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Reversible inactivation of rostral nucleus raphe pallidus attenuates acute autonomic responses but not their habituation to repeated audiogenic stress in rats

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Abstract

The medullary nucleus raphe pallidus (RPa) mediates several autonomic responses evoked by acute stress exposure, including tachycardia and hyperthermia. The present study assessed whether the RPa contributes to the decline/habituation of these responses observed during repeated audiogenic stress. Adult male rats were implanted with cannulae aimed at the RPa, and abdominal E-mitters that wirelessly acquire heart rate and core body temperature. After surgical recovery, animals were injected with muscimol or vehicle (aCSF) in the RPa region, followed by 30 minutes of 95-dBA loud noise or no noise control exposures on 3 consecutive days at 24-hr intervals. Forty-eight hours after the third exposure, animals were exposed to an additional, but injection-free, loud noise or no noise test to assess habituation of hyperthermia and tachycardia. Three days later, rats were restrained for 30-minutes to evaluate their ability to display normal acute autonomic responses following the repeated muscimol injection regimen. The results indicated that the inhibition of cellular activity induced by the GABA_A-receptor agonist muscimol centered in the RPa region reliably attenuated acute audiogenic stress-evoked tachycardia and hyperthermia, compared with vehicle-injected rats. Animals in the stress groups exhibited similarly attenuated tachycardia and hyperthermia during the injection-free fourth audiogenic stress exposure, and displayed similar and robust increases in these responses to the subsequent restraint test. These results suggest that cellular activity in neurons of the RPa region is necessary for the expression of acute audiogenic stress-induced tachycardia and hyperthermia, but may not be necessary for the acquisition of habituated tachycardic responses to repeated stress.

Keywords

Adaptation; Restraint Stress; Hyperthermia; Tachycardia; Muscimol; Telemetry

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Declaration of Interest

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Introduction

Acute psychological stressors induce multiple autonomic responses that gradually decline when the same stressor is experienced repeatedly, a phenomenon known as stress habituation (Konarska et al., 1989a, Carter et al., 2004, Masini et al., 2008, Grissom and Bhatnagar, 2009). Although disrupted stress habituation is associated with several mood disorders (Malmo et al., 1951, Lader and Wing, 1964, Brierley and Jamieson, 1974, Roth et al., 1990, Metzger et al., 1999, Rothbaum et al., 2001, Campbell et al., 2014) and may contribute to several cardiovascular disorders (Pasternac and Talajic, 1991, Stotz-Potter et al., 1996, Vanitallie, 2002), the central mechanisms responsible for habituation-related autonomic response suppression are not fully understood. Stressful situations selectively activate multiple interconnected limbic and hypothalamic brain regions, which in turn ultimately innervate well-defined “premotor” centers that control specific behavioral, neuroendocrine, and autonomic reactions elicited by stress (Herman et al., 2003, Sarkar et al., 2007, Vianna et al., 2008). The medullary raphe pallidus (RPa) region is one of these premotor recipients targeted by stress-responsive regions including the prefrontal cortex, amygdala, bed nuclei of the stria terminalis, and dorsal posterior hypothalamus (Hermann et al., 1997, Kataoka et al., 2014, Nyhuis et al., 2016), which is necessary for the expression of stress-induced autonomic responses, including tachycardia and hyperthermia (Zaretsky et al., 2003b, Pham-Le et al., 2011, Kataoka et al., 2014). Neurons of the RPa in turn mostly provide descending projections to sympathetic preganglionic spinal neurons (Loewy, 1981, Nakamura et al., 2004, Stornetta et al., 2004) responsible for heart rate and core body temperature modulation, respectively (Morrison et al., 1999, Morrison, 2001, Cao and Morrison, 2003, Zaretsky et al., 2003a, Morrison, 2004, Nakamura et al., 2004). Limbic and hypothalamic regions contribute to the modulation of neuroendocrine response habituation (Grissom and Bhatnagar, 2011, Herman, 2013), but few studies have focused on autonomic response habituation. Because of the caudal premotor location of the RPa region in a circuit mediating acute stress-induced autonomic responses, it possibly integrates habituation-related information and significantly contributes to the development of habituated autonomic reactions to repeated stress. Furthermore, whether the expression of autonomic responses are necessary for the acquisition of habituated responses to repeated stress is not well established.

To assess the possibility that the RPa region is a necessary site of neural modifications in the acquisition of habituated autonomic responses to repeated stress, the GABA_A receptor agonist muscimol was used to reduce cellular activity (Martin and Ghez, 1999) in this region while monitoring heart rate and core body temperature in rats. This neurochemical approach was favored based on its reversibility and prior reports that RPa muscimol consistently prevents stress-induced tachycardia and hyperthermia with minimal basal modifications of these responses, as compared to GABA_A or excitatory amino acid receptor antagonists (Cao and Morrison, 2003, Zaretsky et al., 2003a, Zaretsky et al., 2003b, Cao and Morrison, 2006, Vianna et al., 2008). These studies were performed using repeated audiogenic stress, which consistently elevates both core body temperature and heart rate, and readily habituate upon repeated exposures (Masini et al., 2008, Masini et al., 2011).

Experimental Procedures

Subjects

Forty-four male Sprague-Dawley rats, two months of age and weighing 275–300 g upon arrival, were obtained from Harlan Laboratories (Indianapolis, IN, USA). All animals were housed in the same animal colony room for the duration of the experiments, and were allowed to acclimate to the colony for one week prior to any experimental manipulations. Animals had *ad libitum* access to rat chow and water, and were kept under a 12:12 hr light/dark cycle, with lights on at 7:00 am, under constant humidity and temperature conditions. Rats were initially group housed three to five per cage in clear polycarbonate cages (54×29×20 cm) containing wood shavings, with wire lids. Seven to twelve days following their arrival from the supplier, animals underwent surgical cannula and telemetric sensor implantation (see below). Following surgery, rats were single-housed in clear polycarbonate cages (46×25×22 cm) with wire lids and weighed daily. All experimental procedures were performed between 8:00 am and 12:30 pm during the inactive phase of the rodent circadian cycle to reduce autonomic variations. All procedures were reviewed and approved by the University of Colorado Boulder's Institutional Animal Care and Use Committee and conformed to the National Research Council of the National Academies' Guide for the Care and Use of Laboratory Animals (8th Ed.).

Surgery

E-mitter (Mini Mitter Respironics, Bend, OR) telemetric sensors were surgically implanted under halothane anesthesia as previously described (Masini et al., 2008). Briefly, E-mitter Transponders (E4000) were implanted in the abdominal cavity and the leads were threaded subcutaneously (10 G trocar) and stitched to the muscle layer using stainless steel suture that increased the conductance for the lead tips. The negative lead was placed over the right clavicle and the positive lead placed anterior to the diaphragm in a 45 – 60° angle in the transverse plane of the heart. The abdominal muscle was closed with absorbable (3–0) thread and the skin incisions closed with surgical stainless steel staples. The rat was then placed in a Kopf stereotaxic instrument equipped with blunt ear bars with the incisor bar set to –3.3 mm (Zaretsky et al., 2003a). A skin incision was then made over the skull, the skin retracted, and a small hole drilled through the skull to allow implantation of a chronic guide cannula (23 gauge cut 9mm below pedestal; #C317G - Plastics One, Roanoke, VA) above the RPa (2.2 mm posterior from *lambda*, centered on the midline and 9.4 mm ventral from dura). These coordinates were initially determined empirically in 3 rats by injecting 0.2 µl of 0.1% methylene blue with a 10 µl Hamilton syringe (Reno, NV). The cannula was held in place with dental cement anchored on 3 jeweler's screws fixed to the animal's skull. An obturator attached to a dust cap (Plastics One), which was flush with the tip of the guide cannula, was inserted to keep the cannula free of organic substances. Rats were given buprenorphine (analgesic) and Baytril™ (antibiotic) postoperatively and monitored daily during recovery from surgery for at least 7 days prior to additional experimental manipulation.

Noise apparatus

Loud noise was presented inside acoustic chambers made of ventilated double wooden (2.54 cm plywood board) panels, with the outer chamber lined internally with 2.54 cm insulation (Celotex, Suffolk, England). The inner chamber was painted black, and its internal dimensions were 59.69 cm (w) × 38.10 cm (d) × 50.80 cm (h), which allowed placement of an ER-4000 Energizer receiver (Mini Mitter) and a polycarbonate rat cage. Each chamber had a single 15.24 cm × 22.86 cm Optimus speaker (#12-1769 - 120 W RMS [Radio Shack, Fort Worth, TX]) centered on the ceiling. Lighting was provided by a fluorescent lamp (15 W) covered in red tissue paper located in the upper left corner of the chamber. Noise was generated by a General Radio (#1381 [Concord, MA]) solid-state random-noise generator with the bandwidth set at 2 Hz-50 kHz. The output of the noise generator was amplified (Pyramid Studio Pro #PA-600X [Brooklyn, NY]) and fed to the speakers. Noise intensity was measured daily by placing a Radio Shack Realistic Sound Level Meter (A scale; #33-2050) in an empty single-housed clear polycarbonate cage at several locations and taking an average of the different readings. The ambient/no noise level inside the chamber was approximately 60 decibels-A scale (dBA) and approximately 55 dBA in the rat colony.

Restrainers

Restrainers were constructed from 0.71 mm wire mesh (0.64 cm² mesh size). The mesh was formed into 7.6 cm diameter cylinders that were 30.5 cm long. A 5.1 cm wide, 0.64 cm thick piece of white painted wood was placed at the bottom of the mesh cylinder to form a platform for the rat to sit on. The mesh was stapled to the wood on the outside of the cylinder. The ends of the cylinders were plugged with 7.6 cm diameter plastic atrium grates. Sections of the grates were removed to allow the rats' tails to protrude from the cylinders. With the grates in place, the internal dimensions of the wire mesh restrainers were similar to those of standard Plexiglas restrainers (17.8 cm length and 6.4 cm diameter) and have produced comparable stress-induced ACTH and corticosterone hormone release (Masini et al., 2012).

Measurement of heart rate and core body temperature

Heart rate (beats per minute - bpm) and core body temperature (degrees Celsius) were measured simultaneously when the rat's home cage was placed over the ER-4000 Energizer receivers in the acoustic chambers. The receivers captured the responses at 1-min intervals. The data were digitized and saved using VitalView Data Acquisition Software (version 4.1; Mini Mitter) and analyzed offline.

Experimental Procedures

At least seven days following recovery from surgery, rats were transported to a behavioral room, handled and habituated to the injection procedures daily for 5 days. The rats in their home cages were then placed in the acoustic chambers on the telemetric receivers for 4 hours on 5 consecutive days prior to testing, without presentation of noise, for further acclimation to the procedures. Forty-eight hours following the fifth pre-exposure, rats were exposed to the first of three consecutive daily 30-min loud noise exposures (95 dBA), or a similar duration exposure to the chambers without noise (no noise control), 24 h apart. On

these days, rats were transported in their home cages from the colony to the behavioral room and placed in the acoustic chambers for 2 h prior to injections, in order to normalize baseline heart rate and core body temperature values prior to any experimental manipulations. Then an experimenter gently handled the rats and removed the dust caps/obturator. An injector (30 gauge, #C317I - Plastics One), connected via PE tubing to a 10- μ l syringe fixed to a precision pump (#53220, Stoelting, Wood Dale, IL), was then inserted into the guide cannula. Muscimol (200 nl – Sigma – 1 mg/ml) dissolved in artificial cerebrospinal fluid (aCSF — NaCl, 140 mM; KCl, 3.35 mM; MgCl₂, 1.15 mM; CaCl₂, 1.26 mM; Na₂HPO₄, 1.2 mM; NaH₂PO₄, 0.3 mM, adjusted to pH: 7.4) or aCSF alone (vehicle) was injected at a constant rate of 200 nl/min. The injector was left in place for an additional 1 min after injection to allow for drug diffusion. The injector was then removed, obturator/dust cap replaced, and the rats returned to their home cages and placed immediately in the acoustic chambers on the ER-4000 Energizer receivers. Loud noise (95 dBA) or a similar duration of chamber exposure (no noise) was presented 1 h following the end of the injection for 30 min, and upon noise termination, the rats were removed from the chambers and transported back to the colony room. Similar procedures were followed 24 and 48 h later for the second and third loud noise/no noise exposures. Forty-eight hours after the third injection and noise/no noise exposure, rats were returned to the behavioral room, handled exactly as on the 3 prior exposure sessions, with the exception that no injections were performed (i.e. no aCSF or muscimol), and exposed to a fourth and final 30-min loud noise or no noise exposure. RPa muscimol injections were expected to reversibly inhibit acute heart rate and core body temperature responses to stress exposure, with minimal effects on basal values, as previously reported (Zaretsky et al., 2003b, Pham-Le et al., 2011, Kataoka et al., 2014). This reversible muscimol-induced cellular inhibition during the repeated stress exposures would also be expected to reduce or block activity-dependent modifications that may underlie the plasticity associated with habituation in the RPa region, which would translate in responses during the Test Day that would be as large as the initial aCSF-injected rats' responses on Day 1, with muscimol inhibition now removed. Because autonomic responses are also expected to be significantly reduced by RPa muscimol injections, the lack of these responses might also be expected to disrupt the acquisition of habituation, but this possibility has seldom been tested.

Seventy-two hours after the noise Test Day, a subset of animals (n=5 for vehicle; n=5 for muscimol; all previously repeatedly exposed to loud noise) were again placed in the same acoustic chambers, within their home cages, on the ER-4000 Energizer receivers previously experienced, for two hours. Rats were then removed from their home cages, placed in wire-mesh restrainers without injections, and returned to their home cages within the acoustic chambers and Energizer receivers for a 30-min restraint test. Following the restraint test, rats were immediately removed from the restrainers and returned to their home cages and animal colony. The restraint test was used to assess whether repeated RPa muscimol injections may have induced permanent damage, which would be expected to reduce autonomic responses to any inducing situations indiscriminantly. Some data obtained from these procedures were previously published in abstract form (Masini, Nyhuis, Day & Campeau, 2012).

Cannula placement verification

Twenty-four to 48 hours after the final experimental manipulations, animals were brought to the behavioral room and injected with 200 nl of either 0.1% methylene blue or 1.0 mg/ml BODIPY®TMR-X muscimol conjugate (Life Technologies™, Grand Island, NY) in order to visualize guide cannulae placement and injectate spread. Immediately (for the dye-injected animals) or 1 hour (for the BODIPY-muscimol injected animals) following this injection, animals were euthanized and brains were subsequently sectioned (35 µm) on a cryostat (Leica 1850, Germany), and cannula placements were verified under brightfield (dye-injected) or epifluorescence (BODIPY-muscimol-injected) microscopy (Imager Z1, Zeiss, Germany).

Data analysis

The initial 120 1-min heart rate and core body temperature values obtained were initially averaged into 60 2-min values for each animal included in this analysis, to allow repeated measures analyses on a PC SPSS platform (Ver. 23; maximum of 99 repeated values/factor). Four-way analysis of variance (ANOVA - Greenhouse-Geisser repeated measures statistic) were then employed on these heart rate and body temperature values, respectively, with Day (4 exposures) and Time (60 X 2-min averages) as repeated factors, and Stress (loud noise or no noise) and Drug (vehicle or muscimol) as between-subjects factors, to determine if stress or drug treatment induced anticipatory responses over their repeated administration. These initial analyses were followed by 3-way ANOVAs on each day to more clearly determine the source of interactions obtained in the initial analyses, and to assess possible initial group differences, especially on Day 1 (prior to any drug or stress manipulations). Similar analyses were conducted on the values obtained during the 1-hour (1-min values) interval after injections but before loud noise/no noise exposure to assess putative drug effects or repeated stress or drug treatments on these measures, over days. These analyses were restricted to the last 53 min of this interval due to several missing data points immediately after injections that would otherwise require removal of too many animals to provide meaningful repeated measures analyses.

To help isolate the effects of repeated drug and stress on habituation to repeated loud noise, raw data for each measure were modified by first averaging the 10 minute period immediately prior to the loud noise/no noise period, and then determining the change from these averages during each of the 30 1-min epoch of loud noise or no noise on the first (Day 1) and fourth (Test Day) exposures. Heart rate and body temperature changes were examined in successive 1-min epochs of time for each subject during loud noise or no noise exposure. Heart rate and body temperature changes were analyzed with 3-way ANOVAs, with Time (30 X 1 min) as a repeated measures factor, and Stress (loud noise or no noise), and Drug (vehicle or muscimol), as between-subjects factors ($p < 0.05$). Additional repeated and 2-way ANOVAs were used to evaluate group differences on the first and fourth exposure days and the restraint test. Statistical significance was set at a p value = 0.05.

Results

Localization of injection sites

Of the 44 rats used in this study, 27 received either aCSF ($n=13$) or muscimol ($n=14$) injections centered in the RPa region between -11.2 and -12.0 mm from bregma. As shown in Figure 1, the injections (as indicated with the red fluorescent BODIPY-conjugated muscimol) were centered in the RPa region without encroachment to ventrolateral medullary nuclei, but variable diffusion up the cannula tract to the dorsally located nucleus raphe magnus (RMg), in some cases. Other animals were excluded from the study due to the development of pathologic motor conditions ($n = 2$), severe anorexia that necessitated euthanasia ($n = 3$), failure of E-mitter transponders ($n = 2$), loss of cannula ($n = 3$) and missed cannulae placements ($n = 7$).

Basal heart rate and core body temperature prior to injections

To assess putative effects of repeated drug, stress, or group differences during the initial 2 hours of cage placement daily, a four-way ANOVA with Day (4), Time (60), Stress (2), and Drug (2) factors were employed on heart rate's and body temperature's 2-min averages, representing the two hours after initial transport and whole cage placement in the acoustic chambers, prior to injections (Figures 2 and 3, 1–120 min), respectively. Inspection of Figure 2 suggests significant heart rate increases daily upon transportation and cage placement. This impression was supported by a significant main Time effect ($F_{59,885} = 55.91$, $p < 0.001$). A possible interaction with Stress exposure over Day approached significance with the Day X Time X Stress ($F_{177,2655} = 1.66$, $p = 0.10$) and overall between-subjects effect of Stress ($F_{1,15} = 4.05$, $p = 0.06$). The individual days 3-way ANOVAs with Time, Stress, and Drug as factors all provided significant Time effects, respectively (all p 's < 0.001), with only Day 3 providing a significant Time X Stress interaction ($F_{59,885} = 4.50$, $p = 0.002$), and the Test Day displaying an overall between-subjects effect of Stress ($F_{1,15} = 5.34$, $p = 0.035$). These results suggest that compared to non-stressed rats, repeated loud noise exposure maintained the heart rate increase initially induced by transport and/or cage placement compared to non-stressed groups, irrespective of drug treatment.

Figure 3 suggests a modest rise in core body temperature that generally peaks 15–20 minutes after transport and cage placement, and slowly drifts lower thereafter. This impression was supported by a significant overall effect of Time ($F_{59,885} = 6.17$, $p = 0.002$). This overall analysis also revealed a Time X Stress interaction ($F_{59,885} = 4.06$, $p = 0.015$), perhaps indicating again an overall effect of stress on the regulation of body temperature over these initial 2-hours periods. Additional Day ($F_{3,45} = 4.74$, $p = 0.014$), Day X Drug ($F_{3,45} = 5.42$, $p = 0.008$), and Day X Drug X Stress interactions ($F_{3,45} = 5.48$, $p = 0.008$), together with a significant overall effect of Drug ($F_{1,15} = 11.09$, $p = 0.005$) were obtained from the initial four-way ANOVA. These complex regulations over days, especially with the Drug effect, appear to support the observations that repeated drug treatment over days increased core body temperature, irrespective of stress treatment. However, some of these effects can be attributed to group differences on body temperature from the very first day, for which the analyses revealed a significant main effect of Time ($F_{59,1357} = 12.92$, $p < 0.001$), and overall between-subjects Drug X Stress interaction ($F_{1,23} = 16.92$, $p < 0.001$). Day 2

also displayed an effect of Time approaching significance ($F_{59,885} = 2.71, p = 0.066$), without additional effects. Day 3 did not reveal any significant effects over Time, but all between-subjects main and interactions effects were significant, likely due to the muscimol no stress animals displaying relatively high body temperature; Drug ($F_{1,15} = 53.15, p < 0.001$), Stress ($F_{1,15} = 16.94, p = 0.001$), and Drug X Stress ($F_{1,15} = 23.21, p < 0.001$). The Test Day ANOVA provided a significant main Time effect ($F_{59,885} = 2.84, p = 0.025$), and again a main Drug effect approaching significance ($F_{1,15} = 3.71, p = 0.073$). The general pattern of these combined results suggest that repeated RPa muscimol injections lead to a modest anticipatory rise in core body temperature over days.

Heart rate and core body temperature regulation after RPa injection, but prior to stress

To assess the effects of repeated drug, stress, or group differences after RPa injection of aCSF or muscimol, a four-way ANOVA with Day (4), Time (53), Stress (2), and Drug (2) factors were employed on heart rate's and body temperature's 1-min responses, representing the interval after injection, but prior to loud noise/no noise exposure (Figures 2 and 3, 1–60 min, to the right of Injection), respectively. Only the last 53 values were employed in these analyses due to several animals missing some of the initial values immediately after injections. The initial four-way ANOVA on heart rate values revealed a main effect of Time ($F_{52,780} = 29.92, p < 0.001$), Time X Stress interaction ($F_{52,780} = 19.74, p < 0.001$), and significant between-subjects effects of Stress ($F_{1,15} = 7.96, p = 0.013$) and Drug X Stress ($F_{1,15} = 5.14, p = 0.039$) interaction. Inspection of Figure 2 indicates that simply removing and handling rats, even without injections (as demonstrated on the Test Day), can induce a significant heart rate increase, an effect that declines in non-stressed rats, but is maintained in rats undergoing repeated noise, whether they receive aCSF or muscimol. The only other significant result from this initial analysis indicated a significant interaction of Day X Stress ($F_{3,45} = 5.40, p = 0.013$), further underscoring the ability of loud noise to maintain large tachycardic responses to handling over days, as compared to the reduced responses in no noise groups. Individual 3-way ANOVAs on each day confirmed the results of the initial ANOVA on heart rate, with each day reporting significant Time effects (all p 's < 0.003). Beginning on Day 2 and all subsequent days, significant Time X Stress interactions were obtained (all F 's $F_{52,780} = 4.26 - 9.27, p$'s < 0.01). The only other effects were from between-subjects factors, with a significant Stress X Drug interaction on Day 1 ($F_{1,23} = 13.56, p = 0.001$), and a significant Stress effect on Day 4 ($F_{1,15} = 15.94, p = 0.001$). While the Stress X Drug interaction of Day 1 may indicate initial group differences, the significant overall Stress effect on Day 4 (in the absence of injections) strongly suggest significant regulation of heart rate by prior loud noise exposures.

The four-way ANOVA on core body temperature values indicated a significant Day X Stress interaction ($F_{3,45} = 4.16, p = 0.02$), and Time ($F_{52,780} = 16.90, p < 0.001$), Time X Drug ($F_{52,780} = 4.48, p = 0.01$), Time X Stress ($F_{52,780} = 11.42, p < 0.001$), and Time X Drug X Stress ($F_{52,780} = 4.97, p = 0.006$) interactions. These more complex effects reflect the observations that, similar to heart rate, handling generally induces a rise in core body temperature that also generally declines over time, but in this case, muscimol injections also induce an hypothermic response, and these effects appear to differ based on stress exposure and over days (especially on the Test Day, without muscimol treatment). These impressions

were supported by the 3-way ANOVAs on each day, with Day 1 indicating significant effects of Time ($F_{52,1196} = 20.24$, $p < 0.001$), Time X Drug ($F_{52,1196} = 4.98$, $p = 0.009$), and Time X Stress interactions ($F_{52,1196} = 4.29$, $p = 0.017$), and a between-subjects main Drug effect ($F_{1,23} = 9.93$, $p = 0.004$), clearly indicating the hypothermic effects of RPa muscimol injections. Significant Time effect ($F_{52,780} = 4.78$, $p = 0.008$), Time X Stress ($F_{52,780} = 6.34$, $p = 0.002$) and Time X Stress X Drug interactions ($F_{52,780} = 5.73$, $p = 0.003$) were obtained on Day 2, again indicating a significant reduction of body temperature by RPa muscimol, but mainly in the group exposed to loud noise. Day 3's 3-way ANOVA reported significant Time effect ($F_{52,780} = 10.53$, $p < 0.001$), a Time X Drug interaction ($F_{52,780} = 5.80$, $p = 0.006$), and an overall between-subjects Stress effect ($F_{1,15} = 5.18$, $p = 0.038$), likely due to the relatively high initial body temperatures in the muscimol no noise group. The only significance obtained from the Test Day's 3-way ANOVA was an overall between-subjects Drug effect ($F_{1,15} = 8.26$, $p = 0.012$), suggesting a reliable hyperthermic response in the groups previously receiving repeated RPa muscimol, supporting the hyperthermic responses obtained during the initial 2-hours acoustic chamber placement.

Body temperature and heart rate responses on the first loud noise exposure

To better isolate the effects of repeated stress and drug on stress-induced heart rate and core body temperature, especially in view of some of the pre-existing and/or drug-induced differences in autonomic reactions described above, changes in these responses were computed by subtracting the average values for the 10-min period immediately preceding loud noise or no noise exposure from each minutes' values during loud noise or control exposures for each rat. A 3-way ANOVA on heart rate changes with Time (30 X 1 min) as a repeated measure factor, and Stress and Drug as between-subjects factors on the first loud noise exposure (Figure 4A) revealed significant Time ($F_{29,667} = 3.89$, $p = 0.008$), Time X Drug ($F_{29,667} = 4.75$, $p = 0.002$), Time X Stress ($F_{29,667} = 2.72$, $p = 0.039$), and an overall Time X Stress X Drug interaction ($F_{29,667} = 3.12$, $p = 0.022$), with significant between-subjects effects of Stress ($F_{1,23} = 22.42$, $p < 0.001$) and Drug ($F_{1,23} = 18.49$, $p < 0.001$). The Time X Stress X Drug interaction can be explained by the observation that loud noise stress induced a significant rise in heart rate over the 30-min session, compared to no noise group, but that this difference over time is suppressed by muscimol treatment. This outcome was supported by two-way ANOVAs (Time X Stress) on the aCSF only treated animals, providing a significant Time X Stress interaction ($F_{29,319} = 3.30$, $p = 0.033$), which was not significant in the muscimol only treated rats (Time X Stress interaction; $F_{29,348} = 1.29$, $p > 0.05$). Likewise, two-way ANOVAs (Time X Drug) on loud noise only treated rats indicated a significant Time X Drug interaction ($F_{29,319} = 5.18$, $p = 0.004$), which was not significant in the no noise only groups (Time X Drug interaction $F_{29,348} = 2.30$, $p > 0.05$). These results suggest that significant tachycardia was observed in the vehicle-treated group exposed to loud noise, but that RPa muscimol injection significantly attenuated this response.

Similar analyses on Day 1's body temperature changes (Figure 4B) revealed significant interactions of Time X Drug ($F_{29,667} = 15.30$, $p < 0.001$), and Time X Stress X Drug, ($F_{29,667} = 3.24$, $p = 0.045$), together with significant between-subjects effects of Stress ($F_{1,23} = 5.93$, $p = 0.023$), Drug ($F_{1,23} = 31.78$, $p < 0.001$), and Stress X Drug interaction ($F_{1,23} = 10.24$, $p = 0.004$). The interpretation of the triple interaction is similar to that observed with

heart rate, in that that loud noise stress induced a significant rise in core body temperature over the 30-min session, compared to no noise group, but that this difference over time is prevented by muscimol treatment. This interpretation was supported by two-way ANOVAs (Time X Stress) on the aCSF only treated animals, providing a Time X Stress interaction approaching significance ($F_{29,319} = 2.83$, $p = 0.065$), together with a significant between-subjects Stress effect ($F_{1,11} = 15.34$, $p = 0.002$), but these effects were not observed in muscimol only treated rats (Time X Stress interaction: $F_{29,348} = 0.52$, $p > 0.05$; and Stress effect: $F_{1,12} = 0.31$, $p > 0.05$). Additional 2-way ANOVAs (Time X Drug) on loud noise only treated rats indicated a significant Time X Drug interaction ($F_{29,319} = 11.72$, $p < 0.001$). A similar Time and Drug ANOVA in the no noise only groups indicated a Time X Drug interaction that approached significance ($F_{29,348} = 3.56$, $p = 0.052$), without a significant between-subjects Drug effect ($F_{1,12} = 2.32$, $p > 0.05$). Inspection of Figure 4B suggest that this result may be explained by a continued reduction in core body temperature by RPa muscimol during this period. Overall, however, these results suggest that a significant hyperthermic reaction was observed, but only in the loud noise exposed vehicle-treated rats, while RPa muscimol significantly reduced this response at or below levels observed in the no noise groups.

Body temperature and heart rate responses to the fourth loud noise exposure

A 3-way ANOVA on heart rate changes was conducted with Time as a within-subjects factor, and Stress and Drug as between-subjects factors on the Fourth exposure day (Figure 4C). The only significant effect was obtained for Stress ($F_{1,15} = 12.44$, $p = 0.003$), without any additional effects. These results suggest that significant tachycardia was observed overall in the loud noise exposed rats, with or without prior RPa muscimol injections. The lack of prior drug effect or interaction further suggested that the previously vehicle or muscimol treated loud noise groups had similar tachycardic responses to the repeated loud noise on the fourth exposure.

A similar analysis on the Fourth exposure day's body temperature changes (Figure 4D) indicated significant Time ($F_{29,435} = 2.92$, $p = 0.05$), and Time X Stress interaction, ($F_{29,435} = 3.07$, $p = 0.044$), indicating an increase in body temperature in the loud noise exposed groups, with or without prior RPa muscimol injections. No additional within- or between-subjects effects were obtained on this measure (all F 's < 1.72 , p 's > 0.05). These results suggest that as in the case of heart rate change, significant hyperthermia developed over time in the loud noise exposed rats, with or without prior RPa muscimol injections.

To determine whether significant habituation developed in the repeatedly vehicle-injected group between the first and fourth loud noise exposure, a repeated measures ANOVA between these two days (First vs. Fourth noise exposure days) was carried out in the vehicle/loud noise treated rats at the maximum of the respective responses (0–10 min for heart rate, and 15–30 min for body temperature responses). The heart rate analysis revealed significantly lower mean tachycardic responses on the Fourth loud noise exposure day (Days effect - $F_{1,5} = 8.23$, $p = 0.035$), without a significant interaction with Time (Figure 4A vs. C). These result suggest significant habituation of tachycardia under the current experimental conditions in the vehicle group. A similar analysis on body temperature did not

reveal significant effects or interactions (all F 's > 3.52 , p 's > 0.05), suggesting that the hyperthermic responses were similar on the first and fourth loud noise exposures (see Figure 4B vs. 4D). Similar analyses using the entire 30 min period of noise exposures likewise failed to indicate significant differences between the first and fourth noise exposure (all p 's > 0.05). These analyses could not be performed meaningfully in the muscimol treated group due to the significant attenuation of acute responses to loud noise observed on the first loud noise exposure.

Heart rate and body temperature responses to restraint test

To test whether prior repeated RPa muscimol injections interfered with autonomic responses to stress, a subset of rats previously exposed to repeated audiogenic stress (vehicle-loud noise, $n = 5$; muscimol-loud noise, $n = 5$) were restrained for 30 min 72-hrs after the fourth exposure day. A one-way ANOVA showed no between-groups difference for basal heart rate ($F_{1,8} = 2.473$, $p > 0.05$; group previously treated with vehicle: 342.3 ± 14.2 bpm, group previously treated with muscimol: 315.6 ± 9.3 bpm) or body temperature ($F_{1,8} = 0.004$, $p > 0.05$; group previously treated with vehicle: 36.45 ± 0.19 °C; group previously treated with muscimol: 36.46 ± 0.08 °C) on the 10-min averaged values immediately prior to restraint exposure. A repeated measures ANOVA revealed no differences between previously vehicle- or muscimol-injected groups during the drug-free restraint test for heart rate ($F_{1,8} = 0.14$, $p > 0.05$) and body temperature ($F_{1,8} = 0.33$, $p > 0.05$) response changes (Figure 5). These results suggest that prior repeated injections with muscimol did not reduce the expression of heart rate responses by simply inducing non-reversible damage in the RPa region, as indicated by similar responses to restraint stress between the muscimol and aCSF injected groups.

Discussion

The current study was primarily designed to assess whether reversible inhibition of normal RPa cellular activity with the GABA_A receptor agonist muscimol during repeated audiogenic stress would reduce or prevent the acquisition of habituated tachycardic and hyperthermic responses. The location of the RPa within a circuit controlling multiple autonomic reactions responsive to stress (see Introduction) suggested this region as a potential site in which autonomic habituation-related modifications could take place. If stress-dependent cellular activity in RPa neurons was necessary for habituation-related modification of autonomic responses, reversible inhibition of this activity by RPa muscimol during repeated stress exposure would be expected to reduce the development of habituated responses, that is, responses would remain elevated even after repeated loud noise exposures. Simultaneously, the suppression/inhibition of autonomic responses by RPa muscimol injections may also be expected to disrupt the acquisition of habituation. The main results suggested that although reversible RPa cellular inhibition was likely obtained, as observed by the significant suppression of stress-induced autonomic responses during the initial 3 loud noise exposures, their habituation-related reductions during the injection-free test were indistinguishable from that of control rats. These results imply that while normal RPa activity is necessary for the expression of at least a subset of autonomic responses, stress-induced

activity in this region, and the acute expression of autonomic responses, are not necessary for the acquisition of habituated autonomic responses.

In addition to these primary results, repeated stress was found to reduce the adaptation to transport and handling normally observed in non-stressed animals, and this adaptation was also independent of RPa activity, as aCSF- and muscimol-injected stressed rats maintained similar high responses over days, as compared to the reduced influence of transport and handling in non-stressed rats. This finding may be associated with the phenomenon of stress sensitization often reported in response to repeated heterotypic stress (Konarska et al., 1989b, Herman, 2013), but also to repeated homotypic stress regimens, as in the current study (Weinberg et al., 2009). Repeated RPa muscimol injections raised core body temperatures over days, as observed in both the RPa muscimol injected groups, irrespective of stress treatment. Whereas this effect was apparent only after the first few RPa muscimol injections, it was most marked following handling on the Test Day, which may have provided more proximal cues for this anticipatory response to arise. This observation may suggest that the repeatedly induced hypothermic responses previously induced by RPa muscimol injections were anticipated by these rats, especially after the simulated injection procedures, resulting in a moderate hyperthermic response. These results are reminiscent of findings from repeated systemic drug injections that modify body temperature and induce conditioned tolerance effects to injection-related environmental stimuli (Miksic et al., 1975, Siegel, 1978, Mansfield and Cunningham, 1980, Crowell et al., 1981, Cunningham et al., 1983, Schwarz-Stevens and Cunningham, 1993). Because of the lack of body temperature measurements from the colony room where animals were housed between experimental manipulations, it is difficult to determine if these temperature regulations were limited to contextual cues or were more pervasive and generalized.

As previously shown with other stressors (Zaretsky et al., 2003b, Pham-Le et al., 2011, Kataoka et al., 2014), muscimol-induced inhibition of RPa activity reliably attenuated the audiogenic stress-induced tachycardic response, without consistently modifying basal heart rate in the treated groups. The tachycardic response following repeated audiogenic stress in previously muscimol-treated rats on the injection-free day was similar to the vehicle-injected rats. Importantly, a within-subjects comparison of the vehicle-injected animals confirmed that tachycardia was significantly reduced between the first and fourth audiogenic stress exposure, indicating habituation of that response. Comparisons with the additional control no noise groups on the fourth audiogenic stress exposure helped to rule out possible conditioned effects of prior repeated muscimol on tachycardia because no prior drug or interaction effects were obtained. Furthermore, assessment of putative repeated muscimol-induced RPa damage, using a novel restraint test three days after the last audiogenic stress exposure, provided no evidence of prior drug treatment effect on restraint-induced tachycardia or hyperthermia. It should be noted that a non-injected group might reveal reduced response capacity in both aCSF- and muscimol injected groups if volume injections induced RPa damage, but this appears unlikely, as both the amplitude and duration of the responses to restraint were well within the range of non-injected stressed rats (Aydin et al., 2011). Together, these results suggest that rats in the muscimol-injected stressed group exhibited a similarly reduced and habituated tachycardic response compared to the aCSF-injected rats on the fourth audiogenic stress exposure.

The hyperthermic results were similar to the tachycardic results in many respects with some notable exceptions. As previously reported, acute muscimol injected in the rostral RPa region significantly reduced basal core body temperature compared to vehicle-treated rats (Zaretsky et al., 2003a). Upon acute audiogenic stress exposure, muscimol treated animals did not exhibit hyperthermia, but instead continued to show a slight decline in core body temperature following muscimol injection, compared to aCSF-injected rats. The direct comparison of muscimol's effects on body temperature during audiogenic stress and the no noise control condition indicated no additive effect of loud noise superimposed on lower basal temperature values. Overall, these results indicate that the RPa region is important for controlling rodent's basal core body temperature, as well as the hyperthermic responses to an increasing number of stress situations. In view of the relative basal hyperthermic state observed in previously RPa-muscimol injected rats, they displayed a significant and additional hyperthermic response to the fourth audiogenic stress exposure, which was similar to that in the previously aCSF-stressed rats. For this response, however, rats in the vehicle-treated group did not show significant habituation of the hyperthermic response between the first and fourth audiogenic stress exposure. We have previously shown that audiogenic stress-induced hyperthermia is attenuated over repeated exposures, but 6–10 presentations were used in these studies (Masini et al., 2008, Masini et al., 2011), rather than the 4 employed in the current study to limit the number of daily intracranial injections. The relatively limited number of exposures and high level of variation in the vehicle-treated group of the current study may have precluded the detection of statistically significant hyperthermic habituation in this group.

Overall, these results are consistent with the interpretation that neither the expression of normal stress-evoked autonomic responses, nor normal activity, at the level of the RPa region, are necessary for the acquisition of habituated responses induced by repeated stress exposure. The acquisition of habituated responses in the absence of initial responses is relatively novel; prior work suggested that inhibition of stress-induced responses by muscimol in the medial geniculate body or the rostromedial posterior hypothalamic nucleus prevented the acquisition of habituated neuroendocrine responses (Day et al., 2009, Nyhuis et al., 2016). The caudal and mostly efferent location of the RPa within a circuit responsible for autonomic responses regulated by stress may suggest that more centrally positioned regions are more proximally associated with habituation-related modifications induced by repeated stress. Although few studies have focused on the substrates of habituation of autonomic reactions, evidence from measurement of neuroendocrine responses suggest that limbic and hypothalamic regions may contribute to the response modifications induced by repeated homotypic stress (Bhatnagar et al., 2002, Weinberg et al., 2010, Grissom and Bhatnagar, 2011, Nyhuis et al., 2016). Some of these regions provide direct RPa innervation, and may help explain the various neurotransmitter receptor mediated regulation of autonomic functions obtained at the level of the RPa region, including excitatory amino acids, serotonin, orexin, glycine, and GABA_A receptors (Nakamura and Morrison, 2007, Pham-Le et al., 2011, Tupone et al., 2011, Kataoka et al., 2014). The role of these potential systems in habituation to stress remains to be clearly determined.

The results of this study suggest that although the RPa region importantly controls tachycardia following acute psychological stress exposure, this region may not be necessary

for the acquisition of habituated tachycardia following repeated stress. This interpretation is based on the assumption that the inhibition induced by muscimol at the injection site would prevent activity-dependent modifications requiring depolarization of post-synaptic neurons, which is frequently observed for either excitatory or inhibitory synaptic modifications (Gaiarsa et al., 2002, Kullmann and Lamsa, 2011, Mendez and Bacci, 2011). However, it is possible that activity-independent modifications at the injection site are responsible for the changes associated with habituation of autonomic responses. Additional studies will be required to assess putative intrinsic, depolarization-independent, mechanisms at the level of the rostral RPa region in the acquisition of autonomic response habituation. Although possible, it appears unlikely that habituation-related long-lasting biochemical or physiological changes would occur at the level of this region, as acute autonomic responses following exposure to different stressors are necessary for survival, and many other non-autonomic responses also undergo habituation to repeated stress.

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Figure 1.

A. Brainstem regional outline derived from Paxinos & Watson “The Rat Brain in Stereotaxic Coordinates”, 6th Edition, Plate 128. All animals included in this study had cannulae placements between -11.28 and -12.0 mm from bregma, with the injectate centered in the nucleus Raphe Pallidus (RPa). B. Placements were verified by microinjecting 200 nl of 1mg/mL muscimul conjugated to BODIPY TMR-X (red) over 1 min 1 hr prior to sacrifice. Scale bar (lower right): 500 μ m. Abbreviations: 4V: fourth ventricle; Gi: gigantocellular reticular nucleus; GiA: gigantocellular reticular nucleus, alpha region; LPGiA: lateral paragigantocellular nucleus, alpha region; ml: medial lemniscus; mlf: medial longitudinal fasciculus; py: pyramidal tracts; RMg: nucleus raphe magnus; RPa: nucleus raphe pallidus.

Figure 2.

Average heart rate (beats per minutes +SEM) responses during the four experimental day for the initial 2 hours (120 min - left side of figure), injection of vehicle or muscimol into the rostral raphe pallidus region (60 min after injection), and loud noise or no noise exposure (30 min, as indicated by gray area). The left side of the figure shows that daily transportation and cage placement increased heart rate quickly, but this response decreased over each session (overall Time effect, $F_{59,885} = 55.91$, $p < 0.001$). An interaction with Stress exposure over Day approaches significance (Day X Time X Stress interaction, $F_{177,2655} = 1.66$, $p = 0.10$, and overall effect of Stress, $F_{1,15} = 4.05$, $p = 0.06$). Respective daily analyzes suggested that compared to non-stressed rats, repeated loud noise exposure maintained the heart rate increase initially induced by transport and/or cage placement compared to non-stressed groups, irrespective of drug treatment. This effect that was observed again in response to the handling/injection procedures, as indicated on the right of the injection arrow (main effect of Time, $F_{52,780} = 29.92$, $p < 0.001$, Time X Stress interaction, $F_{52,780} = 19.74$, $p < 0.001$, and Stress, $F_{1,15} = 7.96$, $p = 0.013$).

Figure 3.

Average core body temperature (degrees Celsius +SEM) responses during the four experimental days for the initial 2 hours (120 min - left side of figure), injection of vehicle or muscimol into the rostral raphe pallidus region (60 min after injection), and loud noise or no noise exposure (30 min, as indicated by gray area). A modest rise in core body temperature peaking 15–20 minutes after transport and cage placement, slowly drifted lower thereafter, and was supported by a significant overall effect of Time, $F_{59,885} = 6.17$, $p = 0.002$. A Time X Stress, $F_{59,885} = 4.06$, $p = 0.015$, and Day X Drug X Stress interactions, $F_{3,45} = 5.48$, $p = 0.008$, suggested that repeated muscimol treatment over days increased core body temperature, in a stress-dependent manner. However, some of these effects can be attributed to group differences on body temperature from the very first day, as indicated by a Day 1 Drug X Stress interaction, $F_{1,23} = 16.92$, $p < 0.001$. Core body temperature after injection but prior to loud noise or no noise indicated a significant Time X Drug X Stress interaction, $F_{52,780} = 4.97$, $p = 0.006$. This more complex interaction suggest that handling generally induces a rise in core body temperature that also decline over time, but in this case, muscimol injections also induce an hypothermic response, and these effects appear to differ based on stress exposure and over days (especially on the Test Day, without muscimol treatment).

Figure 4.

Average change from averaged 10-min values immediately prior to loud noise or no noise conditions for heart rate (bpm +SEM) and core body temperature (°C +SEM) responses during 30 minutes of loud noise or no noise on the first (A and B, respectively) and fourth (C and D, respectively) exposures. Animals that received previous injections of aCSF in the RPa region during repeated loud noise exposures exhibited significant habituation of heart rate ($*F_{1,5} = 8.23$, $p = 0.035$), but not body temperature. There were no significant differences between the previously treated aCSF and muscimol groups on the fourth, drug free, loud noise exposure for either heart rate or core body temperature changes (all p 's > 0.05).

Figure 5.

Average change from averaged 10-min values immediately prior to restraint for A. heart rate (bpm +SEM) and B. core body temperature (°C +SEM) responses during 30 minutes of restraint stress. There were no significant differences in these responses between the previously treated vehicle (aCSF) and muscimol groups (all p 's > 0.05).