Animal Model of Sensorineural Hearing Loss Associated with Lassa Virus Infection

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ABSTRACT
Approximately one-third of Lassa virus (LASV)-infected patients develop sensorineural hearing loss (SNHL) in the late stages of acute disease or in early convalescence. With 500,000 annual cases of Lassa fever (LF), LASV is a major cause of hearing loss in regions of West Africa where LF is endemic. To date, no animal models exist that depict the human pathology of LF with associated hearing loss. Here, we aimed to develop an animal model to study LASV-induced hearing loss using human isolates from a 2012 Sierra Leone outbreak. We have recently established a murine model for LF that closely mimics many features of human disease. In this model, LASV isolated from a lethal human case was highly virulent, while the virus isolated from a nonlethal case elicited mostly mild disease with moderate mortality. More importantly, both viruses were able to induce SNHL in surviving animals. However, utilization of the nonlethal, human LASV isolate allowed us to consistently produce large numbers of survivors with hearing loss. Surviving mice developed permanent hearing loss associated with mild damage to the cochlear hair cells and, strikingly, significant degeneration of the spiral ganglion cells of the auditory nerve. Therefore, the pathological changes in the inner ear of the mice with SNHL supported the phenotypic loss of hearing and provided further insights into the mechanistic cause of LF-associated hearing loss.

IMPORANCE
Sensorineural hearing loss is a major complication for LF survivors. The development of a small-animal model of LASV infection that replicates hearing loss and the clinical and pathological features of LF will significantly increase knowledge of pathogenesis and vaccine studies. In addition, such a model will permit detailed characterization of the hearing loss mechanism and allow for the development of appropriate diagnostic approaches and medical care for LF patients with hearing impairment.

Lassa fever (LF), caused by Lassa virus (LASV), is a major public health threat in certain parts of Africa and is characterized by acute hemorrhagic disease and neurological complications (sequela). Up to 500,000 infections are reported annually, and mortality rates for hospitalized patients can be up to 25% (1, 2). In severe cases, the condition of the patient deteriorates rapidly, with severe pulmonary edema, acute respiratory distress, clinical signs of encephalopathy (sometimes with coma and seizures), and terminal shock (3). Sudden sensorineural hearing loss (SNHL) is commonly observed in patients and occurs in the late stages of disease or in early convalescence in survivors (4). Approximately one-third of patients develop an acute hearing impairment upon release from the hospital, and approximately two-thirds of these subjects are left with some degree of permanent hearing loss (4, 5).

Hearing loss is not an uncommon complication of viral infections. Virus-induced hearing loss can be the result of a congenital and/or acquired infection and can be uni- or bilateral (6). Lymphocytic choriomeningitis virus (LCMV), the prototypic arenavirus, has been associated with congenital hearing loss (7, 8), while viruses such as herpesvirus, HIV, measles, and mumps have been associated with hearing loss after acquired infection.

Regardless of the route of initial infection, there are three suggested mechanisms of infection-mediated hearing loss: immune mediated (congenital cytomegalovirus infection) (9, 10), direct viral damage to the inner ear (acquired measles and mumps and congenital or acquired herpes), or a combination of both (congenital Rubella and congenital or acquired HIV) (11). LCMV-induced hearing deficiency has been replicated in a rat model and measured using the acoustic startle response methodology. Newborn rats infected intracerebrally had higher elicitation and inhibition thresholds than control animals (12). However, the mechanisms of hearing loss associated with viral infections still remain largely elusive (6, 8, 12–15).

Previously, we published that mice lacking a functional STAT1 pathway (Stat1−/−) are highly susceptible to infection with LASSV and develop lethal disease with pathology similar to that reported for humans (16). With these findings and knowledge of LCMV-

related hearing loss studies (12), we aimed to develop a reproducible murine model of hearing loss resulting from LASV infection.

In this study, we report for the first time that two LASV isolates obtained from lethal and nonlethal human cases during the 2012 LF outbreak in Sierra Leone induce SNHL in surviving Stat1−/− mice. The SNHL is associated with pathological changes in the inner ear and auditory nerve, which, to our knowledge, has not been demonstrated to date in any other animal model of LF.

**MATERIALS AND METHODS**

**Cells, viruses, and biosafety.** Vero cells (American Type Culture Collection) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1% penicillin-streptomycin, and l-glutamine. LASV isolates were obtained directly from serum samples from fatal LV2384-NS-DIA-1 (LV2384) and nonfatal LV2350-NS-DIA-1 (LV2350) LF cases that were collected during a 2012 outbreak in Sierra Leone. Viral working stocks were generated by infecting Vero cells and collecting virus-containing cell culture supernatant (TCS) at 96 h postinfection (p.i.). All work with infectious LASV was performed in the University of Texas Medical Branch (UTMB) biosafety level 4 (BSL-4) facility in accordance with institutional and safety guidelines.

**Animal experiments.** Six- to 8-week-old mice deficient in alpha/beta interferon receptor (IFN-α/βR−/−) were bred at UTMB. 129S6/SvEv-Stat1tm1Rds (Stat1−/−) and control mice (WT129S6) were purchased from Taconic (Hudson, NY). All animals were housed in a pathogen-free environment. All virus infections were performed in the BSL-4 facility in the Galveston National Laboratory (GNL), UTMB. All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee at UTMB and were carried out according to the National Institutes of Health guidelines. Animals were anesthetized using an isoflurane precision variable-bypass vaporizer prior to virus inoculation by the intraperitoneal (i.p.) route with 10^7 PFU (trial 1) and 10^8 PFU (trials 2 and 3). Standardized recording of death and disease symptoms was performed using the following classifications: encephalitis; development of discoordination, ataxia, or transient seizures (with the ability to drink and feed); paralysis; and hind limb (hemiplegic) or quadriplegic paralysis (with the inability to reach the feeder or water bottle). Telemetric monitoring of body temperature and measurement of body weight also were performed during the course of the study. For telemetry, animals were anesthetized and implanted subcutaneously with BMDS IPTT-300 transponders (chips) obtained from Bio Medic Data Systems, Inc. (Seaford, DE), using a trocar needle assembly. Animals were monitored for signs of infection or transponder migration for 2 days prior to transfer to the BSL-4 facility. Temporal bones then were transferred to 4°C for long-term storage for later use (up to 6 months). The whole temporal bone was embedded in paraffin, thin sectioned at 4- to 7-μm slices, and subjected to labeling. The thin sections were stained with hematoxylin and eosin for routine observation of the inner ear structures. Slides were labeled with primary antibody and treated with DAB-conjugated secondary antibody for visualization. The primary antibodies used in the study were rabbit anti-Lassa GP antibody (IBT Bioservices, Gaithersburg, MD) and rabbit anti-CD3 antibody (Dako, Carpinteria, CA). The slides then were observed with an Olympus IX 71 microscope, and images were obtained using the DP controller software (Olympus Corporation).

**Determining the percent hair cell loss.** The cochlear duct tissue was dissected from the temporal bones and incubated in phosphate-buffered saline (PBS) for 10 min for cleaning. The cochlea then were incubated in 0.5% Triton X-100 in PBS for 30 min, washed 3 times with PBS for 5 min, and then incubated in a blocking buffer (0.3% bovine serum albumin, 0.5% fish gelatin, and 0.2% Tween 20 in PBS). The cochlea were incubated overnight at 4°C with the hyperimmune ascites fluid (HIAF) diluted 1:500 in blocking buffer. The cochleae were incubated with Alexa Fluor 488 anti-mouse antibody (Ab; Invitrogen) and phalloidin-Alexa Fluor 568 (Invitrogen) diluted 1:250 in blocking buffer, respectively, for 1 h shielded from light at room temperature. The nuclei were stained with Hoechst 33258 (Invitrogen) diluted 1:1,000 in PBS for 5 min shielded from light at room temperature. The fluorescently labeled cochleae were mounted using either SlowFade or ProLong kits (Invitrogen) on glass slides and coverslips and observed using a Nikon TE2000 epifluorescent microscope, where inner and outer hair cells were counted for each turn of the cochlea (apical, middle, and basal). The number of dead (missing) cells and the number of viable (stained) cells were counted. The percentage of missing hair cells was determined by dividing the number of dead hair cells by the total number of hair cells counted for each turn. A Student’s t test (two-tailed) was done to compare the percent hair cell loss between mock-infected and infected animals for each turn of the cochlea.

**RESULTS**

Lethal and nonlethal human LASV isolates induce either lethal or sublethal disease in Stat1−/− mice. We infected Stat1−/− mice (n = 5/group) via the intraperitoneal (i.p.) route with 10^6 PFU of recent LASV isolates that were obtained directly from serum samples from fatal (LV2384, lethal isolate; we have previously evaluated the virulence of this virus for Stat1−/− mice [16]) and nonfatal (LV2350, nonlethal isolate) LF cases that were collected during a recent outbreak in Sierra Leone in 2012. Four out of five mice infected with the lethal isolate reached the monitoring criteria that required euthanasia between days 7 and 8 p.i. (Fig. 1Ai). All mice in this group developed a disease characterized by ruffled fur, hunched back, and reduced activity on day 5 (DS) p.i., and this lasted until day 15 p.i. in the survivor. All infected animals had...
fever around day 5 p.i., followed by rapid weight loss (Fig. 1Aii) and hypothermia (Fig. 1Aiii) around day 6 p.i., with the exception of 1 survivor. All animals that succumbed to challenge were viremic and had high titers of infectious virus in the brain and visceral organs at the time of death (Fig. 1B). In contrast, all Stat1−/− mice infected with the nonlethal isolate developed milder disease and survived the challenge (Fig. 1Ai). However, similar to the mice infected with the lethal isolate, these animals developed disease on day 5 p.i. that lasted until day 9 p.i. accompanied by weight loss (Fig. 1Aii), but no fever or hypothermia was detected (Fig. 1Aiii). Interestingly, all survivors in both groups developed a chronic infection (Fig. 1B).

When we increased the inoculation dose to 10⁵ PFU, 8 out of 10 (80%) Stat1−/− mice infected with the lethal isolate and 1 out of 10 (10%) animals infected with the nonlethal isolate became terminally ill and were euthanized between days 7 and 10 p.i. (Fig. 1Ci). Animals showed signs of disease until day 17 (Fig. 1D).

Overall, the results demonstrated that human LASV isolates cause either lethal or prolonged, severe (sublethal) disease in Stat1−/− mice and do not require prior adaptation.

LASV-infected Stat1−/− mice develop late-onset hearing loss. To investigate whether LASV infection induces hearing loss in Stat1−/− mice, we performed a preliminary evaluation of the ability of infected mice to respond to an auditory stimulus. Stat1−/− mice infected i.p. with 10⁴ PFU of either the lethal or nonlethal human LASV isolate were subjected to a hearing assessment in which a series of sound stimuli were delivered by a metal clicker with at least 60-s intervals between each click. Hearing was evaluated at least once weekly in this manner beginning 1 week postinfection through the end of study at 60 dpi. Mice that did not

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**FIG 1** Virulence of a lethal and nonlethal LASV isolate in Stat1−/− mice. Mice were inoculated with either 10⁴ (n = 5/group) (A) or 10⁵ (n = 10 and 7/group, respectively) (C and D) PFU of either lethal (LV2384, black) or nonlethal (LV2350, gray) human LASV isolates. Survival (i), body temperature (Temp.) change (ii), and weight change (iii) were recorded throughout the course of study. Statistical analysis of survival curves was performed using the log-rank chi-square test (P values are shown). Asterisks indicate statistical significance. (B) Serum and tissue samples were collected from infected mice either at the time of death or at the end of study (60 dpi). Virus titers were determined by plaque assay. Horizontal dashes indicate average titers.
show any behavioral changes, such as ear or body twitch, in response to two or more click stimuli were considered to have impaired hearing. Out of 5 animals infected with the lethal human isolate LV2384, only 1 survived the challenge, and this animal developed hearing loss at 33 dpi. All 5 Stat1<sup>-/-</sup> mice inoculated with the nonlethal human isolate LV2350 developed a mild transient disease between days 5 and 9 p.i. and survived the challenge. One of these mice developed hearing loss on day 33 p.i. In both cases, hearing deficiency seemed to be permanent, as it persisted for at least 1 month, when the study was terminated (Fig. 2A).

To test whether an increase in the inoculum dose would lead to greater incidence of hearing loss, Stat1<sup>-/-</sup> mice infected with 10<sup>5</sup> PFU of lethal and nonlethal isolates also were subjected to the hearing test. Intriguingly, all animals that survived the challenge in both groups developed permanent hearing loss. Thus, two mice infected with the lethal isolate and nine mice infected with the nonlethal isolate stopped responding to auditory signals on day 25 and between days 12 and 24 p.i., respectively.

To obtain a more detailed characterization of the phenotypic hearing loss, we repeated inoculations with the nonlethal isolate (<i>n = 7</i>) at a dose of 10<sup>5</sup> PFU (Fig. 1D). All survivors stopped responding to sound stimuli between days 16 and 45 p.i. (Fig. 2B), as measured by ASR. Startle amplitude was measured prior to infection and between days 16 and 59 p.i. Any increased amplitude from background indicated that the animal was able to process the sound stimulus at that decibel level. Amplitude was compared over time. Statistically significant impairments at processing decibel signals at 100, 110, and 120 dB were observed compared to the baseline to D29 p.i. (<i>P = 0.021</i>, <i>P = 0.0006</i>, and <i>P = 0.043</i>, respectively), baseline to D45 p.i. (<i>P = 0.007</i>, <i>P < 0.0001</i>, and <i>P < 0.0001</i>, respectively), and baseline to D59 p.i. (<i>P = 0.002</i>, <i>P = < 0.0001</i>, <i>P = < 0.0001</i>, respectively).

As a control, we infected 4 mice deficient for alpha/beta and gamma interferon receptor (IFN-a/bgR<sup>−/−</sup>) with the lethal human isolate LV2384, which grows to higher titers in these animals than in Stat1<sup>-/-</sup> mice but causes a very mild disease. As expected and previously reported, these mice only developed a transient disease between days 11 and 16 p.i. with no detectable hearing deficiency at any time point (Fig. 2C).

We also have assessed the involvement of antibody responses in the development of hearing loss in infected mice. Therefore, blood samples were collected from all surviving animals at the end of the observation period, and the presence of neutralizing antibodies in sera was assayed by the plaque reduction neutralization test (PRNT). The analysis showed that the Stat1<sup>-/-</sup> mice developed only minimal antibody responses to LASV infection, and we could not detect any neutralizing antibodies in the sera of IFN-a/bgR<sup>−/−</sup> animals (data not shown). This result suggests that it is unlikely that antibody responses played a significant role in the mechanism of LASV infection-associated hearing loss.

The obtained data clearly demonstrate that LASV induces hearing loss in Stat1<sup>-/-</sup> mice surviving the infection but not in IFN-a/bgR<sup>−/−</sup> mice despite a better ability of the virus to replicate in tissues of these animals.

**LASV infection induces pathological changes in the inner ear.** We next investigated whether any damage to the inner ear-
specific cells was present in these mice. Whole-mount inner ear preparations from LASV-infected mice were prepared at 60 dpi. Hair cells were labeled for visualization, and the morphology of the cells was observed under fluorescence microscopy (data not shown). We evaluated hair cell loss in the apical, middle, and basal turns of the cochlea in control (n = 5) and LV2350-infected (n = 4) Stat1−/− mice (Table 1). Mild damage to the three layers of outer hair cells (OHC) and the inner hair cells (IHC) was observed in Stat1−/− mice infected with LASV but not in control Stat1−/− mice. Significantly fewer hair cells were seen in infected mice when counts were performed for IHCs (P = 0.014) and layer 2 (OHC2) and layer 3 (OHC3) of the OHCs (P = 0.026 and 0.024, respectively) (Table 1). We observed the diffuse absence of outer hair cells along the cochlear duct, while the inner hair cells were mostly intact.

The most striking damage was observed in thin sections. Additionally, we observed significant degeneration and hemorrhagic changes in the spiral ganglion cells of the auditory nerve in Stat1−/− mice (Table 1). Mild damage to the three layers of outer hair cells (OHC) and the inner hair cells (IHC) was observed in Stat1−/− mice infected with LASV but not in control Stat1−/− mice. Significantly fewer hair cells were seen in infected mice when counts were performed for IHCs (P = 0.014) and layer 2 (OHC2) and layer 3 (OHC3) of the OHCs (P = 0.026 and 0.024, respectively) (Table 1). We observed the diffuse absence of outer hair cells along the cochlear duct, while the inner hair cells were mostly intact.

In contrast, these changes were not observed in infected IFN-a/bgR−/− mice (Fig. 3, right). Viral antigen was detectable in thin-section preparations of both Stat1−/− and IFN-a/bgR−/− mice. Prominent LASV antibody staining was observed in vascular-rich regions such as the spiral limbus, stria vascularis, and spiral ganglion (Fig. 4). We next investigated whether the destruction and hemorrhagic changes were associated with CD3-positive lymphocytes. Indeed, the CD3 antibody labeling was strong in areas in a pattern similar to that of LASV antibody labeling, including in the spiral ganglion, stria vascularis, and also within the scala tympani cell infiltrates in Stat1−/− mice infected with LASV. On the other hand, CD3-positive cells were much fewer in IFN-a/bgR−/− mice infected with LASV (Fig. 5).

In summary, in the Stat1−/− mice infected with LASV, we observed mild damage in the hair cells, with the main damage visualized in the spiral ganglion neurons and vascular-rich cells within the cochlea. LASV antigen and CD3-positive lymphocytes were present in the area of damage. This damage was not present in mock-infected Stat1−/− mice. Alternatively, in IFN-a/bgR−/− mice infected with LASV, while LASV antigen and CD3-positive lymphocytes were present in identical areas, there was no damage to the structures within the inner ear.

**DISCUSSION**

For the first time, we were able to successfully replicate the SNHL afflicting LF survivors using two human isolates, lethal (LV2384) and nonlethal (LV2350), in the Stat1−/− mouse model. We observed hearing loss in surviving animals in the late stages of infection or early convalescence. This is consistent with what has been described in humans (4, 5). Additionally, an increased viral dose of the nonlethal isolate (LV2350) led to an increased number of survivors with hearing loss, which makes this model very useful for future studies (Fig. 2). Damage to the inner ear hair cells and auditory nerve, each of which are required for transmitting sound stimuli, correlated with the hearing loss. Hearing loss was not present in IFN-a/bgR−/− mice inoculated at the same doses, im-

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**TABLE 1 Percent hair cell loss**

<table>
<thead>
<tr>
<th>Mouse and infection type</th>
<th>Hair lossa (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stat1−/−, LV2350</td>
<td>IHC 4.5 ± 1.7</td>
</tr>
<tr>
<td>Stat1−/−, mock</td>
<td>0.4 ± 0.4</td>
</tr>
<tr>
<td>P value</td>
<td>0.014</td>
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</tbody>
</table>

a Data are expressed as means ± standard deviations (n = 4 for LV2350 and n = 5 for mock infection). P values were determined by two-tailed Student’s t test. IHC, P = 0.014; OHC2, P = 0.026; OHC3, P = 0.024.
Stat1<sup>−/−</sup> patients (24). Also, the presence of T cells in the severely damaged inner ear nerve is destroyed while the adjacent facial nerve is not, which also correlates with clinical symptoms described in humans and in the model presented that there is no facial palsy associated with the LASV infection. It is possible that a combination of the two mechanisms is required, but further studies are needed to determine the exact mechanism of hearing loss.

A mechanism of hearing loss development in LF patients has been proposed in the literature (25). According to this mechanism, LASV particles compete with extracellular matrix (ECM) lamin for its primary receptor, alpha dystroglycan (DG), which leads to ECM lamin displacement and membrane instability. Thus, perturbation of ECM laminin-DG interaction on Schwann cell membranes and downstream signaling led to disruption of myelin and laminin deposition around axons (26). This can result in the interruption of neuronal signal transmission. This mechanism corroborates with the observed hearing loss in Stat1<sup>−/−</sup> mice; however, it does not allow us to explain why we couldn’t detect any hearing deficiency in IFN-a/bg<sup>−/−</sup> mice despite a better ability of LASV to replicate in tissues of these animals, specifically in the brain.

The cell-mediated immune response is essential for the resolution of LASV infection. However, T lymphocytes also play a key role in LF pathogenesis (27). One possible explanation for the observed hearing loss in Stat1<sup>−/−</sup> mice is that the transcriptional factor STAT1 that has been demonstrated to mediate the antiproliferative effect of type I and II IFNs on T cells (28–30) is absent from these animals. Thus, the unchecked expansion of effector CD8 T cells induced in response to LASV infection could lead to both the observed damage of the auditory nerve and, as a consequence, the loss of hearing in infected mice.

In contrast, we observed only minimal T cell infiltration in the auditory nerve without detectable damage and no hearing loss in IFN-a/bg<sup>−/−</sup> mice infected with LASV. Intriguingly, previous cor pulmonale is the importance of the immune response in the pathology found in this model. However, we do not exclude the possibility that the differences in the genetic background between these two mouse strains plays a role in the observed differential presentation of LASV infection in Stat1<sup>−/−</sup> and IFN-a/bg<sup>−/−</sup> mice (19).

There are several routes for viral particles to enter the inner ear: the round window/oval window route, the hematogenous route, and the cerebrospinal fluid (CSF) route. The round window/oval window route is unlikely to be related to the damage seen in the Stat1<sup>−/−</sup> mice, as this route indicates that the agent would have entered from the ear canal and an ear infection and earaches would be present (20). The hematogenous route is linked to systemic viremia, while the CSF pathway typically is characterized by meningitis and a high leukocyte count in the CSF (21).

Two mechanisms related to hearing loss resulting from LASV infection have been proposed in the past, a virus-induced immunological injury (4, 22) and a virus-mediated injury to the inner ear (23). Little evidence to support these hypotheses existed outside clinical descriptions, which do not allow for evaluation of the inner ear postmortem. Hearing loss being present only in infected Stat1<sup>−/−</sup> mice and not in the IFN-a/bg<sup>−/−</sup> mice suggests at least partial involvement of immunopathological mechanisms in LASV-induced hearing loss in our Stat1<sup>−/−</sup> mouse model due to the differences in the functionality of the interferon response. An immunological mechanism is further supported by the development of hearing loss even in the presence of ribavirin treatment, which very efficiently reduces the systemic virus load in human patients (24). Also, the presence of T cells in the severely damaged spiral ganglion in LASV-infected Stat1<sup>−/−</sup> mice correlates with the development of SNHL. It is also very interesting that the auditory nerve is destroyed while the adjacent facial nerve is not, which also
studies have demonstrated that signaling from the T cell anti-
gen receptor complex can selectively activate phosphorylation of STAT1 at the Ser72 residue but not the Tyr701 residue in peripheral blood lymphocytes independently of the classical IFN signaling pathway (31, 32). Since the phosphorylation of STAT1 on Tyr701 is required for binding to DNA and, therefore, its transcriptional (33), the obtained results suggest an alternative mechanism by which STAT1 regulates the function of T cells in vivo.

Interestingly, STAT1 signaling was shown to be required for efficient clonal expansion of activated CD8 T cells by promoting their survival in vivo in response to vaccinia virus infection (34). Therefore, efficient proliferation of activated CD8 T cells in the presence of type I IFN (30) in immunocompetent patients upon LAVS infection could lead to immunopathological damage of the auditory nerve and development of SNHL.

In conclusion, the Stat1−/− mouse model of LAVS infection reproduces the features of human LF, including pathogenesis and neurological manifestations not previously reported in any animal model of LF. This model opens the opportunity to study the SNHL syndrome associated with LAVS infection in an animal model of LF for the first time and potentially to test drugs and/or therapeutic strategies for treatment or prevention of SNHL.

ACKNOWLEDGMENT

This work was funded by UC7 5UAI094660 for operational support of the Galveston National Laboratory and the Institute for Human Infections and Immunity (IHII) (UTMB, Galveston, TX).

We thank Linsey A. Yeager for critical reading of the manuscript. We thank The Research Histopathology Core (UTMB, Galveston, TX) for immunohistochemistry staining.

FUNDING INFORMATION

HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID) provided funding to NadezhdA E. Yun under grant number UC7 5UAI094660. HHS | NIH | National Institute of Allergy and Infectious Diseases (NIAID) provided funding to Slobodan Paesler under grant number UC7 5UAI094660. HHS | NIH | National Institute on Deafness and Other Communication Disorders (NIDCD) provided funding to Tomoko Makishima under grant number K08DC011540.

S.R. was supported by a fellowship from IHII, the Center for Addiction Research, and the Department of Neuroscience and Cell Biology (UTMB, Galveston, TX). This study was conducted with the support of the Institute for Translational Sciences at the University of Texas Medical Branch, supported in part by a Clinical and Translational Science Award NRS1 (TL1) Training Core (TL1TR001440) from the National Center for Advancing Translational Sciences, National Institutes of Health.

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