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Sensory system development influences the ontogeny of hippocampal associative coding and trace eyeblink conditioning

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Abstract

Until recently, it was believed that hippocampal development was the primary rate-limiting factor in the developmental emergence of hippocampal forms of learning, such as trace eyeblink conditioning (EBC). Indeed, hippocampal neuronal activity shows an age-related increase in both complexity and task responsiveness during trace EBC. However, recent work from our laboratory suggests that sensory system development may also play a role. Training with the earlier-developing somatosensory system results in an earlier emergence of trace EBC in rats, suggesting that the development of sensory input to the hippocampus may influence the development of trace EBC. The goal of the current study was to examine the activity of hippocampal CA1 pyramidal cells during acquisition of trace EBC with an early-developing somatosensory CS. Rat pups were trained with a vibration CS on postnatal days (P) 17–19, P21–23, and P24–26 while CA1 pyramidal cell activity was recorded. Results indicated that CA1 neurons show an age-related increase in responsiveness to trial events. Although the magnitude of neuronal responding showed age-related increases in activity, all three age groups demonstrated learning-related increases in firing rate magnitude and peaks in firing rate were evident both at CS onset and offset. These findings suggest that the ontogeny of trace eyeblink conditioning is related to both hippocampal and sensory system development.

Keywords

development; trace eyeblink conditioning; hippocampus; learning; somatosensory; Pavlovian conditioning

1. Introduction

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

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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, Elkin, & Freeman, 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, Paczkowski, & Stanton, 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, 1987). 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, Cacucci, Burgess, & O’Keefe, 2010). Most importantly, work from our laboratory has shown that hippocampal neuronal activity is correlated with the developmental emergence of trace EBC (Goldsberry, Kim, & Freeman, 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, 2016). 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.

2. Materials and methods

2.1. Subjects

Subjects were 9 Long-Evans rat pups ($n = 3$ per age group) from 7 different litters (2 males and 1 female in the P17–19 group, 2 females and 1 male in the P21–23, and 2 females and 1 male in the P24–26 group). 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.

2.2. 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 Ω . 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). Using an in-brain reference tetrode minimizes the electrical artifact which typically occurs when using a periorbital stimulation US.

2.3. 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).

2.4. 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 ($\times 2000$), filtered (500–5000 Hz), and integrated (20 ms time constant).

2.5. Conditioning procedures

Training began 2 days after surgery, on P17–19, P21–23, or P24–26 and consisted of 2 training sessions per day for 3 days. 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 vibrating grid floor CS (144 Hz, 2.4 m/s²), 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, Kehoe, & Marshall, 1983). During paired training, trials were separated by a variable intertrial interval averaging 30 s. 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, Thompson, & Weisz, 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.

2.6. Neuronal analyses

Following data collection, KlustaKwik (Kadir, Goodman, & Harris, 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 (Goldsberry et al. 2015).

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, $\alpha = 0.05$) 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).

2.7. 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 μ 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.

3. Results

3.1. 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 (Fig. 1). Learning-rate differences between the three age groups were

revealed when examining the percentage of adaptive (Fig. 1), 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.

3.2. 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. An ANOVA showed that 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) ($F(2, 455) = 1.475$, $P = 0.230$). 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 1).

The proportion of excitatory responsive neurons was greater when pups were given unpaired training than paired training. When collapsed across age, 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%) ($\chi^2(1, N = 462) = 11.91$, $P = 0.0006$). Unfortunately, because unpaired training always occurred on session 1 whereas paired training occurred on session 2 we are unable to conclude whether the differences found between paired and unpaired training are due to the training type or presentation (session) order.

3.2.1. 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 ($\chi^2(2, N = 890) = 4.15$, *n.s.*). This held true, even when examining only session 2 of paired training (thus matching the unpaired training analysis) ($\chi^2(2, N = 248) = 3.69$, *n.s.*) (Fig. 2). 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 $\chi^2(2, N = 890) = 9.33, P = 0.009$ (Fig. 2). 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) $\chi^2(2, N = 890) = 9.60, P = 0.008$ (Fig. 2). 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 $\chi^2(2, N = 890) = 7.90, P = 0.019$ (Fig. 2). 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 $\chi^2(2, N = 890) = 1.89, n.s.$ 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 $\chi^2(2, N = 890) = 1.24, n.s.$ 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.

3.2.2. 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. For example, a cell would fall into the “responsiveness changed” category if during No-CR trials a cell was responsive to the US but during CR trials it was responsive to both the CS and the US. 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 ($\chi^2(2, N = 890) = 2.89, P = 0.236$). When broken down by session, only session four showed group differences in responsiveness between CR and No-CR trials ($\chi^2(2, N = 165) = 9.32, P = 0.009$). This difference, however, did not follow any clear developmental pattern. The other four paired sessions failed to show any between age-group differences ($\chi^2(2, N = 115) < 3.73, n.s.$). 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.

3.2.3. 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 ($X^2(2, N = 214) = 7.75, P = 0.021$) (Fig. 2). 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 ($X^2(2, N = 214) = 18.90, P = .00008$).

3.2.4. Changes in neuronal responsiveness across sessions—When collapsed across age, there was a significant decrease in the proportion of responsive neurons across the 5 sessions of paired training ($X^2(5, N = 890) = 26.88, P = 0.00002$) (Fig. 3). 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. When further broken down by age group and trial event, only the oldest two age groups showed significant decreases in CS-, trace-, and US-responsiveness across paired training session ($ps < 0.05$). The youngest age group however, did not have significant decreases in responding to trial events across session.

3.2.5. 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 (Fig. 4) 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 ($\alpha = 0.05$). 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. Interestingly, bins that differed significantly between CR and No-CR trials were clustered together, forming peaks in activity at CS onset and offset, with the older animals showing the greatest number of bins (Fig. 4).

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. Importantly, these significant bins tended to be clustered together during or immediately following US presentation (Fig. 4). 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.

3.2.6. 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 (Fig. 5). 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.

3.2.7 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).

4. 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. Surprisingly, this trend was even more dramatic when pups received unpaired training with a vibrotactile CS. There was a substantial increase in responsive neurons in the oldest age group when compared to the younger two age groups. 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). It is unclear how the vibration, but not the tone, CS may be increasing neuronal responsiveness during unpaired training. However, regardless of CS modality, hippocampal CA1 activity shows age-related increases in activity. Moreover, we see very few CA1 neurons which show inhibitory responses. This is somewhat surprising because other studies examining CA1 responsiveness in adult animals show inhibitory neuronal responses to trial events (Green & Arenos, 2007; McEchron & Disterhoft, 1997, 1999). While it is unclear why neither our previous developmental electrophysiology study (Goldsberry et al., 2015) nor the current study found a significant number of neurons with inhibitory responses, it is possible that this variation in responsiveness increases with age and hippocampal development.

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, trace interval, and US 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 had similar firing rates. 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. However, when examining US responsiveness we found that only the oldest age group had increases in neuronal responding to the US during CR trials. This increase in responding was well-timed in that it primarily occurred during or immediately following the US. Likewise, during the CS 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 ($F_{13.66, 26647.89} = 4.536, P < 0.0001$) (Greenhouse-Geisser correction for sphericity) (Fig. 6). 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 observed a significant bin X CS modality X age interaction ($F_{21.39, 46933.45} = 4.530, P < 0.0001$) (Greenhouse-Geisser correction for sphericity). Although the P21–23 age groups had somewhat similar firing rate magnitudes across CS modality (12 of 60 bins were significantly different), the P24–26 age group had a far greater magnitude when trained with a vibration CS (27 of 60 bins were significantly different) (Fig. 6). Moreover, as previously mentioned, 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 and found a significant bin X CS modality X age interaction ($F(36.21, 53357.39) = 2.233, P < 0.0001$) (Greenhouse-Geisser correction for sphericity). 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 (17 of 60 bins were significantly higher). However, P21–23 pups had substantially higher magnitudes of responding when trained with a vibration CS (28 of 60 bins were significantly higher). 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 the oldest two 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 (McEchron & Disterhoft, 1997; Takehara, Kawahara, & Kirino, 2003). Specifically, hippocampal neuronal activity is believed to play a critical role both during and immediately following consolidation of trace EBC (Hattori, Chen, Weiss, & Disterhoft, 2015). 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). Interestingly, this early increase in hippocampal neuronal activity, which was primarily seen in the oldest two age groups, appears to occur regardless of whether or not the stimuli are presented in an associative context. The current study revealed that the highest rates of neuronal responsiveness occurred during the first training session, which consisted of unpaired presentations of the vibration CS and stimulation US. Moreover, there appears to be an age-related increase in hippocampal responsiveness during this first session. However, because session order is confounded with training type in this study, it is unclear whether these increases in activity are due to the unpaired training context or because this is the first session.

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 (Goldsberry et al., 2015). Taken together, these data demonstrate that training with an earlier-developing sensory modality is indeed able to increase not only conditioned responding, but also hippocampal neuronal activity.

There are a number of developmental changes which could account for the facilitation of hippocampal learning and neuronal activity observed when using a somatosensory CS. Whereas in delay eyeblink conditioning depends on subcortical sensory thalamus projections the pontine nucleus, which then sends it to the cerebellum, trace conditioning requires the sensory cortex (Galvez, Weible, & Disterhoft, 2007; Galvez, Weiss, Weible, & Disterhoft, 2006; Steinmetz, Harmon, & Freeman, 2013; Ward, Flores, & Disterhoft, 2012). CS information is relayed from the sensory thalamus to the sensory cortex before projecting to other forebrain structures including the medial prefrontal cortex and hippocampus, (Takehara-Nishiuchi, 2014; Weiss & Disterhoft, 2011; Woodruff-Pak & Disterhoft, 2008). The medial prefrontal cortex then projects to the pontine nucleus, providing CS input that bridges the trace interval (Kalmbach, Ohyama, Kreider, Riusech, & Mauk, 2009). Therefore, the development of learning and corresponding neuronal activity observed in the current study could be due to the development of sensory cortex, sensory inputs to the hippocampus (via the entorhinal cortex), or projections from the prefrontal cortex to the pontine nucleus. Developmental differences in neural CS processing between somatosensory and auditory CSs may make the vibration CS more salient to developing rats than the tone CS, resulting in earlier and faster learning with the vibration CS.

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Highlights

- Prevalence of trial event-responsive neurons in CA1 increases with age
- Magnitude of CA1 trial event-related activity is greatest during learning
- Magnitude of CA1 trial event-related activity increases with age
- Proportion of event-responsive neurons decreases across training days
- Early-developing CS modalities facilitate learning and hippocampal responding

Fig. 1.

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 (UP) (session 1) and paired (P) training (sessions 2–6). (A) Scatterplot and mean conditioned response (CR) percentage in rat pups given trace eyeblink conditioning with a vibration CS. (B) Scatterplot and mean adaptive conditioned response (CR) percentage in rat pups given trace eyeblink conditioning with a vibration CS. Mean (SEM) for CR Percentage for the P17–19 group were 6.63 (3.38), 14.25 (6.86), 27.53 (1.73), 20.99 (6.73), 34.72 (3.30), 38.91 (9.22), for the 21–23 group were 14.62 (6.56), 40.02 (17.40), 30.96 (17.88), 33.33 (14.91), 45.28 (15.88), 43.28 (15.65), and for the 24–26 group were 17.33 (11.63), 45.40 (16.91), 53.36 (18.71), 75.29 (7.01), 85.70 (4.46), 78.14 (7.93).

Fig. 2.

Hippocampal CA1 neurons respond differentially to trial events when trained with a vibration CS during paired (PR) and unpaired (UP) trials. (A) Example of Nissl-stained brain section with CA1 layer tetrode placement (B) There were age-related changes in the proportion of cells that respond to trial events (vibration CS, trace interval, and/or periorbital stimulation US) during unpaired but not paired training. (C) There was a significant age-related increase in the proportion of cells that responded to the CS-only during unpaired, but not paired training (D) 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). (E) The proportion of US-only responsive neurons increased across age. (F) 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).

Fig. 3. Session by session changes in neuronal responsiveness. When collapsed across age, the proportion of neurons that were responsive to trial events decreased as training continued.

Fig. 4.

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. The US onset and offset are indicated with dotted lines. All age groups showed significant neuronal firing rate increases during the CS and trace periods during CR trials compared to No-CR trials. When comparing firing rate magnitude during CR trials across age group, there was an age-related increase in firing rate during the CS and trace periods.

Fig. 5.

Paired and unpaired presentation of the 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), Trace (500 ms), and US (250 ms) periods for all responsive neurons were calculated for each age group during unpaired (session 1) and paired (sessions 2–6) training. 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) The oldest age group had a significantly higher firing rate than the younger two groups. (C) There was a significant age-related increase in the firing rate during the US period. (D) The oldest age group had a higher firing rate than the two younger groups.

Fig. 6.

CA1 pyramidal cells show different firing rate profiles when trained with vibration versus tone CS modalities. In order to examine population-level changes in activity during unpaired training, the average neuronal firing rate during the baseline, CS (250 ms), Trace (500 ms), and US (250 ms) periods for all responsive neurons were calculated for each age group during unpaired (session 1) and paired (sessions 2–6) training. Firing rates were normalized to pre-CS baseline activity. (A) Unpaired training with a vibration CS resulted in increased firing rate magnitude during the CS (B) CA1 firing rate magnitude was similar in the P21–23 groups trained with vibration and tone CSs. (C) In the P24–26 group vibration-trained pups had greater CA1 firing rates than those observed in tone-trained pups.

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”.

Paired Training	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%)
Grand Total	254 (100%)	303 (100%)	333 (100%)	890 (100%)
<i>Combination Neurons</i>	<i>71 (28.0%)</i>	<i>81 (26.7%)</i>	<i>102 (30.6%)</i>	<i>254 (28.5%)</i>

Unpaired Training	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%)
Grand Total	64 (100%)	55 (100%)	95 (100%)	214 (100%)