undergo less learning-related plasticity (Freeman, 2010). Conversely, if pontine input to the cerebellum is increased, younger animals should have facilitated acquisition of EBC. By using a pontine stimulation CS, as opposed to a tone CS, we were able to bypass late-developing sensory systems and thereby facilitate acquisition of EBC (Campolattaro & Freeman, 2008; Freeman et al., 2005). In fact, pups trained at P12-13, which do not normally acquire EBC, showed high

levels of conditioned responding when trained with pontine stimulation as a CS. Increased pontine input to the cerebellum, therefore, reversed developmental deficits in EBC. Because these data suggest that sensory system development is in fact largely responsible for the delayed development of EBC with tone and light CSs, employing a sensory modality that emerges earlier in ontogeny could increase pontine neuronal activity, and thus increase pontine input to the cerebellum and facilitate learning in younger animals.

In contrast to the auditory and visual systems, the rat somatosensory system develops prenatally (Narayanan et al., 1971; Smotherman & Robinson, 1988). In fact, research using the somatosensory system in other learning paradigms has demonstrated that animals are able to learn at a much younger age than observed with auditory and visual stimuli. For example, in the case of Pavlovian fear conditioning, one-day-old infant rats were able to form an association between a vibrotactile stimulus applied to the chest region and a foot shock (Caldwell & Werboff, 1962).

The goal of Experiment 1 was to investigate the development of EBC in rat pups using a vibration or tone CS. Pups were given paired or unpaired training on P14-15, 17-18, 21-22, 24-25. If pups are able to learn EBC with a vibration CS earlier ontogenetically than with a tone CS, it will further strengthen the hypothesis that the ontogeny of EBC is mediated by the development of sensory inputs to the pontine nuclei. Experiment 2 examined whether or not the sound of the vibration CS contributed to CRs during EBC. Experiment 3 examined extinction of EBC with a vibration CS.

## **General Methods**

### **Subjects**

The subjects were Long-Evans rat pups born and reared in the colony in Spence Laboratories of Psychology at the University of Iowa. The colony was maintained on a 12/12-hr light/dark cycle, with light onset at 7 am. Male and female breeders were pair housed in polycarbonate cages with wire lids. Each day cages were checked for births, with the day of birth being designated P0. On P2 litters were culled to eight pups. Subjects remained in their home cage until P19, at which time they were transferred to separate cages with same-sex littermates. Experimental groups included no more than two animals from the same litter (one male and one female). All training occurred from 7 am to 7 pm. All procedures were approved by the Institutional Animal Care and Use Committee at the University of Iowa.

### **Surgery**

Surgery was performed two days prior to training, on P12, P15, P19, or P22 to allow one day for the pup to recover. Isoflurane (1.5-3%) was used for anesthesia. Prior to surgery the rat's head was fixed in a mouse stereotaxic head holder and aligned. During surgery rats were fitted with differential EMG electrodes to record blink activity and a bipolar stimulating electrode for US delivery. Two EMG electrodes were threaded through the left upper eyelid and a ground wire was attached to the skull with a small stainless steel skull screw. All three wires (two recording and one ground) terminated in small gold pins that were secured in a plastic connector. The plastic connector was cemented to the skull using bone cement (Zimmer, Warsaw, IN), leaving only the gold pins exposed. The bipolar stimulating electrode for US delivery was placed subdermally immediately caudal to the left eye. The bipolar electrode terminated in a small plastic connector which was cemented to the skull using bone cement.

## **Conditioning Apparatus**

Rat pups were trained in a conditioning chamber that was contained within a sound-attenuation chamber. One side of the conditioning chamber was fitted with two small speakers, which delivered the tone CS (2 kHz, 82 dB). The floor of the chamber, which delivered the vibration CS, consisted of a custom-built vibrating grid floor placed on top of a piece of bench paper that protected a thin foam pad. The vibrating grid floor was made of square wire that was attached to a vibration motor (model number C1030B028F; JinLong Machinery, Zhejiang, China). When completely assembled and placed on the bench paper and foam pad in the chamber, the vibration CS vibrated at 144 Hz, with an acceleration of  $2.4 \text{ m/s}^2$ . The foam pad placed under the grid floor ensured that the vibrating grid floor produced a minimum amount of sound. This method of stimulus delivery has been used in previous studies of learning in rat pups (Markiewicz, Kucharski, & Spear, 1986; Spear & Smith, 1978). Lightweight cables with connectors for both the recording EMG and the bipolar stimulating electrode were attached to a commutator above the conditioning chamber and threaded through a hole in the ceiling of the chamber. Computer software controlled the delivery of both CS and US while simultaneously recording differential eyelid EMG activity (sampling rate = 250 Hz). All EMG activity was amplified (x 2000), filtered (500-5000 Hz), and integrated (20 ms time constant).

## **Conditioning Procedure**

Conditioning occurred over the course of two days. All pups received 6 sessions (3 per d) of either paired or unpaired delay EBC training with either a tone or vibration CS. Pups in the paired group received 100 trials per session of delay EBC with a 400 ms vibration or tone CS and a 25 ms periorbital stimulation US. Each session was divided into 10 equal blocks of 10 trials, each with an intertrial interval of approximately 30s. The first 9 trials of each block were

paired CS-US presentations and the 10th trial of each block was a CS-alone probe trial. The probe trials were used to evaluate conditioned responding (CR) in the absence of the UR (Gormezano, Kehoe, & Marshall, 1983). Pups in the unpaired group received 200 trials per session, 100 CS-alone trials and 100 US-alone trials. Each trial consisted of an unpaired presentation of either the CS or the stimulation US. Unpaired trials were separated by intertrial intervals of approximately 15 s.

### **Data Analysis**

Behavioral data were examined offline. CRs were defined as any blink response during the CS that crossed a .4 unit threshold above the pre-CS baseline EMG activity. Any responses that occurred within 80 ms of CS onset were considered startle responses. Trials with EMG activity that crossed the threshold prior to the CS onset were omitted from the analysis. A repeated measures ANOVA was performed on session data related to CR percentage, amplitude, onset latency, and peak latency. CR amplitude, onset latency, and peak latency measures were examined on CS-alone trials in which a CR occurred. Because group sizes were approximately 7-8 pups, pup sex was not analyzed. Significant group effects were further analyzed with the Bonferroni Test. An alpha level of 0.05 was used for all statistical tests.

### **Experiment 1**

The purpose of Experiment 1 was to determine if sensory system development plays a role in the ontogeny of EBC. If sensory system development is a rate-limiting factor in the ontogeny of EBC, then training with an earlier-developing sensory system, the somatosensory system, should result in an earlier onset of conditioning. Pups were trained with either a vibration or tone CS on P14-15, 17-18, 21-22, 24-25.

## Methods

The subjects were 108 Long-Evans rats pups derived from 58 different litters. The experimental design included 2 conditions (paired or unpaired), 2 CS modalities (tone or vibration), and 3 age groups (P17-18, P21-22, or P24-25). For vibration training only, there was an additional group trained on P14-15.

## Results

Regardless of CS type, pups that were trained in the paired condition showed significantly more CRs than those trained in the unpaired condition (Figure 1). Furthermore, during paired training age-matched pups trained with the vibration CS showed far greater levels of conditioned responding than those trained with the tone CS (Figure 1). When trained with the vibration CS, significant levels of conditioned responding were observed as early at P14-15. Importantly, these increased levels of responding were not due to non-associative factors; levels of unpaired responding did not significantly differ across CS type or age.

A repeated measures ANOVA on the CR percentage data from all but the P14-15 age group confirmed these observations with an Age X CS-type X Condition (paired or unpaired) X Session interaction  $F(7.08, 290.36) = 2.592, p = 0.013$  on CR percentage (Greenhouse-Geisser correction for sphericity). A repeated-measures ANOVA on the CR percentage for the vibration-trained pups only showed main effects of age  $F(10.53, 189.53) = 2.883, p < 0.001$  and condition  $F(3.51, 189.53) = 27.595, p < 0.001$  (Greenhouse-Geisser correction for sphericity). When data of paired, vibration-trained pups were further analyzed, *post hoc* tests indicated that the oldest three age groups trained with a vibration CS had a similar CR percentage ( $p > 0.05$ ), but differed significantly from the youngest (P14-15) age group ( $p < 0.0001$ ). Further comparisons confirmed that these differences were observed during all six training sessions ( $p < 0.01$ ). When the data of

paired tone-trained pups were further analyzed, *post hoc* tests examining the above-mentioned interaction indicated that the P24-25 and P17-18 groups had significantly different CR percentages on sessions 4-6 ( $p < 0.05$ ). However, the P21-22 group performed intermediately and was not significantly different from either group.

In order to determine whether the youngest age group (P14-15) was able to learn the association between the vibration CS and the periorbital stimulation US, *post hoc* tests were performed on CR percentage between paired and unpaired groups across sessions. The paired vs. unpaired groups differed only on sessions 4-6 ( $p < 0.05$ ).

The amplitude of the CR was also influenced by age, session, and condition (paired vs. unpaired) (Figure 2). However, CS type did not influence response amplitude. There was an age- and session-related increase in CR amplitude during paired, but not unpaired training. A repeated measures ANOVA on all but the P14-15 age group (with Greenhouse-Geisser correction for sphericity) indicated a Session X Age X Condition (paired or unpaired) interaction  $F(6.86, 219.63) = 2.09, p = 0.047$ . *Post hoc* tests on paired data indicate that there was an age-related increase in CR amplitude across sessions with both the P21-22 and P24-25 age groups having higher amplitude CRs than the P17-18 age group ( $p < 0.05$ ). A repeated-measures ANOVA on the vibration-trained pups indicated a Session X Age X Condition (paired or unpaired) interaction  $F(9.85, 144.49) = 2.49, p = 0.009$ . *Post hoc* tests on the paired data from the vibration groups confirmed that there was an age-related increase in CR amplitude. The P14-15 group was significantly lower than the P21-22 and P24-25 groups and the P17-18 group was significantly lower than the P24-25 group.

The UR amplitude, CR onset, and peak CR latencies did not differ between CS modalities or across age groups. Mean (+/- SD) session 1 UR amplitudes are as follows for



vibration- and tone-trained pups, respectively: P14-15: 1.59(.78), P17-18: 1.94(.45), 2.6(1.08), P21-22: 3.34(1.02), 3.21(1.44), P24-25: 3.21(1.43), 2.87(1.07).

## **Experiment 2**

In order to determine whether or not the sound of the vibration CS motor influenced CR production, we performed a control experiment. The goal of Experiment 2 was to determine whether the sound of the vibration CS had an impact on the CR. If the sound of the vibration CS contributed to the previously-observed level of conditioned responding, its presentation alone should also elicit a CR.

### **Methods**

Subjects were 7 pups from 4 different litters trained on P17-18. Because Experiment 1 found robust and nearly identical learning at all but the youngest age group trained with a vibration CS, this experiment only utilized the P17-18 age group. The conditioning apparatus and methodology were identical to that described in Experiment 1 for CS-US acquisition sessions 1-5. However, during session 6 the vibration motor was suspended from the ceiling of the chamber by a lightweight cable where it could not vibrate the cage.

### **Results**

As observed in Experiment 1, pups in the P17-18 age group readily showed CRs when trained with a vibration CS. Furthermore, their levels of CRs did not appear to be influenced by the sound of the vibration motor (Figure 3). During session 6, when the sound of the CS was presented alone, pups showed an immediate drop in CRs (block 1 mean = 38.89 and blocks1-10 mean = 26.11). A repeated measures ANOVA across all six sessions confirmed these results  $F(1.87, 11.22)=29.72, p < 0.0001$  (Greenhouse-Geisser correction for sphericity). Post hoc tests

showed that there was a significant difference between the level of CRs on sessions 5 and 6 ( $p = 0.001$ ), but not between the level of CRs on sessions 1 and 6 ( $p > 0.05$ ).

### **Experiment 3**

The goal of Experiment 3 was to investigate whether pups trained on P17-18 with a vibration CS were able to show extinction. Previous research in auditory EBC with this age group has yielded low levels of conditioned responding (Stanton et al., 1992). Therefore, to date, there have not been any studies examining extinction in this age group. Assessing extinction with the vibration CS further assesses whether conditioning with a somatosensory CS has similar properties to conditioning with a visual or auditory CS.

### **Methods**

Subjects were 8 pups from 5 different litters. Pups received 6 CS-US acquisition training sessions on P17-18, as in Experiment 1. The 2 CS-alone extinction sessions took place the following day, on P19.

### **Results**

As in Experiments 1 and 2, P17-18 pups showed rapid acquisition of EBC using a vibration CS. However, this level of responding dropped off substantially during extinction training session 7 (Figure 4). Overall, both extinction sessions showed similar levels of CRs. However, when examining block data within the two sessions, it became evident that sessions 7 and 8 had very different session profiles. Responding during session 7 started out high but dropped rapidly until halfway through the session, at which point responding leveled out. The profile of session 8 was substantially flatter, dropping only slightly across blocks. These observations were confirmed with a repeated-measures ANOVA across all 8 sessions  $F(7, 49) = 13.22, p < 0.0001$  and post hoc tests indicated that there was a significant difference between the final session of acquisition

training and the first session of extinction training ( $p = 0.003$ ). A repeated-measures ANOVA across the block data of session 7, the first extinction session, showed a significant effect of training block  $F(9, 63) = 3.46, p = 0.002$ . Post hoc tests examining block data changes across session 7 showed that blocks 4-7 and 9-10 were significantly different from block 1.

## **Discussion**

EBC was faster and ontogenetically earlier with a vibration CS when compared to EBC with a tone CS. When trained with a tone CS, pups showed only modest levels of learning when trained on P17-18, a finding consistent with previous studies of the developmental trajectories of auditory and visual EBC (Paczkowski et al., 1999; Stanton et al., 1992). In contrast, training with a vibration CS resulted in robust learning by P17-18. In fact, pups as young as P14-15 showed low levels of EBC when trained with a vibration CS. This developmental increase in CRs does not appear to be the result of increased non-associative responding to the CS. Data from similar studies investigating the development of delay EBC with auditory and visual CSs show nearly identical levels of non-associative responding to those observed during unpaired presentation of the vibration CS (Ivkovich et al., 2000).

The auditory CS pathway includes projections from the cochlear nucleus, superior olive, nucleus of the lateral lemniscus, and inferior colliculus to the medial auditory thalamus, directly and in series (Freeman & Steinmetz, 2011). Medial auditory thalamic neurons project to the lateral pontine nuclei which then project to the cerebellar cortex and deep nuclei (Campolattaro et al., 2007; Halverson & Freeman, 2010b; Halverson, Lee, & Freeman, 2010; Halverson, Poremba, & Freeman, 2008). Development of auditory inputs to the lateral pontine nucleus plays a major role in the ontogeny of auditory EBC. Electrical stimulation of the pontine nuclei as a CS results in much faster conditioning in P17 pups, almost as fast as pups trained on P24 with a

stimulation or tone CS (Freeman et al., 2005). This finding has been extended to pups trained with pontine stimulation as the CS on P12, also resulting in robust associative learning (Campolattaro & Freeman, 2008). These pontine stimulation studies demonstrate that the cerebellum is capable of EBC as early as P12 if it receives sufficient sensory input (Freeman, 2010). However, recordings of pontine neuronal activity in rat pups show a substantial developmental increase in sensory responses to a tone CS between P17 and P24 suggesting that auditory inputs to the pontine nuclei are continuing to develop past P17 (Freeman & Muckler, 2003). Furthermore, stimulation of the cochlear nucleus or medial auditory thalamus as the CS does not result in earlier learning in rat pups, which indicates that there are developmental changes in the auditory pathway projecting to the lateral pontine nuclei (Freeman & Campolattaro, 2008; Freeman & Duffel, 2008). Neuronal recordings from the medial auditory thalamus indicate that the thalamus, like the pontine nuclei, show substantial developmental changes in responsiveness to a tone CS and less learning-related activity (Ng & Freeman, 2012). In sum, these findings concerning the auditory pathway indicate that there are developmental changes in the strength of sensory information upstream of the pontine nuclei.

The neural pathway for a floor vibration CS has not been identified but probably includes tactile, vestibular, and proprioceptive afferent projections to the cerebellum. Vibration stimulation in the body activates spinal afferents that project to the dorsal column nuclei. The dorsal column nuclei then project to the medial pontine nucleus (Kosinski, Azizi, Border, & Mihailoff, 1986; Kosinski, Neafsey, & Castro, 1986). Previous studies in rabbits and ferrets have shown that EBC using stimulation of the body with vibration or weak electrical shocks depends on the middle cerebellar peduncle (Hesslow, Svensson, & Ivarsson, 1999; Lewis et al., 1987). Thus, although there are direct projections from the dorsal column nuclei to the cerebellum

through the inferior cerebellar peduncle (Bengtsson & Jorntell, 2009), the most likely CS pathway for a vibration CS is the dorsal column nuclear projection to the pontine nuclei. In contrast to the auditory and visual CS pathways (Halverson & Freeman, 2010a, 2010b), there do not appear to be direct projections from somatosensory thalamus to the pontine nuclei. In fact, the steep acquisition curve for somatosensory EBC in P17-18, P21-22, and P24-25 pups strongly resembles acquisition curves seen in pups that receive pontine stimulation as a CS (Campolattaro & Freeman, 2008; Freeman et al., 2005). Thus, the dorsal column projections to the cerebellum via the pontine nuclei appear to be mature by P17, whereas the auditory projections to the pontine nuclei are not mature until P24 or so.

Previous studies demonstrated associative learning with a vibration CS and an electrical stimulation US in neonatal rats (Bachevalier & Blozovski, 1980; Caldwell & Werboff, 1962). Conditioned limb flexion was measured in these studies of neonatal conditioning, which like EBC, depends on the cerebellum (Mojtahedian, Kogan, Kanzawa, Thompson, & Lavond, 2007; Voneida, 2000). The findings of these limb flexion studies are consistent with the early EBC in the current study with a vibration CS. Neonatal rats showed only modest levels of conditioning (15-32%) in the previous studies relative to adult rats, comparable to the P14-15 group in the current study. A similar pattern of results was found using a shock-shock (US-US) conditioning paradigm in rat pups (Schreurs, Burhans, Smith-Bell, Mrowka, & Wang, 2013). Somatosensory inputs to the cerebellum from the dorsal column nuclei may therefore continue to develop postnatally until P17, even though rat pups are clearly responsive to tactile stimuli prior to birth (Smotherman & Robinson, 1988). It is also possible that US pathway development (Nicholson & Freeman, 2003) plays a significant role in the early development of cerebellar learning, i.e., before P17.

In conclusion, this study demonstrated that using a vibration CS results in facilitated acquisition of delay EBC in rat pups relative to EBC with an auditory or visual CS. Pups are able to learn faster and at earlier ages than observed previously with other peripheral CSs. Furthermore, non-associative responding (as seen during unpaired training) does not differ from previously reported data using other CS modalities. The findings of the current study support the hypothesis that the ontogeny of cerebellar learning depends on the development of sensory systems and their inputs to the pontine nuclei.

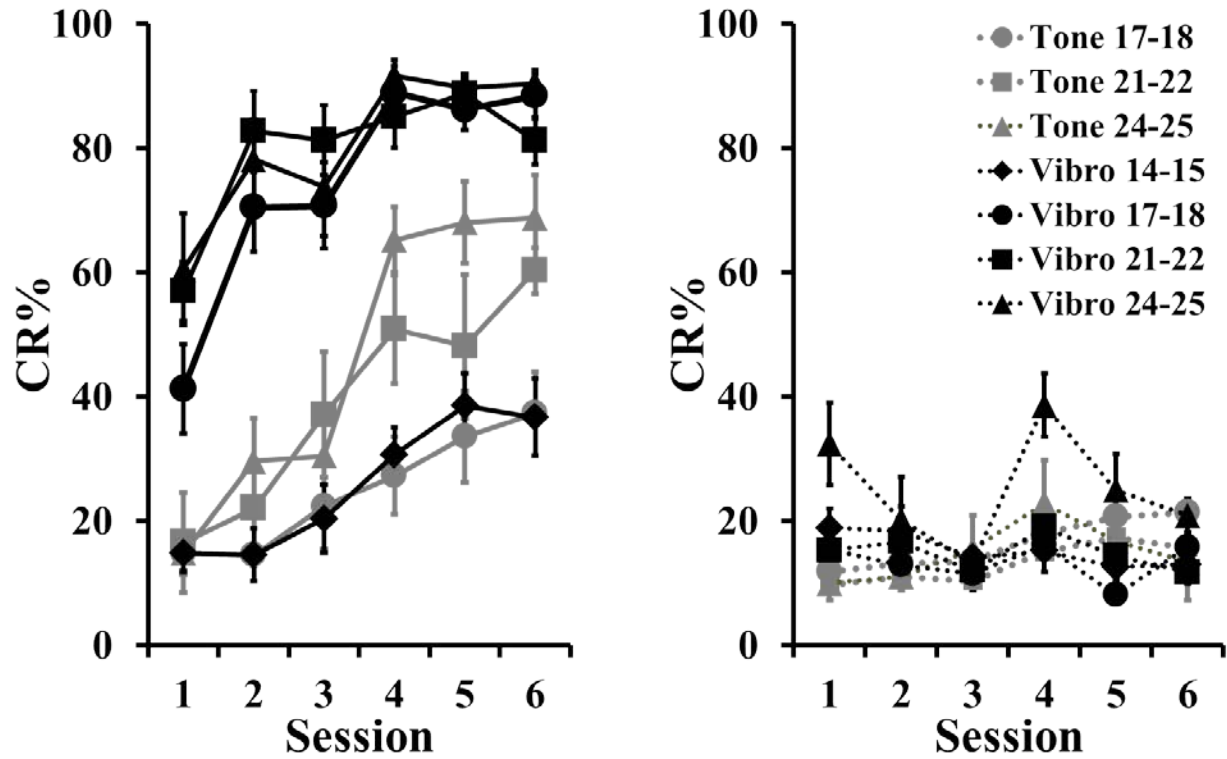


Figure 1. Conditioned response percentage during paired and unpaired delay EBC with a tone and vibration CS.

Mean ( $\pm$  SEM) eyeblink conditioned response (CR) percentage for rat pups given paired (left, solid lines) or unpaired (right, dashed lines) training with a tone (grey) or vibration (black) conditioned stimulus (CS). Pups were trained with the tone on postnatal days (P)17-18 ( $n = 8$  paired, 8 unpaired), 21-22 ( $n = 7$  paired, 9 unpaired), or P24-25 ( $n = 8$  paired, 6 unpaired). Pups were trained with the vibration CS on P14-15 ( $n = 7$  paired, 7 unpaired), P17-18 ( $n = 8$  paired, 8 unpaired), P21-22 ( $n = 8$  paired, 8 unpaired), or P24-25 ( $n = 8$  paired, 8 unpaired).

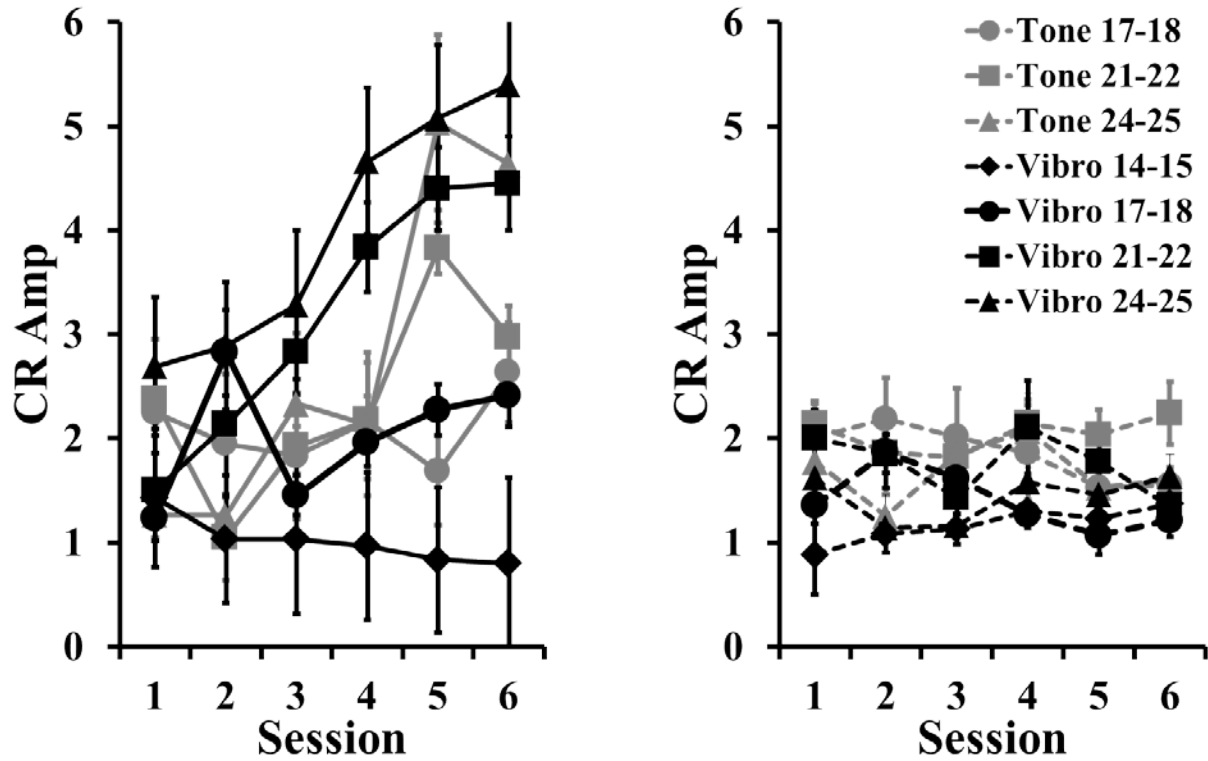


Figure 2. Conditioned response amplitude for rats given paired and unpaired delay EBC with a tone and vibration CS.

Mean (+ SEM) eyeblink conditioned response (CR) amplitude for rat pups given paired (left, solid lines) or unpaired (right, dashed lines) training with a tone (grey) or vibration (black) conditioned stimulus (CS).



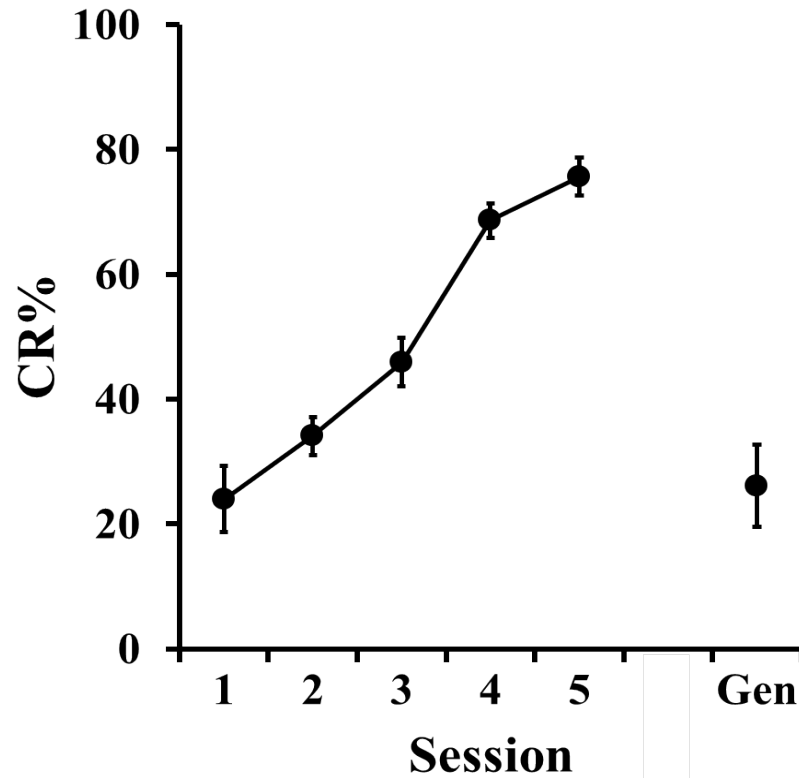


Figure 3. Acquisition and generalization test for vibration-trained P17-18 pups.

Mean (+ SEM) eyeblink conditioned response (CR) percentage for rat pups given paired training with a vibration conditioned stimulus (CS) on P17-18 (sessions 1-5) followed by a CS-alone generalization test (Gen) for the sound of the vibration device.

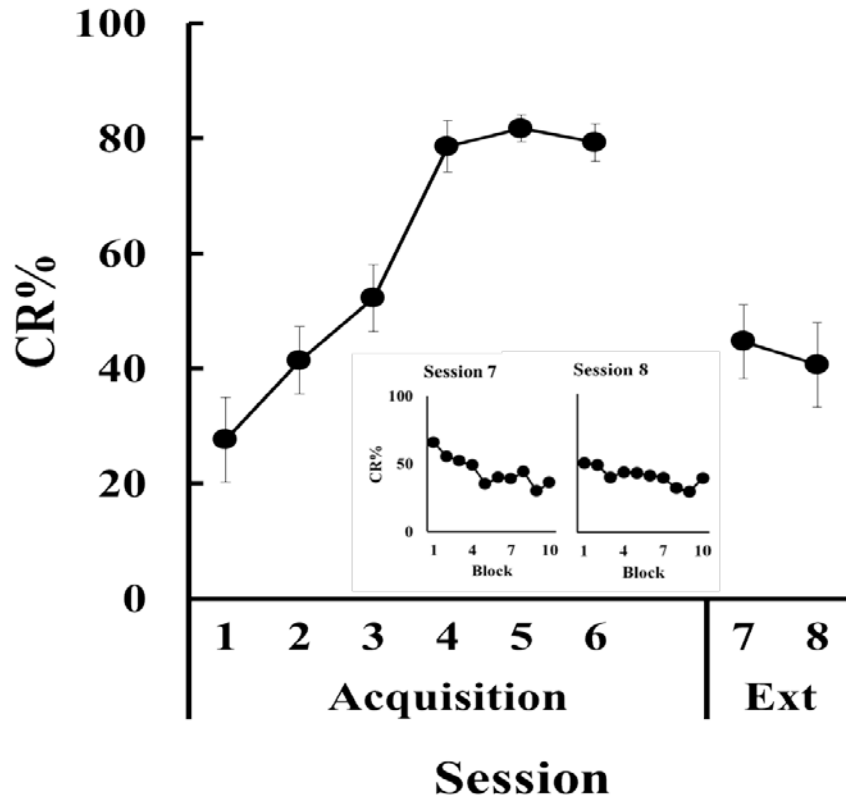


Figure 4. Acquisition and extinction of delay EBC in vibration-trained P17-18 pups.

Mean (+ SEM) eyeblink conditioned response (CR) percentage for rat pups given paired training with a vibration conditioned stimulus (CS) on P17-18 (Acquisition) followed by 2 CS-alone extinction sessions (Ext). Graph insert shows block data for sessions 7 and 8, the extinction sessions.

## **CHAPTER 2: SENSORY SYSTEM DEVELOPMENT INFLUENCES THE ONTOGENY OF TRACE EYEBLINK CONDITIONING**

Eyeblink conditioning (EBC) is a type of Pavlovian conditioning in which a conditioned stimulus (CS) is paired with stimulus that elicits eyelid closure, referred to as unconditioned stimulus (US). Because the unconditioned response (UR) is relatively simple (i.e., eyelid closure), even very young animals are able to perform the response as well as adults. This provides a significant advantage over other learning tasks in that it limits the influence of response system development when comparing behavior across ages. Moreover, the EBC paradigm has been studied extensively and there is a vast amount of research concerning both the behavior and neural circuitry, thus making it an ideal paradigm for studying the development of learning and memory in young animals (Stanton et al., 1992). Another advantage of the EBC paradigm is its flexibility. Whereas delay conditioning involves presenting the CS and the US together, trace conditioning involves the addition of a stimulus-free trace interval in between CS and US presentation. Trace conditioning and delay condition further differ in that trace conditioning emerges later than delay (Ivkovich & Stanton, 2001). Auditory and visual trace EBC emerge simultaneously between P19 and P30 in rats (Ivkovich et al., 2000; Ivkovich & Stanton, 2001). This age closely matches the developmental timeline observed in another Pavlovian conditioning paradigm, trace fear conditioning (Barnet & Hunt, 2005; Moye & Rudy, 1987a).

Because of the parallel developmental emergence of trace fear and eyeblink conditioning paradigms, the development of forebrain systems, and specifically the hippocampus, has been considered to be a major limiting factor in both trace fear and eyeblink conditioning development (Ivkovich et al., 2000). Indeed, work on hippocampal place cell development showed that

although rat pups as young as P16 had place cells, stability of place fields continued to develop until at least P30 (Langston et al., 2010; Wills et al., 2010). This continued development applies to learning as well. *In vivo* CA1 pyramidal cell recordings during auditory trace EBC in P21-23, P24-26, and P31-33 rat pups demonstrated both age- and learning-related changes in neuronal activity (Goldsberry et al., 2015). Neuronal firing differed not only in magnitude, but also in complexity. The youngest age group had fewer cells that responded to tone and trace intervals.

Overall, the above data demonstrate that hippocampal neuronal activity is correlated with the developmental emergence of trace EBC. However, the factors influencing hippocampal activity remain unclear. Although the hypothesis that hippocampal development is the principle factor limiting trace conditioning is reasonable, an alternative hypothesis is that the development of sensory input to the hippocampus could limit acquisition of trace conditioning. If it is the case that sensory pathways to the hippocampus are immature, then it would not receive sufficient CS-related information, thus weakening its ability to associate the CS with the US. Training pups with an earlier-developing sensory modality may increase input to the hippocampus, thus facilitating trace conditioning and increasing associative neuronal activity in the hippocampus.

The concept that sensory system development can play a role in the ontogenetic emergence of learning is not a new one. In fact, previous work from our laboratory has shown that the development of delay EBC is limited by sensory pathway development to the pontine nucleus (Campolattaro & Freeman, 2008; Freeman, 2010; Freeman & Campolattaro, 2008; Freeman & Duffel, 2008; Freeman & Rabinak, 2004; Freeman et al., 2005; Goldsberry et al., 2014; Ng & Freeman, 2012). The auditory and visual sensory systems that feed CS-related information to the pontine do not come online until relatively late in developing rat pups (Freeman & Muckler, 2003). The meatal canal of the ear and eyelids remain closed in rat pups

until postnatal days (P) 12 and P14. Moreover, learning with a given modality does not emerge until after that sensory system has become functional (Hyson & Rudy, 1984). In the case of auditory delay EBC, learning with a tone CS is not evident before P17-P18 (Ivkovich et al., 2000; Stanton et al., 1992).

One possible way to facilitate the developmental emergence of delay EBC is to bypass late-developing sensory systems. Indeed, pups as young as P12-13 are capable of showing nearly adult-like levels of delay EBC when trained with a pontine stimulation CS (Campolattaro & Freeman, 2008). An alternative way to bypass late-developing sensory input to the pontine nucleus is to use an early-developing sensory system. The somatosensory system is the earliest developing sensory system (Alberts, 1984). In fact, tactile stimulation of the forepaw results in evoked responses in pups as young as embryonic day 16 (Narayanan et al., 1971). As expected, learning with a somatosensory CS emerges somewhat later. By P0 there is evidence that pups are able to show low levels of conditioned fear as measured by limb flexion (Bachevalier & Blozovski, 1980; Caldwell & Werboff, 1962).

Based on the above-mentioned work, our laboratory recently investigated the developmental emergence of delay EBC with a vibrating grid floor somatosensory CS (144 Hz with an acceleration of 2.4 m/s<sup>2</sup>) (Goldsberry et al., 2014). Pups aged P14-15, P17-18, P21-22, and P24-25 were trained in somatosensory delay eyeblink conditioning for a total of six sessions over two days. All but the youngest age group showed asymptotic levels of conditioned responding by session four. Moreover, employing a vibration CS at these ages completely eliminated the developmental differences in conditioned responding previously observed in auditory and visual eyeblink conditioning. Even the youngest pups, those trained on P14-15, showed significant increases in conditioned responding across sessions; albeit the overall rate of

learning was substantially lower than that observed in older animals. Importantly, this observed increase in conditioned responding was not accompanied by changes in nonassociative responding. Animals trained with unpaired presentations of the CS and US in the vibration condition performed similarly to animals trained with a tone CS, therefore suggesting that the vibration CS does not elicit a greater likelihood of nonassociative blinking than the tone CS does. Therefore, the use of a vibration CS not only increases conditioned responding, but it results in a completely different pattern of ontogenetic emergence. When trained with a tone CS, pups show a gradual age-related increase in conditioned responding from P17 to P25. However, when trained with a vibration CS these same age groups perform almost identically to one another. Only the youngest vibration-trained age group, P14-15, differed from the oldest animals. Overall, these data lend support to the hypothesis that the ontogenetic emergence of delay eyeblink conditioning is dependent on the development of sensory input to the pontine nucleus (Goldsberry et al., 2014).

If sensory system input is capable of playing such a large role in learning with an early-developing brain structure, such as the pontine nucleus, it is plausible that the same could be true for a late-developing structure, such as the hippocampus. Therefore, the goal of the current study was to test the hypothesis that sensory system development could play a role in the development of trace eyeblink conditioning. Rat pups aged P17-18, P21-22, or P24-25 were trained in both paired or unpaired trace eyeblink conditioning for a total of six sessions over two days, with either a tone CS or a vibrating CS.

## **Methods**

### **Subjects**

Subjects were 116 Long-Evans rat pups. All pups were born and reared in the Spence Laboratories animal colony at the University of Iowa. Cages were checked daily for new births and the day of birth was designated as P0. Litters were culled to eight pups each on P2. Pups remained with the dam until P19, when they were weaned and housed with same-sex littermates. Each experimental group contained no more than two pups from each litter, one male and one female. All behavioral training occurred during the light portion of the light/dark cycle (12/12-hr light/dark cycle, light onset at 7am). All procedures were approved by the Institutional Animal Care and Use Committee at the University of Iowa.

### **Surgery**

Behavioral surgery was conducted 2 days prior to training, on P15, P19, or P22. Isoflurane anesthesia (1.5-3%) was administered and the pup's head was fixed and aligned in a mouse stereotaxic apparatus. Surgery involved implanting differential EMG electrodes that were used to record eyelid activity and a bipolar stimulating electrode for US delivery. The EMG electrodes consisted of a plastic connector which housed 3 small gold pins. Each pin was soldered to an individual wire, 2 thin recording electrodes and a ground wire. The ground wire was attached to the skull with a stainless steel skull screw and the recording electrode wires were implanted in the upper orbicularis oculi muscle. During surgery the plastic connector was cemented to the pup's skull with bone cement (Zimmer, Warsaw, IN), leaving the gold pins exposed. The bipolar stimulating electrode was implanted following the EMG electrodes. The tips of the stimulating electrode were placed subdermally, just caudal to the left eye. The wires of the bipolar electrode terminated in a small plastic connector which was also cemented to the skull using bone cement.

## **Conditioning Apparatus**

The conditioning apparatus has been previously described in detail (Goldsberry et al., 2014). Briefly, pups received behavioral training in a clear Plexiglas conditioning chamber which was housed within a larger sound-attenuation chamber. The tone CS (2kHz, 82 dB) was delivered with 2 small speakers, which were fitted on one side of the Plexiglas chamber. The vibration CS (114 Hz frequency, acceleration of 2.4 m/s<sup>2</sup>) was delivered via a custom-build vibrating grid floor, which was placed on a foam pad to minimize sound. Vibrations were delivered with a small vibration motor (model number C1030B028F; JinLong Machinery, Zhejiang, China). A similar vibration CS has been used in previous papers investigating early learning in rat pups (Goldsberry et al., 2014; Markiewicz et al., 1986; Spear & Smith, 1978). Prior to training, the recording EMG and bipolar stimulating electrodes were connected to lightweight cables which were threaded through small holes in both chambers and connected to a commutator suspended above the outer sound-attenuating chamber. Both CS and US delivery were controlled with computer software which simultaneously recorded differential eyelid EMG activity (sampling rate = 250 Hz). All EMG activity was amplified (x 2000), filtered (500-5000 Hz), and integrated (20 ms time constant).

## **Conditioning Procedure**

Conditioning occurred over the course of 2 days. All pups received 6 one-hour sessions (3/day) of either paired or unpaired training. Pups in the paired group received 100 trials per session of trace EBC with a 250 ms vibration CS, a 500 ms stimulus-free trace interval, and a 25 ms periorbital shock US. The 100 trials per session were divided into blocks of ten trials each, with the tenth trial of each block acting as a probe trial, thus containing only the vibration CS. Probe trials were used to examine parameters of the CR in the absence of the UR (Gormezano et al.,



1983). Paired trials were separated by an average ITI of 30 s. Pups in the unpaired group received 200 trials per session. Each trial consisted of an unpaired presentation of either the vibration CS or the shock US (pseudo-random presentation, 100 of each stimulus). Unpaired training trials were separated by an average ITI of 15 s.

### **Data Analysis**

Following conditioning, behavioral data were examined offline. Learning was indicated by the presence of a CR. CRs were defined as blink responses which occurred 80 ms after CS onset but prior to US onset (threshold was set at .4 units above pre-CS baseline EMG activity). Trials were excluded from further analysis if excessive pre-CS activity contaminated the baseline EMG activity.

Session data concerning CR percentage, adaptive CR percentage, CR amplitude, onset latency, and peak latency were compared across age groups and training conditions (paired and unpaired) using repeated measures ANOVA. Greenhouse-Geisser corrections for violations of sphericity were applied when appropriate. Adaptive CR percentage differed from CR percentage in that adaptive CRs occur closer to the UR and appear to be more influenced by hippocampal manipulations (Solomon, Vander Schaaf, et al., 1986). Only CRs which occurred during the final 250 ms of the trace interval were included in the analysis of adaptive CRs. CR amplitude, onset latency, and peak latency measures were performed on data from all paired trials in order to ensure that we had a sufficiently large sample size. When ANOVA revealed significant group effects Bonferroni *Post Hoc* tests controlling for multiple comparisons were used to determine significant differences between groups. Significance was defined as an alpha level of 0.05 for all statistical tests.

## **Results**

Rat pups that received paired EBC had a higher percentage of CRs than those that received unpaired training (Figure 5). Moreover, when comparing CS modality, pups trained with a vibration CS showed a higher percentage of CRs than those trained with a tone CS during paired, but not unpaired training (Figure 5). Regardless of modality, paired training resulted in age-related increases in CRs, with older pups showing more CRs than younger pups. These findings are similar to observations from previous work in delay EBC, which demonstrated that training with a vibration CS results in an age-related increase in associative, but not non-associative responding (Goldsberry et al., 2014).

A repeated-measures ANOVA on the CR percentage session data confirmed these observations with a session X CS-type (tone or vibration) X condition (paired or unpaired) interaction  $F(3.264, 146.596) = 3.225, p = 0.020$ . When broken down to look at paired training only, a repeated-measures ANOVA on CR percentage showed that there was a session X age interaction  $F(5.984, 339.507) = 2.345, p = 0.034$  and main effects of both age  $F(2, 49) = 6.773, p = 0.003$  and CS-modality  $F(1, 49) = 26.911, p = 0.000004$ . Post hoc tests examining these effects with sessions collapsed across age groups reveal that although the oldest and youngest groups were significantly different from one another ( $p = 0.014$ ) when trained with a tone CS, there were no between-age differences in the percentage of CRs for vibration-trained pups. Examining differences that occurred session by session during tone training revealed that the oldest age group had significantly more CRs than the youngest group on the last three sessions of training ( $p < 0.01$ ).

The percentage of adaptive CRs showed a similar pattern. A session X CS-type X condition interaction was found  $F(3.372, 350.711) = 5.731, p < 0.000001$ . When examining only

paired data, there were session X age  $F(6.744, 165.239) = 4.226, p = 0.0003$  and session X CS interactions  $F(3.372, 165.239) = 5.173, p = 0.001$ . *Post hoc* tests collapsed across sessions comparing age group further showed that although there was a significant difference between the oldest and youngest age groups when trained with a tone CS ( $p = 0.0004$ ), there were no significant age-group differences when pups were trained with a vibration CS. Comparing adaptive CR percentage on individual tone-trained sessions revealed that P24-25 pups outperformed P17-18 pups on sessions 2-6 ( $p < 0.05$ ) and P21-23 pups on sessions 4-6 ( $p < 0.05$ ). The P17-18 and P21-23 age groups differed on sessions 3-6 ( $p < 0.05$ ).

The amplitude of the CR was influenced by age and session, but not by CS modality (Figure 6). A repeated-measures ANOVA revealed a session X age interaction  $F(6.468, 158.477) = 2.519, p = 0.02$ . *Post hoc* tests showed that there was an age-related increase in the amplitude of the CR with the oldest and youngest groups differing significantly from one another ( $p = 0.004$ ). The middle age group had intermediate CR amplitudes (Figure 6).

CR onset latency was not influenced by session, but there were main effects of age  $F(2, 49) = 4.163, p = 0.021$ , CS modality  $F(1, 49) = 30.409, p = 0.000001$ , and an age X CS interaction  $F(2, 49) = 3.656, p = 0.033$ . Although tone-trained pups had an age-related increase in CR onset latency, there was no clear pattern for vibration trained pups. When comparing tone-trained age groups, the youngest group has significantly shorter onsets than both the middle ( $p = 0.019$ ) and oldest ( $p = 0.0002$ ) groups, whereas the oldest two age groups did not differ significantly.

The peak latency of the CR showed a session X CS modality interaction  $F(5, 245) = 3.407, p = 0.005$ . Whereas pups that were trained with a tone CS had an age-related increase in peak latency  $F(2, 24) = 8.853, p = 0.001$ , there were no differences across sessions. The oldest

age group had a significantly higher peak latency than the youngest age group ( $p = 0.001$ ) and the middle age group performed intermediately. Pups trained with a vibration CS performed similarly across age groups. However, there was a main effect of session  $F(5, 125) = 5.961, p = 0.00006$ , with all age groups showing increased latency as training continued.

Finally, although the UR amplitude did not differ between CS modalities or across sessions, there was a main effect of age  $F(2, 49) = 5.977, p = 0.005$ . Bonferroni *post hoc* tests showed an age-related increase in UR amplitude, with the youngest age group having lower amplitudes than the oldest age group ( $p = 0.004$ ). The middle age group performed intermediately and did not significantly differ from the other two groups.

## **Discussion**

Rat pups trained with a vibration CS showed stronger levels of conditioning that emerged ontogenetically earlier than those observed with a tone CS. Whereas the youngest age group trained with a tone did not show any increases in CRs across training, that same age group, when trained with a vibration CS had CRs increasing from 27% in session one to 53% by session six. This age group therefore outperformed even the oldest age group trained with a tone, as their CRs only reached 46% by session six. These increases in CR percentage were not due to non-associative factors, such as overall increased responding to the vibration CS. When comparing response levels observed during unpaired training, there were no differences between pups trained with a vibration versus a tone CS.

Our interpretation of the earlier emergence of trace EBC when using a vibration CS is that it is likely due to the development of sensory pathways that project either directly or indirectly to the trace EBC circuitry. To date, the neural pathway utilized by the floor vibration CS has not been established. Sensory input could be received via exteroceptive or proprioceptive

pathways, or both. Exteroceptive sensory input from vibration stimulation arrives at the level of the dorsal column nuclei via spinal afferents. The dorsal column nuclei then project to the medial pontine nucleus (Kosinski, Azizi, et al., 1986; Kosinski, Neafsey, et al., 1986). Despite the fact that there are additional direct projections from the dorsal column nuclei to the inferior cerebellar peduncle and cerebellar cortex, these routes are not believed to be the principle pathway for the vibration CS. Instead, the medial cerebellar peduncle, which receives input from the pontine nucleus, has been found to be necessary to conditioned responding to a vibration CS in delay EBC (Hesslow et al., 1999; Lewis et al., 1987; Solomon, Lewis, et al., 1986). Thus, at least in the case of delay EBC, the hypothesized CS pathway for a vibration CS is from the dorsal column nuclei to the pontine nucleus.

It is currently unclear how the inclusion of the trace interval may affect this circuitry. Although work in adult animals has determined that trace conditioning requires the additional involvement of forebrain structures, such as the hippocampus, medial prefrontal cortex, and sensory cortices (Galvez, Weible, & Disterhoft, 2007; Galvez, Weiss, Weible, & Disterhoft, 2006; Kronforst-Collins & Disterhoft, 1998; Moyer et al., 1990; Solomon, Vander Schaaf, et al., 1986; Steinmetz, Harmon, & Freeman, 2013; Takehara et al., 2003; Ward, Flores, & Disterhoft, 2012; Weible et al., 2007; Weiss et al., 1996), it is unclear how these are integrated into the already-established delay EBC circuitry (Woodruff-Pak & Disterhoft, 2008). For instance, in both trace and delay EBC, CS information likely arrives at the level of the pontine nucleus prior to projecting to the cerebellum and related learning circuitry. However, in the case of trace EBC, parallel sensory input arrives at the sensory thalamus before projecting to the sensory cortex (Galvez et al., 2007; Galvez et al., 2006; Ward et al., 2012). The sensory cortex then projects to other forebrain structures, such as the hippocampus and medial prefrontal cortex, before once

again projecting to the pontine nucleus and related cerebellar circuitry (Takehara-Nishiuchi, 2014; Weiss & Disterhoft, 2011; Woodruff-Pak & Disterhoft, 2008). Because of this, the earlier emergence of trace EBC with a vibration CS could be due to the development of any one of the above-mentioned structures or their projections.

Although hippocampal development undoubtedly plays a critical role in the ontogenetic emergence of trace EBC, data from the current study suggest that sensory system development upstream from the hippocampus may also contribute to the developmental trajectory of trace EBC. Future studies could test this hypothesis by recording neuronal activity from the hippocampus in vibration-trained pups, to determine whether using an earlier developing sensory modality would increase neuronal responsiveness in pups trained in trace EBC.

Although using an early-developing vibration CS facilitates the developmental emergence of trace EBC, there is some evidence that it too may be developmentally limited. One potential developmental limitation to the ontogenetic emergence of somatosensory trace EBC could be the development of communication between the somatosensory cortex and hippocampus. Although the hippocampus does show somatosensory cortex-evoked activity as early as P7 (Mohns & Blumberg, 2010), work examining the development of the somatosensory cortex suggests that it does not begin to show topographic organization until P15 (Seelke, Dooley, & Krubitzer, 2012). Moreover, it does not reach adult-like levels of organization until P20 (Seelke et al., 2012). These findings suggest that although the hippocampus and the somatosensory cortex are functionally connected at a relatively young age, the nature of this connection may be undergoing substantial development as the somatosensory cortex continues to reach adult-like levels of topographic organization. Indeed, previous work examining how sensory system development influences the ontogeny of learning and memory show that learning

with a given modality does not emerge until after the given sensory system is fully functional (Hyson & Rudy, 1984).

In conclusion, this study demonstrated that using a vibration CS results in facilitated acquisition of trace EBC in rat pups relative to those trained with an auditory CS. In fact, to our knowledge, this is the earliest demonstration of trace EBC in developing rat pups. Moreover, this data set, in combination with previous work examining delay EBC, suggests that this increase in conditioned responding is not simply due to non-associative factors. Rather, pups show increased learning when trained with earlier-developing sensory systems. This would suggest that even late-developing hippocampal forms of learning can be modulated by sensory system development.

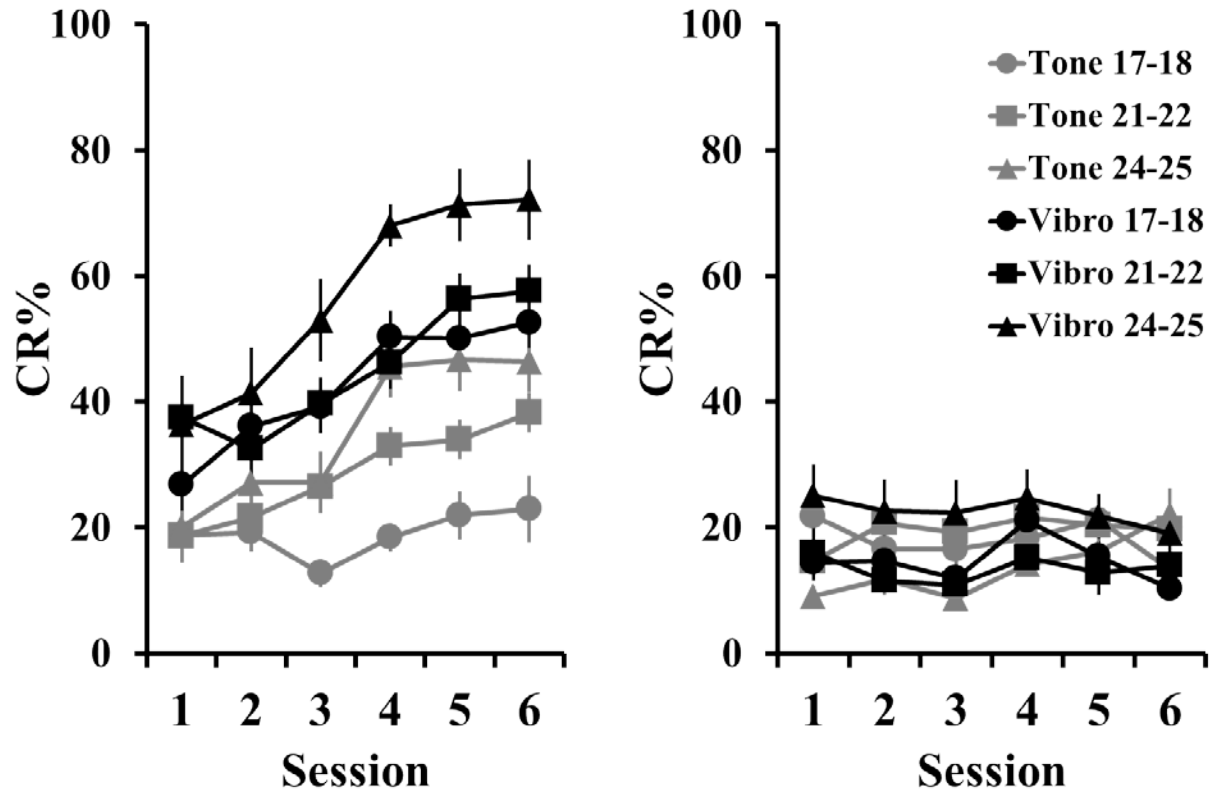


Figure 5. Conditioned response percentage during paired and unpaired trace EBC with a tone and vibration CS.

Mean (+ SEM) eyeblink conditioned response (CR) percentage of rat pups given paired (left) or unpaired (right) training with a tone (grey) or vibration (black) conditioned stimulus (CS). Pups were trained with the tone on postnatal days (P)17-18 (n = 10 paired, 8 unpaired), 21-22 (n = 9 paired, 10 unpaired), or P24-25 (n = 8 paired, 9 unpaired). Pups were trained with the vibration CS on P17-18 (n = 8 paired, 10 unpaired), P21-22 (n = 9 paired, 12 unpaired), or P24-25 (n = 10 paired, 12 unpaired).



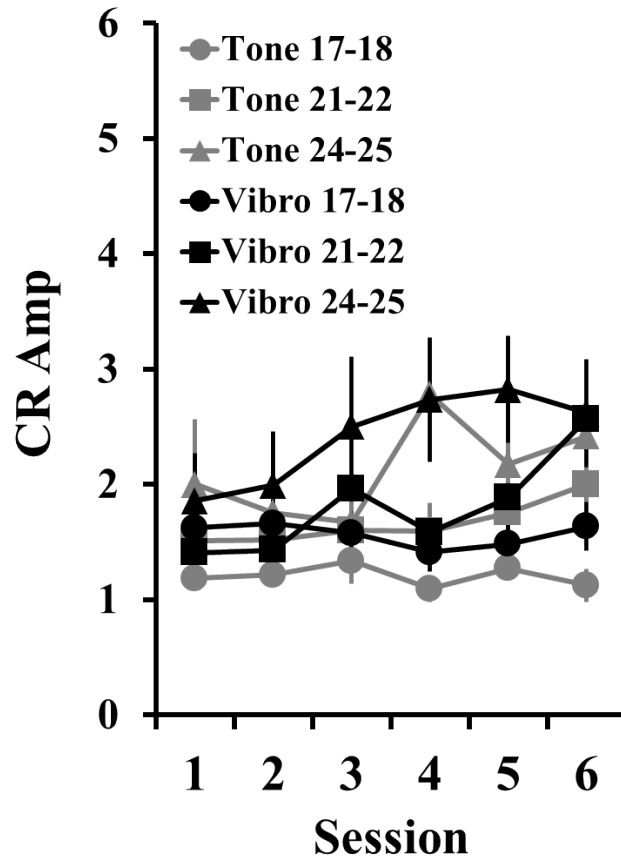


Figure 6. Conditioned response amplitude for rats given paired trace EBC with a tone and vibration CS.

Mean (+ SEM) eyeblink conditioned response (CR) amplitude for rat pups given paired training with a tone (grey) or vibration (black) conditioned stimulus (CS).

## **CHAPTER 3: DEVELOPMENTAL CHANGES IN HIPPOCAMPAL ASSOCIATIVE CODING**

Developmental studies of learning and memory indicate that hippocampus-dependent memory develops after hippocampus-independent memory in altricial mammalian species such as humans, monkeys, and rats (Stanton, 2000; Stanton et al., 2009). Learning that is independent of hippocampal function tends to emerge much earlier in development, in some cases even prenatally (Smotherman, 1982). However, due to the relatively late maturation of forebrain structures, such as the hippocampus (Frotscher & Seress, 2007), other types of learning do not begin to emerge until much later in development (Ivkovich & Stanton, 2001). Several approaches have been used to examine the development of learning, including behavioral analyses of hippocampus-dependent tasks such as spatial learning. Spatial learning emerges relatively late in altricial species and parallels some aspects of hippocampal anatomical development (Freeman & Stanton, 1991; Green & Stanton, 1989; Rudy & Paylor, 1988). Rudy and Paylor (1988) found that although postnatal day (P) 22 rat pups successfully learn the water maze place-learning task, P19 pups are unable to. One possible explanation for the delayed development of place-learning could be that it is limited by the development of hippocampal function. Recent work characterizing the development of place fields in the hippocampus has indirectly supported this hypothesis by demonstrating that the development of place fields occurs between the ages of P16 and P28 (Langston et al., 2010; Wills et al., 2010). However, these studies did not directly examine the relationship between hippocampal physiological development and the ontogeny of memory. Moreover, the behavioral development of complex memory processes such as spatial navigation can be difficult to compare across ages due to the concurrent development of sensory processing and motor control.

One possible solution to these limitations is to employ a learning task that is less affected by motor and sensory development, such as EBC (Stanton et al., 1992). The primary goal of the current study was to examine the relationship between hippocampal physiological development and the ontogeny of a hippocampus-dependent task, trace EBC. In trace EBC a conditioned stimulus (CS, e.g., a tone) is followed by an unconditioned stimulus (US) that elicits a reflexive blink. The CS and US are temporally separated by a brief stimulus-free “trace” interval. The inclusion of this trace interval makes the task hippocampus-dependent (Clark, Manns, & Squire, 2002; Moyer et al., 1990; Solomon, Vander Schaaf, et al., 1986). Adult hippocampal CA1 pyramidal cells respond to the events within the trace conditioning trials including the CS, trace interval, and the US (Green & Arenos, 2007; McEchron & Disterhoft, 1997; Weible et al., 2006). The late emergence of trace conditioning parallels the developmental trajectory of spatial learning and is likewise believed to be due to delayed development of the hippocampus and other forebrain structures (Ivkovich et al., 2000). If the developmental trajectory of this learning task is indeed due to the late development of the hippocampus we would anticipate age- and learning-related changes in the responsiveness of hippocampal neurons.

## **Methods**

### **Subjects**

Subjects were 15 Long-Evans rat pups (7 females and 8 males), all from different litters. For paired training (six sessions) there were three pups used in each age group. For unpaired training (also six sessions per animal) there were 3 pups in the oldest age group, 2 pups in the middle age group, and 1 pup in the youngest age group. For neuron counts per group, please see Table 1. All pups were born and reared in the Spence Laboratories of Psychology animal colony at the University of Iowa. The colony was maintained on a 12/12-hr light/dark cycle, with light onset at

7 am. All procedures were approved by the University of Iowa Institutional Animal Care and Use Committee.

## **Surgery**

Detailed surgical methods have been previously published (Ng & Freeman, 2012). We implanted rat pups with microdrives for neuronal recording with multiple tetrodes on postnatal day (P)19, P22, and P29, two days prior to training. Pups were anesthetized using 1.5-3% isoflurane gas. During surgery rats were fitted with a microdrive for neuronal recording, differential EMG electrodes to record blink activity, and a bipolar stimulating electrode for US delivery.

For microdrive implantation, a small hole was drilled in the skull directly above the right dorsal hippocampus (AP, -4.0 mm; ML, -2.5 mm). The microdrive was lowered until the tetrodes touched the brain surface. Any additional space between the drive and the skull was filled with a low-viscosity silicone gel (Kwik-Sil, World Precision Instruments, Sarasota, FL). The microdrive was then grounded with a stainless steel screw fixed to the skull. Immediately following implantation, the tetrodes were lowered approximately 0.6 mm into the brain.

In order to record differential EMG activity from the eyelid, two stainless steel electrodes were threaded through the left upper orbicularis oculi muscle and a ground wire was attached to the skull with a screw. The bipolar stimulating electrode for US delivery was placed subdermally immediately caudal to the left eye.

On the day following surgery spike activity was monitored as the recording tetrodes were lowered into the CA1 layer (approximately DV, -2.1mm) and the reference tetrode was lowered into the cortex dorsal to the hippocampus (approximately DV, -0.9).

## **Spike data acquisition**

Spike data acquisition has been previously described in detail (Ng & Freeman, 2012). Pups were surgically implanted with either an 8- or 16-channel microdrive array for either 2 or 4 recording tetrodes, respectively. Each drive had a separate, independently-moving reference channel tetrode. Prior to implantation, each tetrode was gold-plated until the tetrode impedance was approximately 350 k $\Omega$ .

During data collection and tetrode lowering the microdrive was connected to a spike acquisition system (Neuralynx, Bozeman, MT). The spike signal was then amplified at a gain of 10,000-25,000 and band-pass filtered between 0.6 – 6.0 kHz. Signals were digitized and stored at 32 kHz (Cheetah, Neuralynx, Bozeman, MT).

## **Conditioning apparatus**

The conditioning apparatus has been previously described in detail (Ng & Freeman, 2012). Rat pups were trained within a conditioning chamber that was contained within a sound-attenuation chamber. Lightweight cables with connectors for both the recording EMG and the bipolar US electrode were attached to a commutator above the conditioning chamber and threaded through a hole in the ceiling of the chamber. Computer software controlled the delivery of both CS and US while simultaneously recording differential eyelid EMG activity (sampling rate = 250 Hz). EMG activity was amplified (x 2000), filtered (500-5000 Hz), and integrated (20 ms time constant).

## **Conditioning procedures**

In order to parse out non-associative responding to the CS from learning, pups were given either paired or unpaired presentations of the CS and US. Pups in the paired group

received 100 trials per session of trace EBC with a 250 ms (2 kHz) tone CS, a 500 ms trace interval, and a 25 ms periorbital stimulation US (2-3 mA) (Figure 7). In adult animals, this training paradigm results in a learned association between the CS and US. Each paired session was divided into ten blocks of ten trials. The first nine trials of the block were paired CS-US presentations and the tenth trial of each block was a probe trial, containing only the tone CS. The probe trials were used to evaluate conditioned responding (CR) in the absence of the unconditioned response (Gormezano et al., 1983). Paired trials were separated by a variable intertrial interval that averaged 30 s. Learning was demonstrated by the presence of a conditioned blink response (CR) after CS onset but before US onset on a given trial. The CR threshold was 0.4V above the amplified and integrated baseline EMG activity. Pups in the unpaired group received 200 trials per session of either the CS or the US. In adult animals unpaired training does not result in a learned association because the CS and US are presented separately. Unpaired trials were separated by a variable intertrial interval that averaged 15 s. Any response that occurred during the first 80 ms of the CS was considered a startle response and omitted from future analyses.

### **Neuronal recording analyses**

Offline neuron separation was initially performed automatically with KlustaKwik (Kadir, Goodman, & Harris, 2013). Separated neurons were then manually inspected and refined using MClust-3.5 (Redish et al., 2010). Neurons were classified as pyramidal cells if they (1) showed a bursting pattern of activity as demonstrated by a peak in the autocorrelogram at 3-8 ms, (2) had a baseline (500 ms sample duration prior to CS onset) firing rate of less than 10.5 spikes/second, and (3) had at least 300 spikes during the training session.

Neuronal activity was then analyzed in relation to trial event responsivity using NeuroExplorer (Madison, AL). First, neurons were classified according to the trial event(s) they responded to (CS, trace interval, US). To accomplish this, the trial was divided into nine 125 ms intervals. The nine time intervals included baseline (125 ms), CS (250 ms), trace (500 ms), and US periods (250 ms). Firing rate for each neuron was normalized to the pre-CS baseline using z-score values in NeuroExplorer. Intervals that had values exceeding the pre-established 99% confidence limits based on the Poisson distribution (two-tailed,  $\alpha < 0.05$ ) were considered to be statistically significant from the baseline firing rate, thus showing either excitatory or inhibitory responses to trial events. Based on which intervals were different from baseline, neurons were classified as unresponsive, CS-responsive, trace-responsive, US-responsive, or a combination thereof. Therefore, categories were overlapping and combination units could be considered as belonging to more than one category. For example, a unit that showed increased activity during both the CS and the US periods would be categorized as a CS-responsive unit and a US-responsive unit (Figure 8). The proportion of neurons that fell into each response category was compared across age groups and sessions with chi-square analyses.

The magnitude of the neuronal response was also examined. Peristimulus-time histograms of neuronal activity were generated with 12.5 ms bins and normalized (normalized bin = (bin mean – 125 ms baseline mean)/standard deviation of 125 ms baseline)). The normalized bin values of responsive neurons were then compared across age and CR/no-CR trials with a repeated-measures ANOVA and the Tukey HSD post hoc test to examine age-related differences in the magnitude of neuronal responding during the trial.

## **Histology**

Histological methods have been previously described in detail (Ng & Freeman, 2012). Tetrode placement was determined by creating small electrolytic lesions after the last session of training. Brains were placed in a 30% sucrose-formalin solution upon removal, sectioned at 50  $\mu\text{m}$ , mounted on slides, and stained with thionin. Histology was then examined with a light microscope to determine tetrode placement. Only placements confirmed to be in the CA1 layer of the hippocampus were included in the analysis.

## **Results**

### **Behavioral Data**

Behavioral analyses of learning, as demonstrated by conditioned responding across age and training type revealed that there were significant differences between groups (Figure 7). When pups received paired training, there was an age-related increase in learning, with older pups learning the association better than younger pups. However, there were no significant differences between age groups when pups received unpaired training. These observations were confirmed with a repeated measures ANOVA examining differences in the percentage of CRs across sessions (sessions 1-6), training condition (paired vs. unpaired) and age (P21-23, P24-26, or P31-33), which found a session X training condition X age interaction ( $F(10, 45) = 2.654, P = 0.012$ ) (Figure 7). This interaction was further examined with Tukey HSD post hoc tests. During paired training the P31-33 age group had a significantly greater CR percentage on sessions 1-6 than the P21-23 group ( $P < 0.01$ ). The P31-33 age group also had a significantly higher CR percentage on sessions 1-4 when compared to the P24-26 age group ( $P < 0.01$ ). Finally, the two youngest age



groups differed from each other on sessions 5-6 ( $P < 0.01$ ). For unpaired training there were no significant differences in the percentage of CRs across age.

### **Neuronal Responsiveness**

Recorded pyramidal neurons from the CA1 field of the hippocampus were categorized (Figure 8) according to their firing rates as either responsive or unresponsive to trial events ( $n = 1812$ ). Responsiveness was further categorized as either excitatory (showing increases in activity) or inhibitory (showing decreases in activity). The proportion of neurons that showed inhibitory responses was extremely low, and therefore excluded from further statistical analyses. The proportion of neurons that showed either excitatory or no response were then compared across categories using chi-square analyses (Table 1). The proportion of excitatory responsive neurons was greater when pups were given paired training than unpaired training ( $X^2(1, N = 1812) = 18.49, P = 0.0001$ ) (Figure 9). These differences in neuronal responsiveness across paired and unpaired training indicate that the associative nature of the CS and US during paired training leads to an increase in neuronal recruitment.

Within the paired training group there were no differences across age for the proportion of responsive versus unresponsive neurons ( $X^2(2, N = 1310) = 1.30, P > 0.05$ ). However, this analysis only examined overall responsiveness and did not take into account responsiveness to individual trial events (e.g., the US). When trial components were broken down into CS, trace, and US components several age-related changes were found.

#### Proportion of responsive neurons during paired training

When CS responsivity was examined, an age-related increase in the proportion of neurons that responded to the CS was observed  $X^2(2, N = 1310) = 14.71, P = 0.0006$  (Figure 9A). However,

the proportion of neurons that were responsive to the CS alone, as opposed to those responsive to the CS in combination with other trial events, was too low to statistically compare between age groups. During paired training, there were no significant differences across age in the proportion of neurons that showed responses to the US  $X^2(2, N = 1310) = 0.71, P = 0.70$  (this category included combination neurons that were also responsive to the CS and trace interval) (Figure 9B). However, when examining the proportion of neurons that showed increases in activity uniquely during the US (therefore not US combination neurons), a significant effect of age was found, with the proportion of US-only responsive neurons decreasing with age  $X^2(2, N = 1310) = 9.61, P = 0.008$  (Figure 9C). Thus, although all age groups showed similar levels of US-responsivity, older pups tended to have neurons that responded to the US in combination with other trial events whereas younger pups were more likely to have neurons that responded only to the US. In younger animals the increased proportion of neurons that were responsive to the US alone could be interpreted as a neuronal representation of surprise to the US presentation due to weaker associative prediction (Rescorla & Wagner, 1972). A second interpretation of these results is that the increased activity during the US is learning-related and serves as a precursor to the overt learned response (Berger, Alger, & Thompson, 1976).

There was also an age-related difference in the proportion of neurons that showed responding to a combination of trial events  $X^2(2, N = 1310) = 6.64, P = 0.036$  (Figure 9D). Younger animals had a lower proportion of cells that responded to a combination of trial events when compared to older animals. Thus, the older a pup is, the more likely its hippocampal neurons are to respond to a combination of trial events (Figure 9).

### Learning-related changes in the proportion of responsive neurons during paired training

In order to examine how learning affected neuronal activity, the proportion of neurons that showed changes in responsiveness between CR and no-CR trials were compared across age and sessions. Only neurons that showed responsiveness to trial events were included in the analysis. Depending on whether the responsiveness category was same or different between CR and no-CR trials, two different categorical values (e.g., 1 for same and 0 for different) were assigned to each neuron. These values were compared across sessions as well as across the age group with a Pearson's chi-square test. Note that we excluded sessions in which the CR percentage was less than 5% or more than 95% to have minimum numbers of samples in both CR and no-CR conditions.

The proportion of neurons that responded differentially during CR and no-CR trials did not differ significantly when compared across age groups  $X^2(2, N = 648) = 1.210, P = 0.546$ . Thus, although all three age groups had a large number of neurons that differed in responsiveness between CR and no-CR trials, there were no significant age-related differences (Figure 10A). When broken down by session as well as age group, between-group differences in responding were found during session 2 ( $X^2(2, N = 122) = 7.098, P = 0.029$ ). However, during the other sessions, the proportions were not statistically different across age groups ( $X^2s(2, N \geq 84) < 2.297, P_s > 0.317$ ) (Figure 10B). Lastly, the proportion of units that showed differential responses between CR and no-CR trials was examined across sessions separately for each age group. In the youngest two age groups the proportion of neurons that showed differential responsiveness was not statistically different across sessions ( $X^2s(2, N \geq 140) < 7.268, P_s > 0.201$ ). In the P31-33 age-group however, the proportion of neurons that responded differentially

to CR and no-CR trials significantly decreased across sessions  $X^2(5, N = 294) = 16.755, P = 0.005$ .

#### Proportion of responsive neurons during unpaired training

In contrast to paired training, during unpaired training the proportion of responsive neurons differed significantly across age, with an age-related decrease in responsivity ( $X^2(2, N = 502) = 22.84, P = .00001$ ). When examining trial events during unpaired training, there was a difference in US responsiveness across age (Figure 9B). The youngest age group had a greater proportion of responsive neurons compared to the oldest two age groups ( $X^2(2, N = 502) = 21.73, P = .000003$ ). This increased proportion of US-responsive neurons was primarily due to the extremely high proportion of US-responsive neurons during the first three sessions of P21-23 unpaired training (session1: 88%, session 2: 58%, session 3: 64%). Indeed, there was a significant decrease in the proportion of US-responsive neurons as unpaired training continued ( $X^2(5, N = 169) = 39.21, P = .0000002$ ). By the end of training, the P21-23 group neurons showed a similar level of US responsiveness to that seen in the older groups.

#### Changes in neuronal responsiveness across sessions in all age groups

When collapsed across age-group, differences in neuronal responsiveness across sessions were found. There was a significant decrease in the proportion of responsive neurons across sessions during both paired ( $X^2(5, N = 1310) = 26.82, P = 0.00006$ ) and unpaired ( $X^2(5, N = 502) = 24.81, P = 0.0002$ ) training (Figure 11). When neuronal responsiveness during unpaired sessions was further examined, chi-square analyses showed that the significant decrease in responding across sessions was most likely

due to the US responsive neurons. The proportion of US responsive ( $X^2(5, N = 502) = 21.57, P = 0.0006$ ), but not CS-responsive ( $X^2(5, N = 502) = 10.35, P = 0.066$ ) neurons significantly decreased across training sessions. When neuronal responsiveness across sessions was examined during paired training chi-square analyses revealed that the proportion of both CS ( $X^2(5, N = 1310) = 24.25, P = 0.0002$ ) and US ( $X^2(5, N = 1310) = 24.95, P = 0.0001$ ) responsive neurons decreased significantly as training continued. The proportion of trace responsive neurons, however, did not change across sessions ( $X^2(5, N = 1310) = 10.20, P = 0.07$ ).

### **Magnitude of Neuronal Responses to Trial Events**

#### Magnitude of neuronal response during paired training

In order to determine how the magnitude of the neuronal response changed across age, learning, and stimulus type, activity during trials was normalized to the pre-CS baseline (see methods section). Normalized neuronal activity of responsive neurons (Figures 12-14) was then examined with repeated measures ANOVA to determine which time bins were significantly affected by age and whether or not the animal showed a CR. Thus, the magnitude of the response was compared across age groups and learning. Overall, there was a significant age X CR X time bin interaction,  $F(41.87, 19196.93) = 1.486, P = 0.022$ . Tukey post hoc tests controlling for the number of comparisons and the Tukey-Kramer approach to unequal “n” were run on individual bins. These post hoc tests indicated that the oldest two age groups showed greater neuronal activity during the CS and trace periods on CR trials relative to no-CR trials (Figure 14). Specifically, in the P24-26 and 31-33 age groups there were 30 and 45 (respectively) out of 60 total bins that were significantly lower in the no-CR trials compared to the CR trials during the CS and trace periods. In contrast, when examining this averaged activity, all age groups had a decrease in the magnitude of responding to the US during CR trials when compared to no-CR trials, with this

effect being most evident in the youngest age group with 13 of 20 bins showing significant decreases (Figure 14). Learning was therefore associated with an age-related increase in the magnitude of the neuronal response during the CS and trace interval, but a decrease following the US. Additionally, there were age-related differences in the magnitude of neuronal responding across groups during CR trials. Specifically, four of 20 bins were significantly lower in the youngest age group than the two older age groups during the CS ( $P_s < 0.05$ ). During the trace interval, 6 of 40 bins were significantly lower in the youngest age group when compared to the older two age groups, and during the US period 11 of 20 bins were significantly lower in the youngest age group than the older two age groups ( $P_s < 0.05$ ).

#### Magnitude of neuronal response during unpaired training

The magnitude of the neuronal response was also evaluated during unpaired training (see Figure 12 for an example of single-unit neuronal activity). Normalized neuronal activity of responsive neurons during either CS- or US-alone unpaired trials was compared across age groups. When the magnitude of responding for unpaired CS-responsive neurons was examined, there was no effect of age on the magnitude of the neuronal response. This is in stark contrast to the robust age-related changes in response magnitude to the CS observed during paired training, a finding that suggests that the presence of the associative context is sufficient to produce increased activity during the CS presentation.

When the magnitude of responding for unpaired US responsive neurons was examined, there was a significant effect of age on the magnitude of the neuronal response ( $F(178, 18245) = 2.175, P = 0.0000001$ ). Post hoc tests showed that differences in the magnitude of responding were observed only in the first 150 ms after the US onset ( $P < 0.05$ ). Specifically, 4 of 20 bins were significantly higher in the P21-23 than the P24-26 group, 3 of 20 bins were significantly

higher in the P21-23 than the P31-34 group, and 2 of 20 bins were higher in the P31-34 group than the P24-26 group. However, unlike the differences observed in the magnitude of responding during paired training, no clear overall developmental changes were evident in these differences.

#### Changes in the magnitude of neuronal response across session

When the magnitude of responding for different age groups was compared across session, a repeated-measures ANOVA on responsive cells found an age X session X time bin interaction  $F(219.927, 14735.123) = 1.664, P = 0.0000001$  (Figure 13). Tukey post hoc tests ( $P_s < 0.05$ ) indicated that the majority of age-related changes in activity to the CS and trace interval occurred during the first two sessions of training (Figure 13). Although there were significant group differences in the magnitude of the neuronal response for individual bins during the US period, a clear developmental trend was not apparent. Table 2 depicts the number of significant bins between age groups during the CS, trace, and US intervals. During both sessions 1 and 2 the oldest age group had a significantly increased magnitude of responding during the CS period when compared to the younger two age groups. Although there were a few bins that showed increases in magnitude during the trace interval when compared to the younger two age groups, this effect was far more noticeable during session 2.

#### Discussion

As seen in previous studies (Ivkovich et al., 2000), rat pups trained in trace EBC did not show robust levels of conditioned responding until nearly the 4<sup>th</sup> postnatal week. This developmental trajectory is very similar to that seen in studies of spatial learning (Freeman & Stanton, 1991; Green & Stanton, 1989; Rudy & Paylor, 1988). However, just as observed in place cell development (Langston et al., 2010; Wills et al., 2010), the development of neuronal activity

preceded the development of the behavior. Even at the youngest age tested here (P21-23), hippocampal CA1 pyramidal cells showed changes related to associative learning. There was significantly greater responsivity to trial events when presented in the paired context than when presented in the unpaired context. Thus, even though the youngest rats were not showing overt behavioral signs of learning, they did, at the neuronal level, recognize the difference between associative and nonassociative contexts. However, the pattern of neuronal responsivity in younger rats differed substantially from older rats. This difference in CA1 responsiveness may be one of the underlying factors contributing to the developmental change in trace EBC. During paired sessions, neurons of younger rats were more likely to respond to a single-trial event, such as the US, whereas neurons of older rats were more likely to respond to a combination of trial events. The CA1 pyramidal cells of younger pups also showed a lower magnitude of responding during conditioning trials. This pattern of responsiveness suggests that the hippocampus of older rats is sufficiently developed to bind the US with other trial events, thus providing the basis for richer associative coding.

CA1 pyramidal cells also showed changes in responsivity across training sessions. Just as seen in adult animals, there was a decrease in responding to both the CS and the US as sessions progressed (McEchron & Disterhoft, 1997). Moreover, these changes were most evident in the oldest age group. These results support the current hypothesis that hippocampal involvement may be most critical early in training (Takehara et al., 2003).

All age groups showed differences in the magnitude of CA1 pyramidal cell responding to the US between learning and non-learning trials. However, in older animals learning was associated with an increase in neuronal responsiveness to the CS and trace intervals. This increased responding could be a neuronal representation of the pups attending to CS and trace



intervals as salient predictors of the US. Conversely, the increased magnitude of neuronal responding to the US during no-CR trials could indicate that the pup is “surprised” by the arrival of the US (i.e., a prediction error) (Rescorla & Wagner, 1972).

A number of developmental changes in both neurogenesis rates and cellular function occur during these ages that might explain the developmental changes in CA1 activity. There is an age-related increase in the strength of CA3 - CA1 synapses due to an increase in the probability of transmitter release (Dumas & Foster, 1995) which, in turn, could be a result of age-related changes in adenylyl cyclase expression (Matsuoka, Suzuki, Defer, Nakanishi, & Hanoune, 1997). Moreover, there is an overall increase in synaptic connectivity across development as indicated by an age-related increase in synaptic number and density (Harris, Jensen, & Tsao, 1992; Hsia, Malenka, & Nicoll, 1998). There is also some evidence that there is a postsynaptic decrease in LTP induction threshold, therefore contributing to greater levels of depolarization (Dumas, 2012). These and other changes in synaptic connectivity result in overall changes in synaptic plasticity. In addition to synaptic plasticity mechanisms, the rate of neurogenesis in the dentate gyrus could play a role in the development of hippocampal forms of learning (Akers et al., 2014). High rates of neurogenesis, as seen early in the postnatal development of altricial species, such as rats, are related to weaker memory and blocking dentate gyrus neurogenesis in young animals results in better retention (Akers et al., 2014). Developmental changes in dentate gyrus function related to changes in neurogenesis might have downstream effects on CA1 function that influence CA1 responses during associative learning. Thus, developmental changes in synaptic function and neurogenesis rates in the dentate gyrus may be reflected in the development of learning-related neuronal activity in CA1 pyramidal cells described in this report.

Developmental changes in hippocampal associative coding identified in the current study provide a more mechanistic understanding of the development of learning and memory relative to previous studies that relied on inferred mechanisms from developmental changes in learning or deficits produced by lesions. Previous studies of hippocampal function found that some of the quantitative features of place fields developed as a nearly linear function of postnatal age (Langston et al., 2010; Wills et al., 2010). Our findings suggest, however, that developmental changes in hippocampal associative coding are more abrupt developmentally, with a substantial transition between P21 and P24. The developmental increases in the strength and complexity of hippocampal coding may underlie the development of other hippocampus-dependent processes, such as episodic memory, which requires integration of object, spatial, and temporal information.

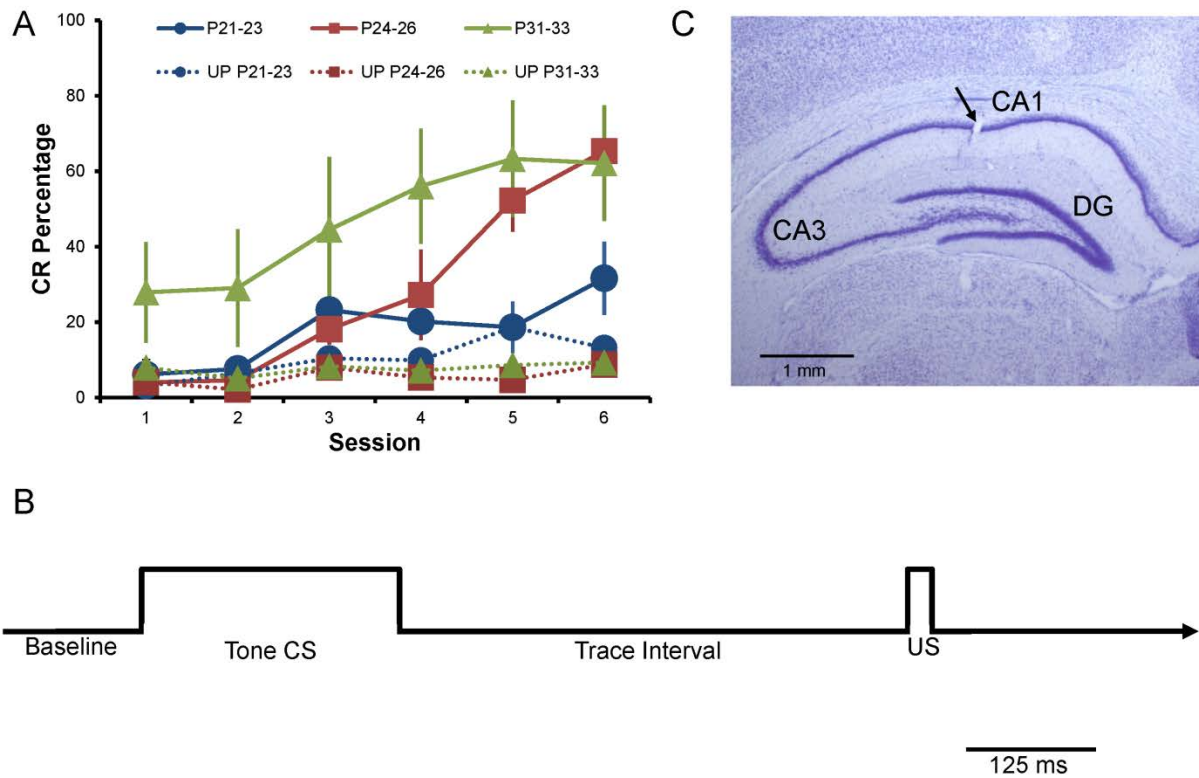


Figure 7. Hippocampus-dependent learning increases as a function of age.

(A) Mean ( $\pm$  SE) conditioned response (CR) percentage in rat pups given trace eyeblink conditioning on postnatal days (P) 21-23 (paired group,  $n = 3$ ; unpaired group,  $n=1$ ), P24-26 (paired group,  $n = 3$ ; unpaired group,  $n=2$ ), or P31-33 (paired group,  $n = 3$ ; unpaired group,  $n=3$ ). There was an increase in CR percentage as a function of age in the pups given paired training but not in the pups given unpaired training. (B) The paired trace conditioning procedure involved a 250 ms tone CS, a stimulus-free 500 ms trace interval, and 25 ms periorbital stimulation US. (C) Representative nissl-stained coronal brain section with tetrode placement in CA1 layer of hippocampus, tetrode marking lesion indicated by arrow.

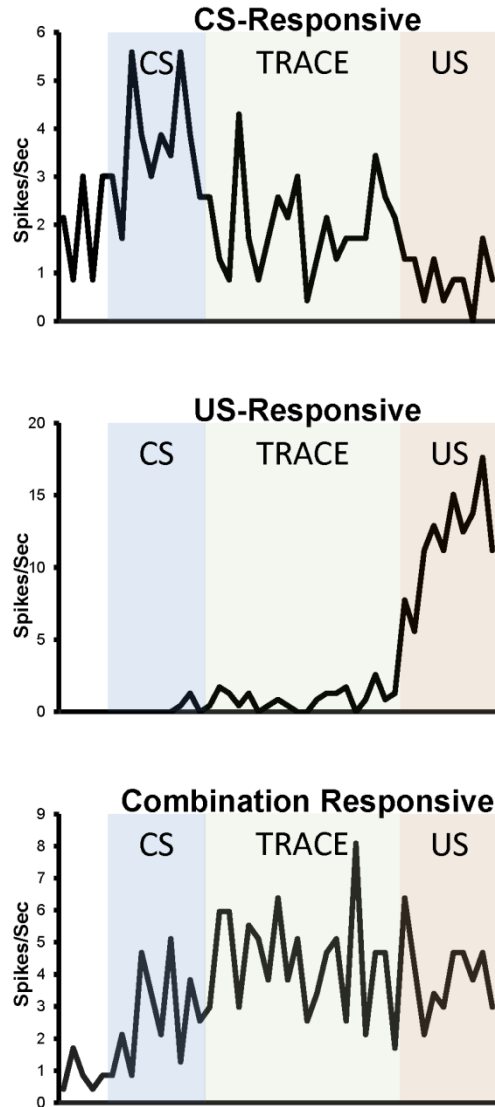


Figure 8. Example CA1 pyramidal cell firing rate profiles during trial presentation.

All figures include pre-CS baseline (125 ms), CS (250 ms), trace (500 ms), and US (250 ms) intervals. Z-score analyses were used to categorize neurons according to the responsiveness to trial events ( $P < 0.01$ ). The proportion of neurons in each category was compared across training conditions and age groups.

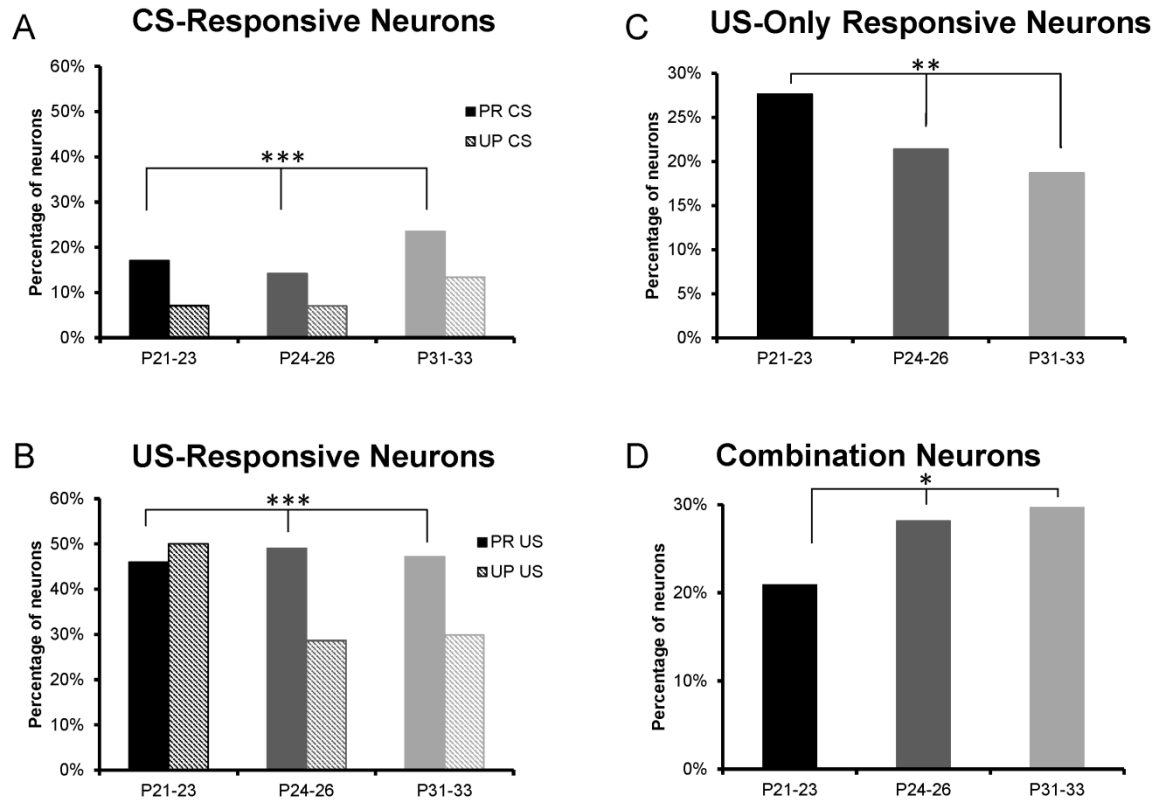


Figure 9. Hippocampal neurons respond differentially to trial events.

(A) Age-related difference in the proportion of cells that respond to the tone CS during paired but not unpaired training (this category includes neurons that were also responsive to other trial events, i.e., CS neurons that were also responsive to the trace interval). The difference in activity between paired and unpaired training is an index of associative activation. (B) Age-related difference in the proportion of neurons that responded to the US during unpaired but not paired training (this category includes neurons that were also responsive to other trial events, i.e., US neurons that were also responsive during the trace interval). The drop in US-related activity during unpaired training with age is related to learning that the CS does not predict the US. (C) Proportion of US-only responsive neurons decreased with age, which reflects an age-related increase in the proportion of neurons that respond to the US in combination with other learning-related events. (D) An age-related increase in the percentage of neurons that respond to a combination of trial events (CS, trace interval, and US) was also found. These findings indicate that there was a developmental increase in the percentage of neurons that showed complex coding of learning-related events.

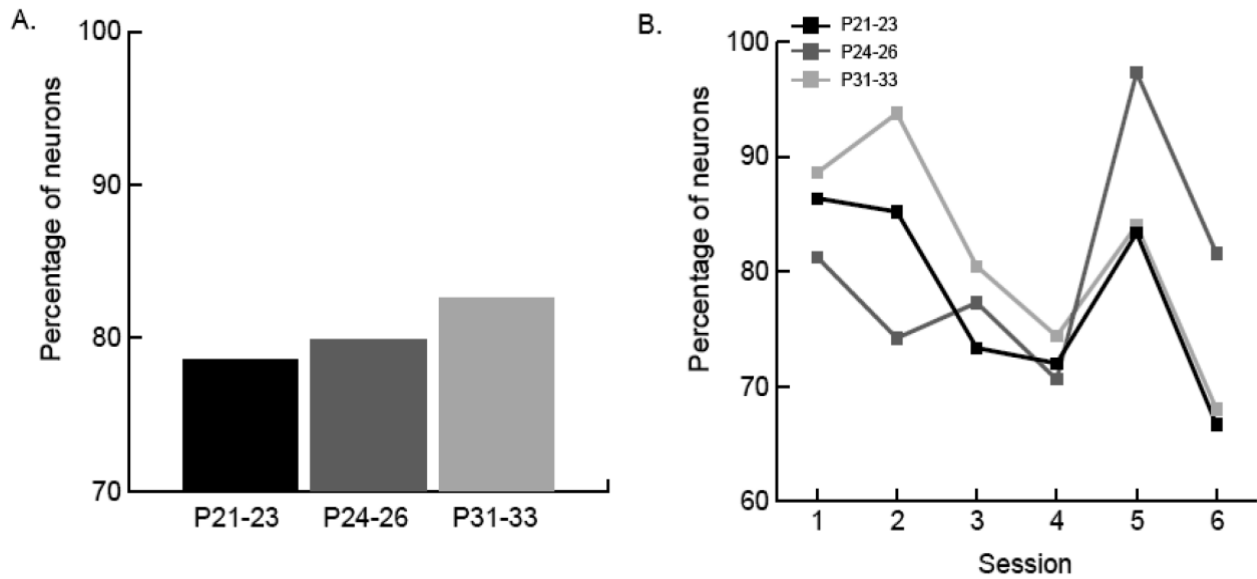


Figure 10. Learning-related changes in neuronal responsiveness.

(A) The percentage of cells that responded differently during CR and No-CR trials was similar across age group (total number of significantly responding neurons used as denominator). (B) When broken down by session, there were age-related differences in the proportion of neurons that responded differently during CR versus No-CR trials (total number of significantly responding neurons used as denominator). Although the younger two age groups tended to show similar proportions of differently responding neurons throughout training, the oldest age group had a steep decrease in the proportion of neurons that responded differently.

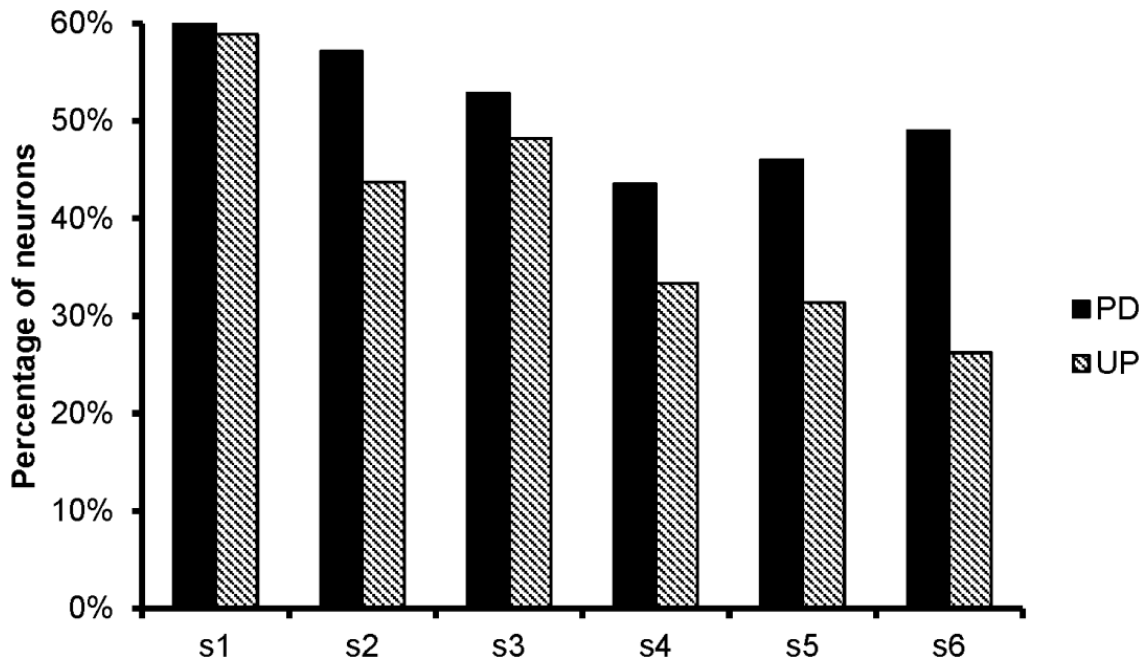


Figure 11. Session by session changes in neuronal responsiveness.

The proportion of neurons that were responsive to trial stimuli significantly decreased during both paired and unpaired training.

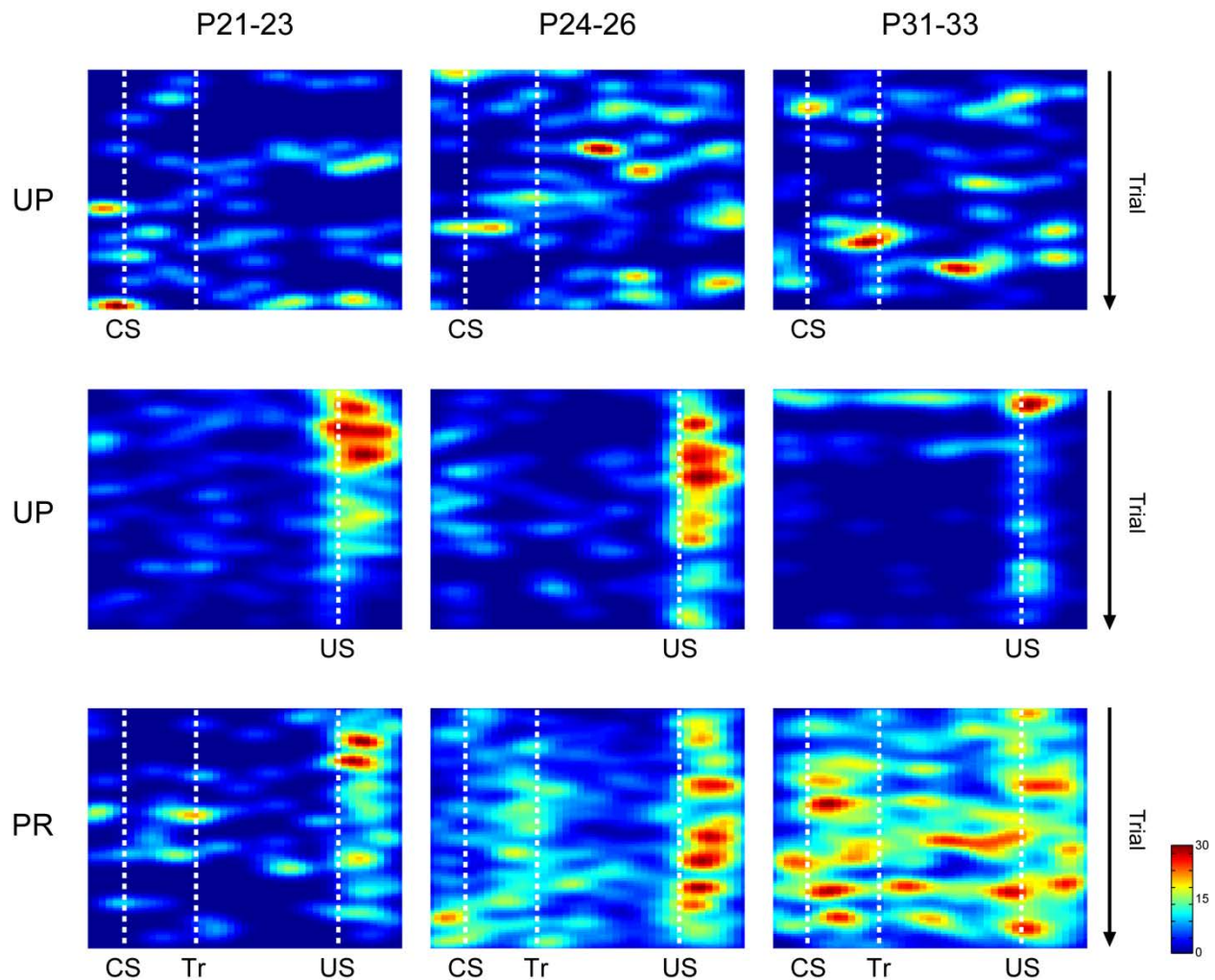


Figure 12. Color map depicting single representative CA1 neuronal firing rate profiles during unpaired and paired training.

Firing profiles of six different single CA1 neurons recorded from rats in the P21-23, P24-26, and P31-33 groups during a single session of either paired or unpaired training. Pups received CS alone and US alone trials during unpaired training. During trace conditioning the CS and US were separated by a 500ms trace (Tr) interval. In each color map the x-axis indicates the CS and US onset time and the y-axis represents trial numbers (from top to bottom). Unpaired trials (UP) had low levels of neuronal responding to the CS. However, during paired training (PR) there was an age-related increase in the magnitude of responding during the CS and trace intervals. Firing rates are shown during the baseline (125 ms), CS (250 ms), trace (500 ms), and US (250 ms) periods. Color map scaling ranges from 0 to 30 spikes/second. Dashed lines indicated onset of trial event.



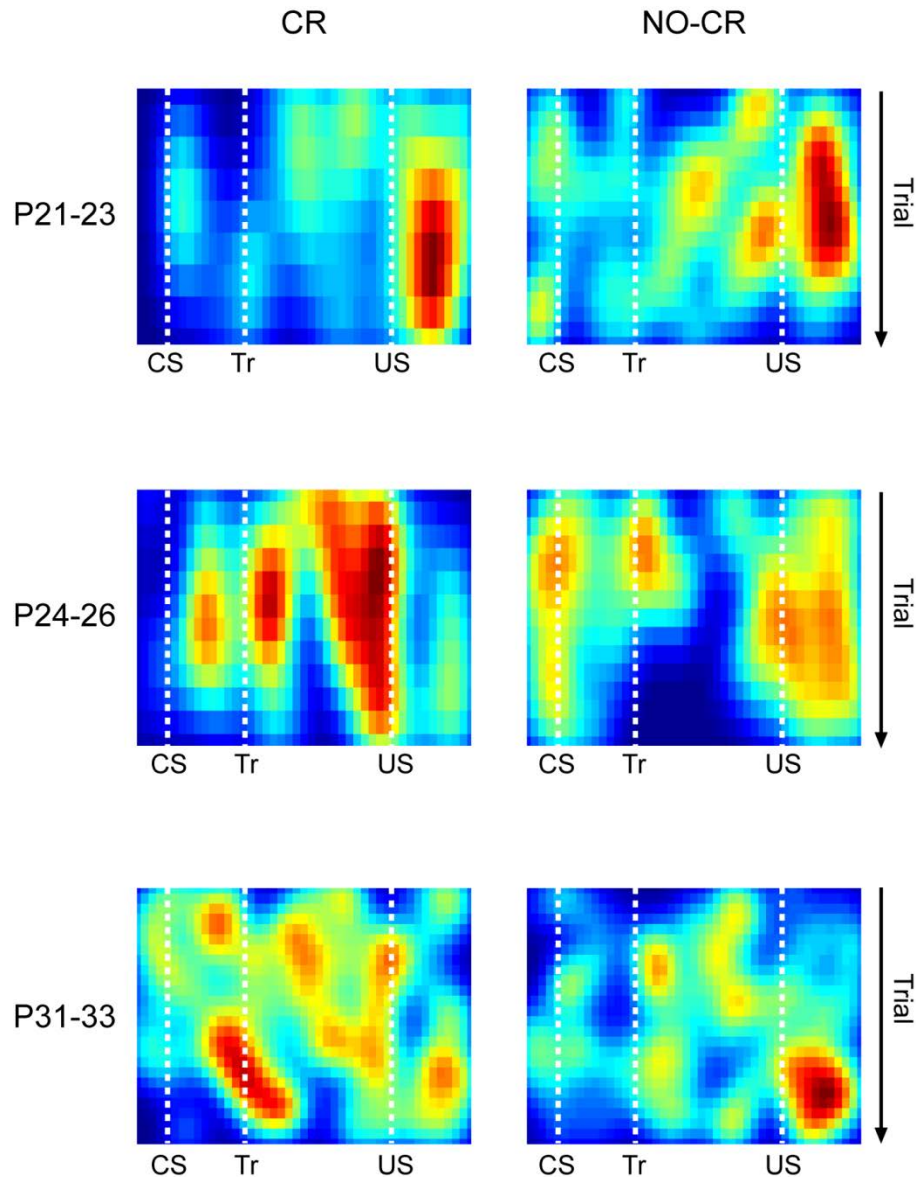


Figure 13. Color map of representative single CA1 pyramidal cell firing rate profiles comparing No-CR and CR trials across age.

Firing profiles of three different single CA1 neurons recorded from rats in the P21-23, P24-26, and P31-33 groups during CR and No-CR trials in a single session. On the colormap figures the x-axis indicates the event time onset and the y-axis indicates time (from top to bottom). Firing rates are shown during the baseline (125 ms), CS (250 ms), trace (500 ms), and US (250 ms) periods. The neurons recorded from the older pups show greater activity during the CS and trace interval during CR trials compared to no-CR trials. These neurons also show a stronger response to the US on no-CR trials. In contrast, the neuron recorded from the youngest pup shows very little change in responding between CR and no-CR trials. Color map scaling is based upon the maximum value of each image. Dashed lines indicated onset of trial event.

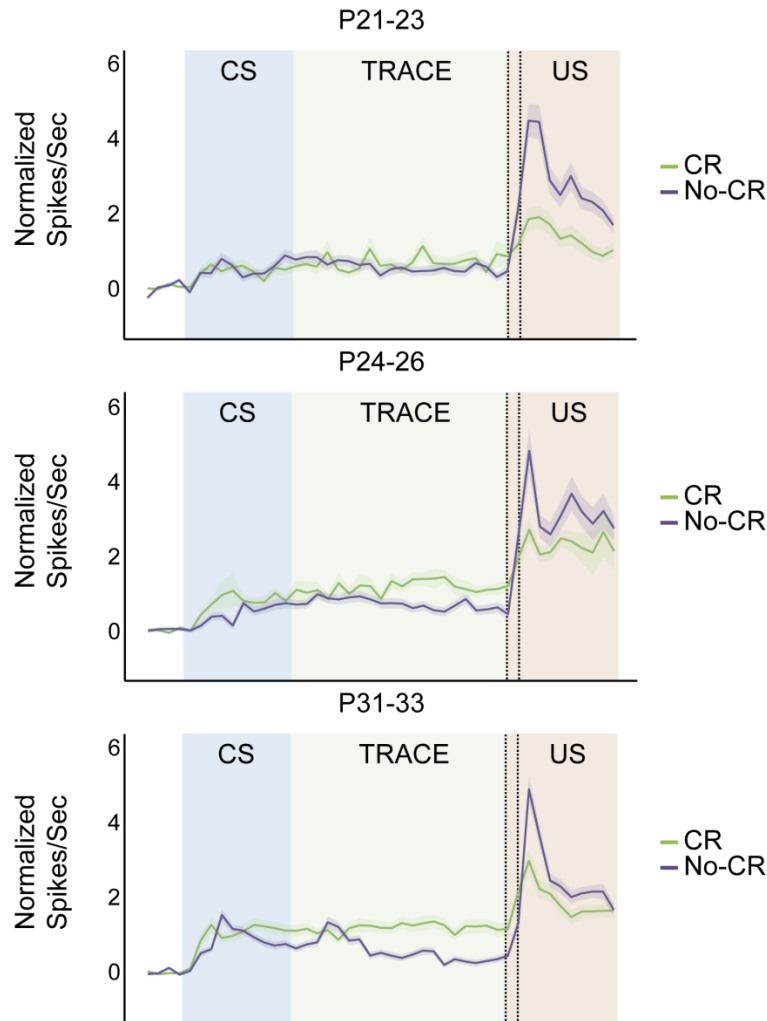


Figure 14. CA1 pyramidal cells show age-and learning-related changes in neuronal activity.

In order to examine population-level changes in activity, the average neuronal firing rates during the baseline, CS (250 ms), trace (500 ms), and US (250 ms) periods for all responsive neurons were calculated for each age group across all paired sessions. Each graph compares trials with a CR and trials without a CR for a given age group. The shaded areas along the lines indicate the standard error of mean across neurons. Firing rates were normalized to pre-CS baseline activity. Hippocampal neurons in the pups trained on postnatal days 24-26 and 31-33 showed greater activity during the CS and trace interval on CR trials than on no-CR trials, an associative effect not seen on days 21-23. Conversely, across all ages the presence of a CR was associated with a decrease in the neuronal firing rate in response to the US. This difference was greatest and most prolonged in the youngest age group. These findings suggest that the magnitude of associative activity during the CS and trace interval increased between days 21 and 24. Moreover, the magnitude of responsiveness to the US may be inversely related to performing a conditioned response.

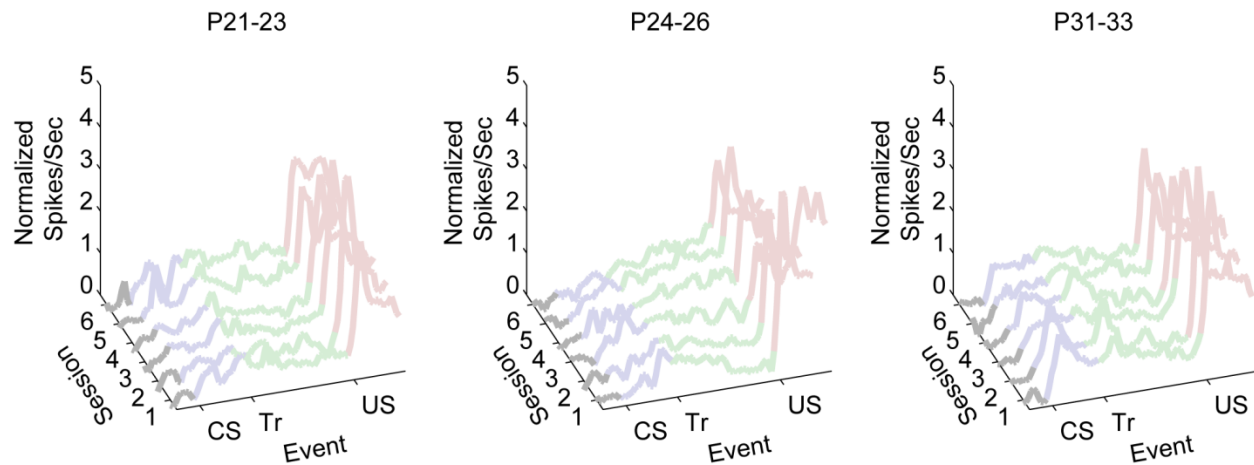


Figure 15. CA1 neuronal firing rate magnitude across sessions and age groups.

The average magnitude of population CA1 neuronal activity from responsive cells was compared across session and age group. The x-axis shows the event onset time, the y-axis indicates normalized spikes/sec values, and the z-axis represents the session numbers. During trace conditioning the CS and US were separated by a 500 ms trace (Tr) interval. There was an age-related increase in firing rate during the CS and trace intervals. Importantly, this developmental change was primarily evident during the first two sessions. See Table 2 for details. Firing rates are shown during the baseline (125 ms), CS (250 ms), trace (500 ms), and US (250 ms) periods.

Table 1. Count and percentage of neurons in categories of responsiveness.

During paired training CA1 neurons were categorized in one of eight categories according to the trial events to which they were responsive. During unpaired training neurons were categorized in one of four categories. The number and percentage (in parentheses) is noted for each category and age group. Based on these values, the number (and percentage) of units that responded to more than one trial event was calculated and labeled as “combination neurons”.

<b>Paired Training</b>	P21-23	P24-26	P31-33	All Ages
Nonresponsive	152 (48.9%)	188 (45.2%)	282 (48.4%)	622 (47.5%)
CS-Responsive	2 (.6%)	1 (.2%)	11 (1.9%)	14 (1.1%)
Trace-Responsive	6 (1.9%)	21 (5%)	8 (1.4%)	35 (2.7%)
US-Responsive	86 (27.7%)	89 (21.4%)	109 (18.7%)	284 (21.7%)
CS- & Trace-Responsive	8 (2.6%)	2 (.5%)	7 (1.2%)	17 (1.3%)
CS- & US-Responsive	3 (1.0%)	7 (1.7%)	27 (4.6%)	37 (2.8%)
Trace- & US-Responsive	14 (4.5%)	59 (14.2%)	47 (8.1%)	120 (9.2%)
CS-, Trace-, & US-Responsive	40 (12.9%)	49 (11.8%)	92 (15.9%)	181 (13.8%)
<b>Grand Total</b>	<b>311 (100%)</b>	<b>416 (100%)</b>	<b>583 (100%)</b>	<b>1310 (100%)</b>
<i>Combination Neurons</i>	<i>65 (20.9%)</i>	<i>117 (28.1%)</i>	<i>173 (29.7%)</i>	<i>355 (27.1%)</i>
<b>Unpaired Training</b>				
Nonresponsive	77 (45.6%)	132 (66.3%)	86 (64.2%)	295 (58.8%)
CS-Responsive	7 (4.1%)	10 (5.0%)	8 (6.0%)	25 (5.0%)
US-Responsive	80 (47.3%)	53 (26.6%)	30 (22.4%)	163 (32.5%)
CS- & US-Responsive	5 (3.0%)	4 (2.0%)	10 (7.5%)	19 (3.8%)
<b>Grand Total</b>	<b>169 (100%)</b>	<b>199 (100%)</b>	<b>134 (100%)</b>	<b>502 (100%)</b>

Table 2. Session differences in the magnitude of responding between age group.

The magnitude of responding was broken down by session and compared across age group (see Figure 14). The number of bins that differed significantly between age groups for sessions 1-6 is depicted in columns. The age group and the trial interval (CS, trace, US) during which the significant bins occurred are depicted in rows. The majority of changes that occurred during the CS and trace intervals occurred during the first two sessions. The magnitude of responding during the US did not show any clear age- or session-related patterns.

	Interval	Session 1	Session 2	Session 3	Session 4	Session 5	Session 6
P21-23 vs. P24-26	CS	0	0	0	0	1	0
	Trace	0	0	0	3	0	0
	US	18	7	5	1	5	1
P21-23 vs. P31-33	CS	2	3	0	1	0	0
	Trace	1	3	0	2	0	0
	US	9	5	5	1	3	2
P24-26 vs. P31-33	CS	7	5	0	1	0	1
	Trace	2	3	0	2	0	0
	US	13	5	8	5	1	0

## **CHAPTER 4: CS MODALITY INFLUENCES HIPPOCAMPAL NEURONAL ACTIVITY DURING TRACE EYEBLINK CONDITIONING ONTOGENY**

The development of learning and memory processes which depend on the hippocampus has consistently been found to emerge ontogenetically later than learning which is considered to be independent of the hippocampus. This developmental dichotomy is very well illustrated in the Pavlovian conditioning task, eyeblink conditioning. Whereas early-developing delay EBC is generally regarded as a simple associative task, late-developing trace EBC is more complex in that it requires the organism to form an association between two stimuli that are temporally separated by a stimulus-free “trace” interval. Neurobiological research concerning the development of delay eyeblink conditioning suggests that its ontogeny is dependent on sensory input to the pontine nucleus. Therefore, most pups can show sufficient levels of learning to an auditory or visual CS as early as P17-18, but as early as P14-15 if trained with an earlier-developing vibrotactile CS (Goldsberry et al., 2014).

Because trace EBC emerges later than delay EBC, at P21-22 with a tone or light CS, sensory system development has not been viewed as a potential contributor to its development (Ivkovich et al., 2000; Ivkovich & Stanton, 2001). Moreover, due to the parallel emergence of trace fear conditioning and trace eyeblink conditioning, the developmental time course of trace conditioning has traditionally been believed to be dependent primarily on hippocampal development (Ivkovich & Stanton, 2001; Moye & Rudy, 1987b). This hypothesis has been further supported by work concerning the development of hippocampal place cells. Although pups as young as P16 have place cells, the stability of their place fields continues to develop until at least P30 (Langston et al., 2010; Wills et al., 2010). Most importantly, work from our laboratory has shown that hippocampal neuronal activity is correlated with the developmental

emergence of trace EBC (Goldsberry et al., 2015). We recorded CA1 pyramidal cell activity while rat pups were trained in auditory trace eyeblink conditioning. Neuronal firing differed not only in magnitude, but also in complexity. The youngest age group, which showed low levels of trace conditioning, had fewer cells that responded to the tone and trace intervals. Moreover, of the cells that did show responding, very few exhibited changes in activity across training, regardless of whether the animal demonstrated learning. Although the traditional explanation for these results is that late development of the hippocampus is the primary contributing factor to age-related changes in its activity, an alternative hypothesis could explain these results. Specifically, development of sensory input to the hippocampus could be limiting neuronal responsiveness in auditory trace conditioning. If this is the case, then just as seen in delay conditioning, training pups with an earlier-developing sensory modality may facilitate trace conditioning and the associated neuronal activity.

Indeed, recent work from our laboratory shows that when pups are trained with a vibrotactile CS they are capable of learning trace EBC as early as P17-18 (Goldsberry & Freeman, in prep). These data suggest that hippocampal development is not the sole limiting factor in the development of trace EBC. Rather, it would seem that both the CS pathway and hippocampal development play roles in trace eyeblink conditioning. The goal of the current study was to elucidate the role of CS modality on the ontogeny of hippocampal neuronal activity during trace eyeblink conditioning. If sensory input to the hippocampus is not playing a role in the development of trace EBC, then pups trained in somatosensory trace eyeblink conditioning should show identical levels of neuronal responsiveness to those trained in auditory trace conditioning (Goldsberry et al., 2015). However, if the development of sensory input to the hippocampus is limiting the ontogenetic emergence of trace EBC, then we would anticipate that

training pups with an earlier-developing sensory modality would result in increased hippocampal neuronal responsiveness to trial stimuli.

## **Methods**

### **Subjects**

Subjects were 9 Long-Evans rat pups ( $n = 3$  per age group) from 7 different litters (6 females and 3 males). All pups were born and reared in the Spence Laboratories of Psychology animal colony at the University of Iowa with a 12/12-hr light/dark cycle (light onset at 7 am). The University of Iowa Institutional Animal Care and Use Committee approved all procedures.

### **Surgery**

Detailed surgical methods have been previously published (Ng & Freeman, 2012). Rat pups were implanted with multiple-tetrode microdrives for neuronal recording on postnatal day (P)15, P19, or P22. Each microdrive contained an 18-channel electronic interface board which allowed for 4 independently-moving recording tetrodes and 1 independently-moving reference tetrode.

Immediately prior to implantation, each tetrode was gold-plated to an impedance of approximately 350 k $\Omega$ . During surgery pups were anesthetized using 1.5-3% isoflurane gas and fitted with a microdrive, differential EMG electrodes to record blink activity, and a bipolar stimulating electrode for US delivery.

As previously described (Goldsberry et al., 2015), microdrive implantation involved drilling a small hole in the skull directly above the right dorsal hippocampus (AP,  $-4.0$  mm; ML,  $-2.5$  mm). The base of microdrive was lowered through the hole until the tetrodes touched the brain surface. The microdrive was then cemented into place and grounded with a stainless steel screw fixed to the skull in the P19 and P22 pups and a stainless steel skull hook in the P15 pups.



Tetrodes were lowered approximately 0.6 mm into the brain immediately following microdrive implantation.

Pups were then fitted with differential EMG electrodes to record eyelid activity. This consisted of two stainless steel electrodes that were threaded through the left upper orbicularis oculi muscle and a ground wire was attached to the skull with a screw in the P19 and P22 pups and a stainless steel skull hook in the P15 pups. Finally, the bipolar stimulating electrode for US delivery was placed subdermally immediately caudal to the left eye.

Recording tetrodes were lowered to the CA1 layer of the hippocampus (approximately DV, -2.1 mm) on the day following surgery. A separate, independently-moving reference tetrode was lowered to a neuronally quiet area slightly further into the cortex, dorsal to the hippocampus (approximately DV, -0.9).

### **Spike data acquisition**

The microdrive was connected via a tether to a spike acquisition system (Neuralynx, Bozeman, MT). The recorded spike signal was then amplified at a gain of 10,000-25,000 and band pass filtered between 0.6 – 6.0 kHz. Signals were digitized and stored at 32 kHz (Cheetah, Neuralynx, Bozeman, MT).

### **Conditioning apparatus**

The conditioning apparatus has been previously described in detail (Ng & Freeman, 2012). Briefly, pups were trained in a clear Plexiglas conditioning housed within a sound-attenuation chamber. The recording EMG and the US stimulating electrode were attached to cables that were threaded through a hole in the ceiling of the chamber to the recording hardware. Computer software controlled CS and US delivery while simultaneously recording differential eyelid EMG

activity (sampling rate = 250 Hz). EMG activity was amplified (x 2000), filtered (500-5000 Hz), and integrated (20 ms time constant).

### **Conditioning procedures**

Training began 2 days after surgery, on P17-19, P21-23, or P24-26. In order to parse out the role of nonassociative factors from learning, pups were given both paired and unpaired presentations of the CS and US. Unlike paired training, unpaired presentation of the CS and US does not result in a learned association. Unpaired training (session 1) consisted of 100 CS-alone and 90 US-alone trials separated by a variable intertrial interval averaging 15 s. Paired training (sessions 2-6), which results in learning an association between the CS and US, consisted of 100 trials per session of trace EBC with a 250 ms vibration CS (144 Hz, 2.4 m/s<sup>2</sup>), a 500 ms trace interval, and a 25 ms periorbital stimulation US (2-3 mA). Each paired session was divided into 10 blocks. In each block the first nine trials were paired CS-US presentations and the tenth was a probe trial, containing only the vibration CS. The probe trials were used to evaluate parameters of the conditioned response (CR) in the absence of the unconditioned response (UR) (Gormezano et al., 1983). During paired training, trials were separated by a variable intertrial interval averaging 30 s. During both paired and unpaired training, any response that occurred during the first 80 ms of the CS was considered a startle response and omitted from further analyses. Learning was demonstrated by the pup showing a conditioned blink response after CS onset, but before US onset. We also examined the timing of this response as an additional method because in adults a well-timed (adaptive) CR is generally indicative of better learning (Solomon, Vander Schaaf, et al., 1986). We defined an adaptive CR as one in which the maximum amplitude of the response occurred within 250 ms of the US onset. This period also corresponds to the last half of the trace interval.

## Neuronal analyses

Following data collection, KlustaKwik (Kadir et al., 2013) was used to run an initial automatic separation of spike activity into individual clusters. These clusters were then manually inspected and refined using MClust-3.5 (Redish et al., 2010). Neurons were classified as pyramidal cells if they (1) showed a bursting pattern of activity as demonstrated by a peak in the autocorrelogram at 3-8 ms, (2) had a baseline (500 ms sample duration prior to CS onset) firing rate of less than 10.5 spikes/second, and (3) had at least 100 spikes during the training session.

Neuronal responsiveness to trial events was examined using NeuroExplorer (Madison, AL). First, neurons were categorized according to their firing rate profile (responsive to CS, trace interval, US, or a combination thereof) (Goldsberry et al., 2015). To accomplish this, each trial was divided into nine 125 ms intervals. The nine time intervals included baseline (125 ms), CS (250 ms), trace (500 ms), and US periods (250 ms). Firing rates across the trial were normalized to the pre-CS baseline by using modified z-score values in NeuroExplorer. Intervals that had values exceeding the pre-established 99% confidence limits (based on the Poisson distribution, two-tailed,  $\alpha \leq 0.05$ ) were considered to be statistically significant from the baseline firing rate, thus showing either excitatory or inhibitory neuronal responses to the given trial events. Based on these values, neurons were classified as unresponsive, CS-responsive, trace-responsive, US-responsive, or a combination-responsive. For example, a combination unit that showed increased activity during both the CS and the trace periods would be categorized as a CS-responsive neuron and a trace-responsive neuron. The proportion of neurons that fell into each response category was then compared across age groups and sessions using Chi Square analyses.

The neuronal firing magnitude was also examined. Unresponsive neurons were not included in these analyses in order to prevent differences in the proportion of responsive neurons

from exaggerating potential magnitude differences. In order to control for potential differences in the baseline (pre-CS) magnitude of responding, all neuronal activity was first normalized to pre-CS baseline levels. Normalized peristimulus-time histograms of neuronal activity were produced using 12.5 ms bins (normalized bin = (bin mean – 125 ms baseline mean)/standard deviation of 125 ms baseline)). These normalized bin values were then compared across age, session, and CR/No-CR trials with a repeated-measures ANOVA (for responsive neurons only) and the Tukey HSD post hoc test to examine age-related differences in the magnitude of neuronal responding during the trial (Goldsberry et al., 2015).

## **Histology**

Histological methods have been previously described in detail (Ng & Freeman, 2012). Immediately following the final training session, tetrode placement was determined by creating small electrolytic lesions. The following day, brains were removed and placed in a 30% sucrose-formalin solution, sectioned at 50  $\mu\text{m}$ , mounted on slides, and stained with thionin. Histology was examined with a light microscope to determine tetrode placement. Only placements confirmed to be in the CA1 layer of the hippocampus were included in the analysis.

## **Results**

### **Behavioral Data**

Learning was assessed in two different ways: the overall percentage of CRs and the percentage of adaptively timed CRs. Overall, all age groups showed an increase in CRs across sessions (Figure 16). However, learning-rate differences between the three age groups were revealed when examining the percentage of adaptive (Figure 16), but not overall, CRs. Specifically, the oldest age group had a higher level of adaptive CRs compared to the other age groups during the

final three sessions of training. Repeated-measures ANOVAs supported these observations. When the percentage of CRs was compared across sessions (sessions 1-6) and age (P17-19, P21-23, and P24-26), a main effect of session ( $F(5, 30) = 11.531, P = 0.000003$ ) but not of age ( $F(2, 6) = 3.824, P = 0.085$ ) was found. When a session X age repeated-measures ANOVA was used to examine adaptive CR percentage, a main effect of age ( $F(2, 6) = 6.432, P = 0.032$ ), session ( $F(5, 30) = 13.831, P = 0.000001$ ), and an age X session interaction ( $F(5, 30) = 11.531, P = 0.000468$ ) were found. This interaction was further examined with Tukey HSD post hoc tests. The P24-26 age group had a significantly greater percentage of adaptive CRs on sessions 4-6 relative to both the P17-19 and P21-23 groups ( $P < 0.01$ ). Finally, the two youngest age groups did not differ from each other on any of the sessions.

### **Neuronal Responsiveness**

A total of 1104 neurons passed our CA1 pyramidal cell exclusion criteria (see methods section for details) to be included in further analyses. Baseline firing rates of responsive neurons did not differ between age groups (P17-18 = 1.26 Hz, P21-23 = 1.59 Hz, P24-26 = 1.61 Hz). CA1 pyramidal cells were categorized as either responsive or unresponsive to trial events (i.e., CS, trace, and US) (Goldsberry et al., 2015). If categorized as responsive, the direction of responsiveness was classified as either excitatory (increased activity during trial) or inhibitory (decreased activity during trial). As found in our previous paper (Goldsberry et al., 2015), there were very few neurons that showed inhibitory responses. These cells were therefore excluded from further statistical analyses. Chi square analyses were used to compare the proportion of cells that showed an excitatory response to trial events across category (Table 3).

The proportion of excitatory responsive neurons was greater when pups were given unpaired training than paired training (for a breakdown by age group see Figure 17). Chi square

analyses confirmed these observations, showing that sessions 1 and 2 differed significantly from one another with unpaired responsiveness (76.64%) being greater than paired responsiveness (61.69%) ( $X^2(1, N = 462) = 11.91, P = 0.0006$ ).

#### Proportion of responsive neurons during paired training

Although there was a slight increase in the proportion of responsive neurons across age, when pups received paired training there were no differences across age for the proportion of responsive versus unresponsive neurons ( $X^2(2, N = 890) = 4.15, P > 0.05$ ). This held true, even when examining only session 2 of paired training (thus matching the unpaired training analysis) ( $X^2(2, N = 248) = 3.69, P > 0.05$ ) (Figure 17). Importantly, this analysis focused only on overall levels of responsiveness, not on responsiveness to individual trial events, such as the CS, trace or US.

When CS responsiveness was examined, an age-related increase in the proportion of CS-responsive neurons (this category included units that were responsive to all CS-responsive units, even if they were also responsive to other trial events) was found  $X^2(2, N = 890) = 9.33, P = 0.009$  (Figure 17). A similar pattern was observed when comparing US responsiveness. There was an age-related increase in the proportion of neurons that showed responsiveness to the US (this category included combination neurons)  $X^2(2, N = 890) = 9.60, P = 0.008$  (Figure 17). Moreover, when comparing the proportion of neurons that showed increases in activity only during the US (therefore not combination neurons), a significant effect of age was found, with the proportion of US-only responsive neurons being greater in the oldest age group than the younger two age groups  $X^2(2, N = 890) = 7.90, P = 0.019$  (Figure 17). Together, these data are in agreement with the behavioral data, suggesting that there is a developmental step in neuronal

processing that separates the youngest two (P17-19 and 21-23) age groups from the oldest P24-26 group.

When the proportion of trace period-responsive units was compared across age there was not a significant effect of age  $X^2(2, N = 890) = 1.89, P > 0.05$ . These results are similar to those seen when pups are trained with a tone CS (Goldsberry et al., 2015). Also, the proportion of units that responded to a combination of trial events did not differ across age groups  $X^2(2, N = 890) = 1.24, P > 0.05$ . Instead, all three age groups showed relatively robust levels of responsiveness to a combination of trial events. The P17-19 group had 28%, the P21-23 group had 27%, and the P24-26 group had 31% of neurons classified as combination neurons. This is in contrast to our previous findings that showed that hippocampal neurons of older pups are more likely to respond to a combination of trial events.

#### Learning-related changes in the proportion of responsive neurons during paired training

In order to determine how learning affected changes in neuronal responsiveness to trial events we compared responsiveness during CR trials to responsiveness during No-CR trials. Neurons could fall into one of two possible categories; one that indicated that responsiveness changed between CR and No-CR trials and one that indicated the responsiveness was identical on CR and No-CR trials. The proportion of neurons that fell into each category was compared across age and session using a Pearson's Chi-Squared test (Goldsberry et al., 2015).

A large proportion of neurons showed changes in response profile between CR and No-CR trials. Specifically, 59.84%, 66.34%, and 61.26% of neurons showed changes in the P17-19, P21-23, and P24-26 age groups, respectively. However, there were no differences in the proportion of neurons between age groups ( $X^2(2, N = 890) = 2.89, P = 0.236$ ). When broken

down by session, only session four showed group differences in responsiveness between CR and No-CR trials ( $X^2(2, N = 165) = 9.32, P = 0.009$ ). This difference, however, did not follow any clear developmental pattern. The other four paired session failed to show any between age-group differences ( $X^2s(2, N \geq 115) < 3.73, Ps > 0.155$ ). In a final analysis, the proportion of units that showed changes between CR and No-CR trials was compared across sessions for each separate age group. Only the youngest age group had a significantly different proportion of neurons that showed changes across sessions ( $X^2(2, N = 254) = 12.365, P = 0.015$ ). However, there was no clear pattern across the training sessions.

#### Proportion of responsive neurons during unpaired training

During unpaired training (session 1) the proportion of responsive neurons differed significantly across age, with an age-related increase in responsivity ( $X^2(2, N = 214) = 7.75, P = 0.021$ ) (Figure 17). This responsiveness was then broken down by specific trial events. Results showed a significant difference in CS and US responsiveness across age. The oldest age group had substantially more CS-responsive neurons than the younger two groups ( $X^2(2, N = 214) = 15.01, P = .0006$ ). When US responsive neurons were analyzed, the oldest age group once again had a far greater proportion of responsive neurons compared to the youngest two age groups ( $X^2(2, N = 214) = 18.90, P = .00008$ ).

#### Changes in neuronal responsiveness across sessions

There was a significant decrease in the proportion of responsive neurons across the 5 sessions of paired training ( $X^2(5, N = 890) = 26.88, P = 0.00002$ ) (Figure 18). When neuronal responsiveness during these sessions was further broken down by trial event type, Chi square analyses showed that the significant decrease in responding across sessions was found across all



trial events. The proportion of US responsive ( $X^2(4, N = 890) = 31.49, P = 0.00004$ ), CS-responsive ( $X^2(4, N = 890) = 25.61, P = 0.00003$ ), and trace-responsive ( $X^2(4, N = 890) = 26.17, P = 0.000002$ ) neurons significantly decreased across training sessions.

## **Magnitude of Neuronal Responses to Trial Events**

### Magnitude of neuronal response during paired training

In addition to comparing the proportion of responsive neurons, the magnitude of the neuronal response shows the strength with which neurons respond to trial events. In order to understand how the magnitude of the neuronal response varied across age, learning, stimulus type, and training session, the neuronal activity of responsive neurons was first normalized to the pre-CS baseline (see methods section for details). This normalized neuronal activity of responsive neurons (Figure 19) was then compared across age and CR (whether or not the pup showed a learned response on a given trial) with a repeated-measures ANOVA (Greenhouse-Geisser correction for sphericity) to determine which time intervals (bins) were significantly different. Results showed a significant age X CR X bin interaction  $F(52.05, 23108.43) = 1.547, P = 0.007$  and, a significant age X bin interaction  $F(52.05, 23108.43) = 3.964, P < 0.00001$ . In order to establish which bins were significantly different between CR and No-CR trials for a given age group, post hoc tests using the Tukey-Kramer approach to unequal “n” were run on individual bins ( $\alpha \leq 0.05$ ). Results showed an overall age-related increase in the magnitude of the neuronal response during the CS and trace periods. In the youngest age group (P17-19) only 5 of 60 bins had significantly higher magnitudes during the CR than No-CR trials. The middle age group (P21-23) performed intermediately with 13 of 60 bins showing higher magnitudes during CR than No-CR trials. The oldest age group (P24-26) had the highest number of bins (20 of 60) that differed between CR and No-CR trials.

The magnitude of responding during the US period provided a different picture. Whereas in the youngest age group 7 of 20 bins were significantly higher during No-CR trials, the middle age group had very similar magnitudes on CR and No-CR trials, with only 2 of 20 bins being higher during CR trials. Finally, the oldest age group showed the opposite pattern of activity from the youngest age group in that there were 11 of 20 bins that were significantly higher during CR than No-CR trials. Learning was therefore associated with an age-related increase in the magnitude of responding during the CS, trace, and US periods.

When looking at age-related differences in the magnitude of responding during CR trials, results showed an overall age-related increase in the magnitude of the neuronal response during the CS, trace, and US periods. Specifically, during the CS and trace intervals post hoc tests showed that 5 of 60 bins were lower in the P17-19 than the P21-23 group, 5 of 60 bins were lower in the P21-23 than the P24-26 group, and 7 of 60 bins were lower in the P17-19 than P24-26 group. During the US, 5 of 20 bins were lower in the P17-19 than P21-23 group, 8 of 20 bins were lower in the P21-23 than P24-26 group, and 12 of 20 bins were lower in the P17-19 than P24-26 group.

#### Magnitude of neuronal response during unpaired training

The magnitude of the neuronal response was also compared during unpaired training (session 1). Normalized neuronal data from CS trials was compared across age group and time interval (bin) with a repeated-measures ANOVA. Results showed that the oldest age group had the greatest magnitude of responding during the CS, however there were not clear differences between the youngest two age groups (Figure 20). An ANOVA confirmed these observations with a bin X age interaction ( $F(19.23, 845.94) = 1.758, P = 0.023$ ) (Greenhouse-Geisser correction for sphericity). Post hoc tests comparing age groups showed that 2 of 20 bins differed between the

P17-19 and P21-23 groups, 1 of 20 bins differed between the P17-19 and P24-26 groups, and 15 of 20 bins differed between the P21-23 and P24-26 groups. When comparing magnitude differences during the US, an age-related increase in the magnitude of responding was found. These observations were confirmed with a repeated-measures ANOVA showing an age X bin interaction ( $F(8.63, 630.32) = 2.336, P = 0.015$ ) (Greenhouse-Geisser correction for sphericity). Post hoc tests comparing age groups revealed that 7 of 20 bins differed between the P17-19 and P21-23 groups, 9 of 20 bins differed between the P17-29 and P24-26 groups, and 2 of 20 bins differed between the P21-23 and P24-26 groups.

#### Changes in the magnitude of neuronal response across sessions

The magnitude of the neuronal response on all trials (both CR and No-CR) was also compared across age and sessions. There was not a significant age X session X bin interaction ( $F(105.782, 5857.669) = 1.121, P = 0.189$ ) (Greenhouse-Geisser correction for sphericity).

#### **Discussion**

Although rat pups in all the age groups showed an increase in CRs across training session, there was a clear developmental step in learning that occurred between the P21-23 age group and the P24-26 age group. Previous work from our laboratory using a vibration CS during trace EBC has shown a similar trend, with a jump in CR percentage between P21-23 and P24-26 (Goldsberry et al., 2014). These behavioral data indicate that the ontogenetic emergence of trace EBC with a vibration CS does not follow the same developmental trajectory as trace EBC with a tone CS. Using an earlier-developing sensory modality has pushed the previously-observed developmental boundaries of trace EBC.

One of the primary goals of this experiment was to determine if training with an earlier-developing CS modality would result in not only increased associative learning, but also increased CA1 neuronal responsiveness to trial events. Our findings indicate that even when training with an early-developing sensory modality, CA1 neuronal activity is highly correlated to both age and learning. When comparing CS- and US-responsiveness across age groups during paired training, we found that neuronal responsiveness mapped onto the behavioral data, with the oldest age group having substantially greater responsiveness than the younger two age groups. This same trend was observed when pups received unpaired training. Previous work from our laboratory using a tone CS has shown similar age-related increases in CS-responsiveness during paired, but not unpaired training (Goldsberry et al., 2015). Therefore, regardless of CS modality, hippocampal CA1 activity shows age-related increases in activity.

Firing rate magnitude during trial events was affected by age, learning rate, and CS modality. Overall, there was an age-related increase in the magnitude of the neuronal response during the CS and trace intervals in paired training. This effect appeared to stem from age group differences during CR trials, but not No-CR trials. Specifically, although older animals had higher firing rates during CR trials, on No-CR trials all age groups performed similarly. When comparing CR and No-CR trials within a given age group, results showed that all age groups had increased neuronal responding during the CS and trace intervals on CR, compared to No-CR trials. Interestingly, the temporal pattern of the neuronal response during CR trials differed from that observed during No-CR trials. In all three age groups CR trials were marked by a peak in activity both following CS onset and prior to CS offset. Although No-CR trials also showed increases in neuronal firing rate (when compared to baseline) during the CS and trace intervals, there were no clear peaks in activity to mark CS onset and offset. These observations suggest

that even though the youngest two age groups have lower levels of conditioned responding, hippocampal CA1 activity shows learning-related increases in activity in all age groups. Moreover, firing rate peaks at CS onset and offset may indicate that the hippocampus is processing temporally relevant trial events.

When comparing the firing rates during unpaired training, the oldest age group (P24-26) had a substantially higher magnitude of responding during the CS when compared to the youngest two age groups (P17-19 and P21-23). These data show a very different pattern from what we observed in our previous study using a tone CS (Goldsberry et al., 2015). Tone-trained pups did not show any age-related changes during unpaired training. However, when trained in an associative context, we observed age-related increases in hippocampal CA1 activity during tone training. In fact, the P24-26 age group, but not the P21-23 age group, had significant increases in the magnitude of responding during the tone CS and trace periods on CR, but not No-CR trials (Goldsberry et al., 2015). These data suggest that, regardless of CS modality, there may be an age-related jump in hippocampal processing that occurs between the P21-23 and P24-26 age groups.

We compared firing rate data reported in the current study to that reported in our previous study which utilized a tone CS with ANOVA (Goldsberry et al., 2015). Overall, we found that training with an earlier-developing sensory modality did indeed increase hippocampal responsiveness, regardless of whether the pups received paired or unpaired training. During unpaired training we observed that the magnitude of the response was substantially greater in P24-26 pups trained with a vibration CS than those trained at the same age with a tone CS. In fact, responding in the P24-26 tone-trained group was nearly identical to responding in the P21-

23 vibration-trained group. Therefore, using an earlier-developing sensory modality may actually shift the developmental emergence of CS-related neuronal activity in the hippocampus.

When comparing neuronal activity across CS modality during paired training, two different patterns emerged, depending on whether we examined all trials, or only trials during which the pup showed a learned response (CR trials). If neuronal magnitude was compared across CS modality for all trials, regardless of whether or not the animal showed a CR, we saw that although the P21-23 age groups had similar firing rate magnitudes across CS modality, the P24-26 age group had a far greater magnitude when trained with a vibration CS. Moreover, the temporal pattern of the neuronal response in P24-26 vibration-trained pups had clear peaks in activity both following CS onset and immediately prior to CS offset. These same peaks were not present in either tone trained or younger pups trained with a vibration. Some of these differences could be due to the high percentage of CRs in the P24-26 vibration trained group.

In order to control for learning-rate differences between vibration- and tone-trained pups, we compared neuronal responsiveness across modalities during CR trials only. When comparing CR trials across CS modality, we found that P24-26 pups had similar increases in the magnitude of responding regardless of CS type. However, P21-23 pups had substantially higher magnitudes of responding when trained with a vibration CS. Moreover, peaks in neuronal activity following CS onset and prior to CS offset were evident in the vibration-trained condition when examining CR trials only. Therefore, even when learning is controlled for, training with a vibration CS results in changes in both the magnitude and the shape of CA1 activity as early as P21-23.

The hippocampus is thought to bind trial events together such as the CS, trace interval, and US during trace EBC (Woodruff-Pak & Disterhoft, 2008). This hypothesis is consistent with previous work from our laboratory using a tone CS, which showed age-related increases in the

proportion of CA1 neurons that respond to a combination of trial events (Goldsberry et al., 2015). A different pattern emerged, however, when pups were trained with a vibration CS. Rather than showing an age-related increase in the proportion of combination neurons, all three age groups, even those trained on P17-19, had proportions of combination neurons that matched those observed in the oldest tone-trained age group. These data suggest that the hippocampus is sufficiently developed at P17-19 to encode a combination of trial events. Therefore, there may be additional factors, such as the magnitude of firing rate increases during trial events, which contribute to driving the age-related differences observed in learning between the youngest and oldest groups.

The CA1 neurons of all age groups also showed overall decreases in responsiveness to trial events across training sessions. These results are very similar to that seen in previous studies in both adults and juveniles (Goldsberry et al., 2015; McEchron & Disterhoft, 1997). These data lend further support to the hypothesis that the hippocampus is most involved early in learning (Hattori, Chen, Weiss, & Disterhoft, 2015; Takehara et al., 2003). Specifically, hippocampal neuronal activity is believed to play a critical role both during and immediately following trace EBC. However, with memory consolidation, retrieval eventually becomes somewhat independent of hippocampal functioning, depending instead on the medial prefrontal cortex (Takehara-Nishiuchi, 2014; Takehara-Nishiuchi, Maal-Bared, & Morrissey, 2011; Takehara-Nishiuchi & McNaughton, 2008; Takehara et al., 2003).

Overall, the current results support our previous findings that developmental changes in hippocampal associative coding develop somewhat abruptly, with the greatest transition in behavior and neuronal responsiveness occurring between P21 and P24 (Goldsberry et al., 2015). Although both the P17-19 and P21-23 groups had low levels of conditioned responding, overall

neuronal activity, as measured by firing rate and the proportion of responsive neurons, tended to be lower in these age groups than in the oldest age group. Despite relatively low levels of neuronal responding, when trained with a vibration CS, even pups as young as P17-19 showed learning-related changes in neuronal activity, modest levels of CS-responsiveness, and a high proportion of neurons that responded to a combination of trial events. In fact, vibration-trained P17-19 pups had either similar or higher levels of hippocampal CA1 activity compared to P21-23 pups trained with a tone CS. Taken together, these data demonstrate that training in an earlier-developing sensory modality is indeed able to increase not only conditioned responding, but also hippocampal neuronal activity.



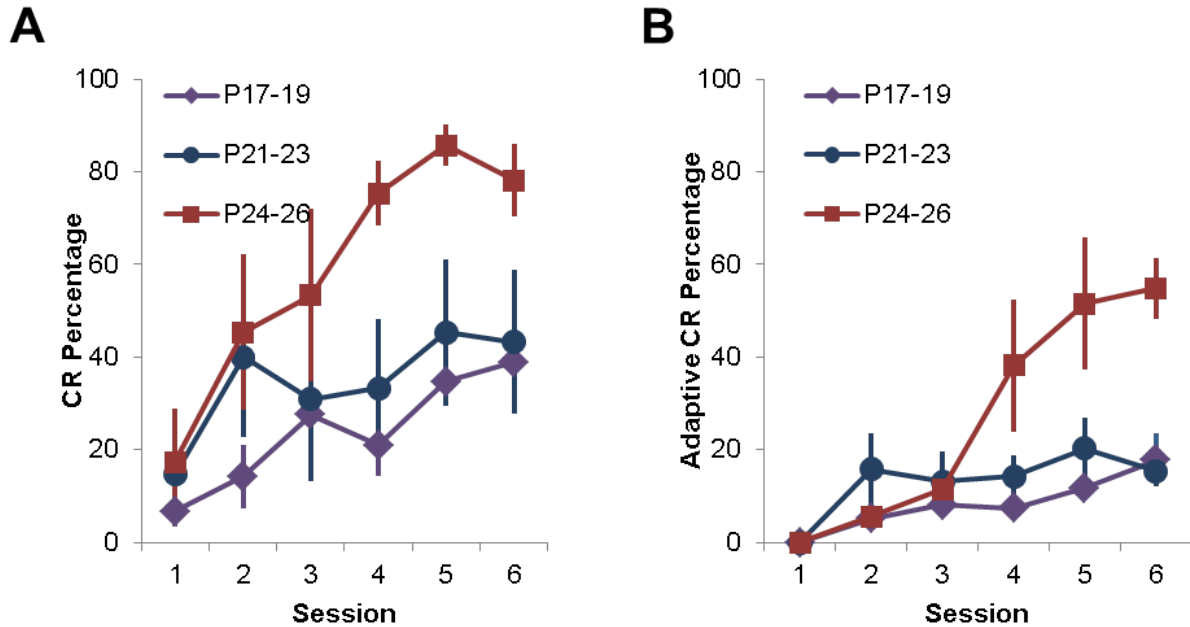


Figure 16. Trace eyeblink conditioning with a vibration CS increases as a function of age.

Training occurred on postnatal days (P)17-19, 21-23, and 24-26. All pups received unpaired (session 1) and paired training (sessions 2-6) . (A) Mean (+/- SE) conditioned response (CR) percentage in rat pups given trace eyeblink conditioning with a vibration CS. (B) Mean (+/- SE) adaptive conditioned response (CR) percentage in rat pups given trace eyeblink conditioning with a vibration CS.

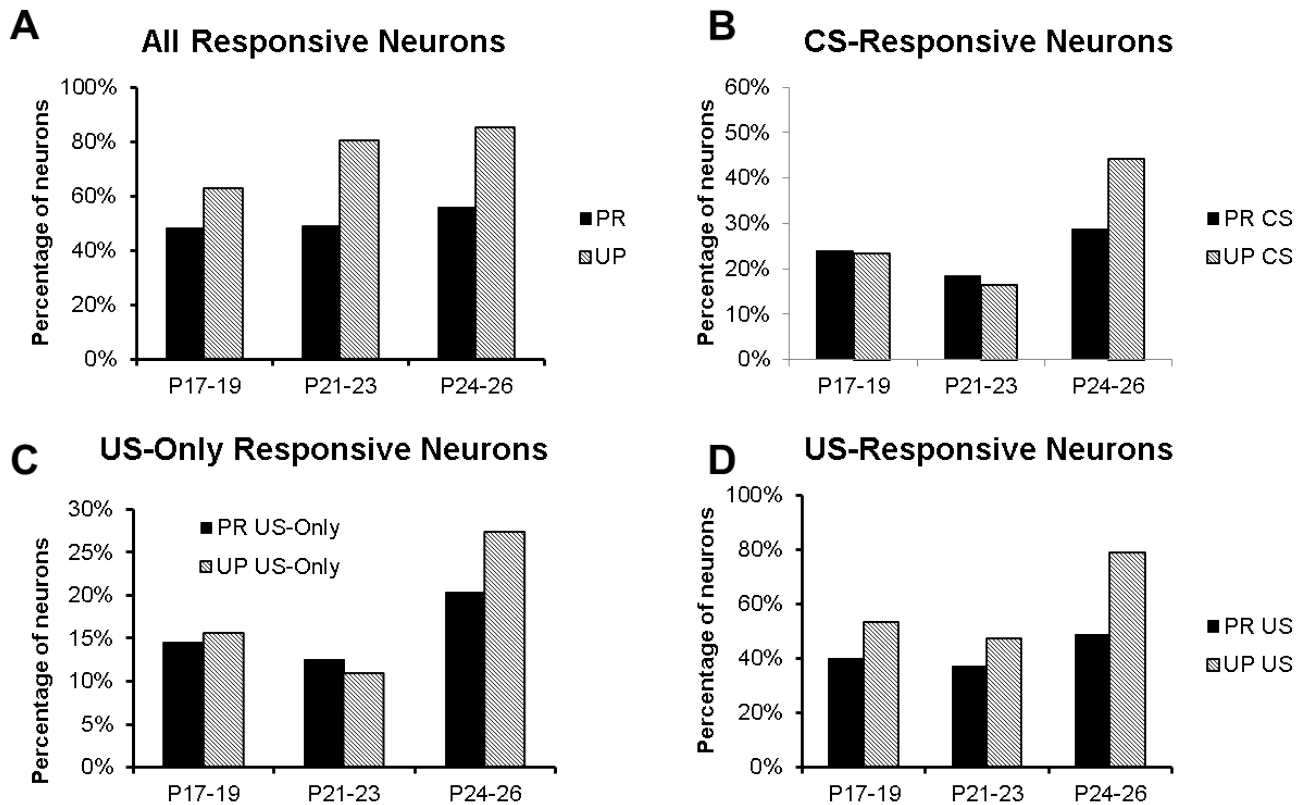


Figure 17. Hippocampal neurons respond differentially to trial events when trained with a vibration CS.

(A) There were age-related changes in the proportion of cells that respond to the vibration CS during unpaired but not paired training (this category includes neurons that were also responsive to other trial events). (B) There were age-related difference in the proportion of neurons that responded to the CS during unpaired and paired training (this category includes neurons that were also responsive to other trial events). (C) The proportion of US-only responsive neurons increased across age. (D) There was an age-related increase in the percentage of neurons that respond to the US (this category includes neurons that were also responsive to other trial events).

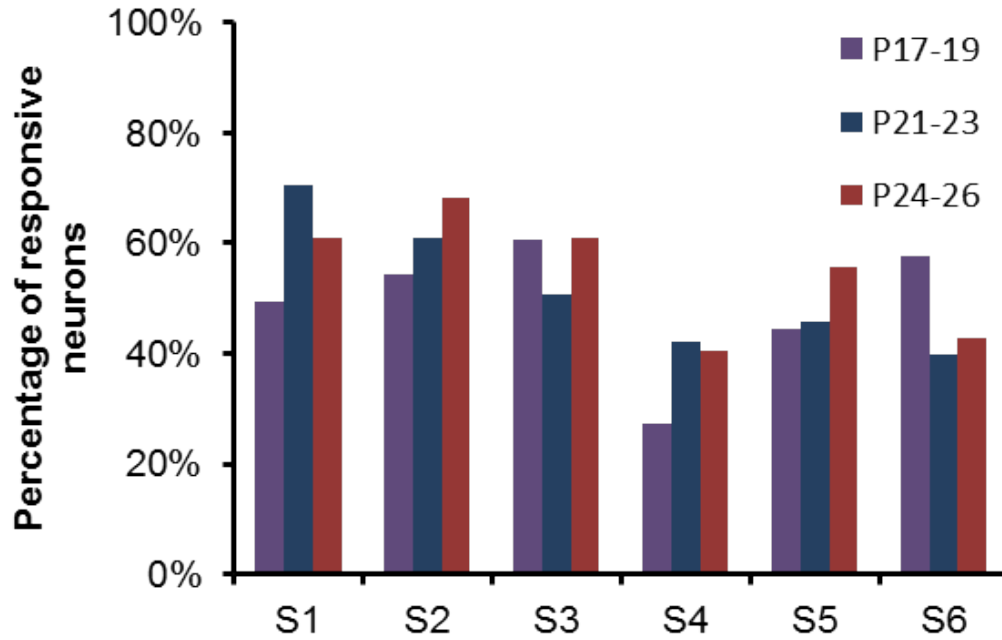


Figure 18. Session by session changes in neuronal responsiveness across age group when trained with a vibration CS.

The proportion of neurons that were responsive to trial events decreased as training continued.

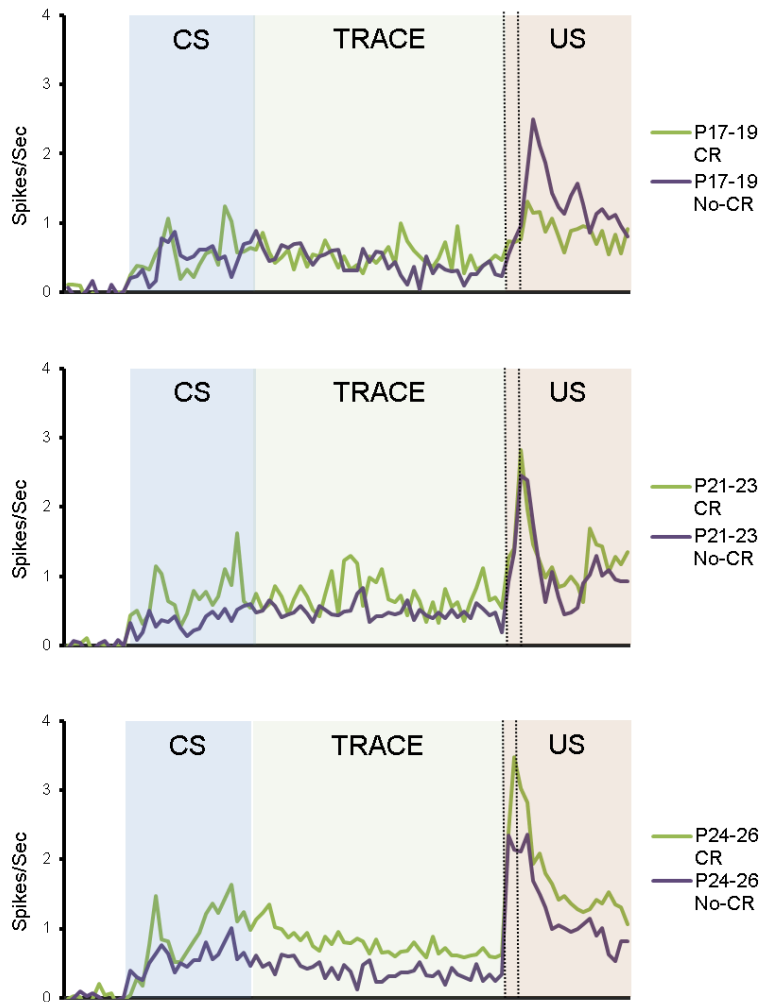


Figure 19. When trained with a vibration CS, CA1 pyramidal cells showed age-and learning-related changes in neuronal activity during paired training.

In order to examine population-level changes in activity, the average neuronal firing rates during the baseline, CS (250 ms), trace (500 ms), and US (250 ms) periods for all responsive neurons were calculated for each age group across paired sessions (sessions 2-6). All firing rates were normalized to pre-CS baseline activity. All age groups showed significant firing rate increases during the CS and trace periods during CR compared to No-CR trials. When comparing CR amplitude across age group, there was an age-related increase in firing rate during the CS and trace periods.

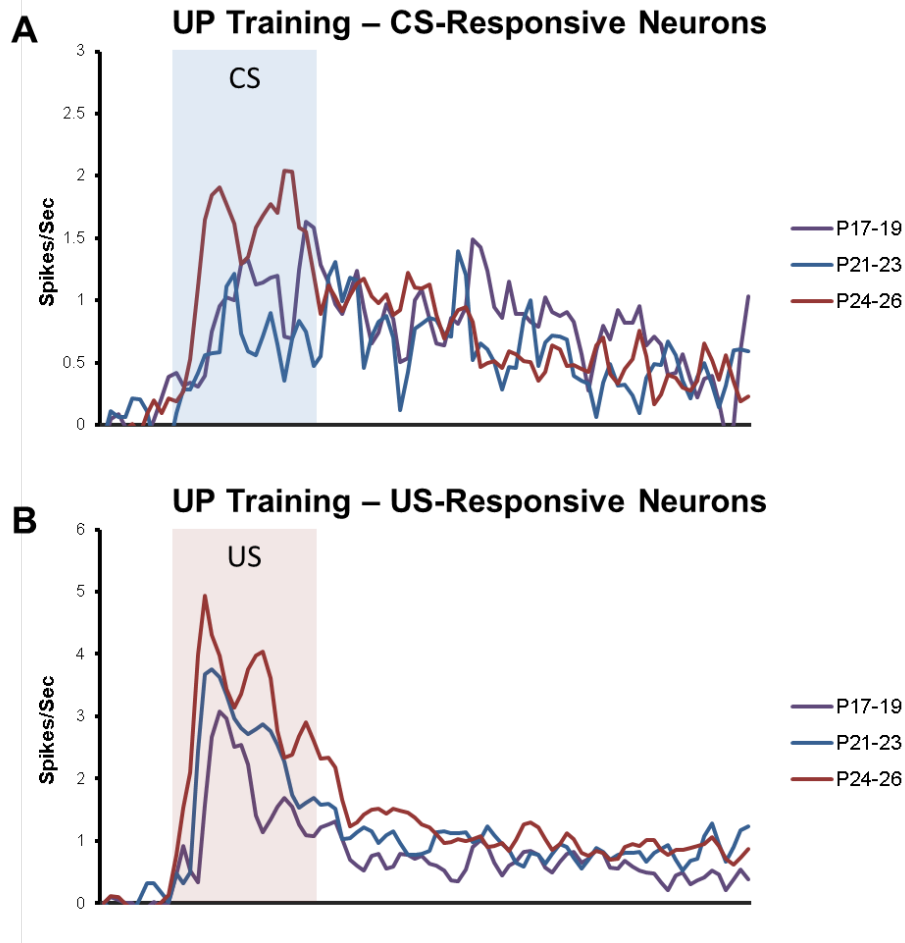


Figure 20. Unpaired presentation of the vibration CS and the US resulted in age-related increases in CA1 pyramidal cells activity.

In order to examine population-level changes in activity during unpaired training, the average neuronal firing rate during the baseline, CS (250 ms) and US (250 ms) periods for all responsive neurons were calculated for each age group during unpaired training (session 1). Firing rates were normalized to pre-CS baseline activity. (A) The oldest age group had a significantly higher firing rate during the vibration CS than the younger two age groups. (B) There was a significant age-related increase in the firing rate during the US period.

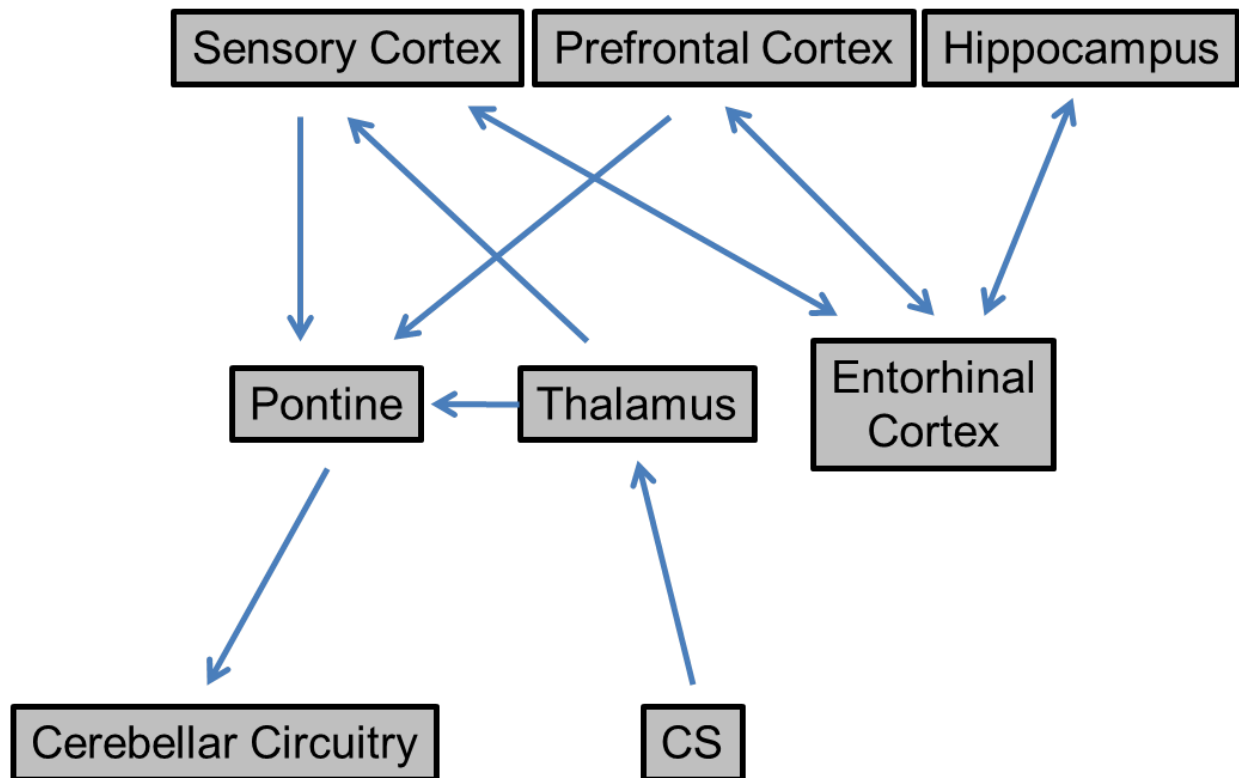


Figure 21. Possible trace EBC circuitry.

A possible trace EBC circuitry could be described as follows: CS input from a given modality arrives at the thalamus before projecting to the appropriate sensory cortex. From the sensory cortex information is relayed to the hippocampus and prefrontal (anterior/rostral cingulate) cortex via the entorhinal cortex. As the acquisition phase progresses into the consolidation phase, the relay from the entorhinal cortex to the hippocampus becomes less critical. Instead, the projection from the entorhinal cortex to the prefrontal cortex (prelimbic area) becomes crucial to the expression of the learned association. Finally, the prefrontal and sensory cortex project to the pontine nucleus, which provides the cerebellar circuitry with CS-related information.

Table 3. Count and percentage of neurons in categories of responsiveness.

During paired training CA1 neurons were categorized in one of eight categories according to the trial events to which they were responsive. During unpaired training neurons were categorized in one of four categories. The number and percentage (in parentheses) is noted for each category and age group. Based on these values, the number (and percentage) of units that responded to more than one trial event was calculated and labeled as “combination neurons”.

<b>Paired Training</b>	P21-23	P24-26	P31-33	All Ages
Nonresponsive	131 (51.6%)	154 (50.8%)	147 (44.1%)	432 (48.5%)
CS-Responsive	6 (2.4%)	5 (1.7%)	8 (2.4 %)	19 (2.1 %)
Trace-Responsive	9 (3.5%)	25 (8.3%)	8 (2.4%)	42 (4.7%)
US-Responsive	37 (14.6%)	38 (12.5%)	68 (20.4%)	143 (16.1%)
CS- & Trace-Responsive	6 (2.4%)	6 (2.0%)	7 (2.1%)	19 (2.1%)
CS- & US-Responsive	7 (2.8%)	7 (2.3%)	17 (5.1%)	31 (3.5%)
Trace- & US-Responsive	16 (6.3%)	30 (9.9%)	14 (4.2%)	60 (6.7%)
CS-, Trace-, & US-Responsive	42 (16.5%)	38 (12.5%)	64 (19.2%)	144 (16.2%)
<b>Grand Total</b>	<b>254 (100%)</b>	<b>303 (100%)</b>	<b>333 (100%)</b>	<b>890 (100%)</b>
<i>Combination Neurons</i>	<i>71 (28.0%)</i>	<i>81 (26.7%)</i>	<i>102 (30.6%)</i>	<i>254 (28.5%)</i>

<b>Unpaired Training</b>	P21-23	P24-26	P31-33	All Ages
Nonresponsive	32 (50.0%)	25 (45.5%)	28 (29.5%)	85 (39.7%)
CS-Responsive	15 (23.4%)	9 (16.4%)	42 (44.2%)	66 (30.8%)
US-Responsive	34 (53.1%)	26 (47.3%)	75 (78.9%)	135 (63.1%)
CS- & US-Responsive	18 (28.1%)	9 (16.4%)	32 (33.7%)	59 (27.6%)
<b>Grand Total</b>	<b>64 (100%)</b>	<b>55 (100%)</b>	<b>95 (100%)</b>	<b>214 (100%)</b>

## CONCLUSIONS

Overall, these studies suggest that sensory system development plays a critical role in the developmental trajectory of both delay and trace EBC. Using an early-developing CS modality facilitated the developmental emergence of delay EBC and resulted in a steeper acquisition curve than observed in tone-trained pups (Goldsberry et al., 2014). In fact, training with a vibration CS resulted in learning curves that closely matched those of pups trained with a pontine stimulation CS (Campolattaro & Freeman, 2008; Freeman et al., 2005).

Neuronal recordings from the CA1 layer of the hippocampus during auditory trace EBC revealed both age- and learning-related changes in neuronal activity (Goldsberry et al., 2015). Furthermore, the development of hippocampal activity mirrored the behavioral development of trace EBC. Specifically, both showed a relatively abrupt developmental step between P21 and P24. Although this study supported the hypothesis that hippocampal neuronal activity is related to the development of trace EBC, it did not rule out the possibility that the functional development of sensory input to the hippocampus is contributing to the delayed developmental emergence of trace EBC.

In order to address potential sensory system contributions to the ontogenetic emergence of trace EBC, we trained pups with an early-developing somatosensory CS modality. Results showed that pups trained with a vibration CS learned trace EBC at a younger age than those trained with a tone CS. Furthermore, hippocampal neuronal recordings during somatosensory trace EBC revealed increased activity to trial events compared to that observed during auditory trace EBC. Finally, even when controlling for different learning rates between CS modalities, there was a substantial increase in the magnitude of learning-related activity in vibration-trained pups.



These results suggest that although hippocampal maturation may be playing a role in the development of trace EBC and corresponding hippocampal activity, it is not the sole factor to consider. In fact, inputs to the hippocampus could also be playing a role. It is currently unclear how sensory system input is integrated into the trace EBC circuitry. One possibility is that parallel projections from the sensory cortices project to both the hippocampus and the pontine nucleus. The hippocampus then sends projections to the anterior cingulate cortex, which also projects to the pontine nucleus (Woodruff-Pak & Disterhoft, 2008). If this proposed pathway is accurate, then developmental changes in the sensory cortices or their projections to the hippocampus via the lateral entorhinal/perirhinal cortices (Takehara-Nishiuchi, 2014) may play a critical role in the development of trace EBC.

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