Published in final edited form as:

Dev Psychobiol. 2014 July ; 56(5): 999–1007. doi:10.1002/dev.21180.

Amygdala Inactivation Impairs Eyeblink Conditioning in Developing Rats

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Abstract

The amygdala facilitates acquisition of eyeblink conditioning in adult animals by enhancing conditioned stimulus (CS) inputs to the cerebellum and the unconditioned response circuitry. Ontogenetic changes in amygdala modulation of eyeblink conditioning have not been investigated directly. We examined the effects of amygdala inactivation on the ontogeny of eyeblink conditioning and conditioned freezing in rat pups. Rat pups received bilateral infusions of saline or bupivacaine into the central nucleus of the amygdala before each of the first 5 training sessions, which consisted of paired CS-US trials on postnatal days (P)17-19, P21-23, or P24-26. The final session consisted of CS-alone test trials to assess the effect of amygdala inactivation during training on conditioned freezing. Amygdala inactivation impaired acquisition of eyeblink conditioning in all of the age groups and impaired freezing to the context during the extinction test. The results indicate that the amygdala modulates cerebellar learning as soon as it begins to emerge ontogenetically.

Keywords

rat pup; associative learning; eyeblink conditioning; amygdala; eyelid; cerebellum

INTRODUCTION

Eyeblink conditioning is a form of associative learning that involves repeated pairings of a conditioned stimulus (CS) such as a tone with an unconditioned stimulus (US) that elicits an eyelid closure unconditioned response (UR). Continued pairing of the CS and US leads to the development of an eyelid conditioned response (CR) that occurs after onset of the CS but before onset of the US. Acquisition and storage of the memory underlying delay eyeblink conditioning occurs within the cerebellum (Freeman & Steinmetz, 2011; McCormick et al., 1982).

Although the cerebellum is the site of memory storage underlying eyeblink conditioning, forebrain systems modulate the rate of acquisition and retention. Lesions or inactivation of the amygdala impair acquisition of eyeblink conditioning in adult rabbits and rats

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NOTES

This work was supported by National Institutes of Health grant NS038890 to J.H.F.

(Blankenship et al., 2005; Burhans & Schreurs, 2008; Lee & Kim, 2004; Weisz et al., 1992). The deficits in eyeblink conditioning with amygdala inactivation or lesions may be due to the loss of modulatory input to the UR circuitry or to the CS pathways projecting to the cerebellum. Evidence for amygdala modulation of the UR circuitry comes from studies that found that inactivation or lesions of the central nucleus (CeA) impair reflex facilitation (in the presence or absence of the CS) and stimulation of the CeA facilitates the UR (Burhans & Schreurs, 2008; Weisz et al., 1992; Whalen & Kapp, 1991). Evidence for amygdala modulation of the CS pathway comes from a study showing that inactivation of the amygdala blocked conditioning-specific increases in pontine nucleus neuronal activity (Taub & Mintz, 2010). Neurons in the CeA recorded during eyeblink conditioning show a substantial increase in firing during the CS that increases in parallel with CR acquisition and is primarily seen on trials in which a CR occurred (Rorick-Kehn & Steinmetz, 2005). Moreover, this learning-specific activity decreases during extinction as the CR decrements. These neuronal changes do not occur as robustly in the basolateral nucleus, suggesting that the CeA is the primary source of modulation of cerebellar learning mechanisms.

The ontogeny of delay eyeblink conditioning has been studied extensively in rats, which start to show robust CRs between postnatal days (P)17 and 24 (Stanton et al., 1992). Developmental changes in the CS and US pathways have been identified that limit plasticity within the cerebellum during eyeblink conditioning (Freeman, 2010). However, developmental changes in amygdala modulation of cerebellar learning could also play a role in the ontogenetic emergence of eyeblink conditioning. Fear conditioning, which is mediated by the amygdala, develops earlier than eyeblink conditioning when freezing is measured but not when fear-potentiated startle is measured (Stanton, 2000; Richardson & Hunt, 2010). Amygdala modulation of the cerebellum may therefore be present early in the ontogeny of eyeblink conditioning when freezing is evident or emerge later in the ontogeny of eyeblink conditioning when fear-potentiated startle is evident. The current study used reversible inactivation to examine the role of the amygdala in the ontogeny of eyeblink conditioning. Rat pups were trained on eyeblink conditioning on P17-19, 21-23, or 24-26 with bilateral infusions of bupivacaine or vehicle into the CeA prior to each training session. Freezing was assessed from video records along with eyeblink conditioning to examine the effects of CeA inactivation on fear conditioning as a way of verifying the efficacy of the amygdala inactivation.

METHODS

Subjects

Seventy seven Long-Evans rat pups (38 female and 39 male) from 34 litters between the ages of postnatal day (P) 17-19 ($n = 27$), P21-23 ($n = 23$), and P24-26 ($n = 27$) were used. Rats were housed in Spence Laboratories of Psychology at the University of Iowa with ad libitum access to food and water, and maintained on a 12 hr light/dark cycle. P21-23 and P24-26 pups were weaned on P19 and housed with littermates. P17-19 pups were housed with their dam and were weaned after the last session of training. All procedures used in this study were approved by the University of Iowa IACUC.

Surgery

Pups received surgery 2 d before training. While anesthetized with isoflurane (1-3%), a craniotomy was performed for bilateral implantation of 27 gauge stainless steel cannula guides 1.0 mm above the CeA. The stereotaxic coordinates for P24-26 pups were 1.5 mm posterior to bregma, 3.8 mm lateral to midline, and 5.7 mm ventral to the skull surface. The stereotaxic coordinates for P17-19 and P21-23 were 1.5 mm posterior to bregma, 3.6 mm lateral to midline and 5.5 mm ventral to the skull surface. Stainless-steel electromyography (EMG) electrodes for recording eyelid activity were implanted into the upper left orbicularis oculi muscle. A bipolar stimulating electrode for delivering the US was implanted subdermally, caudal to the left eye. Bone cement (Zimmer) was used to secure EMG, bipolar electrodes, and cannula guides to skull screws.

Conditioning Apparatus

A detailed description of the apparatus can be found in a previous report (Ng & Freeman, 2012). The conditioning chamber had a metal grid floor and was illuminated by a red light. There was a fan outside each chamber that generates white noise. The CS was delivered through a speaker on one side of the chamber. The US was delivered by a stimulus isolator (model number 365 A; World Precision Instruments). Presentation of the stimulus and recording of the eyelid EMG activity was controlled by computer software (JSA Designs). Differential EMG activity was filtered (500-5000 Hz), amplified (2000x), and integrated (JSA Designs).

Conditioning Procedures

Standard delay conditioning procedures with a 2.0 kHz 85 db tone CS that lasted for 400 ms, and a 2.5 mA periorbital shock US that lasted for 25 ms were used. Rats were trained twice daily with a 4 h interval between sessions, for a total of 6 sessions. In the first 5 sessions, rats were given bilateral infusions of either bupivacaine (1.2%, 0.3 μ L per side) or vehicle at the rate of 6 μ L/hr. The infusion cannula was left in place for 2 min after infusions and then replaced with a stylet. Bupivacaine was used to inactivate the amygdala in the current study because of its efficacy in a previous developmental study of conditioning (Kim & Richardson, 2008) and its shorter duration than muscimol. After infusions, the pups were given 45 trials of CS-US paired training with 5 CS-alone trials, which occurred every 10th trial. In the 6th session rats were presented with 50 CS-alone extinction trials with no infusions. The extinction session was used primarily to assess fear conditioning in the absence of the US. We did not expect the younger pups to acquire eyeblink conditioning at the same rate as the older pups, so assessment of extinction in eyeblink conditioning was confounded by different levels of acquisition between age groups. Extinction was analyzed for eyeblink conditioning but the data of primary interest for extinction were the fear conditioning data. The inter trial interval for both paired and extinction sessions ranged from 20 to 40 s (mean = 30 s).

Eyeblink Conditioning Measures

Eyeblink CRs were defined as EMG activity that exceeded a threshold of 0.4 units (amplified and integrated units) above the baseline mean during the CS period after 80 ms.

EMG activity that exceeded the threshold during the first 80 ms of the CS period were defined as startle responses. On CS-alone probe trials, the duration for scoring CRs was extended beyond the CS to the end of the trial period (1.0 s). URs were defined as EMG activity that crossed the threshold after the offset of the US. EMG activity was not recorded during the US to avoid the shock artifact.

Freezing Measures

All training sessions were videotaped with a Panasonic BL-C1A network camera that was secured outside the conditioning chamber. Two small LED lights were placed outside the conditioning chamber (not visible to the rats) to indicate the occurrence of the CS for the video recordings. Video files were imported into MATLAB to perform automated frame-by-frame coding of movement. A threshold was set to ensure movements that produced by respiration were not counted. The absence of movement throughout an entire 1 s period for a given time interval was counted as freezing. Analysis of movement occurred 10 s before and 11 s after CS onset for each trial. Baseline activity for the extinction session was taken from the 210 s immediately before the first CS. The 210 s period was divided into 10 “trials” that were 21 s in duration to equate time intervals between baseline, CS-US, and CS-alone trials.

Histology

At the end of training, rats were deeply anesthetized with sodium pentobarbital. They were then perfused with PBS and 10% formalin. Their brains were then extracted and fixed with 30% sucrose formalin. After sectioning at 50 μ m, tissues were mounted on glass slides and stained with thionin. Histology was used to examine cannula placement using a light microscope and a stereotaxic brain atlas (Swanson, 2004).

RESULTS

Cannula Placement

All data used in the subsequent analyses were from rat pups with bilateral cannula placements in the CeA (Fig. 1). Among a total of 77 pups used in the study, 15 were excluded because of unilateral or bilateral cannula misses. This included 8 out of 27 for P17-19 (3 bupivacaine; 5 saline), 3 out of 23 for P21-23 (3 bupivacaine; 0 saline), and 4 out of 27 for P24-26 (2 bupivacaine; 2 saline).

Eyeblink Conditioning

Eyeblink conditioning was assessed by examining the percentage of trials with CRs across training sessions (Fig. 2). As in previous studies, there was an age-related increase in CR percentage across training sessions in the control groups. Amygdala inactivation severely impaired acquisition in all of the age groups. The developmental changes in learning in the control groups and deficits in the inactivation groups are most clearly evident on the last CS-US training session (Fig. 3). A repeated-measures ANOVA on the CR percentage data indicated a treatment by age by session interaction, $F(8,224) = 8.74, p < .01$. Post-hoc examination with the Tukey HSD test showed that pups in the P17-19 bupivacaine group showed a significantly lower CR percentage than their saline controls on session 5, pups in the P21-23 bupivacaine group showed a lower CR percentage than their saline controls on

sessions 2-5, and pups in the P24-26 bupivacaine group showed a lower CR percentage than their saline controls on sessions 3-5 (all comparisons $p < .05$). Comparisons between saline groups indicated that the P21-23 and P24-26 pups had a higher CR percentage than the P17-19 pups on sessions 3-5 ($p < .05$). No significant developmental differences in CR percentage were observed between bupivacaine groups. The results indicate that there was a developmental increase in conditioning, as seen in previous studies, and bupivacaine inactivation of the amygdala impaired conditioning in all the age groups.

A two-way ANOVA on averaged CR percentage during the extinction session indicated a significant main effect of age, $F(2,56) = 5.32, p < .01$, and a significant main effect of treatment, $F(1,56) = 32.96, p < .01$. Post-hoc tests of the main effect of age showed that the P24-26 group had a higher CR percentage than the P17-19 group ($p < .05$), but no significant differences were seen between the P24-26 and P21-23 groups or between the P17-19 and P21-23 groups. Post-hoc tests of the treatment main effect indicated that bupivacaine groups showed a significantly lower CR percentage than saline groups ($p < .05$) (Fig. 2). The CR percentage across 10-trial blocks within the extinction session was also examined to assess within-session extinction. A repeated-measures ANOVA on the block data during the extinction session (data not shown) indicated a significant treatment by block interaction, $F(4,224) = 8.63, p < .01$ and a main effect of age, $F(2,56) = 4.99, p < .01$. Post-hoc tests of the treatment by block interaction showed that the saline groups had a higher percentage of CRs than the bupivacaine groups on every block during extinction ($p < .05$).

Analyses of CR amplitude and latency were not performed because the frequency of CRs in the bupivacaine groups was too low for valid statistical comparisons of these variables.

A previous report showed that the percentage of startle responses increases with associative learning in rat pups and this learning-related increase may be driven by the amygdala (Ng & Freeman, 2012). In the current study, amygdala inactivation impaired the associative increase in startle responses (Fig. 4). A repeated-measures ANOVA on startle percentage data across paired training indicated a significant treatment by session interaction, $F(4,224) = 5.70, p < .01$. Post-hoc test showed that the saline groups had a significantly higher startle percentage than the bupivacaine groups on sessions 3 to 5 ($p < .05$). In addition, a two-way ANOVA on averaged startle percentage during extinction showed a significant main effect of treatment, $F(1,56) = 4.48, p < .05$, which was due to a higher startle percentage in the saline groups than the bupivacaine groups.

Because previous reports indicated that the amygdala modulates the US pathway we also examined the effects of bupivacaine inactivation of the amygdala on UR parameters. A repeated measures ANOVA on the peak UR latency across training sessions indicated a main effect of treatment, $F(1,56) = 5.64, p < .05$, which was due to an increase in UR latency with amygdala inactivation. A repeated measures ANOVA on UR amplitude found no effect of treatment. This finding is consistent with a previous study that examined UR amplitude from eyelid EMG activity in adult rats that had received amygdala inactivation with muscimol shortly before training (Lee & Kim, 2004).

Fear Conditioning

Analysis of freezing behavior was used to assess fear conditioning. It is important to note that the current analysis of fear conditioning from eyeblink conditioning sessions differs from standard fear conditioning studies in rat pups or adult rats (e.g., Kim & Richardson, 2007; Kim & Richardson, 2008). The CS and US durations, ISI, and ITI are considerably shorter than in fear conditioning studies. Moreover, hundreds of CS-US trials were given during eyeblink conditioning whereas most fear conditioning studies use just a few CS-US trials. The difference in the number of training trials is due to a substantial difference in the rate of acquisition, with eyeblink conditioning requiring hundreds of trials for acquisition and fear conditioning requiring as few as 1 trial for acquisition. Thus, the fear conditioning in the current eyeblink conditioning study is weaker and more phasic (i.e., short-duration within trials) than the fear conditioning seen in more traditional studies of fear conditioning.

Contextual freezing was examined for the extinction session in the training context during which no infusions were administered (Fig. 5). Freezing was first examined during the baseline period before the first extinction trial. Thus, retention of contextual fear conditioning following training with or without amygdala inactivation was assessed in this analysis. The 21 bins (1 s each) of freezing data during the baseline period were averaged into one interval for each age and treatment condition. A two way ANOVA on this averaged baseline freezing data indicated a significant age by treatment interaction, $F(2,56) = 3.29$, $p < .05$. Post-hoc tests indicated that the P21-23 and 24-26 bupivacaine groups had significantly less baseline freezing than respective age matched saline controls, but the P17-19 bupivacaine group did not differ significantly from their respective age matched saline group. However, t-tests indicated lower freezing in the bupivacaine groups relative to the saline groups in the P17-19 ($t(17) = 3.45$, $p < 0.01$), P21-23 ($t(18) = 2.57$, $p < 0.02$), and P24-26 ($t(21) = 5.71$, $p < 0.001$) groups. To further examine the apparent group differences seen at each age, separate repeated measures ANOVAs were done on each age. There was a significant main effect of treatment for the P21-23 and P24-26 groups, $F(1,18) = 6.62$, $p < .05$ and $F(1,21) = 32.56$, $p < .01$, respectively. A significant treatment by bin interaction was found for the P17-19 group, $F(20,340) = 1.92$, $p < .05$. Post-hoc tests showed that the saline group had a higher freezing percentage than the bupivacaine group on all bins except 7, 8 and 13.

Freezing during the first and last 20 trials of the extinction session was analyzed to examine age-related and inactivation-related effects on fear and fear extinction (Fig. 6). Freezing during the pre-CS period was thought to primarily reflect fear conditioned to the training context. After CS onset, a drop in freezing occurred in the saline groups, which was due to a head jerk and subsequent shuffling movements. These responses were presumably the result of eyeblink conditioning in sessions 1-5 and countered the freezing response. Freezing then returned to the pre-CS level after a few seconds. A repeated measures ANOVA on the freezing percentage data during the initial 20 trial block and the final 20 trial block indicated a block by treatment by bin (1 s) interaction, $F(20,1120) = 2.73$, $p < .01$. For the initial 20 trial block during extinction, post-hoc tests showed that saline groups had a higher percentage of freezing during the pre-CS period (all bins) and after CS onset (bins 4 - 10) than the bupivacaine groups ($p < .05$). Saline pups also showed a bigger drop in freezing

immediately after CS onset (bins 0-2; $p < .05$). For the last 20 trial block during extinction, the saline groups showed significantly more freezing than the bupivacaine groups during the pre-CS baseline period (all pre-CS bins except -7 and -2; $p < .05$). Saline groups also showed a bigger drop in freezing immediately after CS onset (bins 0-4; $p < .05$).

Comparison of the saline groups between the initial and final 20 trial blocks of extinction showed that they had a higher percentage of pre-CS freezing during the initial block relative to the final block (all pre-CS bins). Saline animals also showed a bigger drop in freezing immediately after CS onset (bin 0) in the initial 20-trial block than the final 20-trial block of extinction ($p < .05$). Comparison of the bupivacaine groups between the initial and final 20-trial blocks of training showed that they had a higher percentage of freezing during the pre-CS period in the final block (all bins, except bin -7). These results indicate that bupivacaine inactivation of the amygdala during eyeblink conditioning impaired acquisition of fear conditioning, which was indexed by freezing during the pre-CS period and a drop in freezing caused by a head jerk during the CS.

Freezing was also examined on the 5 CS-alone trials for session 1 of eyeblink conditioning to assess acquisition of fear before eyeblink conditioning emerged (data not shown). The percentage of time freezing was compared for 10 s before and 10 s after CS onset.

Bupivacaine inactivation impaired conditioning-related movement during the CS and freezing during the baseline period. A repeated measures ANOVA on freezing percentage revealed a significant treatment by time period (baseline vs. CS + post-CS) interaction, $F(1,56) = 20.12$, $p < .01$, and a time period by bin (1 s) interaction, $F(9,504) = 5.13$, $p < .01$. Post-hoc tests showed that saline groups had a significant drop in freezing from baseline after CS onset, but bupivacaine groups did not show the same drop in freezing. In addition, the saline groups showed more freezing during the baseline period than the bupivacaine groups ($p < .05$).

Analysis of freezing on the 5 CS-alone trials for session 5 indicated a significant treatment by time periods by bin interaction, $F(9,504) = 2.85$, $p < .01$. Post-hoc tests showed that saline groups had a larger drop in freezing in bins (0-6) after CS onset than bupivacaine rats, indicating a loss of conditioned movement with amygdala inactivation ($p < .05$). There was no inactivation-induced deficit in baseline freezing during session 5.

DISCUSSION

Amygdala inactivation severely impaired acquisition of eyeblink conditioned responses in rat pups trained on P17-19, 21-23, or 24-26. Deficits were also seen in conditioning-specific increases in acoustic startle responses and in the UR latency. The deficits in eyeblink conditioning suggest that the amygdala modulates the CS and UR neural circuitry in rat pups as young as 17-19-days-old. Deficits were also seen in contextual freezing and conditioned body movements during the CS-alone extinction test.

The age-related increase in the acquisition rate of eyeblink conditioning replicated previous studies (Ng & Freeman, 2012; Stanton et al., 1992). All saline groups showed an increase in CRs across training sessions; even the youngest group (P17-19) showed a modest increase in CRs. Inactivation of the CeA blocked eyeblink conditioning as soon as it began to emerge

ontogenetically. Thus, even the modest level of eyeblink conditioning seen in pups trained on P17-19 was impaired by CeA inactivation. Previous studies found that adult rats with cerebellar lesions can acquire eyeblink conditioning with more than 800-1000 trials (Blankenship et al., 2005; Lee & Kim, 2004) and we would expect the same to be true of pups that started training on P21 or 24. Amygdala modulation of cerebellar learning mechanisms is therefore present very early in development. An implication of this finding is that amygdala modulation of the cerebellum, although clearly important at all ages tested, does not play a major role in the ontogenetic emergence of eyeblink conditioning. We would have concluded that amygdala modulation plays an important role in the ontogenetic process if inactivation of the CeA impaired a subset of the age groups; for example, the P21-23 and P24-26 groups. Our findings suggest, however, that amygdala inputs to the CS and US pathways boost eyeblink conditioning as early as cerebellar learning is possible but developmental changes in the US and CS pathways themselves appear to be the primary developmental mechanisms underlying the ontogeny of eyeblink conditioning (Freeman, 2010).

Acoustic startle responses increased across eyeblink conditioning sessions in all of the age groups, which replicated the findings of a previous study (Ng & Freeman, 2012). Deficits in the percentage of eyelid acoustic startle responses in the groups given amygdala inactivation are also consistent with the findings of studies on the neural mechanisms underlying fear-potentiation of whole body startle responses in adult rats (Davis, Falls, Campeau, & Kim, 1993). These findings suggest that fear conditioning resulting from eyeblink conditioning potentiates the eyelid startle response through amygdala projections to the brainstem circuitry that produces the eyelid reflex (Burhans & Schreurs, 2008; Pellegrini et al., 1995; Trigo et al., 1999; Weisz et al., 1992; Whalen & Kapp, 1991).

Amygdala inactivation increased the peak latency of the UR in all of the age groups. The CeA projects to the UR pathway and has been shown to facilitate the UR in the presence or absence of the CS (Burhans & Schreurs, 2008; Weisz et al., 1992; Whalen & Kapp, 1991). Deficits in conditioning-specific facilitation of the UR in the absence of the CS following amygdala inactivation suggest that the amygdala input to the UR circuitry can boost the responsiveness of the circuitry (Burhans & Schreurs, 2008). Amygdala facilitation of the UR appears to occur relatively early in the development of eyeblink conditioning since inactivation effects were seen in all of the age groups in the current study. Amygdala modulation of the UR neural circuitry therefore plays an important role in eyeblink conditioning but may not contribute to the ontogenetic processes underlying the developmental emergence of eyeblink conditioning.

Freezing was measured to verify that the amygdala inactivation was effective, which was a necessary part of the experimental design since we originally hypothesized that eyeblink conditioning would be impaired at P21-23 and P24-26 but not at P17-19. Deficits in freezing were seen in all age groups during the extinction test. Freezing during the baseline period, before any CSs were delivered, is an index of contextual fear conditioning. Freezing during CS presentations was a combination of contextual and cued fear conditioning since it was assessed in the training context. For the purposes of verifying amygdala inactivation it was not necessary to do separate assessments of cued and contextual fear conditioning in the

current study. The fear conditioning assessed in the current eyeblink conditioning study differed in several important ways from standard fear conditioning studies. First, the durations of the CS, US, ISI, and ITI were considerably shorter than in most fear conditioning studies. Second, hundreds of CS-US trials were given during eyeblink conditioning whereas most fear conditioning studies use just a few CS-US trials. Fear conditioning in our eyeblink conditioning experiment was therefore weaker and more phasic (i.e., short-duration within trials) than the fear conditioning seen in standard fear conditioning experiments. Nevertheless, deficits in fear conditioning were seen in all of the age groups with CeA inactivation, verifying that the manipulation was effective.

A novel finding in the current study is the presence of a drop in freezing immediately after CS onset. This is not a measurement error because the changes in activity persist for several seconds before returning back to baseline. The drop in freezing with CS presentations is amygdala-dependent as it was not seen in the groups given CeA inactivation. Moreover, the decrease in the magnitude of the drop in freezing during the last 20 trials of the extinction test shows that it is learning-related. The CS-related drop in freezing was acquired earlier than the eyeblink CR, much like conditioned freezing. Fear conditioning with eyeblink conditioning parameters therefore produced two responses, freezing to the context and movement to the CS. Although the behavioral mechanisms underlying the drop in freezing during CS presentations have not been determined, the most relevant finding for the goals of the current study is that CeA inactivation blocked this effect in all of the ages tested, further confirming that inactivation was successful in all of the age groups that were given eyeblink conditioning.

The findings of the current study are consistent with a two-factor model of eyeblink conditioning in which an early emotional conditioning component facilitates acquisition of a later developing motor CR (Thompson et al., 1987; Weisz et al., 1992; Neufeld & Mintz, 2001; Taub & Mintz, 2010). The early emotional conditioning is thought to be mediated by the amygdala and the motor CR is thought to be mediated by the cerebellum. We demonstrated that the initial stage of emotional conditioning and later acquired eyeblink conditioning are impaired by amygdala inactivation in all of the age groups tested. Amygdala modulation of cerebellar learning is therefore present as soon as the cerebellar circuitry is mature enough to start producing eyeblink CRs. These findings suggest that amygdala modulation of the cerebellar circuitry plays a significant role in eyeblink conditioning but does not play a role in the developmental processes underlying the ontogenetic emergence of eyeblink conditioning. Rather, the ontogeny of eyeblink conditioning is primarily attributable to developmental changes in the CS and US pathways (Freeman, 2010).

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Figure 1.

Coronal section of the rat pup brain showing representative cannula placements. Two 27 gauge guide cannulas were implanted bilaterally to allow concurrent infusion into both sides of the central nucleus of the amygdala (CeA) with a 32 gauge cannula prior to each paired training session. BLA, basolateral amygdala; LA, lateral amygdala. No infusions were given prior to the extinction session.

Figure 2.

Mean $\pm$ SEM eyeblink conditioned response (CR) percentage in rat pups given saline (Sal) or bupivacaine (Bup) infusions into the central nucleus of the amygdala and trained on postnatal days (P)17-19, P21-23, or P24-26. The first 5 sessions consisted of paired CS-US (P) trials and the 6th session was an extinction sessions (Ext) that consisted of CS-alone trials.

Figure 3.

Example traces of eyelid EMG signals from each trial from representative pups in each age group (P17-19, P21-23, P24-26) and treatment condition (Sal, saline; Bup, bupivacaine) during the last CS-US paired session. The shaded area indicates the CS period.

Figure 4.

Mean $\pm$ SEM eyeblink startle response (SR) percentage in rat pups given saline (Sal) or bupivacaine (Bup) infusions into the central nucleus of the amygdala and trained on postnatal dyas (P)17-19, 21-23, or 24-26. The first 5 sessions consisted of paired CS-US (P) trials and the 6th session was an extinction sessions (Ext) that consisted of CS-alone trials.

Figure 5.

Mean + SEM freezing during the extinction session prior to CS presentations in pups given saline (Sal) or bupivacaine (Bup) infusions into the central nucleus of the amygdala before each of the CS-US training sessions on postnatal days (P) 17-19, 21-23, or 24-26. No infusions were given before the extinction session. Baseline freezing for the extinction session was taken from the 210 s immediately before the first CS. The 210 s baseline period was divided into 10 “trials” that were 21 s in duration. Top graphs show freezing in 1 s bins. Bottom graphs show freezing averaged across bins.

Figure 6.

Mean + SEM freezing during the extinction session before, during, and after CS presentations (gray bar) in pups given saline (Sal) or bupivacaine (Bup) infusions into the central nucleus of the amygdala before each of the CS-US training sessions on postnatal days (P) 17-19, 21-23, or 24-26. No infusions were given before the extinction session. Freezing was measured 10 s before the CS and 11 s after CS onset.