Abstract

The two-factor theory postulates that classical conditioning proceeds through two stages, which support successive acquisition of emotional and motor responses. Emotional conditioning is thought to facilitate the subsequent acquisition of the motor response. This form of interaction between the two stages of learning can be investigated while considering the central role of the amygdala and the cerebellum in emotional and motor conditioning, respectively. Rats with bilateral lesions of the amygdala or the cerebellar interpositus or intact rats were subjected to a fear conditioning session followed by four eyeblink conditioning sessions. Another group of intact rats was subjected to eyeblink conditioning only. The CS in the fear conditioning session was a 73 dB tone, paired with a 100 dB noise-US. The same CS was paired with a periorbital electroshock-US during eyeblink conditioning. Results showed that fear preconditioning facilitated the subsequent eyeblink conditioning among the intact groups. Amygdaloid lesions abolished this facilitatory effect of fear conditioning. These findings demonstrate that amygdala-mediated emotional conditioning facilitates the subsequent acquisition of cerebellum-mediated motor responses.

1. Introduction

The two-factor theory of learning advocates the idea that classical conditioning proceeds through two separate stages of learning [8,12]. The two stages are most often demonstrated in the context of the aversive conditioning paradigm, which supports acquisition of emotional responses (emotional CRs) in the first stage and acquisition of discrete motor responses (motor CRs) in the second stage. Acquisition of emotional CRs is fast, typically after just a few presentations of paired CS–US trials. Emotional CRs are defined as ‘preparatory’ and ‘nonspecific’ in the sense that they feature an energized state of the organism but not an actual action that may alleviate the impact of the impending aversive US, e.g. a conditioned suppression of ongoing activity. On the other hand, acquisition of motor CRs is typically slow. The motor CRs are considered to be ‘specific’ since they involve discrete skeletal movements of an organ that is best equipped to antagonize the effects of the noxious US, e.g. a conditioned eyeblink preceding a corneal airpuff [5,8,13].

The two-factor theory of conditioning also advocates a possible interaction between the two stages of learning whereby acquisition of motor CRs benefits from the preceding acquisition of emotional CRs [8]. Neurobiological testing of the above hypothesis was made possible after realization that distinct brain sites mediate the two stages of learning. The amygdala, and particularly its lateral and central nuclei, seems to be a key component in acquisition and expression of conditioned fear [1,7]. Lesions of the central nucleus abolished conditioned heart rate, blood pressure, and freezing responses [1,4,14]. The cerebellum is involved in acquisition of discreet motor responses and lesions of the interpositus abolished the acquisition and
retention of the eyeblink CRs [5,6,11,16]. These findings point to the amygdala and the cerebellum as prototypical structures involved in emotional and motor conditioning, respectively. Manipulation of these sites may help in revealing the nature of interaction between the two stages of learning.

Weisz et al. [13] showed that lesions of the central amygdala, performed in order to abolish the emotional conditioning, retarded the acquisition of cerebellum-related eyeblink CRs. These findings suggest that the two learning stages do interact in an intact brain, and that this interaction features some beneficial effects of the amygdala on the conditioning process in the cerebellum. Interestingly, this form of interaction between the two stages of learning was revealed only when the CS intensity (65 dB) was set to trigger sub-maximal rates of CR acquisition. Lesions of the amygdala had no effect on the fast acquisition of eyeblink CRs when the tone CS intensity was raised to 85 dB.

The present study attempts to demonstrate that cerebellum-related eyeblink conditioning benefits from the preceding acquisition of amygdala-based fear responses. To achieve this goal, rats were exposed sequentially to two procedures, each preferentially supporting the acquisition of fear and eyeblink CRs, respectively. Contribution of the fear CRs to motor conditioning could then be revealed in comparison to rats in which the fear-inducing procedure was omitted and to rats with amygdaloid lesions. Throughout the two procedures, we relied on findings by Weisz et al. [13] which suggest that amygdala effects could be revealed only when experimental parameters supported sub-maximal rates of eyeblink conditioning. Thus, the CS was set to moderate intensity levels throughout the two procedures and the US was set to low-intermediate levels during the motor conditioning procedure.

2. Methods

2.1. Subjects

Subjects were male Wistar rats, weighing 320–380 g at the time of surgery. They were housed individually with free access to food and water and were kept under a reversed dark/light schedule with all experiments performed during the dark phase.

2.2. Surgery

Animals were anesthetized with Equithesine (3 ml/kg, i.p.). Standard stereotaxic procedures were used to induce bilateral electrolytic lesions of either the cerebellar interpositus or the central nucleus of the amygdala. An insulated insect pin, exposed for 0.5 mm at the tip, was inserted into the target area. A DC current of 0.5 mA was applied for 10 s at four locations of the interpositus on each side (AP $-2.7$ to $-3.3$; ML $1.4$ to $2.2$; DV $6.1$) [9]. The same current was delivered for 20 s at two locations of the central amygdaloid nucleus on each side (AP $6.2$ to $6.8$; ML $4.1$; DV $8.4$) [9].

Two wire electrodes of 0.3-mm-diameter were implanted subcutaneously over the nasal and temporal canthi of the orbicularis oculi muscle of the right eye. These leads were used alternately for recording of eyeblink-related EMG and for delivery of an electric shock. Amphenol connectors, attached to the end of the leads, were fixed over the skull with dental acrylic and three mini-screws. Rats were allowed to recover from surgery for three to five days before behavioral procedures commenced.

2.3. Conditioning procedures

The experimental sequence consisted of daily sessions in the following order: habituation to the chamber, habituation to the CS, fear preconditioning and eyeblink conditioning. The above sequence was applied to three groups of rats: lesioned in the cerebellum, lesioned in the amygdala and intact rats. An additional group of intact rats was subjected to the entire procedure except the fear preconditioning session.

2.3.1. Habituation to the experimental chamber

Rats of all groups were introduced to a dimly illuminated experimental cage (24×25×45 cm) for 30 min on 2 successive days.

2.3.2. Habituation to the CS

Rats of all groups were exposed to 40 tone CS+ and 40 light CS− stimuli, presented alternately with a random inter-trial interval ranging from 25 to 35 s. The CS+ was a 0.4-s-long, 2.8 kHz tone of 73 dB intensity, emitted by a solenoid. The CS− was a 0.4-s-long white light, emitted by a 3 W bulb.

2.3.3. Emotional preconditioning (EP)

Cerebellum ($n=9$) and amygdala lesioned rats ($n=11$), and intact rats (intact+EP, $n=14$) were exposed to a procedure known to support fear conditioning [1,13]. Rats of an additional intact group were placed in the chamber without being exposed to any stimuli (intact, no-EP, $n=13$). The procedure consisted of 10 paired trials delivered with a random inter-trial interval of 25–35 s. Each trial consisted of the tone CS+ (0.4-s-long) co-terminating with a 0.1-s-long US in the form of a 100-dB-loud white noise pulse. Pulses of this intensity were applied in our laboratory and were found to cause a freezing response, preceded on some occasions by a startle response. No attempt was made to measure emotional CRs.

2.3.4. Eyeblink conditioning

Rats of all groups were exposed to eyeblink conditioning on four successive days. Sessions opened with a
presentation of 40 light-alone (CS−) trials, followed by 40 paired eyeblink conditioning trials. Trials were delivered at a random inter-trial interval ranging from 25 to 35 s. Paired trials consisted of the previously experienced 0.4-s-long tone CS+, co-terminating with a 0.1-s-long periorbital electric shock as the US. The US was a train of rectangular pulses of 0.2-ms duration, delivered at 50 Hz. The intensity of the shock was 0.5 mA for half of the rats at each group that triggered only a merely visible unconditioned eyeblink response. The other half of the rats were conditioned with a 1.0 mA shock which triggered a distinct eyeblink response, usually associated with a mild head jerk. Rats which did not display a blink response at these US intensities were discarded from the experiment.

The periorbital electrodes were alternately attached through an electric swivel to an AC amplifier (Grass 7C, 10–500 Hz) or to a constant-current stimulator (Grass S5), thus permitting recording of blink-related EMG and delivery of periorbital shocks in the freely moving rat. Trials were monitored and controlled by a PC-based CED1401 interface.

2.3.4.1. Analysis of eyeblink conditioning. The amplified EMG signal was amplitude-discriminated in order mark the events when EMG amplitude exceeded at least twice the baseline level. These events were counted on each trial during a baseline period of 50 ms just before the CS onset and during five successive periods of 50 ms each preceding the onset of the US. Each trial was tested for presence of eyeblink CRs, which was defined as a significant increase in the number of events in at least one of the post-CS periods, in comparison to the baseline number of events (Z-score test with $P<0.025$). Trials with a noisy baseline, i.e. showing more than 10 events during a period of 600 ms just preceding the CS onset were discarded from analysis.

2.4. Histology

Rats were deeply anesthetized (Nembutal 0.8 ml/kg, i.p.) and were perfused intracardially with saline, followed by 10% formalin. Brains were submerged in 10% formalin and were sectioned on a cryostat into 40 μm coronal sections. Stained sections were analyzed under a light microscope for the location and extent of cerebellar and amygdaloid lesions. The borders of the lesions were reproduced through a camera lucida on images of brain sections.

3. Results

3.1. Conditioning

Fig. 1 shows the rate of eyeblink CRs across the four conditioning sessions. Intact controls with emotional preconditioning (intact+EP) showed the highest rate of conditioning, followed by the intact controls with no emotional preconditioning (intact, no-EP). Rats with either amygdaloid or cerebellar lesions showed virtually no acquisition of eyeblink CRs. The rate of CRs was analyzed using MANOVA with between-Ss variables of Group (four groups) and intensity of US shock (0.5 mA or 1.0 mA), and within-Ss variable of Sessions (four conditioning sessions). The analysis confirmed a significant Group effect $[F_{1,36}=11.8, P<0.001]$. A borderline significant Group-by-Intensity interaction $[F_{1,36}=2.7, P=0.06]$ directed us to consider the samples conditioned with a US of either 0.5 mA or 1.0 mA separately. Animals trained with a 1.0 mA US (Fig. 1A) showed a significant group effect $[F_{3,19}=10.3, P<0.01]$, with the intact controls+EP showing a significantly higher rate of CRs than the other three groups (Tukey, $P<0.05$). This difference was also significant when the groups were compared on the fourth session ($P<0.05$). The intact group with no-EP showed higher rates of CRs than the two lesioned groups, but these
differences were not significant. The Session effect reached a borderline level of significance $[F_{3,57}=2.2, P=0.09]$. Similar differences between the groups were observed in the sample trained with 0.5 mA US intensity (Fig. 1B). However, the Group $[F_{1,57}=2.3, P=0.11]$ and Session $[F_{3,57}=0.3, P=0.85]$ effects were not significant.

To control for possible nonspecific sensitization effects of emotional preconditioning on the acquisition of eyeblink response by intact rats, the CR rate in response to the light CS− were analyzed in the two intact control groups.

MANOVA included the between-Ss variable of Groups (two intact groups) and the within-Ss variable of Sessions (the CS habituation session before EP vs. the first eyeblink conditioning session after the EP). The rate of CRs to the CS− was 46% and 48% on the habituation session and 29% and 43% on the eyeblink conditioning session, for the group without EP and with-EP, respectively. The Group effect was not significant $[F_{1,17}=0.1, P=0.75]$, while the Session effects confirmed a reduction of responding across sessions of borderline significance $[F_{1,17}=3.4, P=0.08]$ with no significant interaction. This analysis suggests that the nonspecific effects of fear preconditioning training did not increase the rate of eyeblink CRs to the CS−.

3.2. Histology

Fig. 2 illustrates the extent of cerebellar lesions, which included large portions of the interpositus and medial deep nuclei in all rats. The interpositus was spared unilaterally over its most rostral extension and bilaterally over its most ventral extension. The medial nucleus was spared bilaterally over its medio-ventral portion. The lateral nucleus was spared bilaterally in all rats. In some rats the lesion extended to the cortex overlying these deep nuclei.

Fig. 3 shows the amygdaloid lesions, which included the central nucleus, sparing its anterior part, in all rats. The lesions extended most significantly beyond the posterior parts of the central nucleus, thus including the intra-amygdaloid division of the bed nucleus, some of the ventro-medial portion of the lateral nucleus and small portions of the fimbria.

4. Discussion

Experimental procedures were designed to support preferential conditioning of fear and eyeblink responses in successive sessions. Fast acquisition of fear CRs was attempted during the first session by exposing rats to tone CS, paired with a loud noise US (EP) [1]. Acquisition of eyeblink CRs was attempted during subsequent sessions by pairing the same tone CS with a novel US in the form of a periorbital electric shock. Among intact rats, those that experienced the EP had a significantly higher rate of eyeblink CRs in comparison to those with no previous EP. These results confirm the hypothesis that motor conditioning is enhanced by the antecedent acquisition of emotional responses [12].

Lesion procedures were applied to test the involvement of the cerebellar hemispheres and the amygdala in acquisition of the eyeblink CRs. Eyeblink conditioning was practically abolished by interposital lesions which extended occasionally to the overlying cortex. Bilateral lesions were applied based on reports that unilateral lesions in rats are not sufficient to prevent acquisition of eyeblink CRs [10]. These findings are in line with the suggestion that the
Amygdala imply that emotional preconditioning results in enhancement of the CS and US inputs to the cerebellum which in turn may account for accelerated eyeblink conditioning.

Involvement of the amygdala in eyeblink conditioning may sound paradoxical in light of the notion that the cerebellum is both necessary and sufficient for short delay eyeblink conditioning [6,11]. This paradox may be reconciled, however, by findings showing that the enhancing effects of amygdala on motor conditioning are expressed only when experimental parameters support sub-maximal levels of conditioning. This may be inferred from Weisz et al. [13], who showed that amygdala lesions effectively reduced the number of eyeblink CRs when the intensity of the tone-CS was 65 dB but not when it was 85 dB. These findings support the suggestion that cerebellum is sufficient for reliable acquisition of eyeblink CRs under conditions of intense CSs but it requires amygdala’s enhancing effects to process a weak CS. We also hypothesized that the cerebellum may require the amygdala’s enhancing effects to process a weak intensity US. In line with this reasoning we applied a moderate intensity tone CS of 73 dB paired with a low intensity electroshock US of 1 mA. Under these conditions, amygdaloid lesions caused a nearly complete loss of eyeblink conditioning. Thus, we conclude that demonstration of amygdala involvement in motor conditioning requires application of CS and US events at low intensities that support submaximal motor conditioning.

Fig. 3. Extent of bilateral amygdaloid lesions. The darker shade indicates lesion area shared by all animals which was characterized by small cavitation surrounded by gliosis. In some animals gliosis extended to the areas marked by lighter shading.

interpositus is essential for the acquisition and expression of the defensive eyeblink CRs [6,11,16]. Amygdala lesions were applied based on the notion that this structure is involved in acquisition of fear CRs [1,7,13]. Lesions were directed at the central nucleus which gives rise to divergent efferents controlling the expression of conditioned fear responses [7]. Some of these efferents presumably interact with the eyeblink conditioning circuitry since amygdala lesions effectively erased the enhancing effect of fear preconditioning on the subsequent acquisition of eyeblink CRs. Cumulatively, these findings confirm the hypothesis that amygdala mediates the enhancing effect of emotional conditioning on cerebellum-mediated motor conditioning.

Mechanisms mediating the influences of the amygdala on motor conditioning were discussed by Weisz et al. [13]. This author advocates the possibility that the amygdala enhances the effectiveness of the CS, which is consistent with the amygdala-related conditioned arousal and attention response [2,3]. The amygdala seems to enhance also the effectiveness of the US as reflected by an increase in the US-elicited neuronal activity in the interpositus and in amplitude of the unconditioned eyeblink response at an early stage of conditioning, before the appearance of the conditioned eyeblink CRs [13,15]. These functions of the amygdala imply that emotional preconditioning results in enhancement of the CS and US inputs to the cerebellum which in turn may account for accelerated eyeblink conditioning.

In context of the two-factor theory of learning, the present findings confirm that the amygdala and cerebellum may be considered as prototypic learning sites, representing the fast and the slow stages of conditioning, respectively. It seems that interaction between the two sites has some adaptive value as emotional conditioning facilitates the subsequent motor conditioning. The anatomical counterpart of this interaction is yet to be studied. In a broad sense these findings operationalize the experimental conditions under which adaptive learning requires interactive processing among multiple learning systems rather than independent processing within any one system.

References


