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Research Article

Behavioral Animal Model of the Emotional Response to Tinnitus and Hearing Loss

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ABSTRACT

Increased prevalence of emotional distress is associated with tinnitus and hearing loss. The underlying mechanisms of the negative emotional response to tinnitus and hearing loss remain poorly understood, and it is challenging to disentangle the emotional consequences of hearing loss from those specific to tinnitus in listeners experiencing both. We addressed these questions in laboratory rats using three common rodent anxiety screening assays: elevated plus maze, open field test, and social interaction test. Open arm activity in the elevated plus maze decreased substantially after one trial in controls, indicating its limited utility for comparing pre- and post-treatment behavior. Open field exploration and social interaction behavior were consistent across multiple sessions in control animals. Individual sound-exposed and salicylate-treated rats showed a range of phenotypes in the open field, including reduced entries into the center in some subjects and reduced locomotion overall. In rats screened for tinnitus, less locomotion was associated with higher tinnitus scores. In salicylate-treated animals, locomotion was correlated with age. Sound-exposed and salicylate-treated rats also showed reduced social interaction. These results suggest that open field exploratory activity is a selective measure for identifying tinnitus distress in individual animals, whereas social interaction reflects the general effects of hearing loss. This animal model will facilitate future studies of the structural and functional changes in the brain pathways underlying emotional distress associated with hearing dysfunction, as well as development of novel interventions to ameliorate or prevent negative emotional responses.

Keywords: tinnitus, distress, anxiety, depression, hearing loss, sound exposure, salicylate

INTRODUCTION

Patients seeking treatment for tinnitus or hearing loss often report negative effects on lifestyle, emotions and mood, sleep, social function, and general health (Arlinger 2003, Erlandsson et al. 1992, Gates and Mills 2005, Li et al. 2014, Tambs 2004, Tyler and Baker 1983, Welch and Dawes 2008). Some patients experience very strong adverse emotional responses, including increased stress, irritability, feelings of helplessness, anxiety, or depression (Andersson and Kaldo 2004, Arlinger 2003, Davis and Rafaie 2000, Gates and Mills 2005, Jakes et al. 1985, Tyler and Baker 1983). We use the term *emotional distress* for convenience to refer to the constellation of adverse psychological states associated with tinnitus and hearing loss in both animal models and humans.

In cases of hearing loss, emotional distress may contribute to social isolation and cognitive decline (Dawes et al. 2015, Gates and Mills 2005, Mener et al. 2013). In general, greater perceived tinnitus severity is linked to greater emotional distress, but tinnitus loudness and the amount of hearing loss experienced by tinnitus patients are not always correlated with tinnitus annoyance or distress (Andersson and Vretblad 2000, Erlandsson et al. 1992, Erlandsson

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and Holgers 2001, Hallberg and Erlandsson 1993, Hesser et al. 2015, Hiller and Goebel 2006).

The prevalence of emotional distress related to tinnitus or hearing loss is difficult to estimate because people with who are not bothered by the conditions may be less likely to seek treatment. Current estimates of tinnitus-related distress range from 4–20 % of tinnitus sufferers (Andersson and Kaldo 2004, Davis and Rafaie 2000). Approximately, 11 % of adults in the USA with self-reported hearing loss have moderate to severe depression (Li et al. 2014). Despite the prevalence of emotional distress related to hearing dysfunction and the serious negative impact on quality of life, the underlying mechanisms remain poorly understood. For instance, it is unclear if the negative emotional response to tinnitus is a direct result of tinnitus, or if pre-existing personality traits or psychological factors predispose tinnitus sufferers to high levels of tinnitus-related distress. Furthermore, it is uncertain how much of tinnitus distress can be explained by hearing loss since many studies do not include tinnitus-negative and tinnitus-positive patients matched for age, gender, and hearing loss. These questions are difficult to address in human patient populations in which longitudinal data are typically unavailable and potentially confounding factors are uncontrolled.

Several studies have measured behaviors that reflect anxiety or depression in animals after exposure to conditions that can cause tinnitus and hearing loss, primarily damaging sounds or salicylate. Behavioral patterns vary across studies and tests. Two studies showed on average no effect of acoustic overexposure on elevated plus-maze behavior, a test commonly used to screen for group differences in the effects of anxiolytic and anxiogenic conditions (Pace and Zhang 2013, Zheng et al. 2011). Inspection of individual behavioral patterns in one study showed that the majority of animals with the highest anxiety scores on an elevated plus-maze test also tested positive for tinnitus, even though group differences were not statistically significant (Pace and Zhang 2013). Social interaction behavior, another common measure of anxiety in rodents, was abnormal after acoustic overexposure or repeated salicylate injections (Guitton 2009, Zheng et al. 2011). Acoustic overexposure was also associated with a slight increase in locomotion in an open field arena (Zheng et al. 2011). The effects of hearing loss associated with the manipulations to induce tinnitus were only considered in one of these studies. Tinnitus-positive rats showed more hearing loss than tinnitus-negative rats (Pace and Zhang 2013), raising the possibility that increased hearing loss could explain the observed increase in anxiety in some subjects. No studies have explicitly investigated emotional distress in animal models of hearing loss, but rodents chronically exposed to loud noise show reduced open field exploration (Naqvi et al. 2012, Wang et al. 2016).

In the present study, we evaluated three rodent "anxiety" tests as potential screens for tinnitus-related and hearing loss-related distress in a rat model. Typically, the literature refers to them as anxiety tests, but the tests have been used to model anxiety, depression, and learned helplessness. It is difficult to unambiguously differentiate these emotional states in animals. Elevated plus-maze, open field, and social interaction tests were assessed for stability over repeated test sessions in control animals. Animals exposed to emotional distress in individual animals, and a subset of animals previously screened for tinnitus was analyzed for associations between tinnitus and emotional distress.

METHODS

Subjects and Handling

A total of 72 male Sprague-Dawley rats were obtained from Harlan Laboratories, Inc. (now Envigo) when they were approximately 2 months of age (225–250 g). This strain is known to have a docile disposition (Slawecki 2005). Animals were housed socially in pairs or triplets to avoid stress from social isolation. Interactions between cage mates were frequently observed by lab personnel to ensure that they were not aggressive. Cages were kept in a quiet room to reduce potentially confounding effects of environmental noise exposure (Lauer et al. 2009). Animals were each handled for 5 min once a day for 1 week after arrival to habituate them to the experimenter and reduce acute stress from transportation and adaption to a new housing environment. Animals were randomly assigned to groups with the assumption that there would be some natural variability in baseline anxiety levels across the sample. A total of 26 unexposed controls (2 to 7 months of age), 16 rats injected with salicylate or saline (2 to 9 months of age), and 30 monaurally sound-exposed rats (2 to 10 months of age) were tested. The number of animals tested in each experiment is specified in the Results. A single experimenter performed all anxiety tests while blind to sound exposure condition, salicylate injection condition, or tinnitus status, and a second experimenter performed tinnitus screening and auditory brainstem response (ABR) measurements to prevent the rats from associating the first experimenter with anything but the anxiety tests. The experimenter who performed the anxiety tests was instructed to handle the animals as consistently and calmly as possible, to avoid wearing perfumed prod-

ucts, and to wear the same lab coat during test sessions. All procedures were approved and performed in accordance with the Guide for the Care and Use of Laboratory Animals, Johns Hopkins University Animal Care and Use Committee.

To evaluate performance stability over repeated test trials for each anxiety test, a cohort of control animals was tested multiple times with at least 2 weeks between test sessions. Cohorts of monaurally sound-exposed and salicylate-treated rats were also tested on repeated sessions to establish behavioral changes related to the induced tinnitus or hearing loss. Most of the subjects were tested behaviorally as part of other studies investigating tinnitus (e.g., Ropp et al. 2014, Jones and May 2016, Jones and May 2017, and unpublished data), and, as such, were not always tested on all conditions due to death or diversion to other experiments. Additional cohorts of control and sound-exposed rats were tested on a single trial to verify the observed differences in behavior and determine if similar patterns of behavioral differences would be evident using a shorter test protocol. Additional cohorts of salicylate-treated animals were not tested due to the complications in interpreting the observed behavioral changes described below in the Discussion. Data are reported as individual data points and, for controls tested across multiple test sessions, as averages.

Elevated Plus Maze

The elevated plus-maze measures an anima's tendency to explore open arms versus arms enclosed with sides in an elevated platform (Pellow et al. 1985). It is a well-established measure of anxiety in rats that is affected by a range of anxiolytic and anxiogenic treatments (Hogg 1996, Pellow and File 1986). The maze consisted of 50-cm long by 10-cm wide arms elevated by 50-cm high legs (Stoelting, Inc.). Two arms were enclosed with 40-cm walls on three sides. The maze was housed in a dimly lit room. At the start of a trial, the rat was placed in the center of the maze at the junction of the arms facing an open arm. Activity was recorded for 5 min using Anywaze software (Stoelting, Inc.). The experimenter was hidden from view from the subject. This curtain also served to limit extraneous visual cues. The animal was removed from the maze at the conclusion of the test and returned to the home cage. Animals were tested between the hours of 10 AM and 4 PM, using similar test times across test trials for each subject. The maze was cleaned between each test trial.

Performance was analyzed offline using the Anymaze software's automated tracking features. Percent entries into open arms, percent time spent in open arms, percent distance traveled in open arms, total number of arm entries, total time spent in arms, and total distance traveled were measured. A subset of analyses was reviewed for accuracy by a second observer.

Open Field Test

The open field test measures an animal's spontaneous exploration of a novel environment (Walsh and Cummins 1976). Anxiogenic stimuli and drugs reduce exploratory activity in an open field test arena (Prut and Belzung 2003). The animal was placed in its home cage in the dimly lit test room half an hour prior to testing to acclimate it to the environment. The test arena consisted of a 72 cm × 72 cm × 58 cm industrial plastic tub. A Fujinon YV5x2.7R4B-2 Varifocal 2.7 to 13.5 mm f/1.3 lens camera was mounted 100 cm above the center of the tub. The illumination in the arena was set at 0 lx to avoid light-induced anxiety. The experimenter was hidden from view from the subject. The animal was placed into the bottom left corner square of the arena (always the same corner for each animal) and released just as its front paws touched the floor. The animal's behavior was videotaped for 3 min using Anymaze software. At the conclusion of the test session, the animal was removed from the arena and returned to its home cage. The arena was cleaned after each test trial. Animals were tested between the hours of 10 AM and 4 PM, using similar test times across test trials for each subject.

Test sessions were analyzed offline using the Anymaze software's automatic tracking function (tracker follows the center of the body of the animal by comparing the animal's color to the background color). The number of entries, time spent, and distance traveled in the arena perimeter or in a center square area of 1296 cm^2 was calculated for each subject. A subset of analyses was reviewed for accuracy by a second observer.

Social Interaction Test

The social interaction test measures the subject's interactions with an unfamiliar, age-matched rat (File and Seth 2003). Anxiogenic stimuli and drugs reduce social interaction (File and Seth 2003). The animal was placed in the test room half an hour prior to testing to acclimate it to the environment. The tests were performed in the same arena used for the open field test, but on the following day to reduce the potential effects of experiencing a novel environment on social interaction. Animals were tested at the same times as for the open field tests. The animal was placed in a corner of the arena

along with an unfamiliar, age-matched, unmanipulated male rat. Social interactions were recorded for 20 min using Anymaze software. The experimenter was hidden from view. At the conclusion of the test session, both rats were removed from the arena and placed in their home cages. The arena was cleaned after teach test trial.

The total number of social interactions and time spent interacting were calculated automatically using Anymaze software (Stoelting). Specific behaviors that were counted included: sniffings, followings, groomings of partner, crawlings over/under partner, and wrestling. No aggressive behaviors (biting, boxing, kicking, sidling, etc.) were observed.

Salicylate Injections

Animals were briefly anesthetized with isofluorane, and then received 100, 200, 250, or 300 mg/kg sodium salicylate or similar volumes of saline-injected intraperitoneally. The animals receiving 100, 200, and 300 mg/kg of salicylate or saline were screened on the anxiety tests 1 h after injection. The animals receiving the 250 mg/kg dose were screened 2 h after injection. The first range of doses and testing time course was chosen because it is similar to other reports in the literature. However, animals tested 1 h after injection performed poorly in pilot tests of behavioral tinnitus screening. Thus, we adopted a second dose of 250 mg/kg with a longer wait period between injection and testing to be consistent with Jones and May (2016), in which these animals were able to perform the tinnitus screening task.

Sound Exposure

Rats were monaurally exposed to a 120 dB 16 kHz tone for 2 h while awake (Jones and May 2016, Ropp et al. 2014). Animals were placed in an acoustically transparent wire mesh cage 25 cm below two Pyramid Gold series (#TW57) speakers mounted from the ceiling of a small sound-attenuating chamber. The cage was rotated slowly throughout the duration of the exposure to prevent the animal from adopting positions that shielded the ear. The sound field was calibrated before and after exposure. Nine sound-exposed rats (three tinnitus-negative, six tinnitus-positive) previously screened for tinnitus using the final conditioned lick suppression procedure (Jones and May 2016, described briefly below) were tested for anxiety in the present study.

Tinnitus Screening

Nine rats were screened for tinnitus with a conditioned suppression procedure. Details of the training and testing methods have been previously described (Jones and May 2016). Briefly, the rats were trained to drink from a spout during periods of silence (safe trials) and to suppress drinking during sound presentations (warning trials). A spout in the test cage delivered water during safe trials and mild electrical shocks during warning trials. The rats were sound-exposed or treated with salicylate when they showed strong suppression for a variety of sounds that included broadband noise, narrow-bands of noise, and pure tones. Training after the tinnitus induction procedure was limited to silent safe trials and broadband noise warning trials. If induction was successful, rats heard the "sound" of their own tinnitus during silent trials, and therefore learned to associate the tinnitus percept with safe drinking. If induction was not successful, the rats failed to acquire safe sound behaviors.

The rats were tested with unreinforced pure-tone probes to establish the presence of safe sound behaviors. Tinnitus-positive rats were expected to drink when probes matched salient features of the tinnitus percept. Tinnitus-negative rats were expected to suppress drinking during all probe presentations. The lick rates elicited by each probe frequency were assigned a tinnitus score that ranged from 0 (suppression equivalent to broadband noise warning trials) to 1 (drinking equivalent to silent safe trials). Rats that produced the upper quartile of tinnitus scores were classified as tinnitus-positive outcomes. Rats with the highest tinnitus scores showed the strongest behavioral indications of tinnitus.

Auditory Brainstem Response Audiometry

Hearing was screened using ABR audiometry in monaurally sound-exposed animals as part of prior studies (Ropp et al. 2014; Jones and May 2016). The monaural sound exposure induces a 20 to 60 dB threshold shift for frequencies above 10 to 12 kHz in the exposed ear, while the unexposed ear retains normal or near-normal hearing.

Statistical Analysis

Statistical analysis was performed using SigmaStat software. To determine the consistency of various test measures across test sessions, one-way analysis of variance tests (ANOVAs) were performed on data from animal tested in multiple test sessions to test for significant main effects. In cases where the data were not normally distributed or showed unequal variances, nonparametric Kruskal-Wallis tests were performed to test for statistically significant main effects. Post hoc comparisons were performed using two-tailed Holm-Sidak tests to identify statistically significant specific comparisons if the main effects were

significant. In cases where the data were not normally distributed or showed unequal variances, Mann-Whitney U tests were used for pairwise comparisons performed independently of Krusal-Wallis tests. *T* tests or Mann-Whitney U tests were also used to test for specific group differences. Pearson's product moment correlation analyses were used to test for relationships between tinnitus and anxiety measures or age and anxiety measures. Main effects were considered statistically significant if $p < 0.05$ (two-tailed). Data are shown as individual points to highlight the range of phenotypes of observed, with a line depicting the average for control animals.

RESULTS

Elevated Plus Maze

Controls

Elevated plus performance measured across repeated test sessions is shown for individual control rats ($n = 6$), 2 to 5 months of age at the time of testing, and averaged across rats in Fig. 1. Four rats were tested for three sessions and two rats were tested for two sessions. Greater activity in open arms is typically interpreted as lower anxiety. Previous elevated plus-maze studies variably report entries, time spent, or distance traveled in open arms, so all three measures of anxiety are considered here. Total number of entries for both closed and open arms, total time spent in arms, and total distance traveled in arms are used as measures of overall locomotion/exploration. Percent entries into open arms (Fig. 1a), percent time spent in open arms (Fig. 1b), and percent distance traveled in open arms (Fig. 1c) decreased substantially in four rats after the first test session. Two rats showed no open arm activity in the first test session. Paired *t* tests performed on the data from T1 and T2 identified no statistically significant differences in percent entries into open arms [$t(5) = 1.563$, $p = 0.179$, $d = 0.670$], percent time spent in open arms [$t(5) = 2.191$, $p = 0.08$, $d = 0.964$], or percent distance traveled in open arms [$t(5) = 3.794$, $p = 0.109$, $d = 0.841$]. The lack of significance was presumably due to the two subjects that showed no open arm activity across all three trials, underscoring the importance of evaluating individual behavioral phenotypes in addition to group differences. The reduced open arm activity could not be explained by reduced locomotor activity, since total number of arm entries (Fig. 1d), total time spent in arms (Fig. 1e), and total distance traveled in arms (Fig. 1f) were either unchanged or slightly increased across trials. Paired *t* tests showed no statistically significant differences in the total number of arm entries [$t(5) = -0.131$,

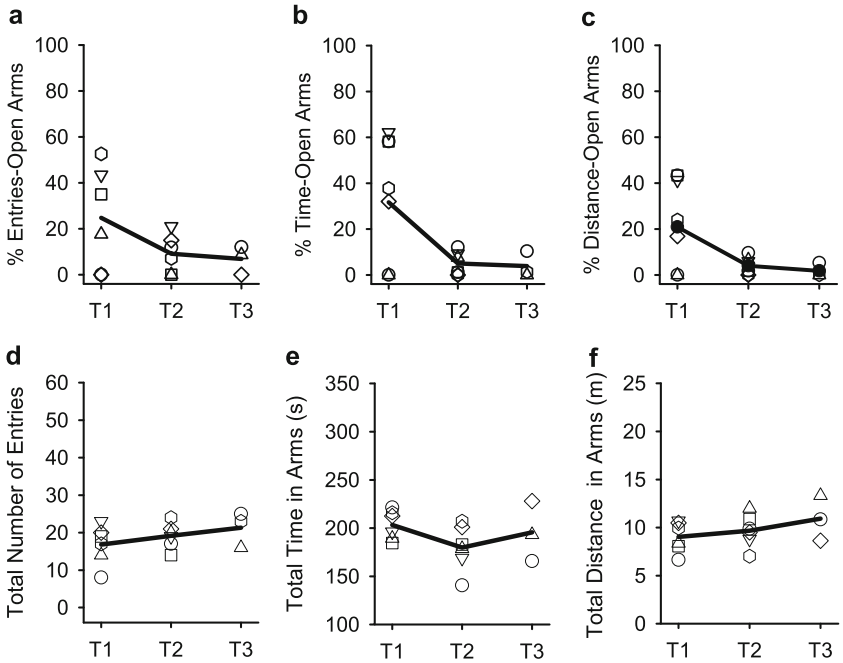

Fig. 1. Elevated plus-maze activity in the open arms decreases when measured across repeated test sessions (T1, T2, T3) in control rats. Percent entries (**a**), percent time spent (**b**), and percent distance traveled in the open arms (**c**) were highest in T1 for all but two rats, which showed no open arm activity in T1. Locomotor activity indicated by total number of open plus closed arm entries (**d**), total time spent in arms (**e**), and total distance traveled in arms (**f**) were constant or increased slightly across test sessions. Data are shown for individual subjects (open symbols) and the average across subjects (thick line). Different symbol shapes correspond to different individual rats

p = 0.901, d = 0.054], total time spent in arms [$t(5)$ = 0.435, p = 0.682, d = 0.217], or total distance traveled in arms [$t(5)$ = − 0.345, p = 0.744, d = − 0.141]. We determined that the elevated plus maze would not be sensitive to detecting behavioral changes between pre- versus post-sound exposure or before versus during salicylate treatment due to floor effects. No further experiments were conducted using this test.

Open Field Test

Controls

Open field test performance was measured across repeated test sessions is shown for individual control rats (n = 7), 2 to 7 months of age at the time of testing, and averaged across rats in Fig. 2. Two rats were tested for three sessions and five rats were tested for four sessions. Previous open field studies variably report area entries, time spent, or distance traveled, so all three measures of anxiety are considered here. Total distance traveled is used as a measure of overall locomotion. Percent of entries into the center of the test arena (Fig. 2a), percent time spent in the center (Fig. 2b), and percent of distance traveled in the center (Fig. 2c), and total distance traveled (Fig. 2d) were consistent across test sessions in control rats. One-way repeated measures ANOVAs revealed no statistically significant differences across test sessions for percent entries into the center [F(3, 6) = 0.837, p = 0.493, η^2 = 0.083], percent time spent in the center [F(3,6) = 0.486, p = 0.697, η^2 = .053], percent distance traveled in the center [F(3,6) = 0.666, p = 0.585, η^2 = 0.055], or total distance traveled [F(3,6) = 2.481, p = 0.098, η^2 = 0.196]. These results demonstrate the stability of the test over multiple sessions.

Sound-exposed

Pre- and post-exposure open field performance was measured in 18 monaurally sound-exposed rats, nine of which were previously screened for tinnitus using a conditioned lick suppression paradigm (Jones and May 2016). Four rats were tested twice following noise exposure to assess the stability of the behavioral changes. A range of anxiety phenotypes was observed, indicated by different patterns of percent center entries before and after noise exposure (Fig. 3a). Two rats (11 %) showed no center entries prior to sound exposure, but only one of these rats retained this high-anxiety phenotype post-exposure. Two rats (11 %) showed a change from normal percent center entries pre-exposure to no percent entries post-exposure, indicating a change from a low anxiety to

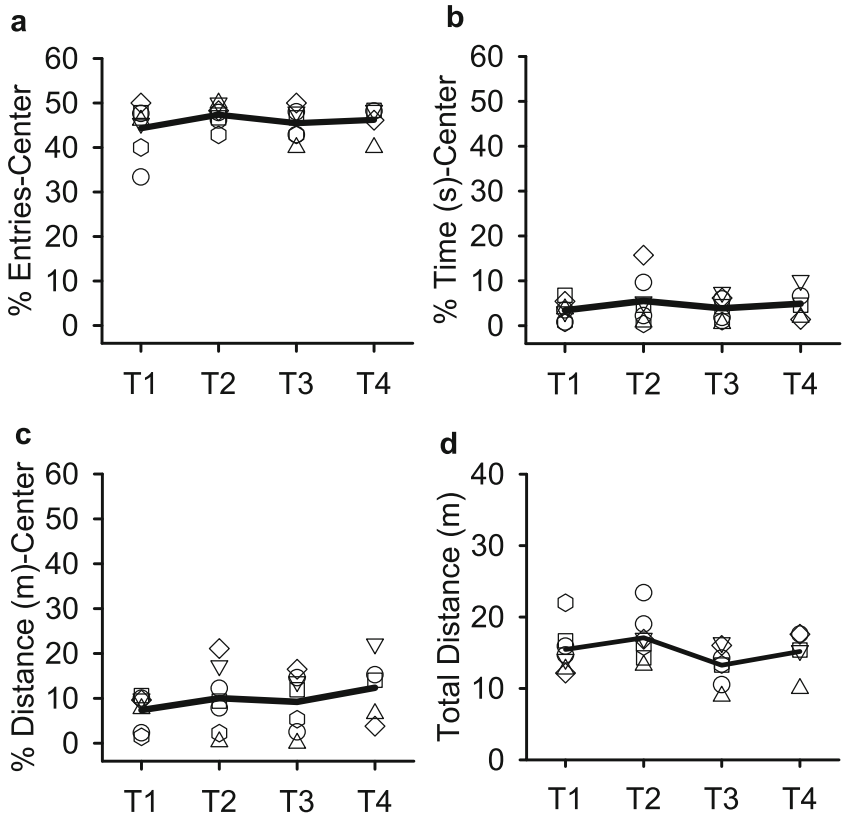

Fig. 2. Open field behavior is consistent across multiple test sessions in control rats. Percent center entries (**a**), percent time spent in the center (**b**), percent distance traveled in the center (**c**), and total distance traveled (**d**) are shown for individual subjects (open symbols) and the average across subjects (thick line). Different symbol shapes correspond to different individual rats

a high-anxiety phenotype. Three rats (17 %) showed slightly reduced percent entries post-exposure compared to pre-exposure, indicating a moderate increase in anxiety. The remaining 11 rats (61 %) showed no change between pre- and post-exposure test sessions. Percent time spent in the center (Fig. 3b) and percent distance traveled in center (Fig. 3c) were somewhat reduced in sound-exposed animals, but percent time spent and distanced traveled in the center was low overall even during pre-exposure test sessions. This indicates that the animals tended to pass through the center quickly regardless of hearing status. Total distance traveled was reduced in many rats after noise exposure (Fig. 3d). One-way repeated measures ANOVAs performed on the data from the two pre-exposure test sessions and the first post-exposure test session revealed statistically significant main effects of percent time spent in the center [$F(2,17) = 4.256$, $p = 0.022$, $\eta^2 = 0.084$] and total distance traveled [$F(2, 17) = 8.347$, $p = 0.001$, $\eta^2 = 0.168$]. There were no statistically significant effects of percent center entries [$F(2,17) = 2.764$, $p = 0.077$, $\eta^2 = .131$] or percent distance traveled in the center [$F(2,17) = 2.888$, $p = 0.069$, $\eta^2 = 0.096$]. Post hoc comparisons identified significant differences in Pre1 and Post1 percent time in center [$p = 0.048$], Pre2 and Post1 percent time in center [$p = 0.034$], Pre1 and Post1 total distance traveled [$p = 0.008$] and Pre2 and Post 1 distance traveled [$p = 0.001$] test sessions. Data from the second post-exposure test session was not included in the statistical analysis because only four subjects were tested twice after sound exposure. Rats were 2 to 3 months of age during pre-exposure testing and 4 to 10 months of age during Post1. Time since exposure was between 3 weeks and 1 month. Age correlations are described below for a larger sample size of sound-exposed animals.

Correlations between pre- and post-exposure behavior and tinnitus score were investigated in the nine animals previously screened for tinnitus to identify whether or not pre-existing anxiety levels predicted tinnitus behavior, and whether tinnitus behavior was predictive of anxiety. Pre2 and Post1 test sessions were used for this analysis. Pre- and post-exposure percent time in the center [$r(9) = 0.434$, $p = 0.243$] and total distance traveled [$r(9) = 0.613$, $p = 0.0795$] were not correlated in animals screened for tinnitus, indicating that pre-existing anxiety levels did not predict performance on the tinnitus behavior. Pre-exposure [$r(9) = 0.254$, $p = 0.510$] and post-exposure [$r(9) = -0.209$, $p = 0.590$] percent time in the center were not significantly correlated with tinnitus score.

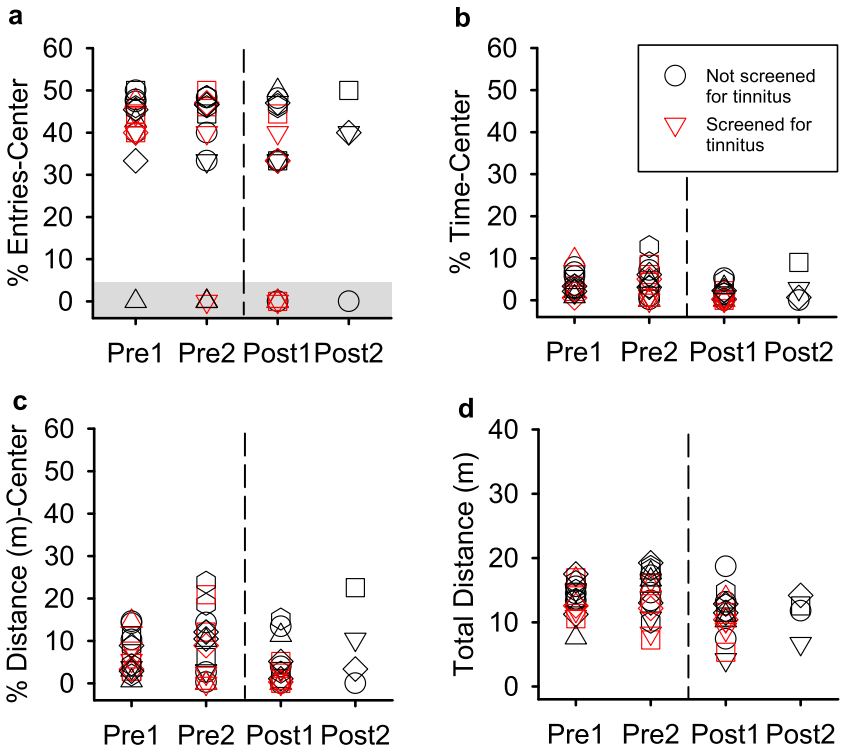

Fig. 3. Open field behavior measured across test sessions in rats prior (Pre1, Pre2) to and after (Post1, Post2) damaging monaural sound exposure revealed a range of anxiety phenotypes. Percent center entries were low in some rats prior to or after sound exposure (**a**). Percent time spent in the center was reduced overall (**b**), percent distance traveled in the center was unchanged (**c**), and total distance traveled was reduced overall (**d**) after sound exposure. Data are shown for individual subjects screened for tinnitus (red symbols) and not screened for tinnitus (black symbols). The gray area in **a** highlights subjects with extremely high-anxiety phenotypes. Different symbol shapes correspond to different individual rats

Pre-exposure total distance traveled was not correlated with tinnitus score in the nine animals screened for tinnitus [$r(9) = -0.540$, $p = 0.133$], but post-exposure distance traveled was negatively correlated with tinnitus score [$r(9) = -0.902$, $p = 0.000886$].

A second cohort of control ($n = 19$) and monaurally sound-exposed ($n = 12$) rats were tested in a single session to determine the repeatability of the phenotypic differences observed in the first cohort, and to determine the potential for using a shorter test protocol to identify the effects of sound exposure on anxiety and locomotor activity. A shorter test protocol may be desirable in cases where animals are being tested daily on a conditioned task in order to induce minimal disruptions to the conditioned behavioral paradigm. Percent entries into the center (Fig. 4a) showed similar patterns to the first cohort of sound-exposed rats, with several rats showing no center entries at all, several others showing reduced percent center entries compared to control, and others showing control-like percent entries. Also similar to the first cohorts of sound-exposed and control rats, percent time spent (Fig. 4b) and percent distance traveled in the center (Fig. 4c) in the center were low overall, and total distance traveled was lower in sound-exposed rats. Mann-Whitney U tests were used to test for differences between control and sound-exposed rats because the normality or equal variance tests failed for each comparison. Percent entries into the center [$U = 74.50$, $p = 0.111$], percent time spent in the center [$U = 92.0$, $p = 0.383$], and percent distance traveled in the center [$U = 103.0$, $p = 0.670$] were not statistically different between control and sound-exposed rats. Total distance traveled was significantly lower in sound-exposed rats compared to controls [$U = 30.0$, $p < 0.001$]. These behavioral differences are similar to the differences in pre- and post-exposure behavior observed in the first cohort of sound-exposed mice.

The possibility that age or time since sound exposure affected performance or response to sound exposure was investigated by pooling data from all sound-exposed rats (post-exposure). Percent distance traveled in the center was moderately correlated with age [$r(= -0.406$, $p = 0.0259$), but not with percent center entries [$r(30) = -0.0681$, $p = 0.721$], percent time spent in center [$r(30) = -0.335$, $p = 0.0704$], or total distance traveled [$r(30) = -0.324$, $p = 0.0811$].

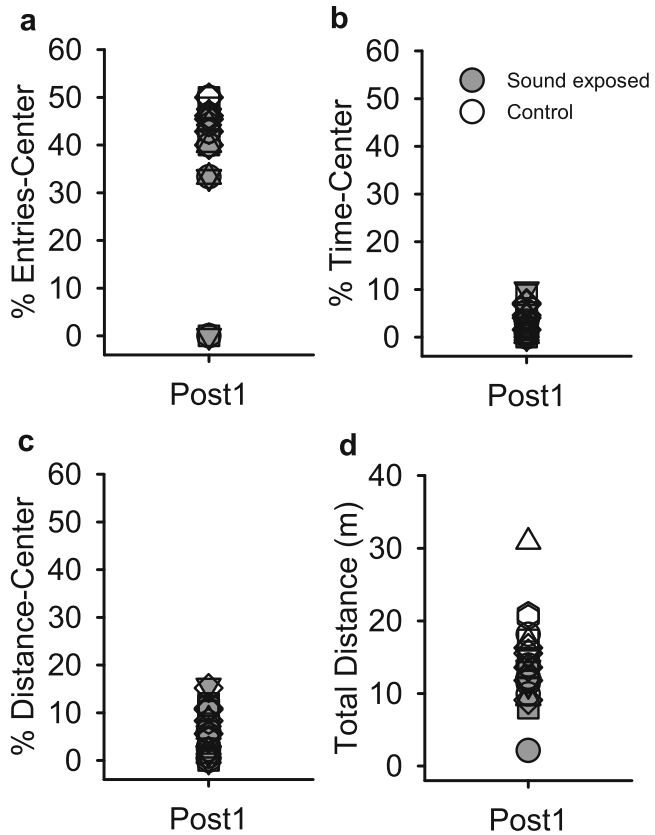

Fig. 4. Open field behavior measured in one test session in a second cohort of control rats (open black symbols) and in monaurally sound-exposed rats (filled gray symbols) is consistent with results from the first cohorts. Percent entries into the center (**a**), percent time spent in center (**a**), percent distance traveled in center (**c**), and total distance traveled (**d**) are shown for individual subjects. Different symbol shapes correspond to different individual rats

Percent center entries [$r(30) = -0.0723$, $p = 0.704$], percent time in the center [$r(30) = -0.293$, $p = 0.116$], percent distance traveled in center [$r(30) = -0.313$, $p = 0.092$], and total distance traveled [$r(30) = -0.359$, $p = 0.0511$] were not significantly correlated with time since sound exposure.

Salicylate

Open field test performance in rats tested before (Pre), during (SS/saline), and after (Post) injections of salicylate or saline in Fig. 5. Three rats received 200 mg/kg salicylate and then were tested again later with a lower dose of 100 mg/kg; seven rats received 200 mg/kg; two received 250 mg/kg; two received 300 mg/kg. Five animals (three receiving 200 mg/kg and two receiving 250 mg/kg) were only tested on the Pre and SS/saline conditions before being used in other experiments. Animals were 2 to 9 months of age at test. As with sound exposure, salicylate and saline injections resulted in a range of anxiety phenotypes indicated by percent entries into the center (Fig. 5a). One rat (7 %) showed no center entries prior to treatment with salicylate. In six rats (42 %), center entries were reduced to zero during salicylate injections. Three rats (21 %) showed moderate decreases in center entries, while four rats (29 %) showed no change. In one animal that received saline, center entries were reduced to zero, while the other's behavior remained normal. Percent time spent in center (Fig. 5b) was low in all subjects in conditions, but showed decreases during the salicylate treatments.

Percent distance traveled in the center was decreased in some rats and remained unchanged or increased in others (Fig. 5c). Total distance traveled decreased with both salicylate and saline injections

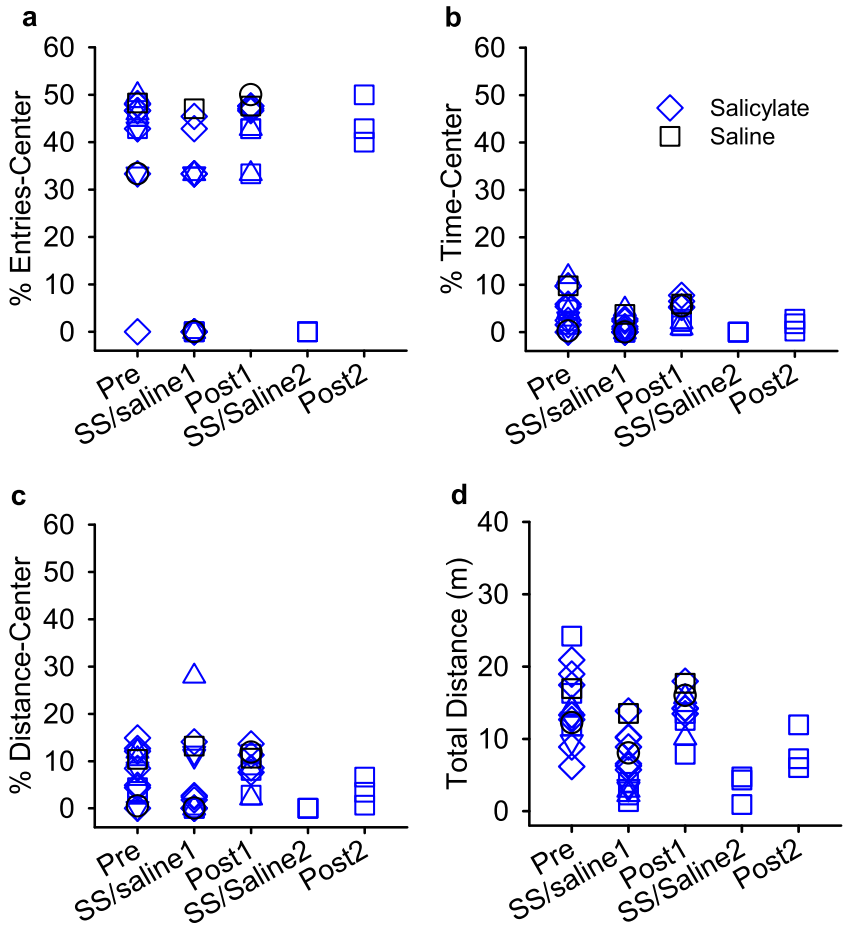

Fig. 5. Open field behavior measured across test sessions in rats prior to (Pre1), during (SS/saline1, SS/saline2), and after (Post1, Post2) injections of 100 to 300 mg/kg sodium salicylate (SS) (blue symbols) or saline (black symbols). Squares—rats tested with 200 mg/kg and again with 100 mg/kg salicylate; triangles—300 mg/kg; inverted triangles—250 mg/kg; diamonds—200 mg/kg; circles—saline. All three rats tested with 100 mg/kg salicylate showed no center entries; therefore, the points overlap and appear as one symbol in (**a**–**c**). Percent entries into the center were reduced in some rats during treatment (**a**). Percent time spent in center was reduced overall (**b**), percent distance traveled in center was reduced in some rats (**c**), and total distance traveled was reduced overall (**d**) during treatment. Data are shown for individual subjects. Different symbol shapes correspond to different individual rats

(Fig. 5d). One-way repeated analysis of variance performed on the Pre, SS/saline, and post-test session data revealed significant effects of percent entries into the center [$F(2, 18) = 15.161$, $p < 0.001$, $\eta^2 = 0.342$], percent time spent in the center [$F(2,18) = 7.054$, 0.004, $\eta^2 = 0.272$], and total distance traveled [$F(2,18) = 15.747$, $p < 0.001$, $\eta^2 = 0.403$]. The main effect of percent distance traveled in the center was not significant [$F(2,18) = 2.342$, $p = 0.117$, $\eta^2 = 0.099$]. Post hoc analysis showed that Pre versus SS/salicylate test sessions were significantly different for percent center entries ($p < 0.001$), percent time spent in the center ($t = 3.309$, $p = 0.009$), and total distance traveled ($p < 0.001$). Post versus SS/salicylate test sessions were also significantly different for percent center entries ($p < 0.001$), percent time spent in the center ($p = 0.011$), and total difference traveled ($p < 0.001$). Salicylate dose was not correlated with percent center entries [$r(19) = 0.123$, $p = 0.617$], percent time in center [$r(19)$=0. 0852, $p = 0.729$], percent distance traveled in center [$r(19) = 0.268$, $p = 0.268$], or total distance traveled [$r(19) = -0$ .355, $p = 0.136$]. Age at time of injection was correlated with total distance traveled [$r(19) = -0.589$, $p = 0.008$], but not percent center entries [$r(19) = -0.261$, $p = 0.281$], percent time in center [$r(19) = -00.326$, $p = 0.173$], or percent distance traveled in center [$r(19) = -0.105$, $p = 0.669$]. Younger animals tended to show more locomotor activity during the injection test sessions.

Social Interaction

Controls

Individual control rats aged 2–7 months ($n = 9$) showed consistent numbers of sniffing, following, crawling over, grooming, and wrestling (playing, never biting) with their social partners across four test sessions, but the number of instances of each specific behavior varied across animals. For this reason, specific social interaction behaviors were totaled, and this total was more consistent across animals (Fig. 6a). A one-way repeated ANOVA showed no statistically significant effect of test session on the number of interactions [$F(3, 8) = 0.496$, $p = 0.689$, $\eta^2 = 0.048$]. Aggressive and escape behaviors were rarely observed. These results demonstrate the stability of the test over multiple sessions.

Sound-exposed

Pre- and post-exposure social interaction behavior was measured in 18 rats, 9 of which were previously screened for tinnitus (Fig. 6b). Four rats were tested twice following noise exposure to assess the stability of the behavioral differences. Animals were 2 to 10 months of age at test. Social interaction was reduced after noise exposure compared to pre-exposure levels, and a one-way ANOVA two pre-exposure test sessions and the first post-exposure test session found a significant main effect of test session [$F(2, 17) = 19.335$, $p < 0.001$, $\eta^2 = 0.327$]. Post hoc analysis revealed significant differences between Pre1 and Post1 ($p < 0.001$), Pre2 and Post1 ($p = 0.004$), and Pre1 and Pre 2 ($p = 0.007$). Tinnitus score was not significantly correlated with the number of social interactions before [$r(9) = 0.00462$, $p = 0.991$] or after [$r(9) = -0.196$, $p = 0.614$] sound exposure.

The significant decrease in social interaction observed between the two pre-exposure test sessions suggested that the additional decrease in the first post-exposure session might be due to habituation to the test, as observed in one of the control animals (Fig. 6a). To determine whether the effect of sound exposure was repeatable in rats that were not subject to repeated testing experience, and to evaluate the efficacy of a shorter test protocol, additional cohorts of control ($n = 15$) and sound-exposed ($n = 11$) rats were tested once (Fig. 6c). The number of social interactions was lower in sound-exposed animals compared to controls ($U = 0.500$, $p < 0.001$), indicating that reduced social interaction in the first cohort was due to the effects of sound exposure and not habituation to repeated testing. Post-exposure data from the first sound-exposed cohort was pooled with data from the second cohort to test for correlations between age at test [$r(29) = 0.0589$, $p = 0.762$] or months since exposure [$r(29) = -0.0411$, $p = 0.833$] and social interaction performance, and neither correlation was significant.

Salicylate

Seven rats aged 2 to 9 months were tested on social interaction before and during salicylate treatment (Fig. 6d). Three rats received 200 mg/kg salicylate 1 h before testing, two received 100 mg/kg 1 h before testing, and two received 250 mg/kg 2 h before testing. Rats often showed signs of disorientation or immobility when tested on social interaction the day after open field testing, requiring two injection days, so testing was limited to a small cohort of subjects. Observation of the videos indicated that most salicylate-injected rats stayed in the corner in which they were released. At this point, it was determined that salicylate injections are not suitable for emotional distress tests due to reasons described in the Discussion. Social interaction significantly decreased in injected animals compared to pre-injection [$t(6) = 7.994$, $p < 0.001$, $d = 3.336$]. Performance during salicylate treatment [$r(7) = -0.306$, $p = 0.505$] was not correlated with age.

Several studies in humans have attempted to determine the relationship between pre-existing per-

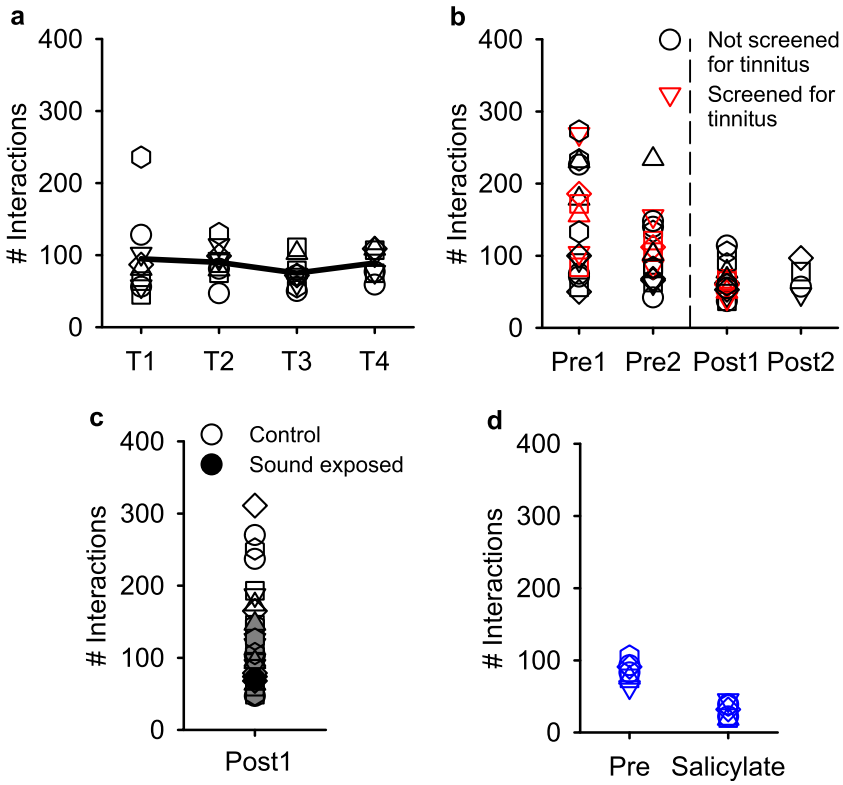

Fig. 6. The number of social interactions was consistent measured across repeated test sessions in control rats (**a**). Social interactions decreased after sound exposure compared to pre-exposure test sessions (**b**). Red symbols in **b** indicate animals previously screened for tinnitus, and black symbols indicate animals not screened for tinnitus. Social interactions were also decreased in a second cohort of sound-exposed rats (filled gray symbols) compared to a second cohort of controls (open black symbols) (**c**). Sodium salicylate-treated rats (blue symbols) also showed reduced social interaction compared to before treatment (**d**). Different symbol shapes correspond to different individual rats

sonality or psychological traits and tinnitus (Pattyn et al. 2016). Thus, we examined the pre-test behavioral data for tinnitus-positive and tinnitus-negative to determine if pre-existing anxiety levels were different in animals that eventually develop tinnitus. Mann-Whitney U tests found no significant differences between pre-test behavior in tinnitus-positive compared to tinnitus-negative rats.

DISCUSSION

We have demonstrated that sound exposure and salicylate produce behaviors consistent with emotional distress. Control rats showed a range of elevated plus-maze activity in the first test session, including performance consistent with high, moderate, and low emotional distress. Open arm activity in the second test session was substantially reduced, which could be interpreted as high anxiety, habituation to the test, or loss of novelty seeking (Carobrez and Bertoglio 2005). These findings suggest that the elevated plus maze may only be a useful indicator of individual emotional phenotypes when using a single trial in rat models of tinnitus and hearing loss. In contrast, open field and social interaction performance were consistent across sessions in control rats, indicating that these tests are appropriate for identifying changes in behavior in individual animals exposed to manipulations that induce tinnitus and hearing loss. The results of the present study also show that the open field test provides a selective measure of tinnitus-related emotional distress, and social interaction behavior reflects emotional distress associated with hearing loss. Previous studies have identified different patterns of open field and social interaction activity in juvenile or senescent rats compared to young adult and middle age rats (Altun et al. 2007, Gage et al. 1984, Salchner et al. 2004, Soffié and Bronchart 1988, Valle 1970), underscoring that age is important when interpreting the results of these tests. The results of our experiments in adult control rats did not depend on age, but age effects may differ across strains and species.

Tinnitus-related Distress

A subset of sound-exposed and salicylate-treated rats showed phenotypes consistent with high emotional

distress, indicated by no entries into the center of an open field. Several other rats showed slightly reduced center entries, indicating a milder distress phenotype. Among animals screened for tinnitus, about half of the animals showed moderate or high distress, but percent entries to the center of the open field was not correlated with tinnitus score. This result is consistent with reports in humans that only a subset of patients with tinnitus find it distressing and that tinnitus loudness is not necessarily correlated with perceived tinnitus severity (Andersson and Vretblad 2000, Budd and Pugh 1995, Dineen et al. 1997, Erlandsson et al. 1992, Erlandsson and Holgers 2001, Hallberg and Erlandsson 1993, Hesser et al. 2015, Hiller and Goebel 2006). Though salicylate-treated rats were not screened for tinnitus in the present study due to the limited effective time period of single doses of salicylate, previous studies have demonstrated behavioral evidence of tinnitus in rats treated with salicylate (Jones and May 2016, Lobarinas et al. 2004, Yang et al. 2007). We presume that the animals from the present study also experienced tinnitus during the anxiety screening. Interestingly, two salicylate-treated rats continued to show decreased open field center entries in the recovery test session, indicating that the experience of temporary tinnitus had lasting effects on emotionality. Other rats showed little to no effect of salicylate treatment or sound exposure on center entries, whereas several rats showed high distress prior to tinnitus-inducing manipulations. The varying phenotypes across individual animals may explain why a previous study found no significant differences in open field exploration when performance was averaged across subjects (Zheng et al. 2011). Our results demonstrate that the open field test can be used to identify individual subjects with low, moderate, and high distress, and also to test the effects of pre-existing distress levels on tinnitus outcomes.

The behavioral phenotype observed in salicylate-treated animals must be interpreted with caution. First, it is not possible to separate the effects of salicylate-induced tinnitus from effects on cochlear function on emotional behaviors (Chen et al. 2013). Second, salicylate is associated with side effects such as gastrointestinal distress, fatigue, and headache in humans. The high doses of salicylate used in the present study may have made the animals feel too nauseous or tired to initiate exploratory and social behaviors, although dosage was not correlated with performance. Third, one of the saline-injected animals and all three animals injected with the low salicylate dose showed high-anxiety phenotypes, indicating that the injection procedure itself may have induced lasting stress despite the 1–2 h wait period. Additionally, salicylate-mediated effects were correlated with the age of the animals, indicating an interaction between age and emotionality. While the salicylate manipulations provide a useful step in validating the sensitivity of emotional tests to manipulations that affect auditory function, the results cannot be unequivocally interpreted as reflecting distress that is specifically associated with tinnitus hearing loss, or the injection. For experiments intending to investigate causal mechanisms of emotional distress, sound exposure may be the preferable method for inducing hearing dysfunction.

Our experiments suggest that tinnitus distress can arise as a result of tinnitus in some rats. However, our findings do not preclude the possibility that increased negative affect at the time of exposure to tinnitus-inducing conditions may increase the likelihood of developing tinnitus or result in more severe tinnitus. Future experiments using larger sample sizes and manipulating the pre-existing behavioral state of the animals prior to tinnitus induction can address this issue. Intriguingly, tinnitus scores were correlated with reduced distance traveled in the open field. Since animals with higher tinnitus scores, indicating a positive tinnitus phenotype, do not have significantly worse hearing than animals with lower tinnitus scores (Jones and May 2016), this effect is likely related to the tinnitus itself. Reductions in open field exploration also occur in rats exposed to chronic or inescapable stress (Katz et al. 1981; Rygula et al. 2005; Van Dijken et al. 1992). Thus, the present results may indicate that tinnitus is experienced as a chronic, inescapable stressor in some rats.

Hearing Loss-related Distress

Our experiments indicate that there are negative emotional responses that are specific to tinnitus and responses related to the general effects of hearing loss. The number of social interactions was not correlated with tinnitus score, but reduced social interaction was observed in nearly all animals sound-exposed and salicylate-treated rats. We interpret reduced social interaction as reflecting the general effects of hearing loss, whereas reduced open field exploration is specifically related to tinnitus distress experienced by a subset of animals. In contrast to previous studies in mice and Wistar rats (Guitton 2009, Zheng et al. 2011), salicylate-treated and sound-exposed Sprague-Dawley rats did not show increased aggressive interactions in the present study, possibly indicating species and strain differences in the emotional response to manipulations that induce hearing dysfunction. These differences may be exploited in future studies to explore genetic or dispositional contributions to the emotional outcomes of hearing dysfunction.

Interestingly, reduced social interaction has been hypothesized to contribute to the increased cognitive decline observed in aging adults with hearing loss (Lin et al. 2013). Our animal model of reduced social interaction associated with hearing loss facilitates controlled studies of the causative mechanisms underlying this relationship. Reduced social interaction in rodents is associated with numerous anxiogenic conditions (File and Seth 2003). Hearing loss may reduce social interaction simply because acoustic communication is impaired when one rat in a pair cannot hear the other rat's vocalizations. However, the rats exposed to monaural sound in the present study maintained normal hearing in one ear. While monaural hearing loss may compromise localization of vocalizations, the animals were confined to a small arena where they were within no more than ~ 90 cm from each other. More likely, changes to limbic structures associated with acoustic overexposure contributed to increased distress (Kraus et al. 2010, Kraus and Canlon 2012).

Potential Mechanisms

Emotional distress related to tinnitus and hearing loss may be due to changes in limbic networks and neuromodulatory pathways that are associated with mood disorders. Hyperactivity in limbic and cerebellar networks is implicated in tinnitus (Bauer et al. 2013, Chen et al. 2015, Husain and Schmidt 2014, Leaver et al. 2011, Rauschecker et al. 2010). Animals with lesions to components of these networks show increased open field activity, suggesting that they normally inhibit open field exploration (Schwartzbaum and Gay 1966, Supple et al. 1987). From this, one would predict that excessive activation of these circuits in tinnitus-positive animals would reduce open field exploration as observed in the present study.

Hippocampal dysfunction related to sound exposure or salicylate treatment could also contribute to reduced locomotion (Deacon et al. 2002a, Deacon et al. 2002b). Damaging sound exposure reduces neurogenesis, induces cell death, and alters neural firing patterns in hippocampus (Goble et al. 2009, Kraus et al. 2010, Liu et al. 2016, Säljö et al. 2011). Chronic noise stress alters hippocampal dendrite morphology in (Manikandan et al. 2006), and environmental noise exposure can induce morphological changes (Cheng et al. 2016). Changes to neuromodulatory systems implicated in anxiety and depression may in turn exacerbate hippocampal dysfunction.

Psychomotor deficits and reduced exploratory behavior are associated with anxiety, depression, and serotonergic dysfunction (Caligiuri and Ellwanger 2000, Geyer 1995, Gould et al. 2009). Conversely, dorsal and median raphe lesions increase open field activity (Asin et al. 1979, Jacobs and Cohen 1976), and serotonergic neurons form substantial connections with auditory brain regions (Fitzpatrick et al. 1989, Hurley et al. 2002, Klepper and Herbert 1991, Papesh and Hurley 2012, Willard et al. 1984). These pathways have been hypothesized to play a role in tinnitus and hyperacusis (Marriage and Barnes 1995, Norena et al. 1999, Simpson and Davies 2000), and both sound exposure and stress alter serotonergic projections to the inferior colliculus (Hall et al. 2010, Hall et al. 2012, Papesh and Hurley 2012). Serotonergic pathways also interact considerably with extra-auditory pathways that have been implicated in tinnitus, and could conceivably influence emotional distress through modulation of these pathways (Leaver et al. 2011, Rauschecker et al. 2010). Dopaminergic and noradrenergic pathways may also modulate these pathways (Rauschecker et al. 2010), and both are known to affect open field activity (Fink and Smith 1980, Svensson and Ahlenius 1983). Social behavior is also linked with serotonergic modulation in the inferior colliculus (Hall et al. 2011). Thus, social interaction and locomotor behavior in sound-exposed animals may be useful for probing the relationship between cognitive decline, hearing loss, and social function.

CONCLUSIONS

We demonstrated emotional distress related to tinnitus and hearing loss in a rat model. These results provide a foundation for studies investigating more refined questions about the behavioral and neurophysiological relationship between tinnitus, hearing loss, and emotional distress. These assays will also be useful in preclinical screening of emerging therapies.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest The authors declare that they have no conflicts of interest.

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