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Reversible Inactivation of the Auditory Thalamus Disrupts HPA Axis Habituation to Repeated Loud Noise Stress Exposures

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Abstract

Although habituation to stress is a widely observed adaptive mechanism in response to repeated homotypic challenge exposure, its brain location and mechanism of plasticity remains elusive. And while habituation-related plasticity has been suggested to take place in central limbic regions, recent evidence suggests that sensory sites may provide the underlying substrate for this function. For instance, several brainstem, midbrain, thalamic, and/or cortical auditory processing areas, among others, could support habituation-related plasticity to repeated loud noise exposures. In the present study, the auditory thalamus was tested for its putative role in habituation to repeated loud noise exposures, in rats. The auditory thalamus was inactivated reversibly by muscimol injections during repeated loud noise exposures to determine if brainstem or midbrain auditory nuclei would be sufficient to support habituation to this specific stressor, as measured during an additional and drug-free loud noise exposure test. Our results indicate that auditory thalamic inactivation by muscimol disrupts acute HPA axis response specifically to loud noise. Importantly, habituation to repeated loud noise exposures was also prevented by reversible auditory thalamic inactivation, suggesting that this form of plasticity is likely mediated at, or in targets of, the auditory thalamus.

Keywords

adaptation; corticosterone; acute stress; restraint; muscimol; rat

2. Introduction

Acute stress situations evoke a variety of neuroendocrine, autonomic, and behavioral reactions that help organisms respond more efficiently to the specific internal or external challenges encountered [3,25,44]. However, repeated or sustained stress reactions are also associated with the development or precipitation of several pathologies in animals and humans [17,30]. Under many conditions, the responses to repeated stress are reduced through adaptive mechanisms, generally termed habituation [4,6,13,18,28,29,34]. Thus, habituation to stress could be argued to play an important role in reducing the impact of repeated or sustained stress.

Habituation of the hypothalamo-pituitary-adrenal (HPA) axis response is frequently observed to the relatively predictable and repeated exposures to the same (homotypic) stressor in

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experimental animals [26,29]. Importantly, the HPA axis response is either normal or facilitated in habituated animals exposed to a different (heterotypic) novel stress situation [5, 7,26,29]. These observations may suggest that the habituation-related plasticity occurs in the central limb of stress-responsive circuits [1], thus allowing the HPA axis to respond normally, or even become sensitized, to new challenges. Alternatively, this pattern of results could be explained by habituation-related plasticity in the ascending sensory limb of the specific stressor afferents activated by the challenging situation. Although this alternative would be somewhat difficult to support given the more psychological, multisensory nature of many stress situations, and the likely dire consequences of toned down sensory processes [32], this alternative has only been functionally tested in a very limited way in the context of habituation to stressful situations [28,40].

In order to help define and locate the putative brain regions subserving habituation of the HPA axis response to intermittent homotypic stress, several studies have measured brain activation using immediate-early gene induction [13,16,20,31,41,43,45]. *c-fos* induction is most reliably reduced in the paraventricular nucleus of the hypothalamus (PVN), which ultimately controls the activity of the HPA axis, in rats exposed to homotypic intermittent stress. Interestingly, some of these studies have reported significant habituation in sensory cortices and thalamic relay nuclei associated with specific stress stimuli [13,20], suggesting that perhaps the plasticity associated with stress habituation takes place in the sensory afferents providing inputs to more central stress-responsive regions. However, whether immediate-early gene reduction in these areas corresponds to functional sensory changes, or whether these regions are anatomically and functionally responsible for the observed immediate-early genes reduction in PVN activity, is unknown.

In the present study, a specific sensory system involved in loud noise stress was tested for its putative role in habituation to repeated loud noise exposure. Loud noise has repeatedly been employed as an effective stressor evoking reliable HPA axis activation [4,8,10,14,19,21,23, 38], and is associated with immediate-early gene induction in several forebrain regions [14]. In turn, a number of these regions project directly to the PVN [15], and are highly correlated with *c-fos* mRNA induction in the PVN [11]. In addition, many of these forebrain regions receive auditory information from the ventromedial aspect of the auditory thalamus [15], which is necessary for loud noise stress-induced HPA axis activation [12]. Along sensory afferents of the auditory system, several brainstem, midbrain, thalamic, and/or cortical auditory processing areas could support habituation-related plasticity to repeated loud noise exposures. Inactivation at the level of the auditory thalamus during the repeated loud noise exposures was thus performed to determine if activation of lower brainstem or midbrain auditory nuclei would be sufficient to support habituation to this specific stressor. However, because permanent auditory thalamic inactivation blocks HPA axis response to acute loud noise [12], muscimol-induced reversible inactivation [27] of this region was performed only during the first two loud noise exposures. This permitted the measurement of an unimpeded HPA axis response during the third and final noise exposure under drug free conditions. Our results indicate that HPA axis habituation to repeated loud noise exposures is prevented by auditory thalamic inactivation, suggesting that this form of plasticity is likely mediated at the level of, or in targets of, the auditory thalamus.

3. Results

3.1 Cannulae placements

A cross section of a rat's brain implanted bilaterally with indwelling cannulae at the level of the medio-ventral auditory thalamus is shown in Figure 1B. As can be seen in Figure 2, the majority of injector tips were located in the vicinity of the ventral medial division of the medial geniculate body and the dorsal posterior intralaminar nucleus. The majority of cannulae

placements were in the mid-rostrocaudal levels of the medial geniculate body (approximately -5.4 to -5.9 mm from Bregma), with only a few cannulae found at very anterior (-5.1 mm from Bregma) or posterior (-6.3 mm from Bregma) levels.

Of the 54 rats that underwent cannulae implantation, 11 rats were lost due to guide cannulae obturation or loss of guide cannulae. One rat lost his cannulae during the second injection, so while the response of this rat to the initial injection was retained, no data were collected on the final noise exposure test. Therefore, the results of the current study is based on 43 rats for the initial injection day, and 42 rats for the third and final loud noise exposure. The following number of rats were therefore included in the 6 main experimental groups. The first 2 groups included ACSF ($n = 5$) and muscimol ($n = 4$) injected rats exposed to the quiet chambers both during the first 2 experimental days (training), and during the third and final test day (testing), to control for putative effects of ACSF or muscimol injections on corticosterone release during the final test. The next 2 groups included ACSF ($n = 5$) and muscimol ($n = 5$) injected rats exposed to the quiet chambers during the first 2 experimental days (training), but exposed to loud noise during the third and final test day, to evaluate if 2 prior muscimol injections alter corticosterone release to an acute loud noise under drug free conditions. The final 2 groups included ACSF ($n = 12$) and muscimol ($n = 11$) injected rats exposed to loud noise on the first 2 experimental days, and again on the third and final test day, to determine if habituation to noise was evident on the final drug free loud noise exposure.

3.2 Corticosterone Responses to Acute Loud Noise

Figure 3 depicts the individual plasma corticosterone levels of rats treated with intra-thalamic ACSF or muscimol injections to the first loud noise (Noise) or control chamber exposure (Quiet). The means and standard errors of the means for the ACSF-Quiet ($n = 10$), ACSF-Noise ($n = 12$), muscimol-Quiet ($n = 10$), and muscimol-Noise ($n = 11$) groups were 1.75 (0.44), 18.35 (1.54), 7.80 (3.90), 13.76 (5.74), respectively. An initial ANOVA indicated a significant effect of loud noise ($F(1,39) = 15.07, p < .001$), and a significant interaction between noise exposure and drug condition ($F(1,39) = 8.84, p = 0.005$). To better characterize this interaction, additional ANOVAs on the quiet and noise exposed rats, respectively, indicated no differences between the ACSF and muscimol groups in the quiet no noise condition ($F(1,18) = 2.37, p > 0.05$), but a significant reduction of corticosterone levels in the muscimol injected group in response to 95 dB noise ($F(1,21) = 7.95, p = 0.01$). The significant reduction in acute loud noise-induced corticosterone release in the auditory thalamic muscimol-injected rats thus replicates earlier findings with permanent auditory thalamic lesions [12].

3.3 Corticosterone Responses to Repeated Loud Noise

Figure 4 shows that two prior loud noise exposures produced a sizable reduction in corticosterone release in ACSF treated rats, but this reduction was blocked in rats treated with muscimol at the level of the auditory thalamus. This impression was supported statistically with a reliable drug effect ($F(1,21) = 11.61, p = 0.003$), from an ANOVA on the corticosterone levels from the third and final loud noise exposure under drug free conditions in the groups that were exposed to loud noise on all days (right set of bars, Figure 4). However, there were no group differences in rats that received the loud noise for the first time ($F(1,8) = 0.25, p > 0.05$) under drug free conditions (middle set of bars, Figure 4). There were likewise no differences between the groups ($F(1,7) = 1.38, p > 0.05$) that never received loud noise (left set of bars, Figure 4). A repeated measures ANOVA on the corticosterone levels of the ACSF-treated rats exposed repeatedly to loud noise indicated a significant reduction in corticosterone levels on the third loud noise exposure test ($F(1,11) = 23.23, p = 0.001$). This test was not performed in the muscimol-treated rats because of the significant reduction produced by auditory thalamic inactivation on the initial noise exposure, as presented above. The range of corticosterone values on the first injection day, especially in the muscimol injected rats, may

have predicted their corticosterone responses on the test day, but correlation coefficient analyses (Pearson's) did not reveal reliable correlations when all rats ($r = 0.019$, $p=0.91$) or only the muscimol-treated rats ($r = 0.222$, $p=0.35$) were analyzed.

3.4 Corticosterone Responses to Acute Restraint

Figure 5 displays individual plasma corticosterone levels of ACSF- and muscimol-injected rats following a 30-min restraint stress test. An ANOVA on restraint-induced plasma corticosterone levels indicated the specificity of auditory thalamic muscimol injections on the acute corticosterone response to loud noise, as the muscimol- (mean: 20.93 ± 2.06 $\mu\text{g/dl}$) and ACSF-treated (mean: 19.30 ± 2.45 $\mu\text{g/dl}$) rats' mean corticosterone values did not differ reliably ($F(1,15) = 0.26$, $p > 0.05$).

4. Discussion

Injection of the GABA_A receptor agonist muscimol, a proven technique employed to reversibly inactivate different brain regions [9,27,33,35,37,39,42], was employed to determine if disruption of auditory signaling at the level of the auditory thalamus would modify habituation of corticosterone release in response to repeated loud noise exposures. Our results suggest that compared to control rats that displayed significant habituation of corticosterone release to repeated loud noise exposures, rats treated with intra-thalamic muscimol during the initial two loud noise exposures showed little signs of corticosterone habituation on the third and final loud noise test exposure. This effect was likely not due to irreversible damage to the auditory thalamus by the two prior injections of muscimol, as the acute corticosterone responses to loud noise on the drug free test were comparable in rats that were or were not previously presented with loud noise, and were comparable to levels observed in ACSF-treated rats exposed to loud noise for the first time. Likewise, intra-thalamic muscimol treatment did not reliably induce the release of corticosterone in the control chamber exposure condition, but it did significantly reduce corticosterone release in response to an acute loud noise exposure, which supports our prior findings with permanent excitotoxic damage of the auditory thalamus [12]. As observed in this prior study, the reduction in corticosterone release induced by acute loud noise exposure did not generalize to a different stressor, restraint, indicating the specificity of the auditory thalamic manipulation to loud noise stress. Future studies will test the specific role of the auditory thalamus in loud noise habituation by repeatedly inactivating this brain region during a repeated restraint stress regimen, for example.

Overall, therefore, the results of the current study strongly suggest that the habituation-related plasticity induced by repeated loud noise exposures is unlikely to be mediated at lower auditory processing regions of the brainstem and midbrain. This is based on the assumption that auditory thalamic muscimol-induced inactivation would not disrupt normal processing at lower auditory levels. This assumption, however, might be challenged by findings indicating that corticofugal and thalamofugal feedback [22] upon collicular and lower level auditory neurons are necessary for some forms of plasticity at these lower auditory levels [24,46]. It is therefore possible that reversible inactivation of the auditory thalamus disrupted habituation by blocking plasticity at lower auditory levels. Whether the behaviorally-relevant plasticity associated with habituation to stress might also be disrupted in lower processing regions due to inactivation in higher brain regions is unknown. However, the induction of the immediate early gene *c-fos* evoked by loud noise exposure was not found to differ in auditory processing brainstem and midbrain regions in permanently auditory thalamic lesioned rats [12], arguing against a significant change in cellular functions and responses in lower auditory regions to thalamic inactivation. Thus, combined with our previous functional findings that habituation to repeated loud noise exposures does not modify auditory evoked brainstem potentials [13], or the acoustic startle reflex and its prepulse inhibition [28], the present results provide additional evidence that the

plasticity associated with habituation to stress does not take place in the more peripheral sensory regions of the systems providing information about a stressful event. The results further suggest that the plasticity associated with stress habituation takes place at, or rostral to, thalamic levels, but before the paraventricular nucleus of the hypothalamus, given the specificity of habituation. Because many forebrain systems regulating innate tendencies, perception, memory, comparator, controllability and predictability functions have been suggested to be intimately associated with the triggering and elaboration of stress responses [2], this likely dictates the involvement of complex forebrain circuits in stress habituation. The auditory thalamus has a highly divergent projection pattern that goes far beyond its lemniscal projections to the auditory cortex, and include many subcortical regions of the hypothalamus, extended amygdala, basal forebrain, and septum [15], many of which, in turn, project to the paraventricular hypothalamic nucleus. Which of these multiple auditory thalamic targets, or the auditory thalamus itself, provides the plastic substrate underlying habituation to repeated loud noise exposures remains to be unambiguously determined.

5. Experimental procedures

5.1 Subjects

Fifty-four male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 275 – 300 g and approximately 2 months old were used, in three independent replications. They were housed in a dedicated colony facility and initially grouped four to five in clear polycarbonate tubs (54 × 29 × 20 cm) containing wood shavings, with wire lids providing food (rat chow) and water ad libitum. Animals were housed for a period of 7–12 days after arrival from the supplier, before surgical cannulae implantation were performed (see below). They were kept on a controlled 12:12 hrs light/dark cycle (lights on 7:00 am), under constant humidity and temperature conditions. All procedures were performed between 8:30 am and 1:00 pm to reduce variability due to normal circadian hormonal variations. All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Colorado and conformed to the United States of America National Institute of Health Guide for the Care and Use of Laboratory Animals.

5.2 Surgery

Under halothane anesthesia, rats were shaved and placed in a Kopf stereotaxic instrument equipped with *blunt* ear bars. After appropriate disinfections, an incision was made over the skull, the skin retracted, and small burr holes drilled through the skull bone to allow implant of bilateral chronic guide cannulae (26 g, Plastics One, Roanoke, VA) above the auditory thalamus (5.8 mm posterior, 3.1 mm lateral, and 5.8 mm ventral to Bregma, according to the flat skull coordinates of Paxinos & Watson, 1998 - [36]). These coordinates were initially determined empirically in 4 additional rats, while simultaneously testing the volume of injection required to invade the relatively large auditory thalamic region. Of three injection volumes tested (0.1, 0.25, and 0.5 µl/side), 0.5 µl/side was found to invade most of the auditory thalamus without much encroachment of surrounding regions, as observed with 0.1% methylene blue injection (see Figure 1A). The cannulae were held in place by dental cement anchored on 3 jeweler's screws solidly fixed to the rat's skull. Stylets, extending 0.5 mm below the guide cannulae tips, were inserted to keep the cannulae free of organic substances, and externally closed with dust caps. Rats were given buprenorphine (analgesic) and Baytril™ (antibiotic) postoperatively, and thereafter housed individually in smaller (46 × 24 × 20 cm) clear polycarbonate tubs. Rats were observed daily during recovery from this surgery for at least 7 days prior to additional experimental manipulations.

5.3 Noise Apparatus

The acoustic chambers used in this experiment consisted of ventilated double wooden (2.54 cm plywood board) chambers, with the outer chamber lined internally with 2.54 cm insulation (Celotex™). The internal dimensions of the inner box were 59.69 cm (w) × 38.10 cm (d) × 38.10 cm (h), which allowed placement of the rats' home cages. Each chamber was fitted with a single 15.24 cm × 22.86 cm Optimus speaker (#12-1769 - 120 W RMS) in the middle of the ceiling. Lighting was provided by a fluorescent lamp (15W) located in the upper left corner of the chamber. Noise was produced by a General Radio (#1381) 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), and fed to the speakers. The speaker characteristics allowed relatively flat delivery between 20 and 27,000 Hz, rolling off quickly (20 dB/octave) at both ends. Noise intensity was measured by placing a Radio Shack Realistic Sound Level Meter (A scale; #33-2050) in the rat's home cage at several locations and taking an average of the different readings. The ambient/quiet noise level inside the chamber was approximately 60 dBA, and approximately 55 dBA in the rat colony.

5.4 Loud Noise Procedures

At least seven days following recovery from cannulae implantations, rats were handled and habituated to the injection procedures, and placed in the acoustic chambers for 30 min, without noise exposure, for 4 consecutive days. The next day, rats were exposed to the first of two consecutive daily 30-min loud noises (95 dBA) or control chamber exposures, 24 hrs apart. On these days, rats were brought from the colony to the laboratory for at least 30 min prior to injections. They were then gently handled by an experimenter while the dust caps/stylets were removed. Bilateral injectors (28 g, Plastics One), connected via PE tubing to 10- μ l syringes fixed to a precision pump (#53220, Stoelting, Wood Dale, IL), were then inserted into the guide cannulae. Muscimol (0.5 μ l/side - Sigma - 1 mg/ml) mixed 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 (control) were injected at a constant rate of 0.25 μ l/min. Muscimol was chosen because it offers a relatively specific synaptic inactivation mechanism through the ubiquitously located GABA_A receptors, leading to rapid, long-lasting, and reversible cellular hyperpolarization [27]. Injectors were left in place for an additional 60 sec after the injection to allow for drug diffusion. Injectors were then removed, stylets and dust caps replaced, and the rats returned to their home cages and placed immediately in the acoustic chambers. Loud noise (95 dB SPL – A scale) was turned on 10 min following the end of the injections, for 30 min. Immediately upon noise termination following the first noise exposure, rats were removed from the acoustic chambers and a blood sample via tail bleed was collected for corticosterone determination. The rats were then transported back to the colony room. Rats undergoing control chamber exposures spent the same period of time in the chambers, without noise exposure. Similar procedures were followed 24 hrs later for the second loud noise or control chamber exposure, with the exception that no blood samplings were performed. Forty-eight hours after the second noise or control chamber exposure, rats were brought back to the laboratory, placed in the acoustic chambers without any drug injections (i.e. drug free), and exposed to a 30-min loud noise, or a 30 min control chamber exposure. Immediately upon noise termination or chamber exposure, rats were sacrificed by decapitation, trunk blood collected in chilled EDTA-containing Vacutainer tubes, and brains were harvested and frozen in -30 to -40° C isopentane. Brains were subsequently sectioned (40 μ m) on a cryostat (Leica 1850), and cannulae placements were histologically verified following Nissl staining (cresyl violet), under brightfield microscopy (Nikon E800).

5.5 Restraint Procedure

A subset of rats ($n = 17$) randomly chosen were sampled for blood via tail bleed during the third and final chamber exposure test (with or without noise), so these rats were available for an additional restraint test. At least 48 hrs after the loud noise or control chamber exposure test, rats were brought to an experimental room and injected with ACSF ($n = 8$) or muscimol ($n = 9$) as described above, and were immediately confined in circular $21 \times 8 \times 8$ cm clear Plexiglas open frames for 30 min. Of the 8 rats that received ACSF, 6 rats had previously received ACSF, and half of the 8 rats had received noise at least once previously; of the 9 rats that received muscimol, 6 had previously received muscimol, and 5 of the 9 rats had received noise at least once previously. Holes at both ends of the open frames allow for air circulation. This was performed on a tabletop in a different experimental room. Immediately following the 30 min restraint procedure, rats were sacrificed by decapitation, and trunk blood and brains were collected as described above.

5.6 Tail Blood Sampling Procedure

Immediately after the initial loud noise exposure (or control chamber exposure for the no noise groups), rats were gently restrained using a clean towel. A small incision was then made in a lateral tail vein with the corner of a razor blade. Approximately 225 μ l of blood was collected, using Heparin-coated hematocrit capillaries, and deposited into empty 1.5 mL microcentrifuge tubes on ice. The whole procedure lasted less than 3 min from removal to return of rats to their home cages. Blood samples were centrifuged at 2000 rpm for 2 min, the plasma pipetted into 0.5 ml Ependorf microcentrifuge tubes, and stored at -80° C until assayed. Because of the small plasma samples collected and the heparin interference with ACTH assays, ACTH levels were not determined in this study.

5.7 Corticosterone Enzyme Linked ImmunoSorbent Assays (ELISA)

The corticosterone assay was performed according to the manufacturer's instructions (kit #901-097 – AssayDesigns, Ann Arbor, MI). Ten μ l of plasma plus steroid displacement reagent in the standard buffer were used. Levels were then quantified on a BioTek Elx808 microplate reader and calculated against a standard curve generated concurrently.

5.8 Data Analysis

Values for corticosterone on Day 1, the test day, and the restraint test were statistically evaluated with analyses of variance (ANOVA), with drug (ACSF or muscimol) and exposure condition (quiet chamber or 95 dB noise) used as between-subjects variables when appropriate. An additional repeated measures ANOVAs from the initial and last exposure sessions was performed on the ACSF-treated rats' corticosterone levels to test for the development of habituation. The level of statistical significance was set at $p = 0.05$.

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Figure 1.

A. Extent of diffusion of a volume of 0.5 μ l of a 0.1% methylene blue water solution, injected in the ventromedial region of the medial geniculate body, used to approximate diffusion of ACSF and muscimol. The white arrow indicates the approximate location of the injector tip. Abbreviations: MGD, dorsal division of the medial geniculate body; MGM, medial division of the medial geniculate body; MG, ventral division of the medial geniculate body; PIL, posterior intralaminar nucleus. B. Representative example of a rat brain coronal section stained with cresyl violet at the level of the mid-to-caudal medial geniculate body displaying bilateral cannulae placements and approximate injector tips (black arrows) in the ventromedial aspect of the medial geniculate body.

Figure 2.

Reconstruction of injector tip placements from the rats retained in the study. Approximate tip placements are represented by small black circles on composites from four different coronal sections reproduced from Paxinos and Watson, 1998 [36], at four different coronal levels through the rostro-caudal extent of the medial geniculate body. Plates 76, 80, 82, and 86 correspond to 5.1, 5.4, 5.9, and 6.3 mm posterior to Bregma (negative numbers by sections at right), from top (most rostral) to bottom (most caudal), respectively.

Figure 3. Individual plasma corticosterone levels (µg/dl) measured after intra-thalamic injections of ACSF (empty symbols) or muscimol (filled symbols) and immediately following an acute 30-min, 95-dBA loud noise (squares) or quiet chamber exposure (circles). The lines in each column indicate the respective group means. ^astatistically different from ACSF-injected group also exposed to noise, $p = 0.01$.

Figure 4.

Mean plasma corticosterone levels ($\mu\text{g/dl} + 1$ standard error of the mean) measured immediately following 30 min, 95-dBA loud noise (testing +), or quiet chamber (testing -) exposure test (48 hrs interval), in rats previously treated with ACSF (grey bars) or muscimol (black bars) and exposed to loud noise (training +) or quiet chambers (training -). *statistically different from ACSF-injected group repeatedly exposed to loud noise, $p = 0.003$.

Figure 5. Individual plasma corticosterone levels ($\mu\text{g/dl}$) measured after intra-thalamic injections of ACSF (circles) or muscimol (squares) and immediately following an acute 30-min restraint stress session. The lines in each columns indicate the respective group means.