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Inactivation of the central nucleus of the amygdala blocks classical conditioning but not conditioning-specific reflex modification of rabbit heart rate

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Abstract

Heart rate (HR) conditioning in rabbits is a widely used model of classical conditioning of autonomic responding that is noted for being similar to the development of conditioned heart rate slowing (bradycardia) in humans. We have shown previously that in addition to HR changes to a tone conditioned stimulus (CS), the HR reflex itself can undergo associative change called conditioning-specific reflex modification (CRM) that manifests when tested in the absence of the CS. Because CRM resembles the conditioned bradycardic response to the CS, we sought to determine if HR conditioning and CRM share a common neural substrate. The central nucleus of the amygdala (CeA) is a critical part of the pathway through which conditioned bradycardia is established. To test whether the CeA is also involved in the acquisition and/or expression of CRM, we inactivated the CeA with muscimol during HR conditioning or CRM testing. CeA inactivation blocked HR conditioning without completely preventing CRM acquisition or expression. These results suggest that the CeA may therefore only play a modulatory role in CRM. Theories on the biological significance of conditioned bradycardia suggest that it may represent a state of hypervigilance that facilitates the detection of new and changing contingencies in the environment. We relate these ideas to our results and discuss how they may be relevant to the hypersensitivity observed in fear conditioning disorders like post-traumatic stress.

Keywords

classical autonomic conditioning; heart rate conditioning; bradycardia; rabbit; fear conditioning; post-traumatic stress disorder

1. Introduction

Conditioning of autonomic responses such as heart rate (HR) develops rapidly and is thought to reflect an affective or emotional component of classical conditioning that facilitates the acquisition of more slowly acquired adaptive somatomotor or skeletal responses (Buchanan & Powell, 1993; Lennartz & Weinberger, 1992). This early emotionally driven part of the conditioning process may represent an important

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physiological aspect of the acquisition of abnormal or maladaptive fear responses because clinical research on conditioned fear based disorders such as post traumatic stress disorder (PTSD) and phobias have detailed abnormal cued cardiovascular responses in patients (Bedi & Arora, 2007; Busscher, van Gerwen, Spinhoven, & de Geus, 2010; Ginsberg, Ayers, Burriss, & Powell, 2008; Lang & McTeague, 2009; Peri, Ben-Shakhar, Orr, & Shalev, 2000). Noted for being very similar to the conditioned HR responses observed in humans, conditioned HR deceleration (bradycardia) in rabbits is a widely used model for studying classically conditioned autonomic responding in animals (Ghelarducci & Sebastiani, 1996; Lavond, Lincoln, McCormick, & Thompson, 1984; McCabe et al., 1992b; McEchron, Tseng, & Disterhoft, 2000; Pascoe, Supple, & Kapp, 1991; Powell et al., 1997; Schneiderman, Smith, Smith, & Gormezano, 1966). A thorough understanding of the neural substrates of HR conditioning in rabbits may therefore offer important insight into the mechanisms responsible for altered physiological responding in human fear disorders.

Conditioned HR changes in rabbits were first documented in the 1940s when it was shown that rabbits develop conditioned bradycardia to a bell conditioned stimulus (CS) following pairings of the bell with the unconditioned stimulus (US) ammonia (Kosupkin and Olmsted 1943). Early experiments established that HR conditioning in rabbits is parasympathetically mediated through the vagus and critically dependent on the amygdala (reviewed in McCabe et al., 1992b), a structure well known for its role in the acquisition, expression, modulation, and extinction of a variety of conditioned emotional responses (Davis & Whalen, 2001; Gabriel, Burhans, & Kashef, 2003; Kim & Jung, 2006; LeDoux, 2000; Maren, 2001; McGaugh, McIntyre, & Power, 2002; Shors, 2006). The suggested neural pathway by which the amygdala can influence HR conditioning is through connections of the central nucleus of the amygdala (CeA) to the dorsal motor nucleus of the vagus and nucleus ambiguus which give rise to vagal efferents to the heart and also less directly via connections to the nucleus of the solitary tract that can affect the baroreceptor reflex (McCabe et al., 1992b; Schwaber, Kapp, Higgins, & Rapp, 1982). Stimulation of CeA can produce responses that mimic conditioned bradycardia (Applegate, Kapp, Underwood, & McNall, 1983), and damage to or neurochemical manipulation of the CeA can disrupt the acquisition of HR conditioning (Gallagher, Kapp, Frysinger, & Rapp, 1980; Kapp, Frysinger, Gallagher, & Haselton, 1979; McCabe, Gentile, Markgraf, Teich, & Schneiderman, 1992a; Powell et al., 1997) and also retention (Gentile, Jarrell, Teich, McCabe, & Schneiderman, 1986). In addition, studies using extracellular recordings from the CeA during HR conditioning have found correlations between CS-related neural activity and the conditioned bradycardia response (Applegate, Frysinger, Kapp, & Gallagher, 1982; Pascoe & Kapp, 1985).

These previous studies on HR conditioning in rabbits have mainly focused on the neural mechanisms behind the development of conditioned HR changes to a CS such as an auditory tone that is paired with a US, often shock. However, work in our laboratory has shown that the conditioning procedure can also modify unconditioned HR responses to the US when it is presented in the absence of the CS following conditioning (Burhans, Smith-Bell, & Schreurs, 2008; Burhans, Smith-Bell, & Schreurs, 2010; Schreurs et al., 2007; Schreurs, Crum, Wang, & Smith-Bell, 2005; Schreurs, Smith-Bell, & Burhans, 2011b). These changes are not observed in rabbits receiving explicitly unpaired CS-US presentations (Schreurs et al., 2005) and therefore represent a learning-related cardiovascular change in the unconditioned response to the US that we refer to as conditioning-specific reflex modification (CRM). CRM of rabbit HR is measured in an ABA design in which rabbits are first exposed to varying intensities and durations of a periorbital shock US to obtain a baseline of physiological responsiveness (Pretest) followed by two days of HR conditioning and then another US test identical to the Pretest (Post Test). HR CRM is characterized by a Pre- to Post Test increase in interbeat interval to the US (representative of a decrease in HR) to US intensities that are equal to or approach that of the intensity utilized during

conditioning. The observed HR change closely resembles the conditioned bradycardia that develops to the tone CS, suggesting that HR CRM may in part be a generalized conditioned response (Burhans et al., 2008). However, we have shown that there are certain conditions that can extinguish HR CRM while leaving the HR conditioned response intact, so there is also evidence for a dichotomy between the two types of responding (Burhans et al., 2010). HR CRM has also been shown to generalize across USs with overlapping features and is enhanced, along with HR conditioning, by dietary cholesterol (Schreurs et al., 2007; Schreurs et al., 2011b).

PTSD is an example of a fear disorder that involves exaggerated fear responses to discrete stimuli associated with previous trauma *in addition to* abnormal responding to innately stressful stimuli (DSM IV, 1994). This latter symptom represents a hypersensitivity in reflexive responding that can occur in the absence of any specific trauma-associated cues. CRM of rabbit HR, therefore, may be an important unexplored aspect of conditioned autonomic responding that might be relevant to the physiological reactivity found in fear disorders. To our knowledge, we are the only laboratory to explicitly document this phenomenon in rabbit HR, although we and others have more extensively reported CRM in a different system, the rabbit nictitating membrane response (Burhans et al., 2008; Gruart & Yeo, 1995; Wikgren, Ruusuvirta, & Korhonen, 2002). Our next step is to study the neural substrates of HR CRM and how they relate to those already established for HR conditioning to a discrete CS. Because of the well established critical role of the amygdala in conditioned bradycardia, the CeA is a potential neural substrate for the development of HR CRM, which consists of a similar bradycardic response. In addition, it has been shown that during HR conditioning, some CeA neurons are responsive to both the CS and US (McEchron, McCabe, Green, Llabre, & Schneiderman, 1995), suggesting that the CeA receives converging CS and US information and thus may be in a position to mediate responding to both types of stimuli. Taken together, these findings suggest that the CeA may also play a critical role in HR CRM.

To test whether CeA plays a role in HR CRM acquisition or expression, the current study used reversible inactivation of the CeA using muscimol during HR conditioning or CRM testing. In agreement with previous studies using permanent electrolytic or chemical lesions, it was expected that temporary inactivation of the CeA during HR conditioning would block the development of conditioned bradycardia to the CS. Because HR CRM closely resembles conditioned bradycardia to the CS and does not occur in rabbits receiving explicitly unpaired training, one hypothesis being tested was whether blocking the acquisition of HR conditioning would also block the acquisition of HR CRM. Since the CeA is known to be involved in the expression of many fear behaviors, it was also important to test whether the CeA inactivation would interfere with CRM expression.

2. Methods

2.1 Subjects

The subjects were 35 male, New Zealand White rabbits (*Oryctolagus cuniculus*) weighing approximately 2.0–2.2 kg upon delivery from the supplier (Harlan, Indianapolis, IN). The rabbits were housed in individual cages on a 12 hour light-dark cycle and given *ad libitum* access to food and water. They were maintained in accordance with guidelines issued by the National Institutes of Health, and the research was approved by the West Virginia University Animal Care and Use Committee.

2.2 Surgical Procedure

After a minimum period of one week for adaptation to living conditions, rabbits underwent stereotaxic surgery for bilateral implantation of chronic guide cannulae targeted at the CeA.

The rabbits were anesthetized using a subcutaneous injection (0.6 ml/kg) of a solution containing ketamine HCl (83.3 mg/ml) and xylazine (16.7 mg/ml) with additional 0.25 ml injections of ketamine given every 30–60 minutes to maintain anesthesia. Rabbits also received a single subcutaneous dose (0.1 ml) of the analgesic Flunixin Meglumine (50 mg/ml) prior to the start of surgery in addition to an injection of 2% lidocaine HCl (0.25 ml) at the incision site, with a second dose administered at the end of surgery prior to suturing. Stainless steel 22 gauge guide cannulae (length: 13.8 mm), designed to custom fit an internal 28 gauge injection cannula with a 1 mm projection (Plastics One Inc. Roanoke, VA), were implanted bilaterally into the amygdala, targeting the central nucleus: AP = +0.5, ML = $\pm$ 5.5; DV = 11.0. The stereotaxic coordinates were obtained from the atlas of Girgis and Shih-Chang (1981) and are consistent with morphological characterization of amygdalar nuclei in rabbits (Jagalska-Majewska et al., 2001). The guides were also fitted with protective dummy cannulae that were only removed for subsequent intra-amygdalar infusions. Two small stainless steel screws (Small Parts Inc., Miami Lakes, FL) were partially screwed into the skull to anchor the cement (Jet Repair Acrylic, Wheeling, IL) used to secure the cannulae in place.

2.3 Apparatus

The apparatus has been detailed elsewhere (Schreurs & Alkon, 1990; Schreurs, Tomsic, Gusev, & Alkon, 1997) and is modeled after those developed and described by Gormezano (Coleman & Gormezano, 1971; Gormezano, 1966). Briefly, rabbits were restrained in a Plexiglas box placed inside a sound-attenuating, ventilated chamber (Coulbourn Instruments, Allentown, PA; Model E10–20). Inside the chambers, a stimulus panel containing a speaker and houselight (1W LED, 12 V) was mounted at a 45° angle 15 cm anterior and dorsal to the rabbit's head. An exhaust fan created a constant ambient noise level of 65 dB inside the chamber. Periorbital electrical stimulation (ES) served as a US, which was delivered by a programmable two-pole stimulator (Colbourn Instruments, Model E13–35) via stainless steel Autoclip wound clips (Stoelting, Wood Dale, IL) that were positioned 10 mm ventral and 10 mm posterior to the dorsal canthus of the right eye.

For the recording of HR, three wound clips were placed in shaved skin, two on either side of the breast bone (20 mm lateral of the apex) and one on the right shoulder. For each rabbit, the three clips were coupled to an individual custom amplifier providing a 10,000-fold amplification of the electrocardiogram (ECG) signal which was then routed to an analog-to-digital converter (1-ms sampling rate) and stored on a trial-by-trial basis for subsequent inspection and analysis. A custom circuit isolated the US from the ECG amplifier during US presentations to prevent high-voltage artifacts and subsequent electronic ringing from saturating the system. The custom circuit was crucial for resolving components of the ECG waveform during trials where the US was presented, allowing detection of all heartbeats with the exception of those during or immediately following the US. Stimulus delivery, data collection, and analysis were all accomplished using the LabVIEW software system (National Instruments, Austin, TX).

2.4 Procedure

One week following recovery from surgery, rabbits received one session per day starting with adaptation, US pretesting (Pretest), two sessions of HR conditioning, and two sessions of US post testing (Post1, Post2). For adaptation, subjects were prepared for ES delivery and ECG recording and then adapted to the training chambers for an amount of time equivalent to subsequent training sessions (80 min). For pretest and post tests, subjects received 80 trials of US presentations with an average inter-trial interval (ITI) of 60 s (range 50–70 s). Each US presentation was one of 20 combinations of ES intensity (0.1, 0.25, 0.5, 1.0, or 2.0 mA) and duration (10, 25, 50, or 100 ms), and these 20 unique USs were presented in four

separately randomized sequences with the restriction that the same intensity or duration could not occur more than 3 times in succession during the session. For HR conditioning, paired trials consisted of presentations of a 1000 ms, 1 kHz, 82 dB tone CS followed by a 100 ms, 2 mA ES US (1000 ms inter-stimulus interval, 0 ms trace) that were interspersed with CS-alone test trials following every 9th paired trial. For the first session of HR conditioning, 20 CS-alone trials were presented in order to habituate the bradycardic orienting response that occurs in rabbits to a novel tone. The remaining 60 trials of the first session and all 80 trials of the second session were paired trials interspersed with CS-alone trials, yielding a total of 54 and 72 paired CS-US presentations on the first and second days of conditioning, respectively. Average ITIs for HR conditioning sessions and all other sessions were the same as US testing, 60 s (range 50–70 s).

2.5 Heart Rate Data Collection and Analysis

For all sessions, the ECG signal was recorded for a total of 6 s per trial. For paired trials and CS-alone trials, heartbeats were recorded starting 2200 ms prior to the onset of the CS, and for US-alone trials, 2200 ms prior to the onset of the US. Details for detection of heartbeats and components of the ECG signal have been described in detail elsewhere (Schreurs & Smith-Bell, 2005). Briefly, heartbeats were detected in the filtered ECG signal with a template-matching algorithm created in LabVIEW software. Visual inspection of the data corrected any false positives or negatives as a result of artifacts such as those related to movement. Heart beat detection was relatively straightforward because the R component of the PQRST waveform of the ECG had amplitudes approaching 1 mV, typically 10–20 times greater than the noise level. Data were expressed as a change in interbeat interval (Δ IBI) to the CS or US from a pre-stimulus baseline. There is continued debate in the field of neurophysiology about the appropriate measurement of HR change, but we have selected to measure and report on change in IBI since it is consistent with our previously published studies and a good deal of the HR conditioning literature (Burhans et al., 2010; McEchron, Tseng, & Disterhoft, 2003; Powell, Churchwell, & Burriss, 2005; Schreurs et al., 2007; Schreurs et al., 2005; Schreurs & Smith-Bell, 2005; Supple, Jr. & Kapp, 1993).

Previous studies have shown that the maximal conditioned HR deceleration to the CS during HR conditioning takes place in the first 2000 ms following CS onset whereas conditioning-specific changes to the US (CRM) occur within the first one or two heart beats post-US best captured by a 1000 ms post-US time period (Burhans et al., 2010; Schreurs et al., 2007; Schreurs et al., 2005; Schreurs et al., 2011b). Based on this precedent set in our previous published HR CRM studies, analyses of Δ IBI included the first 2000 ms following CS onset and the first 1000 ms following US onset with respect to a 2000 ms pre-stimulus baseline. Unless otherwise indicated, change in IBI data were analyzed by repeated measures analysis of variance (ANOVA; SPSS 14.0) with violations of the sphericity assumption addressed by using Huynh-Feldt corrections for degrees of freedom and p values. Planned and follow up comparisons were Bonferroni corrected for the number of comparisons. In addition to IBI measurements, topographies of the HR responses during US testing and HR conditioning were also generated to examine the shape and timing of the HR response. Topographies were plotted as average change in IBI over time with respect to the 2000 ms pre-stimulus baseline.

2.6 Temporary Inactivation of the Amygdala

The procedure used for intra-amygdalar muscimol infusion in rabbits is based on the technique validated in a number of publications by Gabriel and colleagues (Poremba & Gabriel, 1999; Poremba & Gabriel, 2001; Talk, Kashef, & Gabriel, 2004) and our own work (Burhans & Schreurs, 2008). Approximately 20 minutes prior to the start of each session (Pretest, HR conditioning, Post1, and Post2), subjects received bilateral intra-amygdalar

injections of either the GABA_A agonist muscimol (1 µg/µl, dissolved in 0.9% saline) or vehicle (0.9 % saline). For all injections, dummy cannulae were removed and 0.50 µl of muscimol or vehicle was infused at a rate of 0.25 µl /min into each side of the amygdala simultaneously through injection cannulae that when inserted, projected 1 mm from the tip of the guide cannulae. Each injection cannula was attached to a 10 µl Hamilton microsyringe via polyethylene tubing, and the rate of infusion was controlled by an infusion pump (KD Scientific, Holliston, MA) that was modified to allow four rabbits to be infused simultaneously. Following microinfusion, the injection cannulae remained in place for 2 minutes and were then replaced with the dummy cannulae.

Rabbits were randomly assigned to one of three groups based on their scheduled order of injections for Pretest, Habituation + HR conditioning, and Post1. The groups were as follows: 1) SSS, saline during all three phases; 2) SMS, saline during Pretest and Post1 and muscimol during habituation + HR conditioning; and 3) MSM, muscimol during Pretest and Post1 and saline during habituation + HR conditioning. All groups received saline injections during Post2. Group SSS was a control for the effects of cannula implantation and the infusion procedure. Group SMS was designed to test the effects of amygdala inactivation on the acquisition of HR conditioning and HR CRM whereas group MSM tested the effects of inactivation on CRM expression.

2.7 Histology

After the completion of training, rabbits were anesthetized with a solution containing Ketamine HCl (83.33 mg/ml) and Xylazine (16.67 mg/ml), followed by a lethal dose of Euthasol (sodium pentobarbital, 390 mg/ml) and then transcardial perfusion with 0.9% saline and 10% formalin. The brains were post fixed in the 10% formalin and prior to sectioning, cryoprotected in a 20% sucrose-formalin solution for a minimum of 24 hours. Brains were then frozen, and coronal sections were cut at 40 µm using a microtome. Sections were placed on slides and digitally imaged while wet (1.5X) with an Olympus BX51 microscope equipped with a computer assisted camera and software (monochrome Olympus FV10, Olympus MicroSuite). Once dried, the sections were stained with a metachromatic Nissl and myelin stain using formal-thionin (Donovick, 1974). The digital images and stained slides were then used to determine the placement of the guide cannulae and verified by consulting two rabbit brain atlases (Girgis & Shih-Chang, 1981; Urban & Richard, 1972) and published anatomical characterization of the amygdaloid nuclei in the New Zealand rabbit (Jagalska-Majewska et al., 2001).

To estimate the spread of muscimol, a subset of subjects received intra-amygdalar injections of 0.2% cresyl violet dye (n = 4) or florescent muscimol (n=3; BODIPY TMR-X muscimol conjugate, Molecular Probes) dissolved in 0.9% normal saline at the same amounts and with the same procedures as injections during training. After a maximum period of 90 minutes following dye or florescent muscimol injection, rabbits were then anesthetized and perfused, and the brains were sectioned and imaged as described above.

3. Results

3.1 Histology

Six rabbits were determined to have at least one cannula placement outside of the CeA. A reconstruction of the location of the guide cannula tips for the remaining 29 rabbits is illustrated in Figure 1, and shows that the majority of placements were either inside or just dorsal (> 1 mm) to the CeA, suggesting that the injection cannulae, which when inserted for drug delivery projected 1 mm ventral from the guide tips, would likely have been within the CeA. Examination of the unstained slides from the subset of rabbits receiving cresyl violet dye injections prior to perfusion revealed a similar pattern of spread to that reported

previously, consisting of a bolus of dye that extended approximately 1 mm in length ventrally from the tip of the guide with very little lateral diffusion (Burhans & Schreurs, 2008). Additional estimations of muscimol spread were made by inspection of the brain slices of the subset of rabbits injected with fluorescent muscimol. Similar to the dye injections, there was a visible bolus of fluorescent muscimol at the tip of the guide cannulae with no visible spread outside of the amygdala (see Figure 1).

Three of the six rabbits with cannula placement outside the CeA were from the muscimol groups (SMS and MMM), and their data were excluded from statistical analyses. The remaining three rabbits were in the saline control group (SSS), and all had at least one of the bilateral cannulae within the amygdala. Analyses of HR baseline, HR conditioning, and HR CRM indicated that this subset of the SSS group did not statistically differ from the other rabbits, and their data was subsequently included as part of the SSS group. Final n's for each group were 12, 10, and 10 for SSS, SMS, and MMM, respectively.

3.2 Baseline, Orienting, and Unconditioned Heart Rate Responses

Figure 2 illustrates the effects of muscimol infusion into the CeA on baseline HR responding during each training session and suggests that muscimol may have increased baseline HR, indicated by a decrease in the averaged IBI. For example, the group receiving muscimol during Pretest and Post1 (MSM) exhibits the lowest averaged IBI during those sessions, and a similar attenuation is observed when group SMS received muscimol during HR conditioning (Habituation, Day1, Day2). This observation was confirmed by a significant interaction of Session and Drug Group indicated by repeated measures ANOVA [$F(10, 145) = 3.33, p < 0.002$]. Bonferroni corrected planned comparisons indicated there was a trend for a reduced baseline in group SMS during Habituation compared to the control group SSS ($p=0.088$). Possibly related to the variability in baseline regardless of drug infusion, there were no additional differences between drug groups.

To determine if muscimol had any effect on the initial bradycardic orienting response to the tone CS or habituation of that response, HR responding was examined across groups for the first and last trials of habituation (Figure 3). Although all groups showed an initial bradycardic response to the CS, unexpectedly, this response appeared to be attenuated in the group receiving muscimol during habituation and conditioning (SMS) compared to groups receiving saline (SSS and MSM). However, there were no significant effects or trends involving Drug Group after repeated measures ANOVA for the first and last habituation trials or when the first trial was analyzed separately with one-way ANOVA. A planned contrast was then conducted for the first habituation trial in order to compare saline versus muscimol, regardless of previous infusion history (SSS and MSM compared to SMS). Results indicated a trend for an attenuated response due to muscimol ($p = 0.060$).

The effect of muscimol on unconditioned HR responses was examined by looking at responses to each of the five US intensities (0.1, 0.25, 0.5, 1.0, 2.0 mA) during Pretest. Repeated measures ANOVA indicated a significant main effect of Intensity [$F(2.20, 63.92) = 7.50, p < 0.002$] but no Drug Group main effect or interaction of Drug Group with Intensity.

3.3 Heart Rate Conditioning

Figure 4 illustrates the shape and timing of the Δ IBI response topographies for each group during the last trial of the habituation session (Habituation) and the averaged probe trials for each conditioning session (Day1, Day2). The topographies are aligned at the point where the first IBI is measured (the second R peak in the post-CS ECG signal) and therefore do not start at time 0 ms. Figure 5 summarizes the data utilized for statistics and shows the mean Δ

IBI from baseline to the first 2000 ms post-CS averaged across the CS-alone trials during habituation and the CS-alone probe trials during the first and second HR conditioning sessions (Day1 and Day2). Examination of Figures 4 and 5 shows that only groups receiving saline infusions during habituation and HR conditioning (SSS, MSM) exhibited an increase in Δ IBI during both conditioning sessions relative to Habituation, indicative of the development of conditioned bradycardia. However, the level of HR conditioning peaked earlier for group SSS on the first day but not until the second day for group MSM which received muscimol during US Testing. In contrast, HR responses in the group receiving muscimol during habituation and conditioning sessions (SMS) remained at habituation levels across both days.

These results were confirmed statistically by a significant interaction of Drug Group (SSS, SMS, MSM) and Session (Habituation, Day1, Day2) [$F(4,58) = 3.53$, $p < 0.013$] with repeated measures ANOVA. Bonferroni corrected planned comparisons indicated that Δ IBI was significantly lower at Day1 in the SMS group compared to SSS ($p < 0.02$) but did not differ between groups SSS and MSM. In addition, the differing peaks in HR conditioning levels between groups SSS and MSM were indicated by a Habituation to Day 1 increase in Δ IBI for group SSS ($p < 0.004$) that was not significant for group MSM until Day 2 ($p < 0.03$). This finding may be a result of a carry-over effect of the muscimol that was infused in group MSM during the Pretest session occurring one day prior to HR conditioning. Importantly, there were no significant differences in comparisons between Habituation, Day 1, and Day 2 for group SMS.

3.4 Conditioning-Specific Reflex Modification of Heart Rate

HR CRM has been previously characterized as a pretest to post test increase in mean Δ IBI at US intensities at or approaching 2.0 mA, the intensity paired with the tone CS during HR conditioning (Schreurs et al., 2005). Figure 6 is an example of HR CRM from a rabbit in the SSS group and shows the ECG signal to a 2.0 mA US during Pretest and Post1. It can be clearly seen that the first detectable IBI following the periorbital shock US is larger at Post1 compared to Pretest (indicated by double sided arrows). To determine the effects of CeA inactivation on the development of HR CRM, mean Δ IBI was analyzed for the first 1000 ms post-US during the first 20 trials of US testing, where the strongest HR CRM is observed (Burhans et al., 2010; Schreurs et al., 2007; Schreurs et al., 2005; Schreurs, Smith-Bell, & Burhans, 2011a; Schreurs et al., 2011b). Examination of Figure 7 shows that at the 2.0 mA intensity, all groups showed the pattern of increased Δ IBI from Pretest to Post1 indicative of HR CRM, but the change was greatest for group SSS. In addition, all groups showed a decrease from Post1 to Post 2, the session during which all groups received saline in order to measure CRM in the MSM group after CeA inactivation was removed. Repeated measures ANOVA revealed a marginally significant interaction of Test (Pretest, Post1, Post2) and US Intensity (0.1, 0.25, 0.5, 1.0, 2.0 mA) [$F(3,14, 90.96) = 2.61$, $p = 0.054$] but no effects involving Drug Group. To increase the sensitivity to detect CRM and the effects of muscimol, further analysis focused on the first post-US IBI value for Pretest and Post1 at the 2.0 mA intensity (Figure 7, right panel). This analysis revealed a significant effect of Test [$F(1,29) = 4.79$, $p < 0.038$] as a result of the Pretest to Post1 increase in Δ IBI ($p < 0.028$), but once again there were no effects of Drug Group. To evaluate the level of CRM in each group individually, paired t-tests comparing Pretest vs Post1 were conducted and revealed trends for a larger Δ IBI at Post1 compared to Pretest for groups SSS ($p = 0.16$) and MSM ($p = 0.11$) and a weaker trend in group SMS ($p = 0.24$). Additional paired t-tests for muscimol groups demonstrated that Post2 responding levels did not differ from either Pretest or Post1 (all p 's > 0.57). However, in group SSS there was a trend for Δ IBI at Post2 to remain larger than Pretest levels ($p = 0.16$).

4. Discussion

The major finding of the current study was that temporary inactivation of the CeA with muscimol during HR conditioning blocked the acquisition of HR conditioning without completely blocking the acquisition of HR CRM. In addition, it was shown that inactivation of the CeA during CRM testing also did not completely block CRM expression. It did appear, however, that CRM may be slightly attenuated by CeA inactivation, particularly when inactivation occurred during HR conditioning. In summary, these findings corroborate the results of previous HR conditioning studies using permanent CeA lesions (Kapp et al., 1979; McCabe et al., 1992a; Powell et al., 1997) and also show that the CeA may play a modulatory rather than critical role in HR CRM acquisition and expression.

To our knowledge, this is the first study to use temporary muscimol inactivation of the CeA during HR conditioning in rabbits. Previous studies producing similar effects on HR conditioning have utilized radio frequency, electrolytic, or permanent ibotenic acid lesions (Kapp et al., 1979; McCabe et al., 1992a; Powell et al., 1997). Results presented here show that real time disruption of CeA functioning is sufficient to block HR conditioning and provide further proof that previously reported effects are a result of CeA damage and not likely due to destruction of fibers of passage or more widespread damage from non-chemical lesioning techniques. There was also some indication that CeA inactivation increased baseline HR and attenuated the orienting bradycardic response to the tone CS. Although baseline effects have not been reported in other studies, it is difficult to compare our results to the studies with permanent amygdalar lesions where the first evaluation of baseline HR does take place until recovery from lesion surgery, as much as three weeks in some cases (Powell et al., 1997). Stimulation of the CeA in awake rabbits has been shown to produce bradycardia (Applegate et al., 1983), so it is interesting that strong GABA inhibition of the CeA with muscimol may have had an opposite, mild tachycardic effect. In regards to the orienting response, a similar non-significant attenuation of the orienting response on the first habituation trial prior to HR conditioning was reported by Powell and colleagues (1997). Other studies that reported no changes to the orienting response averaged across habituation trials (Kapp et al., 1979), and we likewise saw no drug effect when analyzing across the whole habituation session.

Histological findings from the current study suggest that the muscimol was largely confined to the CeA, although some spread to surrounding adjacent amygdalar areas such as the basolateral and lateral nuclei cannot be completely ruled out, particularly in cases where the cannula was closer to the ventral and medial-lateral CeA borders. Electrolytic lesions centered on the basolateral nucleus have been shown to produce very similar effects on HR conditioning as CeA damage (Powell et al., 1997), but they may be due to damage to fibers of passage. Another concern that must be addressed is whether damage to the CeA caused by the permanently implanted guide cannula affected HR conditioning or HR CRM. The CRM observed in the control group receiving saline only infusions was more variable but at a similar magnitude to that reported in previous HR CRM behavioral experiments (Burhans et al., 2010; Schreurs et al., 2007; Schreurs et al., 2005; Schreurs et al., 2011b). It is possible that amygdala damage from the cannulae or the infusion procedure itself may have contributed to a greater variability in HR responses in the controls, but those effects cannot be determined since the current study did not include a non-infusion control. However, it should be noted that the saline controls still had robust HR conditioning despite the CeA damage.

An alternative substrate underlying acquisition of HR CRM is the medial prefrontal cortex (mPFC). Lesions of the mPFC in rabbits produce the same effects as CeA lesions (Buchanan & Powell, 1982; Powell et al., 1997; Powell, Watson, & Maxwell, 1994). In addition,

stimulation of the mPFC, like the CeA, can elicit bradycardia that closely resembles the response that develops to a CS (Buchanan, Valentine, & Powell, 1985), and recorded single and multi-unit activity in mPFC shows changes correlated with the development of the HR conditioned response (Gibbs & Powell, 1988; Gibbs & Powell, 1991; Maxwell, Powell, & Buchanan, 1994). Anatomically, the mPFC has similar efferent pathways to the downstream areas involved in cardiac adjustments, including connections to the nucleus of the solitary tract, nucleus ambiguus, and dorsal motor nucleus of the vagus (Buchanan, Thompson, Maxwell, & Powell, 1994). Powell and colleagues (Powell et al., 1997) showed that a disconnection lesion consisting of a unilateral lesion of the CeA combined with a unilateral lesion of the contralateral mPFC attenuated but did not abolish HR conditioning unlike bilateral lesions of either structure. The authors concluded that mPFC and CeA are part of independent, parallel circuits mediating HR conditioning but that their interactions may be required to produce optimal performance of the HR conditioned response. The attenuation but not complete blockage of HR CRM in the current study suggests that mPFC–CeA interactions might be critical to producing optimal HR CRM.

Theories behind the biological relevance of conditioned bradycardia may offer insight into the significance of HR CRM. For example, it has been suggested that the cardiac inhibitory response may represent a sensory, attentional process that is activated by new or changing contingencies related to the CS that can enhance the acquisition of new somatomotor responses (Buchanan & Powell, 1993; Powell et al., 1997). When stimuli are found to be irrelevant or nonsignificant, conditioned bradycardia is markedly reduced. For example, in differential HR conditioning greater bradycardia is elicited by the CS+ which is paired with the US compared to the CS- which is not paired with the US (McCabe et al., 1992b). In contrast, conditioned bradycardia can be enhanced under conditions where the CS is made less predictable by partial reinforcement or when it is difficult to discriminate the CS from stimuli that are not reinforced (Buchanan & Powell, 1993). These findings fit Lacey and Lacey's early theoretical account of cardioinhibitory responses as a way to facilitate the processing of "meaningful" signals (Lacey & Lacey, 1980). Also, bradycardia in the rabbit has been described as an aspect of the rabbit's natural freezing response, a "no go" state accompanied by decreased blood flow to limbs, rapid breathing, a pressor response, and vasoconstriction that allows the rabbit to become hypervigilant to the environment (Paredes, Winters, Schneiderman, & McCabe, 2000). Taken together, these ideas raise the possibility that the bradycardia that characterizes HR CRM may represent a state of hypervigilance resulting from a detection of new or changing contingencies in the environment. Indeed, the post test session of unsignaled shock US presentations given to test for CRM represents a contingency change from the previously signaled shock experienced during HR conditioning, particularly when the US presented is the same as that used during conditioning which is where the greatest CRM is actually observed (Burhans et al., 2010; Schreurs et al., 2005). Once this change has been detected, the absence of any new predictive signals may account for the resulting within and across session declines in HR CRM observed here and in previous studies (Burhans et al., 2010; Schreurs et al., 2005). HR CRM does not occur in rabbits that receive unpaired CS-US presentations (Schreurs et al., 2005), presumably because there is no switch from signaled to unsignaled shock during CRM testing.

The role of the amygdala in HR conditioning and CRM may offer insight into the neural mechanisms of conditioned fear disorders like PTSD, which is proposed to be a result of amygdalar hyperactivity and dysfunction of amygdala-mPFC interactions (Patel, Spreng, Shin, & Girard, 2012). One possibility is that in PTSD, which is theorized to be an over generalized fear response that is resistant to extinction (Brewin & Holmes, 2003), the observed hypersensitivity may reflect inappropriate autonomic responses to environmental signals. In the only study to explicitly test the acquisition of HR conditioning in PTSD,

combat veterans with PTSD had markedly reduced bradycardia to a CS paired with an unpleasant corneal air puff compared to non-PTSD veterans (Ginsberg et al., 2008). As mentioned previously, conditioned bradycardia may represent a hypervigilant state that allows one to evaluate the signals in the environment. Perhaps with PTSD, the assessment phase is bypassed and the individual immediately goes to a defensive fight or flight response that would involve autonomic responses like tachycardia, increased blood flow to hindlimbs, and increased respiration (Duan et al., 1997).

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Highlights

- We tested the role of the amygdala central nucleus in heart rate reflex modification
- Inactivation of the central nucleus blocked heart rate conditioning
- Inactivation of the central nucleus attenuated but did not block reflex modification

Figure 1.

Locations of the guide cannula tips for rabbits in group SSS (open circles), SMS (closed circles), and MSM (closed squares) at the indicated number of millimeters anterior (positive values) and posterior (negative values) to Bregma. When inserted, injection cannula projected 1 mm from the tip of the guide. On the right side is a representative coronal rabbit brain section illustrating the spread of a 0.50 μ l infusion of fluorescent muscimol.

Abbreviations: CE: central nucleus; LA: lateral nucleus; BL: basolateral nucleus; BM: basomedial nucleus. [Drawings adapted from Urban & Richard, 1972, *A stereotaxic atlas of the New Zealand Rabbit's brain*, courtesy of Charles C. Thomas Publisher Ltd., Springfield, Illinois].

Figure 2.

Mean interbeat interval (IBI) averaged across the 2000 ms pre-stimulus baseline for unconditioned stimulus test sessions (Pretest, Post1, Post2), habituation, and the two heart rate conditioning sessions (Day1, Day2) for groups receiving saline throughout (SSS, open circle), muscimol during habituation and conditioning (SMS, closed circle), or muscimol during Pretest and Post1 (MSM, closed triangle).

Figure 3.

Mean change ($\pm$ SEM) in inter-beat interval (Δ IBI) from baseline to the tone conditioned stimulus (CS) during the first (Trial 1) and last (Trial 20) CS-alone presentation from the 20 trial habituation session prior to the start of heart rate conditioning.

Figure 4.

Averaged heart rate response topographies of the mean change in inter-beat interval (Δ IBI) from baseline to the tone conditioned stimulus (CS). The topographies are aligned at the point where the first IBI is measured (the second R peak in the post-CS electrocardiogram signal) and therefore do not start at time 0 ms. The overlaid averaged topographies include the last trial of habituation (Habituation, dotted black line) and the first (Day1, solid black line) and second conditioning sessions (Day2 solid gray line) for groups receiving saline throughout (SSS, top graph), muscimol during habituation and conditioning (SMS, middle graph), or muscimol during Pretest and Post1 (MSM, bottom graph).

Figure 5.

Mean change ($\pm$ SEM) in inter-beat interval (Δ IBI) from baseline to the tone conditioned stimulus (CS) averaged across CS-alone habituation trials, and CS-alone probe trials on Day1 and Day2 of heart rate conditioning for groups receiving saline throughout (SSS, open circle), muscimol during habituation and conditioning (SMS, closed circle), or muscimol during US testing (MSM, closed triangle).

Figure 6.

Example electrocardiogram (ECG) signal illustrating conditioning-specific reflex modification in a rabbit from the control group receiving saline throughout all phases of the experiment (SSS). The ECG signal shown is in response to a 2.0 mA periorbital shock presented prior to heart rate conditioning (Pretest) and afterwards (Post1). The open arrow indicates the time of the shock (US) onset, and the vertical double sided arrows draw attention to the increase in interbeat interval indicative of bradycardia at Post1 that was not present during Pretest.

Figure 7.

Mean change ($\pm$ SEM) in inter-beat interval (Δ IBI) from baseline during the first 20-trial block of unconditioned stimulus (US) presentations during Pretest, Post1, and Post2 at the five different US intensities (0.1 mA, 0.25 mA, 0.5 mA, 1.0 mA, and 2.0 mA), collapsed across duration for the groups receiving saline throughout (SSS, open circle), muscimol during habituation and conditioning (SMS, closed circle), or muscimol during Pretest and Post1 (MSM, closed triangle). All groups received saline during Post2. The right panel shows the Δ IBI for the first post-US IBI at the 2.0 mA US intensity.